Protein Tyrosine Kinases
and
The Regulation of Signalling and Adhesion in
Drosophila melanogaster

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To Chance,

and Life Itself...

(I det långa loppet träffar man bara det man siktat på. Så även om man missar sitt mål i första försöket så är det lika bra att sikta högt.)

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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens junction</td>
</tr>
<tr>
<td>AJC</td>
<td>Apical junctional complex</td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AR</td>
<td>Amphiregulin</td>
</tr>
<tr>
<td>BS</td>
<td>Bang sensitivity</td>
</tr>
<tr>
<td>Cas</td>
<td>Crk-associated substrate</td>
</tr>
<tr>
<td>CIN85</td>
<td>Cbl interacting protein of 85 kDa</td>
</tr>
<tr>
<td>Cbl</td>
<td>Casitas B-lineage lymphoma</td>
</tr>
<tr>
<td>CD2AP</td>
<td>CD2-associated protein</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CG</td>
<td>Computed gene</td>
</tr>
<tr>
<td>CMS</td>
<td>Cas ligand with multiple SH3 domains</td>
</tr>
<tr>
<td>Csk</td>
<td>c-Src tyrosine kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DD</td>
<td>Delayed discharge</td>
</tr>
<tr>
<td>Dlg</td>
<td>Discs large</td>
</tr>
<tr>
<td>DLM</td>
<td>Dorsal longitudinal muscle</td>
</tr>
<tr>
<td>DUB</td>
<td>Deubiquitinating enzyme</td>
</tr>
<tr>
<td>DVM</td>
<td>Dorsal ventral muscle</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ECS</td>
<td>Electroconvulsive shock</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethyl methanesulfonate</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>ERBB</td>
<td>Erythroblastic leukaemia viral oncogene homolog</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>F</td>
<td>Failure period</td>
</tr>
<tr>
<td>FA</td>
<td>Focal adhesion</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FAT</td>
<td>Focal adhesion targeting</td>
</tr>
<tr>
<td>FC</td>
<td>Founder cell</td>
</tr>
<tr>
<td>FCM</td>
<td>Fusion competent myoblast</td>
</tr>
<tr>
<td>FERM</td>
<td>Common domain of 4.1 protein, ezrin, radixin and moesin</td>
</tr>
<tr>
<td>FLP</td>
<td>Flippase, a recombinase from <em>S. cerevisiae</em></td>
</tr>
<tr>
<td>FRNK</td>
<td>FAK-related non-kinase</td>
</tr>
<tr>
<td>FRT</td>
<td>Flippase recombination target</td>
</tr>
<tr>
<td>ID</td>
<td>Initial discharge of spikes</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>JAM</td>
<td>Junction adhesion molecule</td>
</tr>
<tr>
<td>Jeb</td>
<td>Jelly belly</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GAL</td>
<td>Name from GALactose metabolism</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>GF</td>
<td>Giant fiber (pathway)</td>
</tr>
<tr>
<td>Grb</td>
<td>Growth factor receptor-bound proteins</td>
</tr>
<tr>
<td>HF</td>
<td>High frequency</td>
</tr>
<tr>
<td>Hrs</td>
<td>Hepatocyte growth factor receptor substrate</td>
</tr>
<tr>
<td>IB</td>
<td>Immunoblotting</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTK</td>
<td>Leukocyte tyrosine kinase</td>
</tr>
<tr>
<td>MAGI</td>
<td>Membrane-associated guanylyl kinase inverted protein</td>
</tr>
<tr>
<td>MAM</td>
<td>Common domain of meprins, A-5 protein and receptor PTP mu</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK and ERK kinase</td>
</tr>
<tr>
<td>METS1</td>
<td>Mesenchyme-to-epithelium transition protein with SH3 domains</td>
</tr>
<tr>
<td>MHC</td>
<td>(Muscle) Myosin heavy chain</td>
</tr>
<tr>
<td>MK</td>
<td>Midkine</td>
</tr>
<tr>
<td>MZB</td>
<td>Marginal zone B</td>
</tr>
<tr>
<td>NRPTK</td>
<td>Non-receptor protein tyrosine kinase</td>
</tr>
<tr>
<td>PAR</td>
<td>Partitioning defective proteins</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDZ</td>
<td>Common domain of PSD-95, Dlg and ZO-1s</td>
</tr>
<tr>
<td>PDMN</td>
<td>Posterior dorsal mesothoracic nerve</td>
</tr>
<tr>
<td>PEST</td>
<td>Proline, glutamic acid, serine, threonine</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PP</td>
<td>Protein phosphatase</td>
</tr>
<tr>
<td>PS</td>
<td>Position specific (integrins)</td>
</tr>
<tr>
<td>PSI</td>
<td>Peripherally synapsing interneuron</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PTN</td>
<td>Pleiotrophin</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>PYK2</td>
<td>Proline-rich tyrosine kinase</td>
</tr>
<tr>
<td>R</td>
<td>Response recovery</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>Ruk</td>
<td>Regulator of ubiquitous kinase</td>
</tr>
<tr>
<td>SETA</td>
<td>SH3 domain-containing gene expressed in tumourigenic astrocytes</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src-homology 3</td>
</tr>
<tr>
<td>SH3KBP1</td>
<td>SH3-domain kinase binding protein 1</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressors of cytokine signalling</td>
</tr>
<tr>
<td>c-SRC</td>
<td>Cellular Src</td>
</tr>
<tr>
<td>v-SRC</td>
<td>Viral Src</td>
</tr>
<tr>
<td>STAM</td>
<td>Signal transducing adaptor molecule</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>TKB</td>
<td>Tyrosine kinase binding</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TTM</td>
<td>Tergotrochanteral muscle</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activation sequence</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UBA</td>
<td>Ubiquitin associated</td>
</tr>
<tr>
<td>UIM</td>
<td>Ubiquitin interacting motif</td>
</tr>
<tr>
<td>UBD</td>
<td>Ubiquitin-binding domain</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VM</td>
<td>Visceral mesoderm</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula occludens</td>
</tr>
</tbody>
</table>
SUMMARY

In order to build a multi-cellular organism and to regulate cellular functions, cells need to communicate with each other, as well as tightly regulate their behaviour in response to environmental changes. For these purposes all eukaryotic cells express a large number of membrane spanning receptors that either themselves contain catalytic activity or via cytoplasmic effector enzymes, function to transmit “signals” from the cell exterior to induce appropriate responses within the cell. Protein tyrosine kinases (PTKs) are important signalling molecules, represented by the transmembrane receptor tyrosine kinases (RTKs) in addition to the cytoplasmic non-receptor PTKs, which alter cell behaviour by phosphorylating target proteins. An additional requirement for proper signalling and multicellular organisation is the adhesion between cells as well as adhesion of cells to the extracellular matrix (ECM).

Adhesion between cells and the ECM is mainly mediated by the integrin family of cell surface receptors, which functions as a structural link between the ECM and the actin cytoskeleton as well as important centres for signalling. Mammalian studies have implicated the cytoplasmic Focal Adhesion Kinase (FAK), as a major transmitter of signalling emanating from integrins, regulating cell migration, survival, proliferation and differentiation. In our studies of the sole FAK family member in Drosophila, Fak56, we have concluded that the deletion of Fak56 from the fruit fly genome causes no obvious defects in integrin-mediated adhesion, migration or signalling in vivo. Consequently, in contrast to the embryonic lethality observed in mouse knockouts, Fak56 mutant flies are both viable and fertile. However, we do find a clear genetic interaction between Fak56 and Drosophila integrins. Additionally, overexpression studies indeed indicate Fak56 as a negative regulator of integrin adhesion, given that excess Fak56 protein phenocopies loss of integrin function, causing phenotypes such as muscle detachment and wing blistering.

In Drosophila, as well as in mammals, FAK family proteins are highly abundant in the CNS and in our studies we have identified a requirement of Fak56 in synaptic transmission at neuromuscular junctions. Lack of Fak56 causes a weakening of action potential conduction, resulting in sensitivity to high-frequency mechanical and electrical stimulation, manifested by epileptic-like seizures and paralysis in Fak56 mutants, a phenotype known as Bang Sensitivity (BS) in flies. We also show that Fak56 phosphorylation is directly modulated in response to alterations in intracellular calcium levels, supporting a role for Fak56 in neurotransmission.

Fak56 is directly activated by the Drosophila Anaplastic Lymphoma Kinase, DALk, receptor which was identified in our lab. We characterised DALk as a novel RTK that is expressed in the embryonic CNS and mesoderm where it drives activation of the ERK/MAPK pathway. Indeed, we found DALk to ectopically induce protein tyrosine phosphorylation and specifically phosphorylation of ERK, resulting in autonomous cell transformation and uncontrolled tissue growth. Subsequently, we identified a requirement for DALk function during Drosophila embryogenesis, where it displays an essential role in gut development. Specifically, we identified the secreted molecule Jelly belly (Jeb) as a ligand for DALk and showed that Jeb-DALk interaction activates an ERK-mediated signalling pathway essential for visceral muscle specification and fusion, and consequently formation of the gut.

The potent ability of PTKs to regulate cell behaviour, together with the strong linkage between RTK dysregulation and tumour formation, renders the negative regulation of kinase activity an important area of research. We have identified the Drosophila homologue of Cbl-interacting protein of 85kDa, dCIN85, an adaptor molecule which in mammalian cells has shown involvement in RTK endocytosis and downregulation, as well as in the regulation of actin cytoskeleton dynamics. In the fruit fly, dCIN85 displays essential functions, given that dCIN85 loss of function mutants display a grand-child less phenotype. Generation of a dCIN85 antibody, together with isoform-specific transgenic flies, have allowed us to observe a punctuate localization pattern of the SH3-domain containing dCIN85 variants, representing Rab5-positive endosomal structures. This, in addition to the confirmation of a direct dCIN85-dCbl interaction, indicates an evolutionary conservation of dCIN85 function. Interestingly, dCIN85 co-localises with dRICH1, a Cdc42 specific RhoGAP, in differentiated photoreceptor cells in eye imaginal discs. This may imply a role for dCIN85 in the regulation of the specialised endocytic recycling processes required for the assembly/maintenance of tight junctions and establishment of cell polarity in epithelial tissues.
PAPERS INCLUDED IN THIS THESIS


PAPERS NOT INCLUDED IN THIS THESIS


**INTRODUCTION**

*Drosophila melanogaster* as a model system

**Historical perspective**

The goal for all biologists is naturally to increase the understanding of birth, life and death and gain insight as to how nature can be manipulated in order to treat illness and improve the quality of life. For many of us, the understanding of higher organisms such as ourselves is based on studies made in a variety of model systems and model organisms, with the belief that results gained in one system will likely be applicable to others. Almost 100 years ago the foundation of using the fruit fly, *Drosophila melanogaster* (Figure 1), as a model system, was laid down. This work was initiated in a lab at Columbia University, where the famous Thomas Hunt Morgan started to grow fruit flies and was the first (in 1910) to isolate a naturally occurring *Drosophila* mutation - *white* - the mutation which causes a red to white switch in eye colour in the fly. During the following years, Morgan and three of his students (A. H. Sturtevant, C. B. Bridges and H. J. Muller) studied fruit flies and managed to demonstrate that genes are carried on chromosomes, thus formulating the revolutionary chromosome theory of inheritance (Morgan et al., 1920; Sturtevant et al., 1919). By investigating multiple mutations, they further discovered “crossing over” and introduced the idea of genetic linkage, a finding that enabled Sturtevant to in 1913 construct the first genetic map. To facilitate the maintenance of lethal mutations, Muller introduced the use of balancer chromosomes in 1918, an invention for which Drosophilists have been truly grateful, ever since. The importance of the work made by Morgan and co-workers was recognised in 1933 when Morgan was awarded the Nobel Prize in medicine or physiology. During the following years many advances in *Drosophila* genetics were made, not least the discovery of polytene chromosomes and the publication of polytene maps (Bridges 1935), which significantly facilitated the mapping of mutations. In addition to studying naturally occurring mutations, X-rays were introduced as a method to induce new mutations in the *Drosophila* genome.

The employment of using the fruit fly to study the genetic control of early embryonic development was not launched until the 1980’s. At this time C. Nusslein-Volhard and E. Wieschaus performed the first genome-wide screen to identify genes involved in embryonic development, leading to the definition of the gap, pair-rule and segment polarity classes of genes (Nusslein-Volhard and Wieschaus, 1980). This effort was recognised and awarded the Nobel Prize in 1995, a prize that was shared with E. Lewis who dedicated his life to the analysis of the genetic basis for homeotic transformations (Lewis, 1978; Lewis, 1982; Lewis, 1985).

An important breakthrough for the manipulation of the *Drosophila* genome was made in 1981, when Alan Spradling and Gerry Rubin elegantly developed a method for making transgenic flies, using the transposable P-element as a genetic carrier (Rubin and Spradling, 1982; Rubin and Spradling, 1983; Spradling and Rubin, 1982). The following two decades saw a huge growth in the quantity of information, materials and tools being assembled, and especially the knowledge of P-elements became indispensable for developing genetic tools in order to manipulate the *Drosophila* genome. These methods include genomic rescue of mutant phenotypes, enhancer trap screens identifying genes based on their expression pattern,
large-scale insertional mutagenesis, site-specific recombination and a two-component system to induce controlled ectopic gene expression, the GAL4-UAS system.

In the year 2000, the entire Drosophila genome was sequenced and this impressive resource is now used to explore every aspect of Drosophila biology (Adams et al., 2000).

**Why Drosophila?**

It is an interesting question as to why certain model organisms become successful and commonly used. I think it is often a matter of chance in addition to the accomplishments of hard-working people. It all starts with a pioneer, in the case of Drosophila – Morgan - who initiates investigations in a certain species. As time goes by and information is gathered, knowledge results in yet more knowledge, new tools are developed and in the end this particular organism will be the system of choice to dig deeper into understanding biology. As a model system, Drosophila has not only the advantage of a long history and a wealth of knowledge, but is also favourable for practical reasons. Fruit flies are small, easy and cheap to keep in large numbers and have a short generation time, allowing results to be scored rapidly. During the last decade the resources of genetic information and techniques have exploded and the relevance of using Drosophila as a model system was greatly emphasised by the release of the Drosophila genomic sequence, revealing that more than 90 percent of the genes found in the fruit fly are similar to the genes found in mice or humans. However, compared with the three billion bases and 20,000-25,000 estimated genes, located on 23 chromosomes composing the human genome, the Drosophila genome, with its four chromosomes, 180 million bases and ~13,600 estimated genes, offers a much simpler system to study (Adams et al., 2000). Unquestionably there are fewer problems with redundancy in Drosophila, compared to mammals. In addition, the fruit fly not only resembles us humans in genetic aspects, but also in regards to complex behaviours such as memory, vision, sleep and addiction, among others. Therefore Drosophila can be valued as one of the most important tools we have today to increase the understanding of ourselves.

**Drosophila genetic tools and techniques**

From being a model in which to investigate inheritance and simple genetics, Drosophila has gained a role as one of the most important model organisms used in order to understand gene function and pathogenesis as well as for the development of new therapeutics. The field has literally exploded the past two decades and today the tools and techniques available are astonishing.

A breakthrough for Drosophila genetics came with the introduction of using the P element for transgenesis in 1982 by Rubin and Spradling (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Essentially, the P element is a mobile piece of (extragenomic) DNA which has the ability to transpose, namely insert and excise itself, within and between genomes. Transposition is mediated by the action of a transposase enzyme which specifically recognises sequences of perfect inverted repeats in the ends of the P. Naturally occurring P elements usually encode an internal transposase gene, while P elements utilised in the laboratory have been engineered to be transposase deficient, in order to enable inducible transposition in a controlled manner, by the addition of an exogenous transposase source (e.g. Δ2.3). To date,
the applications of P elements are numerous and include gene tagging, gene disruption, chromosome engineering and inducible gene expression (Ryder and Russell, 2003).

One of the main goals of a Drosophilist is to disrupt gene function and for this purpose P elements are excellent tools (Ryder and Russell, 2003; Venken and Bellen, 2005). If the investigator is lucky enough, the insertion of a P may directly disrupt the function of his/her favourite gene. If not, since P elements often have a preference for inserting in the 5’ UTR of genes, the simplest way to disrupt gene integrity is to induce imprecise excision of a P inserted in close proximity to your gene. Imprecise excision is the result of an insufficient double-stranded-break (DSB) repair of a P element excision, creating a deletion of variable size in the flanking DNA. In order to generate more precisely targeted deletions, the removal of genetic material between two P elements in trans can be induced, but this method is now often rejected in favour of the FLP/FRT system. The FLP /FRT technique is based on the S. cerevisiae recombinase gene Flippase (FLP), which act on recombination sequences referred to as FLP recombination target (FRT) sites (Beggs, 1978; Broach et al., 1982; Broach and Hicks, 1980). This system has been adapted for use with mobile genetic elements, such as P elements and piggyBacs, and has become a powerful means to generate precisely mapped deletions in the Drosophila genome by inducing recombination between two suitable FRT-containing P elements (Golic et al., 1997). This method has been further refined and is currently being elegantly used by the DrosDel consortium (Ryder et al., 2004) as well as the functional genomics company Exelixis (Parks et al., 2004), to create a battery of deficiencies covering the entire fly genome. All strains generated in these projects are now generously provided to the public, enabling everyone to make their desired deletions. Furthermore, the FLP/FRT system can also be used to produce genetic mosaics of marked loss of function, or gain of function, clones in an otherwise wild-type background (Theodosiou and Xu, 1998). The most recent advance in making targeted deletions in Drosophila is the increasing use of homologous recombination to induce designer deletions and mutations in the genome, a system which appears to function with relatively high efficiency (Gong and Golic, 2003). In addition to these techniques, mutations can also be introduced by chemical mutagens such as EMS, but the use of these is now being out-competed by advanced genetic tools.

Another revolution in the Drosophila field is the ingenious combination of yeast (S. cerevisiae) and Drosophila genetics resulting in the GAL4-UAS system, a technique to selectively induce gene expression in a temporally and spatially controlled manner (Brand and Perrimon, 1993; Phelps and Brand, 1998). This system has a bipartite approach based on a responder and a driver, where one transgenic fly contains a construct where your gene of interest is under control of the inducible upstream activating sequence (UAS) element. This sequence element can subsequently be induced by the transcriptional activator GAL4, carried by a second transgenic fly strain, which in turn is regulated by a cell- or tissue-specific enhancer (Brand and Perrimon, 1993). Since the introduction of the GAL4-UAS system, it has been expanded to virtually all cell types, including the maternal germline. Furthermore it has been combined with the inducible TetOn/TetOff systems in addition to the GAL4-mediated gene activation repressor GAL80, introducing the option of an additional sophisticated level of control to this technique (Duffy, 2002). The GAL4-UAS system can also be utilised in genomic based screens to identify and target specific enhancer regions as well as individual genes, e.g. enhancer trapping and gene trapping, respectively (Ryder and Russell, 2003).
Drosophila life cycle and development

The lifecycle of the Drosophila begins with the newly fertilised egg that within the first 24 hours after egg-laying completes embryogenesis, a process which was divided into 17 stages by Campos-Ortega in 1985 (Campos-Ortega, 1997). During the first two hours the egg is a syncytium, meaning that nuclei divide and migrate in a common cytoplasm. During this time the zygotic nuclei goes through 13 rapid, synchronised cell divisions and as they divide, they migrate out towards the egg surface where they are enclosed by plasma membrane to form the cellular blastoderm (stage 5) (Foe, 1989). After cellularisation, gastrulation is initiated by the invagination of the ventral furrow and results in the formation of the three germ layers. The invaginating cells of the ventral side form the mesoderm which later gives rise to internal structures such as the visceral musculature, fat body, dorsal vessel and the somatic musculature. Most of the cells in the outer layer turn into the ectoderm, which eventually will form the CNS, PNS, epidermis and trachea. The endoderm is formed by the simultaneous invagination of the anterior and posterior midgut primordia from the opposite poles of the embryo. These primordia subsequently migrate towards the middle of the embryo where they fuse to form the midgut. Taken together this layered, invaginated ventral area is referred to as the germ band. During gastrulation the germ band starts to elongate in a process known as germ band extension, a movement that pushes the posterior tip of the germ band upward and then towards the anterior region of the embryo. This process continues until stage 11 and is very important for the morphogenesis of the different germ layers and for the segmentation of the embryo (Campos-Ortega, 1997).

At stage 12-13, the germ band retracts again, giving the embryo its characteristic segmental appearance. At this point cells in most organ primordia also start to differentiate. Germ band retraction is accompanied by the formation of a hole in the dorsal surface of the embryo. This hole becomes immediately covered by the amnioserosa, but is subsequently covered by epidermal cells that during a process known as “dorsal closure” migrate towards the dorsal midline where they fuse to enclose the embryo (stage 15). During the last stages of embryogenesis, head involution is completed and the embryo acquires its final larval morphology.

24 hours after fertilisation, embryogenesis is completed and the Drosophila hatches into a first instar larva, for which the only purpose in life is to eat, grow and molt. During four days, the larva undergoes three larval stages, or “instars”, before it wanders off from the food to find a place to pupariate. During the subsequent 3-4 days of pupal development the fly undergoes a complete metamorphosis, where most of the larval tissues are degraded and the fly is rebuilt mainly from the progenitors laid down during larval stages in structures known as imaginal discs (Brody, 1999; Campos-Ortega, 1997; Grumbling and Strelets, 2006).

Development and attachment of Drosophila muscles

Muscle cells develop from the Drosophila mesoderm during blastoderm stages. The bHLH transcription factor dorsal is initially responsible for inducing the expression of twist and snail, two genes essential for the establishment of mesodermal fates and for mesoderm invagination (Ip et al., 1992; Jiang et al., 1991). Combinations of gene activities induce the formation of unique muscle founder cells (FCs), as well as fusion competent myoblasts (FCMs) which subsequently fuse to give rise to several different muscle types, including the muscles of the
gut (visceral muscles), the body wall muscles (somatic muscles), the heart and the fat body (Bate, 1990; Bate and Rushton, 1993).

Irrespective of muscle type, the morphogenesis of each muscle is a multi-step process involving myoblast specification and fusion, myotube guidance and targeting to specific attachment sites, eventually giving rise to a stereotyped muscle which can be identified by its specific size, shape and position. In the *Drosophila* embryo, the body wall musculature is organised into a stereotyped pattern consisting of 30 uniquely specified syncitial muscle fibers in each abdominal hemisegment (Bate, 1990). In parallel to muscle specification and fusion, tendon precursor cells are produced within the epidermis. Tendon cells are the epidermal attachment sites for *Drosophila* muscles, connecting the muscles to the exoskeleton of the embryo. After myoblast fusion the muscle precursors migrate and stretch out underneath the ectoderm, guided by cues produced by the tendon cell precursors, to the correct insertion sites. When the initial association has been established, bi-directional signalling between the muscle and the tendon cell drives the final differentiation of both cell types and triggers the cytoskeletal reorganisation that is required to withstand the force of muscle contraction and enable the animal to move (Schnorrer and Dickson, 2004; Volk, 1999).

At the *Drosophila* muscle attachment site, rather than interacting directly with each other, both the muscle cell and the tendon cell form hemiadherens junctions by binding to ECM ligands in the dense tendon matrix, consisting predominantly of tiggrin and laminin, which is accumulated in the space between the cells. Molecularly, these junctions are mediated by the *Drosophila* integrin family of adhesion receptors that function as a bridge between the ECM and the intracellular actin cytoskeleton (Volk, 1999).

**Myoblast fusion**

A central event during muscle development is the fusion of a variable number (roughly 3-25) of myoblasts to form multinucleate muscle fibers. During early stages of mesoderm development, muscle founder cells are specified in a process characterised by lateral inhibition, where the muscle progenitors are singled out by the expression of high levels of the transcription factor *lethal of scute* (*l'sc*) which subsequently, though Notch-mediated lateral inhibition, forces the surrounding cells into the default FCM fate (Figure 2) (Carmena et al., 1995). As a result of this process, each founder cell gains all the identity information that is required for muscle formation, whereas the FCMs mainly are believed to contribute to the growth of the muscle. Each muscle FC contains a unique gene expression profile characterised by a combinatorial expression of the so called muscle identity genes, including *Krüppel, slouch, apterous, ladybird, vestigial, nautilus* and *even-skipped*, among others (Schnorrer and Dickson, 2004).

A series of sequential events are required in order to complete fusion of FCs and FCMs. Following myoblast differentiation, FCs express immunoglobulin (Ig) domain transmembrane proteins, which serve as attractants and adhesion molecules to recruit the appropriate number of FCMs. When contact has been established, cells elongate and the membranes of FCs and FCMs align, forming prefusion complexes at the contact sites. These complexes, which in electron microscopy studies have shown to consist of roughly 50 electron-dense vesicles per contact, subsequently transform into electron-dense plaques where membrane breakdown ultimately occur, probably through the fusion of the electron-dense vesicles with the plasma membrane, turning two cells into one (Dworak and Sink, 2002; Paululat et al., 1999).
In order to correctly complete myoblast fusion, a large network of various molecules is required. Extracellular guidance cues and adhesion molecules are needed for recognition and contact establishment, signalling and cytoskeletal molecules are required to induce membrane fusion and regulate the cytoskeletal arrangements needed for fusion. Several screens aiming to molecularly understand fusion have revealed a fundamental asymmetry of genes expressed by the FCs versus the FCMs (Artero et al., 2003; Estrada et al., 2006). Below I will discuss a few of these molecules which are of importance to this thesis.

**Duf/Kirre:** Dumbfounded (Duf), also known as Kirre, is a cell adhesion molecule and a member of the immunoglobulin superfamily. Structurally Duf/Kirre is highly similar to the *Drosophila* protein Roughest (Rst) and is characterised by five extracellular immunoglobulin-like repeats, a single transmembrane domain and a cytoplasmic tail containing a PDZ motif. Duf/Kirre is predominantly expressed on founder cells of the somatic, pharyngeal and visceral muscles, where it functions as an attractant for fusion-competent myoblasts, an event that is essential for muscle fusion. Duf/Kirre mutant animals die at late embryonic stages and are characterised by a complete lack of fusion in both the somatic and visceral mesoderms, creating gaps in the VM (Ruiz-Gomez et al., 2000). Importantly, Duf/Kirre does not appear to affect founder cell specification, given that *duf/kirre* mutant muscles maintain the expression of typical founder cell markers (Ruiz-Gomez et al., 2000). Recent findings furthermore suggest that Duf/Kirre might act as an attractant for FCMs, given that ectopically expressed Duf/Kirre is capable of attracting myoblasts to the induced cells (Ruiz-Gomez et al., 2000). The finding that Duf/Kirre can be proteolytically cleaved in its extracellular domain suggests that it might act as a diffusible guidance cue for FCMs (Chen and Olson, 2001), but could also remain at the membrane and attract cells by stretching out filopodia and/or cytonemes.

**Sticks and stones (sns):** The cell adhesion molecule Sns is specifically expressed by FCMs, in a complementary expression pattern to Duf/Kirre. In *sns* mutants, muscle cell specification and differentiation is intact, but in spite of this, fusion does not occur, resulting in unfused myoblasts (Bour et al., 2000). Similar to Duf/Kirre, Sns belongs to the immunoglobulin superfamily and is composed of eight Ig-like repeats in addition to a single...
fibronectin domain, a transmembrane domain and a cytoplasmic tail that encloses potential PKC and CK-II target sites. In an S2-based binding assay, Sns has shown to interact with Duf/Kirre, a finding which together with the reported co-localisation of these proteins at contact sites, has led to the suggestion that the Sns-Duf/Kirre interaction is an important mediator of the recognition/adhesion between FCs and FCMs (Galletta et al., 2004).

**Org-1:** Org-1, short for optomotor-blind-related gene-1, is a member of the highly conserved family of T-box genes which are characterised by a unique DNA binding domain known as the T domain/T-box (Porsch et al., 1998). In general, T-box proteins function as transcriptional activators (and/or repressors) to regulate key events during animal development, across evolution, controlling among other things, cell fate specification, morphogenetic movements and formation of organs such as limbs, heart and eyes. The *Drosophila* *org-1* gene is most closely related to mammalian *TBX1*, which in humans is linked to the DiGeorge syndrome, manifested by cardiac defects, abnormal facies, thymic hypoplasia, cleft palate and hypocalcaemia (Porsch et al., 1998; Wilson et al., 1993). In the fly, *org-1* is highly expressed during embryogenesis and appear to function during the specification of the visceral mesoderm (Lee et al., 2003a). To date, no *org-1* mutants have been reported, but an essential function for this gene is suggested, given that ubiquitous knock-down of *org-1* using RNAi constructs results in pupal lethality (Porsch et al., 2005).

**Jelly belly:** The *Drosophila* *jelly belly* (*jeb*) gene, originally identified in a screen for Tinman-regulated molecules, encodes a secreted protein containing a single LDL receptor motif, most similar to mammalian Sco-spondin and enterokinase, found in the bovine genome (Weiss et al., 2001). The importance of *jeb* for muscle cell migration and differentiation was established by investigations made in *jeb* mutant embryos, which display a recessive lethal phenotype caused by a total loss of differentiated visceral mesoderm and consequently a functional gut. Given that Jeb normally is expressed in somatic muscle precursors, but is essential for development of the visceral mesoderm, it was suggested that Jeb is required for mediating intercellular signalling between these two cell types, essential for visceral mesoderm specification. Indeed, secreted Jeb molecules bind to and are later taken up by visceral mesodermal cells by a receptor-mediated endocytic mechanism (Weiss et al., 2001). During later stages of development, Jeb is interestingly expressed in a subset of embryonic neurons, mainly distributed along longitudinal axons (Weiss et al., 2001).

