Deciphering the Alk signaling pathway in *Drosophila*

Fredrik Hugosson
The true delight is in the finding out rather than in the knowing

Isaac Asimov
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## Aim of thesis

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## Results and Discussion

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Abstract

In *Drosophila melanogaster* the visceral mesoderm (VM) develops during embryogenesis in a process where myoblasts become specified to generate two distinct cell types, the founder cells (FCs) and the fusion competent myoblasts (FCMs) that consequently fuses. The cell specification is dependent on cell signaling mediated by the receptor tyrosine kinase (RTK) Anaplastic lymphoma kinase (Alk) and its ligand Jelly belly (Jeb), how this further sets up different identity programs that drive myoblasts to differentiate into FCs and FCMs is still not well understood.

We have analysed whether the Midkine (MDK)/Pleiotrophin (PTN) homologues in *Drosophila*, Miple1 and Miple2 activate the Alk RTK *in vivo*. Earlier results from cell culture experiments suggested that vertebrate MDK/PTN is capable of activating ALK, findings that have become controversial with other studies showing contradictory results. We wanted to use *Drosophila* that have conserved homologues of both MDK/PTN and ALK, to address the question *in vivo*. We analysed the contribution of Miple in Alk dependent developmental processes such as visceral mesoderm (VM) specification during embryogenesis and in body size regulation of adult flies. Specification of VM as well as body size are not effected by loss of Miple proteins, and over expression of Miple proteins do not effect VM specification or body size. All together we conclude that there is no evidence that Miple1 or Miple2 can activate Alk *in vivo*. We found that loss of Miple protein effect the median lifespan of the fly which is reduced, interestingly the over expression of Miple proteins can promote an increased median life span in *Drosophila*.

We have also analysed how Alk RTK signaling regulates the Gli-like transcription factor Lame duck (Lmd) *in vivo* on a post-translational level. It has already been reported that Lmd play an essential role in specification of FCMs in the somatic mesoderm during embryogenesis. We detect Lmd protein exclusively in FCMs of VM in control embryos, but in Alk mutants Lmd protein is present in all cells of VM and opposite to this when Alk is activated in all cells in VM by over expression of Jeb this results in total loss of Lmd protein. This suggests that Alk signaling is regulating Lmd, and we additionally show that Lmd persist in FCMs in mutants where VM is specified but where myoblast fusion do not occur, supporting that Alk activity in FCs is regulating the downregulation of Lmd in FCMs upon fusion.

Finally we have characterised the Rap1GEF C3G *in vivo* in *Drosophila*. In cell culture systems, the GTPase Rap1 have been identified to mediate Alk signaling and that this is regulated by the GEF C3G and interestingly the *Drosophila* C3G is expressed in the FCS of VM. We generated deletion mutants of C3G which exhibit semi-lethality and reduced life span, but no defects in visceral mesoderm development during embryogenesis. Instead we detected distinct phenotypes in somatic muscles of 3rd instar mutant larvae, with detachment and mistargeting of muscles, which effect localisation of integrins. We suggest that *Drosophila* C3G regulate Rap1 via inside out signaling to integrins which in turn effect cell adhesion *in vivo* in *Drosophila* larval muscles.
Abbreviations

**ALCL:** Anaplastic Large-Cell non-Hodgkin’s Lymphoma

**Alk:** Anaplastic lymphoma kinase (*Drosophila*)

**ALK:** Anaplastic Lymphoma Kinase (Mammalian)

**Bap:** Bagpipe

**Bin:** Binou

**Boss:** Bride of Sevenless

**Cic:** Capicua

**CNS:** Central nervous system

**Dof:** Downstream of FGFR

**Dpp:** Decapentaplegic

**Drk:** Downstream of receptor kinase

**Duf:** Dumbfounded

**EGFR:** Epidermal Growth Factor Receptor

**En:** Engrailed

**ERK:** Extracellular signal-regulated kinases

**EMS:** Ethyl Methanesulphonate

**ETS:** E26 Transformation Specific

**Eve:** Even-skipped

**FC:** Founder cell

**FCM:** Fusion Competent Myoblast

**FGF:** Fibroblast Growth Factor

**FGFR:** Fibroblast Growth Factor Receptor
**FLP**: FLP recombinase

**FRT**: FLP Recombination Target

**GAPs**: GTPase-activating proteins

**GEFs**: Guanine exchange factors

**GMR**: Glass Multimer Reporter

**GPCR**: G-Protein Coupled Receptor

**Grb2**: Growth factor receptor-binding protein 2

**GSK3**: Glycogen Synthase Kinase 3

**HARP**: Heparin Affin Regulatory Peptide

**HB-GAM**: Heparin binding Growth-Associated Molecule

**HBGF-8**: Heparin Binding Growth Factor-8

**Hh**: Hedgehog

**HSC**: Hematopoietic Stem Cell

**HSPG**: Heparin Sulphate Proteoglycan

**Jeb**: Jelly Belly

**LDL**: Low Density Lipoprotein

**Lmd**: Lame duck

**LRP-1**: LDL-Receptor-related Protein-1

**LTK**: Leukocyte Tyrosine Kinase

**MAPK**: Mitogen Activated Protein Kinase

**MAPKK**: Mitogen Activated Protein Kinase Kinase

**MDK**: Midkine

**Mef2**: Myocyte specific enhancer factor-2
**MAM:** Meprin/A5-protein/PTPmu

**Mib2:** Mind bomb 2

**N:** Notch

**Nf1:** Neurofibromatosis 1

**NMJ:** Neuro Muscular Junction

**NPM:** Nucleophosmin

**ORF:** Open Reading Frame

**OSF-1:** Oestoblast-Specific Factor-1

**PI3K:** Phosphoinositide-3 Kinase

**Pnt:** Pointed

**PS:** Parasegment

**PTN:** Pleiotrophin

**PTK:** Protein Tyrosine Kinase

**Rap:** Ras-proximate

**RIHB:** Retinoic acid-Inducible Heparin Binding factor

**RING domain:** Really Interesting New Gene domain

**RPTP:** Receptor Protein Tyrosine Phosphatase

**RTK:** Receptor Tyrosine Kinase

**Sdc:** Syndecan

**Sev:** Sevenless

**Shc:** SHC-adaptor protein

**Slp:** Sloppy-paired

**SM:** Somatic Mesoderm
**Sns:** Sticks and Stones

**Sos:** Son of sevenless

**TF:** Transcription Factor

**TGF-β:** Transforming growth factor-β

**Tin:** Tinman

**Ttk69:** Tramtrack69

**Ttm2:** Tiny Tim 2

**Twi:** Twist

**UAS:** Upstream Activating Sequence

**VM:** Visceral Mesoderm

**Vrp1:** Verprolin 1

**Wg:** Wingless
Peer-reviewed papers in thesis


Peer-reviewed paper not included in thesis

1. **Drosophila melanogaster** as a model system, how it all started

Nowadays, *Drosophila melanogaster* is commonly regarded as a genetic workhorse that offers a well-established model for developmental studies. The expansive genetic tool box that has been developed allows the research community to conduct elegant *in vivo* experiments to address their scientific questions. In the following sections I will highlight at least a few people that have made great contributions to the development of this model organism for experimental use.

Thomas Hunt Morgan (1866-1945) is considered the founding father for the use of *Drosophila melanogaster* as a model organism and identified the first mutation, located in the *white* locus (Morgan, 1910). Morgan started his work with *Drosophila* with the aim of inducing genetic mutations and was in need of a cheap and easy organism to handle and to culture. He experimented with a variety of treatments to induce mutations, including temperature, acids, sugars, alkalis, radium and X-rays. Eventually the first mutation appeared in his cultures and in form of a white eyed fly, caused by an aberration in the locus that still today is called *white* (Morgan, 1910). From his studies on this mutation and its segregation, he provided proof for the concept of sex-linked genes as well as observing what they interpreted as the exchange between the homologous X chromosomes in females, what we commonly refer to today as “crossing over”. In Morgan’s “fly room” during these ground breaking years,
he was accompanied with his students Alfred Sturtevant, Herman Muller and Calvin Bridges that all contributed to our present day knowledge. Sturtevant presented the first chromosomal maps in 1913 (Sturtevant, 1913), followed by Bridges who first presented the concept of chromosomes and non-disjunction (Bridges, 1914) as the failure of chromosome segregation as the cause of aneuploidy that results in abnormal chromosome numbers, published in the first issue of the new journal Genetics in 1916 (Bridges, 1916). Herman Muller discovered that X-rays induce mutations, published in 1927 (Muller, 1927), for which he received the Nobel prize 1946. He also published the most valuable tool of fly genetics, “the balancer chromosomes” that makes it possible to maintain a recessive lethal mutation (Muller, 1948).

2. The life cycle of Drosophila

One reason that Drosophila melanogaster has been an attractive model organism it rapid life cycle together with its high reproduction capacity. A female fly can produce up to 2000 (Sang, 2014) offspring during its lifetime and a male can contribute to the production of 10000-14000 new flies (Duncan, 1930). The development from fertilised egg to an adult fly takes only ten days (Figure 1). Embryogenesis in Drosophila takes little less than a day, resulting in a small larva, still a rather complex organism with an epidermis, nervous system, muscles, heart and digestive tract. The larva feeds for approximately 5 days, molting 24 and 48 hours after hatching, thereby increasing around 200 times in size (Church, 1965) before entering pupation and metamorphosis. During metamorphosis the entire organism is re-
organised and most larval tissues disintegrate and are rebuilt to form the adult structures of the fly. The eye, wing, leg and haltere imaginal discs develop into their corresponding adult tissues. The adult fly then ecloses and after a few hours its cuticle hardens and wings extend.

