Subcellular localization of LKB1 and characterization of its interactions with the membrane skeleton in *Drosophila melanogaster*

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1 Summary

Germ line mutations in the human *lkb1* gene are the main cause of the Peutz-Jeghers syndrome (Hemminki, 1999) and somatic *lkb1* mutations are associated with mainly epithelial cancers (Sanchez-Cespedes, 2007). The serine/threonine kinase LKB1 (STK11) is involved in different cellular processes like cell proliferation, energy homeostasis and cell polarity (Martin-Belmonte and Perez-Moreno, 2012). Especially the observation that activation of mammalian LKB1 can polarize cells in absence of cell-cell contacts (Baas et al., 2004) has drawn attention to the role of LKB1 in cell polarity. Using *Drosophila melanogaster* and other model organisms various potential downstream targets have been identified, while little is known about its upstream regulation and the mechanisms by which LKB1 controls cell polarity. It has been suggested that LKB1 might be mostly constitutively active and that its involvement in specific responses depends on localization of it to specific subcellular compartments (Sebbagh et al., 2011).

In order to study the role of LKB1 in cell polarity an antibody against LKB1 has been raised for this work that could detect LKB1 in different tissue of *Drosophila*. Remarkably, endogenous LKB1 localizes cortically in asymmetrically dividing embryonic neuroblasts but cytoplasmic in larval neuroblasts. LKB1 has been described to control asymmetric divisions in both of these cell types. How it can exert its functions with different localizations remains to be answered. Furthermore, endogenous LKB1 localizes to the basolateral cortex in embryonic epithelial cells. I observed that a farnesylation deficiency does not alter the localization of LKB1 remarkably in epithelial cells and did not affect its activity towards its downstream target AMPK (AMP-activated protein kinase). A polybasic motif was identified in this work, which interacts with phospholipids found in the plasma membrane and potentially with the newly identified interaction partners α-Spectrin and β-Spectrin. Furthermore, three nuclear localization signals (NLS) of *Drosophila* LKB1 were identified. A construct carrying mutations in all three NLS had a reduced ability to rescue an *lkb1*-KO mutant and revealed a lower basal activity towards its substrate AMPK in embryonic lysates. Moreover, the mutation of the three NLS decreased the phenotype of LKB1 overexpression in the negative regulation of organ size compared to its wild type counterpart.

2 Introduction

2.1 Cell polarity

Cells in tissues of multicellular organisms, but also cells of single-cell organisms, show asymmetries in shape, protein and lipid distribution and cell function, defined as cell polarity. Polarization is initiated by either external or internal polarity cues. Contact to other cells or the extracellular matrix can, for example, serve as external cues for epithelial cells to form junctions and give rise to an epithelia tissue (Nelson, 2003). The establishment and maintenance of cell polarity is fundamentally important for diverse complex cellular functions, like formation of epithelial barriers, cell migration and asymmetric cell division. Most of the key components of cell polarity found in metazoa are well conserved throughout evolution.

2.2 *Drosophila* cell types as a model for cell polarity

Several cell types of *Drosophila melanogaster* have been established as model systems for *in vivo* studies on different aspects of cell polarity:

- The oocyte, similar to the early *Caenorhabditis elegans (C. elegans)* embryo, displays an anterior-posterior polarity.

- The mesodermal follicle cell epithelium surrounding the egg chamber and the ectodermal epithelium surrounding the embryo. Both form monolayers of cuboidal polarized cells. A peculiarity of the follicle cells is that their apical side is not directed towards a lumen or the external environment, instead it forms cell-cell contacts with the germline cells.

- *Drosophila* neuronal stem cells (neuroblasts) are a common model for studying asymmetric cell division.

The use of *Drosophila* as a model system for cell polarity offers diverse tools to study mechanisms and effects of cell polarity *in vivo*. One important question for the understanding of tumor progression is, whether the loss of epithelial polarity observed

in cancer is an epiphenomenon or a cause of cancer (Partanen et al., 2013). Experiments in *Drosophila* suggest a causal role of polarity proteins in tumor progression (Bilder, 2004; Martin and St Johnston, 2003)).

2.3 Epithelial cell polarity in vertebrates and *Drosophila*

Sheets of epithelial cells line the cavities and surfaces throughout the body of multicellular organisms. They function as barriers between compartments and are essential for the transport of molecules between them. In order to function, epithelial cells have to be polarized into apical and basolateral membranes and form junctions with each other. A key feature of all epithelial cells is the polarized assembly of actin filaments at the cytosolic side of the apical and the basal cytoplasmic membrane. In both *Drosophila* and vertebrates, the apical identity of epithelia cells is maintained and regulated by two conserved polarity modules: the Crumbs module and the Par module. The Crumbs module is composed of Crumbs, PALS1 (Stardust in *Drosophila*) and Patj (PALS1 associated tight junction protein), the Par module is composed of Par3 (Bazooka in *Drosophila*), Par-6 and atypical protein kinase C (aPKC). On the basolateral sides of the cells the Scribble polarity module composed of Scribble (Scrib), Dlg (Discs large) and Lgl (Lethal (2) giant larvae) defines basolateral identity. The polarity modules show mutually antagonistic interactions, restricting the activity of each module and regulate positioning of the adherens junctions (Humbert et al., 2008).

While many basic mechanisms of cell polarity are conserved, the junctions along the lateral side of epithelial cells are organized differently in vertebrates and *Drosophila* (Knust and Bossinger, 2002).

In vertebrates the apical domain contains a brush border of microvilli with a network of actin and Spectrin filaments beneath on the cytosolic side. The primary cilium protrudes from the apical surface and has a unique membrane and protein composition. Tight junctions mark the border of the apical and lateral domains; they include homophilic adhesion molecules like Occludin, JAMs (Junctional Adhesion Molecules) and Claudins that form a paracellular diffusion barrier. Adherens junctions are found basally of the tight junctions and are the main connection between neighboring cells. Nectins

and Cadherins localize to the adherens junctions and mediate these homophilic connections (Nelson et al., 2013). Only the basal side of the cell is connected to the extracellular matrix and has integrins and dystroglycan as receptors (Johnston and Ahringer, 2010).

