Exploiting *Drosophila* as a model system for studying Anaplastic Lymphoma Kinase *in vivo*

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Umeå 2010
Till mor och far

Den mätta dagen, den är aldrig störst.

Den bästa dagen är en dag av törst.

Nog finns det mål och mening i vår färd
men det är vägen, som är mödan värd.

Karin Boye
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Abstract

Anaplastic Lymphoma Kinase (ALK) is a Receptor Tyrosine Kinase (RTK) and an oncogene associated with several human diseases, but its normal function in humans and other vertebrates is unclear. Drosophila melanogaster has an ALK homolog, demonstrating that the RTK has been conserved throughout evolution. This makes Drosophila a suitable model organism for studying not only Drosophila ALK function, but also to study mammalian forms of ALK. In Drosophila the ligand Jeb activates ALK, initiating signaling crucial for visceral mesoderm development. The activating ligand for mammalian ALK is unclear, and for this reason Drosophila was employed in a cross-species approach to investigate whether Drosophila Jeb can activate mouse ALK. Jeb is unable to activate mouse ALK, and therefore mouse ALK is unable to substitute for and rescue the Drosophila ALK mutant phenotype. This suggests that there has been significant evolution in the ALK-ligand relationship between the mouse and Drosophila.

In humans ALK has recently been shown to be involved in the development of neuroblastoma, a cancer tumor in children. I have developed a Drosophila model for examining human gain of function ALK mutants found in neuroblastoma patients. The various ALK variants have acquired point mutations in the kinase domain that have been predicted to activate the RTK in a constitutive and ligand independent manner. When expressed in the fly eye, active human ALK mutants result in a rough eye phenotype, while inactive wild type ALK does not, due to the lack of an activating ligand in the fly. In this way several of the ALK mutations identified in neuroblastoma patients could be confirmed to be activated in a ligand independent manner. Moreover, a novel ALK mutant; ALKF1174S, was discovered in a neuroblastoma patient and was in the Drosophila model shown to be a gain of function mutation, and a previously predicted gain of function mutation; ALKI1250T, was shown to be a kinase dead mutation. This fly model can also be used for testing ALK selective inhibitors, for identifying activating ligands for human ALK and for identifying conserved components of the ALK signaling pathway.

Gut musculature development in Drosophila is dependent on ALK signaling, while somatic muscle development is not. Proteins of the Wasp-Scar signaling network regulate Arp2/3-complex mediated actin polymerization, and I have investigated their function in visceral and somatic muscle fusion. I found that Verprolin and other members of this protein family are essential for somatic but not visceral muscle development. Despite fusion defects in both tissues in Verprolin and other examined mutants, gut development proceeds, suggesting that fusion is not crucial for visceral mesoderm development. Hence the actin polymerization machinery functions in both somatic and visceral muscle fusion, but this process only appears to be essential in somatic muscle development.
Papers included in this thesis

This thesis is based on the following publications and manuscripts.


Paper I, IV and V are reproduced with permission from the publishers.
Populärvetenskaplig sammanfattning på svenska

**Bakgrund:** Onkogener, det vill säga gener associerade med cancer, kan studeras i olika modellsystem. I min forskning har jag använt bananflugan (*Drosophila*) till att studera onkogenen ALK (Anaplastic Lymphoma Kinase). ALK-genen kodar för ett receptor tyrosin kinas, vilket är ett protein på cellens yta. ALK-receptorn aktiveras när ett protein, en så kallad ligand, binder till receptorn, detta startar en signalerings-kaskad inne i cellen via interaktioner mellan många proteiner, och slutligen leder detta till att cellen genomgår en förändring, till exempel börjar delta sig. Okontrollerad aktivering av ALK orsakad av specifika mutationer i receptorn kan ge upphov till neuroblastom, en cancerform som drabbar barn. Evolutionen har bevarat ALK i alla flercelliga organismer, således finns ALK-homologer, det vill säga ALK-liknande gener, i människan såväl som i musen och bananflugan. Detta antyder att ALK har en viktig fysiologisk funktion, och mycket riktigt är ALK och dess aktiverande ligand; Jeb, livsviktiga i bananflugan där de behövs för magutvecklingen. I däggdjur är den aktiverande liganden okänd och inte heller den fysiologiska funktionen för ALK är känd.


Jag har också studerat muskelutvecklingen i flugan genom att specifikt analysera cytoskelettet, en struktur i alla celler som bland annat ger cellerna mekaniskt stöd och framkallas cellrörelse. Cytoskelettet är uppbyggt av långa kedjor proteiner, mest aktin-proteiner, medan andra proteiner reglerar cytoskelettets dynamik. Jag har funnit att cytoskelett-reglerande proteiner är viktiga för muskelutvecklingen, särskilt för utvecklingen av kroppsmuskulerna, men de har också en viss funktion i magmuskelutvecklingen. Dessa resultat är viktiga för förståelsen för aktin-cytoskelettets betydelse i organutveckling i allmänhet och muskelutveckling i synnerhet.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Abi</td>
<td>Abelson interacting protein</td>
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<tr>
<td>ALK</td>
<td>Anaplastic lymphoma kinase</td>
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<tr>
<td>Arp2/3</td>
<td>Actin-related protein 2/3</td>
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<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
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<tr>
<td>Bap</td>
<td>Bagpipe</td>
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<tr>
<td>BDGP</td>
<td>Berkeley <em>Drosophila</em> Genome Project</td>
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<td>Blow</td>
<td>Blown fuse</td>
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<td>DFG</td>
<td>Asp-Phe-Gly motif</td>
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<tr>
<td>Dpp</td>
<td>Decapentaplegic</td>
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<tr>
<td>Duf</td>
<td>Dumbfounded</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
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<tr>
<td>FC</td>
<td>Founder cell</td>
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<tr>
<td>FCM</td>
<td>Fusion competent myoblast</td>
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<tr>
<td>G-actin</td>
<td>Globular actin</td>
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<tr>
<td>GEF</td>
<td>Guanine-nucleotide exchange factor</td>
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<tr>
<td>HRD</td>
<td>His-Arg-Asp motif</td>
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<tr>
<td>HSPC300</td>
<td>Haematopoietic stem/progenitor cell protein 300</td>
</tr>
<tr>
<td>IRS1</td>
<td>Anti-insulin receptor substrate-1</td>
</tr>
<tr>
<td>IRSp53</td>
<td>Insulin receptor substrate protein of 53 kDa</td>
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<tr>
<td>JAK3</td>
<td>Janus kinase 3</td>
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<td>Jeb</td>
<td>Jelly-belly</td>
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<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
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<td>MAPK</td>
<td>MAP kinase</td>
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<td>Mbc</td>
<td>Myoblast city</td>
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<td>Mek</td>
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<td>MK</td>
<td>Midkine</td>
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<td>Mtl</td>
<td>Mig-2-like</td>
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<tr>
<td>Nap1</td>
<td>Nucleosome assembly protein</td>
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<tr>
<td>Nck</td>
<td>Non-catalytic region of tyrosine kinase adaptor protein</td>
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<tr>
<td>NF1</td>
<td>Neurofibromatosis type 1</td>
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<td>NPM</td>
<td>Nucleophosmin</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<tr>
<td>PLCγ</td>
<td>Phospholipase C-gamma</td>
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<tr>
<td>PTB</td>
<td>Phosphotyrosine-binding</td>
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<tr>
<td>PtdIns</td>
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<td>PTK</td>
<td>Protein tyrosine kinase</td>
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<td>PTN</td>
<td>Pleiotrophin</td>
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<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<td>Scar</td>
<td>Wasp-related protein</td>
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<tr>
<td>SH2</td>
<td>Src homology-2</td>
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<tr>
<td>SH3</td>
<td>Src homology-3</td>
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<tr>
<td>SM</td>
<td>Somatic mesoderm</td>
</tr>
<tr>
<td>Sns</td>
<td>Sticks and stones</td>
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<tr>
<td>Sra1</td>
<td>Rac1-associated protein 1</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activation sequence</td>
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<tr>
<td>Wash</td>
<td>Wasp and Scar homolog</td>
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<tr>
<td>Wasp</td>
<td>Wiskott aldrich syndrome protein</td>
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<tr>
<td>Wave</td>
<td>Verprolin-homologous protein</td>
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<tr>
<td>VCA</td>
<td>Verprolin homology, Cofilin homology, Acidic</td>
</tr>
<tr>
<td>WH1</td>
<td>Wasp homology domain 1</td>
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<tr>
<td>WH2</td>
<td>WASp Homology domain 2</td>
</tr>
<tr>
<td>Wip</td>
<td>Wasp interacting protein</td>
</tr>
<tr>
<td>VM</td>
<td>Visceral mesoderm</td>
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<td>Vrp1</td>
<td>Verprolin</td>
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Introduction

1. Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are receptor proteins which are generally localized to the plasma membrane of the cells in multicellular organisms and their function is to transduce vital signals from the extracellular environment of the cell across the plasma membrane into the interior of the cell. Ultimately signaling cascades initiated by RTKs regulate key cellular processes such as growth, differentiation, migration and survival. There are 20 subfamilies of human RTKs, which share the common architecture of having an extracellular part consisting of multiple domains, a transmembrane domain consisting of one single helix and an intracellular domain which contains the protein tyrosine kinase domain (figure 1). Intracellular signaling pathways by RTKs are initiated by activation of the receptor; usually by the binding of high affinity ligands to the extracellular domains of two RTKs, which leads to dimerization of the two receptors and consequently activation of the intracellular protein tyrosine kinase domains (PTKs) [1].

Dimeric complex formation of two RTKs is either mediated by ligands that effectively cross link the receptors, or is induced by ligand binding stimulating conformational changes in the receptor molecules which subsequently promote receptor-receptor interactions, or a combination of both. In the inactive state, kinase domain activity is inhibited by cis-autoinhibition, i.e. intramolecular interactions block access between ATP and protein substrates. Upon dimerization of RTKs, key tyrosines become phosphorylated through trans-phosphorylation by its partner
leading to disruption of the cis-autoinhibition and allowing activation of the intracellular kinase domain (figure 2). However, there are exceptions to this model; some RTKs are activated by activating receptors, not ligands, and some RTKs are dimerized also in their inactive states [1, 2].

Figure 1. Human RTK family members. Modified and reproduced with permission from Cell Press [1].

After the initial activation of the RTK, autophosphorylation of additional tyrosines in the cytoplasmic domain follows. Phosphotyrosines function to increase the catalytic activity of the kinase, and to create specific sites for the assembly of downstream signaling molecules. SH2 and PTB domain containing signaling molecules interact directly with the phosphotyrosines, other proteins are recruited to the kinase domain via these adaptor molecules, and proteins that come into close proximity with the kinase domain become tyrosine phosphorylated at multiple
sites to create binding sites for more downstream signaling proteins. In this way activated RTKs can initiate multiple signaling cascades by influencing a large number of signaling molecules. Not only the general architecture of the RTKs has been conserved from *Caenorhabditis elegans* and *Drosophila* to humans, but also the signaling cascades initiated by the individual RTKs. (Cell signaling by RTKs is reviewed in [1]).

**Figure 2.** Illustration of active protein kinase domain. Between the N-lobe and C-lobe interaction between ATP and substrate takes place. Catalytically important residues are Asp of the DFG motif in the activating loop which interacts with ATP, and Asp of the HRD motif in the catalytic loop which interacts with the substrate. Reproduced with permission from PNAS [2].

In short, cell signaling by an RTK can be summarized as follows: Ligand induced receptor dimerization leads to release of cis-autoinhibition in the kinase domain, following trans-phosphorylation of key tyrosines in the intracellular domain. Additional tyrosine phosphorylation then takes place both in the RTK itself and in signaling and adaptor molecules that are recruited to the intracellular domain, resulting in recruitment of multiple downstream signaling molecules, thereby initiating complex downstream signaling pathways.
2. Mammalian ALK

Anaplastic lymphoma kinase (ALK) shares the common domain organization of RTKs (figure 1). ALK and the close homolog Leukocyte tyrosine kinase comprise a separate subfamily of RTKs, which in turn belongs to the insulin receptor RTK family based on kinase domain homology [3, 4]. However, despite high sequence similarity between the kinase domains of ALK and the Insulin receptor, crystal structures have shown that the mechanism for retaining the two RTKs in their inactive states are mediated by different intramolecular interactions. All protein kinases employ highly similar structures when activated, therefore significant structural differences between them are usually detected when analyzing the inactive states [5, 6].

2.1. Expression pattern and ligand

Mammalian ALK is predominantly expressed in the central and peripheral nervous system during embryonic development, although it is also found in the developing sensory organs, skin, internal organs such as the stomach and in the reproductive organs [4, 7]. Although the ALK expression pattern hints that this RTK may have functional roles in embryonic development, in particular in development of the nervous system, the function of ALK in higher organisms remains unclear.

Another unsolved mystery concerning mammalian ALK is the identity of the activating ligand. As an RTK, ALK is expected to be activated by a ligand, or by an activating receptor, although it should be borne in mind that this simple scenario may not be the case. Indeed in *Drosophila* the
ALK homolog is activated by Jeb, a small secreted LDL-domain containing molecule (discussed below). To date no such molecule has been identified in vertebrates, and work presented in this thesis clearly demonstrates that mammalian ALK is unable to respond to the Jeb ligand [8], suggesting that either co-evolution of the ligand and its binding regions in the receptor has occurred or that the activating ligand has changed throughout evolution. Two molecules of great interest are the growth factors Midkine (MK) and Pleiotrophin (PTN), which have been claimed by some investigators [9, 10], but not by others [11], to be able to activate mammalian ALK in vitro. This controversy between different studies may indicate that the mechanism by which MK and PTN activate ALK is not through standard straight-forward ligand-receptor interactions, but via other receptors. For example, both MK and PTN are known to interact with the protein tyrosine phosphatase beta/zeta, which in turn would indirectly activate ALK [12, 13]. Today all avenues of ALK activation remain open, the search for a LDL-domain containing ligand continues, the experiments using MK and PTN to activate ALK carry on and the investigation of finding novel activating ligands or mechanisms persist.