**Figure 1 Schematic overview of The Drosophila life cycle.** The first step of development is the fertilization of the oocyte. This is followed by nuclear cleavage where the embryo will remain as one large cell containing many nuclei. After this blastoderm stage the embryo undergoes gastrulation where patterning and segmentation occurs. Embryogenesis is completed after around 24 hours, resulting in a 1st Instar larvae. The larvae will repeatedly eat, grow and molt into 2nd and 3rd Instar larvae, until it reached its final size and undergoes pupation, followed by metamorphosis. After approximately 10 days, the fully developed fly will hatch from its pupae and the cycle is completed.
3. The genetic toolbox of *Drosophila*

Another key reason for the success of the fruitfly as model system can clearly be found in the strong genetic tool box that makes almost any genetic manipulation possible *in vivo*. Morgans lab members contributed with important early knowledge, the chromosome maps, the first mutants and deficiencies and the “balancer chromosomes” for maintaining these mutants. There is a number of discoveries that are of extraordinary importance, such as the mutagenesis screen by Christiane Nüsslein-Volhard and Eric Wieschaus (Nusslein-Volhard and Wieschaus, 1980), the first transgenic P-elements generated by Allan C. Spradling and Gerald M. Rubin (Rubin and Spradling, 1982; Spradling and Rubin, 1982), the targeted gene expression UAS/GAL4 system (Brand and Perrimon, 1993), the FLP/FRT system (Xu and Rubin, 1993) and the sequencing of the full genome of *Drosophila melanogaster* (Adams et al., 2000). The very ambitious approach of Nüsslein-Volhard and Wieschaus employing the mutagen Ethyl Methanesulfonate (EMS) in a screening approach with aiming to identify all genes required for embryonic development generated an impressive amount of information. They identified three classes: the polarity-, gap- and pair-rule genes (Nusslein-Volhard and Wieschaus, 1980). They suggested that genes generating the same phenotype when mutated, work in the same biochemical pathway and related phenotypes indicate a hierarchy. The generation of the first artificial P-element and subsequently the first transgenic flies by Spradling and Rubin was the first step for development of the tools that today are used in daily fly work for generating transgenic flies (Rubin and Spradling, 1982; Spradling and Rubin, 1982). To be able to express your gene of interest in a defined spatio-temporal
context within an organism allows investigators to perform sophisticated experiments in vivo. This became possible in the fly with the development of the UAS/GAL4 system by Brand and Perrimon (Brand and Perrimon, 1993) and with a constantly growing number of different UAS and GAL4 stocks available, the possibilities offered by this method or modifications of it seem unlimited. Generating one transgenic fly carrying any active enhancer or promotor upstream of the gene encoding yeast transcriptional activator GAL4 and another fly carrying an Upstream Activator Sequence (UAS) upstream of any Open Reading Frame (ORF) allows generation of F1 progeny carrying both elements in their genome, thus driving expression of the ORF in the pattern of the active enhancer/promotor (Figure 2).
In 1929 Sturtevant described analysis of mosaic animals using unstable X-chromosomes (Sturtevant, 1929), and in 1936 Curt Stern reported that crossing over between homologous somatic chromosomes could be used to generate mosaic tissues (Stern, 1936). The real breakthrough for generating mosaic tissues came in 1993, when Xu and Rubin published a method utilising FLP recombinase (FLP) in combination with chromosomes carrying FLP recombination targets (FRTs). Generation of a number of fly stocks carrying FRT containing transgenes for each chromosome arm, located close to the

Figure 2 Schematic overview of the UAS/GAL4 system in Drosophila. The parental flies in P1, in this example a female fly carries a GAL4 P-element with a promotor active in the eye, thus expressing GAL4 protein(green) in cells of the eye, and a male fly carrying a UAS P-element with the UAS sequence upstream of a ORF (red). The F1 progeny fly with both components of the system in all cells, express GAL4 protein in cells of the eye that binds UAS sequence and promote expression of ORF in the eye cells (red)
centromere, enables the generation of mosaics for over 95% of the genes in *Drosophila* (Xu and Rubin, 1993). The most recent contribution to the genetic tool box in the fly, is the efficient genome editing that can be generated utilising the RNA-guided CRISPR/Cas9 technology (Jinek et al., 2012), with this method one can generate site specific mutations and deletions with high efficiency in the genome of the fly (Bassett et al., 2013; Gratz et al., 2013; Yu et al., 2013).

To summarise, a large collection of different fly stocks have been generated, providing the tools for generating deletion mutants, tissue specific miss- and overexpressions, tissue specific RNAi, lineage tracing, mosaic tissues and much more in *Drosophila*.

4. **Embryogenesis and development of muscles in *Drosophila***

About two hours after fertilisation the zygote has developed into a syncytical blastoderm containing a single cytoplasm with around 6000 nuclei that become separated into individual cells (Mahowald, 1963). Next, gastrulation occurs leading to the formation of the three germ layers that will develop into mesoderm, generating muscles, endoderm and ectoderm.
4.1 Subdivision of the embryonic mesoderm in *Drosophila*

In the early *Drosophila* embryo the trunk mesoderm as well as the ectoderm is divided up in units known as parasegments (PS) (Azpiazu et al., 1996; Borkowski et al., 1995; Riechmann et al., 1997). These segments are formed as a result of signals that pattern and specify them. They are divided as domains, named A (Anterior) and (P) Posterior (also named *slp* and *eve* functional domains (Riechmann et al., 1997). These domains contain primordial cells of the mesodermal tissues, visceral, somatic, heart and fat body, that are positioned in anterior-posterior and dorso-ventral positional sub-domains (Azpiazu et al., 1996; Borkowski et al., 1995; Riechmann et al., 1997) (Figure 3A).

When the embryo is segmentally patterned from anterior to posterior and from ventral to dorsal the cells start to differentiate (Borkowski et al., 1995; Riechmann et al., 1997). During *Drosophila* embryogenesis three characteristic types of muscle tissues develop from single cells that migrate, differentiate and undergo fusion to form the final muscle tissue. The somatic muscle precursors form the skeletal or larval body wall muscles from the somatic mesoderm, the visceral muscle precursors form the gut muscles from visceral mesoderm and the cardiac mesoderm forming a heart tube of cardioblasts and surrounding pericardial cells.
**Figure 3** Schematic overview of the patterning and domain organization of the trunk visceral mesoderm. (A) The embryo has 11 segmental clusters of trunk visceral mesoderm primordia (red) each corresponding to a parasegment (PS). Each PS can be divided into a posterior (P) and anterior (A) domain. These domains contain primordial cells for visceral mesoderm, cardiac mesoderm, dorsal mesoderm, ventral mesoderm, fat body and gonadal mesoderm. (B) In the trunk mesoderm, specification of the visceral mesoderm primordia is specified by expression of *bagpipe* (*bap*) and *binou* (*bin*) in dorsal mesoderm. The Dpp signal along A-P axis activates *tinman* (*tin*) and together with Tin, expression of *bap* is initiated that in turn activates *bin* in the dorsal mesoderm (black arrows). Segmental expression of Wg from the ectoderm induces expression of *sloppy-paired* (*slp*) (black arrows) that blocks expression of *bap* at the level of *even-skipped* (*eve*), *hedgehog* (*hh*) and *engrailed* (*en*) (grey arrows). Modified after Lee et al., 2006 and Riechmann et al., 1997.
4.2 Specification of the visceral mesoderm primordia of the trunk mesoderm

The P and A domains are defined by *eve-skipped* (*eve*) and *sloppy-paired* (*slp*), and to some extent by *hedgehog* (*hh*) and *engrailed* (*en*) that regulate *bagpipe* (*bap*) and *biniou* (*bin*) expression (Azpiazu et al., 1996; Borkowski et al., 1995; Riechmann et al., 1997)(Figure 3B).

The dorsal mesoderm receives a signal from the TGF-β family member, Decapentaplegic (Dpp), originating from the dorsal ectoderm (St Johnston and Gelbart, 1987). Dpp signaling activates expression of the transcription factor Tinman (*Tin*) in the dorsal mesoderm (Frasch, 1995). Dpp together with Tin drives the expression of *bap* - an early transcriptional regulator of trunk mesoderm (Azpiazu et al., 1996). A major target of *bap* is the FoxF homolog, *bin* (Perez Sanchez et al., 2002; Zaffran et al., 2001), that in turn regulates *bap* in a positive feedback loop (Zaffran et al., 2001). Bap together with Bin specifies the visceral trunk mesoderm primordia development in the dorsal part of the P domain (Zaffran et al., 2001). Wingless (*Wg*) expressed in ectoderm of the A domain drives expression of *slp* (Azpiazu et al., 1996; Lee and Frasch, 2000) that in turn represses *bap* in the A domain of the dorsal mesoderm (Riechmann et al., 1997).
4.3 Development of the larval somatic muscles in *Drosophila*.

The larval body wall or skeletal muscles are stereotypically arranged muscle fibers, in total 30 in number for each of the parasegments A2-A9 (Beckett and Baylies, 2006). The somatic muscle primordium is specified from the mesoderm after gastrulation by high *twist (twi)* expression (Baylies and Bate, 1996) and during stage 10-11, cells expressing high levels of *lethal of scute (l’sc)* and high levels of *twi* originating from twist-expressing cells, generate cells that will become the muscle progenitors of the somatic muscles (Carmena et al., 1995). These progenitors are selected by Ras-signaling together with Notch (N)-mediated lateral inhibition, where Ras specifies founder cells (FCs) and lateral inhibition the FCMs (Carmena et al., 1995), resulting in expression of a group of FCM-specific genes (Artero et al., 2003). After specification progenitors undergo an additional round of asymmetric cell division, generating another FC or another FCM (Carmena et al., 1998; Ruiz Gomez and Bate, 1997).