Epithelial cells of *Drosophila* lack primary cilia. The adherens junctions of most Drosophila epithelial cells are apical of the septate junctions and contain DE-Cadherin and Nectin (Baumann, 2001). Instead of tight junctions septate junctions function as the paracellular diffusion barrier in *Drosophila* and contain Sinuous (a claudin family member), Cor and Megatrachea (Wu et al., 2004). Unlike in vertebrates, the Spectrin cytoskeleton of *Drosophila* epithelia cells is polarized, with $\alpha_2\beta_{H2}$-Spectrin at the apical and $\alpha_2\beta_2$-Spectrin at the basolateral membrane (Thomas and Kiehart, 1994). The mammalian ortholog of β_H–Spectrin shows no distinct apical distribution (Stabach and Morrow, 2000).

2.4 *Drosophila* neuronal stem cells (neuroblasts) as a model for asymmetric cell division and stem cell induced tumors

During stage 9 of embryogenesis, neural stem cells called neuroblasts (NBs) delaminate from the neuroectodermal epithelium into the interior of the embryo. NBs divide asymmetrically, giving rise to another NB (stem cell renewal) and a ganglion mother cell (GMC), that divides once more to form a pair of neurons or glia cells.

The asymmetric division of a NB requires apical-basal polarity, which is partly inherited from the epithelium (Wodarz and Huttner, 2003). During metaphase the Par module, as well as the Insc/Pins/Gαi complex, is localized to the apical cortex, while cell fate determinants like Prospero, Brat and Numb and their adaptor proteins Miranda and Partner of Numb are localized to the basal cortex. The mitotic spindle is first oriented in parallel to the surface of the epithelium but rotates by 90° during metaphase. As the NB divides, the apically localized daughter cell inherits the apically localized proteins and retains stem cell fate. The smaller basal daughter cell inherits the proteins of the basal cortex and becomes a GMC (Wodarz, 2005).

The apical-basal polarity of the NB cortex is adjusted with the spindle orientation during metaphase and essential for asymmetric division (Lee et al., 2006). Defects in cell polarity of NBs can lead to symmetric divisions, resulting in formation of ectopic NB-like stem cells, which proliferate and finally lead to tumor formation (Wodarz and Näthke, 2007).

2.5 The PAR proteins

The PAR proteins were discovered as regulators of cytoplasmic partitioning in the early embryo of *C. elegans*. PAR stands for ”abnormal embryonic PARtitioning of cytoplasm”, describing the phenotype of *par* mutants, which is a mislocalization of germ line-specific P granules (Kemphues et al., 1988). Following the nomenclature for genes and proteins in *C. elegans*, these six proteins required for early polarity and asymmetric cell divisions were named PAR-1 to PAR-6. During one cell stage PAR-3 and PAR-6 become enriched in the anterior cortex, while PAR-1 and PAR-2 become enriched in the posterior cortex. PAR-4 and PAR-5 are symmetrically localized cortical and cytoplasmic (Goldstein and Macara, 2007).

The biochemical roles of the PAR proteins differ remarkably. PAR-1 and PAR-4 are serine/threonine kinases, PAR-3 and PAR-6 contain PDZ domains (named after the three proteins **P**sd95, **D**iscs large and **Z**O-1, that contain this domain) and PAR-2 is a protein with a “ring finger” zinc binding domain (Kemphues, 2000). With the exception of PAR-2, which seems to be a nematode-specific protein, homologs of the PAR protein have been identified in all bilateral animals investigated (examples in Table 2-1) and have been found to regulate cell polarization in multiple contexts in various animals (Goldstein and Macara, 2007).

Table 2-1: Homologs of PAR proteins

Caenorhabditis elegans	*Drosophila melanogaster*	*Homo sapiens*
PAR-1	Par-1	MARK1
PAR-2	-	-
PAR-3	Bazooka	PAR3
PAR-4	LKB1	STK11/LKB1
PAR-5	14-3-3ε and 14-3-3ζ	14-3-3 protein family
PAR-6	Par-6	PAR6

2.6 LKB1 (STK11, PAR-4) is a multifunctional protein kinase with tumor suppressor activity

Germline mutations of human *LKB1* (also known as *STK11*) are the main cause of Peutz-Jeghers-Syndrome (**PJS**) (Hemminki *et al.*, 1998), a rare autosomal cancer disease. PJS is characterized by intestinal hamartomatous polyposis and mucocutaneous melanin pigmentation (Jeghers, 1949). Patients with PJS have a very high risk of developing cancer (Giardello et al., 2000). Somatic mutations of *lkb1* are associated with a wide variety of mainly epithelial cancers (Sanchez-Cespedes, 2007), especially lung adenocarcinomas (Conde et al., 2007). Some cancer cell lines show severly reduced mRNA levels of LKB1, probably due to hypermethylation of the promoter region of *lkb1*. In HeLa S3 cells (a cervical cancer cell line) and in G361 cells (a melanoma cell line) reintroduction of functional LKB1 suppresses proliferation by growth arrest in G1 phase (Tiainen et al., 1999). On the other hand many malignant tumors appear to have elevated *LKB1* expression levels (Rowan et al., 2000).

LKB1 is a ubiquitously expressed **s**erine/**t**hreonine **k**inase (also called STK11), which is known to phosphorylate the AMPK-family of 13 kinases and therefore termed a "master kinase" (Lizcano et al., 2004). In humans there are two LKB1 isoforms, resulting from alternative splicing. The long LKB1 form (50 kDa) is ubiquitously expressed in adult tissues, with higher expression in epithelia (Rowan et al., 2000). In the shorter form (48 kDa) the C-terminus is replaced by a shorter and unique amino acid sequence (Towler et al., 2008). Both isoform are expressed in human tissues, but the shorter form is especially prevalent in testis, where it has been identified to be crucial for

spermiogeneis (Towler et al., 2008). The catalytic domain of LKB1 is poorly related to other protein kinases, the N-terminal and C-terminal non-catalytic regions are not related to any other proteins and posess no idenfiable domains (Boudeau et al., 2003b).