In addition to the above mentioned molecules, the following genes have also shown to be important in muscle fusion:

- **Irregular-chiasm-C/Roughest (irreC/rst):** A duf/kirre paralogue expressed in both FCs and FCMs, which participates in homotypic interactions and can compensate for loss of *duf/kirre* and rescue *duf/kirre*-mediated muscle defects (Strunkelnberg et al., 2001).
- **Hibris (hbs):** A Sns paralogue which exclusively is expressed in FCMs and interacts with Duf/Kirre. Displays redundant functions in somatic myoblast fusion (Dworak et al., 2001).
- **Rolling pebbles/Antisocial (rols/ants):** Cytoplasmic adaptor protein that binds to the cytoplasmic tail of Duf/Kirre in FCs, is essential for the recruitment of FCMs and thus myoblast fusion (Rau et al., 2001).
- **Myoblast city/Crk/Rac:** This machinery is believed to be responsible for the regulation of actin cytoskeleton dynamics associated with guidance and fusion. Mbc is an SH3-domain containing GEF molecule that - via interaction with Rols - is believed to recruit (and activate) the small GTPase Rac to sites of myoblast fusion (Dworak and Sink, 2002).
**Drosophila Neuromuscular Junctions (NMJs)**

To mediate its essential functions, muscles need to be instructed concerning how, when and where to contract and extend. Naturally, for this purpose, the nervous system exists. Nerves and muscles communicate via specialised centres of synapses called neuromuscular junctions (NMJs).

Simultaneously to *Drosophila* myogenesis, approximately 40 motor neurons send their axons to specifically innervate the 30 uniquely identifiable muscles present in each abdominal hemisegment. Motor axons exit the CNS via two major pathways, the Intersegmental Nerve (ISN) and the Segmental Nerve (SN). At specific branch points, subsets of motor axons defasciculate from the main nerve and steer into their determined target regions. In a process called target recognition, individual growth cones explore potential muscle targets in the region and choose their correct muscle fiber. Once the location of the synapse is determined, synapse assembly begins (Prokop, 1999). The pre- and postsynaptic membranes become enriched in molecular components necessary for synaptic transmission and differentiate/mature into the final synaptic apparatus. Although muscles and neurons develop as individual functional units and autonomously assemble presynaptic active zones and postsynaptic receptor domains, respectively, intercellular signalling is required for the maturation of a synapse. By late embryogenesis many of the anatomical features of the mature larval synapse have been laid down (Featherstone and Broadie, 2000; Prokop and Meinertzhagen, 2006).

The embryonic neuromuscular junctions undergo extensive changes in size and morphology during postembryonic development in order to adjust to the tremendous increase in size. These dynamic structural changes include elongation of branches, generation of new boutons and sprouting of new branches. At the third instar larval stage, individual NMJ boutons can be up to 5 µm in diameter (Figure 3), allowing subcellular resolution within both presynaptic and postsynaptic compartments (Prokop, 1999). In *Drosophila* the NMJs are glutamatergic and many of the proteins required for synaptic function, are conserved from mammalian species. Signal transmission at the synapses is mediated by neurotransmitters that are packaged in synaptic vesicles (SVs) at the nerve terminal. In response to influx of Ca\(^{2+}\), triggered at the arrival of an action potential, neurotransmitters are released in discrete quantal units, by exocytosis. Released neurotransmitters diffuse across the synaptic cleft and bind to postsynaptic glutamate receptors, triggering postsynaptic signalling, depolarisation and subsequently, muscle contraction (Featherstone and Broadie, 2000; Prokop, 1999).
**Cell adhesion**

Cell adhesion is a requirement for intercellular cohesion and communication and consequently for the mere existence of multicellular organisms. The biochemical entities mediating cell adhesion are multiprotein complexes comprising adhesion receptors, ECM molecules and adhesion plaque proteins, many of which are conserved through evolution, although it is clear that differences to meet different biological needs exist. While cell-cell adhesion mainly is mediated by members of the cadherins and immunoglobulin superfamily, cell-matrix adhesions are formed by integrin heterodimers, linking extracellular matrixes to the interior of the cells. Adhesion to a matrix directs cell shape and polarity, cytoplasmic organisation and cell motility, as well as proliferation and survival (Hynes and Zhao, 2000). Deregulation of adhesion may permit cancer cells to migrate into surrounding tissues during the development of malignant disease, or to survive and grow under normally inappropriate conditions.

**Cell-matrix adhesions in general and focal adhesions in particular**

At sites of close contact between the plasma membrane and the underlying ECM, integrins are co-localised together with signalling and cytoskeletal proteins, forming specialised structures known as focal complexes and focal adhesions (Petit and Thiery, 2000; van der Flier and Sonnenberg, 2001). These two can be distinguished by physical appearance and functionality, in the sense that focal complexes are small (∼1 µm²) adhesions found in membrane protrusions of spreading and migrating cells, whereas focal adhesions are larger (up to several µm²), elongated and more stable adhesions that are associated with the termini of actin stress fibers, mediating strong adhesion to the substrate. In addition, integrins are also involved in the formation of distinct adhesive structures such as fibrillar adhesions, podosomes and hemidesmosomes (Danen, 2006). Interestingly, there are differences in the constituents and the state of activation of the different focal adhesions, even within the same cell (van der Flier and Sonnenberg, 2001).

Upon integrin aggregation, focal adhesion components are assembled at the site of adhesion in a hierarchal fashion to anchor and stabilise the actin cytoskeleton and to participate in intracellular signalling and modulation of the integrin activation state. Importantly, cell-matrix adhesions are highly dynamic, a focal complex can rapidly (within a few seconds to a few minutes) mature into a focal adhesion (Zaidel-Bar et al., 2003) and correspondingly, focal adhesions can disassemble equally fast. The maturation of focal complexes into focal adhesions is mediated by Rho-dependent actin-myosin contraction and requires the Rho target p140mDia (Bershadsky et al., 2006).

A strict regulation of focal adhesion turnover is crucial for the reorganisation of adhesive contacts, for example during cell migration. For instance, at sites of wounding, enrichment of extracellular proteins such as trombospondin and tenascin promotes cell migration by inducing disassembly of stress fibers and focal adhesions (Huttenlocher 1995). In addition, focal adhesion disassembly can be induced by growth factors and requires the downregulation of Rho activity (Petit and Thiery, 2000).
**Extracellular matrix (ECM)**

The substance that fills the intercellular space in an organism is composed of a well organised, complex network of high molecular weight proteins such as laminins, collagens and fibronectin which is stabilised by the addition of various polysaccharides, all secreted and assembled by the surrounding cells, to form the ECM. ECM molecules are usually fibrillar in nature and provide a complex structural and functional network serving as a structural support for cells and in addition acts as a physical barrier to, or as selective filter for (e.g. glomerular basement membranes), soluble molecules. The integrity and composition of the ECM, which appears to be both tissue- and developmentally-specific, is vital for maintaining proper cellular functions in the mature organism as well as during development and tissue repair. The ECM moreover functions to sequester growth factors and probably plays a critical role in the differentiation and growth of a number of cell types (Miranti and Brugge, 2002). Furthermore, the ECM additionally exerts mechanical tension on cells, important for the regulation of cell adhesion complexes, cell shape, polarity and the manifestation of differentiated cell functions (Miranti and Brugge, 2002).

**Integrins**

Integrins are a widely expressed family of dimeric cell adhesion molecules which are formed by the non-covalent association of two type I transmembrane glycoproteins, the α- and the β-subunit. The main function of integrins is to mediate cell-matrix and in some cases cell-cell adhesion, but integrins also form signalling centres important for the regulation of cell migration, proliferation, differentiation and survival (Figure 4) (Danen, 2006). Integrins are evolutionary conserved, although their complexity and redundancy increase in higher organisms, with 24 α and nine β-subunits estimated in the human genome (Venter et al., 2001), compared to five α- and two β-subunits in *Drosophila* (Adams et al., 2000; Rubin et al., 2000). In mammals at least 24 different heterodimeric combinations, which are differentially expressed and show varying ligand specificity, have been identified. Some recognise the typical RGD motif displayed by fibronectin, vitronectin and some of the laminins, while others bind specifically to collagen or cell adhesion molecules such as ICAMs or VCAMs, present on target cells. Integrins are additionally utilised as receptors for a number of bacteria, parasites and viruses (van der Flier and Sonnenberg, 2001).

Structurally, integrins are characterised by a large extracellular domain, a single transmembrane domain and a, in general, quite small cytoplasmic tail, usually less than 75 amino acids in length. The extracellular ligand-binding site is commonly formed by a seven-repeat motif, folding into a seven-bladed propeller structure, referred to as the integrin globular head domain, which in some cases are accompanied by a MIDAS motif (Mg$^{2+}$-dependent adhesion motif) critical for ligand binding (van der Flier and Sonnenberg, 2001). Ligand binding is believed to cause a conformational change, unmasking the β-subunit cytoplasmic tail and thus facilitating the interaction with downstream cytoskeletal and signalling proteins. Since integrins do not contain any enzymatic activity or protein-interaction modules, all of the events regulated by integrins are presumably mediated by other proteins, recruited to the site of adhesion. To date a growing number, exceeding more than 50 proteins, have been shown to localise stably or transiently at focal adhesions, including SFKs, Abl, Syk/ZAP, Csk, Ras, Raf, Mek, Erk, PI3K, PKC, Jnk, Cbl, Pyk2, PKA, Etk, ck-2, LAR, PEST, Crk, Nck and Grb-2, among others (Miranti and Brugge, 2002; van der Flier and Sonnenberg, 2001).
The multitude of signalling pathways controlled by integrins participate in the regulation of many cellular responses. Firstly, integrins participate in the assembly of ECM and has the ability to mediate stable attachment as well as regulate dynamic adhesion during cell migration (Danen, 2006) (see below). Secondly, multiple steps of cell cycle progression require matrix attachment. Integrins are known to directly modulate the expression and/or activation of cyclin A and D as well as G1/S-induced CDKs, in addition to mediating the degradation of p27<sup>kip1</sup> and p21<sup>kip1</sup> (Miranti and Brugge, 2002). Moreover, integrins are also essential mediators of cell survival, preventing cells to undergo detachment-induced cell death (also known as anoikis). Mechanistically, integrins promote survival by boosting the PI3K/Akt, FAK and Ras/ERK signalling pathways and has additionally been shown to directly inhibit pro-apoptotic genes such as Bcl-2, Flip and IAPs (Miranti and Brugge, 2002).

Studies of mice deficient of various integrin subunits have clearly emphasised the importance of integrin function for mammalian development. Deletion of the ubiquitously expressed β1 subunit (which participates in the formation of at least 12 integrin heterodimers) causes peri-implantation lethality (Fassler and Meyer, 1995). Furthermore, tissue-specific targeting of integrins has revealed integrin functions in haematopoiesis, for haemostasis, during immune responses, for migration of several cell types, neural organisation, organ development, the formation and maintenance of the vasculature, integrity of skeletal and cardiac muscle, skin, bone and cartilage (Miranti and Brugge, 2002). In humans, different mutant integrin variants have been linked to diseases such as LAD (Leukocyte adhesion deficiency), the bleeding disorder Glanzmann’s thrombasthenia, the skin blistering disease PA-JEB (junctional epidermolysis bullosa associated with pyloric atresia) and mild forms of muscle dystrophy. In addition, many tumours show altered patterns of integrin expression which is believed to contribute to anchorage-independent growth and neovascularisation in tumours (van der Flier and Sonnenberg, 2001).

Figure 4. Integrin signalling. Integrins cluster at focal adhesions by binding to ligands in the extracellular matrix. At the cytoplasmic surface, integrins recruit a large number of cytoskeletal adaptors and signalling molecules, important for the regulation of cell migration, survival and proliferation, among other things. (Reprinted from Hynes, 2002, Cell, with permission from Elsevier.)
Integrins in *Drosophila*

The *Drosophila* integrin family consists of two β (βPS and βν [beta-nu]) and five α (αPS1-5) subunits (Adams et al., 2000). The denotation PS is historic since integrins were first identified as position-specific, rather than cell-specific, surface antigens (Wilcox et al., 1981). The majority of studies regarding integrin function in *Drosophila* have focused on integrins containing the βPS subunit. The reason for this is dual, firstly βPS is widely expressed and secondly, it is believed to form heterodimers with all five α subunits, even though to date an interaction with only αPS1-3 has been documented. βPS is regarded as the fly homolog of vertebrate β1 (47% identity) and is encoded by the *myospheroid* (*mys*) gene (Wright, 1960). Together with αPS1, encoded by the *multiple edematous wing* (*mew*) locus and αPS2, encoded by the gene *inflated* (*if*), βPS forms heterodimers specifically binding to laminin and RGD-containing ligands, respectively. Interestingly, αPS1βPS and αPS2βPS heterodimers are frequently expressed by opposing tissues separated by an intervening extracellular matrix, a phenomenon observed both in the *Drosophila* wing as well as at muscle attachment sites (Bogaert et al., 1987; Brower and Jaffe, 1989; Leptin et al., 1989; Wilcox et al., 1981). The αPS3-5 subunits are closely related and appear to originate from a more recent gene duplication event. In fact, αPS3 and αPS4 are adjacently located in the *Drosophila* genome and are suggested to be controlled from the same cis-regulatory element. αPS5 is yet to be characterised.

Almost all null mutations in PS integrin genes cause lethality at late embryonic or early larval stages. The lethality is commonly due to failure of integrin-mediated adhesion, resulting in phenotypes such as detachment of somatic body wall muscles, failure of dorsal closure and defective germband retraction (Bokel and Brown, 2002; Brown, 1994). Importantly, flies deficient of the *mys* gene develop in a relatively, though not completely, normal fashion during early embryogenesis. However, when the first muscular contractions occur at late embryonic stages, the somatic muscles detach from their sites of attachment, round up and become spheroidal, hence the name *myospheroid* (Wright, 1960). Clonal loss-of-function analysis of integrins in adult tissues has further identified roles for βPS in wing morphogenesis, organisation of the photoreceptors in the fly retina and formation of the indirect flight musculature (Zusman et al., 1993). Deletion of individual α subunits generally causes subsets of the phenotypes described for βPS with a certain level of redundancy, particularly between αPS1 and αPS3.

In addition to mediating adhesion, PS integrins have also shown to display essential roles in the development and physiology of the nervous system. βPS, together with αPS1 and αPS2 are highly expressed in larval NMJs where they play a role in regulating NMJ branching, bouton formation, synaptic architecture and targeting specificity (Beumer et al., 1999). Furthermore, the two αPS3 isoforms, encoded by the *scab/volado* locus, are highly expressed in mushroom body cells, where they appear to display essential functions in the physiology underlying memory (Connolly and Tully, 1998; Grotewiel et al., 1998; Rohrbough et al., 2000). Since αPS3 mutants display impaired olfactory memory, these integrins are proposed to act as dynamic regulators of synapse structure involved in the establishment of short-term memory (Connolly and Tully, 1998; Grotewiel et al., 1998).

The second *Drosophila* β-subunit, βν, is not an obvious orthologue of any of the vertebrate β subunits, but shares 33% identity with βPS. In the embryo, βν is exclusively expressed in the endoderm of the developing midgut, an expression that is maintained during larval and pupal...
stages (Devenport and Brown, 2004; Yee and Hynes, 1993). Targeted deletion of βν has recently shown it to be non-essential for viability and fertility in the fly and to date the only documented function for βν is a compensatory role for βPS in the midgut, since removal of βν enhances the phenotype of βPS mutations, in this tissue (Devenport and Brown, 2004). In βPS null mutants midgut migration is delayed, but in the absence of both β subunits migration is completely blocked (Devenport and Brown, 2004). βν is known to form heterodimers with αPS3 and, based on sequence similarity, is also likely to interact with αPS4 and αPS5.

**Integrin-associated proteins**

Due to the overwhelming number of proteins associated with integrins and cell-matrix adhesion, only a few, with importance to this thesis, will be described.

**Actin**: Actin is one of the most abundant proteins found in eukaryotic cells and can exist as either monomeric G-actin (globular) or as F-actin (filamentous actin). In response to external stimuli, ATP-bound actin monomers assemble, by a reversible endwise polymerisation driven by ATP-hydrolysis, into filaments in order to induce changes in cell morphology and motility. Actin filaments that do not arise from existing free barbed ends depend on the Arp2/3 complex and nucleation-promoting factors (NPFs), to induce de novo formation of new filaments. In the cytoplasm, a multiplicity of proteins interact directly or indirectly with actin to influence its dynamics or state, directing actin to form loose networks (cross-linking proteins), tight bundles (bundling proteins) or simply to anchor actin filaments to the plasma membrane (cytoskeletal linker proteins). In addition, there are actin capping proteins responsible for preventing further elongation of filaments and severing proteins which induce actin depolymerisation. Tight regulation of actin dynamics is essential for almost all processes, on a cellular as well as organismal level. It is important for the regulation of normal organogenesis, muscle contraction, migration of immunological cells and wound repair, and additionally plays an important role in the metastasis of tumours (reviewed by (Revenu et al., 2004; Welch and Mullins, 2002)).

**Src-family kinases (SFKs)**: v-Src is the founding member of the SFK family of cytoplasmic non-receptor protein tyrosine kinases. It was originally isolated as the transforming protein in cells infected by the Rous sarcoma virus (RSV) and was, in fact, the first oncogene to be defined (Hunter, 1980; Hunter and Sefton, 1980). The cellular counterpart of v-Src, c-Src, shares the same structural and functional characteristics as the accompanying family members Fyn, Yes, Blk, Hck, Lck, Lyn, Fgr and Yrk. The SFKs are composed of, from N- to C-terminus, a myristoylation sequence (anchoring Src at the plasma membrane) followed by a unique sequence, an SH2, an SH3, a kinase and a regulatory domain. In the resting cell, Src is kept in a closed inactive conformation which is mediated by an inhibitory phosphorylation on Tyr527, catalysed by the c-Src tyrosine kinase (Csk) (Figure 5). This conformation is mediated by the intramolecular interaction between phosphorylated Tyr527 and the
SH2 domain in Src, and is further strengthened by an additional intracellular binding between the SH3 domain and a proline-rich sequence. Importantly, Tyr527 is absent in v-Src, causing a constitutive activation of the protein. In response to stimuli such as cellular adhesion, among other things, Src is recruited to contact sites where it is released from its inactive conformation and gets fully activated by an autophosphorylation event (for review see (Boggon and Eck, 2004)). Activated Src subsequently phosphorylates target proteins such as FAK, p130Cas, paxillin and integrins, thus regulating adhesion, spreading and migration. Interestingly, studies in Src-/- fibroblasts have shown that while adhesion only requires the SH2 and SH3 domains, migration depends on Src kinase activity (Kaplan et al., 1995).

Aberrant regulation of Src activity is an underlying cause of many diseases, including cancer and bone resorption disorders. Indeed, active Src displays potent transforming activities, including the activation of signalling pathways driving proliferation, promoting cell migration and invasion by altering integrin function and FAK activity, regulating epithelial cell junctions (supporting EMT) as well as supporting cell survival (reviewed by (Frame, 2004; Playford and Schaller, 2004).

The *Drosophila* genome encodes two Src family kinases, *Src64* and *Src42A* (Adams et al., 2000). Clearly, the role of Src in regulating actin dynamics is preserved in the fly, given that Src64 has been shown to function during ring canal morphogenesis (Dodson et al., 1998) whereas Src42A is accumulated at cell-cell and cell-matrix sites of adhesion and is essential for dorsal closure (Takahashi et al., 2005). In the fly, further information on Src function has originated from *dCsk* mutants, in which elevated levels of Src activity causes phenotypes such as cellular transformation in imaginal discs, disruption of tissue architecture in the eye and has recently implied a role for Src64 in the actin dynamics associated with the formation of ring canals (O'Reilly et al., 2006; Pedraza et al., 2004; Read et al., 2004).

**p130Cas**: p130Cas, or Crk-associated substrate, is a large multidomain adapter protein, characterised by an SH3 domain, proline-rich regions and a substrate-binding (SD) domain (containing multiple phosphoacceptor tyrosines and serines in addition to a proline-rich region) which is followed by a C-terminal Src-binding domain. p130Cas functions as a scaffolding protein at sites of adhesion, to which it gets recruited as well as tyrosine phosphorylated by the FAK-Src complex. Interaction partners such as Crk, Nck, Grb2, PI3K, C3G and 14-3-3 reveal functions of p130Cas in the regulation of cellular adhesion, migration, apoptosis and transformation (Bouton et al., 2001). Mice lacking *p130Cas* die at embryonic day E11.5-12.5, showing systemic congestion and growth retardation. In agreement with a converging importance for a p130Cas/FAK/Src complex, p130Cas deficient cells display phenotypes similar to FAK-/- cells, including a rounded morphology, impaired stress fiber formation and defective cell motility (Honda et al., 1998). In *Drosophila* I have identified and characterised the *p130Cas* homologue (CG1212) and have generated deletion mutants, which are viable and fertile with no gross defects during development (CG, unpublished results).

**Talin**: Talin is a major actin-binding protein consisting of a small globular head domain and a large C-terminal rod domain, forming anti-parallel homodimers in the cell. The head domain comprises a classical FERM domain which is responsible for a direct interaction of talin with the cytoplasmic tail of integrin β-subunits. Formation of the integrin-talin complex plays a critical role in integrin activation, increasing integrin ligand-binding affinity (Campbell and Ginsberg, 2004). Interestingly, tyrosine phosphorylation of the talin-interacting NPxY motif in integrin tails by SFKs displaces talin binding and inhibits cell adhesion (Campbell and Ginsberg, 2004). In mice, talin knock-outs are lethal and talin-deficient ES cells fail to assemble cell-matrix adhesions (Danen, 2006). In *Drosophila*, loss of talin (encoded by *rhea*)
causes failure of germband retraction and massive muscle detachment in the embryo, as well as loss of adhesion in the adult wing (Brown et al., 2002).

**Paxillin:** Paxillin is a focal-adhesion associated protein which is tyrosine phosphorylated in v-Src transformed cells. It consists of multiple protein-protein interaction domains including five N-terminal LD (leucine-rich) motifs, several SH2 binding sites and proline-rich regions, in addition to four LIM domains in the C-terminus. The increasing number of proteins shown to interact with paxillin (including Src, Crk, Csk, FAK, vinculin, phosphatases etc), indicates that paxillin functions by bringing together various regulatory pathways that control the dynamics of cell-matrix adhesions and the organisation of the actin cytoskeleton (Brown and Turner, 2004). The mouse paxillin knockout displays embryonic lethality, indicating specific functions which are not compensated by the paralogs Hic-5 and leupaxin (Hagel et al., 2002). In *Drosophila*, the single paxillin homologue has been shown to co-localise with integrins at muscle attachment sites (Wheeler and Hynes, 2001; Yagi et al., 2001). Although no paxillin mutant has yet been reported, paxillin has shown to interact genetically with Rho and Rac (Chen et al., 2005), as well as physically with vinculin and p130Cas in a yeast-two-hybrid system (Stark et al., 2006), thus implicating a functional conservation of protein function.

**The PINCH-ILK-Parvin (PIP) complex:** A ternary complex consisting of integrin-linked kinase (ILK), the LIM-domain protein PINCH and the actin-binding protein parvin, has recently shown to be important for the regulation of ECM control and cell behaviour. ILK is a multidomain focal adhesion protein and Ser/Thr kinase, consisting of four ankyrin repeats, a central PH domain and a kinase domain. ILK is recruited to focal adhesions via its C-terminal kinase domain, which directly mediates interaction with β1-integrin cytoplasmic tails. In turn, via binding to the ILK N-terminal ankyrin repeats, PINCH is recruited to focal adhesions by constitutively forming a complex with ILK. Additionally, ILK also interacts with the actopaxin family of proteins, the parvins, which are actin-binding proteins that further reinforces the linkage to the cytoskeleton. Disruption of the PIP complex significantly impairs cell shape modulation, motility and ECM deposition and can also function to reduce proliferation and induce apoptosis in mammalian cells (Wu, 2004). In mice, loss of ILK causes embryonic lethality and in *Drosophila* and *C. elegans*, ILK deficiency results in cell-ECM attachment defects that can be attributed to disruption of integrin-actin linkage. Despite the identification of target proteins for ILK kinase activity (including integrins, Akt, GSK3β), the actual role of ILK catalytic activity is an area of controversy since in both *C. elegans* and *Drosophila*, kinase activity is dispensable for protein function (Mackinnon et al., 2002; Zervas et al., 2001). Importantly, the function of ILK appears to be dependent on an intact PIP complex.

**α-actinin:** The α-actinins are a family of actin cross-linking proteins that belong to the spectrin superfamily. In addition to cross-linking actin, anti-parallel α-actinin dimers have a large number of molecular partners and exhibit multiple important roles in the cell, linking various transmembrane receptors (including integrins, ICAMs, cadherins and syndecans among others) to the cytoskeleton (by associating with protein such as titin, zyxin, vinculin and α-catenin), thus regulating receptor activity and mediating cross-talk between diverse signalling pathways (Danen, 2006; Otey and Carpen, 2004). In *Drosophila*, loss of α-actinin protein causes muscle dysfunction and degeneration, progressively limiting mobility and eventually resulting in death during early larval stages (Fyrberg et al., 1998; Fyrberg et al., 1990).
**Vinculin:** Vinculin is one of the most abundant proteins at focal adhesions, where it has shown to interact directly with actin, talin, paxillin, VASP and vinexin (Petit and Thiery, 2000). Vinculin is composed of a large globular head domain and a rod-like tail which is connected by a proline-rich sequence. In the resting cell, vinculin is retained in an inactive conformation mediated by intramolecular interactions. Upon activation, this closed conformation is released when vinculin is recruited to the plasma membrane, where it interacts with PIP$_2$ (Danen, 2006). Vinculin knockout mice die during early embryonic stages due to heart and brain defects, and cultured vinculin -/- fibroblasts extracted from these mice display reduced adhesion and increased motility, indicating important roles for vinculin in the assembly of cell-matrix adhesions (Xu et al., 1998). In *Drosophila*, deletion of vinculin does not result in any obvious defects (Alatortsev et al., 1997).

**Tensin:** The vertebrate tensin protein family (consisting of *tensin 1, 2, 3* and *cten*) encodes multi-domain, cytoplasmic phosphoproteins that rapidly translocate to focal adhesions in response to integrin engagement. Structurally, tensins are characterised by an SH2 and a PTB domain, in addition to a PTP domain which has been demonstrated to directly bind to the cytoplasmic tail of integrin β-subunits (Lo, 2004). Tensin is phosphorylated in response to cell adhesion and growth factor stimulation and has shown to display actin cross-linking activities and to coordinate signalling with cytoskeletal changes. *In vivo* studies in knockout mice have highlighted roles for tensin during the development of bones, lungs and the intestine, as well as for the normal physiology of the kidney (Danen, 2006). The *Drosophila* tensin orthologue, encoded by the *blistery* locus, lacks the PTP domain and does not appear to be essential for development or life in the fly. Rather, the *Drosophila* tensin seems to be required for the stabilisation of adhesion in the wing, as visualised by a wing blister phenotype in null mutants (Lee et al., 2003b; Torgler et al., 2004).

**Tiggrin:** Tiggrin is a large glycosylated ECM molecule in *Drosophila* that lacks counterparts in vertebrate systems. Importantly, it contains a C-terminal RGD cell attachment motif which has been shown to mediate cell spreading by binding to αPS2βPS integrins (Fogerty et al., 1994). In the *Drosophila* embryo and larvae, tiggrin is accumulated in basement membranes and at muscle attachment sites where it contributes to the strength of integrin-mediated adhesion. Loss of tiggrin in the fly results in pupal lethality, although intriguingly a few adults emerge with a relatively normal appearance. Reported phenotypes caused by loss of tiggrin include muscle detachment as well as defects in muscle and wing morphology (Bunch et al., 1998).

Many of the integrin-associated proteins described above are targets for proteolytic cleavage by calpains and caspases, which is a major mechanism regulating the turn-over, or disassembly, of focal adhesions (Schlaepfer and Mitra, 2004).

**Cell-Cell Adhesion – The AJC complex**

Equally important as cell adhesion to extracellular membranes and matrixes, is the adherence of cells to each other. In particular, epithelial cell layers which protect multicellular organisms from the exterior surroundings, are dependent on the membrane integrity provided by intercellular junctions such as adherens junctions (AJ) and tight junctions (TJ), together forming the apical junctional complex (AJC). In parallel to the barrier function mediated by AJC complexes, another group of junctions, the gap junctions, provide a means for cells to communicate (Dejana, 2004).
**Tight junctions (TJs):** TJs form a continuous, circumferential belt around the apical (luminal) end of epithelial cells, building a permeability barrier to the outer environment. Similar to focal adhesions, the core structure of a tight junction is based on membrane-spanning receptors that provide a structural link between the intracellular cytoskeleton and in this case, a neighbouring cell. At the TJs, this adhesion is mediated by the tetraspanning membrane proteins occludins and claudins, in addition to the junction adhesion molecules (JAMs), which are members of the immunoglobulin superfamily (Schneeberger and Lynch, 2004). Adhesion, mediated by these TJ proteins, is established via homophilic interactions in the extracellular space, forming a pericellular zipper-like structure along the cell borders. Despite the lack of conserved protein-protein interaction domains, the cytoplasmic tails of claudins, occludins and JAMs contain multiple Ser/Thr/Tyr residues which can be targeted for phosphorylation by PKCs and SFKs, and/or a variable number of PDZ-interacting motifs. These motifs consequently recruit a large number of cytoplasmic proteins that are believed to be of importance for the regulation of cell proliferation, polarity, differentiation and survival. One group of molecules that clusters on the cytoplasmic face of TJ, are the TJ plaque proteins which commonly comprise one or several PDZ domain(s). This group includes the zonula occludens proteins ZO-1, -2 and -3, the membrane-associated guanylyl kinase inverted proteins (MAGIs) and the partitioning defective proteins (PAR) 3 and 6, which function as scaffolding proteins that cluster adhesion receptors and link them to the actin cytoskeleton, thus mediating stability, dynamic regulation of junction opening and closure, as well as cell shape and polarity (Dejana, 2004; Schneeberger and Lynch, 2004). The assembly of TJs is dependent on a preceding calcium-dependent cadherin-mediated cell-cell contact as well as three evolutionary conserved polarity complexes, including the PAR-3/aPKC/PAR-6, Crumb3/PALS1/PATJ1 and Scrib/mDlg/mLgl complexes, and requires protein phosphorylation (Schneeberger and Lynch, 2004). TJ assembly is accordingly negatively regulated by PPA2, which dephosphorylates claudins, occludins, ZO-1 and aPKC (Nunbhakdi-Craig et al., 2002). Furthermore, TJs contribute to establish the plasma membrane compartmentalisation and the asymmetry of differentially distributed integral membrane proteins and lipids that is required for cell polarity (Schneeberger and Lynch, 2004).