The formation of each muscle fiber follows the same model, a FC fuses with surrounding FCMs with the FC determining the identity of the mature fiber as well as the number of fusion events (Bate, 1990, 1993). This asymmetric fusion is dependent on cell-cell interaction via two immunoglobulin family members, primarily between Dumbfounded (Duf/kirre) (Ruiz-Gomez et al., 2000; Strunkelnberg et al., 2001) exclusively expressed on FCs and Sticks and stones (Sns) (Bour et al., 2000) specifically expressed on FCMs. In mutants where
fusion does not occur, cells still orientate and innervate properly but are smaller and unable to attach to tendon cells (Rushton et al., 1995).

4.4 Lame duck a transcription factor crucial for FCM fate

During *Drosophila* embryogenesis specific genetic programs generate two types of myoblasts, FCs and FCMs that are essential for the formation of larval muscles (Bate, 1990; Dohrmann et al., 1990).

Lame duck/Gleeful/Myoblast incompetent (Duan et al., 2001; Furlong et al., 2001; Ruiz-Gomez et al., 2002) is crucial for FCM fate and one of three members of the Gli/Zic/Glis family of transcription factors in *Drosophila*, the other two are being encoded by *cubitus interruptus* (Orenic et al., 1990) and *odd-paired* (Aruga et al., 1996; Benedyk et al., 1994). Lmd is specifically expressed in FCMs (see previous section) (Artero et al., 2003), and was first identified in a microarray screen as a gene with decreased expression in all developmental stages in *twist* mutants, hence a putative target of Twist. Initial analysis indicated that loss of this gene leads to disorganisation and severe loss of somatic muscles (Furlong et al., 2001). In addition, ectopic Lmd can drive expression of Mef2, and *lmd* mutants show reduced expression of Mef2 (Furlong et al., 2001) as well as complete loss of Sns in somatic FCMs together with a lack of multinucleate muscle fibers (Duan et al., 2001). However, somatic FCs are specified and capable to differentiate into mono nucleated myotubes (Duan et al., 2001). It is essential that the Lame duck protein is degraded after cell fusion in the somatic mesoderm and this requires
expression of the E3 ubiquitin ligase Mindbomb2 in the FC (Mib2) (Carrasco-Rando and Ruiz-Gomez, 2008; Nguyen et al., 2007).

4.5 Development of the embryonic visceral muscle in *Drosophila*

At embryonic stage 10, the primordial cells of foregut and hindgut are located in close proximity to the anterior and posterior margins of the mesoderm respectively (Lee et al., 2006)(Figure 4A). Caudal visceral mesoderm progenitors located in posterior region of the mesoderm give rise to the longitudinal midgut muscles while trunk visceral mesoderm progenitors
Figure 4 Overview of the developmental origin and morphology of the visceral muscles in the Drosophila embryo. (A) Schematic of lateral view of stage 10 embryo with indicated mesodermal primordia, hindgut and foregut visceral mesoderm (blue), caudal visceral mesoderm (green) and trunk visceral mesoderm (red). (B) Schematic of visceral muscles in a stage 16 embryo. Foregut and hindgut are covered by circular visceral muscles (blue), the midgut is covered by an inner layer of circular visceral muscles (red) and an outer layer of longitudinal muscles (green). Adapted with permission from Lee et al. 2006.

located in 11 clusters generate the circular midgut muscles. The progenitors of the caudal visceral mesoderm migrates and at stage 12 they are aligned as two rows of cells dorsal and ventral of the caudal visceral mesoderm (Campos-Ortega and Hartenstein, 1997). Towards the end of stage 12 FCs of
longitudinal origin fuse with remaining cells of the trunk visceral mesoderm while migrating anteriorly (Klapper et al., 2002; Martin et al., 2001).

The gut musculature of the Drosophila larva is a thin layer formed by the VM that covers the inner midgut epithelium. In the midgut region, the visceral muscle is composed of an inner layer of circular muscle with an outer layer of longitudinal muscles. Circular muscles undergo one round of cell fusion resulting in binuclear fibers that span half of the gut tube and are attached by integrins with another muscle fibers to surround the entire gut tube (Brown et al., 2000; Klapper et al., 2001)(Figure 4B). The longitudinal muscles in the midgut are also multinucleated fibers but unlike the circular muscles that span the foregut, midgut and hindgut the longitudinal muscles span only the midgut.

5. Receptor Tyrosine Kinases (RTKs)

Receptor tyrosine kinases (RTKs) mediate the cellular response to extracellular cues and to regulate cell-differentiation, proliferation, migration, survival, cell-cycle control and metabolism. The human genome contains 58 known RTKs divided in 20 subfamilies, with all of them sharing a common molecular structure (Yarden and Ullrich, 1988). The picture is significantly simpler in Drosophila where around 20 RTKs have been identified (Sopko and Perrimon, 2013)(Table 1). RTKs generally exhibit an extracellular ligand binding domain, a single spanning trans-membrane domain and an intracellular catalytic kinase domain with additional regulatory regions such
as the juxtamembrane domain. Commonly the activation of a RTK is dependent on ligand binding that induces dimerisation of the receptor.

### Table 1. *Drosophila* RTKs with corresponding identified ligands and homologues mammalian receptor. Adapted from Sopko and Perrimon 2013

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<td>Ret oncogene (Ret)</td>
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<td>Rearranged during Transfection (RFT) Tyrosine Kinase</td>
</tr>
<tr>
<td>OFFtrack (ORX)</td>
<td>Wing oncogenic analog (Woa)</td>
<td>Tyrosine kinase-related kinase (Trk)</td>
</tr>
<tr>
<td>PDGF- and VEGF-receptor related (Pyr)</td>
<td>PDGF- and VEGF-related Factor 1-3 (PVR-3)</td>
<td>Flakel Derived Growth Factor and Vascular Endothelial Growth Factor Receptor (VGRF and VEGFR)</td>
</tr>
<tr>
<td>Kpp receptor tyrosine kinase (Kpp)</td>
<td>Ephrin, Vap6 and Epp</td>
<td>Ephrin and EphrinB (Kpp6 and EphB)</td>
</tr>
<tr>
<td>One of two Ret kinases (Ror)</td>
<td>Receptor tyrosine kinase-like orphan receptor 1 and 2 (Ror1 and Ror2)</td>
<td>Muscle-Specific Kinase (MusSK)</td>
</tr>
<tr>
<td>Sevenless (Sve)</td>
<td>Bricke of sevenless (Bom)</td>
<td></td>
</tr>
<tr>
<td>Torso (Tor)</td>
<td>Trunk (Trk)</td>
<td></td>
</tr>
<tr>
<td>Stitcher</td>
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<tr>
<td>Tie-like receptor tyrosine kinase (Tie)</td>
<td></td>
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### 5.1 RTKs in *Drosophila melanogaster*

The *Drosophila* genome contains approximately 20 RTKs (Sopko and Perrimon, 2013) with the majority having a human counterpart, for example FGFR (Heartless and Breathless), INSR/IGF1R (Insulin-like receptor), EGFR (Epidermal Growth Factor Receptor), RET (Ret oncogene), VEGFR/PDGFR (PDGF-and VEGF receptor related) and ALK (Anaplastic lymphoma kinase) (Lorén et al., 2001). *Drosophila* RTKs such as Torso, EGFR, Heartless and Breathless, Sevenless and Alk, are important for distinct cell fate determination events, decisions that are irreversible. Experiments with hybrid *Drosophila* RTKs in which the cytoplasmic domains of Breathless and Heartless were replaced with the equivalent domains of Torso and EGFR led to the rescue of phenotypes caused from loss of the receptors themselves,
suggesting that RTKs in *Drosophila* do not themselves activate specific MAPK signaling pathways (Dossenbach et al., 2001).

5.2 MAPK signaling downstream of the RTK Sevenless in *Drosophila*

The power of *Drosophila* as a genetic model system for analysing cell signaling *in vivo* has helped us unravel complex signaling networks as well as defining their functions. Extension of this knowledge to human RTKs have further increased our understanding of how these networks regulate cellular processes and development *in vivo*.