Diverse effects of LKB1 have been observed on the cellular level, such as inducing cell cycle arrest (Tiainen et al., 1999), mediating apoptosis through p53 (Karuman et al., 2001 and Cheng et al., 2009), AMPK activation in energy metabolism (Shackelford and Shaw, 2009) and cell migration (Zhang et al., 2008). The observation that activation of mammalian LKB1 can polarize cells even in the absence of cell-cell contacts (Baas et al., 2004*)* has drawn attention to the role of LKB1 in cell polarity (2.6.6). While there are several downstream processes described, upstream regulation is poorly understood and might be mediated by localization rather than activation of kinase activity (Sebbagh *et al.*, 2011).

2.6.1 The heterotrimeric complex LKB1-STRAD-Mo25 is the active unit of LKB1

LKB1 activity is highly increased by binding to the pseudo kinase STRAD (STe-20 Related Adaptor) (Baas, 2003). This interaction is stabilized by Mo25 (mouse protein25) (Boudeau, 2003). Together they form the LKB1-STRAD-Mo25 heterotrimeric complex, which is generally considered as the biologically active unit of LKB1. In the presence of STRADα and Mo25, LKB1 relocalizes from the nucleus to the cytoplasm as observed in several overexpression studies on mammalian cell cultures (Baas et al., 2003; Boudeau et al., 2003; Dorfman and Macara, 2008; Dension et al., 2009). The structure of the LKB1-STRAD-Mo25 complex has been determined, showing that STRAD and Mo25 promote the active conformation of LKB1 (Zeqiraj et al., 2009).

2.6.2 The LKB1-STRAD-Mo25 complex is probably conserved in lower model organisms

Lower organisms also express STRAD and Mo25 homologs, which contain the critical residues for the interaction with LKB1, like the STRAD C-terminal WEF motif (Zeqiraj

et al., 2009). In *C. elegans* the STRAD homolog STRD-1 is required for the phosphorylation of AMPK by the LKB1 homolog PAR-4 under reduced insulin signaling conditions to regulate cell growth and proliferation, but the phosphorylation of key proteins like PAR-1 by PAR-4, which is needed for establishment of early embryonic polarity, is independent of STRD-1 (Narbonne et al., 2010). Mo25 has been shown to interact with LKB1 in *Drosophila* neuroblasts and both could be co-immunoprecipitated reciprocally from embryonic lysates (Yamamoto et al., 2008). The *Drosophila* STRAD homolog Ste20-like kinase has not yet been investigated, but lacks key residues for kinase activity, indicating that it is also a pseudokinase (Anamika et al., 2009). Although the LKB1-STRAD-Mo25 complex is not established for these organisms yet, these findings suggest that it is probably conserved.

2.6.3 Posttranslational modification and regulation of LKB1 activity

The observation that LKB1 can phosphorylate and activate almost all known members of the AMPK kinase family (Lizcano et al., 2004) suggests that it has to be tightly regulated to facilitate specific responses.

Several phosphorylation sites have been found on mammalian LKB1. Four threonines (T) were identified as autophosphorylation sites, which are known to be positively correlated with activation of its catalytic activity (Baas et al., 2003). ATM mediates phosphorylation of LKB1 at T366 (Sapkota et al,, 2002b), which has no effect on the catalytic activity of LKB1, but seems to be required for inactivation of CRTC2, a coactivator of CREB, in B cell proliferation (Sherman et al., 2010). Phosphorylation of S428 by p90(RSK) and PKA (cAMP-dependent protein kinase) does not affect LKB1 kinase activity but has been described to be essential for LKB1 to suppress cell growth (Sapkota et al., 2001). Other phosphorylation sites have been described, but the respective kinases and their function are not known. Their substitution with alanine, however, had no observable effect on LKB1 kinase activity (Sapkota et al., 2002a).

Until now little knowledge is established about the regulation of the kinase activity of LKB1. Remarkably, there is no LKB1 complex activating factor known (Sebbagh et al., 2011), which lead to the hypothesis that the LKB1 complex could be constitutively

active and that specific responses are regulated by its intracellular localization (Fogarty and Hardie, 2009; Sebbagh et al., 2009). Two chaperone complexes have been suggested to control the cellular level of LKB1 protein: The chaperone heat shock protein 90 (Hsp90) and its co-chaperone Cdc37 inactivate LKB1, while Hsp/Hsc70 and CHIP trigger LKB1 degradation, which finally controls the cellular level of LKB1 protein (Gaude et al., 2012).

2.6.4 LKB1 in Drosophila

The *lkb1* gene (also known as *dlkb1)* of *Drosophila* was first identified in a genetic screen for mutants that disrupt the localization of Staufen in germline clones (Martin & St Johnston 2003). Due to its stronger homology to the human tumor-suppressor LKB1 than to *C. elegans* PAR-4 it was named *lkb1*. In the same study, *lkb1* is described to be important for the establishment of cell polarity in the follicle cell epithelium (Martin & St Johnston 2003). The oocyte phenotype resembled that of hypomorphic mutants in *Drosophila par-1* and a strong genetic interaction between these genes has been observed. A *par-1* hypomorphic mutation could be rescued by GFP-LKB1 overexpression, but not by a kinase dead version of GFP-LKB1, while *lkb1* mutant germline clones could not be rescued by PAR-1 overexpression. This and *in vitro* experiments indicated PAR-1 is not a substrate for LKB1. Instead, the amino-terminal half of LKB1 was identified to be phosphorylated by PAR-1, which led to the idea that LKB1 acts downstream of PAR-1 (Martin & St Johnston 2003). But Wang et al. could identify that LKB1 phosphorylates PAR-1 at the threonine residue at position 408 (T408) *in vitro* and *in vivo* (Wang et al., 2007). Through biochemical experiments as well as genetic studies of *Drosophila* retinal phenotypes they have shown that LKB1 acts upstream of PAR-1 and tau in a cytotoxic pathway in a model for neurodegenerative diseases (Wang et al., 2007).