**Adherens junctions (AJs):** The molecules that organise AJs are different from those found in TJs, although many features are shared. The adhesive molecules at AJs are members of the cadherin family, cell-type specific calcium-dependent adhesion proteins that mediate cell-cell contact via homophilic interactions. In the cytoplasmic tail, cadherins contain motifs that mediate interaction with, among other proteins, the catenins, linking cadherins to the actin cytoskeleton (Dejana, 2004). β-catenin can additionally function as a transcription factor for cell-cycle proteins such as cyclin D and myc. By binding and retaining β-catenin at the plasma membrane, cadherins are suggested to be important regulators of contact-induced growth inhibition. This is in agreement with the decreased level of cadherin (VE- and E-cadherin, not including N-cadherin) expression which often is associated with EMT and tumour invasion (Larue and Bellacosa, 2005). Furthermore, AJ cadherins recruit and activate PI3K, thus initiating signalling that protects cells from apoptosis (Pece et al., 1999).

During recent years the view of TJs and AJs as pure structural elements regulating cell-cell adhesion and mediating barrier functions, have evolved into a picture of AJCs as dynamic entities, which constantly are modified to meet the requirements of a cell. The AJCs themselves are constitutively being remodelled by processes involving endocytic recycling and delivery of critical components to the microdomains in the plasma membrane where AJCs are formed. Critical components of the endocytic machinery regulating AJCs include Rab13,
vesicle associated protein 33 (VAP-33) and the Sec6/8 multiprotein complex (Ivanov et al., 2005). It is tempting to speculate that specialised vesicles are delivering AJC components, directed by guidance cues provided by polarity proteins and that this process is required for the maintenance of cell polarity.

Cell Signalling

In order to build a multi-cellular organism and to regulate cellular functions, cells need to communicate with each other and be able to respond to changes in the environment. For this purpose all eukaryotic cells express a large number of membrane spanning receptors which function by transmitting “signals” from the cell exterior to induce the appropriate responses within the cell. These receptors are the origins of a complex network of signalling pathways that collaborate and in many cases converge, to properly regulate processes such as cell survival, proliferation, differentiation and cytoskeletal architecture. The molecular basis for a signalling pathway is to, in a hierarchal fashion, bring proteins together, modulate their enzymatic or scaffolding activity and target them to the correct subcellular location at the accurate time point. Signalling proteins are often built in a modular fashion, containing different protein-protein or protein-lipid interaction motifs/domains that in different combinations bring specificity to the pathway. In many cases signals are transmitted by posttranslational modifications such as phosphorylation, ubiquitination and acetylation that can be rapidly added to different amino acids within a polypeptide chain, resulting in changes concerning interaction properties, enzymatic activity and subcellular localisation.

Protein Kinases and Phosphorylation

The most common way, or at least the to date most studied way, of protein modification is the addition of a phosphate group to either a Serine (Ser, S), Threonine (Thr, T) or Tyrosine (Tyr, Y) residue within a polypeptide chain. Phosphorylation, which was first described by Burnett and Kennedy in 1954 (Burnett and Kennedy, 1954), is mediated by protein kinases that function by catalysing the transfer of the gamma phosphate of ATP to the hydroxyl group of the above mentioned amino acids in the target proteins (Pawson, 1994). Kinases are in general specific for either Ser/Thr or Tyr phosphorylation, although some kinases display dual specificity. Of the 518 kinases encoded by the human genome, 90 proteins belong to the protein tyrosine kinase (PTK) family (Manning et al., 2002). The PTKs can further be subdivided into two major classes, the receptor tyrosine kinases (RTKs) (58) and the non-receptor protein tyrosine kinases (NRPTKs) (32) (Manning, G, 2002). The RTKs are single transmembrane proteins consisting of an extracellular ligand-binding domain that in response to stimulation mediates receptor dimerisation, allowing autophosphorylation and activation of the intracellular kinase domain, subsequently inducing downstream signalling events (Hubbard, 1999). Non-receptor PTKs are generally maintained in an inactive state in the cytoplasm of the cell and can be activated by modifying enzymes or protein-protein interactions, which allow the active conformation to be assumed.

The highly conserved structure of PTKs is characterised by a small N-terminal lobe and a larger C-terminal lobe that are connected by a hinge region that contributes to the structural flexibility necessary for catalytic function. Positioned between the lobes is the catalytic cleft which accommodates the ATP- and substrate-binding domain. A high degree of conservation
can be observed in the residues regulating catalysis or correct architecture, like the P-loop (glycine-rich or nucleotide-binding loop) and the catalytic loop, while other regions such as the A-loop is more variable, thus contributing to substrate specificity (Cowan-Jacob, 2006). In most cases the addition of a phosphate group mediates activation of target proteins, but in other cases phosphorylation functions to negatively regulate protein function, which nicely can be exemplified by the inhibitory phosphorylation of Tyr527 in Src, which induces a closed, inactive protein conformation (Boggon and Eck, 2004).

To counteract the activity of kinases and coordinate signalling outcome, the human genome also encodes protein phosphatases that dephosphorylate target proteins via direct hydrolysis of the phosphate group, often resulting in the down-regulation of signalling. In fact, kinases and phosphatases can be viewed as obligatory partners, controlling the amplitude and the rate/duration of a signalling response, respectively (Tonks, 2006).

In the Drosophila genome, only 32 genes (among a total of 236 kinases) are predicted to encode PTKs. Among these, 21 appear to be transmembrane RTKs while 11 encode cytoplasmic non-receptor PTKs (Manning et al., 2002).

Focal Adhesion Kinase

Focal Adhesion Kinase (pp125\textsuperscript{FAK} or FAK) is a cytoplasmic, non-membrane associated PTK and the founder member of the FAK family of PTKs, which also includes Pyk2 (Figure 6). FAK was first described in 1992 (Hanks et al., 1992; Schaller et al., 1992) as a novel PTK of 125kDa that is associated with focal adhesions where it becomes phosphorylated on tyrosines in response to cell attachment to fibronectin. Initially, FAK was identified as one of the target proteins of pp60\textsuperscript{v-src}, showing increased tyrosine phosphorylation in transformed chicken embryo cells (Schaller et al. 1992). Soon afterwards FAK was also identified in rat (Hanks et al., 1992; Schaller et al., 1992). FAK is expressed in most tissues and cell types, is predominantly localised to sites of adhesion and is highly phosphorylated throughout embryogenesis.

![Focal Adhesion Kinase structure and function](image)

**Figure 6.** Focal Adhesion Kinase structure and function. FAK family kinases are composed of a FERM domain, a kinase domain and a FAT domain. Identified interaction partners and residues targeted by post-translational modifications are indicated. (Reprinted from Mitra et al. 2005, Nat Rev Mol Cell Biol, with permission from Nature Publishing Group.)
Structural features of FAK

The FAK family of PTKs appears to be structurally highly conserved through evolution. It is present in higher mammals as well as in lower eukaryotic organisms such as Drosophila, C. elegans and zebra fish. In all species examined, FAK contains a centrally located kinase domain which is flanked by large non-catalytic regions containing multiple sites involved in protein-protein interactions.

The N-terminal FERM domain

In the N-terminal region, FAK contains a domain that shares sequence homology with band 4.1/FERM domains. FERM domains are structurally conserved regions of around 300 amino acids that can be found in many cytoskeletal proteins such as talin and the ezrin, radixin, moesin (ERM) proteins, as well as in signalling proteins including the JAK family of tyrosine kinases and multiple PTPs (Girault et al., 1999b). In general, FERM domains have been shown to mediate either intermolecular interactions with partner proteins and phospholipids at the plasma membrane, often providing a link between transmembrane glycoproteins and the actin cytoskeleton, or to participate in intramolecular regulatory interactions. Among the PTKs encoded by the human genome, FERM domains are highly uncommon and can in fact only be found within the FAK and Janus family of kinases. In Janus kinases, the FERM domain has been shown to mediate a direct interaction with the cytoplasmic tail of cytokine receptors (Hilkens et al., 2001). In vertebrate FAK, the FERM domain has been suggested to interact with several target proteins including the cytoplasmic tail of β-integrins (only shown in vitro), the PH domain of the Tec-family kinase Etk/Bmx and the FERM domain of ezrin (Chen et al., 2001; Poulet et al., 2001; Schaller et al., 1995), implying a role of the FAK FERM domain in the regulation of cell adhesion and motility. Indeed, an intact FERM domain is required for the association of FAK with activated growth factor receptors, in addition to the promotion of cell migration in response to growth factor induced signalling (Sieg et al., 2000).

Recently the crystal structure of the FERM domain in avian FAK was solved, revealing that the overall tri-lobed architecture of ERM family FERM domains is preserved also in FAK (Ceccarelli DF, 2006). However, the FAK FERM domain displays a unique orientation of the F3 lobe that disrupts any ability of FAK to interact with phosphoinositides. Furthermore, these structural studies additionally provided evidence for a potential protein interaction site in the F3 lobe. Interestingly, the corresponding site in talin and radixin FERM domains has been shown to bind the cytoplasmic tails of β integrins and ICAM-2, respectively. This site is speculated to either mediate interaction with target proteins, or participate in intramolecular inhibitory interactions, possibly with the two linker regions on either side of the FAK kinase domain (Ceccarelli DF, 2006).

Interestingly, deletion of the N-terminal region of FAK has in several studies shown to elevate FAK catalytic activity in vitro, resulting in increased tyrosine phosphorylation of FAK in addition to enhanced phosphorylation of downstream targets such as paxillin (Cooper et al., 2003). In agreement, a direct interaction between the FAK FERM domain and the catalytic domain has been observed, thus further reinforcing the presence of an intramolecular inhibitory function of the FAK FERM domain (Cooper et al., 2003). Contradictory to these findings, another study has exposed a requirement for certain residues in the FERM domain for efficient FAK activation and downstream signalling (Dunty et al., 2004). The exact
function of the FAK FERM domain is consequently still an area of controversy that needs to be further studied.

**The catalytic kinase domain**

Structural studies of a large number of both RTKs and NRPTKs have revealed a highly conserved overall architecture of kinase domains (Hubbard and Till, 2000). The FAK kinase domain, spanning from residue 411 to 686 in the human FAK sequence, displays the common bi-lobar organisation of kinase domains and is characterised by an open conformation where the αC helix is rotated away from the C-terminal lobe and the activation loop is disordered (Nowakowski et al., 2002). This is in agreement with a lack of phosphorylation in the solved structure. Remarkably, cysteins 456 and 459 in the N-terminal lobe of the FAK kinase domain have been found to be involved in the formation of an intramolecular disulphide bond, an event rarely observed in kinase domains. The function of this bond is not fully understood but is implicated in the fine-tuning of the orientation of the αC helix (Nowakowski et al., 2002).

In response to a broad range of stimuli, including integrin-mediated adhesion, two FAK molecules are thought to transiently dimerise to enable the in trans transfer of the γ phosphatase of ATP to the autophosphorylation tyrosine Y397 by the kinase domains. The subsequent recruitment of SFKs and the phosphorylation of Y576 and Y577 in the FAK kinase domain activation loop facilitate the full activation of FAK and induction of downstream signalling (Mitra et al., 2005).

**The Focal Adhesion Targeting (FAT) domain**

In the very C-terminus, FAK contains a conserved domain, spanning over residues 921-1046, which has shown to be necessary, as well as sufficient, for the targeting of FAK to focal adhesions. This domain was identified already in 1993, is termed the FAT domain (short for focal adhesion targeting domain) (Hildebrand et al., 1993) and contains binding sites for the cytoskeletal proteins paxillin and talin, which both appear to be important for the recruitment of FAK to focal adhesions.

Recently, structural studies utilising both NMR and X-ray crystallography have shown the FAK FAT domain to be organised into a highly compact, symmetrical four-helix bundle that is held together by an evolutionary conserved hydrophobic core (Gao et al., 2004; Hayashi et al., 2002). This is interesting in light of the discovery of similarly organised domains in a number of cell adhesion molecules (e.g. vinculin, p130Cas and α-catenin). The integrity of this bundle structure was further shown to be required for the interaction of FAK with two specific Leucine-rich “LD”-motifs in paxillin. In contrast, the previously reported interaction of the FAK FAT domain with talin (Chen et al., 1995) only requires the 41 most C-terminal residues of the domain, indicating the presence of two independent routes for recruitment of FAK to focal adhesions (Hayashi et al., 2002).
**Additional conserved motifs in FAK**

The region between the central kinase domain and the C-terminal FAT domain in FAK is dominated by two conserved proline-rich regions (Schaller et al., 1992). These regions serve as binding sites for SH3 domain-containing proteins such as the adhesion molecule p130Cas (Harte et al., 1996; O'Neill et al., 2000; Polte and Hanks, 1997) and two regulators of small GTPases, GRAF and ASAP1, which are GAPs for Rho and Arfs 1 and 6, respectively (Hildebrand et al., 1996; Liu et al., 2002). In light of the identification of these binding partners, the proline-rich regions of FAK appear to be important for FAK-mediated regulation of the actin cytoskeleton.

In addition to the above described protein modules, FAK contains a minimum of six conserved phospho-acceptor tyrosine residues. The most well characterised tyrosine is the autophosphorylation site Y397 (Calalb et al., 1995), which in its phosphorylated state functions as a docking site for the SH2 domains of SFKs, the p85 subunit of PI3K, Phospholipase C-γ and Grb7 (Schlaepfer et al., 1999). Furthermore, Y576 and Y577, located in the kinase domain activation loop, are phosphorylation targets of SFKs and essential for maximal activation of FAK kinase activity and downstream signalling (Owen et al., 1999). In the C-terminal region, situated on the first turn of helix α1 in the FAT domain (Hayashi et al., 2002), Y925 has been shown to function as a binding site for the SH2 domain of the adaptor protein Grb2-SOS complex, thus providing a link to the Ras/MAPK pathway (Schlaepfer DD, 1994). The Grb2-SOS complex is also known to interact with phosphorylated Y397. Two additional tyrosines in FAK, namely Y861 and Y407, are also known to be phosphorylated, but the functional implications of these events have not been well established (Mitra et al., 2005).

In addition to the above described tyrosine residues in FAK, a number of additional tyrosines as well as multiple serine and threonine residues have been identified as putative phosphorylation sites. Recently, an approach utilising FAK immunoaffinity purification followed by mass spectrometry identified 30 sites, established as well as novel, in the FAK sequence which appear to be modified by phosphorylation (Grigera et al., 2005). Many of these sites, which are spread throughout the entire protein, are evolutionarily conserved, leading to the suggestion that coordinated phosphorylation of FAK by serine/threonine- and tyrosine-specific kinases will be an important aspect of the regulation of FAK function. To date however, the significance of serine and threonine phosphorylation of FAK is poorly understood.

**FRNK (FAK-related non-kinase)**

A C-terminal, non-catalytic variant of FAK, termed FRNK or pp41/43FRNK (for FAK-related non-kinase), has been shown to be autonomously expressed in some cell types, possibly acting as a negative regulator of FAK activity (Schaller et al., 1993). FRNK consists of the C-terminal region of FAK, including the proline-rich motifs followed by the FAT domain, and does not possess any catalytic activity. Ectopic expression of FRNK has been shown to inhibit integrin-stimulated tyrosine phosphorylation of FAK as well as of paxillin and tensin (Richardson and Parsons, 1996), thus proposing a dominant negative mechanism where FRNK inhibits FAK signalling by displacing FAK from integrin-containing focal contacts (Schlaepfer and Mitra, 2004). In contrast to the ubiquitously expressed full-length FAK, FRNK expression appear to be tightly regulated, only showing expression in a limited number
of tissues, such as the lung, intestines and muscles (chicken). In addition, FRNK expression is induced in response to vascular injury (Schlaepfer and Mitra, 2004). The physiological importance of FRNK is still an area of controversy, with many studies reporting contradictory results regarding the effect of FRNK expression. However, this might be explained by stringent cell-type and context specific roles for FRNK. Currently, the general view is that FRNK counteracts FAK function, blocking growth-factor induced cell proliferation and motility, inhibiting cell adhesion and activating caspase-mediated cell death (Schlaepfer and Mitra, 2004).

**FAK function**

**Regulation of FAK activity**

FAK has been shown to be activated in response to a number of different stimuli, including integrin engagement to extracellular matrix, growth factor receptor stimulation, neuropeptides, reagents that stimulate G-protein coupled receptors and mechanical stimuli, among other things (Mitra et al., 2005). Integrin-mediated activation of FAK involves the recruitment of FAK molecules to the site of integrin clustering, enabling FAK oligomerisation and a resulting trans-autophosphorylation of tyrosine 397. This autophosphorylation event occurs within the linker region between the FERM domain and the kinase domain, thus creating a binding site for SH2-domain containing proteins such as SFKs, the regulatory p85 subunit of PI3K, PLCγ and Grb7. Upon recruitment, SFKs mediate further phosphorylation of FAK on Y576 and Y577 in the kinase domain activation loop, as well as on other tyrosines. These events function both to reinforce FAK kinase activity as well as to create binding sites for multiple PTB-domain containing proteins which are important for inducing appropriate downstream signalling. Consequently, FAK phosphorylation promotes the ability of FAK to assemble a number of multi-protein complexes, thereby coordinating several signalling cascades, including the Ras/ERK, PI3K/Akt and the Crk/Dock180/Rac pathways (Gelman, 2003; Mitra et al., 2005; Schlaepfer et al., 1999; Schlaepfer and Mitra, 2004).

Since FAK appears to direct a multitude of signalling events and cellular processes it is of highest importance to strictly regulate its activity. Indeed several mechanisms to negatively regulate FAK have been proposed. Firstly, FAK is clearly a target for tyrosine phosphatases such as SHP2, PTEN, PTP-PEST and PTP1B, which acts by removing phosphate groups from tyrosine residues and subsequently abolish the ability of FAK to interact with its target proteins (Manes et al., 1999; Mitra et al., 2005; Tamura et al., 1999). Certainly, cells with deregulated levels of SHP2 or PTEN display defects in cell spreading and motility, highly similar to FAK-/- cells (Feng, 1999; Leslie et al., 2005). Secondly, FAK has been observed to be catalytically targeted by caspases and calpains for cleavage, creating a large N-terminal fragment of 85-100 kDa that subsequently becomes cleaved into smaller fragments, in addition to a C-terminal fragment of approximately 35 kDa, consequently separating the catalytic domain from the FAT domain. It has also been suggested that these FAK fragments may actively transmit aberrant signals that promote cell cycle arrest or induce apoptosis (Schlaepfer et al., 1999). Thirdly, FIP200 (FAK family interacting protein of 200kDa), a novel protein isolated in a yeast-two hybrid screen for Pyk2-interacting proteins, has also shown to directly counteract FAK activity. The mechanism of inhibition is not clear, but it has been suggested that FIP200 by directly binding to the kinase domain of FAK and Pyk2, either can inactivate the kinase activity directly or function by displacing the interaction with SFKs.
and inhibit the phosphorylation events required for full FAK activation (Abbi et al., 2002; Ueda et al., 2000).

**FAK and adhesion**

Given the rounded morphology and increased number of focal adhesions observed in FAK-/- fibroblasts (Ilic et al., 1995), it is obvious that FAK displays important roles in the regulation of cell adhesion. Since FAK is rapidly recruited and activated in response to integrin stimulation, it was long believed that FAK functions in the assembly of focal adhesions. However, today it is generally agreed that FAK in fact exerts the opposite role, driving focal adhesion disassembly (Fincham et al., 1995). One elegant study monitored the localisation of a GFP-tagged α-actinin during cell spreading and showed that in cells deficient for FAK, focal adhesions assembled in a normal fashion, but in contrast to wild-type cells, the focal adhesions failed to disassemble (Ren et al., 2000). In FAK+/+ cells, focal adhesion turnover is a rapid process with a focal adhesion half-life of approximately 2 min, while focal adhesions in FAK-/- cells can last for times exceeding 20 min (Ren et al., 2000).

During focal adhesion turnover there appears to be a tight relationship between FAK and the small GTPase Rho, where Rho activity can be inversely correlated to focal adhesion turnover. When plating cells onto fibronectin, Rho is transiently inhibited during the time of initial cell spreading, but returns to higher levels at later time points, when focal adhesions are being formed (Ren et al., 2000). In FAK-/- cells, Rho is constitutively activated and focal adhesion disassembly is consequently blocked. This indicates the existence of a regulatory circuit where Rho activation initially induces focal adhesion formation and activation of FAK. In turn, when activated, FAK transiently functions to induce Rho down-regulation. This regulatory loop appears to be essential for the regulation of focal adhesion turnover and might provide an explanation for the cell migration defects and embryonic lethality observed in the FAK knockout (Ren et al., 2000). It is further tempting to speculate that FAK-dependent Rho regulation is mediated via the RhoGAP GRAF, which has been shown to interact directly with FAK (Hildebrand et al., 1996).

Activated FAK has additionally been shown to promote focal adhesion turnover by directly phosphorylating cytoskeletal proteins, including α-actinin, on tyrosines, thus triggering their release from the actin cytoskeleton and subsequently the disassembly of focal adhesion complexes (Izaguirre et al., 2001). At sites of adhesion, FAK is furthermore highly regulated by tyrosine phosphatases such as Shp2. In fact, Shp2-induced inhibition of FAK is necessary for the maturation of focal adhesions at initial sites of contact. This is clear from observations in Shp2-deficient cells, which display hyper-phosphorylation of FAK and a dramatic change in α-actinin assembly, accompanied by increased focal adhesion turnover in addition to defective cell spreading and migration (von Wichert et al., 2003). Furthermore, a feedback loop where phosphorylated α-actinin and PTP1B disrupts the FAK/Src complex, thus promoting focal adhesion disassembly and cell migration, was recently reported (Zhang et al., 2006).
Cell migration is a highly dynamic process that requires the integration of multiple signalling pathways in order to properly orchestrate the assembly and disassembly of actin filaments, causing cells to move. The importance of PTKs in cell migration, FAK in particular, has been shown in a large number of studies. Firstly, FAK-deficient cells (e.g. fibroblasts) display extensive alterations of actin cytoskeleton dynamics, characterised by increased numbers of focal contacts and abundant stress fiber formation. Consequently, these cells display spreading defects and migrate poorly in response to chemotactic as well as haptotactic signals, defects that can be rescued by the reintroduction of FAK (Ilic et al., 1995; Owen et al., 1999; Sieg et al., 1999). In agreement, the expression of the dominant negative FRNK protein blocks cell spreading and migration (Richardson et al., 1997; Sieg et al., 1999), whereas expression of wild-type FAK instead enhances migration (Cary et al., 1996). Importantly, FAK-mediated rescue of the migration defect observed in FAK-deficient cells is dependent on FAK kinase activity as well as the interaction of FAK with SFKs and p130Cas, mediated by Y397 and proline-rich region I, respectively (Cary et al., 1998; Hall, 2005; Sieg et al., 1999). Since cells deficient for Src family kinases (SYK cells) or p130Cas show similar phenotypes as cells lacking FAK, it is currently believed that FAK, Src and p130Cas form the core platform that regulates integrin-dependent cell movement.

A family of proteins essential for the regulation of actin networks and the formation of filopodia and lamellipodia during cell migration, is the Rho family of small GTPases (Hall, 2005). During migration, activation of the core family members Rac and Cdc42 stimulates de novo formation of F-actin filaments, resulting in the generation of lamellipodia and filopodia, respectively (Nobes and Hall, 1995). Mechanistically, Rac and Cdc42, when bound to GTP, interacts with WASP/Scar proteins, enabling the interaction of WASP/Scar with the multiprotein Arp2/3 complex, which subsequently functions to initiate actin polymerisation by binding to the sides of pre-existing actin and stimulating new filament formation (Machesky and Gould, 1999; Mullins et al., 1998). Behind the leading edge, activation of Rho mediates further organisation of actin into bundles, promoting the formation of focal adhesions, which serve as attachment sites for actin stress fibers as well as support for the contractile forces that eventually will cause the cell to move (Rottner et al., 1999).

Several lines of evidence imply an integration of FAK signalling with the regulation of small GTPases during migration. Certainly, cell migration induced by overexpression of FAK/Src/p130Cas is abrogated by the simultaneous expression of dominant negative Rac, indicating Rac as an important downstream effector of this complex. Additionally, recent studies have implicated a pathway where tyrosine phosphorylated p130Cas forms a complex with the adaptor protein Crk, causing a subsequent recruitment of the DOCK180 and ELMO proteins which together facilitate the GTP loading of Rac (Brugnera et al., 2002; Cary et al., 1998; Vuori et al., 1996). This model provides a mechanism for the localised activation of Rac at newly formed adhesions at the leading edge of migrating cells.

Besides the downstream signalling events governed by small GTPases in response to integrin-mediated adhesion and migration, the interaction of FAK with cytoskeletal proteins such as paxillin, is also of great importance. Paxillin is involved in the recruitment of FAK to focal adhesions and is implicated in the regulation of Rac as well as PAK and is indeed essential for the organisation and turnover of adhesion complexes (Brown and Turner, 2004).
In addition to integrin-dependent signal transduction, pathways involving the ERK and PI3Ks are also important for the regulation of cell migration. Mechanistically, the role of ERK in cell migration is poorly understood but indeed integrin binding to ECM molecules is sufficient to induce ERK activation (Renshaw et al., 1996; Zhu and Assoian, 1995) and in certain cell types inhibition of MEK, using U0126, blocks cell spreading and migration (Cheresh et al., 1999; Fincham et al., 2000). The activation of ERK is postulated to be mediated via the recruitment of the SOS-Grb2 complex, either by FAK or directly by integrin cytoplasmic tails, inducing activation of the Ras-Raf-MEK-ERK pathway. Additionally, a role for PI3K in cell migration is corroborated by the observation that PI3K-specific inhibitors have the ability to inhibit FAK-induced migration (Chen and Guan, 1994a; Chen and Guan, 1994b).

FAK in tumour development and progression

Because of its involvement in signal transduction pathways regulating cellular processes associated with malignancy, such as adhesion, migration and anchorage-independent growth, FAK is strongly implicated in the development of human cancer. Indeed both FAK itself and many of the proteins known to interact with FAK, have been directly linked to cellular transformation and tumour formation. A large variety of tumours with diverging origins, including breast, colon, thyroid, glial and ovary cancer, show elevated levels of FAK expression (reviewed by (Gabarra-Niecko et al., 2003)), in many cases due to amplification of gene dosage, resulting in increased protein levels and activity (Agochiya et al., 1999). Interestingly, the level of FAK expression seem to be stage-specific, showing increasing levels during the progression from benign tumours to invasive carcinomas (Agochiya et al., 1999) and indeed the level of FAK expression is often correlated with the invasive potential of the tumour (Owen et al., 1999). Thus, FAK status could potentially be used as a prognostic tool, allowing more accurate diagnosis and appropriate treatments.

The levels at which FAK could be important for tumour progression are many. First of all, FAK is a major regulator of focal adhesion turnover and cell spreading, and indeed cancer cells have shown to be highly sensitive to alterations in FAK levels. This can be exemplified by studies showing that overexpression of FAK can increase the adhesive properties of cancer cells while treatment with FAK siRNAs accordingly cause cancer cell detachment (Gabarra-Niecko et al., 2003). Secondly, FAK is a potent activator of proliferation and cell survival and already in early studies FAK was reported to cause an acceleration of tumour cell proliferation, when overexpressed (Schlaepfer and Mitra, 2004).

While it is believed that endogenous FAK levels may be permissive for hyperplasia formation, the involvement of FAK in tumour progression and metastasis is of higher importance. This, given that FAK RNAi, or expression of FRNK, promotes apoptosis in certain cancer cells, in addition to the reported correlation of increased FAK expression and the ability of tumour cells to migrate, invade and metastasise (Owen et al., 1999; Schlaepfer and Mitra, 2004). Metastasis is a multi-step process which requires detachment from ECM (e.g. basement membranes), invasion through matrix and tissue barriers, as well as adhesion-independent growth in a foreign environment(Thiery and Sleeman, 2006), all events where FAK is believed to be influential. The importance of FAK during cell migration is well established (see above) and certainly, inhibition of FAK function has in several cases been observed to reduce or even block cancer cell motility. In addition to acquired motility, tumour cell invasion also requires a regulated proteolytic degradation of surrounding matrix and stroma. During cell invasion, FAK is implicated to promote the expression and secretion of
the matrix metalloproteinase MM9 (Hauk 2001, Shibata 1998, Sein 2000). Furthermore, FAK-mediated survival signalling may be important for tumour growth and progression.

Moreover, several lines of evidence suggest that FAK is an important coordinator of the cross-talk between growth factor receptor- and integrin-mediated signalling pathways (Sieg et al., 2000). In conclusion, the multiple implications of FAK in tumour development depict FAK as an attractive target for the development of new cancer therapeutics.