Elegant genetic studies using *Drosophila* have mapped out core components of the MAPK signaling pathway downstream of the RTK Sevenless (SEV) *in vivo*. This pathway is also utilised downstream of other RTKs such as Torso and EGFR in *Drosophila*. The SEV RTK is required for photoreceptor specification during eye development, and while it is expressed in several of the R photoreceptors (R1/R6, R3/R4, R7 and cone cells) (Tomlinson 1987), it is only required for R7 specification. Loss of SEV activity leads to loss of R7 photoreceptors and does not affect other cells in the eye (Harris 1967). In *sev* mutants the R7 cell fate is changed from a photoreceptor to a non-neuronal cone cell (Reinke and Zipursky, 1988; Tomlinson and Ready, 1986). The SEV receptor on R7 photoreceptors is activated by the seven-transmembrane protein Bride of Sevenless (BOSS) (Hart et al., 1990; Kramer et al., 1991) presented by the R8 photoreceptors (Reinke and Zipursky, 1988). This simple system involving only two cells (R7 and R8) and with a receiving cell that only
can choose between two fates (neuronal and non-neuronal) thus allowed study of RTK signaling in vivo (Raabe, 2000). Components of the MAPK pathway were identified in screens using both loss and gain of SEV activity (Basler et al., 1991; Dickson et al., 1992a; Simon et al., 1991). The Rubin group (Simon et al., 1991) used a sev allele with reduced activity (a temperature sensitive allele, sev\textsuperscript{ts}), while the Hafen group (Basler et al., 1991 and Dickson et al., 1992) expressed a ligand independent, constitutive active SEV receptor (sev\textsuperscript{st}, expressed under the sev promoter). Importantly, the levels of SEV activity in the R7 photoreceptor are sensitive to gene dosage, thus enabling screening for loci that when reduced, affect SEV signaling. These initial screens lead to identification to molecules directly downstream of SEV, including the small GTPase, Drosophila Ras1 (DRas1) (Simon et al., 1991) and its activator, the Ras-GEF, Son Of Sevenless (SOS) (Rogge et al., 1991; Simon et al., 1991), that are linked by the SH3-SH2 adaptor protein, Downstream of Receptor Kinases (DRK the Grb2 fly homolog) (Olivier et al., 1993; Simon et al., 1993). The Rubin group added to the pathway further when they identified the negative regulator Drosophila Ras-GAP1, Gap1, which when mutated phenocopies constitutive active SEV (Gaul et al., 1992). The Hafen group were able to place the Drosophila Raf (DRaf, Pole hole) downstream of DRas1 in the Sevenless pathway (Dickson et al., 1992b). Two additional genetic screens identified the Drosophila MAPK Kinase (MAPKK, MEK, DMEK, Dsor) as a dominant suppressors, of lethal DRaf mutations (Lu et al., 1994; Tsuda et al., 1993). The Rubin and Hafen groups performed further screens to identify components downstream of DRas1 (Karim et al., 1996) and DRaf (Pole hole) (Dickson et al., 1996).
Screening using active Raf in only R7 photoreceptors (sev-Raf\textsuperscript{tors}) identified MAPK (ERK, \textit{rolled}) (Biggs et al., 1994; Biggs and Zipursky, 1992) as suppressor, placing it in the Sevenless pathway. Exploiting the fact that activated DRas-1 in R7 photoreceptors mimics SEV signaling (sev-Ras\textsuperscript{V12}) (Fortini et al., 1992), the Rubin group screened for modifiers of the rough eye phenotype and identified core components, DRaf, MEK and MAPK (Karim et al., 1996). Their screen identified also targets of the MAPK pathway, including the ETS DNA binding protein Yan (Lai and Rubin, 1992) that is phosphorylated and inhibited by MAPK (Brunner et al., 1994; O'Neill et al., 1994; Rebay and Rubin, 1995).

To summarise, the core MAPK pathway downstream of the SEV RTK (Figure 5) was revealed by \textit{in vivo} screens in the fruit fly, illustrating the power of genetic screens in understanding complex signaling pathways.
Figure 5 Model of the Sevenless RTK signaling pathway in *Drosophila* R7 photoreceptor cell fate determination. The Sevenless RTK on R7 photoreceptor cells is activated by the seven-transmembrane protein Bride of Sevenless (BOSS) presented by R8 photoreceptor cells. *Drosophila* Grb2 binds via its SH2 domain to Sev and mediates a link to Son of sevenless (Sos) via its SH3 domains. The GEF Sos activates Ras1 by promoting a GTP bound state which in turn anchors the kinase Raf to the plasma membrane where it becomes activated. Raf phosphorylates MEK that phosphorylates MAPK. Activated MAPK translocates to the nucleus and phosphorylates the two antagonistically working ETS DNA-binding proteins Pointed and Yan.

6. Anaplastic Lymphoma Kinase (ALK)

The Anaplastic Lymphoma Kinase (ALK) RTK was first discovered (Morris et al., 1994; Shiota et al., 1994) in anaplastic large-cell Non-Hodgkin’s lymphoma (ALCL) as the fusion protein NPM-ALK in which the catalytic domain of ALK is fused to the amino terminus of nucleophosmin (NPM). In contrast to the membrane location of full length ALK RTK, NPM-ALK is found...
in the cytoplasm of the cell. Since this initial finding, ALK fusions have been reported with over 20 different fusion partners in a variety of diseases (Hallberg and Palmer, 2013), for example EML4-ALK (Rikova et al., 2007; Soda et al., 2007) and KIF5B-ALK (Takeuchi et al., 2009). The mammalian ALK receptor together with the related Leukocyte Tyrosine Kinase (LTK) receptor (Ben-Neriah and Bauskin, 1988) belongs to the insulin receptor family. Structurally, in addition to a signal peptide sequence and a single trans-membrane domain the extracellular domain of the mammalian ALK receptor contains two MAM-(Meprin/A5-protein/PTPmu) domains, present in proteins as meprins and receptor protein tyrosine phosphatases, with suggested function in cell adhesion (Beckmann and Bork, 1993; Jiang et al., 1993). ALK is the only reported RTK with MAM domains. The extracellular domain also contains an LDLa domain and a glycine rich region, and for mammalian ALK, the function of both these domains are unclear. The intracellular part holds a Protein Tyrosine Kinase (PTK) domain, that shares 64% residue identity with LTK (Morris et al., 1997), a juxtamembrane segment and an extra-long carboxy-terminal tail in comparison to other RTKs (Iwahara et al., 1997; Morris et al., 1997). (Figure 6). The ALK receptor as well as the LTK receptor are considered orphan receptors because well described ligands are lacking.
Figure 6 Schematic of ALK and LTK RTK structure and regulatory outputs. The Drosophila Alk and mammalian ALK contain the same domains. (A) The extracellular domain of ALK consists of a Signal Sequence (SS) (green), an LDLα motif (yellow) flanked by two MAM-s (MAM) (blue), and a glycine rich region (G-rich) that resides close to the membrane. A transmembrane domain (TM) (grey) localises the receptor to the membrane. The intracellular domain contains a Protein Tyrosine Kinase (PTK) domain (red). The extracellular domain of LTK consists only of a glycine rich region but share same TM and PTK domains with ALK (B) Drosophila Alk is activated by the LDL domain protein Jelly-belly (Jeb) (yellow) and the resulting signaling regulates dpp, duf/kirre, org-1, hand and flamingo. Subsequently, Alk activity regulates cell specification in embryonic visceral mesoderm, axonal targeting in the optic lobe, body size regulation, ethanol sensitivity and brain sparing during nutrient restriction.

However several studies suggests that the Midkine and Pleiotrophin growth factors can activate ALK in vitro (Bowden et al., 2002; Powers et al., 2002; Stoica et al., 2001; Stoica et al., 2002) however these findings are still are
considered controversial due contradicting data from other studies (Mathivet et al., 2007; Moog-Lutz et al., 2005; Motegi et al., 2004; Mourali et al., 2006).

6.1 ALK signaling in cell culture systems

In different cell culture systems mammalian ALK can be activated artificially by monoclonal activating antibodies (Moog-Lutz et al., 2005) or by fusing intracellular ALK domains with an extracellular domain that induces dimerisation and activation, such as the Fc domain from mouse IgG (Souttou et al., 1997).

Activation of the MAPK pathway by phosphorylation of ERK has been shown to be induced by ALK signaling in several cell systems for example human neuroblastoma cells (SK-N-SH) (Motegi et al., 2004) and PC12 cells (Souttou et al., 1997). Further, the PI3K/Akt pathway is activated by ALK signaling in both U87MG glioblastoma cells and NIH3T3 fibroblasts (Bowden et al., 2002; Powers et al., 2002). Additionally PLC-γ and PI3K are activated by ALK when an ALK chimera with an EGFR extracellular domain is expressed in NIH3T3 cells, resulting in dimerisation and activation of the receptor on the addition of EGF (Piccinini et al., 2002). Finally ALK has also been shown to mediate signaling via the Rap1/C3G axis in PC12 cells and neuroblastoma cells (Schonherr et al., 2010).
6.2 ALK expression and function in vertebrates

When analysing different tissues from humans, ALK mRNA expression is found in adult brain, small intestine, colon, prostate and testis (Morris et al., 1994; Pulford et al., 1997). In mouse, analysis of expression of ALK mRNA and protein expression during embryogenesis, shows prominent expression mainly in developing nervous system, both central, peripheral, enteric and olfactory nerves and in spinal cord (Iwahara et al., 1997; Morris et al., 1997; Vernersson et al., 2006). This expression declines after birth and remains low (Iwahara et al., 1997). In the dentate gyrus in hippocampus of adult mice ALK protein is detected in overlapping pattern with closely related LTK (Weiss et al., 2012). To summarise, mammalian ALK is expressed in several human tissues and its neuronal expression during development, suggesting a function for this receptor in the developing nervous system.

6.3 ALK signaling in cancer

ALK contributes to cancer progression in several ways including chromosomal translocations resulting in fusion proteins and point mutations that alter the kinase activity (Hallberg and Palmer, 2013). Fusions of the ALK tyrosine kinase domain result in dimerisation and constitutive activation. The identification of point mutations in the kinase domain of ALK is a more recent finding first reported in familiar and sporadic neuroblastoma, a neuronal childhood cancer with origins in the developing sympathetic nervous system (Caren et al., 2008; Chen et al., 2008; George et al., 2008; Janoueix-Lerosey...
et al., 2008; Mosse et al., 2008). Activating point mutations -so called gain-of-function mutations (GOF) in the ALK kinase domain have been identified in neuroblastoma, NSCLC and thyroid cancer (Mosse et al., 2008; Murugan and Xing, 2011; Wang et al., 2011)

6.4 Anaplastic lymphoma kinase (Alk) in Drosophila

The Drosophila Alk receptor was first described in 2001 (Lören et al., 2001) as a 200kD protein with clear homology to mammalian ALK and LTK. Like its mammalian homologue Drosophila Alk contains two MAM-domains, one LDLa domain and a glycine rich region (Lören et al., 2001). The second MAM domain is important for receptor activity in Drosophila, as an Alk mutant with a single point mutation in this domain results in a loss of function phenotype (Lören et al., 2003). The function of the LDLa (Low Density Lipoprotein class a) domain in Alk has not directly been proven to be important, but is important for binding of Alk to the ligand Jelly belly (Jeb) (Lee et al., 2003). The glycine rich region consists of three stretches of consecutive glycine, and single point mutations in glycine residues in this region, results in non-functional Alk receptors (Lören et al., 2003), demonstrating the importance of this region. As for mammalian ALK the intracellular part of Drosophila Alk contains a Protein Tyrosine Kinase (PTK) domain, a juxtamembrane segment and a carboxy-terminal region (Lören et al., 2003) (Figure 6B).

