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Characterization of *Drosophila* Lnk – An Adaptor protein involved in growth control

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Summary

One of the essential and most tightly regulated processes an organism has to accomplish throughout development is the control of growth and proliferation in order to reach and maintain the appropriate size. Until today, the underlying mechanisms are still far from being fully understood.

It has previously been shown that, among other signaling cascades, the insulin signaling pathway plays a crucial role in controlling growth. In *Drosophila*, binding of either one of the seven Insulin like peptides initiates the autophosphorylation of the receptor, which in turn leads to subsequent phosphorylation of a variety of downstream signaling molecules such as Chico, PI3K, PKB and PDK1. As a result, the insulin signaling pathway regulates cellular growth, proliferation, apoptosis and transcription. Strikingly, mutants of positive core components of the insulin receptor pathway lead to common phenotypes such as decreased body size due to smaller and less cells, female sterility, developmental delay and increased total lipid levels.

In an unbiased screen for genes affecting organ size based on the *eyFLP-FRT* system, we found that mutations in a gene called *lnk* result in flies with a smaller head, suggesting a growth promoting role for *lnk*. The *lnk* gene encodes an adaptor protein, containing a PH-domain, an SH2-domain and a highly conserved C-terminal tyrosine phosphorylation site. The phenotypes of *lnk* mutant flies were reminiscent of the phenotypes observed in mutants of the insulin pathway, suggesting an important function for Lnk in promoting the insulin signal.

In mammals, three proteins sharing the same protein structure to *Drosophila* Lnk have been described, SH2B1, SH2B2 and SH2B3 referred to as the SH2B family of adaptor proteins. The members of this protein family have been shown to regulate receptor tyrosine pathways either by direct binding to the receptor or by interaction with one of the multiple signaling proteins such as Grb2, PI3K and c-Cbl.

In this work we present the characterization of *Drosophila lnk*. By analysing the mutant phenotypes displayed by homozygous *lnk* animals, genetic interaction experiments and molecular readouts for insulin signaling activity we were able to place *lnk* into the Insulin pathway between the receptor and *PI3K*.

Zusammenfassung

Während der Entwicklung eines mehrzelligen Organismus ist einer der grundlegendsten und am strengsten kontrollierten Prozesse die Regulation des Wachstums. Bisher sind wir jedoch noch weit davon entfernt, den Prozess der Wachstumskontrolle vollständig zu verstehen.
Der Insulinsignalweg nimmt, neben anderen wichtigen Signalkaskaden, eine entscheidende Rolle in der Regulierung von Zellwachstum und –proliferation ein. In *Drosophila* wird durch die Bindung von sogenannten ‚Insulin like peptides' die Autophosphorylierung des Insulinrezeptors ausgelöst, was wiederum die Phosphorylierung einer Vielzahl von nachgeschalteten Signalmolekülen wie Chico, PI3K, PKB und PDK1 zur Folge hat. Der Insulinsignalweg reguliert neben der Zellgröße und Proliferation auch den Programmierten Zelltod und die Transkription bestimmter Gene. Auffallender weise bewirken Mutationen in den Hauptkomponenten des Insulinsignalwegs einheitliche Phänotypen, wie eine reduzierte Körpergröße, hervorgerufen durch kleinere und weniger Zellen, Sterilität der Weibchen, Entwicklungsverzögerung und erhöhte Lipid werte. In einem auf dem *eyFLP-FRT* System basierenden Screen zur Identifikation von neuen Genen, welche die Organgröße beeinflussen, haben wir Mutationen im sogenannten *lnk* Gen gefunden. Diese Mutationen führen zu Fliegen mit einem kleineren Kopf, was darauf hindeutet, dass *lnk* Wachstum positiv beeinflusst. Das *lnk* Gen kodiert für ein Adaptorprotein. welches eine PH Domäne, eine SH2 Domäne und eine hochkonservierten C-terminalen Tyrosin-phoshorylierungsstelle aufweist. Die Phänotypen der *lnk* mutanten Fliegen gleichen denen, die bei Mutanten der Insulin Signalkaskade beobachtet werden konnten. Dies weist auf eine Funktion von Lnk in der Signalkaskade unterhalb des Insulinrezeptors hin.
In Säugetieren weisen drei Proteine dieselbe Proteinstruktur wie *Drosophila* Lnk auf: SH2B1, SH2B2 und SH2B3. Diese in der SH2B Adaptorproteinfamilie zusammengefassten Proteine regulieren Rezeptor-Tyrosin-Kinase Signalwege, indem sie entweder direkt an den Rezeptor binden oder mit einem Signalprotein, wie zum Beispiel Grb2, PI3K oder c-Cbl interagieren.

In der vorliegenden Arbeit wird die Charakterisierung des *Drosophila lnk* Gens beschrieben. Durch genetische Interaktionsstudien und molekulare Indikatoren für die Aktivität des Insulin Signalwegs konnten wir zeigen, dass lnk innerhalb der Insulinsignalkaskade zwischen dem Insulinrezeptor und *PI3K* einzuordnen ist.

Introduction

The fundamental process of growth control

For every living organism, cell growth – defined as increase in biomass – is one of the most fundamental processes and determines the size and the shape of an individual. Except for smaller differences, the overall body size of animals within a certain species is constant and depends on the number and size of the cells it contains. The control of cell number and cell size within an organism is achieved by a tightly regulated interplay of cell growth, proliferation and apoptosis that are coordinated by a preset intrinsic genetic program (Conlon and Raff 1999). Although it has been suggested that a cell needs to reach a 'critical mass' in order to complete the cell cycle, defects in the latter do not influence its ability for normal growth. The analysis of yeast mutants that are blocked in various stages of the cell cycle showed that cells are still able to grow, thus provided evidence that growth is not dependent on proliferation (Johnston, Pringle et al. 1977). Furthermore, in *Drosophila* imaginal discs, clonal over expression of the cell cycle activator *dE2F* accelerated the cell cycle without affecting cell growth, leading to smaller cells. On the other hand, over expression of the *Drosophila* homolog of the retinoblastoma protein family (RBF), an inhibitor of *dE2F* slowed down the rate of cell divisions, which increased cell size (Neufeld, de la Cruz et al. 1998).

Interestingly, alterations in cell size usually do not influence the size of an entire organ as it was shown in *Drosophila*, where perturbations that affect the size of an individual cell such as changes in ploidy or manipulations of the cell cycle did not affect the size of the organ or the entire animal (Weigmann, Cohen et al. 1997; Neufeld, de la Cruz et al. 1998). These results suggest that there is a mechanism that controls compartment size, independent of proliferation.

Such an intrinsic mechanism responsible to determine the size of an organ was already proposed earlier since transplantation of an infant rat heart or kidney was transplanted into adults, resulting in an organ growing at the same rate they would usually grow within the infant rat and attained the correct size (Dittmer, Goss et al.

1974; Silber 1976). Similar results were obtained when limbs or eyes were transplanted from a smaller salamander species to a larger. The organs always attained an organ size characteristic of the donor (Stone 1930).
However, within a single animal, sizes of individual organs and even organs of the same origin can be regulated differently. An impressive example of such apparent organ size differences can be observed in male fiddler crabs, where one of the first pair of thoracic limbs is strongly enlarged. In early stages the two claws are equal in size and in shape and only during subsequent juvenile development become asymmetric (Morgan 1923). Furthermore, the development of claws in the fiddler crab also shows that the size of a specific organ can influence the development of another. Removal of the large claw stimulates the generation of a new large claw on the opposite side after the next mould, suggesting that both claws posses the intrinsic ability to grow larger but get inhibited in growth as soon as the opposite claw develops into the large claw.
Growth and body size of an organism is not only influenced by genetic factors but also by extrinsic or environmental cues such as temperature, nutrition and oxygen. For example, Atkinson estimated that over 80% of ectotermic species exhibit faster growth but smaller overall body size, when reared under higher temperatures and vice versa under cold temperature conditions (Atkinson 1994). He termed the phenomenon that lead to his observation the temperature-size rule. Although the mechanisms still have not been resolved fully, it has been proposed that low oxygen levels (hypoxia) and poor availability of nutrients (starvation) both restrict growth rates by interfering with the Insulin receptor/PI3K and Target of Rapamycin (TOR) pathways. These pathways are highly conserved through evolution and defects in the Insulin signaling cascade can lead to severe systemic disorders such as diabetes, cardiovascular disease, stroke, neurodegeneration and cancer (White 2003; Pollak 2007).
In order to maintain growth, development and reproduction of all organisms depend on energy and nutrients that need to be taken up from the environment. In the progress of evolution it became necessary to develop specialized organs for resorption and digestion and to employ mechanisms that regulate the interplay between availability and uptake of food. Further, the communication between cells

within an organism became indispensable. Cells communicate through a variety of signaling mechanisms, usually triggered by extracellular ligands that bind to specific receptor and subsequently initiate a cascade which transduces the signal within the receiving cell.

One of the most prominent systems regulating growth, metabolism and reproduction that have evolved in higher organisms including humans is the Insulin and TOR signaling pathway.

The Insulin/IGF and TOR pathway

Insulin-like molecules have been identified in vertebrates and invertebrates including the mammalian Insulin and Insulin-like growth factors (IGFs), the *Drosophila* Insulin-like peptides (DILPs), the *Caenorhabditis* Insulins and the lepidopteran bombyxins (Nagasawa, Kataoka et al. 1984; Kawano, Ito et al. 2000; Brogiolo, Stocker et al. 2001; Pierce, Costa et al. 2001).

The peptide hormone Insulin is produced by β-cells of the vertebrate pancreas and composed of an amino-terminal B-chain (30 amino acids) and a carboxyl-terminal A-chain (21 amino acid) that are linked together by disulfide bonds. The precursor of Insulin, so called proinsulin, is composed of one molecule with three domains. During the process of maturation, the peptide gets folded and cleaved by proteases, which leads to the removal of the C-peptide from the center of the proinsulin leaving the remaining A and B chain connected to their ends (Lu, Lam et al. 2005). The biologically active mature insulin is further on found within the secretory granules of the pancreatic β-cells that are important for the storage and secretion of insulin. Insulin is mostly released in response to increased levels of blood glucose and stimulates glucose uptake primarily into cells of adipose tissue and skeletal muscles (Saltiel and Kahn 2001). In opposite to Insulin, the conversion of glycogen into glucose and its subsequent release into the bloodstream is regulated by a hormone called Glucagon which is also produced by the pancreas and released in response to low blood glucose levels, preventing the development of hypoglycemia (Pipeleers, Schuit et al. 1985).

Insulin signaling has an influence on many processes within the organism, which can be divided into cell autonomous and non-autonomous effects (Figure 1) (Garofalo 2002). The cell autonomous effects include the control of cell growth, proliferation, nutrient storage and protein synthesis, whereas non-autonomous effects comprise the modulation of neuroendocrine signaling pathways, which in turn adapt the metabolism, reproduction and lifespan of the organisms to the amount of available nutrients in their environment.

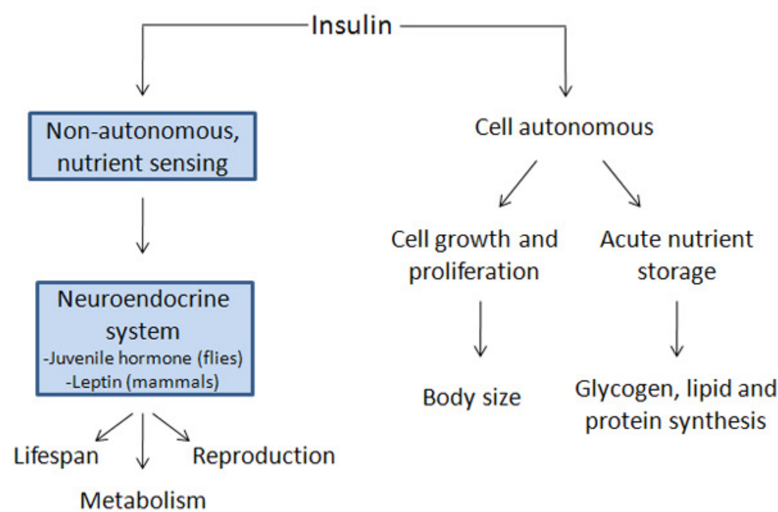


Figure 1: Diverse functions of Insulin signaling

The actions of Insulin signaling can be divided into two distinct categories, the cell autonomous, including the regulation of body size and glycogen, lipid and protein synthesis and the cell non-autonomous, which are important for lifespan, metabolism and reproduction.

The Insulin signal transduction pathway in vertebrates has been studied extensively and many components have been identified, however it is still not solved entirely at which interfaces other regulatory mechanisms are able to intervene.

In addition to Insulin, the vertebrate insulin-related superfamily consists of insulin-related growth factors (IGF-I and IGF-II), relaxin and relaxin-related factors (Hudson, Haley et al. 1983; Dull, Gray et al. 1984; Hudson, John et al. 1984; Rotwein, Pollock et al. 1986; Bathgate, Samuel et al. 2002). Insulin and IGF are ligands for receptor

tyrosine kinases, whereas relaxin and related factors bind to G-protein coupled receptors. The insulin receptor belongs to the group of receptor tyrosine kinases, which also includes the closely related IGF-I-receptor and the IRR-receptor (insulin receptor related receptor) (Patti and Kahn 1998). Schematically, the insulin and IGF-I receptor have an extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic domain displaying the tyrosine kinase activity (Yarden and Ullrich 1988; Ullrich and Schlessinger 1990). After several post-translational processing steps, the mature insulin receptor is a tetrameric protein consisting of two α- and two β-subunits. These function as an allosteric enzyme in which the α-subunit inhibits the kinase-activity of the β-subunit. Upon binding of the ligand to the α-subunit it activates the cytoplasmic tyrosine kinase of the β-subunit through conformational changes. The activated kinase phosphorylates itself exclusively on tyrosine residues and enhances protein tyrosine kinase activity towards exogenous substrates and renders the kinase active in the absence of insulin (Rosen, Herrera et al. 1983).

Signaling downstream of the receptor

Once activated, the insulin receptor mediates its signal downstream through a series of phosphorylation events of intracellular substrate targets. One of these targets, the insulin receptor substrate proteins (IRS1-4) bind to the autophosphorylated tyrosines within the intracellular domain of the receptor via their PTB-domain inducing their phosphorylation. With no intrinsic catalytic capability, the IRS proteins belong to the group of adaptor proteins that mediate protein-protein interactions. In addition to the PTB domain, they contain an N-terminal PH domain and several tyrosine phosphorylation sites. Through these phosphorylation sites, IRS proteins have been shown to connect the insulin signal in mammals to further signaling molecules (White 1998). For example, the SH2 domain of Grb2 binds to a pYXN sequence motif within IRS thus activates the ras/mitogen-activated-protein kinase (MAPK) (Baltensperger, Kozma et al. 1993; Skolnik, Batzer et al. 1993). Furthermore, two functional YxxM motifs allow the IRS proteins to directly interact with the SH2 domain of the p85

regulatory subunit of class I_A Phosphoinositide-3 kinase (PI3K) (Myers, Backer et al. 1992). Subsequently, p85 recruits the catalytic subunit p110, which leads to the activation of the enzyme to catalyze the phosphorylation of specific phosphoinositides, thus converting phosphatidyinositol-4,5-P2 (PIP_2) into the second messenger phosphatidylinositol-3,4,5-P3 (PIP_3) (Britton, Lockwood et al. 2002). This process is counteracted by PTEN (phosphatase and tensin homolog deleted on chromosome 10), a lipid phosphatase that specifically removes phosphates at the D3 position of the PIP_3 inositol ring (Maehama and Dixon 1998; Goberdhan, Paricio et al. 1999; Huang, Potter et al. 1999; Gao, Neufeld et al. 2000). The increase of PIP_3 levels at the cell membrane mediates the activation of the PH domain containing proteins, phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB/AKT) (Alessi, Deak et al. 1997; Andjelkovic, Alessi et al. 1997). Among the phosphorylation targets of PKB/AKT are metabolic enzymes such as GSK-3 (glycogen synthase kinase 3), which subsequent to its phosphorylation by PKB inhibits the phosphorylation of the glycogen synthase leading to synthesis of glycogen (Cross, Alessi et al. 1995). Furthermore, PKB acts on transcription factors of the FOXO family (Brunet, Bonni et al. 1999; Kops, de Ruiter et al. 1999). FOXO transcription factors are highly conserved and important effectors of insulin signaling, involved in the regulation of the cell cycle, metabolism, apoptosis, lifespan and oxidative stress resistance (Burgering 2008). Mammals contain four homologs, FOXO1, FOXO3a, FOXO4 and FOXO6, all of which contain various conserved PKB phosphorylation sites (Corral, Forster et al. 1993; Galili, Davis et al. 1993; Hillion, Le Coniat et al. 1997; Jacobs, van der Heide et al. 2003). Central to the regulation of FOXO activity is a shuttling mechanism that shifts FOXO localization from the nucleus to the cytosol, thereby inhibiting their transcriptional function. In response to the insulin signal, PKB stimulates the phosphorylation of FOXO proteins, thus their exclusion from the nucleus keeping them inactivated within the cytosol. Another protein directly involved in the inactivation of FOXO is 14-3-3, which binds to PKB induced phospho sites in FOXO; thereby leading to the accumulation of 14-3-3 bound FOXO in the cytoplasm (Brunet, Bonni et al. 1999). Unphosphorylated FOXO translocates to the nucleus, where it activates target genes such as *PEPCK*, *p27kip1*, *FasL* and *NPY* (Greer and Brunet 2005).

Another target of PKB is the tumor suppressor TSC2. *TSC1* and *TSC2* were identified by their association with a familial autosomal multisystem disorder known as tuberous sclerosis and encode for proteins called hamartin and tuberin, respectively. In vivo, the two proteins physically associate and function primarily as a complex, furthermore, TSC1 is required to stabilize TSC2 and prevent its ubiquitin mediated degradation (Chong-Kopera, Inoki et al. 2006). It was first established in *Drosophila* that *TSC1* and *TSC2* are involved in the control of cell size and proliferation, since they were identified as growth suppressors in genetic screens for regulators of organ size (Ito and Rubin 1999; Gao and Pan 2001; Potter, Huang et al. 2001; Tapon, Ito et al. 2001). Subsequent biochemical and bioinformatic studies were able to show that the TSC1/TSC2 complex acts downstream of PKB/AKT and that TSC2 is a direct target of PKB/AKT mediated phosphorylation on two residues (Ser^{939} and Thr^{1462}), promoting the disruption of the TSC1/TSC2 complex (Inoki, Li et al. 2002; Potter, Pedraza et al. 2002). The TSC1/TSC2 complex acts as a GTPase-activating protein (GAP) for the small GTPase Rheb (Ras homolog enriched in brain), thereby stimulating the conversion of Rheb-GTP (active) into Rheb-GDP (inactive) (Garami, Zwartkruis et al. 2003; Saucedo, Gao et al. 2003; Stocker, Radimerski et al. 2003; Zhang, Gao et al. 2003). Recent data provide evidence that Rheb in its GTP-bound thus activated state, associates to mammalian Target of Rapamycin (mTOR) and promotes its activation by interfering with binding of FKBP38, an endogenous mTOR inhibitor (Bai, Ma et al. 2007).

TOR, first described in the budding yeast, Saccharomyces cerevisiae, is a conserved Ser/Thr kinase that belongs to the PI kinase-related family (Heitman, Movva et al. 1991; Keith and Schreiber 1995). The yeast *tor1* and *tor2* genes were originally identified by mutants in these genes that were resistant to the growth inhibitory effects of the bacterial antifungal metabolite rapamycin. Rapamycin forms a complex with its intracellular cofactor FKBP12, and this complex then binds to and inhibits TOR (Heitman, Movva et al. 1991). The target of rapamycin kinase is highly conserved among species and every eukaryote genome examined up to now, including yeast, algae, plants, worms, flies and mammals, contains a *TOR* gene (Wullschleger, Loewith et al. 2006). The large TOR proteins contain several HEAT repeats in their amino-terminal half, which enable them to function in a complex with

other proteins. Indeed, biochemical studies in yeast TOR1 and TOR2 led to the identification of two distinct TOR-complexes, TORC1 and TORC2 (Loewith, Jacinto et al. 2002). In mammals, the two TOR complexes contain mTOR and the mammalian homolog of LST8 (mLST8)/Gβ-like (GβL). However, the two complexes differ in their exclusive association to Raptor (TORC1) and Rictor/AVO3 (TORC2) (Wullschleger, Loewith et al. 2006). Importantly, only TORC1 is sensitive to rapamycin, whereas TORC2 is not, or at least not under all conditions (Loewith, Jacinto et al. 2002). Over expression of a rapamycin resistant TOR mutant is sufficient to rescue the growth inhibitory effects of rapamycin, providing evidence that growth inhibition in mammalian cells due to rapamycin treatment is exclusively mediated by its suppressive effects on mTOR (Fingar, Salama et al. 2002). Studies in yeast and mammals have shown that rapamycin associates to the peptidyl prolyl isomerase FKBP12, which subsequently bind to a region close to kinase domain in TOR, termed the FRB (FKBP12-rapamycin binding). Cells lacking FKBP12 are resistant to rapamycin (Heitman, Movva et al. 1991). However, evidence for inhibition of the catalytic activity of TOR through this association remains controversial. An alternative model for how rapamycin/FKBP12 may interfere with TORC1 activity is based on perturbed interaction of raptor and mTOR, which may affect substrate recognition or presentation to mTOR (McMahon, Choi et al. 2002).

The second TOR complex, TORC2 was shown to function as the long-sought PDK2 and is capable to phosphorylate PKB/Akt. Using *Drosophila* S2 cells, they showed that knock down of dTOR significantly decreases phosphorylation of the PDK2 site in dPKB. Furthermore, knock down of the TORC2-specific component Rictor reduced PKB phosphorylation in *Drosophila* and mammalian cells, while knocking down the TORC1-associated molecule Raptor did not. Finally, an in vitro kinase assay provided direct biochemical evidence that TORC2 but not TORC1 can phosphorylate Ser^{473} of Akt in vitro (Sarbassov, Guertin et al. 2005).