LKB1 has been reported to phosphorylate AMPK (AMP-activated protein kinase) to control cell polarity and mitosis under energetic stress in the embryo (Lee et al., 2007) In the retina, LKB1 has been observed to act on an array of targets to regulate polarity remodeling probably independent of AMPK, possibly through PAR-1 and several

AMPK-like kinases (Amin *et al.*, 2009). It has been reported that *Drosophila* LKB1 negatively regulates organ size by inducing caspase-dependent apoptosis without affecting cell size or cell cycle progression (Lee et al., 2006). Silnoon, a monocarboxylate transporter, was identified as a modifier enhancing LKB1- dependent apoptosis, and is reported to be transported to the apical side of polarized cells depending on the kinase activity of LKB1 (Jang et al., 2008). In larval neuroblasts *lkb1* mutations are reported to lead to defects in apical polarity (Bazooka, Par-6 and aPKC localization are not properly localized), suppress asymmetric cell division and disrupt spindle formation resulting in polyploid cells in larval brains (Bonaccorsi et al., 2007). Overexpression of a GFP-tagged LKB1 fusion protein has been observed to reduce the size of embryos and localizes to the cortex of ectodermal embryonic epithelial cells and NBs. In NBs, the overexpression of LKB1 causes a broader distribution of Miranda along the cortex. This effect has been observed to be independent of the kinase activity of LKB1 (Yamamoto et al., 2008). A gain-of-function screen identified that overexpression of LKB1 reduced organ size and extended lifespan (Funakoshi et al., 2011).

2.6.5 AMPK as a downstream target of LKB1

LKB1 has been shown to be the dominant regulator of AMPK (AMP-activated protein kinase) activation in several mammalian cell types by phosphorylating a critical phosphorylation site in its T-loop (T172) (Shaw et al., 2004). Low energy conditions increase the level of AMP and ADP, which interact with the CBS motifs of the AMPKγ subunit of AMPK (Hardie, 2011). This causes conformational changes that activate the AMPKα subunit and promote phosphorylation of the phosphorylation site T172 by inhibiting its dephosphorylation (Sanders et al., 2007). The activation of the AMPKα1 and AMPKα2 catalytic subunits functions to restore energy levels by phosphorylating an immense number of proteins, which are involved in multiple processes like metabolism, cell growth and proliferation (Carling et al., 2011). The net result of AMPK activation is a shift of the balance from anabolic to catabolic function and thereby restoring the cellular ATP levels of the cell.

The tumor suppressor activity of LKB1 is thought to be at least partly mediated by its activation of AMPK, which in turn inhibits mTOR (mammalian target of rapamycin), which is a known regulator of diverse cellular processes including cell growth and proliferation (Shackelford and Shaw., 2009). In ciliated MDCK cells, LKB1 has been found to colocalize with AMPK at the basal body, where it regulates the phosphorylation of AMPK in response to urine flow, which regulates cell size through mTOR (Boehlke et al., 2010).

In spite of its role as a tumor suppressor, LKB1 protects cells from apoptosis in response to elevated AMPK levels (Shaw et al., 2004). The LKB1-AMPK pathway can have a positive role in tumorigenesis by acting to maintain metabolic homeostasis and attenuate oxidative stress and thus enabling the survival of tumor cells by maintaining NADPH levels (Jeon et al,. 2012). This might also explain why many malignant tumors display elevated levels of LKB1 (Rowan et al., 2000).

ampk-null mutant *Drosophila* embryos are reported to be lethal with abnormal cell polarity and mitosis, similar to those in *lkb1*-null mutants and a phosphomimetic mutant of myosin II regulatory light chain (MRLC) rescued the phenotype of *ampk*-null mutants to some extent (Lee et al., 2007). This indicates that the actin-myosin cytoskeleton might be regulated by AMPK under energetic stress conditions. Although Lee et al. could show that AMPK phosphorylates MRCL in *Drosophila* and in the human epithelial cell line LS174T, in mammalian pancreatic cells the inhibition of LKB1 did not affect the phosphorylation status of MRLC (Hezel et al., 2008) and inhibition of AMPK in vascular smooth muscle cells even increased MRLC phosphorylation (Horman et al., 2008). This indicates that MRLC may not be directly phosphorylated by AMPK but that it may mediate the polarity signal from AMPK to the actin cytoskeleton (Mirouse and Billaud 2011). Many aspects regarding an involvement of AMPK in cell polarity like its direct downstream targets associated with this function remain unclear. The cytoplasmic linker protein of 170 Da (CLIP-170) has been proposed to be a target of AMPK involved in cell polarity by affecting the stability of microtubules (Nakano et al., 2010).

2.6.6 LKB1 in the regulation of cell polarity

Activation of LKB1 by overexpression of its adaptor protein STRAD was reported to induce polarization even in the absence of cell-cell contacts in human intestinal epithelial cells, as seen by the formation of an actin-rich brush border on one side of the cell, indicating an apical-like surface (Baas et al., 2004). This suggests that LKB1 is a major regulator of cell polarity. Mst4 and the actin filament binding protein ezrin were later identified as downstream targets of LKB1 in the induction of brush border formation, but had no effect on other polarity events downstream of LKB1, like the formation of lateral junctions (Klooster et al., 2009). The role of LKB1 in actin filament assembly has been further investigated in HeLa cells, which lack endogenous LKB1. In this process LKB1 expression leads to an activation of Rho, mediated by the guanine nucleotide exchange factor Dbl, although the exact mechanism remains unknown. Interestingly, the kinase activity of LKB1 is not required for the induction of stress fibers, but the kinase domain is (Xu et al., 2010). It has also been reported that LKB1 regulates the expression of E-Cadherin and thereby intercellular junction stability through Salt-inducible kinase 1 (Eneling et al., 2012). On the other hand LKB1/STRAD localization has been reported to be localized to the adherens junctions of polarized epithelial cells (MDCK cells) under control of E-Cadherin (Sebbagh et al., 2009).

2.6.7 Localization of LKB1

A nuclear localization signal (NLS) has been identified in the N-terminus of mammalian LKB1 (Nezu et al., 1999; Smith et al., 1999; Tiainen et al., 2002). Localization of LKB1 has mostly been studied in mammalian overexpression systems in cell culture (Table 2-2). In most of these studies, LKB1 has been observed predominantly in the nucleus and to a lower degree in the cytosol (Nezu et al., 1999; Smith et al., 1999; Tiainen et al., 1999; Baas et al., 2003; Boudeau et al., 2003; Denison et al., 2009; Dorfman and Macara et al., 2008; Xie et al., 2008). STRADα has been reported to regulate export of LKB1 through inhibition of the nuclear import of LKB1 and by serving as an adaptor between LKB1 and exportins CRM1 and exportin 7 (Dorfman and Macara, 2008). Some studies have also found localization of LKB1 to the cytocortex (Collins et al., 2000; Sapkota et al, 2001; Xu et al., 2010).