In Drosophila, expression of Alk RNA is first detected in amnioserosa of early embryos followed by expression in the developing visceral mesoderm during its specification, declining after gut development. In the late stage embryo Alk
is expressed in CNS and epidermal patches (Lorén et al., 2001). The requirement for Alk in VM is well characterised, loss of Alk activity or the Jeb ligand results in incorrect specification of cells of the VM that fail to fuse, leading to late embryonic/early larval death. Larva die after embryogenesis due to absence of a gut musculature and thus a functional gut (Englund et al., 2003; Lee et al., 2003; Lorén et al., 2003; Stute et al., 2004). Mechanistically; the Jeb ligand expressed by neighboring somatic mesoderm (Weiss et al., 2001) binds to Alk (Figure 7), leading to robust ERK activation in a layer of precursor cells in the developing VM (Lorén et al., 2003).

![Figure 7 Jelly belly and Alk in developing visceral mesoderm](image)

The ligand Jelly belly (white, white arrow) produced by the neighboring somatic mesoderm, binds Alk (arrowhead). Alk positive cells in the visceral mesoderm (red) include row of columnar shaped founder cells (FCs) and fusion competent cells (FCMs). All cells in the visceral mesoderm are also Fasicilin III positive (green).

This drives specification of FCs and subsequent expression of FC markers such as Hand (Varshney and Palmer, 2006), Org-1 (Lee et al., 2003), Duf/Kirre (Englund et al., 2003; Lee et al., 2003) and Dpp (Shirinian et al., 2007) (Figure 8). The remaining fusion competent myoblasts (FCMs) express FCM markers such as Verprolin 1 (Vrp1/WIP/Sltr) (Eriksson et al., 2010), Lmd
(Stute et al., 2004) and Sticks and stones (Sns) (Bour et al., 2000). These FCMs then fuse with FCs thus forming the visceral musculature.

Activation of Alk by Jeb is employed in different contexts during development. An anterograde Jeb/Alk signaling pathway regulates correct axonal targeting during eye development in Drosophila. Jeb and Alk are expressed in a complementary pattern, Jeb by R-cells and Alk by target neurons when axonal targeting is occurring during late pupae stages. Loss of Alk activity leads to targeting defects of R8-cells but not R1-R6 cells (Bazigou et al., 2007). More recently the mechanism of how Alk is required for this process has been revealed. Alk promotes the survival of L3 dendrites required for proper axonal targeting during development (Pecot et al., 2014). Drosophila Jeb/Alk signaling also regulates anterograde signaling in larval neuromuscular junctions (NMJ). Alk and Jeb were initially found to be enriched in synapses of larval brain and NMJ (Rohrbough and Broadie, 2010). Synaptic transmission at the larval NMJ is regulated by presynaptically secreted Jeb that activates postsynaptic Alk and a Ras/MAPK/ERK cascade (Rohrbough et al., 2013). One study has described a role for Alk and Jeb signaling in a “brain sparing” mechanism, and shows a hierarchy in the growth of polyploid larval tissues under nutrient starvation conditions that protects the brain. This process is more dependent on signaling via Alk during nutrient restricted conditions and not the Insulin Receptor (InR), resulting in activation of PI3-kinase. Alk also genetically suppresses sensing of amino acids via Slimfast/Rheb/TOR1 signaling and sparing of CNS (Cheng et al., 2011).
Neuronal activity of Jeb/Alk signaling regulates body size regulation and associative memory in *Drosophila* (Gouzi et al., 2011). Jeb/Alk signaling has an inhibitory effect on body size as reducing Alk activity leads to increased fly size, while on the other hand both increased Alk activity and increased levels

**Figure 8** Schematic overview of visceral mesoderm specification in *Drosophila* embryo. (A) Activation. Alk is expressed on all visceral mesoderm (VM) progenitors (blue) and is activated by its ligand Jeb (black). (B) Specification. Alk specifies founder cells (FCs) resulting in activation of ERK (pERK) in FCs, remaining cells become fusion competent myoblasts (FCMs). FCs (red) are positive for Org-1, Mind bomb 2 (Mib2), Hand and Dumbfounded/Kirre (Duf/Kirre). FCMs (blue) are positive for Verprolin1 (Vrp1), Lame duck (Lmd) and Sticks & stones (Sns). Both FCs and FCMs are Alk positive. (C) Fusion. One FC undergoes fusion with one FCM and subsequently stretch out and form visceral muscle cells (green) which express both Alk and Fascicilin III.
of Jeb result in reduced fly size. Jeb/Alk functions upstream of the Ras GTPase activating protein (Ras-GAP) Neurofibromatosis 1 (Nf1) in regulation of body size, memory and learning, and pharmacological inhibition of Alk using an inhibitor in Nf1 mutants rescues the Nf1 phenotype (Gouzi et al., 2005). Alk has been identified as a transcriptional target of Drosophila LIM-domain only (dLmo), a factor that regulates sensitivity to ethanol induced sedation in flies, this study also showed that loss of Alk increases resistance to ethanol sedation and that this is conserved in mice (Lasek et al., 2011).

7. ALK signaling regulates Rap1 activity via the GEF C3G

The C3G guanine exchange factor (GEF) and the GTPase Rap1 have been shown to mediate sustained MAPK activity in rat PC12 cells (Kao et al., 2001; Wu et al., 2001; York et al., 1998). ALK signaling is also able to activate Rap1 via C3G, leading to neurite outgrowth in PC12 cells and proliferation in neuroblastoma cell lines (Schonherr et al., 2010).

7.1 GTPases in signaling and their effectors

Small guanosine triphosphatases (GTPases) also referred to as small monomeric G-proteins, are proteins of 20-25 kDa molecular weight that function as molecular switches, cycling between an active or inactive state. Active, GTP bound Ras-like proteins often mediate intracellular signaling downstream of receptor, such as RTKs, with cytosolic and transcriptional outputs. These effects are of wide variety and include cell proliferation, differentiation and migration.
The GTPases are members of the Ras super family named from the founding member Rat sarcoma (Ras) oncogene (Harvey, 1964; Kirsten and Mayer, 1967) and can be further classified in 5 sub-families based on sequence and functional similarity, Ras, Rho, Rab, Sar/Arf and Ran (Wennerberg et al., 2005). The human Ras superfamily consists of at least 167 members (Rojas et al., 2012). The Ras family can be further divided in three branches with Ras, Ras-proximate (Rap) and Ras-like (Ral).

The activity of GTPases is highly regulated by Guanine exchange factors (GEFs) (Figure 9) that release GDP from GTPases, promoting formation of the
Figure 9. Schematic of the regulation of GTPases. The activity of GTPases (blue) is regulated by GEFs (green) via the dissociation of GDP (yellow) by GEFs that facilitate the association of GTPases with GTP (yellow). GAPs (red) accelerates the hydrolysis that results in an inactive GDP bound state of the GTPase.

active state. The GTPase-activating proteins (GAPs) accelerate hydrolysis leading to an inactive GTPase GDP bound state (Boriack-Sjodin et al., 1998) (Scheffzek et al., 1997). Interestingly the affinity for GTP and GDP binding to GTPases is similar, and it is the 10 fold higher concentration of GTP within the cell together with the function of the GEFs that drives activation. This suggests a large set of GEFs and GAPs are required to regulate signaling and specificity, and that several GEFs and GAPs regulate a single GTPase. Indeed for example Rap1 has been shown to be regulated by at least 6 different classes of GEFs,
C3G (Gotoh et al., 1995), EPAC (de Rooij et al., 1998; Kawasaki et al., 1998a), PDZ-GEFs (de Rooij et al., 1999), RapGRPs (Kawasaki et al., 1998b) and DOCK4 (Yajnik et al., 2003).