2.2. Signaling by mammalian ALK

From experimental studies using mammalian cell line systems, ALK has been shown to activate several signaling pathways and has been suggested to have a role in neuronal development. However because no activating ligand is known for mammalian ALK, in these experiments ALK has been activated in alternative ways. In the case of NPM-ALK, an ALK
fusion protein with constitutive ALK tyrosine kinase activity (discussed below), it has been shown that ALK signaling enhances cell proliferation and survival and leads to changes in cell shape [14, 15]. NPM-ALK directly interacts with IRS1, Src kinases, SHC and PLCγ at specific phosphotyrosines in the cytoplasmic domain, this triggers the ERK-signaling cascade which regulates mitogenic activity [16, 17]. Moreover, NPM-ALK activity activates STAT3, either directly or via JAK3, and this signaling is required for the survival mechanism by ALK [18, 19]. NPM-ALK also initiates anti-apoptotic signals by activating PI3K, in turn activating Akt, which enhances cell survival by blocking the function of pro-apoptotic proteins [20] (figure 3).

**Figure 3.** Illustration of mammalian and *Drosophila* ALK signaling.
Using activating monoclonal antibodies specific for ALK, similar results have been gained; ALK activity has been shown to activate signaling cascades such as the ERK-signaling pathway via interactions with IRS-1, Shc, and c-Cbl [21], to activate STAT3 [22] and to activate Rap1 via the Rap1-specific guanine-nucleotide exchange factor C3G [23]. Interestingly, in the neuronal rat cell line PC12, ALK has been shown to induce neurite outgrowth reflecting the process of neuronal differentiation, supporting the hypothesis that ALK functions in the development of the nervous system [8, 21, 22].

2.3. Human diseases
One important motivation for the study of ALK and ALK mediated signaling comes from the involvement of ALK in a number of human diseases. These diseases illustrate how important the regulation of ALK activity is in vivo.

2.3.1. Disease causing ALK fusion genes
ALK was first discovered as part of a fusion gene associated with anaplastic large cell lymphoma development. A chromosomal translocation event results in the fusion of the C-terminal portion of ALK with nuclephosmin (NPM) to give rise to the NPM-ALK fusion gene [24]. NPM-ALK has constitutive ALK kinase activity because of stable dimerization mediated by the NPM portion of the fusion protein [25]. Subsequently ALK has been found to be part of numerous other chromosomal translocation events giving rise to similar oncogenic fusion genes [26]. In addition to being involved in anaplastic large cell
lymphoma pathology, ALK fusion proteins have been identified in various solid tumors, including inflammatory myofibroblastic tumors [27], squamous cell carcinomas [28], non-small cell lung cancers [29, 30] and a subtype of diffuse large B-cell lymphoma [31]. Different ALK-fusion proteins are frequently, but not always, specific for the different diseases, i.e. although NPM-ALK is common in anaplastic large cell lymphoma it also sometimes occurs in diffuse large B-cell lymphoma. Why different ALK-fusion proteins are involved in different diseases is likely to be due to divergence in intracellular localization and cell type expression, leading to slightly different downstream signaling [26, 32].

2.3.2. Neuroblastoma

An additional mechanism in which to activate PTKs, such as ALK, in an uncontrolled manner, at either the somatic or germline level, is through mutation within the locus leading to gain-of-function variants of the PTK. In this manner ALK is involved in the development of neuroblastoma. Neuroblastoma is a childhood cancer, arising in tissues of the sympathetic nervous system. Often the primary tumor appears in the adrenal gland, but other locations within the abdomen and chest are also common, and the tumors may metastasize to lymph nodes, bones and bone marrow. Initially the primary tumor is thought to originate from precursor cells of the neural-crest tissue, which are cells believed to be more active during early embryonic development. Thus neuroblastoma is a disease of developing tissue and this may explain why it generally occurs in very young children (median age is 17 months) [33].
Figure 4. A) ALK crystal structure with mapped point mutations associated with neuroblastoma. Mutations studied in this thesis work are indicated. Original figure published by Lee et al. 2010 [6], here modified and reproduced with permission from Biochemical Journal. B) Neuroblastoma family pedigrees with ALK gain of function mutations. Individuals affected by neuroblastoma are indicated by filled symbols and genotypes are indicated if known. Figure published by Mossé et al. 2008 [34], here reproduced with permission from Nature publishing group.
ALK was first described being overexpressed in neuroblastoma cell lines [35], and in the last year our understanding of the role of ALK in neuroblastoma has increased exponentially. We now know that in numerous cases of neuroblastoma ALK is inappropriately activated in one of several ways; either through genomic amplification of a larger chromosomal region including the ALK gene (chromosome 2p), or through specific amplification of the ALK gene locus, or by generation of gain-of-function mutations in the kinase domain of ALK (figure 4A) [26]. ALK gain-of-function mutations, which are either germ line mutations or somatically acquired, constitutively activate ALK in a presumably ligand independent manner [34, 36-39]. Thus ALK activity clearly plays a role in both sporadic and familial neuroblastoma development (figure 4B).

Other genetic alterations associated with neuroblastoma are; amplification of the oncogene MYCN, loss-of-function mutations in the homebox gene PHOX2B, which is a master regulator of normal autonomic nervous system development [33] and loss-of-function mutations in the tumor suppressor NF1 gene, a mutation that indirectly, via Ras-Mek signaling, inhibits retinoic acid induced differentiation in neuroblastoma and thus complicates treatment [40]. Experimental studies, some of which are presented in this thesis, investigating both wild type and gain-of-function mutants of the full length ALK protein are being carried out in mammalian cell systems and in Drosophila in order to increase our understanding of the molecular mechanisms involved in neuroblastoma development (discussed further in Results and Discussion).
2.4. ALK inhibitors

Small chemical molecules that function as ALK inhibitors are being developed as potential therapeutic drugs against ALK induced cancers. Such inhibitors ought to be highly selective for ALK and they should target ALK itself and not downstream signaling molecules of the activated ALK receptor, given redundancy in signal transduction pathways. So far a couple of ALK selective inhibitors have been identified, and crystal structures of the human ALK kinase domain in complex with such inhibitors have provided information about the ALK kinase active site that should help with the development of more ALK selective inhibitors [5]. TAE684, is a small molecule which effectively blocks ATP binding in the ALK kinase domain, it has been shown to inhibit ALK activity in cell line systems and oncogenic ALK activity in mouse models [41], and in this thesis I can show TAE684 inhibition of ALK activity in a newly developed Drosophila model system for studying oncogenic ALK (see Results and Discussion). However, due to toxicity TAE684 is not considered for any human patient trials at the moment. PF02341066/Crizotinib is another ATP competitor for the ALK (and C-Met) kinase domains [42], which has shown selective inhibition of ALK activity both in vitro and in vivo [43], and has shown good results in patient trials.