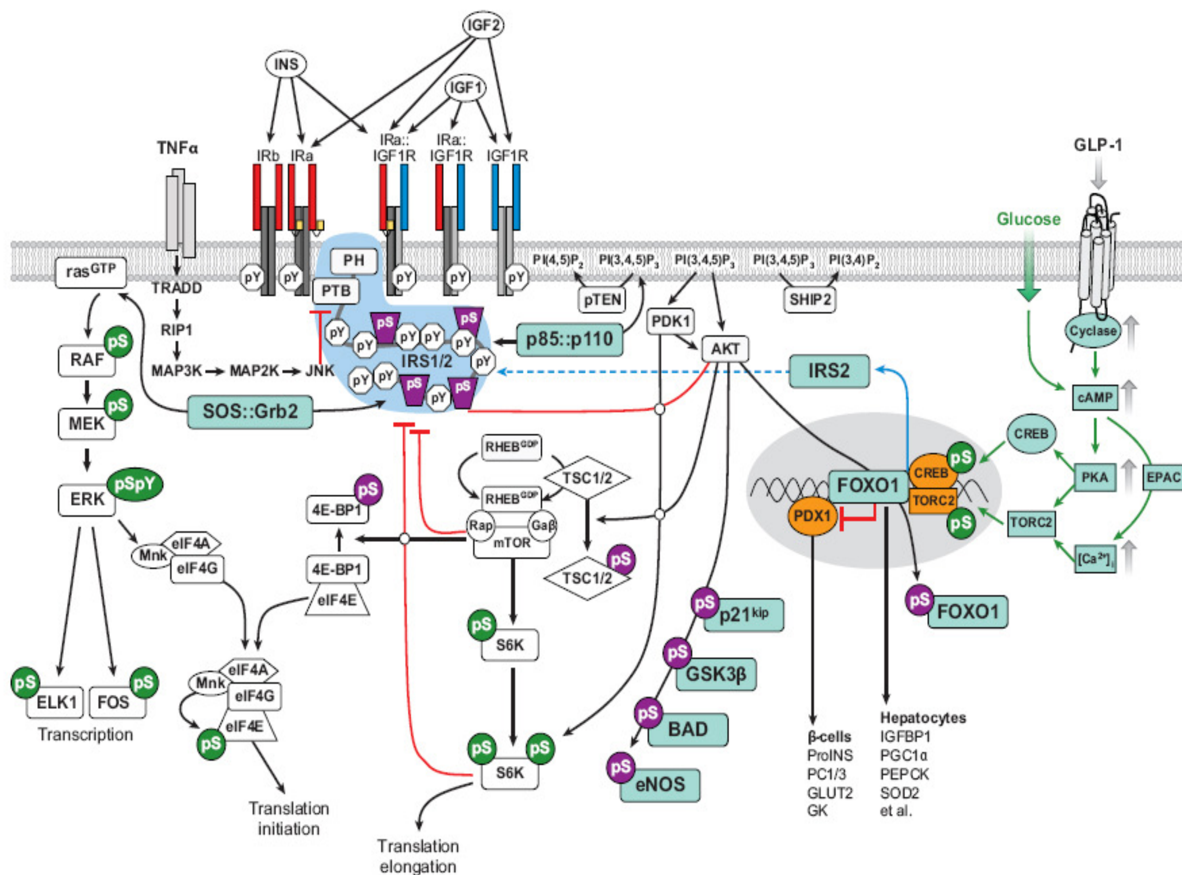
Although it was generally thought that one of the major differences between TORC1 and TORC2 is that TORC1 is sensitive to rapamycin, whereas TORC2 is not, some interesting findings about the inhibition of TORC2 by rapamycin under certain conditions have recently been published. In certain normal and cancer cell lines, prolonged rapamycin treatment lead to a dramatic decrease in phosphorylation of

Akt, which is thought to be due to rapamycin-prevented association of newly synthesized mTOR to Rictor (Sarbassov, Ali et al. 2006).
Downstream of TOR, the two best studied targets are ribosomal S6 kinase 1 (S6K) and the eukaryotic initiation factor 4E binding protein 1 (4EBP1). Mammalian cells contain two similar S6 kinase proteins (S6K1 and S6K2) encoded by two different genes. S6K, a member of the family of serine/threonine kinases, is directly phosphorylated by mTOR on Thr^{389}, which is essential for its activation (Kim, Sarbassov et al. 2002). The p70 S6 kinase was originally identified as a kinase that regulates serine phosphorylation of the 40S ribosomal S6 protein (rpS6) (Avruch, Belham et al. 2001). Phosphorylated rpS6 was long believed to be important for the translational regulation of several mRNAs, in particular the so called TOP mRNAs that are characterized by an oligopyrimidine tract at their 5' termini (5' TOP). However, recent studies using knock-in mice of S6 protein with the S6K phosphorylation site eliminated show that under these conditions, 5' TOP mRNA translation is unaffected but instead total protein synthesis is enhanced (Ruvinsky, Sharon et al. 2005). Interestingly, embryonic fibroblasts (MEFs) of these knock-in mice are significantly smaller than the control, but, unlike wild type MEFs, are not decreased by rapamycin, suggesting that S6 phosphorylation is a crucial step in mediating the effect of mTOR on mammalian cell-size regulation.
Whereas phosphorylation by mTOR results in activation of S6K, it can also have an inhibitory effect as in the case of another downstream target; 4E-BP. Eukaryotic initiation factor 4E binding protein (4E-BP) is an inhibitor of translational initiation. In the unphosphorylated state, 4E-BP binds to eukaryotic initiation factor 4E (eIF4E), preventing the recruitment of the initiation complex eIF4E to the cap structure at the 5' terminus of eukaryotic cellular mRNAs. Phosphorylation of 4E-BP by mTOR releases eIF4E and thus, initiates cap-dependent protein synthesis (Schalm, Fingar et al. 2003).
The TOR pathway integrates both positive and negative signals to regulate cell growth in a coordinated manner. For example, it is activated by growth factors and nutrients such as amino acids but inhibited by a variety of stress conditions such as cellular energy depletion, hypoxia and osmotic stress. Under hypoxic conditions TORC1 mediates the adaptive mechanism activated in cells in order to restrict

energy intense processes such as protein translation. It has been shown that this mechanism involves inhibition of TORC1, which occurs through a novel pathway involving the TSC1/2 complex and the *REDD1* gene, of which recent studies provide evidence to be involved in the release of TSC2 from inhibitory 14-3-3 as an essential mechanism that restricts mTORC1 activity in response to hypoxic stress (DeYoung, Horak et al. 2008).

The mechanism by which amino acids triggers signaling through mTORC1 seems to be independent of TSC2, since studies showed that amino acids modulate mTORC1 signaling in cells lacking TSC2 (smith, em, 2005, j boil chem.; nobukuni, p, 2005, pnas). Instead, another protein has been proposed to be involved in regulation of mTOR by amino acid, namely the type III PI3K Vps34, which acts in a complex with Vps15 to generate PI3P on endosomal membranes and thus regulates protein sorting. Knock down of either Vps34 or Vps15 by siRNA in mammalian cells reduces the amino acid repletion-induced phosphorylation of S6K1 (Nobukuni, Joaquin et al. 2005). However, a clear mechanism is yet to be established.

Exactly to which extracellular stimuli TORC2 responds to is still under investigation, but so far in mammals, TORC2 functions are regulated by growth factors such as insulin. Stress responses that promote cell survival or viability also require the presence of TORC2. Furthermore, TORC2 has been implicated in processes such as the regulation of cell morphology and cytoskeletal reorganization (Jacinto, Loewith et al. 2004; Sarbassov, Ali et al. 2004).



(adapted from Taguchi, 2008, Ann Rev Physiol)

Figure 2: The Insulin signaling cascade in mammals

Insulin signaling in *Drosophila*

In *Drosophila*, the insulin signal transduction cascade is highly conserved. Until today, a large number of homologs of components of the insulin pathway in the fruit fly have been identified and characterized. In this chapter we summarize the *Drosophila* Insulin signaling cascade and emphasize in particular the differences to the vertebrate system.

Although the presence of insulin-like hormones in insects was already speculated more than 30 years ago, it was until 2001 when the seven putative insulin receptor ligands in *Drosophila* were identified, the so-called *Drosophila* Insulin-like peptides

(DILPs) (Brogiolo, Stocker et al. 2001). Of those seven *dilps*, *dilp1-dilp5* are located to a cluster on the third chromosome, whereas *dilp6* and *dilp7* are positioned on different loci of the X-chromosome. The *dilps* encode for insulin precursor proteins, consisting of between 107 and 156 amino acids. Structurally similar to preproinsulin they are composed of the B-chain, the C-peptide and the A-chain. The highest homology to the mammalian insulins has been demonstrated for DILP2 with 35% identity. Over expression of any of the respective *dilps* in *Drosophila* resulted in increased body size in all cases, suggesting an agonistic function towards the insulin receptor (Ikeya, Galic et al. 2002). However, although the *dilps* and the *insulin receptor* genetically interact, direct physical association between the two has never been shown. In situ hybridization using *dilp* antisense probes revealed differential expression patterns in embryos and larvae, suggesting distinct functional specificity (Brogiolo, Stocker et al. 2001). At least three of the seven *dilps* (*dilp2, dilp7 and dilp5*) are expressed in two symmetric clusters containing seven neurosecretory cells of each hemisphere in the larval brain, called the insulin producing cells (IPCs). The IPCs project their axons to the aorta, which allows the recycling of the hemolymph towards the anterior part of the larva and to the corpora cardiaca of the ring gland, a neuroendocrine tissue that produces a glucagon-like hormone. Specific ablation of the IPCs by over expression of the cell death inducing gene *reaper* under the control of the *dilp2* promoter lead to a reduction in cell size and number of the flies, developmental delay, defects in female fertility and elevated carbohydrate levels in the larval hemolymph (Ikeya, Galic et al. 2002; Rulifson, Kim et al. 2002). All these phenotypes can be rescued by over expression of *dilp2*, suggesting a certain level of redundancy between the respective *dilps*. In summary, these results provide evidence that the *dilps* fulfill their postulated function as *Drosophila* insulin homologs and, furthermore, that they carry out both, the growth promoting functions of the vertebrate IGFs and the metabolic functions of the vertebrate Insulins.

In contrast to vertebrates that contain four different receptors for peptides of the insulin family, there is only one *insulin receptor* (*dInR*) homolog present in the *Drosophila* genome (Fernandez, Tabarini et al. 1995). Like its mammalian equivalents, the *Drosophila* Insulin receptor is built up of two α and two β subunits of which the latter contains a kinase domain that is activated upon ligand binding.

Interestingly, the protein sequence of the respective Insulin receptors are highly conserved, in particular around the kinase domain and the ligand binding domain, which are identical to about 40%. Furthermore, the activation of the *Drosophila* Insulin receptor can be triggered by human Insulin (Fernandez, Tabarini et al. 1995; Chen, Jack et al. 1996). However, the most noticeable dissimilarity between the Insulin receptor of the two individual species is an extension of 368 amino acids in the β subunit of the *Drosophila* receptor. The extension contains additional tyrosine phosphorylation sites (Tyr^{1941}, Tyr^{1957} and Tyr^{1978}) that serve as binding sites for downstream components, such as PI3K, and have been shown to allow the *Drosophila* Insulin receptor to transduce its signal without any intermediate adaptor molecules (Yenush, Fernandez et al. 1996). The Insulin receptor in *Drosophila* is essential for normal development, because strong EMS induced *dInR* mutations are recessive embryonic or early larval lethal (Fernandez, Tabarini et al. 1995; Chen, Jack et al. 1996). Nevertheless, some heteroallelic combinations were viable and yielded adults with a severe growth retardation, developmental delay, female sterility and increased lifespan (Chen, Jack et al. 1996; Brogiolo, Stocker et al. 2001; Tatar, Kopelman et al. 2001).

As in vertebrates, activation of the Insulin receptor by its ligands in *Drosophila* results in auto-phosphorylation and subsequent recruitment of the insulin receptor substrate (IRS1-4) homolog, termed Chico. Mutants for *chico* were identified in a screen for mutations causing a reduction in body size (Bohni, Riesgo-Escovar et al. 1999). Homozygous *chico* mutant flies are viable but are severely smaller than their wild type littermates due to a cell autonomous reduction in cell size and cell number. Furthermore, in addition to their size defects, flies lacking *chico* function display a particular set of phenotypes that can also be observed in flies mutant for other components of the insulin signaling pathway (*dInR*, *dp110 PI3K*, *dPKB*), such as developmental delay, increased body lipid levels and female sterility (Bohni, Riesgo-Escovar et al. 1999).

Like its mammalian homologs, the Chico protein is composed of an N-terminal PH domain, a PTB domain and several tyrosines that serve as SH2 binding motifs when phosphorylated. For example, Chico contains two YxxM sites that are recognized by the p60 regulatory subunit of *Drosophila* PI3K (dp60), leading to subsequent

association of dp60 to the catalytic subunit dp110, which is an essential step for the activation of PI3K. However, the C-terminal of the *Drosophila* InR contains several PI3K binding sites, which have been shown to be functional and able to compensate for the absence of IRS proteins (Yenush, Fernandez et al. 1996). In vertebrates, binding of Grb2 to the YxN Grb2/Drk consensus sites of IRS activates the Ras/MAPK pathway in response to Insulin signaling. Although an YxN motif is also found within the Chico protein sequence, depletion of this motif does not interfere with Chico mediated growth and the attainment of wild type body size (Oldham s, 2002, development). So far, no evidence for a direct connection between the Insulin pathway and Ras/MAPK signaling in *Drosophila* could be established.

After binding to Chico and concurrent activation, the *Drosophila* PI3K, as its vertebrate homolog, promotes the conversion of PIP_2 into PIP_3 and the antagonistic phosphatase dPTEN catalyzes the reverse reaction. The cellular accumulation of PIP_3 activates a second messenger pathway that promotes cell growth and proliferation (Oldham, Stocker et al. 2002). The following mechanism transducing the Insulin signal further downstream includes binding of the PH domain of *Drosophila* PKB/Akt (dPKB/dAkt) to PIP_3 and co-localization to dPDK (Scheid and Woodgett 2001). At the membrane, subsequent phosphorylation events by dPDK1 at Thr^{308} (Thr^{342} in *Drosophila*) and by TORC2 at Ser^{473} (Ser^{505} in *Drosophila*) lead to full activation of dPKB/dAkt (Alessi, Andjelkovic et al. 1996; Alessi, Deak et al. 1997; Hresko and Mueckler 2005; Hietakangas and Cohen 2007). Other than in vertebrates, where three PKB proteins have been described (Akt1-3), *Drosophila* only contains one. In *Drosophila*, dPKB/dAkt is of particular importance for the activity of Insulin signaling cascade, as reduced dPKB/dAkt activity is sufficient to rescue lethality due to loss of *dPTEN* function (Stocker, Andjelkovic et al. 2002). Biochemical and genetic studies in *Drosophila* have identified FoxO and TSC2 as two critical downstream targets of dPKB/dAkt. In analogy to the mammalian system, TSC2 functions as a GAP for Rheb, which acts as a positive regulator of TOR signaling (Hafen 2004).

Also in *Drosophila*, TOR is important for the control of cell growth. When activated, it promotes cell growth by enhancing translation and ribosome biogenesis through phosphorylation of the initiation factor 4E-bindingprotein (d4EBP) and ribosomal

protein S6 kinase (dS6K), respectively (Miron, Lasko et al. 2003). Further, TOR promotes bulk endocytosis and the inhibition of the targeted endocytosis of amino acid transporters such as Slimfast in *Drosophila* fat body cells. Potentially, both processes increase the nutrient availability to the cell (Colombani, Raisin et al. 2003; Hennig, Colombani et al. 2006).

Phenotypes associated with impaired Insulin signaling in *Drosophila*

Defects in the insulin signaling cascade usually lead to a severe and specific set of phenotypes. Especially in *Drosophila* these phenotypes have been characterized in detail and were used as a readout to identify new genes involved in Insulin signaling. The most apparent effects of mutations in upstream core components of the Insulin pathway are alterations in the final body size of the animals, whereas differentiation and patterning remains normal. Characteristically, cell size and cell number are affected. Flies mutant for positive components of the insulin pathway are small, due to smaller and less cells, while loss of function of negative components results in bigger and more cells (Hafen 2004). Although many genes involved in insulin signaling, such as *dInR*, *dPI3K*, *dPTEN* and *dPKB/dAkt*, turned out to be recessive embryonic lethal, combination of hypomorphic alleles lead to adult flies in some cases (Garofalo 2002). These flies failed to reach wild type size due to decreased cell size and cell number. Importantly, the effects on cell size and cell number were strictly cell autonomous. It appears that Insulin signaling affects cell size and cell number independently through two distinct pathway branches emerging downstream of dPKB/dAkt. Apparently, the regulation of cell size is achieved through the TSC1/2, Rheb, dTOR, dS6K branch. For example, mutants for *dS6K* display a reduction in cell size but not cell number (Montagne, Stewart et al. 1999). Experiments in which double mutants of *chico* and *dFOXO* were created clearly showed that removing one copy of *dFOXO* is sufficient to dominantly suppress the cell number reduction due to loss of *chico* function but has no effect on cell size, suggesting that the downstream

target of dPKB/dAkt regulating cell number is indeed dFOXO (Junger, Rintelen et al. 2003).

Interestingly, the only component of the canonical Insulin pathway upstream of dPKB/dAkt that is viable in a homozygous mutant situation is the *IRS1-4* homolog *chico*. Consistent with the role of *chico* as a positive regulator of Insulin signaling, homozygous mutant adults are also severely smaller than wild type flies, due to a decrease in cell number and cell size. In addition to the growth reduction phenotype, *chico* mutants further displayed defects that could be assigned to impaired transduction of the Insulin signal such as a developmental delay, female sterility, increased lipid levels and an extended lifespan (Bohni, Riesgo-Escovar et al. 1999; Clancy, Gems et al. 2001). These observations underscore the importance of the *Drosophila* Insulin pathway in normal development, oogenesis, energy homeostasis and longevity. Taken together, the complete set of phenotypes that was repeatedly described for flies lacking proper Insulin signaling activity provides clear evidence that the insulin pathway in *Drosophila* combines the growth regulatory function of IGF and the role in metabolism and energy homeostasis of Insulin mediated signaling in vertebrates.

ey-FLP screen to identify new genes involved in growth control

Within the past 10 years a number of screens based on different strategies have been conducted by many labs in order to identify previously uncharacterized genes implicated in growth control. Whereas early on, most such screens were based on over expression of EP elements in a certain organ of the fly in a wild type or sensitized background, it became more popular later to perform clonal screens for recessive mutations (Rorth 1996; Hipfner, Weigmann et al. 2002; St Johnston 2002). Here, homozygous clones of mutant tissue were generated preferably in the eyes of otherwise heterozygous animals by the mutagen Ethylmethane Sulphonate (EMS), using a *FLP recombinase* expressed under the control of the *eyeless* promoter (*ey-FLP*) (Newsome, Asling et al. 2000). In our lab we made use of this strategy with an additional recessive cell lethal mutation on the homologous chromosome which results in the elimination of the wild type sister cells and thus lead to fly heads that

consist almost entirely of mutant tissue. This approach allowed screening for genes that would cause lethality in ubiquitous homozygous animals and the identification of both, growth-inhibiting and growth promoting genes. In this way, a large number of growth regulators have been identified, among which there were *rheb*, *hippo*, *salvador*, *TSC1* and *dTOR* (Ito and Rubin 1999; Zhang, Stallock et al. 2000; Tapon, Harvey et al. 2002; Stocker, Radimerski et al. 2003; Wu, Huang et al. 2003). From the large number of mutations affecting head size, we were particularly interested in mutations that affect the size of the head without interfering with differentiation. As part of our screen we found mutations causing a severe reduction of head size that we were able to assign to gene called *lnk*, encoding for a *Drosophila* homolog of the mammalian SH2B family of adaptor proteins.

The mammalian SH2B-family of adaptor proteins

The mammalian SH2B-family of adaptor proteins consists of three members, SH2B1 (SH2B/PSM) and its four isoforms (α, β, γ and δ), SH2B2 (APS) and SH2B3 (Lnk). These proteins share a common protein structure with an N-terminal PH domain, an SH2 domain and a highly conserved Cbl recognition motif at the C-terminus (Figure 3) (Huang, Li et al. 1995; Riedel, Wang et al. 1997; Yokouchi, Suzuki et al. 1997).
All three proteins are expressed in a large number of tissues within the organism, and have been implicated in the regulation of various receptor tyrosine kinase signaling pathways such as the Insulin receptor, Insulin like growth factor I receptor as well as receptors for nerve growth factor, hepatocyte growth factor, platelet derived growth factor, fibroblast growth factor and for the Janus-kinase (JAK) family of tyrosine kinase (Rui, Mathews et al. 1997; Wakioka, Sasaki et al. 1999; Koch, Mancini et al. 2000; Riedel, Yousaf et al. 2000; Kong, Wang et al. 2002). However, despite their similar composition and protein structure, each member of the SH2B protein family seems to fulfill individually distinct functions.

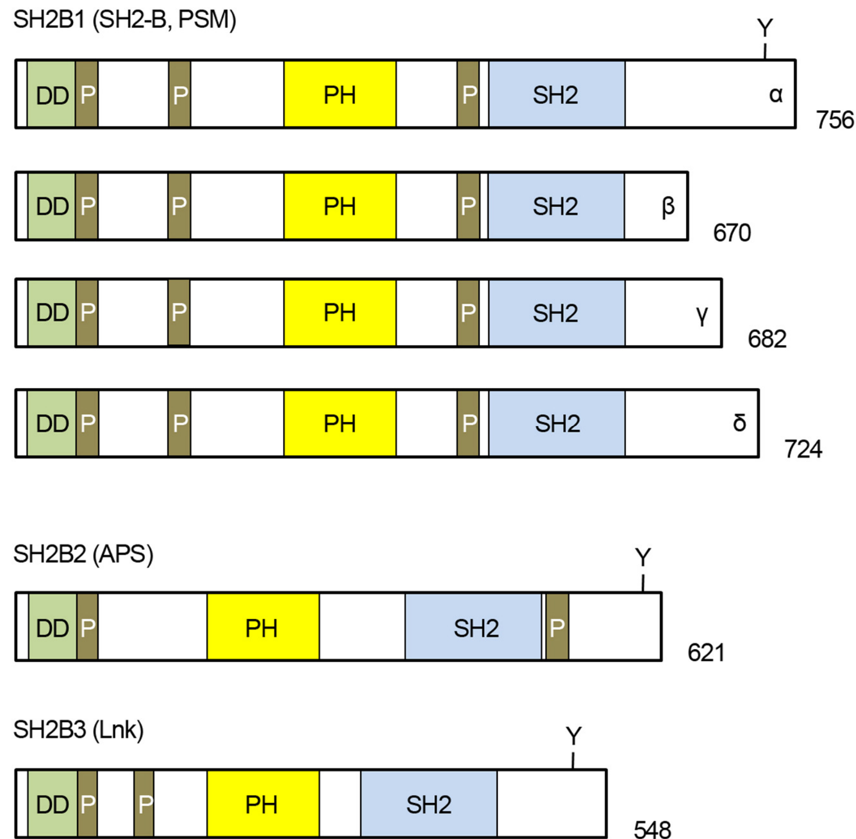


Figure 3: The mammalian SH2B family of adaptor proteins

The members of the mammalian SH2B family consists of four isoforms of SH2B1 (SH2-B), SH2B2 (APS) and SH2B3 (Lnk). All Proteins share a common structure with a dimerization domain (DD) at the C-terminus, proline-rich stretches (P), a PH domain, an SH2 domain and an N-terminal tyrosine (Y) within a c-Cbl consensus binding motif.

SH2B1 was originally termed SH2B and first identified in rat by its association with the activated catalytic domain of the insulin receptor in a yeast-two-hybrid screen or as a substrate of activated JAK2 by coimmunoprecipitation in 3T3-F442A preadipocyte cells (Riedel, Wang et al. 1997; Rui, Mathews et al. 1997). Deletion of SH2B1 impaired leptin stimulated JAK2 activation and phosphorylation of both the transcription activator Stat3 and IRS2 in the hypothalamus which resulted in severe leptin resistance, hyperphagia and obesity (Ren, Li et al. 2005). However, in addition to Leptin and JAK2 related phenotypes, SH2B1 knockout mice also displayed

phenotypes that are connected to defective Insulin signaling such as Insulin resistance and type II diabetes due to attenuated Insulin signaling in the liver, muscle and adipose tissue (Duan, Yang et al. 2004; Li, Ren et al. 2006). Furthermore, mice lacking *SH2B1* function display impaired fertility (Ohtsuka, Takaki et al. 2002).
SH2B2 (APS) was also shown to bind to JAK2 and the Insulin receptor (Moodie, Alleman-Sposeto et al. 1999; Wakioka, Sasaki et al. 1999). However, research of the past years mainly focused on the mechanisms related to the connection of SH2B2 and the proto-oncogenic E3 ubiquitin ligase c-Cbl (Fiorini, Alimandi et al. 2001; Li, Li et al. 2007). Phosphorylation of Tyr^{618} in SH2B2 stimulates binding of c-Cbl and thus mediates GLUT4 translocation and inhibition of erythropoietin dependent activation of Stat5 (Liu, Kimura et al. 2002).
However, the general impact of SH2B2 on receptor tyrosine kinase signaling remains contradictory. Whereas Ahmed et al show that *SH2B2* over expression delays Insulin receptor tyrosine and Insulin receptor substrate dephosphorylation and enhances Akt activation, several other studies, e.g. on *SH2B2* knockout mice suggest a negative regulatory role for *SH2B2* Insulin signaling, which might also be mediated via c-Cbl dependent ubiquitination and subsequent degradation of target kinases (Ahmed, Smith et al. 2000; Fiorini, Alimandi et al. 2001; Liu, Kimura et al. 2002). Interestingly, *SH2B2* knockout mice do not show obvious defects in glucose or lipid metabolism unless *SH2B1* is non-functional, too (Minami, Iseki et al. 2003; Li, Ren et al. 2006). Therefore, these results suggest that, although *SH2B1* and *SH2B2* have been described to predominantly fulfill separate functions, they are still able to compensate for the loss of each other.
So far, the third member of the SH2B family, SH2B3 (Lnk) has been exclusively described as a negative regulator of receptor kinases that are specialized in the development of a subset of immune and hematopoietic cells (Rudd 2001; Velazquez, Cheng et al. 2002). However, *SH2B3* transcripts are also present in non hematopoietic tissue such as testis, brain and muscle, where no specific phenotype correlating to the loss of *SH2B3* function could be revealed so far. These results suggest that *SH2B3* has a function in these tissues that is redundant to the other SH2B family members (Takaki, Sauer et al. 2000; Rudd 2001; Velazquez, Cheng et al. 2002).