**The role of FAK in mammalian systems in vivo**

In 1995, Ilic et al described the generation of FAK knock-out mice, showing that mice deficient of FAK protein start to show abnormalities at day 8.0 post coitum. At day E8.5 FAK knockout mice display dramatic retardation of the anteroposterior axis in addition to general mesodermal defects, such as involution of the head mesenchyme, absence of notochord and somites as well as defects in the differentiation of meso- and endocardium. Furthermore, defects in the neuroectoderm, including aberrant bends and distortions, were observed, although these phenotypes were described as secondary to the mesodermal defects (Ilic et al., 1995). Importantly, no defects in growth or differentiation could be observed in the FAK deficient mice, all mesodermal tissues initially developed normally, but growth was subsequently retarded, leading to tissue regression and lethality at day E8.5. Interestingly, these FAK mutant defects strikingly phenocopy the defects displayed by fibronectin-deficient mice, suggesting that FAK may be an important mediator of fibronectin-integrin interactions in cellular processes at this stage of development (George et al., 1993). Moreover, fibroblasts isolated from FAK knockout mice show a rounded, non-polarised morphology with an increased number of focal contacts and abundant stress fiber formation (Ilic et al., 1995). In addition these cells spread poorly and show reduced rates of migration, suggesting a role for FAK in the turnover, rather than formation, of focal adhesions, during migration.

In recent years, new and more sophisticated technologies to delete gene function have been developed in the mouse. Primarily the Cre/loxP system has been used successfully to study FAK function in different spatial and temporal contexts:

(i) Targeted deletion of FAK in endothelial cells (ECs) using Tie2-Cre mice has revealed an important role of FAK in the formation of vascular networks. Mice with FAK deficient ECs display a disruption of the vasculature. In these mice the vessels are irregularly shaped, frequently dilated and flattened, exhibiting a sheath-like appearance and thin, spiky connections. This phenotype is suggested to be caused by defects in vascular remodelling and maturation, leading to extensive haemorrhaging and subsequently resulting in embryonic lethality at day E10.5/E11.5. Loss of FAK did not cause any defects in differentiation or proliferation, but the survival of ECs was severely diminished (Braren et al., 2006). An essential role of FAK in vasculogenesis is additionally strengthened by its abundant expression in the vasculature at critical times of development (Polte et al., 1994). Furthermore, multiple *in vitro* observations implicate FAK in the transmission of angiogenic signalling from multiple pathways, including VEGF and angiopoietin, mediating EC migration, survival and proliferation (Kanno et al., 2000).

(ii) In the epidermis, loss of FAK, induced by keratinocyte-restricted Cre expression, causes irregularities in hair follicle number and orientation, in addition to hypoplasia in sebaceous glands and thinning of the epidermis. Since epidermal stem cells are thought to contribute to
all lineages of the hair follicle, sebaceous gland and epidermis, this study suggests that the stem cell compartment becomes reduced in the absence of FAK (Essayem et al., 2006).

(iii) A second study targeting FAK removal in the skin epidermis (using keratin-14-driven Cre, K14-Cre) addressed the question regarding a causative role for FAK in chemically induced tumourigenesis. Mice lacking FAK in their epidermis were viable, and upon chemical challenge they showed a 50% suppression of skin tumour formation. By combining the Cre-recombinase with a 4-OHT-regulated promoter, FAK could be deleted directly from preformed benign papillomas in order to examine any causal role for FAK in tumour progression and malignancy. Impressively, in these mice, a total block in malignant progression was observed, further emphasising an important role of FAK in tumour progression (McLean et al., 2004).

(iv) Deletion of FAK, specifically in Purkinje cells (using the L7 promoter to induce Cre expression), has furthermore identified a role for FAK in axonal dynamics. In the absence of FAK, the Purkinje cells display an increased number of axonal terminals and synapses. These findings indicate FAK as a negative regulator of axonal branching and synapse formation, possibly by modulating the activity Rho family kinases in vivo (Rico et al., 2004).

Interestingly, in neither of the above mentioned studies have any essential roles for FAK in cell migration been observed, not in differentiated keratinocytes and endothelial cells, or in epidermal stem cells, in vivo. Importantly, this is in contrast to multiple previous observations made in vitro, ascribing an essential role for FAK in cell migration, particularly during wound healing. Wound healing was notably not affected in K14-Cre/FAK mice (McLean et al., 2004). These studies emphasise the importance of using in vivo and three-dimensional in vitro models, in combination and with multiple experimental approaches, to really understand the function of the genes under investigation.

### FAK - A bridge between Integrins and RTKs?

The regulation of cell behaviour is orchestrated by the integration of signalling pathways emanating from a large number of membrane-anchored receptors. Indeed, integrins do not act autonomously to regulate cell adhesion, migration, proliferation etc., but participate in intensive “crosstalk” with RTKs to regulate many of these cellular processes. In fact, RTKs can activate integrin-related events (Lee and Juliano, 2004) and conversely, in many cases integrin-mediated adhesion is required for full activation of signalling downstream of GF receptors. Most cells are certainly anchorage-dependent for growth and show a concurrent dependence of adhesion for MAPK phosphorylation (Assoian and Schwartz, 2001; Renshaw et al., 1997).

Focal Adhesion Kinase is strongly implicated in the crosstalk between integrins and RTKs. Firstly, integrin-mediated activation of MAPK appears to at least in part be mediated via FAK, given the reported interaction with the Grb2/SOS/RAS and the p130Cas/Crk/C3G/Rap1/Raf pathways. Secondly, FAK is clearly activated in response both to integrin adhesion and growth factor stimulation (Gelman, 2003; Schaller, 2001). Thirdly, FAK has been proposed to engage in physically linking and clustering integrins and RTKs. This linkage is suggested to be achieved by the simultaneous interaction of the FAK FERM domain and RTKs (e.g. EGFR and PDGFRs) on one hand, and the FAK FAT domain and integrin
cytoplasmic tails, on the other (Hildebrand et al., 1993; Sieg et al., 2000). In agreement with an important role in RTK/integrin-crosstalk, constitutive activation FAK indeed supports GF-induced MAPK activation in suspended cells, and conversely MAPK activation is clearly blocked by dominant negative FAK expression (Renshaw et al., 1999).

Pyk2/ RAFTK/ CAKβ

In vertebrates, the FAK family of PTKs includes FAK, together with the “proline-rich tyrosine kinase 2” - Pyk2. Pyk2 was cloned and characterised by three separate groups in 1995 and was thus given three different names, including Pyk2 (Lev et al., 1995), RAFTK for related adhesion focal tyrosine kinase (Avraham et al., 1995) and CAKβ for cell adhesion kinase β (Sasaki et al., 1995). Pyk2 is located on chromosome 8 in the human genome and encodes a polypeptide of 1009 amino acids showing 48% identity (65% similarity) to FAK (Avraham et al., 1995). Structurally Pyk2 resembles FAK, with an N-terminal FERM domain, a central kinase domain and C-terminal FAT domain. However, in contrast to the ubiquitously expressed FAK, Pyk2 shows a more restricted expression pattern, being highly abundant in the brain, in haematopoietic tissues and in osteoblasts, in addition to showing lower levels of expression in the liver, lung, kidney and spleen (Avraham et al., 1995; Lev et al., 1995; Sasaki et al., 1995). Furthermore, Pyk2 occur in two different splice variants, one of which is expressed specifically in the CNS, while the other is located in haematopoietic lineages (Xiong et al., 1998). The subcellular localisation of Pyk2 is currently an area of controversy and seems highly dependent on the antibody used, some generating a diffusely cytoplasmic stain, concentrated in the perinuclear region while others indicate targeting to focal contacts or cell-cell contacts (Gelman, 2003).

Similar to FAK, Pyk2 has been observed to be phosphorylated on tyrosines in response to integrin-mediated adhesion to ECM molecules (Avraham et al., 2000) and has been proposed to facilitate the linkage between integrin receptors and cytoskeletal proteins such as paxillin and Hic-5, in adition to promoting signalling via pathways including p130Cas, PI3K and the JNK/MAPK cascades (Avraham et al., 2000). Pyk2 is furthermore known to be rapidly activated (e.g. phosphorylated on tyrosines) in response to multiple extracellular stimuli that increase intracellular calcium levels, including depolarisation, activation of nicotinic receptors by carbachol, bradykinin, and PMA, as well as by PKC (Lev et al., 1995; Siciliano et al., 1996; Tian et al., 2000). This was thoroughly studied by Lev and co-workers who reported that activation of Pyk2 mediated modulation of ion channel function in addition to activation of the MAPK signalling pathway (Lev et al., 1995). Other studies have identified a connection between Pyk2 activity and JNK signalling in the transmission of signalling emanating from stress stimulation, G-protein-coupled receptors and inflammatory cytokines (Avraham et al., 2000; Tokiwa et al., 1996; Yu et al., 1996).

Pyk2 deficient mice are viable and fertile but display defects in their immunological responses. Most strikingly, macrophages extracted from Pyk2-/- mice show altered cell morphology and impaired migratory potential in response to chemokine stimulation (Okigaki et al., 2003). Further studies have revealed a specific defect in marginal zone B (MZB) cell production in Pyk2 knock-out mice, that is accompanied by defective B cell motility and a reduced production of immunoglobulins IgM, IgG3 and IgF2a, in response to LPS stimulation (Guinamard et al., 2000). This is in agreement with the observed activation of Pyk2 in
integrin-stimulated B cells, which has shown to result in the interaction with p130Cas and paxillin, as well as the induced association of Pyk2 with Fyn and Grb2 following engagement of antigen receptors on T cells, B cells and mast cells (Astier et al., 1997; Avraham et al., 2000; Lev et al., 1995).

**FAK and Pyk2 interrelationship; Redundant and specific functions?**

Given the structural similarities of FAK and Pyk2, together with the identification of both specific and shared interaction partners, it is intriguing to consider how Pyk2 may affect FAK function, as well as ask which of the functions, if any, of the respective proteins that are interchangeable. Indeed, there is redundancy concerning certain functions. Studies in FAK-/- fibroblasts have demonstrated that overexpression of Pyk2 can rescue adhesion-mediated MAPK activation in a Src-dependent manner, but not the haptotactic migration defects displayed by these cells (Sieg et al., 1998). The inability of Pyk2 to rescue cell migration appears to depend on its perinuclear localisation, given that targeting of Pyk2 to focal adhesions restores the cell migration of FAK-/- cells to wild-type levels (Klingbeil et al., 2001).

The two FAK family members also appear to directly influence each other, as indicated by reports describing a FAK-mediated inhibition of Pyk2 activity in fibroblasts, where FAK overexpression displaces overexpressed Pyk2 from focal adhesions, thus suppressing Pyk2-dependent actin reorganisation (Du et al., 2001). In contrast, Pyk2 has instead been shown to directly phosphorylate FAK (Li et al., 1999). Some tissues, such as the nervous system, appear to utilise FAK and Pyk2 for diverging functions in different developmentally regulated contexts (see below), whereas in other cell types FAK and Pyk2 appear to work as a team. This has recently been observed in endothelial cells where VEGF-induced angiogenesis is dependent on cell spreading and migration, which appears to be influenced by both FAK and Pyk2 (Avraham et al., 2003). The importance of cellular context was further emphasised by a recent study, where siRNAs were used to specifically knock-down FAK and Pyk2, respectively, showing a clear divergence in function regarding LPA-induced migration of epithelial cells. In these cells FAK was observed to be highly important for cell migration whereas Pyk2 function appeared to be dispensable (Jiang et al., 2006). Taken together, the FAK family kinases display highly dynamic expression patterns as well as functions, and the balance between the two family members seems to be of great importance for the proper regulation of cellular morphology, cell migration and cell growth.

**FAK and Pyk2 in the nervous system**

Both FAK and Pyk2 are highly expressed in the nervous system, where they are believed to play important roles during development as well as in the adult brain. Interestingly they show alternating expression patterns, with FAK being abundantly expressed in the embryonic CNS prior to downregulation at birth (Menegon et al., 1999), whereas Pyk2, in a complementary fashion, is low in the embryonic CNS but becomes dramatically enhanced in the forebrain around birth (Menegon et al., 1999). As previously discussed, FAK exists in various splice isoforms, some of which appear to be exclusive to the CNS. Most abundant in neuronal tissues is the FAK⁺ isoform which contains a three-amino-acid insertion (Pro-Trp-Ala) in the FAT sequence, together with the FAK⁺₆,₇ isoform, characterised by the addition of alternative exons encoding either six or seven extra amino acids, flanking Tyr397. This insertion has
been shown to increase intrinsic FAK autophosphorylation and augment FAK activation (Burgaya and Girault, 1996; Girault et al., 1999a).

In agreement with their diverging expression patterns, FAK and Pyk2 are implicated in the regulation of different processes within the nervous system. FAK is believed to display important functions during neuronal development, regulating neurite outgrowth as well as neuronal cell migration and survival. Indeed, FAK is concentrated in the highly dynamic contact points formed by migrating growth cones, where it co-localises with focal adhesion proteins such as integrins, vinculin and talin (Xiong and Mei, 2003). Studies in PC12 cells have shown that stimulation of specific growth factor receptors and integrins, together can activate both FAK and Pyk2, subsequently inducing paxillin-dependent signalling events that regulate the morphological changes associated with neurite formation (Haglund et al., 2004; Ivankovic-Dikic et al., 2000). In light of the importance of FAK activity during focal adhesion turnover and migration, it is striking to note that the neuronally enriched FAK isoforms are more readily activated than the ubiquitous isoform, which is in agreement with a regulatory role in dynamic point contacts, as well as the high mobility of growth cones (Burgaya and Girault, 1996). In addition, a requirement of FAK in netrin-induced neurite outgrowth and attractive turning has recently been observed in Xenopus spinal neurons as well as in murine cortical neurons (Li et al., 2004).

In the adult brain, FAK and Pyk2 are most prominent in the hippocampus, cerebral cortex and thalamus, where Pyk2 is apparent both in cell somas and dendrites in contrast to FAK that mainly is localised in cell somas (Menegon et al., 1999). More specifically, proteomic analysis of the adult CNS has shown Pyk2 to be an abundant component of post-synaptic densities, PSDs, a microscopic structure associated with the post-synaptic membrane that contains a variety of signalling proteins (Husi et al., 2000). While neuronal FAK is activated by ECM molecules, growth factors and guidance cues, Pyk2 has been shown in multiple studies to be activated in response to various stimuli that increase intracellular Ca$^{2+}$ or activate PKC, including membrane depolarisation, the action of glutamate/glutamate agonists, bradykinin, LPA, phorbol esters and neurotrophic factors such as NGF (Girault et al., 1999a). These findings have suggested a role for Pyk2 in regulating synaptic plasticity and neuronal cell signalling in the adult brain (Xiong and Mei, 2003). Downstream of Pyk2 phosphorylation, induced by Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels, a signalling pathway involving phosphorylation of paxillin and ERKs are thought to regulate ion channel activity, stress responses, cell adhesion/cytoskeletal reorganisation and vesicle trafficking (Lev et al., 1995; Siciliano et al., 1996; Tian et al., 2000).

PTKs have been shown to be essential for the regulation of synaptic plasticity and long term potentiation (LTP) in the brain. Specifically, SFKs and ERKs display established roles in these processes, but the distribution and activation pattern of Pyk2 suggest that also FAK-family kinases may be involved (Girault et al., 1999a). Indeed, long electronic stimulation that cause membrane depolarisation and LTP have shown to increase Pyk2 phosphorylation. In addition, it has been implied that Pyk2, via activating Src kinases, causes increased tyrosine phosphorylation and subsequently increased activity of NMDA receptor subunits 2A and B (NR2A/B), consequently affecting synaptic plasticity. In fact, LTP is believed to primarily be generated by different patterns of NMDA glutamate receptor activation (Huang et al., 2001).

Given that both FAK and Pyk2 are abundant and display important functions in the CNS, they are naturally thought also to be involved in pathological conditions affecting neuronal tissues. More specifically FAK is implicated in neurodegenerative disorders such as Alzheimer’s
disease, as well as in convulsive and ischemic responses. Moreover FAK involvement is implied in neural malignancies (Girault et al., 1999a).

Besides FAK and Pyk2, integrin-mediated signal transduction in general is clearly important for proper CNS function. A role for integrins in axonal guidance has indeed been determined, given that mutations in the αPS1 or αPS2 subunits cause wide-spread axon pathfinding defects and that the *volado* gene has shown important for mediating olfactory learning in *Drosophila* (Connolly and Tully, 1998; Grotewiel et al., 1998; Rohrbough et al., 2000).

**Drosophila FAK - Fak56**

**Characterisation of the Fak56 gene**

The sole FAK/Pyk2 family member in the *Drosophila* genome, *Fak*56, was cloned independently by three different groups and published in 1999 (Fox et al., 1999; Fujimoto et al., 1999; Palmer et al., 1999). The *Fak*56 locus, spanning over 7 kb, contains 16 exons which give rise to one single transcript encoding a polypeptide of 1200 amino acids, displaying an overall identity of 34% and 29% to human FAK and Pyk2, respectively. Similar to mammalian FAK, *Fak*56 shows a domain organisation characterised by a centrally located kinase domain, flanked by an N-terminal FERM domain in addition to poly-proline-rich regions and FAT domain in the C-terminus. In these conserved domains, the homology to vertebrate FAK is exceptionally high, as can be exemplified by an identity of 61% in the kinase domain. The *Fak*56 PTK domain contains several sequence motifs that are conserved among protein kinases, including a consensus ATP-binding motif (GXGXXG), followed by a downstream AXK sequence. It also shares all of the 14 residues invariant in the tyrosine kinase superfamily. Differing from FAK and Pyk2, *Fak*56 displays a 24-aa kinase insert between the phosphate-anchor containing kinase subdomain I and kinase subdomain II, and has an additional 104 aa C-terminal extension which is absent in mammalian FAK and Pyk2. Furthermore, several of the phosphoacceptor sites important for protein-protein interactions in FAK are also conserved in *Fak*56, including the autophosphorylation motif and YAEI consensus Src SH2 domain-binding sequence Y430 (Y397 in human FAK) and Y956, which aligns with Y925 in FAK and Y881 in Pyk2, respectively. This is interesting, in light of the established interaction between Y397 and the SH2 domains of SFKs and PI3K, in addition to the binding of the SH2/SH3-domain containing adaptor protein Grb2 to Y956. Moreover, the proline-rich region known to interact with p130Cas in vertebrates, but not the GRAF-binding region, is also conserved in *Drosophila* *Fak*56.

*Fak*56 encodes a protein of 140kDa, which displays in vitro kinase activity and is tyrosine phosphorylated in vivo. This phosphorylation is enhanced when plating primary *Drosophila* embryonic cells on ECM molecules such as tigrin, laminin and fibronectin (Fox et al., 1999; Palmer et al., 1999). In response to integrin-dependent adhesion, ectopically expressed *Fak*56 co-localises with integrins and associated focal-adhesion proteins, and has in addition been shown to interact with proteins phosphorylated on tyrosines (Fujimoto et al., 1999). During *Drosophila* embryogenesis, *Fak*56 is ubiquitously expressed, displaying particularly high levels in the developing CNS (e.g. brain and nerve cord), as well as in the epidermis, visceral mesoderm and at muscle attachment sites (Fox et al., 1999; Fujimoto et al., 1999; Palmer et al., 1999). After completion of embryogenesis *Fak*56 levels initially decrease, but has been shown to later on reappear during late larval and pupal stages. These findings strongly...
indicate Fak56 to be involved in developmentally regulated processes. In addition, Fak56 is highly abundant in ovaries where it is enriched in migrating border cells, a cluster of around 8 cells which delaminate from the follicular epithelium, invade the underlying germline tissue and migrate directionally towards the oocyte, to subsequently be involved in the formation of the micropyle (Fox et al., 1999; Montell, 2001), thus displaying characteristics regulated by FAK family kinases.

**Physiological functions of Fak56 in the fruit fly**

Despite extensive efforts to generate mutations in the Fak56 locus, five years after its cloning, this was still not accomplished. In the meanwhile, overexpression studies were used in order to understand potential in vivo physiological roles for Fak56. Already in 1999, Palmer et al observed an importance of a stringent control of Fak56 expression levels, given that ubiquitous overexpression of Fak56 caused 100% pupal lethality. More selective overexpression in the developing wing additionally indicated a role for Fak56 in integrin-mediated adhesion, resulting in the formation of wing blisters, highly similar to the phenotype observed in response to alterations in PS integrin expression levels (both loss of function and gain of function) (Palmer et al., 1999).

The abundance of Fak56 in border cells in the developing egg chamber has prompted investigations of a putative role for Fak56 in their migration. One such study has identified Fak56 as a downstream target of the DWnt4 pathway, given that Fak56 protein levels and localisation is altered in DWnt4 mutant egg chambers. The hypothesis built in this study indicates that DWnt4 promotes motility of apical cells through the regulation of focal adhesions, rendering DWnt4 mutants migration defective (Cohen et al., 2002).

Moreover, Fak56 has recently been shown to be involved in the phagocytic uptake of *E. coli* by *Drosophila* hemocytes. This is suggested in light of the finding that *E. coli*-mediated activation of hemocytes is accompanied by a rapid and transient tyrosine phosphorylation of Fak56, and that treatment of hemocytes with inhibitory anti-Fak56 antibodies reduces phagocytosis by 50% (Metheniti et al., 2001). A role for Fak56 in regulating immune responses in *Drosophila* has been further emphasised by the finding that regulation of Fak56 activity and focal adhesion assembly, mediated via Rac1 and Bsk, is essential for the cellular immune response against wasp parasitation (Williams et al., 2006).
Bang sensitivity

Studies in Drosophila have over the 25 past years identified an intriguing group of mutants that exhibit stress-sensitive seizures and paralysis. Among these, a certain set of mutants, referred to as “Bang Sensitive paralytics” (BS), are characterised by a sensitivity to mechanical and electrical stimulation (Ganetzky and Wu, 1982). Essentially, in response to mechanical agitation such as tapping the culture vial on the bench or vortexing, bang sensitive mutants go through a stereotyped sequence of events referred to as the “bang sensitivity cycle”, characterised by an initial seizure, paralysis, a delayed seizure, activity recovery, and a following period of seizure refractoriness (Figure 7). The seizures are manifested by hyperactivity displayed as uncoordinated movements such as intense abdominal contraction, wing-flapping, proboscis extension and leg-shaking, whereas the paralysis represents a complete cessation of physical activity and responsiveness (Lee and Wu, 2002). In agreement with the knowledge that all higher nervous systems have the capacity to induce seizures after high-intensity stimulation, the bang sensitivity cycle can be observed in wild-type flies as well as in BS mutants. Consequently the defect in BS mutants is not that they have seizures, but rather implicates that the genotype of an organism modifies the seizure threshold, altering the susceptibility for seizures.

Clearly, the genetic background is a critical component for the seizure susceptibility in an individual, but intriguingly no physiological or biochemical defect has been found to be common to the mutations known to cause BS behaviour. Rather, the divergence of the genetic loci linked to BS behaviour suggests a highly complex basis behind this phenotype. The classical BS mutant bang senseless (bss) codes for a HMG-domain-containing transcriptional modulator, namely DSP1 (Tan et al., 2004), easily shocked (eas) is an ethanolamine kinase (Pavlidis et al., 1994) and slamdance (sda) encodes an aminopeptidase (Zhang et al., 2002). Two of the BS mutant gene products have interestingly been shown to display metabolic functions in mitochondria, including technical knockout (tko) which encodes the mitochondrial ribosomal protein S12 (Royden et al., 1987) in addition to knockdown (kdn),

![Figure 7](image)

**Figure 7.** The Bang Sensitivity cycle. In response to mechanical or electrical stimulation, BS mutant flies go through a stereotyped sequence of events, characterised by an initial seizure, paralysis and a delayed seizure, followed by a period of seizure refractoriness. (Reprinted from Tan et al, 2004, Brain Research, with permission from Elsevier.)
encoding the citrate synthetase (Fergestad et al., 2006). This is interesting in light of the finding that all BS mutants show reduced levels of ATP, thus strongly implicating cellular metabolism in the pathophysiology behind seizure susceptibility (Fergestad et al., 2006). In addition, BS mutations have been shown to affect genes encoding ion channels, components of the synaptic machinery, as well as proteins required for the proper generation and transmission of electrical signals in the nervous system (Littleton et al., 1999; Wu and Ganetzky, 1992).

Interestingly, in contrast to BS mutant flies, the literature also describes mutant strains that are protected against seizures (Kuebler and Tanouye, 2000). The best characterised of these is the maleless no-action potential temperature sensitive allele (mle<sup>napts</sup>), which is a gain-of-function mutation in an RNA-helicase-like protein that causes a loss of action potentials and behavioural paralysis at non-permissive temperatures (Hurd et al., 1996; Reenan et al., 2000). The mle<sup>napts</sup> has furthermore been shown to act as a suppressor of BS phenotypes (Kuebler and Tanouye, 2000).

**The Giant Fiber pathway**

The behavioural characteristics shared by all BS mutants are in all identified cases accompanied by a failure of the giant fiber (GF) pathway and can be recorded by electrophysiological measurements (Figure 8) (Fergestad et al., 2006; Kuebler and Tanouye, 2000; Pavlidis and Tanouye, 1995; Royden et al., 1987; Trotta et al., 2004; Zhang et al., 1999).

The GF pathway is the neuronal circuit responsible for the jump and flight escape reflex in *Drosophila*. It functions by integrating inputs from various sources, such as the visual and olfactory system, and subsequently projecting its output to the thoracic ganglion, which forms several synapses inducing jump and flight responses. In fact, the giant fiber (GF) is an interneuron that projects from the brain to the thoracic ganglion where it contributes to several distinct outputs. Firstly, the GF forms mixed electrical and chemical synapses with a peripherally synapsing interneuron (PSI), which in turn projects via cholinergic synapses to the posterior dorsal mesothoracic nerve (PDMN) which contains all five dorsal longitudinal muscle (DLM) motor neuron axons. These subsequently innervate the six contralateral DLMs. Interestingly, the DLM fibers 5 and 6 are innervated by the same motor neuron. Secondly, the GF also forms a direct electrical synapse with the ipsilateral tergotrochanteral muscle motor neuron (TTMmn), which forms a glutaminergic synapse with the TTM. Thirdly, the GF additionally sends an output to the DVM motor neurons, but this circuit is still fairly uncharacterised (Pavlidis and Tanouye, 1995).

In response to stimulation of the GF pathway, all six DLM fibers are activated synchronously via the PSI and the DLMmns. Specifically it seems as the DLM circuits are responsible for initiating seizures since ID and DD patterns (see below) are never generated solely upon TTM stimulation. However, the recovery of the TTM from response failure concurs with the time of DD initiation from DLMs, which coincide with the onset of general bursting activities in the CNS (Lee and Wu, 2002).

The stereotyped behavioural response evoked in BS mutants has been strictly correlated with an electroconvulsion-induced physiological repertoire composed of an initial spasm, paralysis, delayed spasm, activity recovery, and seizure refractoriness. These phases are in
electrophysiological measurements manifested by an initial discharge of spikes (ID), a response failure period (F), delayed discharge (DD), response recovery (R) and a refractory period. All BS mutants examined display a unique and highly reproducible profile regarding ID, DD and F patterns, while the DD may differ (Lee and Wu, 2002).

After completion of the BS cycle, flies enter a refractory period in which they are resistant to HF stimulation and cannot be re-paralysed by a second bang stimulus. The refractory period varies according to genetic background and interestingly, during this period the resistance to seizures is transient and declining. This indicates that behavioural BS varies according to previous experience and that immediately after a seizure the susceptibility is modulated by some process that causes a threshold increase, presumably because a greater number of neurons must be stimulated (Kuebler and Tanouye, 2000).

**Electrophysiology**

Electrophysiology is often performed by stimulating and recording giant fiber (GF)-driven muscle potentials and seizures in tethered flies. Practically, a pair of stimulating electrodes is placed into the eyes of the fly and the recording electrodes into specific flight (DLM) and/or TTM muscles, together with a reference electrode in the abdomen. The placement into identified muscles can be guided by cuticular markers, by the characteristic latency and shape of action potentials displayed by individual muscles, and can further be confirmed using dye stainings through the insertion site (Lee and Wu, 2002).

When applying high-frequency electrical stimulation across the *Drosophila* brain, a seizure-like activity lasting 1-2 s, followed by a failure period, can be recorded in the DLM motor neurons (Kuebler and Tanouye, 2000). Each genotype, whether wild-type or mutant, has a characteristic, or signature, HF stimulus intensity at which seizures occur. Qualitatively, the pattern of seizures and failure of the GF pathway is shared in BS mutants and wild-type flies. However, in wild-type animals the synaptic failure period is significantly shorter and the threshold levels needed to evoke seizures are significantly higher, compared to those displayed by BS mutants. For instance, wild-type flies of the Canton S and Oregon R strains show a seizure threshold at 44.5 ± 4.4 V, compared with 3.1 ± 0.7 V for *bss* mutants, 3.6 ± 0.7 for *eas* and 6.8 ± 1 for *sda*, in response to stimulation at a frequency of 200 Hz (Kuebler and Tanouye, 2000).

The HF threshold stimuli that elicit a seizure for a given genotype behave in an all-or-

![Figure 8](image.png)

**Figure 8.** Electrophysiological recordings of the BS cycle corresponds to the behavioural observations and are represented by an initial discharge of spikes (ID), a response failure period (F) and a delayed discharge (DD). (Reprinted from Tan et al, 2004, Brain Research, with permission from Elsevier.)
nothing manner. Consequently, below the threshold no seizures are observed, whereas above the threshold, seizures are elicited and spread throughout all muscle groups examined. It is thus believed that the seizure threshold defines the minimum number of neurons required to initiate a seizure (Kuebler and Tanouye, 2000).

Detailed electrophysiological studies have identified the site of failure along the GF pathway displayed by BS mutants. In general, the failure occurs at the synapse between the peripherally synapsing interneuron (PSI) and the DLMs, while the DLMmn-DLM neuromuscular junctions and DLMmn action potentials remain functional (Pavlidis and Tanouye, 1995).

**Bang Sensitivity in Vertebrates?**

Although poorly understood, the behavioural similarities between bang sensitivity and human seizure disorders designate BS mutants as relevant models for studying the molecular and cellular mechanisms underlying seizure susceptibility. Indeed, human seizure disorders, such as epilepsy, are a substantial health problem affecting more than 1% of the human population (McNamara, 1994). Similar to the phenotypes observed in BS flies, seizures displayed by humans are characterised by a stereotyped action pattern followed by a refractory period caused by disordered, synchronised firing of large neuronal populations in the brain (Tan et al., 2004). As in flies, vertebrate seizures are evoked at individually diverging threshold values, can be segregated into specific regions of the CNS and are highly sensitive to genetic backgrounds (Tan et al., 2004).