3. Drosophila ALK

Simple organisms like the fruitfly (Drosophila melanogaster, figure 5) and the roundworm (C. elegans) also have an ALK gene, thus the receptor has been conserved throughout evolution. This fact implies
that *Drosophila* is a suitable model organism for studying both the normal function of *Drosophila* ALK as well as analyzing signaling by ALK from other species, simply by expressing any mammalian form of ALK in the fly. *Drosophila* ALK signaling drives ERK activation in the embryo [44] (figure 3), and further downstream transcriptional targets have been suggested to be Duf [45], Org-1 [46], Hand [47] and Dpp [48]. Two major differences between *Drosophila* ALK and mammalian ALKs are that 1) in *Drosophila* an activating ligand (Jeb) has been identified and 2) the physiological function for *Drosophila* ALK has been well characterized, two important details which are, as mentioned before, still unclear in vertebrates. In the developing *Drosophila* embryo ALK is predominately expressed in the visceral mesoderm, and accordingly ALK signaling is required for the development of the embryonic gut making it essential for life (described in detail below) [45, 46, 49]. Moreover, ALK and Jeb are expressed in the nervous system and have roles in the development of the visual system of the adult fly by being involved in targeting of neurons and photoreceptor axons in the brain and retina [50], and in larval locomotion by functioning in neuromuscular junction development [51]. Also in *C. elegans* the ALK homolog (Scd-2) functions in the nervous system by controlling entry into dauer stage, and an activating ligand (Hen-1), which is homologous to *Drosophila* Jeb, has been identified [52] (figure 6).
3.1. ALK and Jeb function in the visceral mesoderm

The expression patterns for ALK mRNA and protein in the *Drosophila* visceral mesoderm in the embryo have been well described. In short, ALK expression arises at embryonic stage 10 in twelve mesodermal clusters, the clusters then migrate longitudinally to form two ALK expressing bands at stage 11, these bands are the early visceral mesoderm, and ultimately the cells of the two bands migrate ventrally to form a closed tube at stage 15 to 17, which makes up the organ structure that after the embryonic stages will function as the larval gut (figure 7) [44].

![Jeb and Hen-1](image)

**Figure 6.** Domain organization of the ALK activating ligands Jeb (*Drosophila*) and Hen-1 (*C. elegans*).

Jelly-belly (Jeb) is the activating ligand for ALK, is expressed at the embryonic stage in the somatic muscle precursors. Jeb, which is a small LDL-domain containing molecule (figure 6), is secreted from the somatic mesoderm to be taken up by the ALK expressing cells in the adjacent visceral mesoderm [45, 46, 49].

ALK-Jeb signaling is crucial for the formation of the circular muscles of the visceral mesoderm, which make up the inner muscle layer of the gut. ALK and Jeb mutant embryos have no gut due to defective circular visceral mesoderm development, a phenotype which is lethal [45, 46]
(figure 7B). The outer layer of the gut, the longitudinal visceral mesoderm, is formed by visceral mesoderm precursors which migrate over the circular muscle layer. What direct role ALK signaling has on longitudinal visceral mesoderm development is unclear and difficult to define experimentally. However, since no longitudinal muscles can be formed unless an inner circular muscle layer is formed first, ALK signaling affects longitudinal muscle formation at least indirectly.

In a wild type Drosophila the circular visceral mesoderm consists of two myoblast subtypes at embryonic stage 11; founder cells and fusion competent myoblasts, which need to attach to each other for the gut to be formed. The fate of these two cell types during gut formation has been studied in detail. Morphologically they are shaped differently; founder cells are columnar and fusion competent myoblasts are round, they express different adhesion molecules on their cell membranes and they express a number of intracellular molecules differently (table 1). Two well characterized adhesion molecules are the molecules Dumbfounded (Duf) and Sticks and Stones (Sns), which are expressed on the cell membranes of the founder cells and the fusion competent myoblasts respectively, where they mediate the attachment between the cell types via their extracellular domains that recognize each other [53, 54]. Attachment between the founder cells and the fusion competent myoblasts is required for the continuation of the gut development, but whether or not the completion of the fusion event, which leads to the formation of binucleated cells, is absolutely essential remains unclear. ALK signaling is required for the specification of the
founder cells in the early visceral mesoderm. In ALK and Jeb mutant embryos the founder cell subtype is absent, and therefore the fusion competent myoblast have no cells to attach to, resulting in the lack of gut formation [45, 46, 49] (Figure 7B).

**Figure 7.** *Drosophila* embryos. Larval organs start to develop during the embryonic stage. Here green fluorescent antibody stainings of the visceral mesoderm show the development of the gut from early stage embryos (top pictures) to late stage embryos (bottom pictures). Only if the gut develops correctly will the larva that hatches out from the egg be able to digest food. **A)** In the wild type embryo visceral mesoderm cells first line up in two rows (arrows), attach to each other and stretch out to form the gut (intestine like structure, bottom picture). **B)** In the ALK mutant embryo one cell type is missing, therefore the cells are unable to line up and attach to each other (arrows indicate unattached, unorganized cells) and consequently no gut is formed (bottom picture).

### 4. Cytoskeletal proteins and muscle fusion in *Drosophila*

ALK function in visceral mesoderm development in *Drosophila* is quite fascinating. In its role as the key regulator of visceral mesoderm founder specification, the ALK signaling pathway by definition regulates the entire fusion process in the developing visceral muscle. The process of
muscle fusion is complex and intricate, and although much studied in the somatic muscle is poorly understood in the visceral muscle. However, in general it can be hypothesized that visceral mesoderm and somatic muscle development are not too dissimilar, since the two muscle types are formed through comparable processes and many proteins are expressed in both tissues (table 1). In my thesis work I have investigated the role of actin cytoskeletal proteins in visceral and somatic muscle development. Thus, while ALK signaling has no known function in somatic muscle development, the study of fusion processes in the visceral muscle inadvertently includes study of the somatic muscle.

4.1. Somatic muscle development
Somatic muscles are used for body movement by the *Drosophila* larvae. Above I have described the process of visceral mesoderm development, interestingly the process of somatic muscle development is very similar. The somatic muscles are also initially formed by founder cells and fusion component myoblasts, here many fusion competent myoblasts fuse with one founder cell to generate multinucleated muscle fibers of appropriate size (figure 8). In somatic muscle development the molecules Duf and Sns also play a central role in attracting the fusion competent myoblasts to the founder cell by the same mechanism as in the visceral mesoderm, and numerous intracellular signaling molecules specific for either cell type have been identified in both somatic and visceral muscles (table 1). In somatic muscle formation fusion between the myoblasts are required, and any defects in the fusion process gives
obvious phenotypes (figure 12B). Importantly, fusion between the cell plasma membranes is dependent on the actin cytoskeleton (somatic muscle development is reviewed in [55]).

**Figure 8.** Somatic muscle development. A) Muscle precursors arise in clusters in the early embryo (grey areas in the embryo). 1) Each cluster consists of many fusion competent myoblasts (white cells) and a few founder cells (dark cells). 2) Several fusion competent myoblasts fuse with one founder cell, giving rise to multi-nucleated cells (big dark cells). 3) Several rounds of fusion take place and in the end stretched out muscle syncytia are formed. B) Somatic muscles of a late stage embryo. Phalloidin staining.

### 4.2. Actin

In eukaryotic cells one of the most abundant proteins is actin, which exists in two forms; either as globular monomeric actin (G-actin) or as filamentous actin (F-actin). Assembly and disassembly of actin filaments are rapid dynamic processeses required for many cellular functions such as morphogenesis, migration, cytokinesis, membrane transport and, as shown in the work presented in this thesis; fusion. Mechanistically the
The actin cytoskeletal system is believed to generate force, give structural support and act as tracks for protein transport within the cell [56].