The localization of LKB1 in cells expressing endogenous levels of LKB1 has not been investigated much, probably due to low expression levels and lack of suitable antibodies (Sebbagh et al., 2009). Surprisingly, in MDCK cells, endogenous LKB1 is reported to localize mainly to the cytosol and membrane and not to the nucleus (Sebbagh et al., 2009). A fractionation study of HEK293 cells, which express moderate amounts of endogenous LKB1, was only able to detect LKB1 in the cytosolic and membrane fractions, but not in the nuclear fraction (Denison et al., 2009). Fractionation of polarized Caco-2 cells revealed an absence of LKB1 in the nuclear fraction, but showed abundance in the cytosolic and membrane fractions (Sebbagh et al., 2009). It can be assumed, that the examined cell lines express enough STRAD to export the endogenous amounts of LKB1 from the nucleus.

Table 2-2: Localization of LKB1 in mammalian cell culture lines

Cell line (type)	LKB1 localization	Method	Expresses endogenous LKB1	Reference
A549 (human alveolar basal epithelial cells)	nuclear	immunocytochemical detection of tagged fusion protein	no	Xie et al., 2008
CCL13 (human hepatocytes)	nuclear +cytosolic (activation by STRAD+Mo25 → cytosolic)	immunocytochemical detection of tagged fusion protein	no	Dension et al.,, 2009
COS-7	nuclear cytosolic	immunocytochemical detection of tagged fusion protein		Smith et al., 1999
CV-1 (kidney fibroblast cells from normal african green monkey)	plasma membrane and internal membranes (farnesylation deficient mutant does not localize to membranes)	fluorescence tagged protein	yes	Collins et al., 2000
G361 (human melanoma)	nuclear cytosolic	overexpression, immunocytochemical anti-LKB1 antibody	impaired endogenous level	Tiainen et al., 1999

Cell line (type)	LKB1 localization	Method	Expresses endogenous LKB1	Reference
HeLa (cervical carcinoma)	nuclear cytosolic (coexpression of STRAD → cytosolic)	fluorescence tagged protein	no	Boudeau et al., 2003; Dorfman and Macara, 2008
HeLa-S3 (cervical carcinoma)	nucleus, fraction at cell membrane	immunocytochemical detection of tagged fusion protein	no	Xu et al., 2010
HUVEC (Human Umbilical Vein Endothelial Cells)	nuclear (treatment with metformin → cytosolic)	immunocytochemical detection of tagged fusion protein	yes	Xie et al., 2008
LS174T (human intestinal epithelial cancer)	predominantly nuclear (coexpression of STRAD → cytosolic)	immunocytochemical detection of tagged fusion protein	Probably not (author statement)	Baas et al., 2003
Rat-2 (fibroblast-like)	mainly cytosolic, small but significant amount at membrane	fractionation	yes	Sapkota et al., 2000

2.6.8 The C-terminus of LKB1 is involved in membrane targeting of LKB1

Numerous mutations that affect only the C-terminus have been identified in tumors, making it an interesting area of investigation (Boudeau et al., 2003b). The C-terminal CAAX-sequence, which is only present in the long isoform of mammalian LKB1 and conserved in most model organisms including *Drosophila* (Figure 2-1), is a farnesylation motif (Collins et al., 2000; Sapkota et al., 2001; Martin and St Johnston, 2003). In CV-1 cells and *Drosophila* oocytes a farnesylation-deficient mutant of LKB1

strongly impairs the cortical localization of LKB1 (Collins et al., 2000; Martin and St Johnston, 2003). The overexpression of a farnesylation-deficient LKB1 mutant in mammalian cell culture, however, had no effect on the ability to suppress cell growth (Sapkota et al., 2001). With a farnesylation-specific antibody the majority of LKB1 was

identified to be farnesylated in wild-type mouse tissues and cultured cells (Houde et al., 2014). A farnesylation deficient version of LKB1 (LKB1^{C433S}) was investigated using mouse knockin analysis (Houde et al., 2014).

Another motif at the C-terminus that is conserved in model organisms including *Drosophila* and *C. elegans* is the PKA/p90RSK phosphorylation motif "RKLS" (Figure 2-1). In human endothelial cells it has been reported, that PKC-ζ phosphorylates LKB1 at S428 and that this would lead to a nuclear export of LKB1 and hence AMPK activation (Xie et al., 2008). Others describe that this phosphorylation site is essential for mammalian LKB1 to suppress cell growth (Sapkota et al., 2001), but is not required for regulation of AMPK or cell cycle arrest (Fogarty and Hardie, 2009). In agreement with the latter mentioned findings, AMPK is activated normally in knockin mice carrying the phosphodeficient mutant LKB1^{S431A} (Houde et al., 2014). Co-expression and mobility shift assays of *Drosophila* LKB1 and PKA in S2 cells revealed a conservation of this phosphorylation site (Martin and St Johnston, 2003). Moreover, a GFP-LKB1 with a phosphodeficient version of this site, when expressed in low amounts, does not rescue localization of Staufen to the posterior oocyte of *Drosphila*, while a phosphomimetic version rescues even more efficiently than the wild type control, indicating a positive regulation of LKB1 by phosphorylation of this site (Martin and St Johnston, 2003).

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STK11[Homo sapiens]                 1…409-R------APNPARKACSASSKIRRLSACKQQ------ 433
LKB1[Mus musculus]                  1…411-RPG----TANPARKVCS-SNKIRRLSACKQQ------ 436
STK11[Danio rerio]                  1…415-SS-----SSNPSRKGLSAASKIRKLSTCKQQ------ 440
XEEK1[Xenopus laevis]               1…411-SS---------QRKASTTGSKVRKLSACKQQ------ 432
LKB1[Drosophila melanogaster]       1…537-PVKKKGSALKRRAKKLTSCISVRKLSHCRTS------ 567
PAR-4[Caenorhabditis elegans]       1…581-GVASASDPPPTAAPGAPPRRRKRNFFSCIFRSRTDSA 617
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Figure 2-1: Alignment of the C-termini of LKB1 homologs. Blue color indicates the PKA phosphorylation motif and green color the farnesylation motif conserved between human LKB1 and homologs in model organisms.