The *Drosophila* genome encodes for a single homolog of the SH2B family, termed Lnk. *Drosophila* Lnk shares a similar protein structure to the mammalian SH2B proteins with the highest sequence homology to SH2B1 and SH2B2 particularly in the PH and SH2 domains, motifs that enable adaptor proteins to interact with their partners.

Adaptor proteins

Many steps in signal transduction require the formation of multi- protein complexes, mediated by proteins summarized in the family of so-called adaptor proteins. These proteins can recruit binding partners to a specific location and regulate interactions between different signal transduction proteins and even establish a connection between two originally distinct signaling pathways. Adaptor proteins lack any enzymatic activity or transcription activation domains. Instead, they usually contain a variety of discrete binding sites and domains that allow protein-protein interactions according to their target binding specificity (Pawson and Scott 1997). The best characterized of these domains comprise src homology 2 (SH2) and phosphotyrosine binding (PH) domains, which bind to phosphorylated tyrosine residues that are part of specific recognition motifs; src homology 3 (SH3) domains that bind to proline rich regions of proteins, and pleckstrin homology (PH) domains that mediate interactions to phospholipids (Harlan, Hajduk et al. 1994; Marengere and Pawson 1994; Pawson 1995). Further examples include the WW domain that associates either with proline rich regions or phosphorylated serine or threonine residues as well as the PDZ domain that bind short hydrophobic residue-containing motifs (Doyle, Lee et al. 1996; Sudol 1996). Additionally, most adaptor proteins contain multiple residues that serve themselves as binding targets for other proteins. Notably, adaptor proteins usually contain only a single phosphotyrosine binding module – except for Shc-related adaptors, containing both a PTB domain and an SH2 domain (Luzi, Confalonieri et al. 2000).

Given the diversity in the presence and combinations of interaction domains within adaptor proteins, a prediction for their cellular roles in signaling is impossible.

Furthermore, in most cases, adaptor molecules are not restricted to a single signal transduction process but rather involved in multiple signaling events. A nice example for such diverse regulatory functions is the adaptor Grb2, which is involved in different signaling cascades and the activation of various effectors. Grb2 is the predominant constitutive binding partner of the Ras activator Sos and bridges the Epidermal growth factor (EGF) receptor directly to Sos (Downward 1994). However, Grb2 can bind other adaptor molecules, e.g. Gab1, which can then activate the effector PI3K (Lock, Royal et al. 2000). Furthermore, in the context of Fibroblast growth factor (FGF) signaling, Grb2 is recruited to the receptor by the adaptor protein FRS2 and then binds to Gab1, thus linking these two adaptor molecules together (Csiszar 2006). Although rather indirectly involved in the transduction of molecular signals, adaptor proteins have been shown to be indispensable for most signaling pathways and cellular processes.

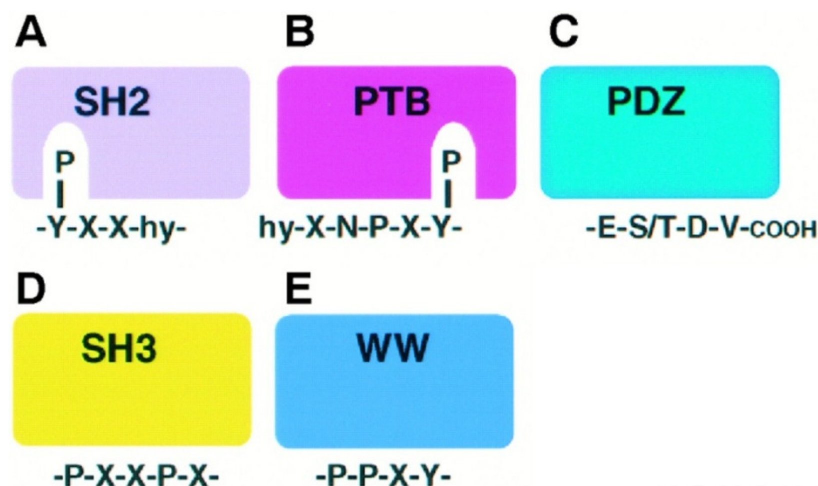


Figure 4: Protein domains that are able to mediate direct protein-protein interactions

Several protein domains have been identified that enable adaptor proteins to recognize specific amino acid sequences in their binding targets and thus mediate their physical interaction.

Results

The results part is divided into the following two parts:

-The manuscript of ‘The *Drosophila* SH2B Family Adaptor Lnk Acts in Parallel to Chico in the Insulin Signaling Pathway’

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-Additional results of the characterization of *Drosophila* Lnk

The Drosophila SH2B Family Adaptor Lnk Acts in Parallel to Chico in the Insulin Signaling Pathway

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Abstract

Insulin/insulin-like growth factor signaling (IIS) plays a pivotal role in the regulation of growth at the cellular and the organismal level during animal development. Flies with impaired IIS are developmentally delayed and small due to fewer and smaller cells. In the search for new growth-promoting genes, we identified mutations in the gene encoding Lnk, the single fly member of the SH2B family of adaptor molecules. Flies lacking *lnk* function are viable but severely reduced in size. Furthermore, *lnk* mutants display phenotypes reminiscent of reduced IIS such as developmental delay, female sterility, and accumulation of lipids. Genetic epistasis analysis places *lnk* downstream of the *insulin receptor* (*InR*) and upstream of *PI3K* in the IIS cascade, at the same level as *chico* (encoding the single fly IRS homolog). Both *chico* and *lnk* mutant larvae display a similar reduction in IIS activity as judged by the localization of a PIP_3 reporter and the phosphorylation of PKB. Furthermore, *chico; lnk* double mutants are synthetically lethal, suggesting that Chico and Lnk fulfill independent but partially redundant functions in the activation of PI3K upon InR stimulation.

Author Summary

The regulation of growth is among the most fundamental processes during development of multicellular organisms. Research over the past years has established a key function of the insulin/insulin-like growth factor signaling (IIS) pathway in ensuring proper growth at the cellular and the organismal level. Impaired IIS has been associated with diseases such as type 2 diabetes, leprechaunism and heart disease, and deregulated IIS often contributes to the development of cancer. Here, we describe the characterization of the *Drosophila* SH2B family adaptor protein Lnk. Mutants in *lnk* are viable but unable to reach the normal size due to a reduction in cell size and cell number. Our characterization of *lnk* mutant flies has revealed phenotypes associated with impaired IIS, such as developmental delay, female sterility, and increased lipid levels in adults. Using a combination of genetic

interaction experiments and molecular readouts for IIS activity, we demonstrate that Lnk acts in parallel to the IRS homolog Chico downstream of the insulin receptor to regulate cellular growth.

Introduction

The control of cell, organ and body size is tightly regulated to ensure proper development of multicellular organisms. A key pathway controlling growth, metabolism, reproduction and longevity is the insulin/insulin-like growth factor signaling (IIS) pathway (Rulifson, Kim et al. 2002). The insulin receptor (InR) and the corresponding downstream core components are conserved in *Drosophila* (Fernandez, Tabarini et al. 1995; Chen, Jack et al. 1996; Garofalo 2002), mediating cell growth and cell division in response to environmental factors such as nutrient availability through a series of protein-protein interactions and phosphorylation events (Ikeya, Galic et al. 2002).
The core components of the *Drosophila* IIS pathway include Chico, the homolog of the insulin receptor substrates (IRS), the lipid kinase PI3K, the lipid phosphatase PTEN, and the serine-threonine kinase PKB (Hafen 2004). Chico gets phosphorylated upon IIS pathway activation, providing binding sites for the SH2 domain of p60, the regulatory subunit of PI3K. Increased PI3K activity leads to the accumulation of PIP_3 at the plasma membrane, which recruits PKB to the membrane via its PH domain. PKB takes a central position in the regulation of multiple cellular processes such as cellular growth, proliferation, apoptosis, transcription and cell motility (Brazil, Park et al. 2002).
In *Drosophila*, mutations in IIS components result in reduced cell, organ and body size with little effect on cell fate and differentiation. For example, hypomorphic mutants of essential IIS components and, in particular, homozygous null mutants of *chico* are viable but only approximately half the size of wild-type flies, due to smaller and fewer cells. Furthermore, characteristic defects caused by reduced IIS activity

include female sterility, increase in total lipid levels of adults, and a severe developmental delay (Bohni, Riesgo-Escovar et al. 1999; Goberdhan and Wilson 2003).

chico encodes an adaptor protein, a group of proteins lacking catalytic activity that usually contain domains mediating specific interactions with other proteins such as an SH2 domain, a PH domain, or a PTB domain. Adaptor proteins play an important role in the formation of protein-protein interactions and thus in the formation of protein networks. The various interaction domains within adaptor proteins and the specificity of those domains provide adaptor molecules with the ability to elicit characteristic responses to a particular signal.

Recently, a novel family of adaptor proteins, the SH2B family, has been identified in mammals. It consists of three members – SH2B1 (SH2B/PSM), SH2B2 (APS) and SH2B3 (Lnk) – that share a common protein structure with an N-terminal proline-rich stretch, a PH domain, an SH2 domain and a highly conserved C-terminal Cbl recognition motif (Huang, Li et al. 1995; Riedel, Wang et al. 1997; Yokouchi, Suzuki et al. 1997). They have been shown to regulate signal transduction downstream of several receptor tyrosine kinases such as the InR, IGF-I receptor as well as receptors for nerve growth factor, hepatocyte growth factor, platelet-derived growth factor, fibroblast growth factor and for the JAK family of tyrosine kinases (Riedel, Wang et al. 1997; Rui, Mathews et al. 1997; Wakioka, Sasaki et al. 1999; Koch, Mancini et al. 2000; Riedel, Yousaf et al. 2000; Kong, Wang et al. 2002). Whereas SH2B3 (Lnk) has been described to function exclusively by negatively regulating receptor kinases that are specialized in the development of a subset of immune and hematopoietic cells, the picture for the other two family members is not as clear yet (Velazquez, Cheng et al. 2002).

Although both SH2B1 and SH2B2 have been shown to be directly involved in the regulation of JAK tyrosine kinases and of IIS, their specificities and physiological functions are complex and remain largely elusive. For example, depletion of SH2B1 in mice leads to severe obesity, leptin and insulin resistance as well as female infertility (Ohtsuka, Takaki et al. 2002; Ren, Li et al. 2005). However, a number of studies suggest that SH2B1 exerts its function predominantly in the association with JAK2 and regulation of related signaling cascades (Maures, Kurzer et al. 2007). For

example, binding of SH2B1 to JAK2 results in an enhancement of JAK2 activation and JAK2-mediated growth hormone signaling (Rui and Carter-Su 1999), and depletion of SH2B1 leads to decreased leptin-stimulated JAK2 activation and reduced phosphorylation of its substrates (Ren, Li et al. 2005).
SH2B2 is also able to bind to JAK2 and to the InR (Moodie, Alleman-Sposeto et al. 1999; Wakioka, Sasaki et al. 1999) but recent research has mainly focused on the mechanisms related to the connection of SH2B2 and c-Cbl (Wakioka, Sasaki et al. 1999; Ahmed, Smith et al. 2000; Liu, Kimura et al. 2002). Phosphorylation of Tyr618 in SH2B2 stimulates binding of c-Cbl and thus mediates GLUT4 translocation and inhibition of erythropoietin-dependent activation of Stat5 (Wakioka, Sasaki et al. 1999; Liu, Kimura et al. 2002). However, the general impact of SH2B2 on receptor tyrosine kinase signaling remains contradictory. Whereas Ahmed and colleagues showed that SH2B2 overexpression delayed InR and IRS dephosphorylation and enhanced Akt activation (Ahmed, Smith et al. 2000), several other studies (e.g. on SH2B2 knockout mice) suggest a negative regulatory role for SH2B2 in IIS, which might also be mediated via c-Cbl dependent ubiquitination and subsequent degradation of target kinases (Fiorini, Alimandi et al. 2001; Li, Li et al. 2007).
Although interactions with the IIS pathway and the InR have been described for SH2B1 and SH2B2, the physiological significance of this connection in mammals appears to be the regulation of metabolism and energy homeostasis rather than the control of cell growth and proliferation (Minami, Iseki et al. 2003; Ren, Li et al. 2005).
In contrast to the mammalian situation, the *Drosophila* genome encodes a single adaptor protein that shares a common domain structure with the SH2B family, termed Lnk. Here, we show that *Drosophila lnk* predominantly regulates cellular and organismal growth in a cell-autonomous way. We observed that loss of *lnk* function leads to a reduction in cell size and cell number, reminiscent of decreased IIS activity. A thorough genetic analysis placed Lnk as a positive regulator of IIS at the level of IRS/Chico.

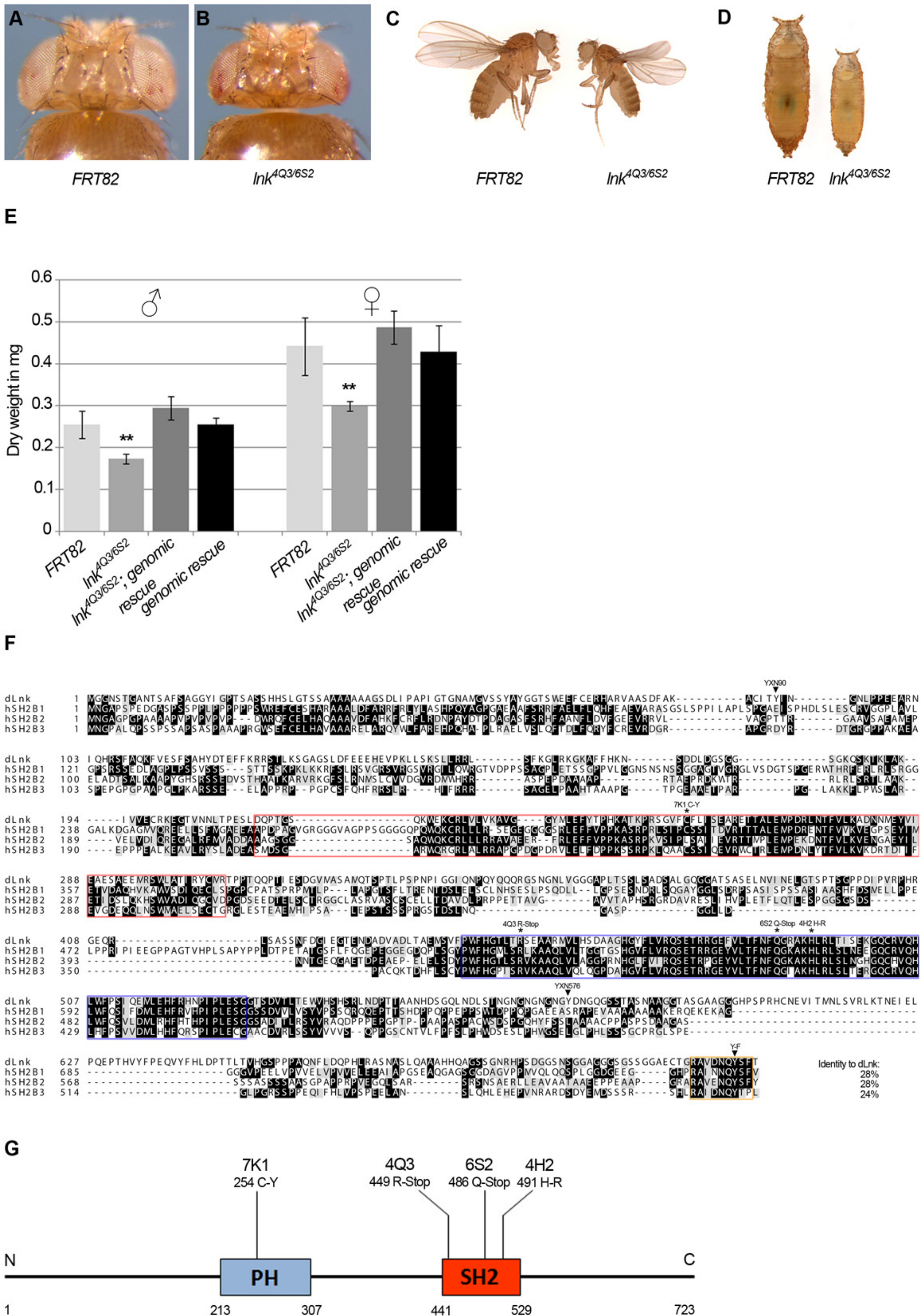
Results and Discussion

Drosophila lnk Regulates Growth and Body Size

We identified *lnk* in an unbiased screen for growth-regulating genes based on the eyFLP/FRT technique in *Drosophila.* In principle, mutations in growth-promoting genes led to flies with smaller heads (the so-called pinheads), whereas negative regulators of tissue growth resulted in larger heads (referred to as bighead mutants). Among others, we identified four mutations causing a pinhead phenotype that fell into a single complementation group on the right arm of the third chromosome (Figure 1B). We mapped the complementation group close to the *lnk* locus (CG 17367) at the cytological position 96F. Subsequent sequencing revealed EMS-induced mutations in the *lnk* coding region for each allele.

Flies homozygous mutant for *lnk* are small but do not show any obvious patterning defects (Figure 1C). Homozygous mutant pupae are also small, indicating that *lnk* is essential for proper organismal growth throughout development (Figure 1D). *lnk* mutant flies are severely reduced in dry weight, as shown for male and female flies (Figure 1E). This defect is fully rescued by introducing a genomic rescue construct comprising the entire *lnk* locus, proving that the mutations in *lnk* are responsible for the growth phenotype (Figure 1E).

The most closely related group of proteins to *Drosophila* Lnk in vertebrates is the SH2B family of adaptor proteins sharing a common protein structure. Alignment of *Drosophila* Lnk with its human homologs (SH2B1, SH2B2 and SH2B3) shows high sequence identity in particular in the conserved PH and SH2 domains (Figure 1F, Figure S2). The four *lnk* alleles recovered in the screen (7K1, 4Q3, 6S2, 4H2) contain a single point mutation in either of these two highly conserved protein domains resulting in a premature stop (4Q3, 6S2) or an amino acid exchange in conserved residues (7K1, 4H2) (Figure 1F and 1G). Since hemizygous and heteroallelic *lnk* mutant animals display identical phenotypes, all *lnk* alleles are genetically null, suggesting an essential role of both the PH and the SH2 domain.


A
B
C
D
FRT82
lnk4Q3/6S2
FRT82
lnk4Q3/6S2
FRT82
lnk4Q3/6S2
E
Dry weight in mg
0.6
0.5
0.4
0.3
0.2
0.1
0
♂
♀
**
**
FRT82
lnk4Q3/6S2
lnk4Q3/6S2; genomic rescue
genomic rescue
FRT82
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F
dLnk
hSH2B1
hSH2B2
hSH2B3
YXN90
7K1 C-Y
4Q3 R-Stop
6S2 Q-Stop
4H2 H-R
YXN576
Y-F
Identity to dLnk:
28%
28%
24%
G
7K1
254 C-Y
4Q3
449 R-Stop
6S2
486 Q-Stop
4H2
491 H-R
N
C
PH
SH2
1
213
307
441
529
723

Figure 1. Flies mutant for *lnk* are viable but small

(A-D) *lnk* regulates organismal size throughout development. In comparison to *ey-flp* induced control clones (*FRT82*, A), *FRT82 lnk*^{4Q3} clones result in a small head phenotype (B). *lnk*^{4Q3}/*lnk*^{4Q3} adult flies (C) and pupae (D) are smaller than the controls. (E) Flies lacking *lnk* function are strongly reduced in dry weight. Introduction of a genomic construct comprising the *lnk* locus rescues the *lnk* growth deficit. Significant changes relative to the control ($p \leq 0.01$, Student's t-test, n=20) are marked by double asterisks; error bars represent the standard deviation. (F) Alignment of the *Drosophila* Lnk protein and its human homologs of the SH2B family of adaptor proteins. *lnk* codes for a 723 amino acid adaptor protein containing a PH domain and an SH2 domain. Black and grey boxes indicate amino acid identity and similarity, respectively. The SH2 domain is highlighted in red, the PH domain in blue, and the highly conserved Cbl binding motif in orange. Asterisks mark the mutations recovered in the screen, and arrowheads indicate the tyrosines of the two potential YXN Drk SH2 binding motifs and the conserved Cbl binding motif. (G) The mutations leading to an amino acid exchange in the PH domain (7K1) and to premature translational stops (4Q3, 6S2) or an amino acid exchange in the SH2 domain (4H2), respectively, genetically behave as null alleles, indicating that both the PH and the SH2 domain are essential for Lnk's function. Genotypes are: (A) *y w ey-flp*/*y w*; *FRT82*/*FRT82 cl(3R3) w*$^{+}$, (B) *y w ey-flp*/*y w*; *FRT82 lnk*^{4Q3}/*FRT82 cl(3R3) w*$^{+}$.

Lnk Is a Component of the IIS Pathway

SH2B1 and SH2B2, two members of the mammalian family of Lnk-related adaptor proteins, have been shown to associate with several signaling molecules including JAK2 and the InR (Riedel, Wang et al. 1997; Li, Ren et al. 2006; Maures, Kurzer et al. 2007). However, the different proteins seem to have distinct impacts on the respective pathways, regulating them either in a positive or negative manner (O'Brien, O'Shea et al. 2002; Li, Ren et al. 2006). Using the new mutations in the single member of the SH2B family in *Drosophila* allowed us to determine whether *lnk* plays an essential role in either of these pathways.

Although the tyrosines in JAK2 and JAK3 mediating their interaction with the SH2B family proteins in mammals are not conserved in the *Drosophila* homolog, we wondered whether Lnk has a function in the regulation of *Drosophila* JAK. Misregulation of JAK/Stat signaling in *Drosophila* results in formation of melanotic tumors and proliferative defects in larval blood cells, held out wings and rough or disrupted eye phenotypes as well as male sterility and fused egg chambers in the vitellarium due to the absence of stalk cells (Zeidler, Bach et al. 2000; Baksa, Parke et al. 2002; McGregor, Xi et al. 2002; Arbouzova and Zeidler 2006). In our characterization of homozygous *lnk* mutant animals, we did not observe any of the

phenotypes that are characteristic for impaired JAK/Stat signaling (data not shown). Moreover, genetic interaction experiments of *lnk* with any of the core JAK/Stat pathway components did not reveal a connection of Lnk to JAK/Stat signaling. These results suggest that in *Drosophila*, Lnk is not involved in the regulation of signaling activity downstream of JAK.

The initial observation that *lnk* mutations reduced organ and body size pointed to a role of Lnk in the IIS pathway. We characterized the growth phenotype of *lnk* mutants further by quantifying ommatidia number and generating tangential sections of mosaic eyes to study the impact of *lnk* on cell number and cell size (Figure 2A-E). SEM pictures of heads of *lnk* mutant adults compared to wild type and quantification of ommatidia number revealed that mutations in *lnk* caused a reduction in cell number by about 30% (Figure 2A-C). Induction of *lnk* mutant clones in the eye resulted in a cell-autonomous reduction of cell size in photoreceptor cells and rhabdomeres, as shown by tangential eye sections (Figure 2D, arrowheads) and subsequent quantification of photoreceptor cell and rhabdomere area in *lnk* mutant tissue compared to wild type (Figure 2E). Therefore, *lnk* function is important to ensure proper regulation of cell number and cell size, similar to IIS components.
It has previously been shown that IIS is required in oogenesis beyond the last previtellogenic stage, thus a reduction of IIS leads to an arrest in oogenesis and female sterility (Drummond-Barbosa and Spradling 2001). Female flies lacking *lnk* function are also sterile and have small ovaries. These ovaries only contain oocytes that developed until the last previtellogenic stage and resemble ovaries of females mutant for *chico*, the homolog of human IRS (Figure 2F and 2G).
A further characteristic phenotype of impaired IIS is the accumulation of lipids in adult flies. The lipid levels in three-day old male *chico* flies are more than twice the level than in the control despite their smaller body size (Bohni, Riesgo-Escovar et al. 1999). Homozygous *lnk* mutant flies reach the same lipid levels as *chico* mutants (Figure 2H). Taken together, these results strongly indicate a role of Lnk in the IIS pathway.