4.3. The Arp2/3 complex

Actin polymerization is mediated by actin nucleators, which are proteins that interact with actin monomers and polymerize them into filaments. One actin nucleator is the Arp2/3 complex, it consists of seven protein subunits, including the actin binding components Arp2 and Arp3, and its activity is regulated by nucleation promoting factors like Wave (Scar in Drosophila) and Wiskott Aldrich Syndrome Protein (Wasp) [57]. Wave and Wasp both have a VCA-region at the C-terminal region, through which they bind to and activate the Arp2/3 complex. In turn, the activity of Wave and Wasp, which are structurally different to each other at their N-terminal domains, are regulated by other proteins (figure 9) [56]. A third Arp2/3 complex regulating protein of the same protein family exists; Wash (Wasp and Scar homolog), however little is known about Wash as it was recently identified [58] and it has not been investigated in this thesis work.

4.3.1 Mammalian Wave

Humans have three isoforms of Wave; Wave1, Wave2 and Wave3. Wave has a N-terminal domain through which it forms a complex with four other proteins; HSPC300, Abi, Nap1 (Kette in Drosophila) and Sra1, and all proteins in the complex are required for Wave function [59]. Signaling to Wave occurs through interactions with the complex; Rac1 binds to Sra1 and Nck to Nap1 [60]. In the central region Wave contains a basic
peptide which binds phospholipid PtdIns(3,4,5)P3 (PIP3) [61] and a proline rich domain which interacts with SH2-domain containing proteins like IRSp53 [62]. These multiple protein interactions regulate recruitment and binding of the VCA region to the Arp2/3 complex (figure 9). Studies in mammalian cell lines have shown that actin polymerization regulated by the Wave protein complex is important for the formation of plasma membrane protrusions and cell motility [63].

**Figure 9.**
Domains and binding partners of Wasp and Wave. Black arrow indicate direct interactions and grey arrows proteins that interact indirectly with Wave via proteins of the Wave-complex.

### 4.3.2. Mammalian Wasp and Wip

Two Wasp genes exist in humans; N-Wasp, expressed ubiquitously, and Wasp, expressed in hematopoietic cells. Wasp is a multidomain protein, containing a WH1-domain for interaction with Wip (discussed below), a basic peptide that interacts with phospholipid PtdIns(4,5)P2, a GTPase binding domain that can bind Cdc42, and a proline rich domain which interacts with SH3-domain containing proteins such as Nck2, Toca1 and
Abi (figure 9). In its inactive state Wasp activity is inhibited by intramolecular interactions which mask the VCA-region, consequently the multiple protein interactions are required for release of the inactive conformation, leading to increase in Wasp activity and affinity for the Arp2/3-complex [56].

Wasp interacting protein (Wip), member of the Verprolin family of proteins, is an important regulator of Wasp activity (Verprolin/Vrp1 in Drosophila). Wip has two distinct functional domains, an N-terminal actin-binding WH2-domain region and a C-terminal Wasp -binding domain, which are separated by a proline rich central core implicated in SH3-domain and Profilin binding [64]. Exactly how Wip functions remains unclear, Wip both inhibits Wasp activity in vitro by stabilizing the inactive conformation of Wasp, and stimulates actin assembly when binding to Wasp in cell line experiments, indicating that Wip may act both as an inhibitor and enhancer for Wasp activity [65, 66]. Furthermore, Wip binding to WASP protects WASP from degradation in vitro, showing that Wip stabilizes Wasp [67]. Actin polymerization mediated by the Wasp-Wip complex is important for actin dynamics at the plasma membrane in cell line experiments [68].

4.3.3. Drosophila Arp2/3, Scar, Wasp and Verprolin.

In the fruitfly the Scar-Wasp regulated actin polymerization is essential for myoblast fusion in somatic muscle development. Drosophila Scar (Wave in vertebrates) and Wasp are believed to function in similar fashions and form the same complexes as their mammalian homologs
(figure 9), however complicated cross-talk between the Scar and Wasp pathways are seen in vivo as components of the Scar-complex, such as Sra1, Kette (Nap1 in vertebrates) and Abi, also are involved in the regulation of Wasp activity [69-72].

*Drosophila* mutant embryos for Arp2/3, Scar, Wasp and Verprolin all show somatic mesoderm fusion phenotypes [72-77]. Moreover, mutants for the Scar-complex protein Kette [69, 77] and the Scar-complex activators Rac1, Rac2 and mtl (in particular the Rac1-Rac2-mtl triple mutant) [78, 79] also display somatic muscle fusion phenotypes. Many of the other signaling molecule mutants with somatic muscle phenotypes, interact genetically with the above mentioned proteins during myoblast fusion, indicating that intracellular signaling regulates the Scar-Wasp mediated actin polymerization [77], i.e. Mbc is an activator for Rac [80] and Blow signals upstream of Kette [77]. Other phenotypes observed in Scar-Wasp signaling network mutants are detected in the nervous system (Scar, Kette, Sra1, HSPC300 and Wasp mutants) [70, 81-84], in sensory organ development (Wasp and Abi mutants) [71, 84] and in endocytosis and trafficking of intracellular molecules (Wasp and Arp3 mutants) [85, 86], demonstrating the importance of the actin cytoskeleton in a variety of additional developmental processes.

In this thesis I have investigated the role of molecules belonging to the Scar-Wasp signaling network in both somatic and visceral mesoderm development, with particular focus on the visceral mesoderm.
Embryonic mRNA expression data from the BDGP database indicate that several subunits of the *Drosophila* Arp2/3 complex are expressed in the visceral mesoderm (subunits Arp3, Arpc1, Arpc2 and Arpc5), while no data is available Scar. Verprolin is expressed in both the visceral and somatic mesoderm [87]. Overall, this suggests that visceral mesoderm development should be dependent on actin polymerization mediated by the Scar-Wasp signaling proteins.

**Table 1.** Genes expressed in fonder cells (FC) and/or fusion competent myoblasts (FCM) of the somatic mesoderm (SM) and/or visceral mesoderm (VM) during myogenesis. Transcription factors are excluded.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cell type</th>
<th>Muscle type</th>
<th>Molecule description</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK</td>
<td>FC, FCM</td>
<td>VM</td>
<td>RTK</td>
<td>[45, 46]</td>
</tr>
<tr>
<td>Antisocial</td>
<td>FC</td>
<td>VM, SM</td>
<td>Multidomain cytoplasmic</td>
<td>[88]</td>
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<tr>
<td>Blown fuse</td>
<td>FCM</td>
<td>VM, SM</td>
<td>Cytoplasmic</td>
<td>[89]</td>
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<td>FC</td>
<td>VM, SM</td>
<td>GEF</td>
<td>[90]</td>
</tr>
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<td>FC, FCM</td>
<td>VM</td>
<td>TGFβ family ligand</td>
<td>[48]</td>
</tr>
<tr>
<td>D-Titin</td>
<td>FC, FCM</td>
<td>VM, SM</td>
<td>Multidomain cytoplasmic</td>
<td>[91]</td>
</tr>
<tr>
<td>Dumbfounded (Duf)</td>
<td>FC</td>
<td>VM, SM</td>
<td>Transmembrane adhesion</td>
<td>[54]</td>
</tr>
<tr>
<td>Hibris</td>
<td>FCM</td>
<td>VM, SM</td>
<td>Transmembrane adhesion</td>
<td>[92]</td>
</tr>
<tr>
<td>Jelly belly (Jeb)</td>
<td>FCM</td>
<td>SM, uptake VM</td>
<td>Secreted ALK ligand</td>
<td>[45, 46]</td>
</tr>
<tr>
<td>Loner</td>
<td>FC</td>
<td>SM</td>
<td>GEF</td>
<td>[93]</td>
</tr>
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<td>FC, FCM</td>
<td>VM, SM</td>
<td>Multidomain cytoplasmic</td>
<td>[94]</td>
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<td>VM, SM</td>
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<td>Transmembrane adhesion</td>
<td>[96]</td>
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<td>Sticks and stones (Sns)</td>
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<td>VM, SM</td>
<td>Transmembrane adhesion</td>
<td>[53]</td>
</tr>
<tr>
<td>Verprolin (Vrp1)</td>
<td>FCM</td>
<td>VM, SM</td>
<td>Multidomain cytoplasmic</td>
<td>[74, 75, 87]</td>
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Overall aims