2.6.9 Potential LKB1 interaction partners

Apart from an activation of of the AMPK family of kinases (Licano et al., 2004), several potential LKB1 effectors have been identified by mass spectrometry and yeast two hybrid assays (Sebbagh et al., 2011). Interactions with the chaperone complexes Hsp90/Cdc37 and Hsp/Hsc79-CHIP have been reported to regulate activity and stability of LKB1 (Gaude et al., 2012). LKB1 has also been reported to interact with LIP1 (LKB1 interacting protein 1), but the functional consequences of this interaction remain unknown (Smith et al., 2001). Similar to this, the interaction of LKB1 with PTEN was observed but no distinct functional consequences were found (Mehenni et al., 2005). Furthermore, an interaction of LKB1 with the transcription factors Estrogen receptor-α and Brahma related gene-1 has been described, but phosphorylation of these partners by LKB1 could not be observed, indicating a potential indirect effect of LKB1 (Marignani et al., 2001; Nath-Sain and Marignani, 2009).

In order to find new potential interaction partners of LKB1, they were co-immunoprecipitated by expressing GFP-LKB1 in *Drosophila* embryos and identified via mass spectrometry (Krahn, so far unpublished). The co-immunoprecipitation of the LKB1 substrate and the homologs of the components of the LKB1 complex (Stlk and Mo25), indicate that this approach is promising for the identification of interaction partners. Among the potential interaction partners are α-Spectrin and β-Spectrin, which are components of the membrane skeleton. In this work the potential interaction of these proteins with LKB1 were analyzed.

Table 2-3: Proteins identified to bind to GFP-LKB1

Protein	# of peptides	Cellular function
α-Spectrin	**5**	**Cytoskeleton linker protein**
β-Spectrin	**2**	**Cytoskeleton linker protein**
AMP-activated kinase	**7**	**Regulation of cellular energy homeostasis**
Ste20-like kinase (Stlk)	**24**	**Co-factor for LKB1 (STRADa homolog)**
Mo25	**26**	**Co-factor for LKB1**

2.7 The membrane skeleton

Actin has critical roles in establishing and maintaining cell morphology, cell motility, cell division and intracellular transport (Pollard and Cooper, 2009). Most actin filaments in animal cells are nucleated at the plasma membrane, where they form a layer at the cytoplasmic side called the cell cortex or membrane skeleton, which is important for the shape and movement of the cell surface.

2.7.1 Spectrin

Spectrin has been studied mostly in human erythrocytes, were it forms a protein network by binding to actin and peripheral membrane proteins (Bennett, 1985; Byres and Brandon, 1985), which builds stiff structures that are important for the flexibility of this cell type. But it is found in all metazoan species and in almost all cells examined (Baines, 2009). In erythrocytes it is forming the Spectrin-based membrane cytoskeleton by crosslinking transmembrane proteins, signaling proteins, membrane lipids and the actin cytoskeleton, while the cortical composition of other cell types appears to be more complex (reviewed in Bennett and Baines, 2001). In the *Drosophila* neuromuscular junction an erythrocyte-like polygonal lattice structure has been reported (Pielage et al., 2006), which has also been proposed for other cell types (Baines, 2010).

Three types of Spectrins exist in invertebrates: α-Spectrin, β-Spectrin and β_H-Spectrin. α-Spectrin is forming heterotetrameric complexes with β-Spectrin and β_H-Spectrin ($\alpha_2\beta_2$ and $\alpha_2\beta_{H2}$) (Dubreuil et al., 1990). The Spectrin cytoskeleton of *Drosophila* epithelial cells is polarized, with $\alpha_2\beta_{H2}$ found in the apical cortex of polarized cells and $\alpha_2\beta_2$ in the basolateral cortex (Thomas and Kiehart, 1994). In a Spectrin dimer, α- and β-chains lie side-by-side and antiparallel. The Spectrin proteins are linking actin fibers and other proteins by numerous interaction motifs. Both α-Spectrin and β-Spectrin are mostly made of triple-helical repeats. α-Spectrin contains a middle SH3 domain and a C-terminal EF-hand binding motif in addition to multiple Spectrin repeats. SH3 domains are protein interaction domains that bind to proline-rich ligands, that play several different roles in the cell including the regulation of enzymes, changing the subcellular

localization of signaling pathway components, and mediating the formation of multiprotein complex formations (Macheler-Bauer et al., 2011).The β-Spectrin N-terminal region contains a pair of CH domains that binds actin, adducin and Phosphatidylinositol-4,5-bisphosphate (PIP_2). Triple helical repeats 14–15 contain an ankyrin binding site. Other triple helices in both chains bind a variety of different ligands, including the region between repeats 9 and 11 (including the SH3 domain) which can bind both proteins and phospholipids (Baines, 2009).

In human epithelial cells loss of β-Spectrin or ankyrin has been shown to lead to loss of the lateral membrane (Kizhatil, 2007). Apical $\alpha_2\beta_{H2}$ is needed in the *Drosophila* follicle cell epithelium for epithelial morphogenesis but not for apicobasal polarity (Zarnescu and Thomas, 1999). Genetic experiments in *C. elegans* suggest a requirement for Spectrins in muscle and nerves (Hammarlund et al., 2000; Moorthy et al., 2000). Analysis in *Drosophila Spectrin* mutants indicate the need of Spectrin and ankyrin in the consolidation and maintenance of synaptic structures (Pielage et al., 2005, 2006 and 2008) and distinct roles of α-Spectrin and β-Spectrin in axonal pathfinding (Hülsmeier et al., 2007).