7.2 The RapGEF C3G

C3G (Crk SH3-domain-binding guanine-nucleotide releasing factor) (Tanaka et al., 1994) was the first identified GEF for Rap1 (Gotoh et al., 1995) and shows sequence similarity to CDC25 and Sos (Tanaka et al., 1994). GEFs together with GAPs regulates the activity of Ras proteins downstream of transmembrane receptors and the activation of Rap1 by a CRK-C3G complex together with Raf have been reported to sustain the activity of MAPK signaling (York et al., 1998). The identification and initial characterisation of a Drosophila C3G homologue showed that it as the human counterpart, it binds Crk and that the overexpression of constitutive active C3G results in phenotypes that mimic those seen from over activation of the MAPK pathway. This effect can be suppressed by reduction of gene dosage of members of this pathway and also of Rap1 (Ishimaru et al., 1999). Little is known about the function of C3G during Drosophila embryonic development, however, C3G transcripts were identified in a study that aimed to identify transcripts differentially expressed in FCs and FCMs in muscle tissues (Artero et al., 2003).
8. Midkine and Pleiotrophin; a family of Heparin binding growth factors

The Midkine (MDK) and Pleiotrophin (PTN) proteins are secreted growth factors. Based on structural similarity and their ability to bind heparin they are classified together in a family of heparin binding growth factors. The first publications describe MDK as a Retinoic acid induced gene isolated from mouse embryonic carcinoma cells and from mice during embryogenesis (Kadomatsu et al., 1988). In 1989 a protein purified from chick fibroblasts named Retinoic acid-inducable heparin binding protein (RIHP) was reported (Vigny et al., 1989), and was later shown to be MDK protein. During 1989-1990 two papers described PTN, in one case isolated from perinatal rat brain and named heparin binding growth-associated molecule (HB-GAM) (Merenmies and Rauvala, 1990), and in another study purified from bovine uterus, then initially named heparin-binding growth factor-8 (HBGF-8) (Milner et al., 1989) (Li et al., 1990). Additionally, a protein isolated in a differential hybridisation screen between osteoblast and fibroblast cells named Osteoblast-Specific Factor 1 (OSF-1) (Tezuka et al., 1990) and another isolated from adult bovine brain named Heparin Affin Regulatory Peptide (HARP) (Courty et al., 1991) were both found to be PTN protein (Table 2).
8.1 Midkine and Pleiotrophin expression and function in vertebrates

The reported expression patterns of vertebrate MDK and PTN are complex. During embryogenesis, MDK mRNA is detected in ectoderm and mesoderm of early mice embryos at embryonic days (E) E5.5-7.5, during which stages PTN mRNA is absent (Fan et al., 2000; Kadomatsu et al., 1990). At later stages (E9.5-15.5) MDK mRNA becomes restricted to regions of the neural tube after which it declines, while PTN mRNA is detected in ventral regions of neural tube as well as in dorsal parts of spinal cord (Fan et al., 2000; Silos-Santiago et al., 1996; Wanaka et al., 1993). MDK and PTN mRNA have also been detected at sites where epithelial-mesenchymal interactions occur during mouse embryogenesis, typically in the teeth, lungs and kidneys which are organs formed through epithelial-mesenchymal transmission (EMT) (Kadomatsu et al., 1990; Mitsiadis et al., 1995).
The embryonic expression patterns together with the in vitro data suggest developmental roles for MDK and PTN.

### 8.2 Midkine and Pleiotrophin function in mouse models

The widespread embryonic expression of MDK and PTN together with the potent effects seen in cell culture systems, suggested important functions for these growth factors in vivo. Despite this, MDK and PTN knock out (KO) mice of develop without gross abnormalities and are viable (Amet et al., 2001; Nakamura et al., 1998). Analysis of single KO mice, reveals developmental abnormalities that are restricted to neuronal tissues, such as postnatal developmental delay of the hippocampus in mdk deficient mice (Nakamura et al., 1998) and thickening of the cortical cerebrum in ptn deficient mice (Amet et al., 2001; Hienola et al., 2004). Mice deficient in mdk show reduced level of both dopamine and dopamine receptors, loss of dopamine neurons and early features of Parkinson’s disease (Ohgake et al., 2009; Prediger et al., 2011). In ptn mutant mice the Hematopoietic Stem Cell (HSC) population in the bone marrow is reduced as well as the regeneration of HSCs after radiation (Himburg et al., 2012; Himburg et al., 2010). PTN inactivation of the RPTPβ/ζ receptor complex, regulates the retention and regeneration of HSCs in the BM niche (Himburg et al., 2012) a process that is Ras dependent (Himburg et al., 2014).

The lack of striking phenotypes in non-neuronal tissues appears to be due to compensatory function of the two proteins. This is supported by analysis of mdk, ptn double mutant mice which display reduced birth rate as well as
reduced body weight at birth. Further, *mdk, ptn* double mutant females are infertile due to abnormal maturation of the follicles of the ovary (Muramatsu et al., 2006).

### 8.3 Midkine and Pleiotrophin mediated signaling in cell culture systems

While the nature of a MDK/PTN-receptor complex *in vivo* remains unclear, it is clear that this family of secreted cytokines is able to exhibit effects through the ligand activation of downstream signaling pathways. The neuroprotective actions of MDK have been studied in PC12 cells, where MDK was shown to activate Extracellular signal-Regulated Kinases 1 and 2 (ERK1 and ERK2) (Owada et al., 1999). In this work, addition of the MEK inhibitor PD98059 prevents the trophic effect of MDK, suggesting that ERK activation is important. Other observations indicate that other MDK-induced events, such as migration in macrophages, can be inhibited by PP1, wortmannin, PD98059 and vanadate, indicating players downstream of MDK include Src, PI3 kinase and ERK (Hayashi et al., 2001). Similar experiments examining migration in osteoblast-like cells have also shown that MDK activates PI3-kinase and ERK (Qi et al., 2001). In addition, experiments in primary neuronal cultures have also shown that MDK stimulates a rapid activation of ERK and PKB/Akt (Owada et al., 1999).

In analysis of the mitogenic activity of PTN using BEL (Bovine epithelial lens)-cells, stimulation with PTN resulted in phosphorylation of ERK1 and EKR2.
Additionally PTN stimulate the phosphorylation of PI3-kinase, which is inhibited by wortmannin. (Souttou et al., 1997). Similarly, in the U87MG glioblastoma cell line, both wortmannin and PY294002 inhibited phosphorylation of the PI3K downstream effector Akt, although in this cell line PTN not did induce phosphorylation of ERK (Powers et al., 2002). PTN induce anti-apoptotic signals (Zhang et al., 1999), and both MAPK and PI3K pathways are activated with PTN in NIH3T3 mouse fibroblasts, where anti-apoptotic signals can be blocked with the MAPK inhibitor U0126 alone (Bowden et al., 2002).

In summary, MDK and PTN are able to activate the MAPK and PI3K pathways in several different cell culture systems.

### 8.4 Midkine and Pleiotrophin related receptors

Given that both MDK and PTN encode small secreted cytokines with heparin-binding properties, they have long been considered to be functional ligands for cell surface receptors. Multiple receptors for the MDK/PTN family of cytokines have been reported, with no clear consensus concerning which receptor responds to ligand activation by MDK or PTN in vivo. Biochemical studies have shown that MDK forms homodimers (Kojima et al., 1997) and that these dimers are able to bind the Receptor-like Protein Tyrosine Phosphatase β/ζ (RPTPβ/RPTPζ) (Maeda et al., 1994). Additional proposed MDK receptors include: N-syndecan (Nakanishi et al., 1997), Low-Density-Lipoprotein (LDL) Receptor-related Protein 1 (LRP1) (Muramatsu et al., 2000), Anaplastic Lymphoma Kinase (ALK) (Stoica et al., 2002), the α4β1 and
α6β1-integrins (Muramatsu et al., 2004) and membrane localised Nucleolin (Take et al., 1994). Similarly, the proposed PTN receptors are: RPTPβ/RPTPζ (Meng et al., 2000), ALK (Bowden et al., 2002; Powers et al., 2002; Stoica et al., 2001), N-Syndecan (Raulo et al., 1994), Nucleolin (Take et al., 1994) and αvβ3-integrins (Mikelis et al., 2009). A number of reports have specifically addressed the role of MDK and PTN signaling through the ALK receptor tyrosine kinase. In these studies it has been reported that activation of ALK by either MDK or PTN leads to activation of both the ERK and PI3K pathways (Bowden et al., 2002; Powers et al., 2002; Stoica et al., 2001; Stoica et al., 2002). However, a number of independent studies present contradictory results showing that MDK and PTN do not activate ALK (Mathivet et al., 2007; Moog-Lutz et al., 2005; Motegi et al., 2004). There is a proposed mechanism of indirect activation of ALK which is not dependent on direct interaction between ALK and PTN but is rather mediated by PTN dependent inhibition of the phosphatase activity of RPTPβ/RPTPζ, promoting autophosphorylation of ALK (Perez-Pinera et al., 2007).

9. The search for ligands for *Drosophila Alk*

Jeb was first identified in a yeast selection screen for Tinman regulated genes (Weiss et al., 2001). Structurally this secreted protein contains a single LDL receptor binding motif, and display similarity to bovine Sco-spondin and enterokinase within this motif (Weiss et al., 2001). During 2003-2004 three studies reported that Jeb and Alk constitute a novel ligand-receptor pair critical for cells specification in the developing embryonic VM (Englund et al.,
2003; Lee et al., 2003; Stute et al., 2004). The *Drosophila* genome contain two genes with significant homology to vertebrate MDK and PTN, named *miple1* (also called *miple* (Choksi et al., 2006)) and *miple2* (Englund et al., 2006). Initial characterisation show that these proteins are indeed secreted and their mRNA expression pattern during embryogenesis reveal distinct expression of *miple1* in developing CNS, and of *miple2* in cells of the developing endoderm (Englund et al., 2006). Their expression pattern is very interesting in relation to the expression of the Alk receptor (Lorén et al., 2001) that is expressed in the developing mesoderm, the tissue adjacent to the endoderm, as well as in the CNS. This observation together with the results from cell culture studies, where MDK and PTN are reported to activate ALK (Stoica et al., 2001; Stoica et al., 2002) implies a potential role for Miple1 and Miple2 as ligands for Alk in the fly.