During the course of my PhD project I have mainly worked with two projects in parallel. One project with the aim to investigating mammalian ALK function using the fruitfly as a model system, and the other project with the aim to characterizing molecules important for the fusion process in fly muscle development. The main body of this work has been published or will be published in the articles and manuscripts presented in this thesis. In the Results and discussion section I will also present and provide context to some additional results that have not been included in the publications.

Specific aims

- To express mammalian form of ALK in *Drosophila* and analyze signaling, function and activation mechanisms using the fly as a model organism.

- To investigate the function of actin polymerization regulating proteins in *Drosophila* muscle development, with particular focus on the role of Verprolin in visceral muscle development.
Results and discussion

Paper 1. The ligand Jelly Belly (Jeb) activates the *Drosophila* ALK RTK to drive PC12 cell differentiation, but is unable to activate the mouse ALK RTK.

This study aimed to examine ligand-receptor relationship between ALK and its ligand Jeb. The driving force behind this study was, and remains, the controversy surrounding the ligand for the vertebrate ALKs (as discussed above). Since the domain organization of ALK has been conserved from *C. elegans* to human, we asked whether mammalian ALK was able to recognize the *Drosophila* Jeb ligand. In vertebrates no Jeb-like molecule has been identified to date. If *Drosophila* Jeb was able to activate mouse ALK this would argue strongly for a Jeb-like ligand in vertebrates.

The results from this study showed that Drosophila Jeb is unable to recognize and activate mouse ALK, suggesting either of the two following scenarios. Firstly, either mouse ALK and any putative mouse Jeb have co-evolved to the extent that *Drosophila* Jeb cannot bind mALK. Alternatively, mALK has evolved such that it is no longer activated by Jeb-like molecules in vertebrates.

Jeb is a small LDL-domain containing protein, which has no obvious homolog in vertebrates. In vitro studies have shown that the LDL domain of Jeb is required for ALK binding [46], moreover the *C. elegans* ALK ligand (Hen-1) has an LDL domain [52] (figure 6), indicating that the LDL motif is likely to be responsible for ALK activation in lower organisms.
Searching for Jeb-like molecules in the vertebrate genomes is difficult, there are numerous LDL-domain containing genes, but because of the occurrence of co-evolution of ligands and their receptor binding regions, it is possible that the activating ligand for ALK may be a completely different molecule in higher organisms. Midkine and Pleiotrophin are two additional candidate ligands for vertebrate ALK, and these do not contain LDL motifs and show no homology to *Drosophila* Jeb, further indicating that the activating ligand for vertebrate ALK may no longer be a Jeb-like molecule. However, matters are complicated by the fact that both Midkine and Pleiotrophin have been identified to be ligands for multiple other receptors [97], thus making it possible that effects recognized as ALK activation may be mediated by other receptors.

**Summary of results paper 1:**

- Mouse ALK is unable to rescue the *Drosophila* ALK mutant phenotype when expressed in the visceral mesoderm of the fly embryo, indicating that the mouse ALK RTK is not active in the fly.
- Expression of a dominant negative form of mouse ALK in the somatic and visceral mesoderm in a wild type background does not block gut development, indicating that mouse ALK does not compete with *Drosophila* ALK in Jeb binding.
- In HeLa cells Jeb is unable to induce trans-phosphorylation of ectopically expressed mouse ALK nor activation of downstream ERK.
- PC12 cell based experiments clearly show that *Drosophila* ALK stimulated with recombinant Jeb protein induces neurite outgrowth, in contrast to mouse ALK stimulated with Jeb.
Paper 2. Appearance of the novel activating F1174S ALK mutation in neuroblastoma correlates with aggressive tumour progression and unresponsiveness to therapy

In this study a novel somatically acquired ALK gain-of-function mutation; ALK F1174S (figure 4A), was identified in a neuroblastoma patient, and the oncogenic properties of this mutation were then further characterized with molecular methods.

An unusual disease progression was observed in the patient examined here; initially DNA recovered from the tumor biopsy showed no ALK mutation and the patient responded to treatment, however this was followed by an abrupt and rapid disease progression accompanied by a loss of patient response to treatment. At this point the tumor DNA had acquired a homozygous ALK F1174S mutation. This is the first described case of an ALK mutation being gained during the course of neuroblastoma, which correlated with the disease exhibiting a more aggressive phenotype. This suggests that neuroblastomas with ALK mutations have worse prognosis than those without alterations in the ALK locus. Furthermore, this case study indicates that neuroblastoma tumors ought to be screened for aggressive mutations, not only at the initiation of treatment, but also at subsequent time points during treatment, as treatment should then be adapted according to genotype.

Important lessons were learned about the nature of the ALK F1174S mutation by the establishment of a Drosophila model for studying human oncogenic ALK mutations in vivo. Given our earlier results (Paper 1), which demonstrated the lack of a vertebrate ALK ligand in the
fruitfly, we could conclude that the ALK F1174S mutation indeed is a ligand independent gain-of-function mutation by expressing it in the *Drosophila* eye. This in vivo data was further reinforced by experiments in cell lines which characterized activation of downstream pathways by the ALK F1174S mutant. Our fly model provides a system to study ALK function in a very clean genetic background, as compared to in tumor derived cell lines with numerous additional immortalizing mutations complicating readouts. One useful application of the *Drosophila* model is the ability to access the potential of small molecule inhibitors in a controlled manner.

**Figure 10**: Demonstration of the use of the *Drosophila* eye as a simple readout for the activity of oncogenic mutations in human ALK. A) Expression of wild type human ALK in the eye causes no phenotype. B) Expression of human ALK F1174S in the eye gives a rough eye phenotype. C) Expression of human ALK R1275Q in the eye too gives a rough eye phenotype (unpublished) D) The ALK inhibitor TAE684 inhibits the ALK R1275Q induced rough eye phenotype when fed to the flies, illustrating that the model system can be used to test potential therapeutic drugs in vivo (unpublished).