In light of the link between BS in flies and mitochondrial metabolism, it is interesting to note that the first genetically transmitted mammalian epilepsy for which the responsible gene was cloned, human myoclonic epilepsy/ragged red fiber (MERRF) disease, showed to encode a mitochondrial ribosomal protein (mitochondrial lysine tRNA) (Shoffner 1990). In subsequent studies epileptic seizures in human and animal models have furthermore been linked to defects in Na⁺, Ca²⁺ and K⁺ channels, CaM kinase II and a NRPTK (Puranam and McNamara, 1999). In addition, mouse knock-outs have identified genes such as 1-isospartyl methyltransferase, neuropeptide-Y, alkaline phosphatase and a Kv1.1 potassium channel, to be involved in seizure disorders (Kuebler et al., 2001).

Because of the similarities between fly and vertebrate seizures, the *Drosophila* BS mutants have been proposed to be excellent tools in the development of new drug therapies for seizure disorders and have indeed been used to screen for compounds that suppress seizure susceptibility (Kuebler and Tanouye, 2002).
Regulation of Cellular Signalling

Receptor Tyrosine Kinases (RTKs)

Receptor Tyrosine Kinases (RTKs) function as receptors for extracellular ligands, so called growth factors, and mediate activation of intracellular signalling pathways regulating cellular proliferation, survival, differentiation and motility. The ligand-induced response elicited in a cell depends on the cell type, ligand and receptor identity, as well as the composition of the supramolecular protein complexes (signalosomes) that are recruited to the site of receptor activation.

The best studied family of RTKs is the ERBB receptors, of which the EGFR is the founder member. In response to ligand binding (EGF, TGFα or AR), EGFRs homo- or heterodimerise via the formation of disulfide bonds in the extracellular “dimerisation loop”. This results in a conformational change and receptor trans-autophosphorylation, consequently leading to receptor activation. The receptor subsequently gets phosphorylated on multiple residues within the cytoplasmic tail, creating docking sites for various adaptor and signalling molecules, which serve to initiate appropriate cellular signalling, thus enabling the cell to correctly respond to the inductive stimuli. The ERBB receptors have shown to regulate a broad range of cellular responses which mainly are transmitted via signalling pathways including the mitogenic MAPK and Phospholipase Cγ pathways and the anti-apoptotic PI3K pathway, in addition to STAT-regulated transcriptional control. To enable a cell to rapidly respond to extracellular stimuli, RTKs are often enriched in membrane microdomains, such as rafts or caveolae, where the levels of many of the key downstream effectors (such as Ras) are also elevated (Hynes and Lane, 2005; Mineo et al., 1996; Warren and Landgraf, 2006).

Dysregulation of RTK-induced signalling is a common cause underlying tumour formation and can be mediated through mechanisms including activating mutations, overexpression (often due to gene amplification), structural rearrangements, disruption of regulatory loops or inactivation of negative regulators. In light of this, RTKs are intensively pursued as therapeutical targets for a variety of cancers and today blocking ectodomain-binding antibodies and small-molecule tyrosine kinase inhibitors (TKIs, e.g. gefitinib and erlotinib) are extensively used in the clinic (Hynes and Lane, 2005).

The ERK/MAPK signalling module

Activation of the evolutionary conserved ERK/MAPK signalling pathway is a common target for many of the RTKs and provides a common route leading to transcriptional regulation of certain genes which are essential for cell growth and differentiation. In general, activated RTKs recruit adaptor proteins such as Shc and Grb2, via docking of SH2 domains to phosphorylated tyrosines on the cytoplasmic tail of the receptor (Kolch, 2005). This consequently leads to the recruitment of SOS (son-of-sevenless), a guanine-exchange-factor (GEF) for the small GTP-binding protein Ras, which predominantly is localised at the plasma membrane due to palmitoylation/farnesylation modifications (Dudler and Gelb, 1996). The exchange of GDP to GTP associated with Ras activates the protein and subsequently triggers the serine/threonine kinase cascade starting off with the Raf proteins, that activate MEKs, which finally activate ERK kinases (Figure 9). The ERK kinases are the main effectors of the ERK/MAPK pathway and have more than 70 identified substrates, including signalling molecules, nuclear transcription factors, cytoskeletal proteins and receptors (Kolch, 2005).
Somewhere along the pathway, there is clearly a branch-point that enables this classical signalling module to diverge and regulate cellular processes as deviating as proliferation, survival, differentiation, apoptosis, motility and metabolism. Although this branch-point is somewhat of an enigma, a picture has emerged where the kinetics of ERK/MAPK activation, the subcellular compartmentalisation of signalling, as well as cell-type specific contexts, strongly influence the outcome of this pathway (Kolch, 2005). Classically, this can be exemplified by the induction of proliferation versus differentiation in neuronal PC12 cells, by transient and sustained ERK/MAPK activation, respectively (Marshall, 1995). To date, five distinct groups of MAPKs have been described, of which all have different substrates and patterns of activation. These include the classical ERKs (extracellular signal-regulated kinases 1 and 2), the JNKs (c-Jun amino-terminal kinases 1, 2 and 3), the p38 MAPK isoforms (α, β, γ and δ), in addition to ERKs 3, 4 and 5 (Roux and Blenis, 2004).

In *Drosophila*, there is only one MEK and one ERK (excluding p38 and JNK), that during development have been shown to be activated in response to RTK-mediated signalling in specific tissues at distinct time points. The *Drosophila* ERK is encoded by the *rolled* locus, which in overexpression studies indeed has shown to be a point of convergence for multiple RTKs. The generation of a phospho-specific anti-ERK antibody (di-phosphoERK, dpERK), specifically recognising the activated form of ERK, has facilitated the generation of an ERK activity atlas, which has been of great assistance in the identification of tissue-specific roles of different RTKs (DER, Torso and the FGF receptors Heartless and Breathless) during *Drosophila* embryogenesis (Gabay et al., 1997).

**Anaplastic Lymphoma Kinase**

ALK, short for Anaplastic Lymphoma Kinase, was first described in 1994 by Morris and co-workers, who identified ALK as one of the fusion partners in a chimeric protein known as the causative agent in a majority of anaplastic large-cell non-Hodgkin’s lymphomas (ALCLs) (Morris et al., 1994). ALCL is an aggressive T-lineage lymphoma, which is characterised morphologically by large cells with pleomorphic cytology and multilobulated “kidney-shaped” nuclei, and biochemically by the expression of the CD30/Ki-1 antigen (Duyster et al., 2001). In their initial studies, Morris et al discovered that the t(2;5)(p23;q35) chromosomal translocation, typically found in ALCL patients, generates a fusion between the N-terminal part (residue 1-117) of Nucleophosmin (NPM) and the C-terminal, catalytic part, of ALK. Consequently, cells where this rearrangement has occurred express an 80 kDa hyperphosphorylated protein with constitutive ALK activity, under control of the strong NPM promoter (Morris et al., 1994; Shiota et al., 1995). Since ALCLs originate from activated T
cells, it is believed that the oncogenic signalling induced by NPM-ALK involves the pathways originating from the IL-2 and TCR receptors, mediating malignant transformation of lymphoid cells (Morris et al., 1994).

A few years after the discovery of NPM-ALK, full length ALK was cloned and characterised (Iwahara et al., 1997; Morris et al., 1997). In its longest version, the ALK locus was shown to encode a 1620 amino acid long polypeptide with an estimated molecular weight of ~175kDa. However, the generation of anti-ALK antibodies revealed a major band of 200/220 kDa on SDS-PAGE gels, indicating extensive posttranslational modifications of the ALK receptor (Iwahara et al., 1997; Morris et al., 1997). Interestingly, sequence analysis of ALK reveals at least 16 consensus motifs for N-linked glycosylation (Asn-X-Ser/Thr), and indeed, treatment with tunicamycin, an inhibitor of N-linked glycosylation, reduces ALK molecular mass to its expected size, thus indicating that the major modifications of the ALK receptor consist of carbohydrate chains (Iwahara et al., 1997).

### Structural Features of ALK

The ALK receptor belongs to the insulin receptor superfamily of RTKs, which also includes the insulin-growth factor-1 receptor and the TRK neurotrophin receptors, MET and cROS, but shows strongest sequence similarity to the leukocyte tyrosine kinase (LTK) (57% amino acid identity, 71% similarity). Also in an evolutionary perspective, the ALK receptor is highly conserved, showing 85% identity between the human and mouse ALK proteins (Pulford et al., 2004).

Similar to the conventional RTK structure, ALK consists of a large ligand-binding extracellular domain (1014 amino acids), a single transmembrane region and an intracellular kinase domain (Figure 10). In its extracellular part, ALK is characterised by multiple conserved protein modules including a signal peptide, an LDL-A domain, two MAM-domains (named after meprins, A-5 protein and receptor protein tyrosine phosphatase mu, believed to function in cell-cell interactions), a glycine-rich region and a conserved cysteine-rich EGF-like motif, believed to mediate ligand specificity. Following the transmembrane domain, the juxtamembrane region of ALK contains a conserved NPYX motif, which is implied both to mediate interaction with IRS-1 and Shc as well as to be involved in ligand-independent receptor endocytosis (Duyster et al., 2001; Pulford et al., 2004). The ALK kinase domain contains several conserved motifs, including the conserved autophosphorylation motif, YY (Morris et al., 1997). Interestingly, C-terminal to the kinase domain, ALK is extended by an unusually long tail, compared to other RTKs, that contains multiple, yet uncharacterised, potential tyrosine docking motifs. The mouse ALK locus appear to be differentially spliced, giving raise to at least four major transcripts, varying in size and expression pattern. The main isoforms are predominantly expressed in the small intestine, brain, colon and prostate, whereas the truncated isoforms show high expression in the testis, placenta and fetal liver (Iwahara et al., 1997; Morris et al., 1994; Vernersson et al., 2006a).
Signalling mediated via the ALK receptor

Similar to other RTKs, ALK activation is induced by ligand-binding, mediating ALK dimerisation and autophosphorylation (in trans) and subsequently the recruitment of a number of PTB-containing downstream signalling molecules. From studies in NPM-ALK transfected cells, the mitogenic capacity of the oncogenic fusion protein has been shown to be mediated through the activation of multiple signalling pathways, contributing to cell survival, proliferation and inhibition of apoptosis. The cytoplasmic part of ALK indeed harbours several tyrosine residues that upon activation get phosphorylated and recruit proteins such as the regulatory p85 subunit of PI3K, JAK2, JAK3, STAT3, Grb2, IRS, and PLC-γ (Pulford et al., 2004).

ALK ligands: The identity of the ligand(s) responsible for ALK activation is still an area of controversy. Initially, Iwahara at al suggested that since ALK possess an EGF-like repeat in its extracellular domain, it might itself act as a ligand (Iwahara et al., 1997). However, while there still is no direct evidence supporting this hypothesis, high receptor concentrations do induce receptor activation. In recent studies Stoica and co-workers have reported the related proteins pleiotrophin (PTN) and Midkine (MK) as putative ALK ligands. PTN is a small heparin-binding growth factor implicated in the regulation of neurite outgrowth, proliferation and angiogenesis during the development of the neonatal brain and is expressed in a pattern which is highly similar to ALK. Initial studies showed a direct interaction between PTN and ALK and provided evidence for an NPM-induced, ALK-dependent activation of both ERK/MAPK and PI3K pathways, resulting in growth stimulation and inhibition of apoptosis (Bowden et al., 2002; Stoica et al., 2001). Similar to PTN, MK is also a small heparin-binding neurotrophic growth factor that is highly expressed in the developing brain. In a competitive manner, MK interacts with the ALK receptor and induces cellular signalling sufficient to support colony formation of SW-13 cells (Stoica et al., 2001). Importantly, both PTN and MK are up-regulated in most human tumours and are thus suggested to function as tumour growth factors, regulating tumour growth, angiogenesis and metastasis in vivo (Muramatsu, 2002). In Drosophila we have recently characterised two members of the MK/PTN family and named them miple 1 and miple 2. These two genes both encode secreted proteins which are expressed in non-overlapping patterns during embryogenesis, with miple 1 being predominantly expressed in the CNS whereas miple 2 is concentrated in the midgut endoderm (Englund et al., 2006). However, the function of these genes and their nature as putative ALK ligands are still an area of intense research.

The PLC-γ pathway: Phospholipase C-γ is a central molecule which has shown to play a pivotal role in the mitogenicity mediated by NPM-ALK. Transfection of NPM-ALK into Ba/F3 or NIH3T3 cells indeed induces tyrosine phosphorylation of PLC-γ, a direct interaction of PLC-γ SH2 domain with Y664 in NPM-ALK and a clear enhancement of PLC-γ activity. PLC-γ functions to hydrolyse membrane phospholipids into inositol triphosphate and diacylglycerol, consequently causing release of intracellular Ca²⁺ and activation of PKC (Pulford et al., 2004). The importance of PLC-γ in NPM-ALK induced tumourigenesis has clearly been demonstrated in studies where Y664 in NPM-ALK has been mutated to phenylalanine, a mutation that not only inhibits the interaction with PLC-γ, but also abolishes the transforming activity of NPM-ALK (Bai et al., 1998).

The RAS/ MAPK pathway: Similar to many other RTKs, both ALK itself and the oncogenic NPM-ALK activate the RAS/MAPK pathway. Tyrosines 156 and 567 have been shown to recruit the adaptor molecules IRS-1 and SHC, respectively (Bischof et al., 1997;
Fujimoto et al., 1996), thus linking the receptor to the RAS/MAPK pathway via the recruitment of the Grb2/SOS-complex which subsequently stimulate the association of RAS with GTP. Activated RAS-GTP triggers the kinase cascade involving Raf, MAPKKK, MAPKK and MAPK which finally stimulates cell proliferation. ALK is additionally believed to directly bind Grb2, given that the transforming activity of NPM-ALK is not abrogated by mutagenesis of Tyrosines 156 and 567 (Duyster et al., 2001).

**The PI3K pathway:** NPM-ALK recruits the regulatory p85 subunit of PI3K, probably indirectly, via binding to multiple adaptor molecules (Bai et al., 2000). This interaction clearly contributes to the mitogenicity, but is more importantly required for protection against apoptosis in transformed cells. Activated PI3K transmits signals via activation of the serine/threonine kinase AKT/PKB which subsequently mediates cell survival by several means. These include the phosphorylation and thus inactivation of the pro-apoptotic molecule BAD, maintenance of mitochondrial integrity, activation of NF-κB in addition to inhibition of Fas ligand transcription. AKT/PKB has also been suggested to mediate cell survival by inhibiting the forkhead family of transcription factors (FOXO3a/FKHRL1) (Gu et al., 2004).

**STATs:** Signal transducer and activator of transcription (STAT) proteins have lately proven to be essential mediators of signal transduction induced by NPM-ALK. In fact, all cell lines and patient biopsies from ALK-positive ALCLs, so far examined, show constitutive activation of STAT3, mediating enhanced expression of the anti-apoptotic proteins BCL-2 and BCL-XL, cell survival and enhanced cell growth (Pulford et al., 2004). In addition, NPM-ALK has been shown to activate STAT5 in a JAK2-dependent manner, an event which putatively is both mitogenic, anti-apoptotic and possibly essential for lymphomagenesis (Nieborowska-Skorska et al., 2001). Interestingly, STAT5 has recently shown to enhance transcription of RAD51, known to function in homology-dependent recombinatorial DNA repair, an event which also may contribute to the oncogenic potency of NPM-ALK (Slupianek et al., 2002).

**CD30:** ALCLs are characterised by the expression of the transmembrane receptor protein CD30, raising the question of CD30 function in tumourigenic cells. Already in 1994 a physical interaction between NPM-ALK and CD30 was established (Shiota et al., 1995), but to date it is unclear whether CD30 contributes molecularly to the development of ALCL or is just an epiphenomenon to NPM-ALK translocation. CD30, which normally is expressed on activated T-cells, belongs to the TNF receptor subfamily, but lacks the characteristic death domain that in the majority of TNF receptors binds death ligands such as FADD and TRADD (Duyster et al., 2001).

Recently the interactome of ALK was extended by a study using a mass-spectrometry based proteomic strategy to identify ALK-binding proteins. In addition to verification of the known binding partners, this study identified a number of adaptor molecules (SOCS, RhoGAP, RAB35), kinases (PKC, MEK kinase 1 and 4, EphA1, EphB, JNK kinase, MLCK, cyclin G-associated kinase, MAPK 1), phosphatases (meprin, PTPK) and heat shock proteins (Hsp60 precursor) as ALK interacting proteins (Crockett et al., 2004; Lim and Elenitoba-Johnson, 2006).

**ALK in oncogenic translocations**

The fusion of the first 117 amino acids of NPM, with the entire cytoplasmic domain of ALK, generated by the classical t(2;5) translocation, results in the expression of an 80 kDa protein
with constitutive PTK activity. The normal NPM protein is able to form hexamers, while a yet uncharacterised oligomerisation domain in its N-terminus serves to induce homodimerisation and activation of NPM-ALK molecules. The NPM piece of NPM-ALK is additionally responsible for the subcellular localisation of fusion proteins, targeting NPM-ALK to the cytoplasm as well as the nucleoplasm and nucleoli of lymphoma cells (Bischof et al., 1997). This NPM-mediated dimerisation and cytoplasmic localisation of NPM-ALK is necessary for its oncogenic activity. The potency of cell transformation is indeed demonstrated by the ability of exogenously expressed NPM-ALK to induce tyrosine phosphorylation of a number of signalling molecules, resulting in malignant transformation of a large number of cell types in vitro (Duyster et al., 2001). The in vivo oncogenic potency of the NPM-ALK chimera was elegantly reported by Kuefer et al who showed that transplantation of NPM-ALK expressing bone marrow into healthy BALB/cByJ mice triggered development of lymphomas within 4-6 months (Kuefer et al., 1997).

**Nucleophosmin:** Nucleophosmin (NPM) (also known as B23, numatin or NO38) is a highly conserved, ubiquitously expressed phosphoprotein that localises to the nucleoli, but constantly shuttles between the nucleus and cytoplasm. Interestingly the interactome of NPM includes a large number of proteins as well as PIP$_3$, DNA and RNA, contributing to the large number of cellular processes in which NPM is believed to be involved, including proliferation, apoptosis, ribosome biogenesis and regulation of genetic stability (Grisendi et al., 2006). In addition to its involvement in tumourigenic chromosomal translocations, NPM itself is frequently overexpressed in solid tumours, and certainly mutations in NPM have been identified as a causative factor for acute myeloid leukaemia (AML) (Grisendi et al., 2006).

**ALK fusion partners mediate oligomerisation and cytoplasmic localisation:** Since the initial identification of the NPM-ALK fusion protein in ALCLs, over 11 variant translocations generating oncogenic ALK fusion proteins have been described. These include fusion with tropomyosin 3 and 4, clathrin heavy chain, RanBP2 and non-muscle myosin heavy chain, among others. Regardless of fusion partner, ALK fusion proteins all (with a few exceptions) share some common features. Firstly, all proteins share the same breakpoint within the ALK gene, thus containing the entire cytoplasmic domain of ALK (amino acid 1058-1620). Secondly, the ALK C-terminus is generally fused to the N-terminal region of a partner with a widespread expression pattern, causing aberrant expression of the protein. Thirdly, the fusion partner is commonly responsible for the subcellular distribution of the chimeric protein, which often is cytoplasmic. In addition, the fusion partner always contains an oligomerisation domain which mediates homodimerisation or oligomerisation of fusion proteins, consequently mimicking ligand-mediated aggregation of ALK which induces constitutive activation of ALK-mediated signalling (Pulford et al., 2004). ALK fusions are recognised as the causative factor of 60-80% of all ALCLs, among which roughly 80% are NPM-ALK positive. Independent of fusion partner, ALK-positive ALCLs are all clinicopathologically identical. Furthermore, ALK fusions are also implicated in the aetiology of non-haematopoietic tumours such as inflammatory myofibroblastic tumours (IMT) (Griffin et al., 1999) and a few tumours of mesenchymal origin.

In addition to oncogenic translocations, full length ALK is also abnormally expressed in a variety of tumours of both haematologic (e.g. B-cell lymphomas) as well as non-hematologic origin, including rhabdomyosarcoma, neuroblastoma, glioblastoma, breast carcinoma and melanomas (Lim and Elenitoba-Johnson, 2006).
ALK in normal physiology

The role of ALK in normal physiology is an area that is still poorly understood. Early studies indicated a predominant expression of ALK in the central and peripheral nervous system, suggesting important roles during development and function of nervous tissues (Iwahara et al., 1997; Morris et al., 1994). Recently, however, a comprehensive investigation of ALK localisation in mouse embryos by Vernersson et al has not only verified ALK expression in specific regions of the central and peripheral nervous system, but has in addition also found ALK enriched in the eye, nasal epithelium, olfactory nerve, skin, reproductive organs and in the tissue surrounding the entire gastrointestinal tract, excluding only the hindgut (Vernersson et al., 2006b). However, the predominant expression of ALK in the neonatal brain suggests a role for ALK in brain development, putatively functioning as a receptor for a neurotrophic factor. This hypothesis is supported by the extensive overlap that has been observed between the expression patterns of ALK and the TRK family of neurotrophin receptors (Duyster et al., 2001). Gene targeting of ALK in mice have to date not provided any evidence for an essential role for ALK. Besides in vivo studies, ALK signalling pathways have been extensively studied in tissue culture experiments. In agreement with its tissue distribution, ALK has indeed been ascribed a role in neuronal differentiation since ALK activation can induce a MEK1/MAPK-dependent differentiation of PC12 cells (Souttou et al., 2001).

Negative regulation of RTKs

The importance of RTK-mediated signalling in most cellular processes has generated a necessity for stringent regulatory mechanisms, ensuring appropriate signalling outputs and avoiding aberrant cell behaviour. There are many ways in which RTKs are negatively regulated, some of which are transient, providing a reversible fine-tuning of the system and others that are definite and irreversibly cause degradation of the receptor itself (Dikic and Giordano, 2003). Transient regulatory mechanisms interfere with the strength and duration of the signal during a defined time period and can be accomplished in several different ways, including steric hindrance (of ligand binding as well as interaction with downstream targets), effector compartmentalisation, the activity of protein tyrosine phosphatases (PTPs) (inhibiting kinase activity in addition to altering protein/lipid docking sites) or by other post-translational modifications (Dikic and Giordano, 2003). In general, the activation of a signalling pathway simultaneously induces a negative feed-back loop that will ultimately terminate the signal. Sprouty proteins are classical examples of ligand-inducible negative feedback regulators of signalling pathways, specifically inhibiting ERK/MAPK signalling without affecting the PI3K or other MAPK pathways (Mason et al., 2006). The level at which Sproutys act appears to diverge depending on the Sprouty family member and the cellular context. Recently, however, the view of Sproutys as undisputed inhibitors has been challenged by observations indicating a permissive role of Sproutys for ERK/MAPK activation, via interaction with Cbl and CIN85 (Haglund et al., 2005) (see below) (Mason et al., 2006). Other examples of negative feedback regulators are the Cdc42-associated kinase ACK which stimulates clathrin-mediated endocytosis (Worby and Margolis, 2000) and kekkon-1, a transmembrane protein which forms heterodimers with EGFR, thus interfering with its autophosphorylation and consequently inhibits downstream signal transduction (Ghiglione et al., 2003). A more definite way of negatively regulating RTK-induced signalling is mediated by the removal of activated receptors from the cell surface via clathrin-dependent endocytosis, followed by degradation in the lysosomal compartment.
Endocytosis

Endocytosis is the process in which cell surface receptors are removed from the plasma membrane, a process which is essential for the downregulation of signalling as well as for regulating signalling output by altering subcellular localisation and composition of signalling complexes (Crosetto et al., 2005). In resting cells there is a low level of constitutive endocytosis and recycling of receptors, a process which in ligand-stimulated cells is strongly enhanced. Several mechanisms of endosome formation have been identified, the classical clathrin-coated-pit formation as well as clathrin-independent mechanisms including lipid-raft endocytosis as well as macropinocytosis and phagocytosis (Maxfield and McGraw, 2004).

Receptor internalisation: The most extensively characterised endocytic process is the internalisation of ligand-bound RTKs via clathrin-coated pits. This process is initiated by ligand-induced reversible modifications of the activated receptor which serve to recruit multimeric protein complexes, subsequently directing the enrichment of ligand-bound receptors in clathrin-coated pits. The main structural component of clathrin-coated pits is clathrin itself, which assembles into polygonal lattices, but the basic endocytic machinery required for receptor internalisation involves numerous accessory proteins, including the clathrin adaptor-binding protein AP-2 (adaptor protein-2), AP180/CALM, synaptotagmin, intersectin, synaptotjanin, epsin and eps15 (Conner and Schmid, 2003; Slepnev and De Camilli, 2000). Following the formation of the clathrin-lattice, endophilin, epsin and amphiphysin are involved in membrane invagination as well as necessary clathrin rearrangements. Subsequently, during the end-stages of endocytosis, the large GTPase dynamin self-assembles as a helical polymer around the neck of the nascent vesicle and induces the scission of the vesicle either by fission or stretching (Le Roy and Wrana, 2005). Dynamin additionally interacts with a number of actin-associated proteins such as profilin, cortactin, N-WASP and the Arp2/3 complex, thus playing a key role in inducing the actin rearrangements required for the endocytic process (Orth and McNiven, 2003).

The endocytic pathway: After internalisation, clathrin-coated vesicles are uncoated and fuse with the early endosome, a mildly acidic compartment where ligands dissociate from the receptors and the receptors subsequently are sorted into three alternative destinations within the cell (Figure 11). Proteins that are destined for recycling are either directly shuffled back to the plasma membrane, or are initially sorted to the so-called recycling endosome to later be returned to the cell surface. The remaining receptors are transported via the endocytic pathway to be degraded in the acidic lysosomal compartment by the action of various hydrolytic enzymes. The vision of the endocytic pathway as a consecutive transmission of internalised cargo to pre-existing compartments is today discarded in favour of the maturation model, in which the lysosomal system is considered as a heterogenous, tubular network where vesicles go through numerous stages of lysosomal maturation, starting off as early endosomes that transform into late endosomes and finally degradative lysosomes (Le Roy and Wrana, 2005).

The endocytic machinery: One of the key proteins in the endocytic machinery, that is recruited to the invaginating endosome, is Hrs (Hepatocyte growth factor receptor substrate). Hrs interacts, on one hand, via its two ubiquitin-interacting motifs (UIMs) with monoubiquitinated proteins, and on the other, through its FYVE domain, with PI3P, a phosphoinositolde enriched in endosomal membranes. At the endosomal membrane, Hrs forms a complex with another ubiquitin binding protein known as STAM (signal transducing adaptor molecule), initiating the recruitment of the three ESCRT complexes which mediate
the sorting of proteins destined for degradation, into multivesicular bodies (MVBs). The ESCRT-I, -II and III are multi-protein complexes (Endosomal Sorting Complex Required for Transport), that sequentially are recruited to and activated at the endosomal membrane. Here they function to concentrate ubiquitinated cargo to membrane microdomains, deubiquitinate the cargo in order to recycle ubiquitin and finally to induce the invagination and budding of vesicles from the limiting membrane of endosomes into the lumen of the compartment, forming MVBs (reviewed by (Babst, 2005; Slagsvold et al., 2006).
The Rab family of small GTPases: Importantly, the Rab GTPase family of regulatory proteins plays key roles in most aspects of membrane transport along the endocytic pathway, regulating fusion of different membrane compartments, the transport of vesicles along actin networks, as well as cargo sorting. Within the distinct endocytic compartments specific Rabs function to determine the fate of the internalised cargo and can consequently be used as markers along the pathway. In the early endosome enrichment of Rab5 ensures delivery of endosomal cargo to the Rab11-containing recycling compartment. Later on Rab7 has been shown to direct the transport of cargo from late endosomes to the lysosomal compartment (Stein et al., 2003).

Membrane dynamics in the endocytic pathway: The process of endocytic trafficking is accompanied by alterations in the composition of phosphatidylinositol phosphates (PtdInsPs) within vesicle membranes. These adjustments are rapidly mediated by the activity of specialised lipid kinases and phosphatases, giving rise to the unique membrane organization displayed by the various endocytic compartments which is required for the proper recruitment of effector proteins that regulate fusion and trafficking. The PtdIns-4,5-bisphosphate (PIP$_2$) is a key regulator in the formation and scission of CCVs and the subsequent dephosphorylation of PIP$_2$ by synaptojanin, generating PI(4)P, is essential for CCV uncoating. In early endosomes it is instead the PI(3)P that is enriched, where it plays an important role in the recruitment of FYVE-domain containing proteins including EEA1, and Rabenosyn-5, regulating endosome fusion (Simonsen et al., 2001).

Recently it has become evident that endocytosis is not merely a conventional mechanism to terminate signalling, but indeed is important for signalling output, affecting signal propagation as well as duration and amplification. The spatial and temporal control provided by compartmentalisation certainly contributes to signalling specificity as well as regulation and it is clear that signals from endocytic compartments may be qualitatively different from those initiated at the plasma membrane (Miaczynska et al., 2004).