**Aim of thesis**

During the time of my Ph.D. my major aim has been to employ the model organism *Drosophila melanogaster* to analyse two evolutionary conserved proteins Miple1 and Miple2 and address whether they activate the Alk RTK receptor *in vivo* (paper I). I have additionally contributed to the analysis on how Alk RTK signaling regulates the Gli-like transcription factor Lame duck *in vivo* on a post-translational level (paper II). Finally I have contributed to the characterisation of the Rap1GEF C3G in *Drosophila in vivo* (paper III).
Results and Discussion

1. Paper I

The *Drosophila* Midkine/Pleiotrophin homologues Miple1 and Miple2 affect adult lifespan but are dispensable for Alk signaling during embryonic gut formation. *(Hugosson et al., 2014)*

1.1 Background

The aim of this study was to use an *in vivo* approach to investigate whether the *Drosophila* Midkine (MDK)/Pleiotrophin (PTN) homologs Miple1 and Miple2 are functional ligands for the Alk receptor. Former results from cell culture experiments suggested that vertebrate MDK/PTN is capable of activating ALK *(Bowden et al., 2002; Powers et al., 2002; Stoica et al., 2001; Stoica et al., 2002)*, a surprising finding since ALK had been considered to be an orphan receptor within vertebrates. Since this reports these findings have become controversial with a number of other studies showing contradictory results *(Mathivet et al., 2007; Moog-Lutz et al., 2005; Motegi et al., 2004)*. *Drosophila* Alk is not an orphan receptor, and interestingly during *Drosophila* embryogenesis, *miple1* and *miple2* mRNA are expressed in cells close to Alk positive tissues, *miple1* is expressed in developing CNS like Alk and *miple2* in developing endoderm just next to the Alk positive visceral mesoderm (VM) *(Englund et al., 2006; Lorén et al., 2001)*. Thus we wished to explore whether either Miple1 or Miple2 could function as Alk ligands in some context.
1.2 Results and Discussion

To analyse this I generated a series of deletion mutants by imprecise excision for the two *Drosophila* MDK/PTN homologs, Miple1 and Miple2. These mutants were employed for phenotypic analysis in developmental processes, with particular focus on Alk dependent developmental processes. In initial attempts to generate deletion of the two *miple* genes we used a novel approach, site specific targeting homologous recombination (Gong and Golic, 2003), resulting in a large number of candidates that were all false positives. Thus I turned to another strategy, employing imprecise excision. In a sensitised background mutant for the DNA helicase DmBlm (Witsell et al., 2010), we succeeded in generating a deletion harboring both *miple* genes allowing us to analyse their contribution to development. The isolated mutant flies are viable and I can also conclude that there is no obvious requirement for the proteins during fly development as we see no decrease in survival during development when we compare homozygous and heterozygous single or double mutants (Hugosson et al. Supplementary Figure 1).

The human proteins MDK and PTN bind heparin, however not all amino acids identified as important for heparin binding are conserved in Miple1 and Miple2, In spite of this these proteins bind heparin *in vitro* (Hugosson et al. Supplementary Figure 3), suggesting a conserved ability to bind heparin.

One type of proposed receptor for MDK and PTN are the Heparin Sulphate Proteoglycan (HSPG) Syndecans (Kojima et al., 1996; Mitsiadis et al., 1995; Nakanishi et al., 1997; Raulo et al., 1994) and one might think that Miple
protein is somehow mediating signaling via a HSPG containing receptor complex in *Drosophila*.

We further have analysed Miple protein contribution in Alk dependent developmental processes, such as the specification of VM during embryogenesis and in body size regulation of adult flies. Careful analysis of the specification of the cells in the developing VM, using antibodies for marker of FCs and FCMs show that both Alk activation (measured by pERK) and FC specification proceed normally in the absence of Miple proteins (Hugosson et al. Figure 2D, F and H). The Alk regulation of body size illustrated with overexpression of Jeb resulting in flies with reduced body size, was not recapitulated upon expression of either Miple1 or Miple2 (Hugosson et al. Figure 3I and J).

We additionally employed overexpression approach in VM of embryos to address if Miple1 and Miple2 are sufficient to drive ERK (pERK) activation downstream of Alk as a read out for receptor activation, concluding that Miple proteins is not sufficient to ectopically activate ERK in VM. These two experiments with different approaches to analyse if Miple proteins can drive Alk activity using body size and activation of ERK as a read out, clearly illustrate that these proteins do not possess this ability *in vivo*.

In addition I ectopically overexpressed both the *Drosophila* Miple proteins and human the Midkine and Pleiotrophin in the *Drosophila* eye to examine whether they interact with the corresponding ALK receptors *in vivo*. These results show that there is no evidence for an interaction between these proteins as measured on eye phenotype (Hugosson et al. Figure 6D-E and 7B-C).
Since *miple* mutants do not show any obvious developmental phenotypes we performed lifespan experiments on *miple* mutants to see if they display any tendency of towards reduced fitness. *miple* mutants show a clear reduced median life span in comparison with controls (Figure 5A), leading us to perform a follow-up analysis overexpressing Miple proteins. This experiment shows that ectopic expression of Miple promotes an increased median life span in *Drosophila* (Hugosson et al. Figure 5B). Taken together the fact that we did not detect any visible effect upon overexpression or depletion of Miple proteins is surprising and suggests that reported roles for MDK and PTN as growth factors affecting processes such as for example cell growth and differentiation, reviewed in (Muramatsu, 2002; Winkler and Yao, 2014) may not be conserved in the fly. We have focused on whether Alk mediates signaling induced by Miple proteins, the question still remains if fly homologues of any of the other reported receptors can mediate such signaling. That fact that even double *miple* mutants of *miple1* and *miple2* are viable may imply that such a receptor is also not essential and that the mutants would also be viable. This is the case for mutants of the Receptor Protein Tyrosine Phosphatase ζ/β homologue (RPTP ζ/β), *ptp99A* and the single LDL-Receptor-related Protein-1 (LRP-1), *lrp-1* that have been reported (Desai et al., 1996; Khaliullina et al., 2009). Since our analyses did not reveal a functional role for Miple proteins and there is little information regarding the requirements for these two receptors, we are unable to conclude at this time whether any of these could serve as receptors for Miple1 and/or Miple2. Among other candidates the single *Drosophila* Syndecan is well characterised. *Syndecan* mutants are lethal, although one study reported a
hypomorphic allele that displays reduced life span and a number of metabolic phenotypes (De Luca et al., 2010). It would be interesting to address if miple mutants phenocopy any of these metabolic abnormalities and whether there are possible genetic or physical interactions between Syndecan and the Miple proteins.

The distinct expression patterns of miple1 and miple2 exhibited during larvae development are intriguing but since secreted proteins do not necessarily target the same tissues they are produced in, or even those they are in close proximity to, the identification of such target tissue is important to address in future studies addressing the function of Miple proteins during larval development. One remaining explanation for why we see such prominent expression of Miple proteins during both embryogenesis and larval stages is that they are required under extreme conditions that a Drosophila may encounter in nature, for example facing bacteria, viruses or parasites, and/or that conditions in a laboratory in cultures with rich source of food masks any phenotypes related to nutrition and immunity. In this regard, both Midkine and also Miple2 have been reported to have antibacterial properties (Svensson et al., 2010). Whether this is also true in vivo would be interesting to analyse in future studies in the fly.

2. Paper II

Jeb/Alk signaling regulates the Lame duck GLI family transcription factor in the Drosophila visceral mesoderm. (Popichenko et al., 2013)
2.1 Background

We already know that the activity of Alk signaling and consequently ERK activation in visceral mesoderm (VM) is crucial for FC specification. How this specification is regulated on the transcriptional level and how this sets up different identity programs that drive myoblasts to differentiate into FCs and FCMs is not well understood yet. This requires key regulators that regulate transcription and one such molecule is Lame duck (Lmd). It has already been reported that Lmd plays a crucial role in FCM specification in the somatic mesoderm during embryonic muscle formation (Duan et al., 2001; Furlong et al., 2001; Ruiz-Gomez et al., 2002) but in addition to its expression in developing somatic mesoderm, it is also known to be expressed in VM (Ruiz-Gomez et al., 2002).

2.2 Results and Discussion

The Gli transcription factors are well known transcriptional mediators downstream of the Hedgehog pathway (Hui and Angers, 2011) but here we show that the Gli-like transcription factor Lmd is also a target of Alk signaling mediated by MAPK, and this mechanistically results in an exclusion of the Lmd protein from the nucleus.

The initial analysis of Lmd protein in the VM of Alk mutant embryos reveals that Lmd is present in all cells of VM, in comparison to only in FCMs of wild type embryos (Popichenko et al. Figure 1G-I). In contrast, activation of Alk signaling in all VM cells by expression of Jeb, results in total loss of Lmd
protein in all VM cells (Popichenko et al. Figure 2A). These two results together suggest that Alk/Jeb signaling regulates Lmd in the VM.

Loss of the founder cell (FC) markers Org-1 and Duf/kirre (Englund et al., 2003; Lee et al., 2003) has been described in Alk mutants suggesting that FC specification does not occur. Moreover all VM cells express fusion competent myoblast (FCM) markers such as Vrp1 and Sns (Eriksson et al., 2010). In contrast to this loss of lmd mutants or embryos in which Alk has been ectopically activated by overexpression of Jeb, express FC markers such as Org-1 (Popichenko et al. Figure 3B, Figure 5B and Figure 11B), Hand (Popichenko et al., 2007; Varshney and Palmer, 2006) and Duf/kirre (Lee et al., 2003; Stute et al., 2004; Varshney and Palmer, 2006) and the loss or reduction of FCM markers as Sns (Lee et al., 2003; Ruiz-Gomez et al., 2002; Stute et al., 2004) and Vrp1 (Popichenko et al. Figure 2C, Figure 3B) in the entire VM. This all together suggests that both Alk signaling and Lmd activity are required for correct cell specification in the VM.

In wild type embryos expression of lmd mRNA can be detected in all cells of VM, but Lmd protein is only stably expressed in FCMs. When we ectopically activate Alk in the VM, we still detect lmd mRNA in all cells of the VM, although Lmd protein is no longer detectable (Popichenko et al. Figure 6D), suggesting that Alk signaling regulates Lmd at a post-translational level.