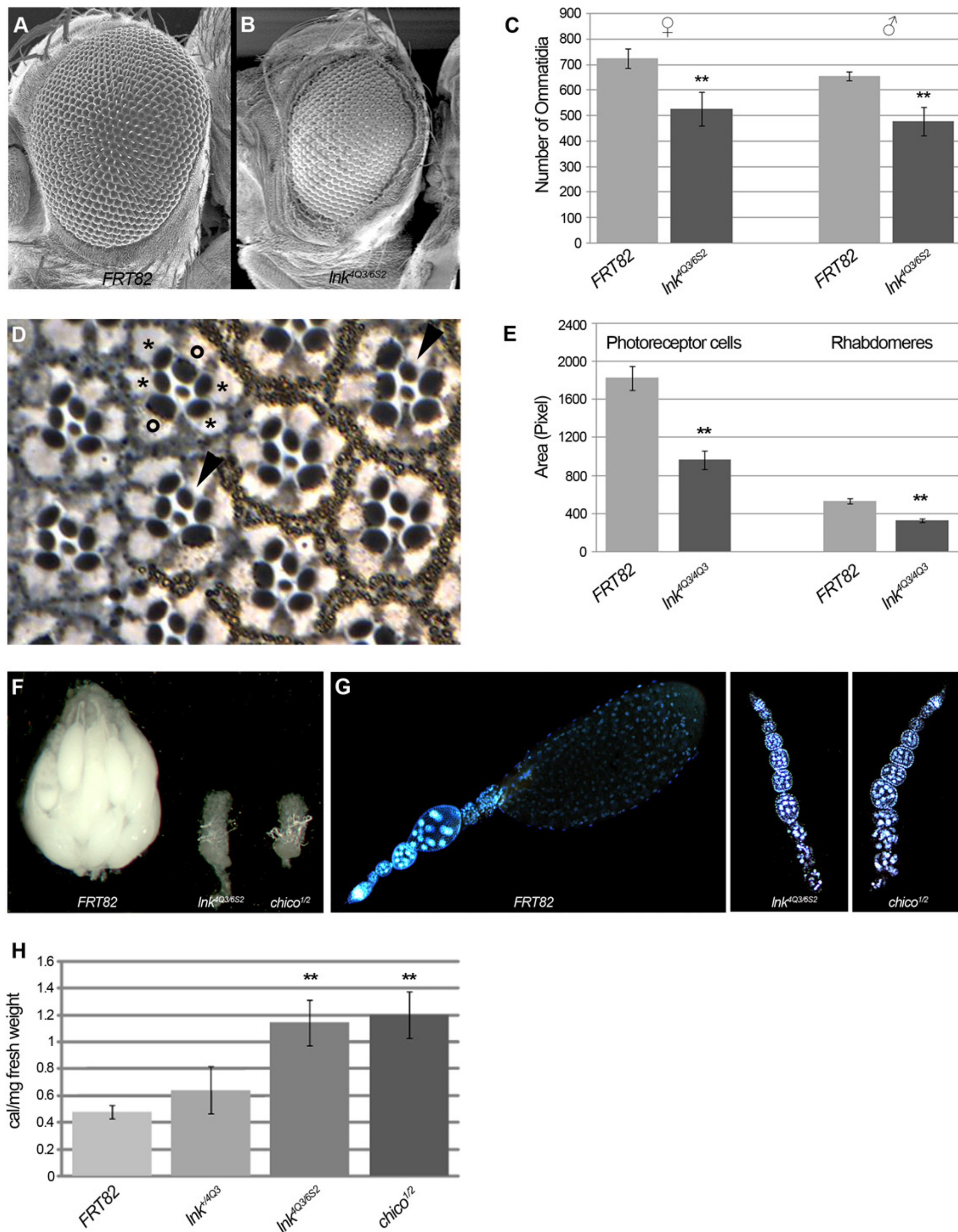

Figure 2. *Drosophila lnk* regulates cell number and cell size, reminiscent of low IIS activity

SEM picture of a wild-type *Drosophila* head (A) compared to the head of a homozygous *lnk* mutant fly (B). The eye of the *lnk* mutant is smaller due to fewer and smaller ommatidia. (C) Whereas wild-type

eyes consist of more than 700 ommatidia, the number of ommatidia is reduced to about 500 in *lnk* mutants. (D) Tangential sections through mosaic eyes consisting of homozygous mutant photoreceptors (marked by the absence of pigment) surrounded by heterozygous tissue. Ommatidia containing both wild-type and small homozygous *lnk* mutant photoreceptor cells (arrowheads) can be observed, pointing to a cell autonomous role of *lnk* in cell size regulation (wild-type cells are marked by circles and mutant cells by asterisks in a representative ommatidium). (E) The sizes of photoreceptor cells and of rhabdomeres are reduced in *lnk* mutant ommatidia compared to wild type. (F) *lnk* mutant females are sterile due to an arrest in oogenesis. Ovaries of homozygous *lnk* females are small and contain only few oocytes developed to previtellogenic stages (G). The ovarioles of *lnk* mutant females resemble those of *chico* mutants (G). (H) The lipid levels of *lnk* mutant males are strongly elevated compared to wild type, similar to the levels measured in *chico* mutant flies. Significant changes relative to the respective controls ($p \leq 0.01$, Student's t-test) are marked by double asterisks; error bars represent the standard deviation; n=8 in (C), n=9 in (E), n=10 in (H).

The phenotypes of homozygous *lnk* mutants suggest that Lnk regulates cellular growth exclusively via IIS. However, the protein sequence of Lnk contains two putative Drk/Grb2 YXN binding sites (Figure 1F). In addition, all SH2B family members, except for the beta, gamma and delta isoform of SH2B1, carry a highly conserved consensus site for binding of Cbl (Hu and Hubbard 2005). The functionality of this Cbl binding site has only been demonstrated in SH2B2 so far (Ahmed, Smith et al. 2000; Liu, Kimura et al. 2002). In order to test the functional significance of the individual binding motifs, we generated rescue constructs consisting of the genomic *lnk* locus but carrying specific mutations that result in amino acid exchanges in the core tyrosine of the respective motifs. These constructs fully rescued the reduction in dry weight in *lnk* mutants, suggesting that neither binding of Drk to the YXN site nor an interaction of Lnk with Cbl through the C-terminal binding motif is important in the regulation of growth (Figure S1A-C). In contrast, both the PH and the SH2 domains of Lnk are essential for its function because the *lnk* alleles disrupting either domain behave genetically as null mutations.

In order to study the consequences of the loss of *lnk* function on cell growth, we performed a clonal analysis in larval wing discs using the *4Q3* allele. We used the hsFLP/FRT system to induce mitotic recombination, thus to generate homozygous *lnk* mutant cell clones (marked by the absence of GFP) adjacent to clones that consist of wild-type cells (marked by two copies of GFP) (Figure 3A). We measured

the area of the respective clones and counted the number of cells within the clones (as visualized by nuclear DAPI staining). All mutant clones were smaller than their wild-type sister clones (Figure 3C), and they contained fewer cells (Figure 3B). Although a clear tendency to a cell size reduction of *lnk* mutant cells, as determined by the ratio of clone area to cell number, was apparent, the relative reduction was not significant in larval wing discs. We thus speculate that the influence of *lnk* on cell size is rather subtle in early stages of development.

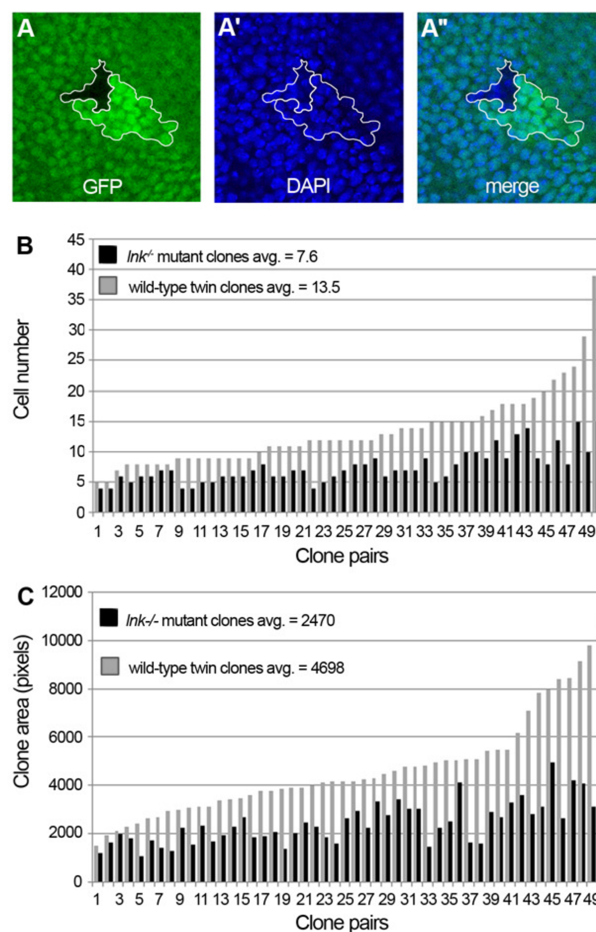

Figure 3. Clones of *lnk* mutant cells are smaller due to fewer and smaller cells

(A) Twin-spot clone in the wing imaginal disc. A clone consisting of *lnk* mutant cells (marked by the absence of GFP) and its wild-type sister clone (marked by two copies of GFP) were induced by mitotic recombination using the hsFlp/FRT system. Nuclei are stained with DAPI (A') and merged with the GFP signal (A''). Clones of *lnk* mutant cells consist of fewer cells (B) and cover smaller areas (C) than

their corresponding wild-type sister clones, indicating that *lnk* is required for proper cellular growth during development.

We further used molecular readouts of IIS activity to investigate the consequences of the loss of *lnk* function. Stimulation of the insulin receptor activates PI3K, which increases the levels of phosphatidylinositol-(3, 4, 5)-trisphosphate (PIP_3) at the plasma membrane (Hafen 2004). Previously, a reporter containing a PH domain fused to GFP (*tGPH*) that localizes to the plasma membrane as a result of PI3K activity was described (Britton, Lockwood et al. 2002). Using this reporter, we monitored PIP_3 levels in wild-type and *lnk* mutant fat body cells as well as in clones of *lnk* mutant cells in the fat body. Whereas the *tGPH* reporter localized to the membrane in wild-type cells (Figure 4A), the GFP signal was predominantly observed in the cytoplasm in *lnk* mutant cells (Figure 4B and 4C), indicating that the loss of *lnk* function causes a reduction of PI3K signaling activity. The impact of *lnk* on tGPH localization is comparable to the effects observed in *chico* mutant cells (Figure 4D). As another molecular readout of IIS activity, we measured the phosphorylation levels of PKB, a downstream kinase of IIS. Lysates of homozygous *lnk* and *chico* mutant larvae were subjected to Western analysis and compared to wild-type controls. Whereas the PKB protein levels were comparable in all genotypes, the amount of phosphorylated PKB was reduced in both *lnk* and *chico* mutant larvae (Figure 4F). Thus, Lnk and Chico contribute similarly to the activity of PI3K.

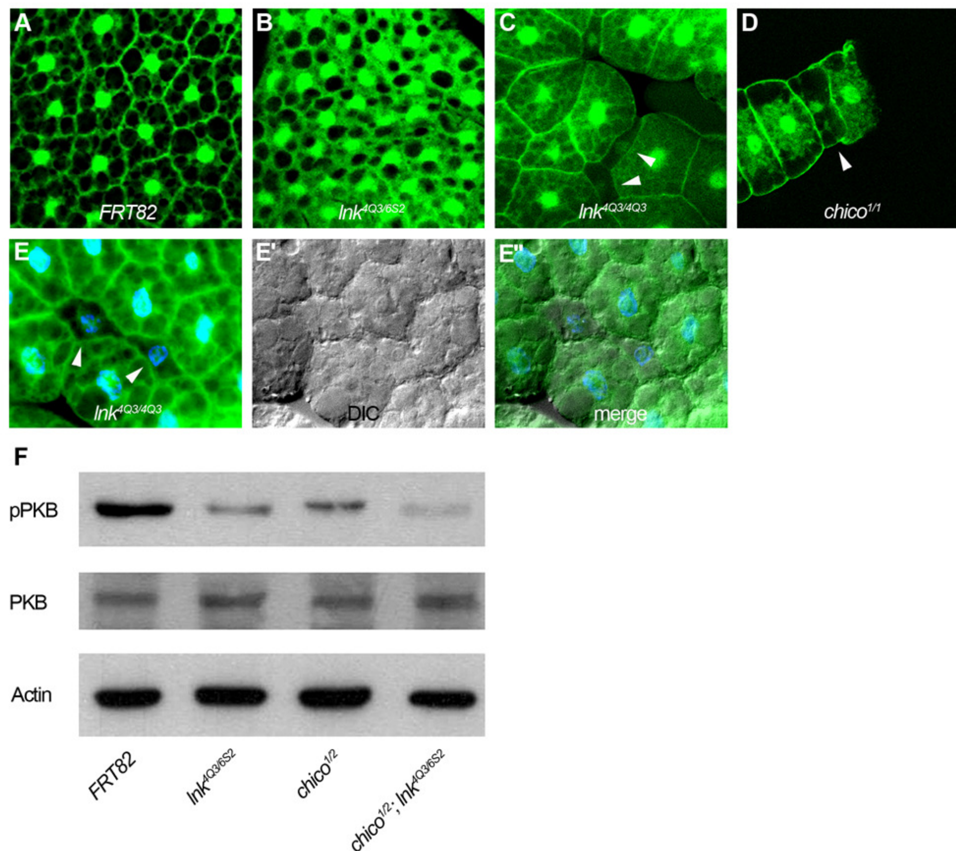

Figure 4. *lnk* affects IIS activity

(A-D) The IIS activity is visualized by the localization of the *tGPH* reporter. Compared to the signal at the membrane of wild-type fat body cells (A), the signal is diffuse and mostly cytoplasmic in *lnk* mutant larvae (B), indicative of low PI3K activity. In clones of lnk^{4Q3} mutant cells (recognizable based on the size reduction and indicated by arrowheads), the signal is also almost absent from the plasma membrane (C). A similar effect is observed in *chico* mutant cells (D). (E-E'') The strong reduction of membrane localized tGPH is not due to structural defects of the *lnk* mutant cells as shown by DIC microscopy pictures (E'-E''). (F) The phosphorylation of PKB is used to monitor IIS activity in larval extracts. Both *lnk* and *chico* mutants display a clear reduction of phosphorylated PKB. Note that the levels of PKB do not change. Actin is used as a loading control.

Lnk Acts Downstream of the InR in Parallel to Chico

In order to establish where *lnk* acts in the IIS cascade, we performed genetic epistasis experiments. We tested the ability of *lnk* to suppress the overgrowth phenotype caused by overexpression of InR during eye development (Figure 5B). In this sensitized background loss of *lnk* function reduced the eye size to almost wild-type size, suggesting that Lnk modulates the IIS pathway downstream of the receptor (Figure 5E). In contrast, homozygosity for *lnk* was not sufficient to suppress the overgrowth caused by a membrane-tethered form of PI3K (Figure 5C and 5F). Thus, Lnk acts between the InR and the lipid kinase PI3K in the IIS pathway.

The phenotypic similarities between *lnk* and *chico* mutants are striking. Both genes encode adaptor proteins with a PH domain and a phospho-tyrosine binding motif (an SH2 domain in the case of Lnk and a PTB domain in the case of Chico, respectively), and both act between the InR and PI3K. Thus, it is conceivable that Lnk is required for proper Chico function, for example by stabilizing the phosphorylated InR and thereby allowing a stable InR-Chico interaction. We attempted to genetically test whether Lnk acts via Chico. If this were the case, *chico; lnk* double mutants would be expected to display similar phenotypes as the single mutants. However, *chico; lnk* double mutants were lethal (Figure 5H). Removing one copy of *PTEN* (encoding the lipid phosphatase that antagonizes PI3K) restored viability of the *chico; lnk* double mutants (Figure 5G and 5H), suggesting that the *chico; lnk* double mutants suffer from reduced IIS activity and thus insufficient levels of the second messenger PIP_3. Reducing the amount of PTEN, the negative regulator of PIP_3 production, allows for PIP_3 levels above a critical threshold for survival but still insufficient to ensure normal growth. These results imply that Chico and Lnk independently act downstream of the InR, and that both adaptors are required for the full activation of PI3K upon InR stimulation. Consistently, we found that the levels of phospho-PKB are further reduced in *chico; lnk* double mutant larvae as compared to single mutants (Figure 4F).

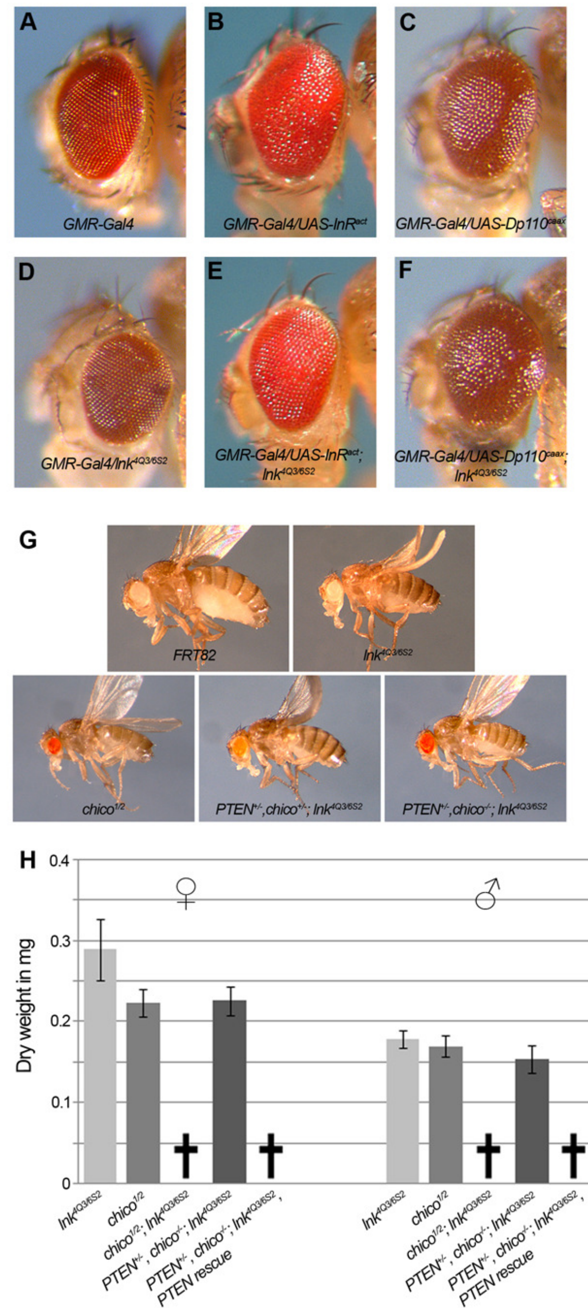

Figure 5. *lnk* genetically interacts with components of the IIS pathway

(A-F): Whereas the loss of *lnk* function suppresses the overgrowth phenotype caused by eye-specific expression of a constitutive active form of InR (compare E with B), it is not sufficient to suppress the overgrowth phenotype caused by an activated form of PI3K (compare F with C). (G and H) *chico* and *lnk* display synthetic lethality. Removing one copy of *PTEN* in a *chico; lnk* mutant background is sufficient to restore viability of *chico; lnk* double mutant flies. Re-introduction of a *PTEN* genomic rescue construct into this background results in lethality. Error bars represent the standard deviation; n=20. Genotypes are: (A) *GMR-Gal4*/+, (B) *GMR-Gal4*/*UAS-InR^{act}*, (C) *GMR-Gal4*/*UAS-Dp110^{CAAX}*, (D) *GMR-Gal4*/+; *lnk^{4Q3}*/*lnk^{6S2}*, (E) *GMR-Gal4*/*UAS-InR^{act}*; *lnk^{4Q3}*/*lnk^{6S2}*, (F) *GMR-Gal4*/*UAS-Dp110^{CAAX}*; *lnk^{4Q3}*/*lnk^{6S2}*, (G) *FRT82*, *lnk$^{4Q3/6S2}$*, *chico$^{1/2}$*, *Df(2L)Exel6026*/+; *lnk^{4Q3}*/*lnk^{6S2}* and *chico^{1}*/*Df(2L)Exel6026*; *lnk^{4Q3}*/*lnk^{6S2}*.

Our data clearly indicate that both Lnk and Chico are required for the full activity of PI3K, with each adaptor being sufficient for a partial stimulation of PI3K activity. This might explain why *chico* and *lnk* are among the few non-essential genes in the IIS cascade. How does Lnk contribute to the activation of PI3K? Probably, Lnk does not exert its function in the same way as Chico. In contrast to Chico, Lnk lacks an YXXM consensus binding site for the SH2 domain of the regulatory subunit of PI3K. One possible mechanism would be that upon activation of the Insulin receptor, Lnk connects the signal from the Insulin receptor with Chico in order to enhance PI3K activation. Interestingly, such a mechanism has been proposed in vertebrates, where, in response to Leptin, SH2B1 promotes IRS1 and IRS2-mediated activation of the PI3K pathway (Duan, Li et al. 2004). However, we favor a model in which Lnk promotes the membrane localization of PI3K by recruiting another binding partner of PI3K or by counteracting a negative regulator of PI3K localization. It will thus be important to identify physical interactors of Lnk.

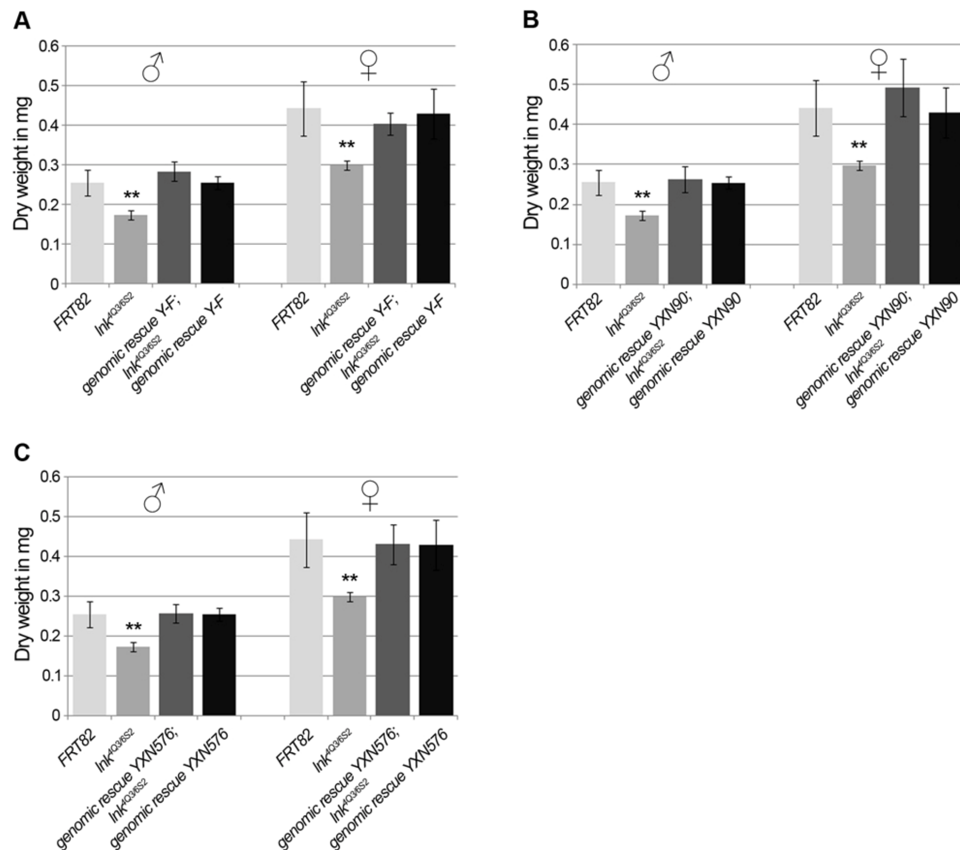

Figure S1. Structure-function analysis of Lnk

Tyrosines predicted to be phosphorylation targets within binding motifs for the SH2 domain of Drk and for Cbl, respectively, were specifically mutated to phenylalanine (see Figure 1D). Genomic rescue constructs carrying the respective mutations were introduced into a homozygous *lnk* mutant background. All mutations were able to complement the loss of *lnk* function with respect to size and weight. (A) Y-F mutation in the Cbl binding motif, (B) mutation in YXN90, (C) mutation in YXN576. Significant changes relative to the control ($p \leq 0.01$, Student's t-test, n=20) are marked by double asterisks; error bars represent the standard deviation.