Ubiquitination

Ubiquitin / Ubiquitination: In 1978, Ciechanover and Hershko described a small heat-stable protein functioning as a co-factor in the non-lysosomal protein breakdown pathway, a discovery that was awarded the Nobel Prize in 2004 (Ciechanover et al., 1980; Hershko et al., 1979). This protein, today known as ubiquitin, is a highly conserved protein consisting of 76 amino acids (8 kDa), that can be covalently attached to a broad range of target proteins, regulating cellular processes including cell-cycle progression, signal transduction, transcription, receptor down-regulation and endocytosis (Hershko and Ciechanover, 1998). Ubiquitin is present in all eukaryotic organisms and is fascinatingly only differing in 3 residues between human and yeast (Wilkinson, 2000). The ligation of ubiquitin to a lysine residue in a target molecule is known as ubiquitination or ubiquitylation and requires the sequential activity of three enzymes. Initially, the ubiquitin activating enzyme E1 activates ubiquitin in an ATP-dependent reaction, generating a high-energy thiol ester intermediate and results in an intermolecular linkage of the E1 to the C-terminal glycine residue in ubiquitin (G76). Subsequently, activated ubiquitin is transferred to a cysteine residue in an ubiquitin-conjugating enzyme, E2. In the final step, a ubiquitin ligase (E3) transfers the activated ubiquitin from the E2 onto an ε-amino group on a lysine residue in the target substrate, an event that can be direct or indirect (Pickart, 2001). In all species examined, the specificity in the ubiquitin system is mediated by E3 ligases of which there are an estimated number of 500-
1000 in mammals, compared to one single E1 and between 20-40 E2s (Goldberg, 2005). In general, E3 ligases recognise short stretches of amino acids known as “degrons” (e.g. the destruction box) in their target proteins, or are alternatively recruited by other post-translational modifications such as phosphorylation (Marmor and Yarden, 2004). This has been shown for a number of proteins including RTKs, regulators of the cell cycle such as cyclins and cyclin kinase inhibitors, transcriptional regulators including IκBα, β-catenin and p53 as well as signalling proteins like SMADs and TGFβ (Wilkinson, 2000).

**Ubiquitin modifications:**
Ubiquitination can occur in three distinct variants (Figure 12). Firstly, the addition of a single ubiquitin molecule to a substrate is known as monoubiquitination and the attachment of ubiquitin to several lysines on the same substrate is subsequently referred to as multiple monoubiquitination or multienzyme ubiquitination. In addition, different types of ubiquitin chains can be formed by the successive conjugation of multiple ubiquitins, via several of the seven lysine residues found within the ubiquitin molecule itself, in a process called polyubiquitination (Haglund and Dikic, 2005). It is well established that polyubiquitin chains formed via lysine 48 in ubiquitin designate target proteins for proteasomal degradation, while ubiquitin chains formed through lysine 63, on the other hand, are involved in endocytosis and DNA repair (Haglund and Dikic, 2005).

**Ubiquitin and protein degradation:** The best studied function of ubiquitin is its role in protein degradation, providing both a regulatory mechanism of cellular functions as well as a way of protein quality control, removing mutant or misfolded proteins in the cytosol and ER (Pickart, 2001). Proteins tagged by polyubiquitin chains are specifically recognised by the 26S proteasome which uses ATP to unfold and translocate proteins through the core proteosomal particle, where the polypeptide is degraded and the ubiquitin moieties subsequently can be recycled (Goldberg, 2005). Multi-ubiquitination of various cell surface receptors additionally has a well established role as an endosomal sorting signal, targeting receptors for lysosomal degradation (Haglund et al., 2003b).

**Ubiquitin and protein-protein interaction:** Besides targeting proteins for degradation, ubiquitin has an important role in mediating protein-protein interactions, and to date nine specialised ubiquitin-binding domains (UBDs) have been characterised. They diverge in sequence and folding but all interact with the hydrophobic Ile44 patch in the ubiquitin molecule and are together believed to participate in a large ubiquitin-network controlling a variety of cellular processes. Monoubiquitination has been shown to regulate target protein subcellular localisation, conformation, activity and protein interactions.

![Figure 12. Ubiquitination can occur in three distinct variants, all regulating specific events in the cell. (Reprinted from Haglund and Dikic, 2005, The EMBO Journal, with permission from Nature Publishing Group.)](image)
In many cases, ubiquitinated proteins themselves have been observed to contain UBDs which appear to participate in intra-molecular interactions, functioning to regulate protein activation status (Hurley et al., 2006).

**DUBs:** Ubiquitination is a reversible process that is counteracted by deubiquitinating enzymes (DUBs) that catalyse the hydrolysis of the peptide bond at G76 of the ubiquitin module. Two main families of DUBs are formed by the ubiquitin C-terminal hydrolases (UCH) and the ubiquitin-specific processing proteases (UBP) (Wilkinson, 2000). In addition to ubiquitin, eukaryotes have evolved a number of ubiquitin-like proteins, like Sumo and Nedd8 that get attached to target proteins by a similar type of machinery (Schmidt and Dikic, 2006).

**Ubiquitin in vivo:** It is clear that the ubiquitin system has crucial roles both in normal physiology, regulating development as well as adult immune responses and apoptosis, and naturally that the deregulation of this system is implicated in various pathological conditions. Genetic disorders such as the Angelman syndrome, a disorder characterised by seizures and mental retardation, the autoimmune syndrome APECED, Fanconi anaemia and neurodegenerative disorders such as Alzheimers and Parkisons have all been linked to defects in specific E3 ligases or proteins within the ubiquitin system. Naturally, since ubiquitin is such an important signal for directing intracellular trafficking and mediating appropriate signalling output, defects are also strongly implicated in cellular transformation and metastasis (Jiang and Beaudet, 2004).

**Cbl**

**Cbl structure and interaction partners:** Cbl, standing for Casitas B-lineage lymphoma, was first identified as the viral oncogene, v-Cbl, causing B-cell lymphomas and myelogenous leukaemia in mice (Langdon et al., 1989), but is today viewed as the key E3 ubiquitin ligase and adaptor protein that directs the internalisation of activated RTKs. The complex structural composition of Cbl is characterised by several protein-interaction modules and provides in itself extensive information concerning Cbl function (Figure 13). The N-terminal region of Cbl is dominated by a TKB domain, consisting of a 4 helix bundle, a Ca\(^{2+}\)-binding EF hand and an SH2 domain, mediating interaction with phosphorylated RTKs (EGFR, PDGF, HGFR etc.) as well as cytoplasmic PTKs (Zap-70 and Syk). Adjacent to the TKB domain is a RING finger domain, which serves to recruit ubiquitin-conjugating enzymes and mediate ubiquitin transfer. In addition, most Cbl variants also contain a UBA (ubiquitin associated) domain in their very C-terminus. The region between the RING finger and the UBA domain varies between different Cbl isoforms, but has been shown to mediate protein-protein interaction via multiple proline-rich regions, binding Grb2, Nck, ArgBP2 and SFKs, as well as multiple Tyr/Ser/Thr sites which, when phosphorylated, can interact with proteins such as Vav, Crk, p85 and 14-3-3. Importantly, the very C-terminal region of Cbl harbours a proline-rich domain known to directly associate with the CIN85/CMS family of adaptor molecules (Dikic et al., 2003; Jozic et al., 2005; Schmidt and Dikic, 2005).

Cbl is a highly conserved protein that in mammals is represented by three family members, including c-Cbl, Cbl-b and Cbl-3, differing mainly in the length of their C termini. c-Cbl and Cbl-b are both ubiquitously expressed and are believed to have crucial and redundant functions *in vivo*, given that both c-Cbl and Cbl-b knock-out mice are viable and generally healthy, whereas the double knock-out is embryonic lethal (Naramura et al., 2002). However,
the c-Cbl and Cbl-b deficient mice do show tissue-specific abnormalities in certain T cell populations, indicating unique as well as common functions for the different Cbl family members (Chiang et al., 2000; Naramura et al., 2002).

**Cbl and RTK-mediated endocytosis:** The involvement of Cbl in RTK internalisation and downregulation was first discovered in *C. elegans* where the Cbl homologue Sli-1 was shown to negatively regulate signalling from Let-23, the *C. elegans* EGFR homologue (Jongeward et al., 1995; Sternberg et al., 1995; Yoon et al., 1995). Upon EGFR activation Cbl is recruited to the phosphorylated receptor indirectly via Grb2 and directly by recruitment to phosphorylated Y1045 on EGFR itself (Schmidt and Dikic, 2005). Here Cbl promotes multiple monoubiquitination of activated EGFRs, initiating the assembly of clathrin-coated pits, thus ensuring efficient lysosomal targeting (Haglund et al., 2002). Following vesicle internalisation, the trafficking of endosomes along the actin and microtubule networks is also influenced by Cbl which interacts with actin-binding proteins and additionally affects the activity of small GTPases such as Cdc42 and Rac1, thus regulating actin polymerisation (Schmidt and Dikic, 2005).

Given the large number of proteins that directly or indirectly are affected by Cbl activity (Schmidt and Dikic, 2005), together with multiple reports on Cbl protein function, it is obvious that Cbl also exerts additional roles, including the regulation of bone resorption, glucose uptake and integrin-mediated cell spreading (Thien and Langdon, 2001).

**Drosophila Cbl:** The *Drosophila* genome contains a single *dCbl* locus that encodes two different isoforms, dCbl-S and dCbl-L. The N-terminus of these variants is shared, consisting of the conserved TKB and RING finger domains, which in dCbl-L is extended by the addition of a long C-terminal tail characterised by conserved proline-rich regions and a UBA domain (Hime et al., 1997; Robertson et al., 2000). Both isoforms are highly maternally contributed and is zygotically expressed at high levels in the embryonic CNS (Hime et al., 2001). The role of Cbl as a negative regulator of RTK-mediated signalling is clearly conserved in the fruit fly, given that dominant negative *dCbl* mutations enhance signalling from the *Drosophila* EGFR as well as activated forms of Ras, giving rise to melanotic tumours (Robertson et al., 2000). Accordingly, a dominant negative dCbl (*UAS-Dv-cbl*) has the ability to rescue phenotypes associated with dominant negative expression of EGFR (*UAS-Egfr·DN*), including loss of wing veins as well as restoration of the adult retina (Robertson et al., 2000). The functional conservation of dCbl is further reinforced by the observed molecular interaction of dCbl with both the *Drosophila* EGFR and PVR, as well as by the established molecular and/or genetic interactions with other components of the ubiquitin/endocytic machinery (including shibire, Dap160 and Gap1), in addition to Argos, a negative regulator of EGFR signalling (Robertson et al., 2000). Loss of *dCbl* leads to embryonic lethality, characterised by pleiotrophic phenotypes including CNS abnormalities and cuticle defects (Robertson et al., 2000). Clonal analysis of *dCbl* LOF has furthermore implied roles of dCbl in establishing polarity of the *Drosophila* egg, as well as in the guidance of migrating border cells during oogenesis (Jekely et al., 2005; Pai et al., 2000; Robertson et al., 2000).

![Cbl domain organisation](image)

**Figure 13.** Cbl domain organisation, phospho-acceptor residues are indicated (Y/S). The star indicates the CIT85-binding proline-rich region.
CIN85/CMS family of adaptor proteins

CIN85 and CMS together form a family of adaptor proteins which are important both for the transmission of signals elicited from RTKs as well as for the regulation of the RTKs themselves. CIN85 was first cloned as a Cbl-interacting-protein of 85 kDa in humans (Take et al., 2000), as Ruk (regulator of ubiquitous kinase) (Gout et al., 2000) or SETA (SH3 domain-containing gene expressed in tumourigenic astrocytes) (Borinstein et al., 2000) in rat and as SH3KBP1 (SH3-domain kinase binding protein 1) in mouse (Narita et al., 2001). Multiple studies have subsequently identified CMS (Cas ligand with multiple SH3 domains) and CD2AP/METS1 (CD2-associated protein/mesenchyme-to-epithelium transition protein with SH3 domains) (Kirsch et al., 1999; Lehtonen et al., 2000), and characterised them as CIN85 family members in human and mouse, respectively. In contrast to the CD2AP/CMS locus, which seems to encode a single transcript, the genetic locus encoding CIN85 is highly complex, comprising 24 exons, spanning over 320 kb on the X chromosome. In addition, multiple promoters as well as alternative splicing give rise to multiple protein isoforms. Several of these are ubiquitously expressed while some display a characteristic tissue-specific and developmentally regulated expression pattern, indicating a divergence in function between the different protein variants (Buchman et al., 2002; Gout et al., 2000).

Both CIN85 and CMS display a quite ubiquitous expression pattern, with CIN85 showing particularly high levels in the brain, thymus and spleen while CMS is concentrated in the kidney. Both proteins have been shown to interact with multiple binding partners, common as well as unique, and have consequently been implicated in processes such as formation of kidney glomerular architecture, organization of specialized junctions in T cells called immunological synapses and to coordinate multiple steps in endocytosis of RTKs (Dikic, 2002). To regulate these processes, a common mode of action has been proposed, whereby CIN85/CMS proteins function by clustering transmembrane receptors and mediating dynamic interaction with the actin cytoskeleton (Dikic, 2002; Haglund et al., 2002; Kowanetz et al., 2004).

The CIN85/CMS family is highly conserved through evolution and also in lower species, family members to display similar functions, given that the yeast homologue Sla1 (Synthetically lethal with ABP1) has been reported to be important for endocytic trafficking and cytoskeletal rearrangements (Stamenova et al., 2004).

CIN85/CMS Protein Structure

Structurally CIN85 and CMS are highly related, sharing 54% amino acid similarity. They are both composed by the same signature domain organisation, characterized by three N-terminal SH3-domains (A, B and C), a central proline-rich (PxxP) region and a coiled-coil domain at the C-terminus (Figure 14). The SH3 domains show high similarities among themselves and have been found to specifically interact with a large number of effector proteins, via an atypical proline-arginine motif (PxxxPR) (Kowanetz et al., 2003). The coiled-coil domain has been shown to mediate oligomerisation, while the PxxP region is the target of other SH3-domain containing proteins (Dikic, 2002). Moreover, both CIN85 and CMS proteins contain a region rich in serine and threonine residues between SH3 domain B and C which potentially can be regulated by phosphorylation, in addition to three Fx Dx F sequences in the N-terminus which may serve as binding sites for the clathrin adaptor protein AP2. Two features that are not shared between CIN85 and CMS is the presence of a C-terminal PEST sequence in CIN85.
and four putative actin-binding domains in CMS, respectively. To date there are no reports of tyrosine phosphorylation of CIN85, however CMS appears to be a target for SFKs (Kirsch et al., 1999).

CIN85/ CMS interaction partners and functional implications

The three SH3 domains in CIN85/CMS have been shown to interact with a multitude of effector molecules containing the PxxxPR consensus recognition motif, many of which are implicated in clathrin-mediated endocytosis, receptor recycling and cytoskeletal rearrangements.

**Cbl:** The most important function of CIN85 identified so far, is the downregulation of RTKs, mediated via the interaction with Cbl. Shortly after ligand stimulation of RTKs, c-Cbl is recruited to the receptor as well as phosphorylated on multiple tyrosines by SFKs, an event that enables the association between Cbl and CIN85. This interaction is accordingly diminished approximately in parallel to dephosphorylation of Cbl (Take et al., 2000) and functions both to assemble signalling complexes in addition to inducing internalisation and downregulation of receptors.

**CD2:** CD2 is an adhesion molecule which is expressed at the plasma membrane of T cells and NK cells. Here it has shown to bind multiple ligands expressed on antigen presenting cells (APCs) and contribute to the formation of a specialised junction (the so called “immunological synapse”), thus facilitating TCR recognition and antigen presentation. In response to T cell activation, the association of CD2AP with the cytoplasmic tail of CD2 is essential for CD2 clustering and T cell polarisation. CD2AP thus appears to function as a molecular scaffold linking the TCR to the actin cytoskeleton (Dustin et al., 1998).

**PI3K, p85α:** Phosphatidylinositol 3-kinases activate one of the most important and well characterised signalling pathways within mammalian cells and are essential for the regulation of cell homeostasis and survival. PI3Ks are formed by a smaller regulatory subunit, p85, and a larger catalytic subunit, p110, that catalyses the formation of various phosphoinositides, subsequently targeting proteins such as Akt/PKB, GSK3 and PLCγ for activation. The CIN85 family member Ruk has shown to interact with the p85α regulatory subunit of class Iα PI3Ks,
acting as a negative regulator of PI3K kinase activity, directly affecting the survival of primary sympathetic neurons in culture (Gout et al., 2000).

**p130Cas and SFKs:** Early studies identified CMS as an adaptor molecule regulating cytoskeletal arrangements and observed a co-localisation of CMS with p130Cas at leading edges and membrane ruffles in resting cells, as well as in cells plated on fibronectin (Kirsch et al., 1999). In the indicated study, CMS was also shown to be a target for tyrosine phosphorylation by c-Abl, Fyn and c-Src. One year later, a physical interaction between CIN85 and p130Cas was established by immunoprecipitation analysis and CIN85 was proposed to participate in the dynamic regulation of the actin cytoskeleton. In addition, a direct interaction between CIN85 and Crk has also been observed (Watanabe et al., 2000).

**AIP1/ALIX:** The apoptosis-linked gene 2 (ALG-2)-interacting protein, or ALG-2 interacting protein X, together with the associated ALG-2, are essential components of the apoptotic machinery. The CIN85 family member SETA has shown to form a complex with AIP1/ALIX, functioning to promote a pro-apoptotic signalling pathway. Indeed increased levels of either SETA or ALG-2 sensitize cells to apoptotic stimuli (Chen et al., 2000). Recently, ALIX/AIP1 was additionally shown to antagonise the CIN85/Cbl complex by competitively binding CIN85 at the activated receptor. Consequently this would inhibit CIN85/Cbl interaction, Cbl phosphorylation as well as EGFR ubiquitination, resulting in an inhibition of EGFR internalisation and a prolonged signalling from activated receptor complexes (Schmidt et al., 2004).

In addition to the above mentioned molecules, the list of verified or implicated CIN85/CMS interaction partners is long and includes proteins such as Grb2, SB1 /SETA binding protein 1, B cell linker protein, polycystine-2, Hip1R, ASAP1, ARAP3, synaptojanin, SHIP-1, STAP-1, p115RhoGEF (Kowanetz et al., 2004; Kowanetz et al., 2003).

**The role of CIN85 in endocytosis**

Most functional data on the CIN85/CMS protein family is available from its role in the regulation and endocytosis of RTKs (EGFR, c-Met, c-Kit and PDGFR). In response to RTK activation, Cbl ubiquitin ligases are associated with the cytoplasmic tail of the receptor, where it mediates monoubiquitination at multiple lysine residues (Dikic and Giordano, 2003; Haglund et al., 2003a; Haglund et al., 2002; Thien and Langdon, 2001). In turn, Cbl is phosphorylated on tyrosines by the activated RTK (as well as by SFKs), which promotes a conformational change in the C-terminal region of Cbl, exposing the CIN85/CMS docking motif (Dikic, 2002). As a result, Cbl recruits CIN85 together with the constitutively associated endophilins to the site of receptor activation. Here, endophilins induce negative curvature and invagination of the plasma membrane, subsequently initiating clathrin-mediated endocytosis of the activated RTK complexes (Figure 15) (Dikic and Giordano, 2003; Haglund et al., 2002; Pawson et al., 2002; Soubeyran et al., 2002). The importance of the Cbl/CIN85/Endophilin complex in the regulation of RTK-induced signalling is evident, since inhibition of complex formation is sufficient to block RTK endocytosis and degradation, but without perturbing the ability of Cbl to ubiquitinate activated receptors (Petrelli et al., 2002; Soubeyran et al., 2002). Interestingly, all three CIN85 SH3 domains have the ability to interact with Cbl, indicating that CIN85 is important for the clustering of Cbl at the site of receptor activation, an event that is crucial for EGFR endocytosis. In addition, CIN85 oligomerisation via the coiled-coil domain is also required for efficient receptor
internalisation. During the endocytic process, both Cbl and CIN85 are themselves also ubiquitinated and accordingly targeted for degradation together with the RTK, in the lysosome (Verdier et al., 2002).

Following receptor internalisation, CIN85 is also implicated in the coordination of vesicle trafficking along the endocytic pathway, in receptor degradation as well as in regulating the cross-talk between endocytic vesicles and the actin cytoskeleton. A model where CIN85 functions by dynamically changing interaction partners during receptor trafficking to temporally and spatially regulate the pathway is suggested, given the observed presence of CIN85 in several high-molecular-weight complexes (during different conditions) and the relatively low affinity by which CIN85 interacts with its target proteins (DiKic, 2002; Kowanetz et al., 2003). Recently, CIN85 was additionally found to interact with Rab4 in early endosomes, an interaction that has been shown to be important for regulating endosome morphology as well as the trafficking between early and late endosomes (Cormont et al., 2003).

While CIN85 acts to negatively regulate EGFR signalling, there are certain molecules that counteract CIN85/Cbl function in order to prolong receptor activity. Recently, it was shown that the above described negative feedback regulator Sprouty 2, can constitutively interact with two of the CIN85 SH3 domains, leaving the third SH3 domain free to associate with Cbl upon RTK activation. Consequently, the binding of Sprouty 2 to CIN85 inhibits CIN85-mediated clustering of Cbl, thus inhibiting EGFR endocytosis and degradation. Intriguingly, the balance of Sprouty 2/CIN85/Cbl complex formation was suggested to modulate signal duration and thus signalling outcome, for example proliferation versus differentiation of PC12 cells (Haglund et al., 2005). Another protein that counteracts CIN85 is ALIX, which similarly to CIN85 constitutively is associated with endophilins, but in contrast does not mediate receptor internalisation. ALIX has been shown to compete with CIN85 for Cbl binding at the receptor and functions to inhibit ubiquitination and internalisation of the receptor, thus augmenting signal transduction (Schmidt et al., 2004).

Figure 15. The role of CIN85 in RTK-mediated endocytosis. Cbl ubiquitin ligases recruit CIN85 and the constitutively associated endophilins, to the site of receptor activation. At the plasma membrane endophilins are proposed to induce negative curvature of the lipid bilayer and act in concert with the endocytic machinery, to induce clathrin-mediated internalisation of the active receptor complex. (Reprinted from DiKic, 2002, FEBS Letters, with permission from FEBS Editorial Office.)
The role of CIN85 in regulation of the actin cytoskeleton

The functions of CIN85/CMS family proteins appear to be performed by clustering transmembrane receptors and mediating dynamic interactions with the actin cytoskeleton, important for the process of cargo delivery along the endosomal pathway (Dikic, 2002). Indeed, confocal microscopy clearly shows an association of CIN85 with endocytic vesicles as well as the cytoskeleton (Schmidt et al., 2003; Soubeyran et al., 2002; Szymkiewicz et al., 2002). CIN85 has additionally been shown to bind directly to known regulators of the actin cytoskeleton including p115RhoGEF, Huntington interacting protein 1 related (HIP1R), Dab2, cortactin and Alix/AIP1 (Kowanetz et al., 2004; Schmidt et al., 2003).

CD2AP, like CIN85, links the actin cytoskeleton to the endocytic trafficking of RTKs via its association with CAPZ (Hutchings et al., 2003) and cortactin (Lynch et al., 2003). CAPZ binds to actin filaments, while cortactin interacts with the actin regulating multi-protein complex Arp2/3, thus regulating actin polymerisation. Additionally, CD2AP has shown to bind dynamin 2, a mechano-chemical GTPase that supports detachment of early endocytic vesicles from the plasma membrane (Lynch et al., 2003).

Recently, CD2AP was shown to localise with dynamic actin in podocytes, suggesting that CD2AP may be involved in endosomal sorting and trafficking events, by regulating actin assembly on vesicles (Welsch et al., 2005). In agreement with this, CIN85 is believed to positively regulate p115RhoGEF and negatively regulate ARAP3 (a RhoGAP), thereby coordinating changes in membranes with actin dynamics, which is necessary for the proper maturation and movement of endosomes (Kowanetz et al., 2004).

In vivo roles of the CIN85/ CMS protein family

The high homology, but divergent patterns of expression, indicate that CIN85 and CD2AP most likely both display unique roles as well as share redundant functions in vivo. In higher mammals, CD2AP has shown to exhibit cell-specific functions in the mouse kidney since homozygous knockouts develop severe renal disease at 3-4 weeks of age and subsequently die of renal failure a few weeks later (Shih et al., 1999). Interestingly the phenotype observed in these mice, very much resemble the pathology displayed by human patients suffering from focal segmental glomerulosclerosis (FSGS), which in some cases have been directly linked to mutations in the human CD2AP gene (Kim et al., 2003). The absolute requirement of CD2AP in these mice is indeed specific to renal functions since the transgenic reintroduction of CD2AP specifically in podocytes, is sufficient to rescue lethality, leaving these mice with only age-related testicular abnormalities and male infertility (Grunkemeyer et al., 2005).

In all organisms examined, CIN85 shows a high abundance in the central nervous system (CNS) (Buchman et al., 2002). Recently, CIN85 was additionally shown to interact with dendrin, a brain-enriched postsynaptic protein implicated in cytoskeletal modulation in dendrites as well as synaptic plasticity. This may suggest that CIN85 and dendrin collaborate in order to regulate endocytosis of synaptic membrane proteins (Kawata A, 2006).

To date, no CIN85 specific knockout animals have been reported, thus leaving the in vivo role of CIN85 as an open question and an area of intense ongoing research.
AIMS OF THE THESIS

The big picture

The scientific interest, for me as well as the Palmer lab in general, has always been based on the urge to understand biological processes and in particular to understand signalling pathways, how the dynamic interactions between molecules can enable a cell to sense the requirements for life and appropriately adjust to it. The aim is to contribute to the understanding of how these pathways function during normal physiological processes and to acquire knowledge of how the deregulation of signalling molecules can become the cause of disease and death. The main theme during the course of these studies has been to understand protein tyrosine kinases in the fruit fly, what they do and how they do it. The strategies have varied, but the main aim has remained the same, to contribute to the understanding for the molecular basis of life.

The smaller picture

The aims of the work presented in this thesis have been to understand the role of the protein tyrosine kinases FAK and ALK, as well as the regulation of RTK activity in the fruit fly. More specifically;

• To utilise the fruit fly, *Drosophila melanogaster*, to understand the role of the Focal Adhesion Kinase family of PTKs, avoiding the putative redundancy problems which has limited the gain of information from higher vertebrates. An additional aim has been to study the requirements of FAK in a tissue-specific context, in the CNS as well as in muscles.

• To understand the functions of Anaplastic Lymphoma Kinase in *Drosophila melanogaster* and to specifically investigate the signalling pathways that mediate these roles.

• To study the negative regulation of receptor tyrosine kinases in *Drosophila melanogaster*. This with the aim of characterising a fly CIN85/CD2AP family member and investigate its function, with emphasis on its putative roles in RTK endocytosis and downregulation.
RESULTS AND DISCUSSION

Focal Adhesion Kinase is not required for integrin function or viability in Drosophila (Paper I).

Fak56 is not essential for viability or fertility in Drosophila

The understanding of the in vivo role of Focal Adhesion Kinase in mammals has been limited for two major reasons. Firstly, loss of FAK expression in the mouse causes lethality at early embryonic stages (Ilic et al., 1995), hampering studies at later stages as well as in more specific contexts, and secondly, the more complex genomes of higher vertebrates contain additional FAK family proteins (such as Pyk2), which raise the issue of overlapping function and redundancy between the different family members. To avoid redundancy problems as well as to utilise the sophisticated array of genetic tools available in Drosophila melanogaster, we set out on a quest to understand the function of the single FAK family member encoded by the genome of the fruit fly.

Early studies identified Fak56 as a ubiquitously expressed PTK which appeared to be activated in response to integrin engagement and to show integrin-related phenotypes when overexpressed. We were interested in creating flies lacking Fak56 function and in order to do this we induced imprecise deletion of a P element inserted in the 5’UTR of the Fak56 locus at 56D. Assuming a deletion of Fak56 would be deleterious to the fly, we initially screened lethal deletion mutants for alterations in the Fak56 coding region. However, when a large amount of putative mutants had been analysed we were forced to consider the possibility that an animal lacking FAK family PTKs could be viable – and indeed, when screening through the homozygous viable stocks established in the screen, a Fak56 loss of function mutant was recovered. Despite being the initiator of screening viable stocks, this result was difficult to believe, until I managed to localise the breakpoints in the Fak56 CG1 deletion mutant, thus confirming the loss of Fak56 sequence – in addition to the absence of Fak56 protein that we could observe in embryo lysates extracted from these mutants.

The revolutionary and controversial finding that Fak56, previously believed to be THE mediator of integrin signalling, appeared non-essential to the fly, prompted us to verify our discovery using an additional approach. For this reason, we utilised the newly released DrosDel battery of FRT-containing P element fly strains (Ryder et al., 2004) to generate an additional deletion in the Fak56 genomic region. The smallest deletion possible, deleting Fak56 in addition to the Calpain A and putatively the hts/adducin loci, was thus generated (Fak56 CG2). Intriguingly this deletion turned out to be lethal, so in order to establish the cause of death in these flies we performed an EMS screen to characterise lethal point mutations in the region uncovered by the Fak56 CG2 deletion. Out of 6000 individual EMS treated flies, 10 lethal hits were recovered and subsequently screened for mutations in Fak56 as well as the Calpain A gene. The sequence analysis performed could not, in any of the lethal mutants, uncover any mutations that would result in either, a frame-shift, a truncation or any crucial alterations in amino acid identities. Having established this fact, we could conclude that the lethality observed in the Fak56 CG2 mutants were not caused by alterations in Fak56 or Calpain A, but rather must be due to yet uncharacterised point mutations in the hts/adducin locus. This was further reinforced by a complementation analysis, where a Fak56 deletion mutant generated in Dr. R. Hynes lab, showed to be viable over the Fak56 CG2 mutant.
**Fak56 is not required for integrin functions in Drosophila**

Even though the viability of Fak56 deletion mutant flies indicated that the Fak56 locus did not display any essential functions, we were intrigued as to why the FAK family of PTKs shows such high conservation from an evolutionary perspective. This, together with the multitude of data implicating FAK functions downstream of integrin receptors and cell adhesion in mammals, as well as the documented activation of Fak56 induced by cell adhesion, led us to perform detailed analysis of Fak56\textsuperscript{CG1} mutant flies, primarily focusing on processes known to be regulated by integrin-mediated functions. Since Drosophila integrin mutants classically display failure of adhesion, frequently resulting in lethality due to detachment of somatic muscles at late embryonic stages (Bokel and Brown, 2002), we first investigated muscle attachment in Fak56 mutant flies. Neither in Fak56\textsuperscript{CG1} or Fak56\textsuperscript{CG2} could we observe any defects in adhesion or displacement of any of the components known to be critical for attachment (including integrins, cytoskeletal scaffold proteins as well as ECM molecules). Furthermore, we investigated the migration of several cell types, which are known to display characteristic stereotyped movements during development as well as oogenesis (including cells of the germ line, trachea, gut endoderm and salivary glands, in addition to borders cells), but were unable to find any obvious defects in animals deficient for Fak56 protein.