3 Material and Methods

3.1 Material

Chemicals (Table 3-1) and kits (Table 3-3) were bought from following companies: Agilent Technologies (Böblingen, Germany), BioVision (San Francisco, USA), Chromotek (Planegg, Germany), Invitrogen (Groningen, Netherlands), Machery-Nagel (Düren, Germany), Merck Chemicals Ltd. (Nottingham, UK), PAN biotech (Aidenbach, Germany), Promega (Mannheim, Germany), Roche Diagnostics Deutschland GmbH and Roche Applied Science (Mannheim, Germany), Roth (Karlsruhe, Germany), Sigma-Aldrich (Steinheim, Germany) and Thermo Fisher Scientific. Chemicals were generally of analytical grade.

Instruments and other material were purchased from Carl Zeiss (Jena, Germany), Echelon Biosciences (Salt Lake City, USA), Eppendorf (Hamburg, Germany), Intas (Göttingen, Germany), Machery-Nagel (Düren, Germany) and Thermo Fisher Scientific.

3.1.1 Reagents

Table 3-1: Reagents

Reagent	Utilization	Note	Company
Albumin fraction V	Blocking Solutions		Roth
Amlyose Resin	Protein Purification of MBP-tagged proteins		New England BioLabs
Aprotinin	Protease inhibiton	used concentration: 2 µg/ml	Roth
Bradford Rothi® Quant	Coomassie Brilliant Blue-G250	Protein concentration measurement	Roth

Reagent	Utilization	Note	Company
FuGENE®HD Transfection Reagent	Transfection of Cells	Blend of Lipids in 80% Ethanol	Promega
GFP-Trap	Co-IP	GFP-Trap® coupled to agarose beads particle size ~80 µm; stored in 20% EtOH	Chromotek
Leptomycin B	Inhibition of CRM1 (exportin 1, embargoed)	Used 1:100	Sigma-Aldrich
Leupetin	Protease inhibiton	used concentration : 2 µg/ml	Roth
Pepstatin A	Protease inhibiton	used concentration : 2 µg/ml	Roth
PfuS Polymerase	PCR	Homemade	-
Phosphatase Inhibitor Cocktail Set, IV	Phosphatase Inhibitors	Phosphatase Inhibitors	Merck
PMSF	Protease inhibiton		Roth
Protein A sepharose	Co-IP	Supplied as 50% slurry in 20% Ethanol/H_2O	BioVision
Protino Glutathione Agarose 4B	Protein Purification of GST-tagged proteins		Macherey-Nagel
T4 DNA Ligase	Ligation		Thermo Fisher Scientific

3.1.2 Solutions

Solutions were prepared with distilled water and sterilized by autoclaving or sterile filtration. Instruments and other material were purchased from Carl Zeiss (Jena, Germany), Echelon Biosciences (Salt Lake City, USA), Eppendorf (Hamburg, Germany), Intas (Göttingen, Germany), Machery-Nagel (Düren, Germany) and Thermo Fisher Scientific.

Table 3-2: Solutions

Name	Composition	Usage
Apple juice agar plates (2%)	1980 ml H_2O 60 g Agar 1020 ml Apple juice 51 g Sugar 10% Nipagin in ethanol	Egg collection, feeding
Blocking Buffer (for Western Blots)	500 ml TBST; 3% skim milk powder; 1% BSA	Western Blot
Blocking Buffer	TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl), 3% BSA	Lipid overlay assay
Buffer A	100 mM Tris-HCl, pH 7.5 100 mM EDTA 100 mM NaCl 1% SDS	Isolation of genomic DNA from adult flies
Buffer G (10x)	10 mM Tris-HCl (pH 7.5 at 37°C); 10 mM $MgCl_2$; 50 mM NaCl; 0.1 mg/ml BSA	Buffer System for restriction enzymes; Thermo Fisher Scientific

Name	Composition	Usage
Buffer O (10x)	50 mM Tris-HCl (pH 7.5 at 37°C); 10 mM $MgCl_2$;100 mM NaCl; 0.1 mg/ml BSA	Buffer System for restriction enzymes; Thermo Fisher Scientific
Buffer R (10x)	10 mM Tris-HCl (pH 8.5 at 37°C); 10 mM $MgCl_2$; 100 mM KCl; 0.1 mg/ml BSA	Buffer System for restriction enzymes; Thermo Fisher Scientific
Buffer Tango (10x)	33 mM Tris-acetate (pH 7.9 at 37°C); 10 mM Mg-acetate; 66 mM K-acetate; 0.1 mg/ml BSA	Buffer System for restriction enzymes; Thermo Fisher Scientific
DAPI	5 µg/ml	DNA staining
Embryo-Glue	adhesive tape; heptane	Mounting of embryos for injection
Fixation Solution	PBS (1x); 4% Formaldehyde	Fixation
Freezing Medium	45% conditioned medium; 45% fresh medium; 10% DMSO	Freezing Cells
LB Medium	10 g Bacto-Tryptone; 5 g Bacto-Yeast Extract; 10 g NaCl	*E.coli* liquid culture medium
LB_{Amp} Medium	10 g Bacto-Tryptone; 5 g Bacto-Yeast Extract; 10 g sodium chloride; 500 µg/ml Ampicillin	Selective liquid culture medium for *E.coli*

Name	Composition	Usage
LB_0 Plates	10% tryptone; 5% Yeast extract; 5% sodium chloride; 15% Agar Agar; pH 7.0	
LB_{amp} Plates	LB_0 Plates + 100 mg/l ampicillin	selective ampicillin plates
LB_{kana} Plates	LB_0 Plates + 50 mg/l kanamycin	selective kanamycin plates
LB_{chl} Plates	LB_0 Plates + 30 mg/l chloramphenicol	selective chloramphenicol plates
LiCl/KAc Solution	4,29 M lithium chloride 1,43 M potassium acetate	Isolation of genomic DNA from adult flies
Loading Buffer (6x)	2.5% Ficoll 400; 11 mM EDTA; 3.3 mM Tris-HCl; 0.017% SDS; 0.015% Bromophenolblue (pH 8.0)	Agarose Electrophoresis
Lysis Buffer	TNT Buffer; Leupeptin; Pepstatin; Pefabloc/PMSF; Aprotinin (1:500 each)	Co Immunoprecipitation
Methylene Blue solution	Methylene blue powder ddH_2O	Agarose gel staining
Mowiol	0.4 g/ml Mowiol; 1 g/ml Glycerol; 2% 0.2 M Tris-HCl	Embedding Medium