A

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dLnk     1  CRKEGTVNNLTPESLD-----------------QPTGSQKWEKCRLVLVKAVGG-----YMLEFYTPHKATKPRSGVFCFLISEARETTALEMPD
hSH2B1   1  VQREELLSFMGAEEAAPDPAGVGRGGGVAGPPSGGGGQPQWQKCRLLLRSEGEGGGGSR--LEFFVPPKASRPRLSIPCSSITDVRTTTALEMPD
hSH2B2   1  IQREGALRFMVADDAA----------------AGSGGSAQWQKCRLLLR-RAVAEERFR--LEFFVPPKASRPKVSIPLSAIIEVRTTMPLEMPE
hSH2B3   1  ALKEAVLRYSLADEAS----------------MDSG--ARWQRGRLALR-RAPGPDGPDRVLELFDPPKSSRPKLQAACSSIQEVRWCTRLEMPD

dLnk    74  RLNTFVLKADNNMEYVIEAESAEEMRSWLATIRYCMRTP          Identity to dLnk:
hSH2B1  94  RENTFVVKVEGPSEYIMETVDAQHVKAWVSDIQECLSPG                       36%
hSH2B2  77  KDNTFVLKVENGAEYILETIDSLQKHSWVADIQGCVDPG                       35%
hSH2B3  77  NLYTFVLKVKDRTDIIFEVGDEQQLNSWMAELSECTGRG                       28%
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B

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dLnk     1  FPWFHGTLTRSEAARMVLHSDAAGHGYFLVRQSETRRGEFVLTFNFQGRAKHLRLTISEK
hSH2B1   1  YPWFHGMLSRLKAAQLVLTGGTGSHGVFLVRQSETRRGEYVLTFNFQGKAKHLRLSLNEE
hSH2B2   1  YPWFHGTLSRVKAAQLVLAGGPRNHGLFVIRQSETRPGEYVLTFNFQGKAKHLRLSLNGH
hSH2B3   1  YPWFHGPISRVKAAQLVQLQGPDAHGVFLVRQSETRRGEYVLTFNFQGIAKHLRLSLTER