Initial experiments show that ALK activity can be inhibited by the small molecular ALK inhibitor TAE684 in *Drosophila* (figure 10D), suggesting that such ALK selective inhibitors, which may be useful in neuroblastoma treatment, can be tested in our fly model system. Furthermore, early
results show that different gain of function mutants are differently sensitive to the TAE684 inhibitor. Thus, potential drug resistant ALK mutations can be studied in our fly model system. Moreover, this fly model will be used to investigate whether Midkine and Pleiotrophin can activate human ALK in vivo.

Summary of results paper 2:

- A young neuroblastoma patient was studied. Tumor samples were taken at two time points; first at diagnosis and secondly at eight months after diagnosis upon disease progression.
- SNP arrays detected a genomic aberration in the ALK gene region in the second tumor sample, but not in the first, indicating that an ALK mutation had arisen during the course of disease.
- DNA sequencing of the ALK gene in both samples, revealed a homozygous single nucleotide mutation in the kinase domain; 3521T>C, in the second tumor sample, but not in the first, indicating that a gain-of-function mutation (F1174S) had been acquired.
- A significant increase in proliferation could be detected by immunohistochemical methods in the second tumor sample as compared to the first, indicating that the tumor phenotype had become worse.
- Expression of human ALK F1174S in the eye of Drosophila generated a rough eye phenotype, suggestive of overproliferation. Given that expression of human wild type ALK does not result in any detectable rough eye phenotype, this result confirms that ALK F1174S indeed is constitutively active in a ligand independent manner in vivo.
- Downstream signaling activation by ALK F1174S was inhibited by TAE684 in mammalian cell line experiments.
**Paper 3. The neuroblastoma ALK(I1250T) mutation is a kinase-dead RTK in vitro and in vivo.**

In this study the model system developed in paper 2 was further utilized to study one particular ALK mutant; ALK I1250T. ALK I1250T was discovered in a neuroblastoma patient and because the I1250T mutation is mapped to a critical region of the kinase domain (figure 4A) it was predicted to be a gain-of-function oncogenic mutation [34].

![Figure 1](image)

**Figure 11.** Human ALK I1250T does not exhibit any activity in vivo. A) Wild type eye. B) Expression of ALK I1250T does not induce a rough eye phenotype, illustrating that it is inactive in vivo. C) Positive control: ALK F1174L is a gain of function mutation that induces rough eye phenotype when expressed in the eye due to constitutive ALK kinase activity.

Surprisingly, our results show that ALK I1250T is not a gain-of-function mutation, in fact it is instead the opposite; a kinase-dead mutant. Human ALK I1250T does not generate a rough eye phenotype when expressed in the fly eye (figure 11B). In agreement with this, ALK I1250T is unable to induce activation of downstream signaling molecules such as ERK in PC12 cells, even after stimulation with activating antibodies, illustrating that the ALK RTK is kinase dead.
When considering the ALK I1250T mutation in the context of the ALK kinase structure we can hypothesize the reason for its inactivity. All protein kinases have a N-lobe and a C-lobe, the lobes form a cleft that serves as a docking site for ATP and the catalytic activity of the kinase can only be mediated when the two lobes are in an accurate spatial position in relation to each other, so that catalytically important residues and ATP contact the substrate. In the C-lobe, such important residues are the DFG motif in the activating loop, in which D interacts with the ATP, and the HRD motif in the catalytic loop, in which D interacts with the substrate (figure 2). These two motifs also interact with each other. Moreover hydrophobic interactions play a significant role in protein kinase functionality because they stabilize the kinase. Hydrophobic residues which form such bonds belong to different parts of the molecule and they play a central structural role, binding together the different parts of the molecule and coordinating their motions. These hydrophobic bonds are referred to as the hydrophobic spine and only exist in active kinases [2].

In human ALK, residue 1250 is situated in the catalytic loop, directly after the HRD motif (position HRD + 1), and the I1250T mutation has been hypothesized to either alter substrate binding or to alter the spatial position of the HRD and DFG motifs towards an activated conformation [34]. Here we instead suggest that that this mutation alters the spatial position of the HRD and DFG motifs towards an inactive, unstable conformation. In wild type ALK, residue 1250 is an I, a hydrophobic residue, suggesting that it may form hydrophobic bonds that stabilize the kinase. We suggest a model in which ALK I1250
interacts with the hydrophobic residues I1233, F1315 and F1376 in order to anchor the HRD with the DFG motif and bring them into their active positions. Consequently, in the ALK I1250T mutant the polar residue T at the 1250 position makes it impossible for these bonds to form, resulting in that the HRD and DFG motifs are positioned incorrectly and the entire active site of ALK is destabilized, hence the kinase dead phenotype. Moreover, the relevance of a hydrophobic residue at the HRD + 1 position is further indicated by the presence of a non-polar amino acid at this position in several receptor tyrosine kinases.

**Summary of results paper 3:**

- Mouse ALK I1254T (the mouse homolog of human ALK I1250T) and human ALK I1250T are inactive in PC12 cells. This is exemplified by their inability to induce tyrosine autophosphorylation of the receptors themselves, or activation of downstream ERK either in the presence or absence of activating antibody, and by their incapability of inducing neurite outgrowth in this cell system. This shows that ALK I1250T is not a constitutively active gain-of-function ALK mutant, in contrast it is a kinase dead.
- Human ALK I1250T does not induce foci in NIH3T3 cells, in contrast to active ALK, further illustrating that this mutant is inactive.
- Expression of human ALK I1250T in the eye of the fly does not induce a rough eye phenotype, demonstrating that this mutant is not a gain-of-function mutation in vivo.
Paper 4. WASP and SCAR have distinct roles in activating the Arp2/3 complex during myoblast fusion

In this study different mutant alleles of the Wasp-Scar signaling network were examined in order to reveal the role of actin polymerization in somatic muscle development. Previous studies have shown that members of this protein network, such as Wasp [72], Verprolin (Vrp1) [74, 75] and Kette [77], indeed have crucial functions in somatic muscle fusion, and this study further investigated the specific roles of these proteins in myogenesis.

**Figure 12:** Somatic muscles of wild type embryo and Scar-Vrp1 mutant embryo. F-actin visualized by phalloidin staining. **A)** Wild type somatic muscles consist of fused and stretched out muscle syncytia. **B)** Scar-Wasp signaling network mutants (Scar-Vrp1 mutant in this figure) display somatic muscle fusion phenotypes.

Our results indicate that actin polymerization is required for proper somatic myoblast fusion, and we proposed a model for the mechanism employed when a fusion competent myoblast (FCM) fuses with a founder cell (FC) or a growing myotube. We speculated that the Duf rings observed at the attachment sites between fusing cells are important for this process. Duf and Sns first mediate attachment
between the FCM and the FC or growing myotube at the site of the Duf-ring, followed by recruitment of additional proteins, such as Blow and Vrp1. Vrp1 recruits Wasp [74, 75], and this presumably leads to an increase of F-actin at the site of fusion, which mechanistically contribute to expanding the fusion pore, and finally the FCM is pulled into the growing myotube. However, it is possible that Vrp1 does not always function together with Wasp, and in fact studies have indicated that Vrp1 is capable of influencing actin polymerization in a Wasp-independent manner in other systems [66]. In myoblast fusion Vrp1 may regulate the first fusion events together with Scar and the later fusion events together with Wasp, suggesting an advanced cross talk between the two nucleation promoting factor pathways.