In mammalian cells FAK has been suggested to be an important molecule mediating cross-talk between integrin adhesion receptors and growth factor-induced receptors, being activated in response to integrin adhesion as well as GF stimulation (Schlaepfer DD, 1994). In order to investigate a putative involvement of Fak56 in RTK-mediated activation of ERK in various tissues during embryonic development, we utilised an antibody specifically recognising the phosphorylated form of Drosophila ERK, described by Gabay and co-workers (Gabay et al., 1997). As indicated in Figure 16, there are no obvious alterations in ERK activation patterns in Fak56\textsuperscript{CG1} mutants. Consequently, Fak56 does not appear to be required for ERK activation at any stages of Drosophila embryogenesis.

Following this comprehensive analysis of Fak56 function, we were convinced that the apparent essential roles displayed by mammalian FAK, are not conserved in Drosophila. These findings are additionally strengthened, and may provide an explanation, as to why the earlier attempts made to target Fak56 for deletion, have failed. In our knowledge, several groups have conducted screens for lethal mutations in this region, as well as screens for integrin-associated loci. However, during the eight years since the identification of Drosophila Fak56, no such studies have managed to isolate Fak56\textsuperscript{!} In light of findings in *C. elegans*, where neither a deficiency uncovering the nematode FAK, *kin-32*, or RNAi-mediated knock-down of *kin-32* result in any obvious phenotypes (Wormbase, Release WS150, date 30 November 2005), it is tempting to speculate that the role of FAK family kinases as downstream mediators of integrin function, has been acquired at a time point evolutionary distal to the divergence of flies and humans.
Fak56 - an evolutionary flaw?

Naturally, unexpected results are always interesting and give rise to a number of questions. In this case questions such as; (i) Why is the FAK family of PTKs so highly conserved through evolution? Even if FAK family kinases have acquired their essential roles late in an evolutionary perspective, the sequence conservation is still a fact. (ii) What important roles, not required for viability, mediated by the Fak56 gene are important enough to preserve

Figure 16. Fak56 is not required for ERK phosphorylation during Drosophila embryogenesis. Fak56^{CG1} and W^{1118} embryos were analysed with anti-cphosphoERK antibodies and compared with the Drosophila atlas of MAPK activation patterns during embryogenesis, in order to analyse the requirement for Fak56 downstream of identified RTKs. (Schematics are reprinted from Gabay et al, 1997, Development, with permission from the Company of Biologists.)

Fak56 - an evolutionary flaw?

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Fak56 in the fly genome? (iii) How are the observed roles reported for vertebrate FAK regulated in Drosophila? (iv) Which, if any, of the 11 reported cytoplasmic PTKs found in the fly genome could potentially rescue the assumed lethality in Fak56 deficient animals?

(i) and (ii) Clearly, Fak56 must display essential roles in Drosophila, otherwise it would have rapidly been lost from the genome, given the short generation time and rapid evolution of the fly. Reasoning that Fak56 probably have functions which would not be apparent in the protective environment provided in a fly lab at UCMP, we addressed this question by subjecting Fak56^{CG1} mutant flies to external stress situations, including challenging of the immune system, wounding, starvation, unfavourable temperatures, UV-light and osmotic stress. Neither of the responses evoked by the above mentioned stresses seemed to have any obvious requirements for Fak56 function, given that Fak56^{CG1} and wild-type flies responded in similar ways. Not until we monitored the life span of Fak56 deficient animals could we observe anything away from the normal. Fak56^{CG1} mutants display a ∼50% reduction in life span when compared with wild-types, a phenotype which was accompanied by neurological defects, which will be further described in Paper II. The observed defects may well be the cause underlying the high evolutionary conservation of FAK family kinases, and would not be deleterious in the absence of natural selection pressure and predators.

(iii) In the mouse, FAK is essential for completion of embryogenesis, whereas in the fly, animals lacking Fak56 survive into adulthood. However, we do observe a reduced viability of Fak56^{CG1} mutant embryos, in approximation only ∼50% manage to survive into adulthood. Since no embryonic defects have been scored, mutant animals presumably die mainly during larval and/or pupal stages, which is in agreement with the observation of many dead pupae in the vials. In our lab we have always questioned the interpretation of the lethal phenotypes observed in FAK knockout mice, knowing that in every case where FAK expression is eliminated, there is an accompanying up-regulation of Pyk2 activity (Sieg et al., 1998). Given the wide range of phenotypes induced by the overexpression of Fak56 in the fly, it is tempting to speculate that the observed phenotypes in FAK deficient mice are in fact a result of aberrant Pyk2 activity. This would be in agreement with early studies, demonstrating that overexpression of Pyk2 in fibroblasts induce a rounded cell morphology, enlargement of focal adhesions and marginalisation of F-actin to the cell periphery (Schaller and Sasaki, 1997), in many ways similar to the phenotype displayed by FAK deficient fibroblasts. In order to address this possibility, it would be necessary to generate a FAK/Pyk2 double mutant mouse, since this would be the only way to, in an unbiased manner, investigate the effect caused by a complete loss of FAK family kinases. This would in addition provide a means to investigate tissue-specific roles of the individual family members and avoid redundancy problems.

(iv) The 11 cytoplasmic PTKs encoded by the Drosophila genome all fall into defined, well characterised families of PTKs, which in general are represented by one single member in the fly. Among these molecules, no other PTK shares the same domain organisation as Fak56. In fact, the only other PTK containing a FERM domain, the Jak kinase, paradoxically contains the kinase domain that is most unrelated to the Fak56 kinase domain. In light of these findings, there is no given PTK candidate that would naturally compensate the absence of Fak56. My feeling is that integrin-mediated functions, in the fly, are mediated by the SFKs and that Fak56 more importantly displays functions in the CNS. In fact, recent studies have implicated SFKs to directly be targeted to integrin tails and to be activated by integrin signalling, independently of FAK kinases (Arias-Salgado et al., 2003). Together with the
finding that SFKs can rescue the cell migration defects displayed by FAK deficient cells (Hsia et al., 2003; Moissoglu and Gelman, 2003), this supports a role of SFKs as the main PTKs mediating integrin signalling within cells. Additionally, this hypothesis would be in agreement with our observation that tyrosine phosphorylation of proteins at embryonic muscle attachment sites is unaffected in Fak56CG1 mutants (Figure 17). Similar tendencies are apparent also in the mammalian FAK field, where the role of FAK as the most important mediator of integrin signalling, has transformed into a view where FAK displays functions of more accessory nature.

The importance of the p130Cas/Fak56 complex is not conserved through evolution (unpublished observations)

In our quest to understand the role of Fak56 and its putative involvement in integrin-mediated functions, we became interested in p130Cas, a cytoskeletonally linked adaptor protein, which in vertebrates physically both can interact with and get phosphorylated by FAK. Consequently, we made a perfect deletion of the Drosophila homologue of p130Cas at 61B1-B2, utilising FLP/FRT-mediated recombination between two FRT-containing transposable elements (Figure 18), obtained from the Exelixis stock collection (Parks et al., 2004; Thibault et al., 2004). To our surprise, we found that flies lacking the p130Cas locus were also viable and fertile, displaying no obvious phenotypes. Accordingly, we were now able to analyse the simultaneous loss of both Fak56 and p130Cas in the fruit fly and could even more surprisingly conclude that flies totally lacking both Fak56 and p130Cas, were viable and fertile. Extensive analysis of these animals is still to be performed, but from only the observed lack of lethality, we can conclude that the requirement of the FAK/p130Cas complex in mammals probably has acquired its essential functions later in evolution, while it in fruit flies only displays accessory roles.

Fak56 is a negative regulator of integrin-mediated adhesion

Given that the majority of knowledge gained concerning FAK function in mammals is based on studies where FAK is either overexpressed or ectopically expressed, we further wished to analyse the effect of excessive Fak56 levels in the fly. Indeed, similar to the phenotypes observed for FAK, high abundance of Fak56 results in the generation of a wide range of
defects, which in many cases can be directly related to integrin function. Ubiquitous overexpression of Fak56 results in lethality, whereas more selective expression in cell-type specific contexts generates phenotypes such as muscle detachment and wing blistering, suggesting that excess Fak56 results in the downregulation of integrin-mediated adhesion. Interestingly, closer examination of detached muscles in Fak56 gain-of-function situations, reveal an unexpected release of integrin binding to ECM molecules. In contrast to observations made in mutants for other cytoplasmic integrin effectors such as ILK and talin, Fak56 overexpression does not cause a failure in the linkage between integrin tails and the actin cytoskeleton, but does rather result in a dissociation of integrins themselves from ECM molecules in the extracellular space (Figure 19). It is highly possible that excess Fak56 causes aberrant protein tyrosine phosphorylation, resulting in an alteration of integrin conformation and thus affinity for ECM ligands, causing the observed adhesion defects. Given the ascribed role of vertebrate FAK in focal adhesion turnover, the increased tyrosine phosphorylation caused by excess Fak56, could also enhance adhesion disassembly in Drosophila, further contributing to the observed failure of strong cellular adhesion.

Overexpression is one thing, but the interesting question still remains, that is; is this implicated role for Fak56 as a negative regulator of integrin function relevant in an in vivo perspective? The answer to this question appears to be YES. Indeed, we have established a genetic interaction between Fak56 and the βPS integrin subunit, given that reduction of integrin function (utilising mys hypomorphs) enhances the wing blistering phenotype observed in response to Fak56 overexpression. This observation clearly confirms that overexpressed Fak56 negatively regulates integrin adhesion in Drosophila. Furthermore, we have also confirmed a requirement of integrin function for the phosphorylation of Fak56 at muscle attachment sites. Taken together, Fak56 appears to display non-essential accessory roles in the organization of integrin-mediated adhesion.

The main scientific contribution of this study, besides demonstrating that the essential role of FAK family kinases is not conserved through evolution, is providing a relevant example of the dangers of studying protein function in a system based solely on overexpression. It is very clear that despite the observation that Fak56 is not required for Drosophila development, the presence of excess Fak56 protein produces a variety of phenotypes, indicating that the overloading of the system is likely to cause neomorphic and aberrant protein functions, which

Figure 16. Generation of a pt30Cas deletion mutant. Drosophila pt130Cas, encoded by CG1212, was removed by FLP/FRT-mediated recombination between RB03132 and WH00069 from the Exelixis stock collection.
are not relevant to normal physiology. It is always the endogenous situation that will provide the truth.

**Mutation of *Drosophila* Focal Adhesion Kinase induces Bang Sensitive behaviour and disrupts axonal conduction and synaptic transmission. (Paper II).**

The abundance of Fak56 expression in the fly CNS suggests a neurological role for Fak56. This would also be in evolutionary agreement with the reported roles of FAK family kinases in neuronal development and survival, as well as modulation of synaptic plasticity in the adult. In light of this, we have recently identified a role for Fak56 in conducting axonal neurotransmission and regulating neuromuscular function. We have found that the reduced life span displayed by Fak56 mutants is accompanied by sensitivity to high-frequency mechanical and electrical stimulation, resulting in a phenotype known as “Bang Sensitivity” in *Drosophila*. In collaboration with Dr. Chun-Fang Wu we have further used electrophysiological methods to demonstrate that Fak56<sup>CG1</sup> mutant flies display a weakening of action potential propagation and an excitatory junctional potential defect. In Fak56<sup>CG1</sup> mutants seizures are evoked at lower threshold values and occur at higher rates than in wild-type controls. Additionally the seizures evoked in Fak56<sup>CG1</sup> mutants, is followed by a long paralytic period which lasts up to several minutes. In support of a role for Fak56 in synaptic regulation, we have shown that in response to increased intracellular calcium levels, endogenous Fak56 protein is dephosphorylated on tyrosines in *Drosophila* cell lines, suggesting that the modulation of Fak56 function is an important step during neuromuscular transmission *in vivo*.

Despite numerous efforts to explain the molecular basis for the BS phenotype in Fak56<sup>CG1</sup> mutant flies, no conclusive results have yet been obtained. Initially we believed loss of Fak56 to cause neurodegeneration due to absence of survival signalling, given that FAK is implicated in the aetiology of neurodegenerative disorders such as Alzheimers (Williamson et al., 2002; Zhang et al., 1994). However, we could not detect any signs indicative of neurodegeneration or excess apoptosis in aged Fak56<sup>CG1</sup> mutants, which together with our
observations that the BS phenotype in $Fak^{CG1}$ mutants declined with age, led us to dismiss this theory in favour of a more direct role of Fak56 in the modulation of neurotransmission.

In order to establish if the BS phenotype is caused by loss of Fak56 in the pre- (neuronal) or post- (muscular) synaptic compartment we utilised the GAL4-UAS system to modulate the level of Fak56 in these tissues, using both overexpression constructs as well as inducible $Fak^{dsRNA}$ hairpins. During our investigations we observed that alterations of Fak56 levels in muscles, but not in neurons, seemed to induce BS, thus implying a failure of the muscles to properly respond to neuronal signal transmission. Given that FAK has been reported to regulate axonal branching in the mouse (Rico et al., 2004), we also performed a detailed analysis of neuromuscular junction morphology in $Fak^{CG1}$ mutant larvae. However, analysis of neither pre- or post-synaptic markers, revealed any obvious disruptions in NMJ morphology, further emphasising a role for Fak56 in the direct regulation of neurotransmission. In addition, when trying to dissect signalling events downstream of Fak56 dephosphorylation in response to increased calcium levels, in $Drosophila$ cell lines, we were unable to make any conclusions regarding Fak56 targets. Since the characterised BS mutants do not display any common features regarding signalling pathways or compartmentalisation and in fact are quite poorly studied in regard to molecular mechanisms, we have not further pursued the hunt for the underlying mechanism behind the BS caused by loss of Fak56.

A role for Fak56 in the modulation of neuronal transmission, possibly reinforcing LTP, would be in agreement with a conservation of Pyk2 functions in $Drosophila$ Fak56, rather than functions mediated by FAK. Pyk2 has been reported to regulate synaptic plasticity and to be activated in response to stimuli that increases intracellular calcium, such as neuronal stimulation. Furthermore, the removal of Pyk2 from the mouse genome is not deleterious to the animal. Indeed, Fak56 protein is modulated by changes in calcium levels, and $Fak^{CG1}$ mutant flies clearly display an inability to properly adjust to alterations of calcium levels. In situations with calcium-enriched environments, $Fak^{CG1}$ mutants display unpredictable scattering of EJPs, occasionally causing a complete failure of neuronal transmission. This is in contradiction to wild-type animals which regularly respond in a highly predictable manner, characterised by a facilitation and step-wise increase in EJP size. The lack of neuronal defects in Pyk2 knockout mice might be explained by the apparent ability of FAK and Pyk2 to compensate for the loss of each other, previously observed in several other contexts. A role for Fak56 in neuromodulation would further also be in agreement with an accessory role of Fak56 in the modulation of integrin function, given that integrins are also implicated in the modulation of neuronal circuits, specifically in the establishment of olfactory memory in fruit flies (e.g. the volado locus) and the regulation of neuronal plasticity at larval NMJs (Beumer et al., 1999).

A few recent studies have addressed the modulation of FAK and Pyk2 in response to electroconvulsive shock (ECS) (Jeon et al., 2001; Kang et al., 2004), and have reported a strong divergence in activation patterns. In the cerebral cortex and in the hippocampus, Pyk2 is rapidly and transiently (peaking at 1 min) tyrosine phosphorylated. This is clearly in contrast to FAK which is initially momentarily dephosphorylated, but within 2 min of stimulation returns to normal levels (Kang et al., 2004). These findings could imply an ECS-mediated activation of an uncharacterised phosphatase at the site of stimulation, causing FAK dephosphorylation, similar to the observed situation in the fruit fly. However, while FAK in vertebrate neurons rapidly becomes phosphorylated again, Fak56 remains dephosphorylated for several minutes. The apparent dynamics of FAK and Pyk2 in neuronal cells in different contexts may well be in concurrence with strong dynamics also in an evolutionary
perspective, allowing different species to divergently develop their own means to utilise the FAK family of PTKs. In this aspect it is also of significance to consider the reported neurotropic effect elicited by ECS (Vaidya et al., 1999), inducing neuronal sprouting. This would be in agreement with the documented mobilisation of activated Pyk2 to plasticity-related regions such as neurites and growth cones, its induced interaction with Src and documented role in actin reorganisation. This may well be a mechanism by which FAK family kinases could function in neuronal plasticity-related signalling, possibly conserved also in *Drosophila melanogaster*.

An intriguing characteristic in some of the reported BS mutants is their ability to physiologically adapt to their defects in neurotransmission, so that they with time in some way can compensate the loss of a critical gene, diminishing the sensitivity to HF stimuli. In *Fak56* mutants clear signs of adaptation can be observed after 2-3 days and at 3 weeks of age, the mutants show behavioural patterns similar to wild-type animals. The mechanism behind neuronal adaptation is poorly understood, but it is tempting to speculate that Fak56 is important in the construction/fine-tuning of neuromuscular communication that is required for adjusting the fly to a life outside the protective pupae. In the absence of Fak56, the maturation of these synapses may be delayed before the loss of *Fak56* can be compensated by other mechanisms possibly mediated by the up-regulation of other PTKs and/or cytoskeletal proteins.

Regardless of the lack of understanding concerning the molecular mechanisms responsible for bang sensitivity, the BS category of *Drosophila* mutants is nevertheless interesting in light of the obvious similarities displayed by BS fruit flies and humans affected by seizure disorders such as epilepsy. Epilepsy is indeed a serious health problem world-wide, but is poorly understood. Hopefully the discovery of additional BS mutations and the utilisation of BS mutants as model systems for human seizure disorders, will contribute to the understanding of the mechanisms underlying disease, as well as the development of new drugs. Indeed, genes encoding both ion channel subunits and cell adhesion molecules have been linked to epilepsy, which further reinforces the relevance of using BS mutants as model systems for seizure disorders (Meisler and Kearney, 2005).

These novel findings of a role for Fak56 in the regulation of neurotransmission in *Drosophila* might also reflect other putative functions for Fak56 in the nervous system, which have not yet been investigated. Accordingly, other BS mutants have been reported to display important roles in additional neuronal functions, as can be exemplified by the observed hearing deficiency in *tko* mutants (Toivonen et al., 2001).

**Implications for Fak56 in DAlk-induced cellular transformation (Unpublished observations).**

FAK family kinases have in all organisms examined been shown to be activated in response to growth factor stimulation of RTKs. In *Drosophila*, we have found Fak56 to be directly activated by the Anaplastic Lymphoma Kinase, DAlk (Figure 20), although the physiological significance of this event remains to be established. Given the enhanced activation of FAK observed in a large number of human tumours, we wished to address the role of Fak56 for DAlk-induced tumourigenesis. Independent of the tissue under examination, induced expression of DAlk always causes malignant transformation and aberrant cell growth. In order
to investigate any contributing role of Fak56 in DAlk-mediated cellular transformation we ectopically expressed DAlk, in the absence or presence of Fak56. Interestingly, in animals deficient for Fak56 protein, cellular transformation appeared to be less extensive, although we did not observe a complete block in tumourigenic growth.

Identification and characterisation of DAlk; a novel Drosophila melanogaster RTK which drives ERK activation in vivo (Paper III).

Identification of a Drosophila Anaplastic Lymphoma Kinase, DAlk

Similar to the discovery of Drosophila Fak56, DAlk was identified by a degenerate PCR-based approach, using primers designed to pick up highly conserved residues within protein tyrosine kinase domains. The full length cDNA we subsequently obtained from Drosophila cDNA libraries was found to encode a new member of the insulin receptor superfamily, consisting of 1701 amino acids, distributed over 10 exons in genomic region 53C10-11. Sharing 34% identity and a similar domain organisation, we concluded the newly identified gene to be a homologue of the mammalian anaplastic lymphoma kinase and consequently named it DAlk. The 8.5 kb DAlk mRNA transcript encodes a polypeptide that is characterised by a signal sequence, an extracellular domain (containing an LDLa domain, two MAM domains and a glycine rich region), a single transmembrane region and a classical cytoplasmic PTK domain. In addition to these conserved signature domains, several of the consensus phosphotyrosine motifs, including the binding site for IRS1 and Shc, are also conserved in the DAlk sequence.

The endogenous DAlk protein, which appears as a 200 kDa phosphoprotein on a Western Blot, was found to predominantly be expressed in the developing visceral mesoderm (VM) and CNS during Drosophila embryogenesis. Specifically, we could first detect DAlk expression in the VM at stage 10/11, initially seen as segmental patches and later on as a continuous waved line. The expression in the CNS appears to be initiated at later embryonic stages (i.e. Stage 13), when DAlk protein is abundant in the developing brain and ventral nerve cord. While the expression of DAlk in the gut (i.e. visceral mesoderm) declines after completion of embryogenesis, the expression in the CNS is maintained throughout larval stages and can also be detected in the adult fly.
Interestingly the *Drosophila* genome does not contain any LTK homologs, rendering *DAlk* the sole member of this branch of RTKs.

**DAlk completes the Drosophila atlas of RTK-mediated ERK-activation**

In the previously described atlas of MAPK activation during *Drosophila* embryogenesis, conducted by Gabay and co-workers (Gabay et al., 1997), most patterns of ERK activation could be ascribed to the activity of known RTKs. Intriguingly, however, we found that one of the uncharacterised patterns, the one in the VM of stage 11 embryos, overlapped with the pattern of DAlk expression. Upon closer examination we could indeed detect a co-localisation of DAlk and phosphoERK at these stages. Due to the unavailability of *DAlk* mutants at this point we could not directly address the question of DAlk requirement for ERK activation in the VM. However, we could certainly observe that ectopically expressed DAlk induces a general tyrosine phosphorylation of target proteins, as well as a specific phosphorylation of ERK. Clonal induction of a *DAlk* transgenic construct furthermore implied a strong proliferative effect of DAlk, given that cells expressing DAlk acquired a transformed appearance, displaying an uncontrolled growth, compared to surrounding wild-type tissue. Consequently, DAlk-expressing cells form tumour-like cell clusters in imaginal discs and disrupt normal disc development, eventually causing lethality. Indeed, the deleterious effect of aberrant DAlk activity was further verified by the embryonic lethality induced by ubiquitous DAlk expression during *Drosophila* embryogenesis. Taken together, these findings strongly suggest that *DAlk* could be the novel RTK responsible for ERK activation in the VM *in vivo*. Given the commonly observed role of RTKs in the development/specification of various tissue systems, we speculated that *DAlk* could play a role in the specification of the VM.

It is of significance to note that signalling from the DAlk receptor appears to be induced when DAlk is ectopically expressed, as observed in larval imaginal discs. This is in agreement with findings in mammalian cell lines and suggests that ALK family kinases have the ability to, under certain circumstances, be activated in a ligand-independent manner. It is also likely that boosting the system with excess amounts of DAlk protein induces spontaneous receptor dimerisation at a threshold level exceeding a certain receptor density.

**DAlk is required for gut development in Drosophila** (Paper not included in this thesis)

To address the question whether the strong expression of DAlk in the VM is relevant for DAlk function *in vivo*, we decided to target the *DAlk* locus for mutagenesis. Since at this time no deficiencies covering the *DAlk* locus were available, we started out by creating a deletion encompassing the *DAlk* gene, together with roughly 30 genes located in the flanking regions. This was accomplished by a simultaneous mobilisation of two P elements that in advance had been recombined onto the same chromosome. By Southern Blot analysis we were subsequently able to identify one fly where the desired recombination event had occurred. Given that the generated deficiency removed *DAlk* as well as multiple other genes, an EMS mutagenesis screen was performed in order to specifically target *DAlk*, using the deficiency to define the target region. Among 4760 EMS treated flies, 68 lethal mutations falling into 8
complementation groups were obtained, indicating 8 essential genes in the region uncovered by the deficiency.

By comprehensive sequencing analysis one complementation group, containing 11 independent lethal hits, was identified as *DAlk* mutants. By studying the nature of the point mutations that caused deleterious effects regarding *DAlk* functionality, we could conclude a requirement for an intact composition of the second MAM-domain, the glycine-rich region, in addition to the kinase domain, for proper *DAlk* activity (Figure 21).

All *DAlk* mutants obtained were phenotypically indistinguishable, displaying lethality at late embryonic or early larval stages. By performing a food coloration assay we discovered the lethality to probably be caused by starvation, given that no midgut structures could be observed in *DAlk* mutants (Figure 22). Closer examination of *DAlk* mutant embryos subsequently revealed that in embryos deficient of *DAlk*, the VM is severely disrupted and fails to maintain the expression of markers for differentiated VM (such as Fasciclin III, Mef2 and MHC), putatively indicating a loss of muscle specification and/or differentiation.

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**Figure 21.**
Schematic representation of domain alterations in *DAlk* mutant alleles. The wild-type *DAlk* domain structure is shown at the top. Importantly, the *DAlk* allele was used for the phenotypic analysis described. *DAlk* was used to investigate *DAlk*-mediated internalisation of active Jeb/DAlk complexes. *DAlk* and *DAlk* are considered as kinase dead alleles.
Furthermore, by analysing activation patterns of activated ERK in DAIlk mutants we could conclude that DAIlk indeed is the RTK which is responsible for ERK activation in the developing VM, given the lack of ERK phosphorylation in the VM of stage 11 DAIlk mutant embryos (Figure 22).

Jeb signals through the Alk receptor tyrosine kinase to drive visceral muscle fusion (Paper IV).

Similar phenotypes suggest a functional relationship between DAIlk and Jeb.

When screening the literature, we noticed that the VM phenotype displayed by DAIlk mutant embryos was strikingly similar to the phenotype observed in embryos deficient of the secreted molecule Jelly belly (Jeb) (Weiss et al., 2001). Both mutants show a loss of intestinal structures and the closer examination we subsequently performed, revealed a loss of the characteristically organised bands of VM cells that normally is present during the time of VM specification, rendering the VM cells to form disorganised groups, scattered in the region. Interestingly, both DAIlk and jeb mutants fail to maintain the expression of proteins considered as markers for differentiated visceral mesoderm, including Fasciclin III, which normally overlaps with DAIlk expressing cells during midgut development.

The explanation of the following findings requires a short introduction to Drosophila VM development, which proceeds as follows. The Drosophila visceral muscles are organised in two sets of muscle types, an inner layer of circular muscles (derived from cells along the trunk of the embryo), which is surrounded by an outer layer of longitudinal muscles (derived from the posterior end of the embryo) (Campos-Ortega, 1997). Initially the presumptive mesoderm forms 12 metameric clusters at stage 10 of embryogenesis. These clusters subsequently migrate, first longitudinally to form two parallel bands along most of the length of the embryo, and then ventrally and dorsally to form a closed tube. This tube simultaneously gets lined by endoderm, which utilises the VM as a template during its migration from the opposite poles of the Drosophila embryo, where the anterior and posterior midgut primordia are formed (Tepass and Hartenstein, 1994). Similar to somatic muscles, the visceral muscles arise from the fusion of FCs with FCMs. In order to generate a functional muscle the FCs initially need to be specified, an event that takes place during stage 11, when the FCs become arranged in an organised row of columnar cells (Figure 23). By performing confocal
analysis we were interested to observe a lack of this critical columnar organisation of FCs in both DAlk and jeb mutant embryos, accompanied by a subsequent loss of muscle fusion.

**Jeb-DAlk interaction activates an ERK-mediated pathway essential for VM fusion.**

In light of the previously observed requirement of DAlk function for ERK activation in the VM, we wished to investigate if this prerequisite was also shared by Jeb. Consequently we analysed jeb deficient embryos with anti-dpERK antibodies and could satisfactory enough conclude that indeed, also jeb mutants displayed a failure of ERK activation in the stage 11 visceral mesoderm.

Since the DAlk and jeb mutant phenotypes imply a failure of muscle specification, and given that RTKs generally are known to mediate these types of signals, we were interested to investigate the expression of typical founder cell identity markers in these mutants. The adhesion molecule duf/kirre is known to be expressed specifically in the FCs of the VM and when we analysed duf/kirre expression in DAlk and jeb mutant animals, we were exited to observe that clearly both DAlk and Jeb are essential for duf/kirre expression in the VM. Instead of being specified as FCs, all VM cells in DAlk and jeb mutant embryos appear to, by default, develop into FCMs. In conclusion, the simultaneous activity of DAlk and Jeb proteins appear to activate an ERK-mediated pathway which induces expression of the FC identity gene duf/kirre and that this pathway is essential for muscle fusion and consequently the formation of a gut (Figure 24). Similar findings have also been reported by other groups (Lee et al., 2003a; Stute et al., 2004).
**Jeb is a ligand for DAlk**

All observations thus far in our analysis of visceral muscle fusion, pointed towards the suggestion that the secreted Jeb molecule functions as a ligand/receptor pair with DAlk. Our additional finding that overexpression of wild-type DAlk partially can rescue muscle specification in jeb deficient animals further emphasised this hypothesis. This would also be in agreement with the formerly discussed ligand-independent (thus Jeb-independent) activation of DAlk receptors, exceeding a certain density.

We reasoned that if we could show a direct interaction between Jeb and DAlk we could be quite sure that our hypothesis is right. Accordingly we found that indeed Jeb and DAlk could be immunoprecipitated in extracts from wild-type embryos, and additionally that Jeb and DAlk showed a direct interaction in a cell-free ELISA-based assay. Our findings were further reinforced by the observation that DAlk-expressing COS-7 cells specifically can recruit recombinant purified Jeb protein added to the cell culture medium. Taken together, these data confirm that Jeb is a ligand for DAlk.