dLnk    61  GQCRVQHLWFPSIQEMLEHFRHNPIP          Identity to dLnk:
hSH2B1  61  GQCRVQHLWFQSIFDMLEHFRVHPIP                       70%
hSH2B2  61  GQCHVQHLWFQSVLDMLRHFHTHPIP                       62%
hSH2B3  61  GQCRVQHLHFPSVVDMLHHFQRSPIP                       65%
```

Figure S2. Homology within PH and SH2 domains

Alignment of the PH domain (A) and SH2 domain (B) sequences of *Drosophila* Lnk with the respective sequences of the human homologs. Sequence identity is marked by black boxes and similarity by grey boxes

Material and Methods

Fly Stocks

Four EMS induced *lnk* alleles on *FRT82B* chromosomes were recovered in a mosaic screen based on the eyFLP/FRT cell lethal technique (Newsome, Asling et al. 2000). The complementation group was mapped close to an y^+ marked transgene in 96E, and the map position was refined to 96F by non-complementation with *Df(3R)Espl3* (96F1; 97B1, Bloomington stock number 5601) and complementation with *Df(3R)ME61* (96F12-14; 97C4-5, Bloomington stock number 5440). The identity of the gene was determined by non-complementation with the P-element allele lnk^{d07478} (Bloomington stock number 19274) and subsequent sequencing of the genetic *lnk* locus. Unless otherwise stated, a heteroallelic combination of *lnk* alleles (lnk^{4Q3}/lnk^{6S2}) was used to characterize the *lnk* phenotypes.
A 6kB fragment spanning from the 3' end of *CG17370* to the beginning of the first exon of *CG5913* was used as genomic rescue. The construct was inserted by means of ΦC31 mediated integration into a landing site on the second chromosome at 51D (Bischof, Maeda et al. 2007).
Constitutive active forms of InR (Bloomington stock number 8248) and of Dp110 (CAAX (Leevers, Weinkove et al. 1996)) driven by *GMR-Gal4* were used for the epistasis analyses. For the generation of *chico; lnk* double mutant flies lacking one copy of *PTEN*, a deletion comprising the *chico* and *PTEN* loci was used (*Df(2L)Exel6026*). To prove that the observed effect on the *chico; lnk* double mutants was caused by the loss of *PTEN*, *PTEN* was re-introduced by means of a genomic rescue construct. The *chico* alleles ($chico^1$ and $chico^2$) have been described (Bohni, Riesgo-Escovar et al. 1999). The heteroallelic combination $chico^1/chico^2$ was used to compare *lnk* and *chico* mutants.

Weight and Lipid Analyses

Flies of the respective genotypes were reared under identical conditions and collected 3 days after eclosion. They were dried at 95℃ for 5 minutes and kept at room temperature for 3 days before weighing on a precision scale (Mettler Toledo MX5).

Three day-old flies were collected and weighted individually. Subsequent analysis of lipid content was performed as described (Van Handel and Day 1988).

Clonal Analysis

Clones of *lnk* mutant cells were induced at 24-36 hours after egg deposition (AED) by heat shocking larvae of the genotype *y, w, hs-flp/y, w; FRT82, w+/FRT82, lnk^{4Q3}* for 1 hour at 37°C. Fixation and tangential sections of the adult eyes were performed as described (Basler and Hafen 1988). For the generation of mutant clones in the wing disc, animals of the genotype *y, w, hs-flp/ y, w; FRT82, Ubi-GFP/ FRT82, lnk^{4Q3}* were exposed to a 5 minute heat shock at 37°C at 54-56 hours AED. Larvae were dissected 48 hours later, fixed in 4% paraformaldehyde on ice for 1 hour, and incubated in PBS containing DAPI (1:2000) for 10 minutes. Discs were dissected and mounted in Vectashield Mounting Medium. Pictures were taken using a Leica SP2 confocal laser scanning microscope.

The quantification of the mutant clones was performed by comparing the size of the area occupied by mutant versus wild-type (pigmented) photoreceptor cells R6 using Photoshop CS2. In the wing discs, the number of nuclei within mutant and wild-type clones was counted and the area was measured using Photoshop CS2.

tGPH Localization and Ovarian Phenotypes

Larvae of the genotype *y, w; tGPH/+; FRT 82, w^{+}/ FRT82, lnk^{4Q3}* were heat shocked 6-8 hours AED for 1 hour at 37°C, collected at wandering stage, fixed for 1 hour at room temperature in 8% paraformaldehyde and stained with DAPI (1:10000 in PBS) for 20 minutes. Fat bodies were dissected and mounted in Vectashield Mounting Medium. Pictures were taken using a Leica SP2 confocal laser scanning microscope (Figure 4A-D) and a Zeiss ApoTome microscope (Figure 4E-E''), respectively.

Ovaries were dissected from 3 day-old wild-type, *lnk^{4Q3}/lnk^{6S2}* and *chico^{1}/chico^{2}* flies, respectively, and subsequently incubated in PBS containing DAPI (1:2000) for 10 minutes. Thereafter, ovarioles were mounted in Vectashield Mounting Medium and pictures were taken using a Leica SP2 confocal laser scanning microscope.

Western Blotting

10 mg of third instar larvae of each genotype were collected, briefly washed in PBS, transferred to 1.5 ml Eppendorf tubes and flash-frozen in liquid nitrogen. Larvae were homogenized in 75 µl of extraction buffer (Stocker, Radimerski et al. 2003). After 15 minutes incubation at 4℃ and centrifugation at 12000 g for 15 minutes, protein concentrations were determined using the RC DC Protein Assay (Bio-Rad).

For the Western blots, 30 µg of protein samples were loaded, blotted and detected with the following antibodies: rabbit anti-Akt (Cell Signaling #9272, diluted at 1:1000), rabbit anti-phospho-Drosophila Akt (Ser 505) (Cell Signaling #4054, diluted at 1:500), and mouse anti-Actin (Sigma A5316, diluted at 1:10000). HRP-conjugated secondary antibodies (Jackson ImmunoResearch) were diluted at 1:10000. Signals were detected using ECL Western blotting detection reagents (Amersham Biosciences).

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Additional Results

Within this chapter, further results are summarized obtained during the characterization of *Drosophila* Lnk, that were not included in the manuscript of 'The Drosophila SH2B Family Adaptor Lnk Acts in Parallel to Chico in the Insulin Signaling Pathway.'

1. Phenotypic analysis

1.1 Loss or reduction of function analysis

1.**1.1 All *lnk* alleles reduce dry weight of adult flies to the same extent**

As mentioned above, we recovered four individual alleles of *lnk* in a screen for genes involved in growth control. Within the *lnk* open reading frame of each allele we were able to identify a mutation causing an amino acid exchange that lead to a severe reduction in dry weight of adult flies (Figure 1A, B). The same phenotype could be observed in flies that contain an EP element (*EP19274*) located to the first intron of *lnk* (Figure 1B). In order to compare the effect on the weight of adult flies of the different alleles and the P- element, we combined them with a novel deletion (lnk^{def29}, Cathy Slack) which deletes the first two exons of *lnk* including the predicted translational start site. Interestingly, all the *lnk* alleles we tested affect the dry weight of the adult flies to the same extend with no statistical significant differences (Figure 1A), showing that disruption of the PH domain affects final weight of the organism as severe as mutations within the SH2 domain. This suggests an essential role in Lnk function for both, proposed membrane localization through the PH domain as well as interaction with phospho-tyrosine containing proteins.

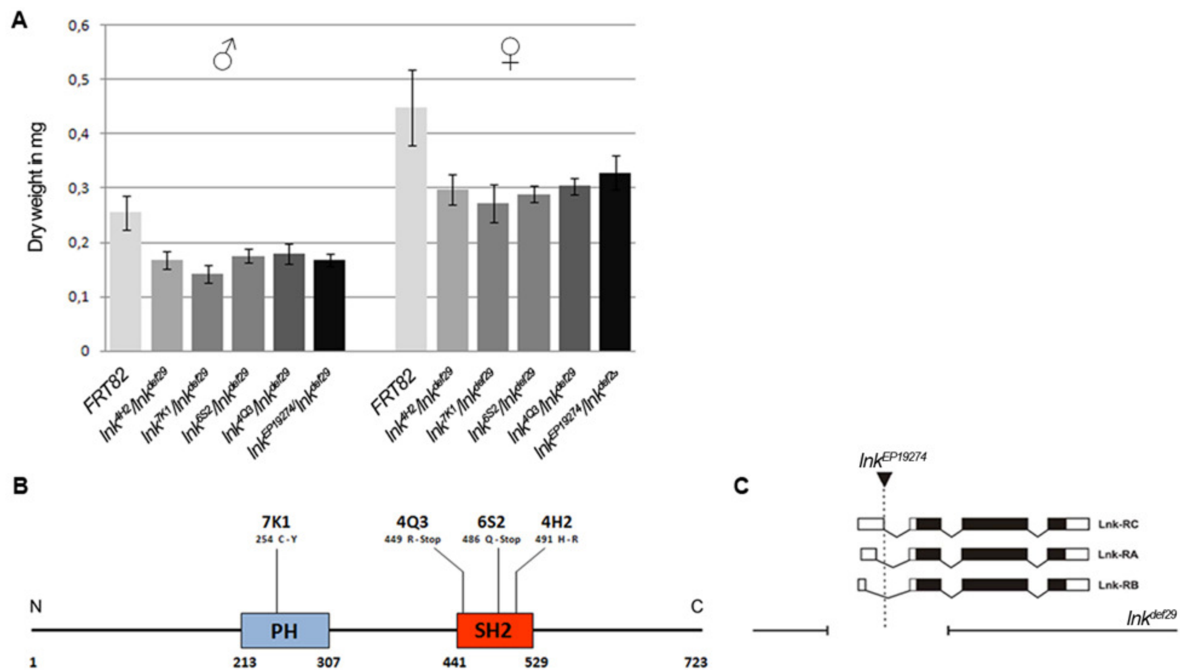

Figure 1: All *lnk* alleles reduce dry weight of flies to the same extent

(A) Shown are the dry weights of flies carrying on of four individual alleles we recovered from the screen, over the *lnk*^{def29} jump-out allele that lacks the translational start site.
(B) Schematic overview of the four individual *lnk* alleles.
(C) Overview of the excision (*lnk*^{def29}) caused by mobilization of the P element (*EP19274*).

1.1.2 lnk RNAi in S2 cells does not affect cell size

In the eye of an adult fly we were able to show that in cell clones homozygous mutant for *lnk*, cell size is reduced by around 40%. In order to gain further insight into the effects of *lnk* on cell size, we performed RNAi experiments on *Drosophila* Schneider cells with dsRNA against *lnk* and assessed cell size using the flow cytometer. Although, compared to untreated cells, *lnk* transcript levels were strongly reduced in cells after three days of incubation with double stranded *lnk* RNA (Figure 2A), no reduction in cell size could be detected (Figure 2B). As a control we also measured the size of cells treated with RNAi against *Rheb* and *TSC 1/2*, which resulted in smaller and larger cells, respectively (Figure 2C, D). One possible explanation for the lack of cell size reduction in S2 cells would be that cells in early stages of

development do not require *Ink* function to reach their proper size. This would be consistent with the results we obtained in the clonal analysis (manuscript Werz et al. Figure 3D), where we could not detect a decrease in cell size in *Ink* mutant clones of larval wing discs.

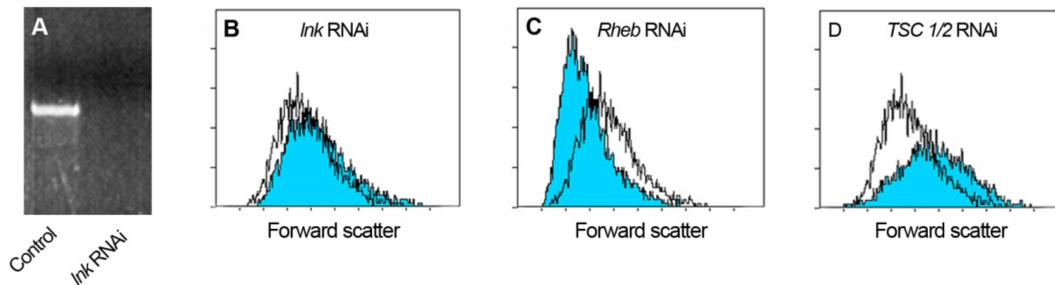

Figure 2: Treatment of S2 cells with *Ink* RNAi does not change cell size.

(A) RT-PCR of cells treated with control or *Ink* double stranded RNA shows down regulation of *Ink* transcripts in *Ink* RNAi treated cells below detectable levels.
(B-D) Flow cytometric analysis of cells treated for 3 days with *Ink*, *Rheb* and *Tsc1/2* double stranded RNA (blue graph) and cells treated with control RNAi (white graph). No significant changes in cell size are detectable in cells treated with *Ink* RNAi (B).

The different *Ink* alleles provided us with the unique opportunity to study the effects of *Ink* loss of function in *Drosophila*. The allelic series clearly showed that all mutations in *Ink* we recovered from the *ey-FLP/FRT* screen behave as genetic null and this represent a situation where *Ink* function is completely missing. However, in contrast to mutant cell clones in the adult eye, we could not detect an effect on cell size in *Ink* mutant clones of third instar larval wing discs. Furthermore, down regulation of *Ink* transcripts by RNAi in S2 cells, originating from *Drosophila* embryos, also did not lead to a reduction in size of the individual cells. Thus, the importance of *Ink* for the control of cell growth seems to be restricted to later stages in development of the organism.

1.2 Gain of function analysis

1.2.1 Over expression of *lnk* rescues the phenotypes displayed by homozygous mutant flies but causes lethality and rough eyes at higher levels

Homozygous *lnk* mutant adult flies are viable, but severely reduced in size (see above). In order to test whether this phenotype could be reverted by ectopic expression of *lnk*, we generated transgenic flies containing UAS-binding sites followed by the *lnk* coding sequence. We recovered two independent insertions, which were both used in the following experiments and subsequently referred to as *UAS-lnk*^{28} and *UAS-lnk*^{45}. First we wanted to determine if ubiquitous over expression of *lnk*, using different driver lines is sufficient to rescue the *lnk* mutant phenotypes, in particular the reduction in dry weight. Whereas driving *UAS-lnk* using the weak driver line *heatshock-Gal4* without heat shock did not result in any changes regarding phenotypes of *lnk* mutant flies, *daughterless-Gal4 (da-Gal4)* in combination with *UAS-lnk*, was able to fully rescue the decrease in dry weight caused by *lnk* loss of function (Figure 3). Both *UAS-lnk*^{28} and *UAS-lnk*^{45} lead to the same results. Importantly, weak ectopic expression of *lnk* by *da-Gal4* in a wild type background did not affect the fly weight, suggesting that under these conditions *lnk* is expressed in physiological levels and the increase in dry weight of the rescued flies is indeed due to full compensation of *lnk* loss of function by the over expression.

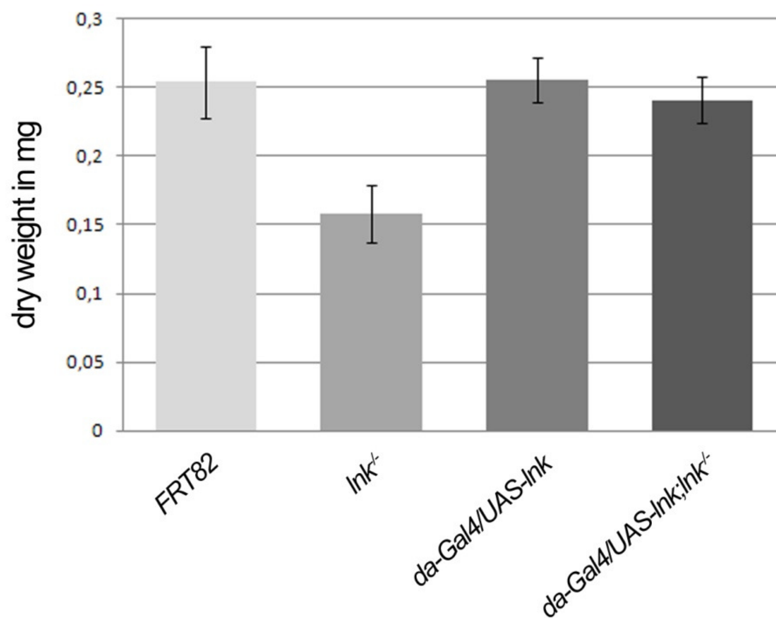

Figure 3: *lnk* loss of function can be rescued by *da-Gal4* driven over expression of *UAS-lnk* transgenes.

Shown are the dry weights of flies over expressing the *lnk* coding sequence under the control of *da-Gal4* in a wild type and *lnk* mutant background.

We then further tested the consequences of high ubiquitous expression of *lnk* transcripts in a homozygous *lnk* mutant and wild type background, using *Actin-Gal4* to activate *UAS-lnk*. In both genetic backgrounds, strong over expression of *UAS-lnk* was lethal, except for 1% escapers that did not show a significant increase in body weight, but slightly rough eyes (Figure 4A-C)

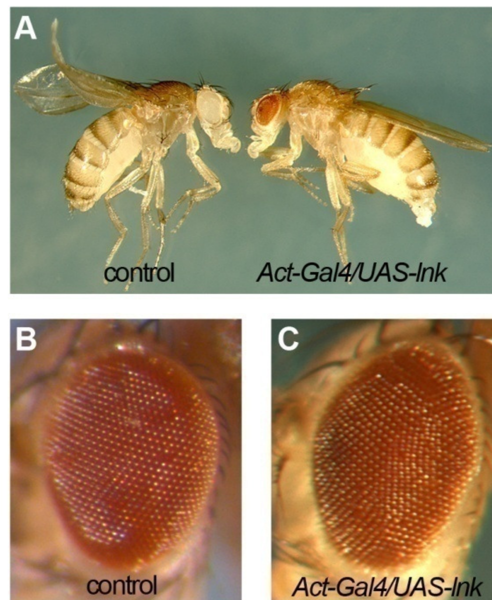

Figure 4: *Actin-Gal4* driven over expression of *UAS-lnk* does not change body size but causes rough eyes.

(A) Shown are adult female wild type flies and flies ubiquitously over expressing *lnk* using the *Actin-Gal4* driver.
(B) Magnification of compound eyes of control flies and eyes of flies over expressing *lnk* by *Actin-Gal4.*

1.2.2 Over expression of *lnk* in specific tissues

Since we observed that *Act-Gal4* driven over expression of *lnk* is lethal in most cases, but escapers show an interesting phenotype in the eye, we wanted to test the effect of ectopic *lnk* expression in specific tissues.
The *UAS-lnk* over expression constructs and the EP insertion provided us with the opportunity to analyze the consequences of high levels of *lnk* expression in specific organs of *Drosophila* to circumvent the lethal effects of the ubiquitous over expression mentioned above. Especially the *Drosophila* eye represents an excellent organ to monitor even the slightest structural differences and changes in cell size and cell number. We induced the *GMR-Gal4* driver to induce ectopic *lnk* expression in postmitotic cells of the eye. Compared to wild type eyes (Figure 5A), the eyes of the flies where *UAS-lnk* was over expressed are only slightly bigger but rough (Figure

5B-C). In higher magnification we were able to investigate the structural alterations on the surface of the eye in better detail. The ommatidia are irregular in size or even fused and therefore not precisely arranged as in the control. Furthermore, the small bristles between the ommatidia are often missing or in rare cases duplicated (Figure 5A'-D'). In order to further clarify the nature of the rough eyes, we performed tangential eye sections, where we were able to observe that the tightly arranged structure of the ommatidia and the photoreceptor cells seen in the control eye, is strongly deranged in eyes over expressing *UAS-lnk* (Figure 5A''-C''). In this situation, the interommatidial space is enlarged and the number of photoreceptor cells is increased, but can vary from 6 to 10 cells per ommatidium. Interestingly, over expression of the EP element (*GE23250*), which is located directly upstream of the *lnk* locus also leads to a larger number of photoreceptor cells, however, the ommatidia are almost normally arranged with no extra space between them. Remarkably, the photoreceptor cells appear smaller and outstretched in shape (Figure 5D'').

We were able to rescue the *lnk* loss of function growth phenotypes by over expressing *UAS-lnk* transgenes, proving that the transgenic constructs are functional. Nevertheless, it cannot be ruled out that the phenotypes we observed in the eye are due to non physiological levels of *lnk* expression rather than over activation of a signaling pathway regulating the eye structure and number of photoreceptor cells. In order to further investigate whether the multiple photoreceptor-cell-phenotype is due to a regulatory function of *lnk* in a signaling pathway involved in this process, we tested for the ability of *lnk* loss of function to influence the activity of the sevenless pathway which has been shown to control the number of R7 photoreceptor cells (Tomlinson and Ready 1986). Constitutive activation of the sevenless receptor leads to an increase in R7 cells, providing a suitable condition to test for genetic interaction between the *sevenless* receptor and *lnk* (Basler, Christen et al. 1991). Although precise quantification of photoreceptor cell number was difficult due to severe structural defects when two copies of *lnk* were removed, we were still able to observe ommatidia with more than one R7 cell (not shown). This result suggests that *lnk* is involved in signaling downstream of the *sevenless* receptor.

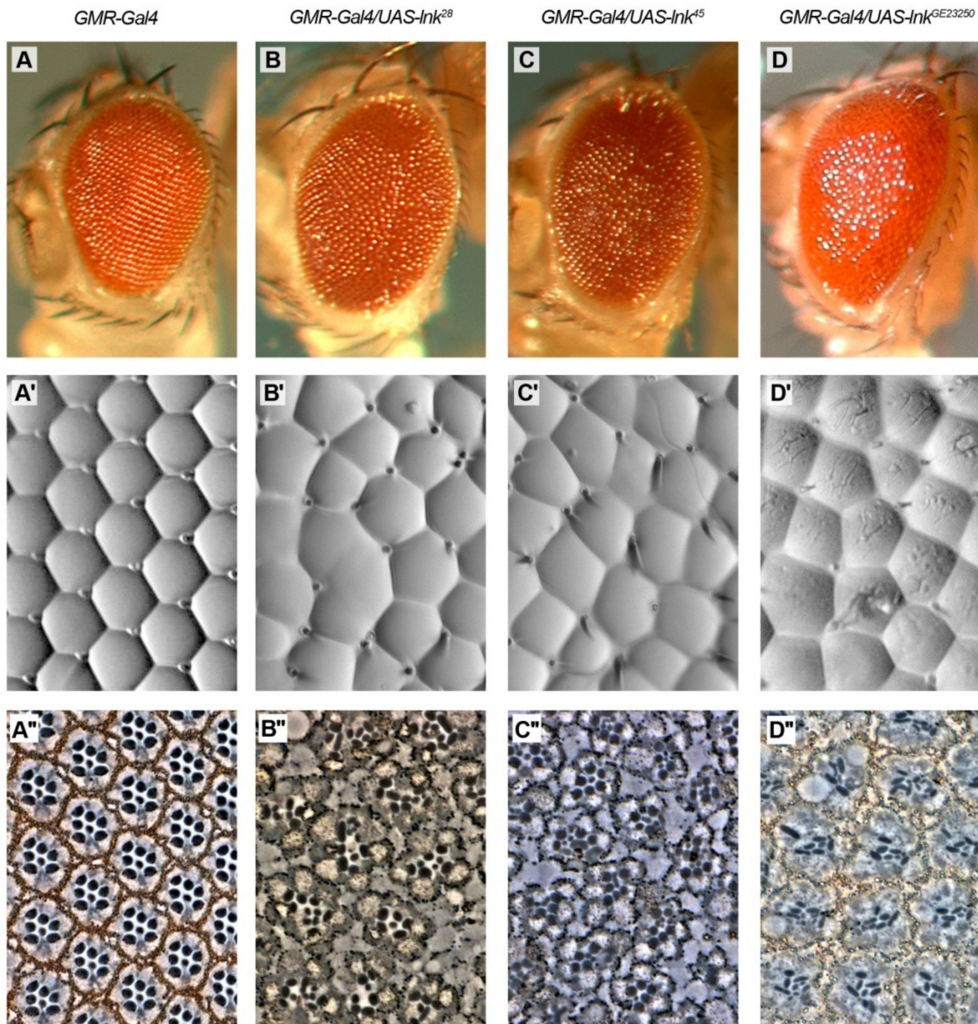

Figure 5: *lnk* over expression in the eye leads to rough eyes and multiplication of photoreceptor cells.

(A-D) Compared to eyes of control flies (A), eyes of flies in which *UAS-lnk* constructs are ectopically expressed by *GMR-Gal4* appear rough and slightly enlarged (B-D).

(A'-D') The surface structure of *Drosophila* eyes in A-D is visualized by Nail polish imprints.

(A''-D'') Tangential eye sections reveal the inner structure of eyes shown in A-D. Whereas photoreceptor cells are regularly arranged and consistent in cell number (A''), the adjustment of ommatidia within *lnk* over expression eyes is disrupted and ommatidia contain more than seven photoreceptor cells in most cases (B''-D'').

We further wanted to assess the effect of *lnk* over expression in another organ to see, whether the effects on differentiation and structure we observed in the eye in response to high *lnk* levels are a common feature of ectopic *lnk* expression. Previous studies showed that over expression of a growth promoting gene using the *patched-Gal4* driver significantly expands the area between wing vein 3 and 4 (Speicher, Thomas et al. 1994). Therefore, we also used *ptc-Gal4* to induce expression of *lnk* in this particular part of the fly wing. However, as shown in Figure 6 (A-E), the size of the area between wing vein 3 and 4 remains unaffected by over expression of *lnk* and is exactly the same as in the control (Figure 6D-E). Moreover, we did not observe any differential and structural defects where *lnk* transcript levels were elevated. Taken together, although the phenotypes in the eye due to high levels of *lnk* were interesting, we think that these do not correlate to a function of *lnk* in a physiological context. It is in fact common to adaptor molecules that their abundance, exceeding wild type levels do not lead to phenotypes related to their normal function.
Thus, we decided not to continue to analyze the over expression phenotypes of *lnk* more thoroughly, since we did not expect to gain further insight into the role of *lnk* in growth regulation.

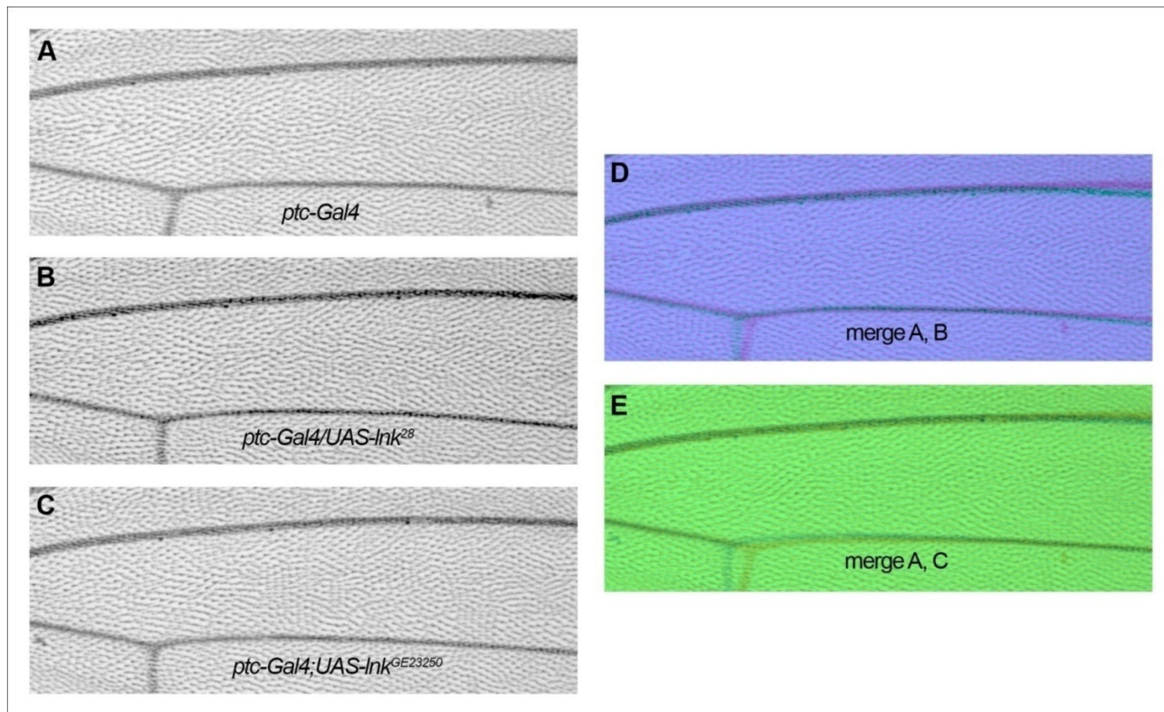

Figure 6: *ptc-Gal4* driven over expression of *lnk* does not increase the area between wing vein 3 and 4.

(A-C) Detail of fly wings showing the area between wing vein 3 and 4 where the *ptc-Gal4* driver is active.
(D-E) Shown is a merge of panel A and B (D) and A and C (E) in order to compare the size of the respective wing area.

In addition to the observation that depletion of *lnk* affects development and size of *Drosophila* flies, another approach to gain insights into the physiological processes and underlying molecular mechanisms *lnk* is involved in is over activation and ectopic expression of *lnk*. Over expression of *lnk* in various different organs did not have an effect on cell and organ size. This observation was not surprising since adaptor proteins are generally not the limiting factors in the transmission of intracellular signals and thus their increased abundance does not change the downstream signal levels.

High levels of *lnk* in the eye lead to an increase in photoreceptor cell number and structural defects. Although these phenotypes suggested a potential role of *lnk* in the regulation of the sevenless pathway, a direct connection could not be revealed in subsequent experiments. These results together with the lack of sevenless signaling related phenotypes in *lnk* mutant animals lead us to the conclusion that the *lnk* gain of function phenotypes are most likely over expression artifacts and will not further contribute to our understanding of the function of *lnk*.

1.3 The genomic rescue construct restores the reduction in dry weight and defects in oogenesis due to *lnk* loss of function

We already described in the manuscript Werz et al. that the genomic rescue construct, comprising the entire *lnk* locus is able to fully rescue the reduction of dry weight caused by homozygosity for *lnk* (Figure 7A, B). Here we show further that introduction of the construct into a homozygous *lnk* mutant background is also capable of restoring infertility and the arrest in oogenesis we observed in *lnk* mutant female flies. Compared to the small and only partially developed ovaries of the *lnk* mutants, rescued flies contain ovaries that are of comparable size with the wild type control and contain a normal number of mature eggs (Figure 7C). Thus, we are not only able to rescue the size and weight dependent phenotypes in homozygous *lnk* mutants by introduction of a genomic rescue construct, but also infertility and defects in oogenesis.

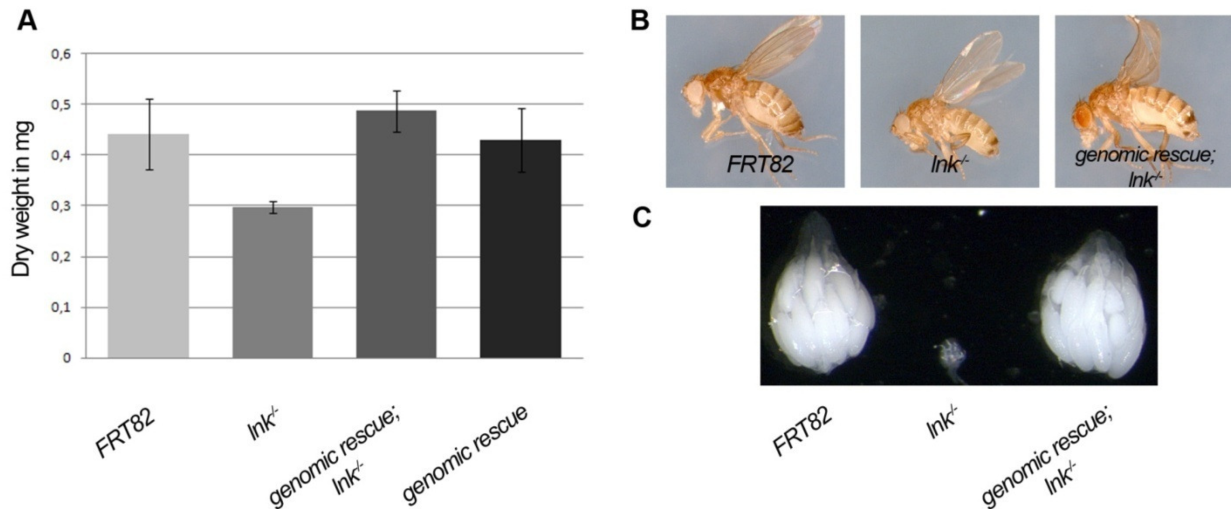

Figure 7: A *lnk* genomic rescue construct restores different *lnk* mutant phenotypes to the wild type situation

(A) Diagram showing the dry weight of adult female flies. *lnk* mutant flies are severely reduced in weight and are smaller, as shown in (B).
(C) Ovaries of *lnk* mutant females are incompletely developed and small compared to the control. Introduction of a *lnk* genomic rescue construct is sufficient to completely restore those defects.

2. Expression and subcellular localization of Lnk

2.1 *lnk* is expressed ubiquitously in early development

In order to examine temporal and spatial expression of *lnk* during embryogenesis, we generated an RNA probe for detection of the *lnk* transcript and performed whole mount in-situ hybridization on *Drosophila* embryos of stage 5 to 14 (Figure 8). We observed a ubiquitous but specific signal already in very early embryos and throughout development until stage 14. It appears that *lnk* is expressed stronger during stages 8 to 11 and slightly accumulated in mesodermal regions of the embryo (Figure 8B, C). The expression pattern suggests a necessity for *lnk* function in a majority of embryonic cells.

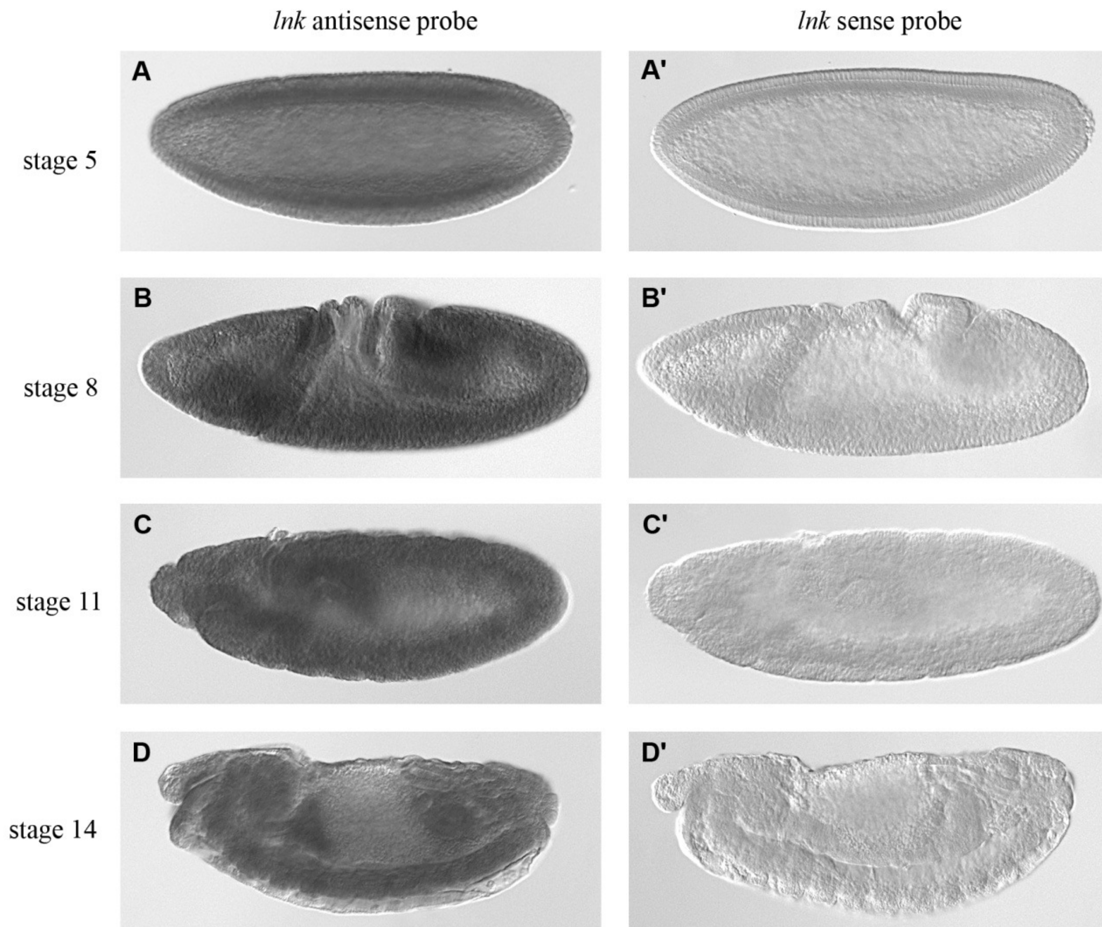

Figure 8: *lnk* transcripts are ubiquitously detectable throughout early development.

(A-D) Whole mount in situ hybridization of wild type embryos was performed using a *lnk* antisense probe to monitor mRNA abundance throughout embryonic development.
(A'-D') The respective sense probe was used as a control and did not lead to a specific signal, as it was expected.

2.2 Lnk localizes to the membrane and intracellular clusters in S2 cells

The intracellular detection of a protein can give valuable insight into its role in signal transduction. We visualized the Lnk protein with an either an N-terminal or a C-terminal GFP tag in order to analyze its intracellular localization (Figure 9A, E). The *UAS-GFP-lnk* transgenes were expressed using the *Actin-Gal4* driver in *Drosophila* Schneider cells (S2 cells). Localization to a particular compartment within the cell and co-localization to other proteins might reveal potential interaction partners and hint to the physiological function of the protein. Expression of *UAS-GFP-lnk* resulted in a clear GFP signal detected as aggregates throughout the cytoplasm and also at some parts of the membrane (Figure 9B). Consistently, both, the N-terminal as well as the C-terminal GFP tagged Lnk protein was detected with the same localization pattern (Figure 9F). After having tested for the functionality of the fusion proteins by assessing their ability to rescue the *lnk* mutant phenotypes in flies, it would be interesting to perform co-localization experiments with established marker proteins for intracellular compartments. These might give further valuable insight to where Lnk exerts its function.

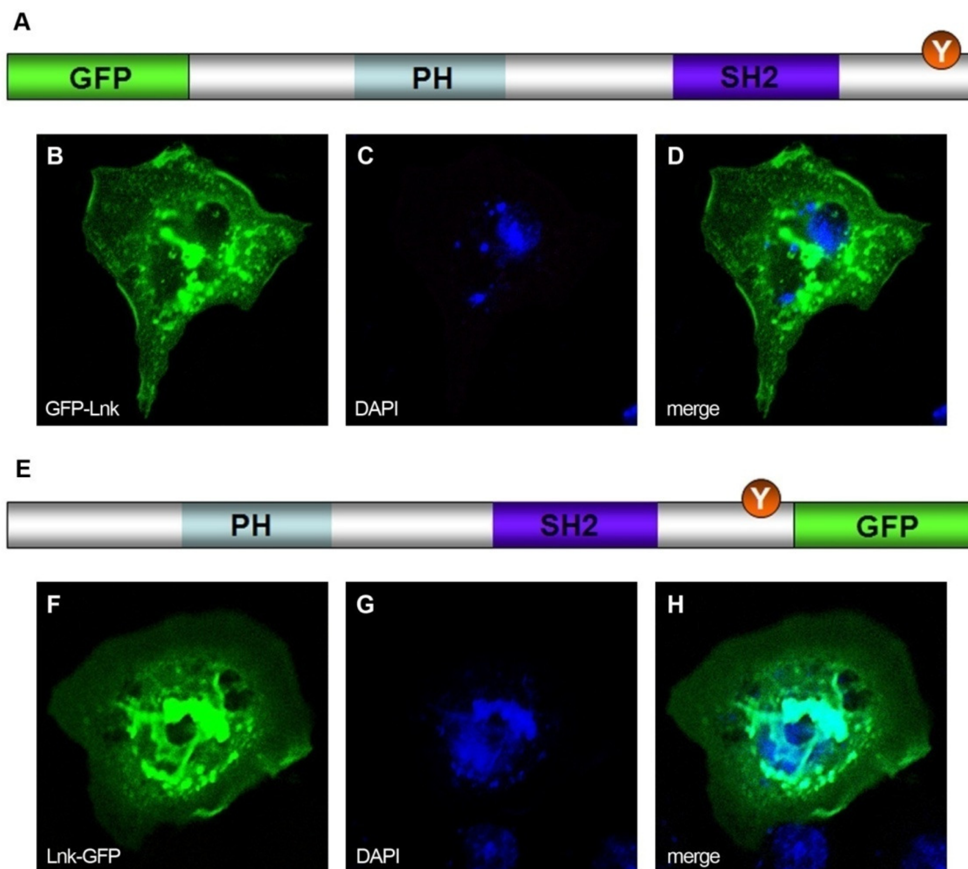

Figure 9: GFP tagged Lnk is localized to intracellular clusters and the membrane.

(A-D) Immunofluorescence of S2 cells expressing N-terminally GFP tagged Lnk protein. GFP-Lnk expression is detected within the cell and partly at the membrane.
(E-H) The same distribution of GFP signal is obtained with GFP fused to the C-terminus of Lnk.

Previous studies were able to show that the respective SH2B family members are preferentially expressed in a variety of different tissues, largely reflecting their individual functions. Whereas SH2B1 is expressed ubiquitously, the expression of SH2B2 is restricted to insulin-sensitive tissue such as adipose tissue, cardiac muscle and skeletal muscle. SH2B3 seems to be mainly expressed within haemopoietic cells. In *Drosophila, lnk* expression can be detected ubiquitously throughout embryonic development, suggesting a general function in the whole organism during