To analyse subcellular localisation of Lmd protein upon Alk signaling we used human embryonic kidney cells (HEK293 cells), common for cell culture experiments, in which we expressed C-terminal tagged Lmd-GFP fusion protein together with the Drosophila Alk (dAlk) receptor and supplemented
with Jeb ligand. In parallel we employed a constitutively active ligand independent human ALK receptor (hALK^{F1174s}), identified in neuroblastoma (Martinsson et al., 2011). In both conditions, dAlk with Jeb ligand and constitutive active hALK^{F1174S}, Lmd-GFP is translocated out from nucleus (Popichenko et al. Figure 7B-C). This translocation can be blocked by the ALK inhibitor NVP-TAE684 and a MEK inhibitor U0126 suggesting that ALK modulates Lmd-GFP via the Ras/MAPK pathway. When we followed cells with live cell imaging, we observed Lmd-GFP translocation to cytoplasm within 20 minutes after removal of NVP-TAE268 (Popichenko et al. Figure 7G).

How is Alk signaling regulating the distribution of Lmd protein in the Drosophila VM tissue? Using mutants where FCs and FCMs are specified but where myoblast fusion does not occur makes it possible to address if Alk activity in FCs might effect and possibly downregulate Lmd in FCMs. The Immunoglobulin protein Sticks and stones (Sns) is required for cell-cell adhesion between FCs and FCMs during myoblast fusion and analysing sns mutants where fusion do not occur, clearly illustrates that when Lmd protein is not exposed to Alk activity it persists in FCMs (Popichenko et al. Figure 8A), supporting the hypothesis of Lmd downregulation.

We also analysed distribution and stability of a number of different Lmd mutant proteins upon overexpression in either Drosophila embryos or in human cells. These results suggests that the N-terminal region consisting of the first 140 amino acids of Lmd protein is required for exclusion of Lmd from the nucleus because a Lmd^{141-866} protein is constitutive active and not responsive to Alk signaling in both embryos and cell culture (Popichenko et
al. Figure 9B and 10C). However the N-terminal region is not sufficient for regulation as Lmd$^{1-140}$-GFP is not affected by Alk activity (Popichenko et al. Figure 10F or G).

Whether phosphorylation of Lmd is a direct result of MAPK activity or occurs indirectly, has been addressed previously on level of Lmd protein (Duan and Nguyen, 2006). We extended this analysis with the identification of novel phosphorylation-sites on Lmd using mass spectrometry. Together with our analysis of the different mutants we suggest that the N-terminal region contains important phosphorylation sites. Generating full length Lmd where only these sites in 140 first amino acids are mutated would be a further way to identify the functional importance of these phosphorylation sites.

We hypothesised that the E3 ubiquitin ligase Mindbomb 2 (Mib2) is involved in targeting the nuclear excluded Lmd for degradation, since Mib2 has previously been reported to be involved in Lmd degradation in somatic mesoderm as well (Carrasco-Rando and Ruiz-Gomez, 2008; Nguyen et al., 2007) We observe a delayed degradation of Lmd protein in the VM of embryos lacking Mib2 in agreement with a role for Mib2 in the VM (Popichenko et al. Figure 11D).

In a recent study the transcriptional repressor tramtrack69 ($ttk69$) as a novel regulator of muscle development identified in Drosophila (Ciglar et al., 2014) the authors show that $ttk69$ is transiently expressed in FCMs of the somatic mesoderm and that a complex balance is required between repression of FC specific genes by Tramtrack69 (Ttk69) and activation of FCM specific genes by Lmd in somatic mesoderm.
It is clear from analysing the combined data of the genomic regions, their cis-regulatory modules (CRMs), bound by Lmd and Ttk69, that Ttk69 predominantly binds and represses enhancers in FCs while Lmd binds and activates enhancers in FCMs (Ciglar et al., 2014). In embryos mutant for ttk69 the FC population is increased and consequently FCMs reduced, supporting a role for Ttk69 as a repressor of FC identity genes. Whether Ttk69 has exactly the same role in VM as shown for somatic mesoderm is not entirely clear, but the data from analysing two Ttk69 bound enhancers with reporter constructs in ttk69 mutants, also show a derepression of these targets in the VM. It has been reported that the FC identity gene hand is repressed by Lmd in FCMs because hand expression is expanded in VM of lmd mutants (Popichenko et al., 2007). This can in fact be explained by Lmd activating Ttk69 expression, which further leads to the repression of hand. If Lmd is lost, then so is also expression of ttk69 and consequently hand is derepressed and detectable in the entire VM.

3. Paper III

The Rap1 guanine nucleotide exchange factor C3G is required for preservation of larval muscle integrity in Drosophila melanogaster. (Shirinian et al., 2010)
3.1 Background

We aimed to identify new components of the Alk RTK pathway downstream of the receptor, and had identified the GTPase Rap1 to mediate MAPK independent ALK signaling and that this is regulated by the GEF C3G (Schonherr et al., 2010) in both a PC12 cell system and in neuroblastoma cells. This prompted us to look in Drosophila where C3G interestingly exhibit distinct expression during embryogenesis in VM FCs (Artero et al., 2003; Ishimaru et al., 1999) (Shirinian et al. Figure 3). This would provide an elegant mechanism with fast regulation of responses during VM development that are not MAPK dependent, such as for example cell shape changes that occurs in the row of FCs upon Alk activation. There is also evidence from Drosophila that GTPases like Rap1 mediates activity of the MAPK cascade of the Torso RTK pathway (Mishra et al., 2005), suggesting that GEFs which regulate the activity of GTPases may additionally be involved in regulating MAPK activity.

3.2 Results and Discussion

The overall domain structure between Drosophila and human C3G is conserved, they both have homologous catalytic CDC25-type GEF domain with 67% identity and a GEFN/REM domain with 39% identity Additionally the region for binding to CRK show strong homology (Shirinian et al. Figure 1A). We generated a deletion mutant for the C3G gene located on the X chromosome by heat shock induced FLP/FRT recombination between two PiggyBac elements flanking the C3G gene locus, resulting in an almost complete deletion of the coding region (Shirinian et al. Figure 1B). Analysis of
C3G mutant flies shows that they are semi-lethal (Shirinian et al. Figure 2C) and the surviving flies exhibit a shortened lifespan (Shirinian et al. Figure 2A and B).

We detect distinct phenotypes with detachment and mistargeting of somatic muscles of C3G mutant 3rd instar larvae (Shirinian et al. Figure 4D). The localisation of integrins at muscle attachment sites seem to be regulated by C3G as we observe that βPS integrins are mislocalised in C3G mutants and become recruited when we overexpress C3G in muscles (Shirinian et al. Figure 5C’ and D’ and Figure 7A’ and B’). This is not likely related to Alk signaling instead we propose this phenotypes link C3G to integrins, possibly by regulation of the activity of GTPases, like Rap1 via inside-out signaling to integrin and affecting cell adhesion. Another GEF the PDZ-GEF/Gef26/Dizzy has been shown to regulate cell shape of migrating macrophages, via the GTPase Rap1 in Drosophila embryos, this study also link Rap1 to Integrin mediated cell adhesion in vitro (Huelsmann et al., 2006), also in agreement with studies in cells such as leukocytes and macrophages (Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000).

Employing an in vitro GDP release assay we analysed the activity of C3G for two putative targets in Drosophila, Rap1 and Rap2, observing that both Drosophila and human C3G can activate Drosophila Rap1 but not Rap2, demonstrating that C3G is a Rap1 specific GEF in vitro (Shirinian et al. Figure 8A, top panels). This demonstrates that C3G is a GEF for Rap1, that effects integrin signaling, suggesting that there might be redundancy between GEFs in Drosophila. Our results suggest that C3G, while potentially involved in Alk signaling in the VM does not play a critical role.
Rap1 has been shown to regulate Ras/MAPK signaling downstream of Sevenless in R7 cells *in vivo*. In addition to Rap1, molecules as PDZ-GEF/Gef26/Dizzy and the Rap1 effector Canoe was found to function in the MAPK pathway downstream of Sevenless (Baril et al., 2014). Although C3G and Rap1 are activated by ALK signaling in both Pc12 cells and neuroblastoma cells (Schonherr et al., 2010), there is at present no experimental evidence for any link between ALK mediated MAPK signaling and C3G *in vivo* in *Drosophila*. We suggest that C3G regulates the correct localisation of integrins and consequently by doing so, sustain muscle integrity. In perspective of Alk signaling it would be interesting to address if C3G interacts with Alk mediated signaling in for example body size regulation or axonal targeting in the optic lobe.
Conclusions

1. Conclusions from Paper I

- *Drosophila* Miple1 and Miple2 are not essential for normal development.

- *Drosophila* Miple1 and Miple2 are not sufficient to activate Alk, either at the level of ERK activation in the VM or in body size regulation.

- *Drosophila* Mipel1 and Miple2 bind heparin *in vitro*.

- Mutants of *Drosophila* miple1 and miple2 show reduced median lifespan.

- Overexpression of *Drosophila* Miple1 and Miple2 is sufficient to extend lifespan.

2. Conclusions from Paper II

- Lame duck is a target of Alk signaling in *Drosophila*.

- Jeb/Alk signaling results in exclusion of Lame duck from the nucleus.

- Jeb/Alk signaling regulates Lame duck on post-translational level.

- N-terminal of Lame duck protein is required for exclusion from the nucleus.
3. Conclusions from Paper III

- Mutant of *Drosophila C3G* are semi-lethal and show a reduced lifespan.

- *C3G* mutant larvae show detached and mistargeted somatic muscles and mislocalised βPS integrins.

- *Drosophila C3G* is GEF for Rap1 *in vitro.*
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