**DAlk and jeb mutant VM cells incorporate into the somatic mesoderm**

Given that jeb and presumably also DAlk mutant VM cells, by default, are specified as FCMs in the absence of the Jeb/DAlk/ERK/Duf-pathway to induce FC fates, we were interested to investigate the destiny of these cells. To do this we analysed DAlk mutant embryos with anti-DAlk antibodies, to follow VM cells, together with anti-DMef2 antibodies, to label muscle cells in a general manner. When analysing late stage embryos we could observe that the DAlk-labelled VM cells appeared to be attracted towards the somatic mesoderm and indeed, later on these cells could be found incorporated into the somatic muscles. In light of this we can conclude that the excess FCMs that are produced in a DAlk mutant retain their fusion competence, and that they in the absence of visceral mesoderm FCs instead fuse with FCs of other muscle cell lineages (i.e. somatic muscles). These findings are furthermore in agreement with the observation of comparable phenotypes in bin and hap mutants, which encode a homeobox transcription factor and a forkhead domain protein, respectively, both required for visceral muscle specification and fusion (Azpiazu and Frasch, 1993; Zaffran et al., 2001).

**Jeb is internalised into FCs via DAlk-mediated endocytosis**

Previous studies have shown that Jeb normally is expressed in the somatic muscle precursor cells (Weiss, 2001). In fact, Jeb molecules are secreted from the somatic mesoderm and have been shown to subsequently be internalised by cells of the VM, via a receptor-mediated endocytic mechanism (Weiss et al., 2001).

Confocal microscopy analysis used to visualise DAlk and Jeb proteins in wild-type embryos allowed us to observe a co-localisation of Jeb and DAlk at the interphase between the Jeb-secreting somatic mesoderm cells and the DAlk-positive VM cells, immediately prior to FC specification. When subsequently performing similar analysis of DAlk mutants, we could observe that this co-localisation was lost, as well as the columnar appearance of FC row of cells, further reinforcing the importance of a direct Jeb-DAlk interaction for FC specification.
Given the reported receptor-mediated internalisation of Jeb into VM cells, we were accordingly interested to investigate if DAlk is the RTK that is mediating this event. In order to address this question, we reasoned that the cytoplasmic tail of DAlk would be necessary for the recruitment of the endocytic machinery required for internalisation, as shown for the EGFR that via specific tyrosine residues recruits the Cbl/CIN85/Endophilin complex prior to endocytosis. Taking advantage of our generated DAlk8 mutant which contains an intact extracellular domain, thus in theory equipped to interact with Jeb, but totally lacks the intracellular tail we could subsequently show that indeed, a functional DAlk is required for internalisation of Jeb into VM cells. In these mutants, Jeb clearly bound to the membrane-anchored DAlk receptor, but remained at the plasma membrane at stages where Jeb protein normally is absent. Given that the endocytosis of previously characterised RTKs is enhanced in response to ligand binding and receptor autophosphorylation, we further investigated the importance of kinase activity for the uptake of Jeb. In order to do this we instead analysed Jeb localisation in DAlk8 and DAlk10 mutants, which contain mutations in amino acids crucial for kinase function. From the results gained in these embryos we could indeed conclude that Jeb-binding to DAlk induces a receptor-mediated endocytosis which is dependent on tyrosine phosphorylation and necessary for visceral muscle fusion and formation of a functional gut. Given that both Jeb and DAlk levels normally decrease at later embryonic stages we assume that internalisation of the Jeb/DAlk complex targets the active complex for degradation in the lysosome. These results are further in agreement with the reported requirement of shibire (Drosophila dynamin) for Jeb accumulation and internalisation in the VM (Weiss et al., 2001).

Figure 24. Signalling downstream of the DAlk receptor in the embryonic visceral mesoderm. Binding of the ligand Jeb to the DAlk receptor tyrosine kinase in the embryonic VM, activates an ERK-mediated pathway that induces transcription of muscle founder cell identity genes such as duf/kirre. This pathway is essential for FC specification, muscle fusion and subsequently formation of a functional gut.
The *Drosophila* homologue of the endocytic regulator CIN85 interacts with the RhoGAP RICH1 (Paper V).

*dCIN85 encodes a Drosophila member of the CIN85/CD2AP family of adapter proteins*

Mechanisms regulating cellular signalling, especially those which protect cells from transformation and tumour formation, are of great interest. As previously described, we have seen that the endocytosis and downregulation of the DAIk/Jeb receptor complex, is not only essential for preventing aberrant cell behaviour, but is also essential for the normal function of an RTK and its resultant signalling output.

To further pursue our interest in RTK downregulation, we identified the *Drosophila* homologue of Cbl-interacting protein of 85kDa, *dCIN85*, an adaptor molecule which in mammalian cells is implicated in RTK internalisation and downregulation, as well as the regulation of actin cytoskeleton dynamics. By searching databases encompassing the *Drosophila* genome, we found a single locus at the tip of the third chromosome right arm (CG31012), with resemblance to the CIN85/CD2AP protein family, *dCIN85*. As commonly observed for vertebrate CIN85/CD2AP family members, *dCIN85* is composed of a signature domain organisation, characterised by three N-terminal SH3 domains followed by proline-rich regions and a C-terminal coiled-coil. The high homology shared with vertebrate CIN85, particularly in the conserved SH3 domains, strongly suggests that *dCIN85* is a true CIN85/CD2AP *Drosophila* homologue. According to the available *Drosophila* gene collection (Stapleton et al., 2002), the *dCIN85* locus appears to utilise multiple promotors as well as alternative splicing, thus giving rise to at least four distinct transcripts, differing both in size and domain composition. Similar to mouse CIN85, *dCIN85* encodes a protein which is ubiquitously expressed, showing particularly high levels in the embryonic CNS. This neuronal expression appears to be maintained also in post-embryonic stages, given the strong *dCIN85* abundance in the photoreceptors of larval eye imaginal discs, as observed using the anti-*dCIN85* antibodies we have generated.

*dCIN85 is essential in the fruit fly*

To learn more about *dCIN85* function we utilised the Exelixis stock collection, inducing recombination events to remove the *dCIN85* locus. The phenotype displayed by flies lacking dCIN85 protein is most intriguing, exhibiting a “grand-child less” phenotype. Apparently the maternal contribution of *dCIN85* is of high importance, since first generation homozygous mutants are reasonably normal, even though they do not follow Mendelian rules, indicating a high degree of developmental lethality. In the second generation the effects are more profound, where the total absence of *dCIN85* function is deleterious, resulting in approximately 98% lethality. However, maternal contribution cannot provide the sole explanation for the rescue of *dCIN85* requirements in first generation homozygotes, given that also third generation individuals are observed to hatch into adult stages (although in very small numbers).

The time of death is also a peculiar feature of *dCIN85* mutant animals. Clearly, most second generation mutants appear to die during embryonic development, but certainly lethality can be observed within a spectrum covering also larval and pupal stages. Closer examination of
mutant embryos implies a general failure of development, showing variable phenotypes both concerning character and degree of severity. After extensive analysis employing the \textit{dCIN85} mutants in order to understand in vivo functions for \textit{dCIN85} we started to realise that probably a simple mutant analysis would not provide the key, but may rather be a way to verify our findings from other systems. We are currently developing a system utilising the FLP/FRT technique to generate \textit{dCIN85} mutant clones with the anticipation that the analysis of wild-type and mutant tissue in adjacent compartments will enable us to uncover subtle changes, as well as compare wild-type and mutant tissue competitiveness. In any case, the generation of \textit{dCIN85} mutant animals has provided evidence that the CIN85/CD2AP family of adapter proteins plays an essential role in \textit{Drosophila}. The ubiquitous expression pattern and the variety of phenotypes observed in deletion mutants, implicate \textit{dCIN85} in dynamic and diverging roles in multiple tissue types and at several time points during development.

\textit{Strong linkage of dCIN85 protein to the endocytic pathway}

In contrast to the lack of definite answers provided by mutational analysis thus far, the studies performed in parallel to characterise \textit{dCIN85} protein have provided more clues as to the physiological function of \textit{dCIN85}. Since mammalian CIN85 plays such a central role in RTK-mediated endocytosis we wished to establish if this function is also preserved in the fruit fly. Classical localisation studies using conventional Rab-GFP reporters and antibodies, revealed a co-localisation of \textit{dCIN85} with Rab5-positive vesicular structures, indicating a targeting of \textit{dCIN85} protein to early endosomal structures. However, the localisation of \textit{dCIN85} does not appear to be restricted to only early endosomes, implying that \textit{dCIN85} may cycle between different compartments of the endocytic system. The additional interaction with \textit{dCbl} observed, both in immunoprecipitation analysis and GST pull-downs, strongly suggests a conserved role for \textit{dCIN85} in endosomal internalisation of activated receptor complexes.

Interestingly, our findings reveal a requirement of \textit{dCIN85} SH3 domains for the recruitment of \textit{dCIN85} to Rab5-positive endosomes, since the short \textit{dCIN85}-RB isoform fails to localise to the vesicular structures where the SH3-domain containing isoforms are enriched. This may indicate that the \textit{dCIN85}-RB isoform performs other functions in the cell, not related to vesicular trafficking. Mammalian studies have indicated the presence of at least seven CIN85 isoforms in the mouse, arising as a consequence of alternative splicing and differential promoter usage. Indeed the different isoforms also show diverging localisation patterns, thus suggesting unique functions for the different CIN85 variants.

Similar to the recruitment of \textit{dCIN85} to vesicular structures, the \textit{dCIN85} SH3 domains are required also for the recruitment of \textit{dCIN85} to \textit{dCbl}-containing protein complexes. Interestingly, in contradiction to findings in mammalian systems, we can observe a constitutive interaction of \textit{dCIN85} and \textit{dCbl}, independent of RTK stimulation. However, this interaction might be explained by the aberrant regulation of phosphorylation known to occur in HEK 293T cells, which were used in the immunoprecipitation analysis performed. We are currently repeating similar experiments using other cell lines to circumvent these problems. However, in either case, the interaction with \textit{dCbl} and the established roles of \textit{dCbl} as a negative regulator of DER signalling in the fly, strongly suggests an involvement of also \textit{dCIN85} in RTK-mediated endocytosis in \textit{Drosophila}.

In order to investigate if the above described implications for \textit{dCIN85} in endocytic events, in reality are relevant to \textit{Drosophila} physiology in \textit{vivo}, we performed an endocytosis assay in
dCIN85 mutant Garland cells. The Garland cells are a group of nephritic cells which form a ring-like structure in front of the proventriculus in the *Drosophila* larvae (Narita et al., 1989). Despite the lack of full understanding regarding the role of Garland cells (possibly functioning as a fly kidney), their high endocytic activity is well established. Consequently these cells are commonly employed to study endocytic uptake of fluid-phase markers, such as dextran, in various mutant backgrounds. Analysis of the endocytic uptake in second generation dCIN85 mutant Garland cells did not show any obvious deviations from normal endocytic trafficking. Dextran was indeed internalised into dCIN85 mutant Garland cells and transported to the lysosome. In addition, these events appeared to occur with similar kinetics as in wild-type animals, suggesting that dCIN85 is not absolutely required for general endocytic trafficking in fly. These findings are not surprising, given that the crude fluid-phase uptake displayed by Garland cells is highly unselective, and in addition mainly is mediated via lipid-rafts, whereas dCIN85 function rather is implicated in far the more selective internalisation of activated transmembrane receptor molecules.

**dCIN85 as a regulator of AJCs and cell polarity?**

Since general endocytosis does not appear to require dCIN85 function, we wished to investigate the role of dCIN85 in more specialised endocytic processes. A recent proteomics study has identified RICH1, a Cdc42-specific RhoGAP protein, as a potential binding partner for mammalian CIN85, and furthermore implicated RICH1 in the regulation of the specialised endocytic recycling processes believed to be essential for the assembly/maintenance of tight junctions, as well as cell polarity (Wells et al., 2006). In light of these findings, we were exited to discover a co-localisation of the *Drosophila* RICH1 homologue and dCIN85 in the nervous system of the fly. Specifically, endogenous dCIN85 and dRICH1 co-localise in the differentiated photoreceptor cells of the larval eye disc. Given that dRICH1 in mammalian cells localises to tight junctions we subsequently analysed dCIN85 localisation with respect to cytoskeletal proteins known to be enriched at apical junctional complexes (AJCs) (including adducin, spectrin and Dlg), and found dCIN85 to indeed be partially localised to these complexes.

To establish whether the interaction of vertebrate RICH1 and CIN85 is conserved also in *Drosophila*, we consequently performed a GST-pull down assay and found that endogenous dRICH1 extracted from wild-type *Drosophila* embryos was clearly present in protein complexes also containing dCIN85. Since the construct used in this assay only contains the N-terminal part (encompassing the SH3 domains) of dCIN85, we suggest that the interaction between dCIN85 and dRICH1 is mediated via the dCIN85 SH3 domains. Whether this binding is direct, or part as a multiprotein complex, remains to be clarified in further studies. However, sequence analysis of the dRICH1 protein reveals the presence of several proline-rich motifs in the C-terminal part of the protein. While none of these represents the classical CIN85 PxxxPR recognition motif, dRICH1 indeed contains a PxxPxR motif, which is the consensus binding motif of class 2R SH3 domains (Cesareni et al., 2002), a class of SH3 domains highly similar to the SH3 domains of the CIN85/CD2AP protein family. The presence of these motifs may consequently indicate a direct interaction between dRICH1 and dCIN85. Given the localisation of dCIN85 at AJCs and the presence of dRICH1 in dCIN85 containing protein complexes, we were interested to investigate whether the targeting of dCIN85 to AJCs was mediated by binding to dRICH1. In order to address this question we expressed the isoform-specific transgenic constructs in the photoreceptors of the eye disc. Again we observed a co-localisation of dCIN85 and dRICH1, but were surprised to find that
all isoforms, including the dCIN85-B variant, strongly localised to dRICH1-enriched structures, indicating an SH3-domain independent recruitment of dCIN85 to AJCs. While this recruitment may be mediated by the dCIN85 proline-rich or coiled-coil domain, it is also possible that dCIN85 can directly bind to actin, given the reported actin-binding motifs present in the mammalian CD2AP protein (Kirsch et al., 1999).

The above implied functions of dCIN85, its interaction with dCbl and the endocytic pathway on one hand, and dRICH1 and AJCs on the other, invite many speculations regarding dCIN85 function. Certainly it is highly probable that dCIN85 displays dual (or more) functions, although indeed both the interaction with dCbl and dRICH1 indicate involvement in endocytic processes. RICH1 has shown to be essential for the maintenance of cell polarity by acting as a GAP for Cdc42, a small GTPase of the Rho superfamily which is responsible for the actin cytoskeletal dynamics associated with cell polarity, as well as the directed endocytosis of AJC components to their appropriate membrane target domains. Indeed it would be extremely exiting, as well as highly plausible, if CIN85 turns out to be the adaptor protein that links the RICH1/Cdc42 actin modulatory complex to the endocytic machinery, ensuring proper regulation of AJC composition and thus cell polarity. This would also be in agreement with our observation that dCIN85 also co-localises with AJC-linked cytoskeletal proteins such as adducin, spectrin and Dlg, indicating the possibility that dCIN85 may function by bringing dRICH1-containing endocytic vesicles to TJs, delivered on actin cables.

Some interesting parallels can be observed when comparing RTK-mediated endocytosis with the less well characterised endocytosis occurring at the AJCs. In the case of RTK-mediated endocytosis, endophilin proteins are important for the induction of plasma membrane invagination and formation of the invagosome/endosome. Endophilins are here believed to function as lipid modifiers, altering lipid composition to induce membrane curvature, but also contains a BAR-like domain. BAR domains have been shown to bind membrane lipids and by doing so induce alterations in membrane conformation. In light of this it is interesting to note that RICH1 also contains a BAR domain and is capable of inducing tubulation of liposomes (Richnau et al., 2004), suggesting the intriguing possibility that endophilin and RICH1 may display corresponding functions, but with different sites of action.

Another interesting speculation, in agreement with the neuronal enrichment of dCIN85 and dRICH1, is that the dCIN85/dRICH1 complex may be involved in exocytosis at nerve endings, given that RICH1 shows 83% identity with the rat Nadrin protein, which is proposed to facilitate membrane fusion of synaptic vesicles by virtue of its GAP domain (Harada et al., 2000).

One final issue to discuss is naturally the importance of these protein complexes in the crosstalk that is needed to properly regulate proliferation of epithelial cell layers. These cells need to complete cell division while simultaneously maintaining epithelial integrity (e.g. barrier functionality), as well as establishing new AJC complexes. In order to accomplish this, a tight communication between the growth factor receptors that instruct the cells to divide, and the machinery that delivers AJC components to appropriate membrane domains, is required. Since CIN85 may be involved in both of these processes it is tempting to speculate that CIN85 may function as an important mediator of this cross-talk, shuttling between RTKs and AJCs, thus integrating proliferation with cell-cell adhesion.
CONCLUSIONS

Focal Adhesion Kinase

- In the fruit fly, FAK family kinases (e.g. Fak56) are not required for life or fertility.

- \textit{Fak56} is not required for integrin-mediated adhesion, for cell migration or for phosphorylation of ERK during \textit{Drosophila} embryogenesis.

- \textit{Fak56} appears to play an accessory role as a negative regulator of integrin functions, and phenocopies integrin mutants when overexpressed.

- \textit{Fak56} is essential for axonal conduction and neuromuscular function in \textit{Drosophila}. Absence of Fak56 protein results in increased susceptibility to seizures.

Anaplastic Lymphoma Kinase / Jelly belly

- The \textit{Drosophila} Anaplastic Lymphoma Kinase, \textit{DAlk}, is a novel \textit{Drosophila} RTK which drives ERK activation \textit{in vivo} in the embryonic visceral mesoderm.

- \textit{DAlk} is required for gut development in \textit{Drosophila}.

- A signalling pathway where the ligand Jeb activates the DAlk receptor, to subsequently induce ERK phosphorylation and transcription of the founder cell identity gene \textit{duf/kirre}, is required for visceral muscle specification and muscle fusion in the \textit{Drosophila} embryo.

- RTK-mediated endocytosis of the activated Jeb/DAlk complex is essential for FC specification in the visceral mesoderm.

CIN85

- \textit{dCIN85} encodes a \textit{Drosophila} member of the conserved CIN85/CD2AP family of adaptor proteins.

- \textit{dCIN85} performs essential, yet uncharacterised, functions in the fruit fly.

- \textit{dCIN85} protein is targeted to Rab5-positive early endosomes and interacts with \textit{Drosophila} Cbl, implicating a role for \textit{dCIN85} in RTK-mediated endocytosis. The SH3 domains of dCIN85 appear to be essential for these functions.

- \textit{dCIN85} interacts with dRICH1 and co-localises with dRICH1 in photoreceptor cells in the larval eye disc. These observations might imply a role for \textit{dCIN85} in the regulation of cell polarity.
FUTURE PERSPECTIVES

The smaller picture

Fak56

The investigations concerning Fak56 function described in this thesis, have been performed with a systematic and comprehensive approach. We have investigated adhesion, migration and signalling and found no obvious defects caused by the absence of Fak56. Regardless of these findings, which have restricted our further interest in Fak56, the FAK field still leaves a multitude of open questions. The most captivating aspect of Fak56 function appears to reside within its neurological roles and it would be interesting to further investigate its role in neurotransmission and to comprehend the mechanism underlying the BS phenotype in Fak56 mutant flies. In order to do this there are a number of options to consider.

• One obvious approach would be to combine our Fak56 deletion with other BS mutations, with the objective of identifying a common pathway or cellular process behind BS behaviour.
• Another approach would be to exploit the different Fak56 mutant constructs available in the lab, to address the question of whether kinase activity and focal adhesion targeting of Fak56 is required for its role in neurotransmission.
• In addition, experiments treating Fak56 mutant flies with chemicals, known to modulate seizure susceptibility, could be informative as to the role of Fak56 in the CNS.
• The rapid dephosphorylation of Fak56 implies Fak56 as a component in a signalling pathway important for the cellular interpretation of neuronal stimulation. Possibly, turning to a mammalian cell culture system, where tools such as antibodies, markers and various assays are more advanced, could be a means in which to more easily identify other players in the pathway. One aim would be to identify the PTP responsible for the dephosphorylation of Fak56 in high calcium environments - possibly RNAi would be a useful technique during such investigations.
• Furthermore, the suggested functional overlap between FAK and Pyk2 is an area of controversy. Indeed it would be interesting to investigate the effect of ectopic Pyk2 expression in the fruit fly, as well as to ask whether Pyk2 can rescue the BS observed in Fak56 mutants. In addition, in light of the reported upregulation of Pyk2 expression in FAK deficient mice, it may be informative to study the effect of ectopically expressed Pyk2 on integrin-mediated adhesion.
• In light of the reported role of the Drosophila volado gene (encoding the αPS3 integrin subunit) in mediating olfactory memory, together with the suggested role for vertebrate Pyk2 in LTP, it would be interesting to investigate additional roles for Fak56 in the nervous system, including memory.

DAlk

Since the discovery of the findings related to DAlk function reported in this thesis, the DAlk-project has been strongly pursued in the lab, but is currently out of my immediate focus. We are currently involved in the identification of downstream targets for DAlk in the visceral mesoderm and are also investigating a role of the PTN/MK homologues miple1 and miple2 as putative DAlk ligands. In light of the strong expression of ALK in the nervous system of all organisms examined, our lab is also interested to investigate the proposed neuronal functions for DAlk. Furthermore, an important aspect of the ALK project, which is also ongoing, is an analysis of the role of ALK in mammalian systems, based on our findings in Drosophila.
**dCIN85**

*dCIN85* is the subject that captures my present focus. Since this project is still in its infancy there are still a multitude of questions to be addressed. We have just now established the tools required to investigate *dCIN85* function (mutants, transgenes, antibodies etc).

- One area of utmost importance is the suggested role of *dCIN85* in endocytosis. The observed interaction with dCbl calls for a follow-up involving *in vivo* investigations concerning not only the interaction with dCbl itself, but also with known components in the EGFR signalling pathway.
- Given the requirement for RTK-mediated endocytosis for Jeb/DAIk functionality it would be exciting to investigate the involvement of the dCIN85/dCbl complex in this RTK signalling pathway.
- The techniques available for studying receptor-specific endocytosis and trafficking in imaginal discs could clearly be used in order to establish the requirement for *dCIN85* in more selective endocytic events, following endocytic trafficking of labelled RTKs in *dCIN85* loss of function situations.
- However, the most intriguing area at the present time must be the putative involvement of *dCIN85* in the endocytic recycling of TJ components and the regulation of cell polarity. Since this is an area which still is poorly understood, I believe studies in the fly will enable the discovery of many novel findings. It is important to examine how and when dCIN85 and dRICH1 interact (considering cell type specificity, interaction in response to different stimulations, modifications etc), to study the dynamics of this interaction and the impact it might have on dRICH1 RhoGAP activity and consequently its impact on Cdc42 activity and actin reorganisation.
- Given the role of RTK-mediated signal transduction in the development of cellular transformation and tumour formation, it is also of high importance to investigate the role of CIN85/CD2AP family proteins in the downregulation of such pathways, with the aim of increasing the knowledge about cancer, as well as developing new and more efficient cancer therapeutics.

**The big picture**

Coming back to the bigger picture is always coming back to the purpose of research, our will to further the understanding of biology and contribute to the improvement of life and the cure of disease. The signalling field is especially important for the development of anti-cancer agents (such as the small PTK inhibitors and inhibitory antibodies). I believe the understanding of selective endocytosis will be essential for the development of new and more efficient therapeutics and has additionally implications for the administration of drugs. This field is also important in the development of anti-viral and anti-bacterial therapies, given that the entry route used by many infectious agents utilise the endocytic machinery of the host cell for infection.
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Dancing crew, no one mentioned, no one forgotten…
Also big thanks to the one and only Stål-Barbro, no gympa - no brain…

Friends who live far away but always are in my heart. Thank you: Ylva, we have a special connection, Christian - for your scientific enthusiasm and for endless e-mail discussions about science in general and life in particular, Mihailo - for your beautiful mind and for really expanding my horizon!
Kristiina – still fun to get news from Estonia!

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Mamma och Pappa; För all kärlek och allt stöd från dag ett i mitt liv, för att ni finns där och tror på mig, vilka galna upptåg jag än ger mig ut på. Jag älskar er – ALLTID! TACK…
PERSONAL PERSPECTIVES

Finishing a PhD is not only finishing an education, concluding a scientific project – it is also about concluding an important chapter of your life, getting ready for turning the page and move on! I’m convinced your PhD project will haunt you for the rest of your life, similar to the impact of your childhood years, but I am even more convinced that the people you meet and the lessons you learn, will have an even greater impact on your future. A great deal happens during the course of a PhD, scientifically as well as personally. Your aims, ideas, perspectives and personality - change in so many ways.

Entering the world of science (or maybe it’s just an island) provides not only a stimulating and interesting employment, but also a multitude of opportunities and possibilities. For me, a key word in the research field is “meetings”, meetings not only with many interesting minds and new discoveries, but also meetings with different cultures, different ways of living and seeing – simply, different views of life. It offers you an excellent opportunity to widening your horizons and appreciating all the fortune into which you were born. Beginning your life in Sweden in the seventies, like me, having few or no worries - with a starting point like that, you have no grounds for complaints, no reason to blame anyone for anything – in no ways could your odds have been any better! I’ve heard that a typical Swede can be defined as “intact, clean and standardized”, and I think in some way the modern way of life delivers people from free will. Maybe that can be a choice, but it inevitably brings the expression “youth is wasted on the young” to my mind. I think there are some clear dangers of living in an environment where everything you need is provided. Firstly, you never learn the ability to fight, something I’ve missed when thrown outside the borders of Sweden. Secondly, with an excess of everything, the lesson of what really matters takes so much longer to learn and finally, sometimes I’ve felt the power of choice to find me too confused to even take off. But I think, if one comes by these obstacles you realise that your life (your “research proposal”) is up to you to propose, and that your funding has already been approved – A big responsibility, but also a great freedom!

Sometimes I like to view life as a game of Monopoly. We all start at given point and throw the dice. Pure luck decides our starting conditions, what genes we get, where we are born, how we will be raised etc. The game will never be fair, the ones with a good start will always have an advantage, but within the scope of the game, everyone has the chance of winning. Sometimes we are thrown back to the beginning by the act of others, by our own stupidity or simply by bad luck – and sometimes we get struck by good fortune even when we don’t really deserve it and when it is least expected. Many people are totally focused on winning, while others care too little to even be bothered – but what it all comes down to, is that most people enjoy the game as long as it is lasting, not when someone suddenly wins and the game is over. Like in the game, when we reach the end, our money is worth as much as that phoney money in Monopoly, our house will be taken over by strangers and no one will really remember who won in the end. So I guess the lesson is to enjoy the game as long as it is lasting and to every once in a while consider your choices/tactics, along the way. Remember that without taking chances you won’t make it far, try not to be limited by fear and remember that everyone has a unique definition of winning. And entering a fight, is the only way to win it!

I have a belief that we all have different purposes in life. Some people are here to ask questions, others are here to provide the answers – while most people struggle in between to make it happen. To be successful as a scientist maybe you require a little bit of everything, the ideas, the hard work and a sense of understanding the answers provided.
I recently got the question if I have any regrets, if I in any way wished that my life would have turned out differently. Without even thinking I replied “That’s a silly question to ask and even think about. The way I’ve lived my life and the choices I’ve made is the reason why I am me, every little moment is put together into the puzzle that shape a personality, so how could I have any regrets - who would want to be somebody else?” Naturally we all have our shortcomings, but imagine walking around in someone else’s skin… I once read an interview with a successful business woman who stated that her role model and ideal was “The best version of me (her)”. This was something I really grasped - role models have their purpose, but just to be the best you can, will take you far and is also what is accomplishable – anything beyond that, will only lead to frustration. To know your limits is a great strength!

Now to something completely different, I was recently highly amused to find a possible explanation as to why the genomes of budding yeast and humans are so similar – you see, our lives might not necessarily be as different as we would like to think! I read a paper concerning cell polarity in budding yeast and the author of this paper wrote; “X are born carrying within them landmark proteins. After a period of uniform growth, all growth becomes polarised towards one and only one site, targeting new X of X to build a bud”, continuing with “Positive feedback loops reinforce inequalities in the local concentrations of X factors, so that stochastic fluctuations are amplified into a single dominating symmetry”, and furthermore finishing up with “When deprived of directional cues – X choose a random axis and commit to it as if they knew where they were going”. Life is life, I guess…

Joking apart… coming to the end of my reflections, I just want to conclude these years as highly important and enjoyable years in my life. I’ve laughed and cried, I’ve talked and listened, worked hard and been distracted – very human years. I guess my dad imprinted a piece of my personality during my early childhood when he instinctively nicknamed me “kärringen mot strömmen” (lady-against-the-flood), based on my predilection to do things in the wrong order (back-to-front). Maybe it is some kind of genetic disorder, never choosing the easy road, seeking out challenging situations, always trying to learn and grow. One of the lessons I will take with me from the past years is the necessity of letting people grow their own characters, I’ve learned the value and joy in getting a view of the multitude of ways in which a common goal can be reached. It’s important to consider that a coin always have two sides, for every win there is a loss, if you wish to see things in that particular way. However, what ever aspect you look into in life, if you really want answers you must be prepared to search for them everywhere and without prejudice, and most importantly, you have to ask the right question! It’s like they say, life is a dance between duty and desire – and the key is to make them find a common rhythm…

Och bara så att du vet... en vältränad forskare rör sig ca 80 gånger snabbare än en sengångare, när han är motiverad...

Just a small summary of my life... Thank you all for being part of it!
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Bakom varje hög av sand finns guld för den som letar... 

(Lars Winnerbäck)