these stages. Notably, the expression pattern of *Drosophila lnk* corresponds to the distribution of Insulin receptor transcripts in early development. In order to further analyze the transcriptional activity of *Drosophila lnk*, it would be required to perform additional mRNA in-situ hybridizations of different larval stages and adult flies. It would be particularly interesting to see, whether *lnk* transcripts are enriched in certain tissues that have been shown to be of particular importance for Insulin function, e.g. adipose tissue and brain.

Although we were able to show that GFP coupled Lnk protein is localized in a distinctive pattern within *Drosophila* S2 cells, evidence for the functionality of Lnk-GFP fusion proteins is still missing. As expected for proteins containing a PH domain, the mammalian SH2B family members have all been shown in different cell lines, to be localized to the plasma membrane and the cytoplasm. This is also true for the *Drosophila* protein, which can at least partly be detected at the plasma membrane. The identity of the intracellular clusters remains to be elucidated in further co-localization studies. Notably, recent studies provide evidence for SH2B1 to have the ability to undergo nucleocytoplasmic shuttling. It would thus be interesting to test whether *Drosophila* Lnk is also shuttling between the cytoplasm and the nucleus by blocking nuclear export with leptomycin B leading to an accumulation of Lnk in the nucleus of S2 cells.

3. Interaction studies

3.1 *chico;lnk* double mutants are severely delayed in development and die in prepupal stage

We previously showed that although homozygous *chico* and *lnk* mutants are viable on their own, double mutants are lethal (see Werz et al. Figure 5). We further investigated the development of *chico;lnk* animals to elucidate survival and to analyze the defects they display. Surprisingly, apart from a delay of two days and reaching only about half the size of *chico* and *lnk* single mutants respectively, *chico;lnk* double mutants develop normally up to larval stage 3 (Figure 10A). However, while *chico* or *lnk* mutants are delayed by two days compared to WT larvae, *chico;lnk* larvae are unable to initiate pupariation until day 14 of development (Figure 10B). Furthermore, in contrast to wild type, in double mutant larvae the induction of pupariation is not a synchronized process and larvae pupariate from day 13 to 36 days after egg deposition. Instead of pupariating, the double mutant larvae continue feeding, undergo enormous mass increase and are finally even bigger than *chico* or *lnk* single mutants before pupariation (Figure 10A). Another noticeable phenotype we observed in *chico;lnk* larvae is their transparent appearance, which is primarily due to an overall reduction in the size of the fat body and also due to structural alterations of the fat body (data not shown). Similar phenotypes can be observed in wild type animals in response to starvation when stored nutrients are precociously mobilized into the hemolymph causing the fat body cells to shrink and become clear. Notably, both, *chico* and *lnk* single mutant larvae respectively, have smaller fat bodies appear slightly transparent, but not as severe as in the double mutants. Interestingly, as Britton et al. previously reported, the dominant negative effect on PI3K activity by induction of *p60* leads to identical alterations of the fat body, indistinguishable from the effects of starvation on larvae (Britton J, 2002, Dev Cell). These results further indicate that mutation of *chico* or *lnk* on their own reduce Insulin signaling activity only to a certain extent, but loss of both genes leads to a further reduction in signaling activity, mimicking a situation in which InR/PI3K activity is suppressed.

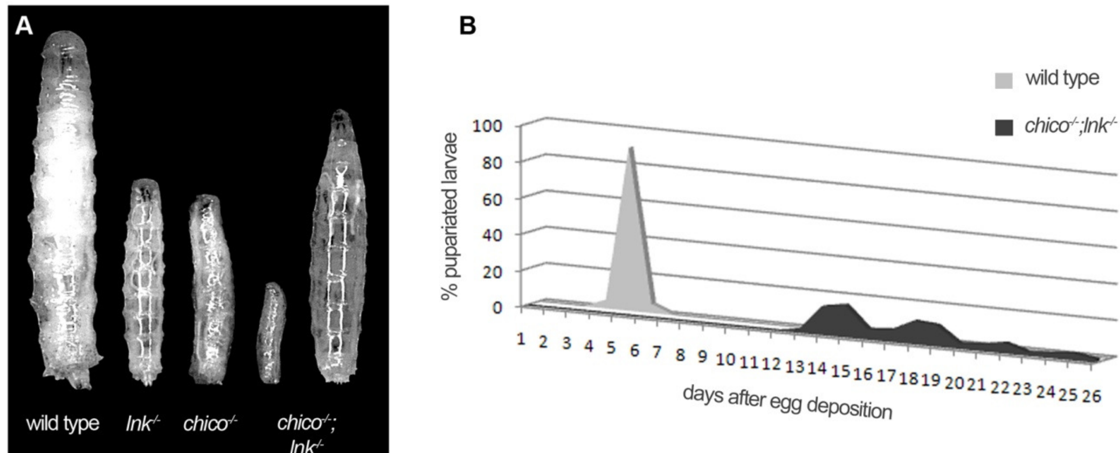

Figure 10: *chico;lnk* double mutants survive until pupariation but are initially dramatically decreased in size and severely developmentally delayed.

(A) Shown are larvae at early 3rd instar stages. Homozygous *lnk* and *chico* mutants are almost half the size compared to WT larvae At the same time of development, *chico;lnk* double mutants are approximately only half the size of the single mutants, but continue growing and are even bigger than single mutant larvae at 20 days after egg deposition.
(B) Graphic representation of pupariation rates in wild type and *chico;lnk* double mutant larvae. Larval development in wild type animals is synchronized so that 5 days after egg deposition 90% of all larvae undergo pupariation. In *chico;lnk* double mutants developmental synchronization is abrogated and severely delayed.

3.2 *cbl* does not interact genetically with *lnk*

In a recent publication, Hu et al., were able to show that besides the highly conserved protein domain structure, SH2B family adaptor proteins are characterized by the presence of a C-terminal c-Cbl consensus binding motif (Figure 11A) (Hu and Hubbard 2005). However, so far only for SH2B2 (APS) it has been proven that the binding site is functional and the association of SH2B2 to c-Cbl is functionally relevant (Liu, Kimura et al. 2002).
The *Drosophila* genome contains a single *cbl* homologue that encodes for a long and a short protein isoform (Hime, Dhungat et al. 1997; Meisner, Daga et al. 1997). Using the cbl^{F165} null-allele and the *eyFLP* technique, we created mosaic fly heads that

were almost entirely composed of homozygous mutant *cbl* tissue. As previously shown by Wang et al., *cbl* mutant heads are larger than the wild type control and contain eyes that appear rough and bulgy with larger ommatidia (Figure 11B, C) (Wang, Werz et al. 2008). We used this phenotype as readout to check for a genetic interaction between *cbl* and *lnk*, and more precisely, if the loss of *lnk* function is sufficient to modify the *cbl* mutant over growth phenotype. However, *lnk* loss of function did not influence the roughness, bulginess or the size of the ommatidia of homozygous *cbl* mutant heads. Furthermore, removing one copy of *cbl* in a heterozygous and homozygous *lnk* mutant background did not change the reduced dry weight of *lnk* mutant male flies (Figure 11E).

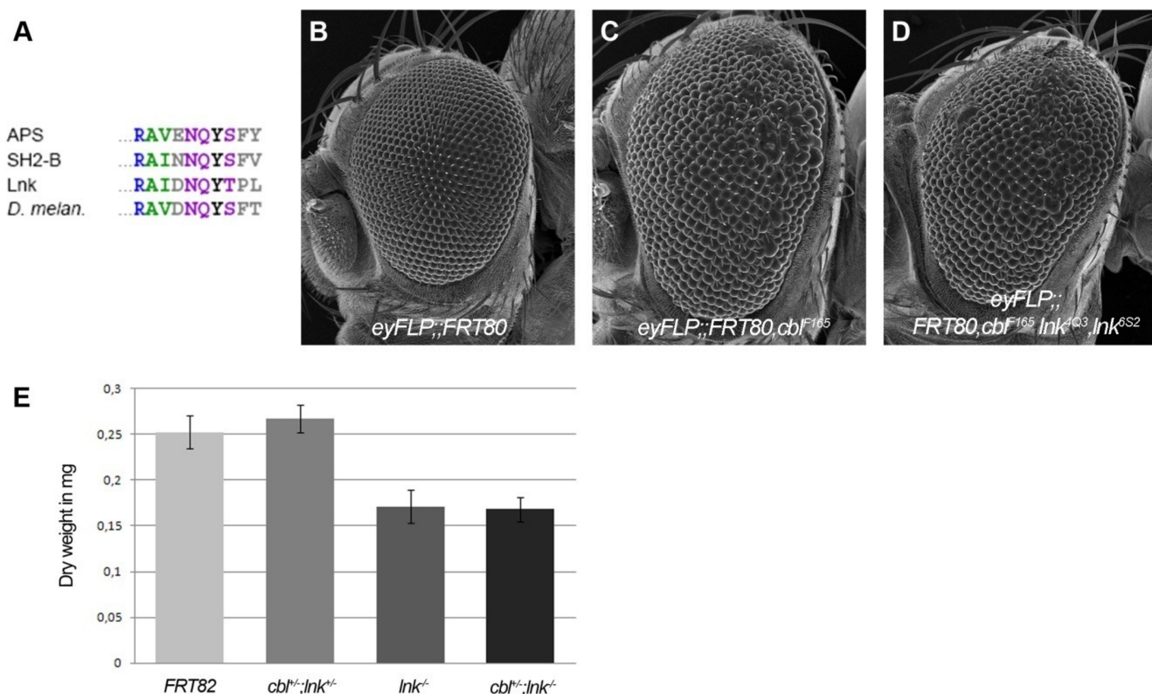

Figure 11: Although a Cbl consensus binding motif is present in Lnk, no genetic interaction is detectable.

(A) A consensus binding motif for Cbl has been shown to be highly conserved in the mammalian SH2B protein family members and in *Drosophila* Lnk.
(B-D) SEM pictures of *ey-FLP* induced *cbl* mutant clones (C) and in a homozygous *lnk* mutant background (D) compared to control heads (B).
(E) Shown is the dry weight of *cbl* and *lnk* double heterozygous adult flies compared to wild type and *lnk* homozygous mutant flies lacking one copy of *cbl* compared to *lnk* mutants.

As already shown in the manuscript Werz et al., (Figure S1A) we generated transgenic flies containing a genomic rescue construct with a mutation in the core tyrosine of the conserved Cbl binding site of *Drosophila* Lnk. We assessed the dry weight of transgenic flies in a *lnk* loss of function background and found that disruption of the Cbl binding site does not interfere with reaching wild type weight (Figure 12A). However, when taking a closer look at the ovaries of the transgenic flies, we noticed that they were smaller and contained less fully developed eggs compared to wild type ovaries (Figure 12B). Thus, it might be possible that *cbl* and *lnk* together execute an important function for proper oogenesis, which cannot be fully compensated by other factors. Nevertheless, the transgenic flies are fertile and produce viable offspring.

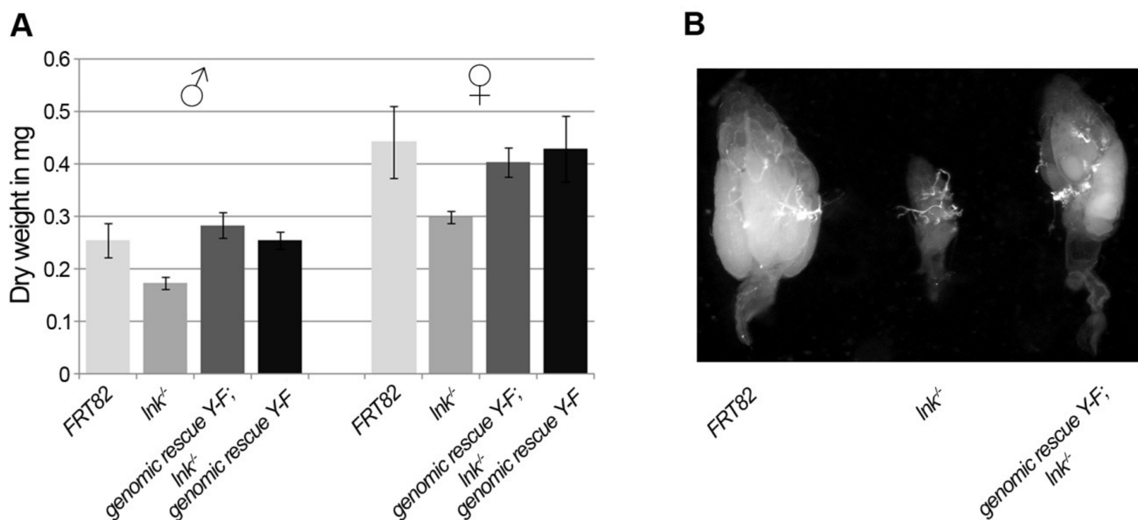

Figure 12: Disruption of the Cbl binding motif in Lnk does not interfere with normal growth but leads to minor defects in oogenesis

(A) A genomic rescue construct including a mutation of the core tyrosine within the Cbl binding motif in Lnk is able to rescue the decreased dry weight of *lnk* mutant flies.
(B) The mutated rescue construct partially restores the defects in oogenesis of homozygous *lnk* females.

Taken these findings together, we conclude that in respect to the growth related phenotypes, no genetic and molecular interaction of *cbl* and *lnk* is detectable. The highly conserved Cbl binding site in Lnk might therefore be not important for growth control in *Drosophila*. Alternatively, other factors could compensate for the disrupted *cbl;lnk* complex, or the interplay between *cbl* and *lnk* is only important for functions other than growth control, such as oogenesis.

3.3 Pull down experiments to identify interactors of Lnk

A sophisticated and unbiased way to detect protein-protein interactions are pull down experiments where an HA-tagged protein of interest is expressed in *Drosophila* cells and subsequently used as a bait to extract and indentify physically interacting proteins (prey) by mass spectrometry.

3.3.1 Identification of proteins that are pulled down with HA-Lnk

In our first pull down experiment, we expressed an HA-Lnk fusion protein in the S2 *Drosophila* embryonic cell line and used an empty vector as control. We were able to identify a total of 144 proteins that were associated to HA-Lnk. Of these, 43 proteins were also found within the control experiment and 63 proteins were sorted out due to appearance in the so called 'blacklist' with potential sticky proteins that were identified in many other pull downs. The 38 remaining proteins were considered interesting although most of them were only identified with one unique peptide (Table 1). These included Clathrin-heavy-chain, Hrs and STAM which have been shown to act in a complex to recognize ubiquinated receptors for further sequential lysosomal sorting and trafficking processes (Komada and Kitamura 2005).

Receptor internalization is known to be an important mechanism in the regulation of receptor tyrosine kinase signaling and is able to mediate, both degradation of receptors as well as sustained signaling activity (Haglund, Sigismund et al. 2003; Sigismund, Argenzio et al. 2008). So far, in *Drosophila* Hrs has been shown to be responsible for down regulation of various receptors such as EGFR, PVR, Ptc and

Smo (Jekely and Rorth 2003). Although there has not been evidence yet for a connection between Hrs and Insulin signaling we did not exclude the possibility that Lnk interacts with the Hrs complex. Thus, we decided to analyze a potential interaction between these proteins in more detail.

Protein name	Molecular function	Unique peptides	Percent coverage
lnk	Protein binding	61	49.0
Fibrillarin	mRNA binding	5	24.1
CG4169	ubiquinol-cytochrome-c reductase activity	3	12.0
His2A: CG33823	DNA binding	3	25.8
CG12264	cystathionine gamma-lyase activity	3	10.4
CG30122	mRNA binding	2	2.4
belle	ATP-dependent helicase	2	3.3
rho-like	GTPase activity	2	13.7
CG4038	rRNA pseudouridylation guide activity	2	7.6
rudimentary	aspartate carbamoyltransferase activity	2	1.0
CG14648	5-formyltetrahydrofolate cyclo-ligase activity	2	5.3
β'-coatomer protein	Protein binding/intracellular protein transport	1	2.5
Clathrin heavy chain	Protein binding/intracellular protein transport	1	1.1
CG12030	UDP-glucose 4-epimerase activity	1	7.7
Ribonucleoside diphosphate reductase	ribonucleoside-diphosphate reductase activity	1	3.6
CG18210	Unknown	1	7.7
stam	JAK pathway signal transduction adaptor activity	1	2.3
vav	Rho GTPase activator activity	1	2.1
CG3251	Unknown	1	3.2
DnaJ-like-2	Heat shock protein binding	1	3.7
CG12013	peroxidase activity	1	5.5
Peroxiredoxin 5037	thioredoxin peroxidase activity	1	5.6
vasa intronic gene	mRNA binding/RNA interference	1	4.7
Coproporphyrinogen oxidase	coproporphyrinogen oxidase activity	1	5.6
hrs	Protein binding/intracellular protein transport	1	2.4
CG5787	Unknown	1	3.7
His2B: CG33874	DNA binding	1	12.2
Septin-1	GTPase/Ubiquitin ligase	1	9.7
mitochondrial single stranded DNA-binding protein	single-stranded DNA binding	1	13.0
Eb1	microtuble binding	1	3.1
septin interacting protein 2	Protein binding	1	5.0
CG12480	Unknown	1	4.9
His4: CG33869	DNA binding	1	7.8
CG8583	Heat shock protein binding/unfolded protein binding	1	1.9
Proteasome 28kD subunit 1	Endopeptidase activity	1	5.6
Death caspase-1	Cysteine endopeptidase	1	3.4
REG	Proteasome activator activity	1	8.2
CG4882	Transcription factor	1	3.4

Table 1: List of proteins that were identified and considered interesting in the pull down using HA-Lnk as bait in S2 cells.

3.3.2 Heterozygosity for *hrs* does not modify *lnk* loss- and gain of function phenotypes

To test for genetic interaction between *hrs* and *lnk* in vivo, we used the hrs^{D28} allele which contains a nonsense mutation at amino acid 270 and behaves genetically like a null mutation to remove one copy of *hrs* in a *lnk* over expression background and in adult flies heterozygous and homozygous mutant for *lnk* respectively (Lloyd, 2002, cell). As described above, *GMR-Gal4* mediated over expression of *lnk* results in slightly bulgy and rough eyes compared to the control (Figure 13A, B). However, removing one copy of *hrs* in this sensitized background does not modify the size or structure of the eye (Figure 13C). We further did not observe any changes in dry weight of adult flies in double heterozygotes for hrs and *lnk* compared to wild type, nor did loss of one copy of *hrs* lead to alterations in the weight of homozygous *lnk* mutants (Figure 13D).

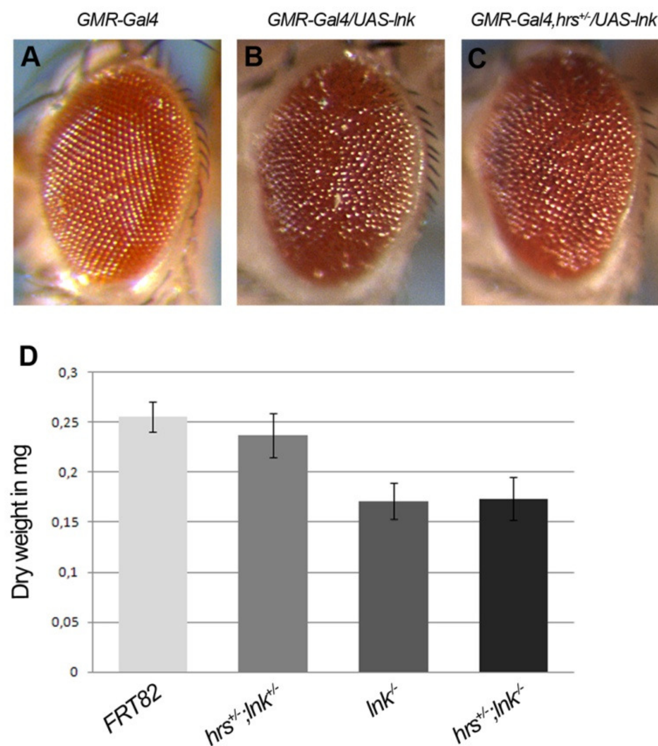

Figure 13: Heterozygosity for *hrs* is not sufficient to modify *lnk* over expression and loss of function phenotypes.

(A-C) Stereomicroscopic pictures of fly eyes show that the rough eye phenotype caused by ectopic expression of *lnk* (B) cannot be suppressed by introduction of a mutant hrs^{D28} allele (C)
(D) Scale bars indicate the dry weight of *hrs;lnk* double heterozygote animals compared to wild type and of *hrs* heterozygotes in *lnk* mutant background compared to *lnk* single mutants

3.3.3 Identification of proteins interacting with HA-Lnk in response to activated Insulin signaling

In a second approach to identify interaction partners of Lnk in a pull down, we used *Drosophila* Kc cells that were transfected with an HA-Lnk construct and control cells containing an HA-GFP expressing vector. This second set of pull downs was performed in two biological replicates. Additionally, this time we added another level of complexity, as we split each of the cell samples into two parts, one of which was starved and the other one, which was stimulated with Insulin for 15 minutes. Therefore, we were able to distinguish between more constitutive interactions or

Insulin independent interactions and binding partners that associate to Lnk in response to Insulin pathway activation.

Although, we performed the two pull down experiments in two distinct cell lines that have been shown to display different protein expression and abundance profiles (Mohanti, S. personal communication), we would have expected to find strongly interacting proteins with Lnk in both experiments. Furthermore, in the Kc cell samples that were starved before protein extraction we only found proteins that were either also identified in the control, in many other pull downs or not considered potentially interesting. There was only one protein that was exclusively identified in the Insulin stimulated cells and thus seemed to be specifically associated to Lnk in response to Insulin pathway activity, called Slik. Slik, a Sterile20 kinase turned out to be particularly interesting to us, because in addition to its potential interaction with Lnk in response to insulin it was also identified earlier in our *eyFLP* based screen for genes involved in growth control (Rottig, unpublished). Furthermore, in previous studies it has been shown that *slik* is able to promote growth by accelerating proliferation in a Raf-dependent manner (Hipfner, Keller et al. 2004). We thus decided to further investigate the potential connection between *slik* and *lnk*.

3.3.4 Heterozygosity for *slik* has no influence on *lnk* over expression phenotypes

First, we tested for genetic interaction using modulation of the rough eye phenotype induced by *GMR* driven over expression of *lnk* in the eye as readout (Figure 14B). In this genetic background we removed one copy of *slik*, which did not have a detectable influence on the surface structure and the size of the eye, respectively (Figure 14). In a second attempt we removed one copy of *slik* in homozygous *lnk* mutant flies and assessed their dry weight and also did not observe a significant difference of homozygous *lnk* flies and flies with additional heterozygosity for *slik*. Based on the in vivo data, we concluded between Lnk and Slik a possible interaction is not essential for the growth promoting function of Lnk. In order to finally confirm or rule out binding of Slik and Lnk, it would be important to perform an in vitro binding assay.

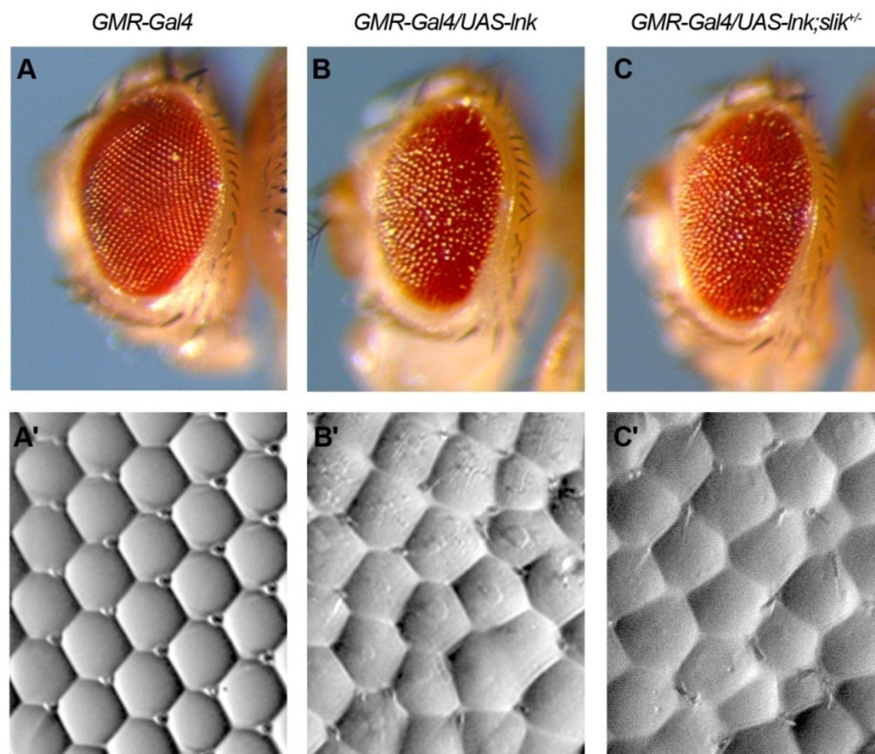

Figure 14: Loss of one copy of *slik* does not rescue *lnk* over expression phenotypes

(A-C) Shown are stereomicroscopic pictures of control eyes (A), eyes of flies over expressing *lnk* (B) and eyes where in a *lnk* over expression background an additional copy of *slik* is removed.
(A'-C') The surface structure of compound eyes in (A-C) is visualized by nail polish imprints.

General Discussion

With the discovery of Insulin by Frederick Banting and Charles Best in 1921, it became possible to treat the symptoms of Diabetes mellitus. Since then, a large number of studies were carried out with regard to Insulin and its effects within the organism (Saltiel and Kahn 2001). The corresponding Insulin signal transduction pathway and its components were discovered, which turned out to be highly conserved throughout evolution. Scientists made use of model organisms such as *Drosophila melanogaster*, *Caenorhabditis elegans* or *Mus musculus*, because these animals possess the same Insulin pathway components such as Humans do (Garofalo 2002). Thereby, many new components of the Insulin pathway have been discovered that could be assigned to homologous human proteins (Hafen 2004). Further understanding of the signaling cascade induced by Insulin and its physiological implications might contribute to the development of therapies against disorders such as diabetes and obesity.

In *Drosophila,* the Insulin pathway and its major components are highly conserved. A single Insulin/IGF receptor exists that regulates size, female fertility and lipid metabolism through a pathway that involves the fly homologues of *IRS1-4* (*chico*), *PI3K* (*dp110*), *PTEN* (*dPTEN*), *Akt/PKB* (*dAkt1/dPKB*), *Foxo* (*dFoxo*) and *S6K* (*dS6K*). In a screen for new genes involved in growth regulation and potential modulators of Insulin signaling, we identified mutations in a gene called *lnk* that caused severe growth retardation on a cellular level and the whole organism. It was the aim of this thesis to characterize *lnk* and to reveal its mode of function by means of detailed analysis of the mutant phenotypes, genetic interaction experiments and biochemical studies.

Mutations in positive components of the Insulin pathway cause a distinct set of phenotypes such as cell autonomous growth reduction, reduced cell number, developmental delay, increased body fat and female sterility. Strikingly, we were able to detect all these phenotypes in homozygous mutant *lnk* animals, strongly suggesting an important role for Lnk in the regulation of growth and metabolism via the Insulin pathway (Hafen 2004). Through genetic interactions with Insulin signaling components and determination of phosphorylation levels of dPKB as a biochemical

read out, we provided additional proof for *lnk* being a positive regulator of the Insulin signaling pathway in *Drosophila*. These results are consistent with the mammalian situation, where the Lnk homologues SH2B1 (SH2B/PSM) and SH2B2 (APS) have already been shown to play a regulatory role in the Insulin pathway via direct binding to the Insulin receptor through their SH2 domain (Kotani, Wilden et al. 1998; Ahmed, Smith et al. 1999). Although it is difficult to assign a clear homolog to *Drosophila* Lnk on the basis of sequence homology, SH2B1 appears to us as the functional homologue, because, other than SH2B2, it has always been reported to regulate Insulin signaling in a positive manner. To further clarify this issue it would be important to see if any of the mammalian SH2B family proteins is able to compensate for the loss of the *Drosophila* protein and to which extend.

Whereas homozygous mutations of core components of the Insulin pathway, e.g. *dInR*, *dPI3K* and *dPKB*, in *Drosophila* are lethal due to their essential requirement in embryonic development, *lnk* mutants are viable and strongly resemble *chico* mutant flies (Garofalo 2002). Furthermore, in epistasis experiments mentioned above, we were able to place *lnk* genetically downstream of the receptor and upstream of *PI3K*. This is exactly where *chico* the *IRS1-4* homolog is placed. Thus, it seems likely that *chico* and *lnk* mutants respectively, are viable due to redundancy between these two proteins. Interestingly, *chico;lnk* double mutants are lethal, suggesting that lacking either *lnk* or *chico* lowers the Insulin signal to a level which is sufficient for survival of the flies but dramatically impairs growth and metabolism. One hypothesis is that removing both proteins at the same time, reduces Insulin signaling levels beneath a threshold required for survival to adulthood. Strikingly, the lethality of *chico;ln*k double mutants can be rescued by removing one copy of the lipid phosphatase *PTEN*, that converts PIP_3 into the inactive PIP_2. This suggests that *chico* and *lnk* have an influence on the levels of the second messenger PIP_3. This was confirmed using the localization of the *tGPH* reporter as a direct measure of the membrane bound PIP_3, showing that the signal of the *tGPH* reporter was reduced in *chico* and *lnk* mutant cells.

The YxxM consensus binding site in Chico allows its binding to the adaptor subunit (dp60) of PI3K, leading to subsequent recruitment of the catalytic subunit (dp110) to

the plasma membrane, a process that results in activation of PI3K (Oldham, Stocker et al. 2002). In contrast, the Lnk protein sequence is missing such a YxxM motif, thus it is rather unlikely that Lnk influences intracellular PIP_3 levels through direct binding to dp60.

In *Drosophila*, embryos containing strong *InR* mutations die before hatching, indicating that Insulin signaling is essential for early embryonic development (Fernandez, Tabarini et al. 1995). Interestingly, loss of function of both, *chico* and *lnk* at the same time also leads to lethality; however, embryos hatch and develop to prepupal stage.
In wild type animals reared under normal conditions the timing throughout larval development is constant in a certain range and regulated by three different size checkpoints, the threshold size for metamorphosis, minimal viable weight and critical weight. Both minimal viable weight and critical weight are assessed in larval stage three. Minimal viable weight is defined as the minimal weight at which the amount of fat body storage is sufficient for survival through metamorphosis, while critical weight is a threshold that controls the regulation of hormonal processes that initiate pupariation. After critical weight is attained the larvae continue to feed and grow in the so called interval to cessation of growth (ICG) (Nijhout, Davidowitz et al. 2006) or terminal growth period (TGP) (Shingleton, Frankino et al. 2007) until a peak of circulating 20-hydroxyecdysone (20HE), a steroid hormone that also triggers earlier moults, causes the termination of feeding and the onset of pupariation. The length of the ICG/TGP is a substantial parameter defining the final size and weight of the animal. By the time entering larval stage 3, *chico;lnk* double mutant larvae are substantially smaller than wild type and even *chico* and *lnk* single mutants (Figure 12A), suggesting that it takes a significantly longer time for them to reach the critical weight checkpoint. However, this observation alone is not sufficient to explain the enormous delay to pupariation, since the *chico;lnk* double mutant larvae do not pupariate at the same size as *chico* and *lnk* single mutants, but grow even larger. It seems more likely that the larvae are rather unable to assess their critical weight correctly and thus continue feeding for a longer period of time. How critical size assessed in *Drosophila* is still not entirely know but it has been suggested that the