Summary of results paper 4:

- Somatic muscle fusion phenotypes of different degrees are detected in Arp3, Wasp, Arp3-Wasp, Scar, Vrp1 and Scar-Vrp1 mutants, with strongest phenotypes observed in the double mutants, indicating involvement of different nucleation promoting factors in somatic myoblast fusion.
- Close examination of the number of nuclei in DA1 muscle precursors at embryonic stage 15 showed that more fusion events take place in the Arp3 mutant than in the Arp3-Wasp mutant, illustrating genetic interaction.
- Electron microscopy examinations indicated that fusion pores between fusing cells are formed normally in the Arp3 mutant, but in Wasp and Vrp1 mutants the membrane breakdown is not completed properly, indicating that the Wasp-Vrp1 complex is required for creation of fusion pores and Arp3 for integrating the FCMs into the growing myotube after fusion pore formation.
- Vrp1 is localized at the tip of the filopodia in migrating FCMs and at the cell-cell contact between fusing cells, indicating that the Wasp-Vrp1 complex increases the formation of F-actin at these sites.
- Duf is expressed at the cell-cell contacts between fusing cells in ring like structures in wild type as well as Arp3, Wasp and Arp3-Wasp mutants, indicating that the fusion defects observed are not due to defects in adherence.
Paper 5. Characterization of the role of Vrp1 in cell fusion during the development of visceral muscle of *Drosophila melanogaster*.

As a continuation of paper 4, this study investigated whether actin polymerization is important for visceral muscle development, as we have shown that it is for somatic muscle development, with particular focus on characterizing the role of Verprolin (Vrp1) in this process. This project started with a deficiency screen for identifying genes involved in visceral and somatic muscle development, and the Vrp1 mutant allele analyzed in this paper was first identified because of its somatic muscle phenotype, however because Vrp1 function in somatic muscle fusion was published by others [74, 75] I decided to focus on investigating the role of Vrp1 in visceral muscle development.

![Figure 13. Vrp1 mutant embryos display severe somatic muscle phenotypes, but embryonic gut development appears to proceed normally. A) Close up of the somatic muscles in Vrp1 mutant, unfused myoblasts are visible (arrow). B) A gut is visible in late stage Vrp1 mutant (arrow).](image)

Vrp1 is highly expressed in the visceral mesoderm at early embryonic stages, indicating a role in the development of this organ. Mutants in Vrp1 were identified and embryonic gut development was found to proceed normally in Vrp1 mutants. Similar results were observed with
other mutants of the Scar-Wasp signaling network, where a well formed gut was visible at late embryonic stages. This lack of organ disruption phenotype in the visceral mesoderm is not surprising, in fact all known somatic muscle fusion mutants (i.e. Rolling Pebbles [95], Antisocial [88], Roughest [96], Blown fuse [98], Lame duck [99], Loner [93] and Sns [100]) develop a gut that appears wild type, indicating that visceral mesoderm development does not require fusion. Founder cells (FCs) and fusion competent myoblasts (FCMs) of the embryonic visceral mesoderm line up, attach to each other, fuse and stretch out to form the gut, however it seems like the fusion event is not crucial, because as long as both cell types are present a gut will be formed despite block in fusion. In an ALK mutant, on the other hand, one cell type is missing resulting in a clear and early gut phenotype [45] (figure 7B). In contrast to ALK and Jeb mutants, this kind of striking phenotype is not observed in mutants in which both FCs and FCMs are specified and present. Consequently, a close examination of the visceral mesoderm is required in order to detect developmental phenotypes in this organ. Indeed, thorough examination of Vrp1 mutants reveals a visceral mesoderm phenotype; the Duf protein is mislocalized, and appears not to be downregulated properly, but is rather accumulated in foci in both the visceral and somatic mesoderm, indicating a developmental defect. This is likely to be a fusion phenotype, but with no impact on embryonic gut development. Duf accumulations are detected in Wasp, Arp3-Wasp and Kette mutants too, suggesting that this subtle phenotype is common for all Scar-Wasp signaling mutants.
It is surprising that a protein which is highly expressed in the visceral mesoderm does not have a more crucial role there, therefore we should not rule out that the detected Vrp1 phenotype may mean more than what we can comprehend at the moment. Possibly the observed Vrp1 mutant phenotype in the gut would have a more profound effect at the larval or adult stages. Rescuing UAS-constructs can with the available GAL4-drivers not be expressed in the somatic mesoderm without being expressed in the visceral mesoderm, therefore we are developing a fly system in which GAL80 will be specifically expressed in the visceral mesoderm using a Bap-promoter. This will allow rescue of the Vrp1 mutant phenotype in the somatic mesoderm alone and the Vrp1 gut mutant phenotype should be possible to study at later stages.

**Summary of results paper 5:**

- Vrp1 mRNA and protein expression is detected in somatic and visceral mesoderm, indicating Vrp1 function in both muscle types. Vrp1 protein is specifically expressed in the FCMs.
- A severe fusion phenotype is detected in the somatic mesoderm, but no strong visceral mesoderm phenotype is evident as the cells fuse and form a gut.
- Closer examination revealed that Duf is accumulated in foci in both the visceral and somatic mesoderm, indicating a fusion phenotype in both organs.
- Additional mutants of the Scar-Wasp signaling network display the same phenotypes, indicating that they all function in muscle development similarly, and that it is not due to functional redundancy between these proteins that the gut manage to develop, but rather that fusion is not essential for visceral mesoderm development.
- We show, using an ALK-Vrp1 double mutant strain, that FCMs of the visceral mesoderm can fuse with FCs of the somatic mesoderm, clearly illustrating that Vrp1 mutant cells are capable of fusing.
- Cell line experiments indicate that the Wasp-binding domain of Vrp1 is crucial for Vrp1 function, indicating that Vrp1 functions in a complex with Wasp in muscle development.
Conclusions

- *Drosophila* Jeb, activating ligand for *Drosophila* ALK, does not recognize mouse ALK, indicating that the activating ligand for the mammalian ALKs has changed extensively throughout evolution from fruitfly to vertebrate.

- *Drosophila* eye tissue can be used as a model system for studying oncogenic mammalian ALKs, because expression of constitutive active forms of ALK induce rough eye phenotypes, while inactive forms do not affect the eye morphology, providing an in vivo system with clean background.

- ALK F1175S is a novel gain of function ALK mutation associated with neuroblastoma.

- ALK I1250T, previously predicted to be a gain of function ALK mutation associated with neuroblastoma, is a kinase dead mutation which should not promote neuroblastoma development in the same aggressive manner as gain of function mutations.

- Several actin cytoskeletal interacting molecules of the Scar-Wasp signaling network, such as Scar, Wasp, Arp3 and Verprolin, interact genetically during somatic muscle fusion.

- Verprolin and several other proteins of the Scar-Wasp signaling network are not essential for visceral muscle development, although visceral mesoderm fusion is affected when those molecules are mutated, indicating that fusion is less important for embryonic visceral muscle development than for somatic muscle development.
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