prothoracic gland is involved as well as growth of imaginal discs (McBrayer, Ono et al. 2007; Stieper, Kupershtok et al. 2008). Defects in the growth of these organs might thus lead to insufficient synthesis of ecdysteroids which in turn would cause the larvae to massively delay pupariation as observed in *chico;lnk* double mutants. In line with this hypothesis is the fact that we were unable to clearly detect imaginal discs in double mutant larvae. To further elucidate the nature of delayed pupariation in *chico;lnk* larvae it would be useful to genetically mark the imaginal disc tissue by GFP or a LacZ reporter and check for their size. Moreover, it would be important to test whether the suggested inability to monitor critical size causes decreased ecdysteroid levels and thus the delay. This could be easily achieved by feeding 20HE or its prohormone Ecdysone which might be sufficient to initiate pupariation of the double mutant larvae at an appropriate time point.

A possible function for Lnk could be deducted from the mammalian situation, where Ren et al show, that Leptin stimulates the formation of a JAK2/SH2B1/IRS1 or IRS2 tertiary complex, dramatically enhancing tyrosine phosphorylation of IRS1 and IRS2. This promotes the association of IRS1 and IRS2 with the p85 regulatory subunit of PI3K to activate Akt (Ren, Li et al. 2005). So far, it is unclear whether this mechanism is specific to Leptin signaling in the brain in mice or if there is also a mechanism present in *Drosophila* in which the two adaptor proteins, Chico and Lnk, would fulfill their function in a complex. However, the results we presented here rather speak for Lnk and Chico acting in parallel, since otherwise the *chico;lnk* double mutant animals would be expected to display the loss of function phenotype of either gene and not synthetic lethality. Furthermore, we could not detect a physical interaction between Chico and Lnk using pull down experiments. The first step to clarify the general ability of the two proteins to bind to each other would be to perform in vitro binding assays, where only Chico and Lnk are present in a reasonable amount.

The question how Lnk and its mammalian homologues are involved in the transduction of the Insulin signal has already been speculated for the mammalian SH2B family members. SH2B1 and SH2B2 have both been shown to directly bind to the Insulin receptor through their SH2 domain, which could stabilize the receptor in

an activated state or protect it from dephosphorylation by protein tyrosine (Duan, Yang et al. 2004). Experimental evidence supporting this hypothesis is still missing, but could be tested in *Drosophila*, assessing the phosphorylation status of an activated Insulin receptor over a certain period of time in a wild type and homozygous *lnk* mutant background. If Lnk is involved in the stabilization of InR phosphorylation, we would expect an initial high level of phosphorylation but a rapid decrease in *lnk* mutant tissue. Assuming, Lnk would protect the Insulin receptor from dephosphorylation, it would be very interesting to check for a possible rescue of the *lnk* loss of function phenotype by generating double mutants of *lnk* and a number of candidate phosphatases. Generally, the inhibition of a negative regulator of Insulin signaling might be a possible function of Lnk. Apart from the above mentioned phosphatases, another potential candidate would be Susi (Wittwer, Jaquenoud et al. 2005), a protein that inhibits PI3K/PKB signaling upstream of PI3K and downstream of the Insulin receptor. Susi regulates intracellular PIP_3 levels, by binding to the regulatory subunit dp60 of PI3K, and thus might exert its inhibitory role by interfering with the recruitment of dp110 or by driving the degradation of dp60 or dp110. Also in this case, in vitro binding assays to detect a physical interaction between Lnk and Susi and genetic interaction studies, e.g. generating *susi;lnk* of double mutants, could give insight into this hypothesis.

Activation of the Insulin pathway leads to binding of SH2B1 and SH2B2 to the Insulin receptor and subsequent phosphorylation of a tyrosine within a c-Cbl consensus binding motif in their C-terminus. The motif is present in all three SH2B family members and conserved in the *Drosophila* protein (Hu and Hubbard 2005). Up to now, functional relevance of this c-Cbl docking site has only been proven for SH2B2. In response to Insulin, SH2B2 couples c-Cbl to the Insulin receptor and facilitates ubiquitination of the receptor (Ahmed, Smith et al. 2000). Although the SH2B2/Cbl complex is involved in the negative regulation of a number of receptor tyrosine kinases (Fiorini, Alimandi et al. 2001), it might still be possible that targeted ubiquitination by Cbl serves as an internalization signal in order to sustain receptor activity. Speaking against this theory in *Drosophila*, are the phenotypes caused by mutations in *cbl* that have clearly been associated to a role of *cbl* as a negative

regulator of the EGF receptor and associated processes such as R7 photoreceptor development and disruption of the dorsoventral axis of the egg and the embryo (Meisner, Daga et al. 1997; Pai, Barcelo et al. 2000). Secondly, we generated transgenic flies containing the *lnk* genomic rescue construct with a mutation in the tyrosine within the potential Cbl consensus binding site and found that those flies in a homozygous *lnk* mutant background do not show any phenotypical defects. Furthermore, removing two copies of *lnk* does not modulate the overgrowth phenotype due to *eyFLP* induced *cbl* mutant clones in the head and finally, heterozygosity for *cbl* does not alter dry weight of *lnk* mutant flies. We thus believe that in *Drosophila*, Cbl does not form a complex with Lnk or that a possible Cbl/Lnk heterodimer plays a role other than the regulation of growth through the Insulin pathway.

Taking into account that the mammalian SH2B family proteins have been implicated in the modulation of a large variety of signaling pathways, it appeared reasonable to test whether *Drosophila* Lnk is also involved in signaling events other than the Insulin pathway. Many studies in vertebrates in the past years established SH2B1 and SH2B2 as important proteins for proper transduction of signals through the JAK/Stat pathway. As mentioned above, SH2B1 binds to JAK2 in response to Leptin and although in *Drosophila* a mechanism analogous to the vertebrate Leptin signal transduction has not been discovered, we tested for genetic interaction of *lnk* with components of the JAK/Stat pathway. In line with the fact that the phenotypes displayed by homozygous mutant *lnk* flies were exclusively related to impaired Insulin signaling, we did not observe a connection between *lnk* and JAK/Stat. This result suggests that Lnk related proteins adopted the ability to bind to JAK2 and, thus to modulate the underlying signaling mechanisms throughout evolution.
Furthermore, SH2B1 mediates mitogenic signals and promotes phosphorylation and activation of ERK1 and ERK2 (Yokouchi, Suzuki et al. 1997; Yokouchi, Wakioka et al. 1999). Although it has been shown that the Insulin pathway in vertebrates stimulates the activation of MAPK initially through association of Grb2 to IRS1 (Ogawa, Matozaki et al. 1998), the link between the Insulin and MAPK pathway in *Drosophila* is still missing. Notably, over expression of an activated InR resulted in MAPK

phosphorylation (Brogiolo, Stocker et al. 2001). Containing two potential YXN Drk SH2 binding sites (Drk represents the *Drosophila* homolog of Grb2) within its protein sequence, Lnk served as a candidate to connect these two pathways. To test whether these motifs promote binding of Drk and if this potential interaction is important for Lnk to exert its growth promoting function, we conducted a structure function analysis in which we specifically mutated the core tyrosines in the motifs, thus disrupted their functionality. In contrast to the PH and the SH2 domain of Lnk, which proved to be essential for normal growth, neither of the potential Drk motifs is necessary for reaching wild type size. From these results and the *lnk* mutant phenotypes, we conclude that the major role of *lnk* in growth control is to modulate the activity of the Insulin pathway, rather than interfering with other signaling cascades.

In a less biased approach to identify binding and interaction partners of Lnk, we conducted pull down experiments using HA-tagged Lnk protein as bait. In our first pull down we expressed HA-Lnk in S2 cells and in the two following experiments we employed a different strategy using Kc cells that were either starved or Insulin stimulated. Next we analysed the list of proteins that we found to be associated to Lnk and picked particularly interesting ones for further experiments to test for their ability to modulate lnk loss of function (dry weight) or gain of function phenotypes (*GMR-Gal4/UAS-lnk*). On the list of proteins of potential Lnk binding partners we identified in S2 cells were proteins such as Hrs, Stam and Clathrin Heavy Chain that act in a complex to regulate sorting of e.g. tyrosine kinase receptors to early endosomes to initiate their degradation (Raiborg, Bache et al. 2002). Although the mutant and over expression phenotypes described for *Drosophila hrs* so far clearly suggest a predominant function in negative regulation of receptor tyrosine kinase mediated signaling, we tested for the ability of *hrs* to genetically interact with *lnk*. However, neither in the *lnk* mutant nor in the *lnk* over expression assay any evidence that *hrs* and *lnk* are functionally connected could be obtained.

Notably, we did not find any of the Insulin pathway components or proteins connected to the Insulin signaling cascade to be pulled down with Lnk, as we would

have expected from our initial results placing *lnk* between the *Drosophila Insulin receptor* and *dPI3K*. Therefore, we decided to pursue a different strategy, expressing HA-Lnk in cells that were either starved or stimulated with Insulin, to discover interactions that depend on activation of the Insulin pathway. However, only one protein called Slik turned out to be identified solely in the Insulin stimulated samples that was not considered to be unspecific as judged by control pull downs using HA-GFP as bait. Interestingly, in previous studies Slik has been reported to be involved in growth control and thus appeared to be a possible interaction partner of Lnk in its function to positively regulate the Insulin signaling pathway (Hipfner, Keller et al. 2004). If this was the case, we would have expected that reducing *slik* levels by taking out one copy of *slik* would rescue or modify the over expression phenotype of *lnk* in the eye. However, we could not detect any differences in the roughness of the eye or eye size, suggesting that *slik* does not act in the same pathway downstream of *lnk*. However, it could still be possible that removing only one copy of slik did not lower its activity beneath a threshold to enable the detection of a genetic interaction in the sensitized background we used.

Summarizing the results from the pull downs with Lnk as bait, we can conclude that we were not able to repeatedly identify the same proteins in different pull downs as specific binding partner of Lnk. There are several possible explanations for the inconsistency in protein identification with the easiest being minimal technical variations. Although we verified that HA-Lnk is expressed in levels that are suitable for a protein pull down, it might be possible that a potential interaction partner of Lnk is only marginally abundant and thus could not be detected in all the relevant samples. In order to solve this problem, by now the protocol for pull down experiments has been improved to increase the sensitivity for weak but specific interactions and will be employed for future analyses. Furthermore, it was surprising for us not to find any known member of the Insulin signaling pathway among the proteins pulled down by HA-Lnk. Nevertheless, proteins that are functionally relevant for Lnk might still be detected but in the course of narrowing down the group of interesting proteins we did not include the ones previously uncharacterized.

Outlook

Within this work we describe the identification and characterization of the adaptor protein Lnk in Drosophila, a homolog of the mammalian SH2B protein family. By means of phenotypic characterization, genetic interaction studies and biochemical read outs were able to show that Drosophila Lnk is involved in the control of cell and organism growth as a novel member of the Insulin signaling pathway at the level between the receptor and PI3K. However, we were not able yet to solve the question about the precise mechanism of Lnk's function. For adaptor proteins with no intrinsic catalytic activity, the answer to this question is always directly connected to the identification of its binding partners in a physiological context. At present, a promising way to discover protein-protein interactions is to conduct a pull down experiment using the tagged protein of interest as bait. Although until now the pull downs with Lnk did not yet lead to the identification of a clear interaction partner, repeating these experiments, using an improved protocol and only parts of the Lnk protein, might increase the sensitivity for specific interactions that could not be detected before. For example, the SH2 domain and parts of the C-terminus that contains the highly conserved tyrosine phosphorylation site would be promising candidates. To further verify binding of Lnk to any protein found in the pull down it would be necessary to conduct in vitro binding or yeast two hybrid assays. In these experiments it might also be interesting to include a set of candidate proteins such as members of the Insulin pathway or proteins that have been shown to associate to the mammalian homologues. Following genetic interaction studies would be valuable to further confirm a functional connection between lnk and the potential candidates on a genetic level and clarify their epistatic relation.

As discussed above, one possible function of Lnk could be the stabilization of InR or Chico phosphorylation, thus we are currently assessing the phosphorylation status of know phospho-peptides of these two proteins in *lnk* mutant larvae and compare them to control larvae using phospho-proteomics. This will give us further insight, if Lnk is indeed having an influence on the phosphorylation and thus activation of the InR or Chico.

In the recent years, known components of the Insulin pathway were subject to a protein interaction analysis in a broader context in order to generate an interaction network surrounding the Insulin pathway (Glatter, T. and Gstaiger, M. unpublished). A large number of pull downs using HA-tagged Insulin components as baits have been conducted and many of the known essential interactions could be detected and verified within this work. Furthermore, the interactome project was also able to reveal previously unknown binding partners of Insulin pathway members and connections to different other signaling cascades. At the moment, these new findings are in the course of confirmation by follow up experiments. The outcome of the interactome project could also provide a useful opportunity to discover the connection of Lnk to the Insulin pathway. The comparison of the results of the pull downs using HA-Lnk as bait to the proteins identified in the interactome project could reveal potential intermediate proteins that would aid to place Lnk in the Insulin signaling cascade.

Material and Methods

General Methods for the work with flies

Standard methods for the keeping and work with *Drosophila* were carried out as described in Ashburner (1989). Information about the genetic nomenclature, the used marker mutations and the balancer chromosomes can be found in Lindsley and Zimm (1992) or in the FlyBase (http://Flybase.bio.indiana.edu). Balancer chromosomes carry multiple inversions and can therefore not recombine with the wild-type homologue chromosome. In order to identify the balancer chromosomes, they also carry marker mutations.

Fly stocks

The following fly stocks were used

y w; Act5C-Gal4/CyOy^{+} (Bloomington Drosophila Stock Center)

y w; GMR-Gal4/CyOy^{+} (Gift of M. Freeman)

y w; ptc-Gal4/CyOy^{+} (Bloomington Stock Center)

y w; chico^{1}/CyOy^{+} (Bohni, Riesgo-Escovar et al. 1999)

y w; chico^{2}/CyOy^{+} (Bohni, Riesgo-Escovar et al. 1999)

y w eyFLP2 glass-LacZ;; FRT80B (Newsome 2000)

y w;; cbl^{F165}/TM6B (Pai, Barcelo et al. 2000)

y w; Hrs^{D28}/CyOy^{+} (Littleton and Bellen 1994)

y w; FRT42D slik^{1}/CyO Kr^{GAL4} UAS-GFP (Hipfner and Cohen 2003)

Scanning electron microscopy images

SEM (Scanning Electron Microscope) images of female fly eyes were analyzed to characterize the eye phenotypes. The flies were anesthetized by ether for 5 minutes and images were directly taken with a SEM JEOL 6060VP.

Nail polish imprints

Adult female flies were decapitated with a sharp razor blade and briefly dipped in a drop of fluid nail polish. The head was then placed on a slide and dried for 10 minutes. The dried layer of nail polish was removed with forceps and a needle and placed on another slide with the imprint facing upright. Subsequently, imprints were

examined and photographed under a standard microscop (Zeiss Axiophot), using 10X magnification. For detailed description see (Arya and Lakhotia 2006).

In situ hybridization

RNA *in situ* hybridization using DIG-labeled probes was performed as described (Brogiolo, Stocker et al. 2001). The probes against lnk were amplified from the EST clone LD10453 (obtained from *Drosophila* Genomics Resource Center) with the following primers, fwd: GAG GAG GAC CTG GAC CAG C and rev: GTA TAA GCA TCA AGT GTG GCC.

Cell transfection

Drosophila embryonic S2 cells were grown at 25°C in Schneider's *Drosophila* medium (Gibco/Invitrogen) supplemented with 10% heat-inactivated fetal-calf serum (FCS), as well as Penicillin and Streptomycin. For the construction of the stably expressing *GFP-Lnk and Lnk-GFP* cell lines, S2 cells were co-transfected with *GFP-Lnk* and *Lnk-GFP*, respectively, *Actin-Gal4* and a vector containing a Blasticidin resistance gene, using effectene transfection reagent (Qiagen). Two days after the transfection, the selection medium (Schneider's containing 10% FCS and 25 μg/ml Blasticidin) was added to the cells. After ten days the selection medium was replaced by Schneider's medium containing 10% FCS and 10 μg/ml Blasticidin. For over expression of *GFP-Lnk* and *Lnk-GFP* in all experiments CuSO4 was added at a concentration of 600 μM for 10 h. Cells were stained with DAPI prior to inspection under the confocal microscope DM5500Q (Leica).

RNAi and flow cytometrie

S2 cells were transfected with dsRNA using Fugene 6 (Roche) with slightly modified protocol: dense cell culture was diluted to 0.8×10^7 cells/ml in big flasks. Next day, 10 μg of dsRNA was diluted with 0.5 ml Schneiders/PenStrep (no FCS) and 15 μl Fugene transfection reagent and the mixture was added to the cells drop wise. On the following day another 5 ml of Schneiders/10%FCS/PenStrep was added to dilute Fugene. The experiment was continued after four days. The silencing efficiency was confirmed using dsRNA amplifying primers and with RT-PCR on agarose gel.

For flow cytometric analyis, cells were detached from the flask and diluted to a concentration of 1,000,000 cells/ml in PBS. Cells were washed twice with 1x PBS and resuspended in 500 µl PBS before analyzing in a flow cytometer (Becton Dickinson). Forward and side light scatter were determined for cell size analysis. Native S2 cells were used as a control. Experiments were performed in triplicates.

Weight Analyses

Flies of the respective genotypes were reared under identical conditions and collected 3 days after eclosion. They were dried at 95°C for 5 minutes and kept at room temperature for 3 days before weighing on a precision scale (Mettler Toledo MX5).

Measurement of wing area

Wings were removed from bodies of adult female flies and washed in 100% Ethanol. They were then placed on a glass slide, air dried and mounted in Euparal for microscopic analysis.

Tangential eye sections

Adult flies were anesthetized and the head removed, cut in half with a razorblade and briefly stored on ice in Ringer's solution or PBS. Subsequently the heads were fixed and further processed as described in (Basler, Christen et al. 1991). Tangential sections were cut with 2050 Supercut (Reichert-Jung) and mounted on a microscope slide.

Construction of fly lines

UAS-Ink was amplified from an EST clone (LD10453) with primers fwd: GAG GAG GAC CTG GAC CAG C and rev: AGT TCC GCT GGT TAA GCC GC and subsequently cloned into pENTR vector. Positive clones were sequenced to exclude PCR errors. Using clonase reaction (Invitrogene) the clone was transferred into the pTW destination vector and injected into *y w* embryos.

Pull down Experiments

Affinity purification

Prior to affinity purification the cells were grown in shaking flasks in Schneider S2 medium. The cells were serum starved in 2% FBS overnight and bait expression was induced using 600µM $CuSO_4$ for at least 16h. The cells were either treated with 100nM insulin for 20min or not treated before harvest. For affinity purification the cell pellets were lysed on ice for 30 minutes in10 ml HNN (50 mM HEPES pH 7.5, 5 mM EDTA, 250 mM NaCl, 0.5% NP40, 1mM PMSF, 50 mM NaF, 1.5 mM Na_3VO_4, protease inhibitor cocktail (Roche)) in the presence of 3mM DSP with ten strokes using a tight-fitting Dounce homogenizer. Reactive DSP was quenched by adding 1ml Tris, pH7,5. Insoluble material was removed by centrifugation and the cleared lysate was precleared using 100uL Protein A-Sepharose (Sigma) for 1h at 4℃ on a rotating shaker. After removal of the Protein A-Sepharose 100uL anti HA-agarose (Sigma) was added to the extracts and incubated for 4h at 4℃ on a rotating shaker. Immunoprecipitates were washed with 4x 20 bedvolumes of lysis buffer and 3x with 20 bedvolumes of buffer without detergent and protease inhibitor. The proteins were released from the beads by adding 3x 150uL 0,2 M Glycine, pH 2.5. Following neutralization using 100 µL 1 M NH_4CO_3 the eluates were treated with 5mM TCEP to reduce Cytein bonds and reduce DSP crosslinker for 30 min at 37℃ and alkylated with 10 mM Iodacetamide for 30 min at RT in the dark. For tryptic digest 1ug trypsin was added to the eluated and incubated at 37℃ overnight. The tryptic digest was acidified to $pH<3$ using TFA and purified using C18 Microspincolumns (Harvard Apparatus) according to the protocol of the manufacturer, resolved in 0.1% formic acid/1% acetonitrile and injected into the mass spectrometer.

LC/MS/MS analysis

LC-MS analysis of affinity purified samples was performed on a LTQ-FT-ICR mass spectrometer (Thermo Electron, Bremen, Germany), which was connected to an online electrospray ionsource. Peptide separation was carried out using an Eksigent Tempo nano LC System (Eksigent Technologies, Dublin, CA, USA) equipped with a RP-HPLC column (75 µm x 15 cm) packed in-house with C18 resin (Magic C18 AQ 3 µm; Michrom BioResources, Auburn, CA, USA) using a linear gradient from 96% solvent A (0.15% formic acid, 2% acetonitrile) and 4% solvent B (98% acetonitrile,

0.15% formic acid) to 25% solvent B over 60 minutes at a flow rate of 0.3 µl/min. The data acquisition mode was set to obtain one high resolution MS scan in the ICR cell at a resolution of 100,000 full width at half maximum (at *m/z* 400) followed by MS/MS scans in the linear ion trap of the three most intense ions (overall cycle time of 1 second). To increase the efficiency of MS/MS attempts, the charged state screening modus was enabled to exclude unassigned and singly charges ions. Only MS precursors that exceeded a threshold of 150 ion counts were allowed to trigger MS/MS scans. The ion accumulation time was set to 500 ms (MS) and 250 ms (MS/MS) using a target setting of 10^6 (for MS) and 10^4 (for MS/MS) ions. After every sample, a peptide mixture containing 200 fmol of [Glu1]-Fibrinopeptide B human (Sigma, Buchs, Switzerland) was analyzed by LC-MS/MS to constantly monitor the performance of the LC-MS/MS system..

Data processing

MS2 peptide assignment

Acquired MS2 scans were searched against the Drosophila Flybase database version 5.7 using the SORCERER-SEQUEST (TM) search algorithm, which was run on the SageN Sorcerer (Thermo Electron, San Jose, CA, USA). *In silico* trypsin digestion was performed after lysine and arginine (unless followed by proline) tolerating two missed cleavages in fully tryptic peptides. Database search parameters were set to allow phosphorylation (+79.9663 Da) of serine, threonine and tyrosine as a variable modification and carboxyamidomethylation (+57.021464 Da) of cysteine residues as fixed modification. Furthermore, a variable modification of lysine residues (+145) from the carboxyamidomethylated cleaved DSP cross-linker was considered. Search results were evaluated on the Trans Proteomic Pipeline (TPP) using Peptide Prophet (v3.0) and Protein Prophet.

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