Name	Composition	Usage
NP-40-lysis-buffer	50 mM Tris, pH7.5 140 mM NaCl 1% NP40/Igepal 1 mM $CaCl_2$ 1 mM $MgCl_2$	Lysis of embryos
P1-Buffer	50 mM Tris-HCl (pH 8.0); 10 mM EDTA; 100 µg/ml RNase A Storage 4°C	Mini-Preparation
P2-Buffer	200 mM NaOH; 1% SDS	Mini-Preparation
P3-Buffer	3.0 M potassium acetate, pH 5.5	Mini-Preparation
PBS (10x)	58.44 g/mol NaCl; 74,55 g/mol KCl; 141.96 g/mol Na_2HPO_4; 136 g/mol KH_2PO_4	Washing Buffer
PBT	PBS (1x); 0.1% Tween20	Immunohistology
Schneider's *Drosophila* Medium	With L-Glutamine; 0.40 g/l $NaHCO_3$	Transfection Medium, PAN biotech
Schneider's *Drosophila* Medium (Complete)	With L-Glutamine; 0.40 g/l $NaHCO_3$; 5% Penicillin/Streptomycin; 10% FCS	Liquid culture medium for *Drosophila* Schneider Cells, PAN biotech
2X SDS Loading Buffer (Laemmli Buffer)	126 mM Tris (pH 6,8); 4% SDS; 0.2% bromophenol blue; 20% glycerol; 200 mM DTT	SDS-PAGE

Name	Composition	Usage
6X SDS Loading Buffer	375 mM Tris (pH 6,8) 9% SDS 0.03% bromophenol blue 50% glycerol 600 mM DTT	SDS-PAGE
SDS Running Buffer (10x)	1.92 M Glycine 250 mM Tris 1% SDS	SDS-PAGE
TE	10 mM Tris-HCl, pH 8.0 1 mM EDTA	
TAE (1x)	40 mM Tris-Base 1 mM EDTA 0.1 % acetic acid	Agarose Electrophoresis
TBS (1x)	50 mM Tris-HCl, pH 7.4 150 mM NaCl	Western Blot
TBST 0,1%	1 mM Tris-HCl; pH 8.0; 150 mM NaCl; 0.1% Tween 20	Western Blot
TNT Lysis Buffer	150 mM NaCl; 50 mM Tris pH 7.5; 1% Triton X-100	Co Immunoprecipitation
Transfer Buffer (10x)	250 mM TRIS-base 1.9 mM Glycine	Western Blot

3.1.3 Commercial kits

Table 3-3: Commercial Kits

Kit	Utilization	Company
BM Chemiluminescence Western Blotting Substrate (POD)	Western Blot Analysis	Roche
In situ Cell Death Detection Kit, TMR red	TUNEL-assay for apoptosis detection	Roche
pEntr/D-Topo Cloning Kit	Gateway Cloning	Invitrogen
Nucleo Bond® PC 100	Midi-Preparation	Macherey-Nagel
Nucleo Spin®Gel and PCR Clean-Up	PCR Purification	Macherey-Nagel
QuikChange	Site-directed mutagenesis	Agilent Technologies
Super Signal West Pico Chemiluminescence Substrate	Western Blot Analysis	Thermo Scientific

3.1.4 Instruments and other material

Table 3-4: Instruments and other material

Name	Utilization	Company
Mikro-Dialysierkapsel QuixSep (max. Volume 1 mL)	Dialysis	Roth
Slide-A-Lyzer Dialysis Cassettes	Dialysis	Thermo Scientific
PIP Strips (P-6001)	Lipid binding assay	Echelon Biosciences
Digital-Microscope VHX-	Imaging of *Drosophila*	Keyence

Name	Utilization	Company
500FC	eyes	
NanoDrop 1000 Spectrophotometer	Measuring DNA concentration	Thermo Scientific
LSM 510 Meta	Microscopy	Zeiss
LSM 710 Meta	Microscopy	Zeiss
UV Transilluminator		Intas
Femptotips®II	Injection for germline transformation	Eppendorf

3.1.5 Antibodies

Table 3-5: Primary antibodies

Target	Species	Utilization	Designation	Origin/References
aPKC (aPKCξ)	Rabbit	IF (1:500)	sc-216	*Santa Cruz*
Actin	Rabbit	IF (1:100)	A2066	Sigma
Phospho-AMPKα (Thr172)	Rabbit	WB(1:200)	40H9	Cell Signaling Technology
α-Spectrin	Mouse	IF (1:20) WB (1:10)	3A9	*Developmental Studies Hybridoma Bank* (DSHB)
Baz Nterm	Rabbit	IF (1:1000)	DE99646-2	Wodarz *et al.*, 1999
β-Spectrin	Rabbit	IF (1:1000)		Christian Klämbt
c-Myc	Mouse	IF (1:100) IF (1:50)	9E 10	DSHB
Crb	Mouse	IF (1:10)		Tepass
DE-Cadherin	Rat	IF (1:10)	DCAD 2	DSHB

Target	Species	Utilization	Designation	Origin/References
Dlg	Mouse	IF (1:25)	4F3	(DSHB
elav	Mouse	IF (1:20)	9F8A9	DSHB
GFP	Rabbit	IF (1:500)	A11120	*Molecular probes (Invitrogen)*
HA	Rat	IF (1:1000) (1:100)		DSHB
MBP	Rat	WB (lipid overlay experiments) 1:5000	1.1 MBP 7G4	Sigma Aldrich
MBP	Mouse	WB (1:500)	sc73416	Santa Cruz
LKB1	Guinea Pig	IF/WB/Co-IP (1:500)	SAC 288 SAC 289	Homemade
LKB1 phospho Thr-312	Rabbit	IF (1:500)		Homemade
LKB1 phospho Thr-460	Rabbit	IF (1:500)		Homemade
Lgl	Guinea Pig	IF (1:500)		
Mir	Rat	IF (1:1000)		Homemade
PATJ	Guinea Pig	IF (1:500)		Homemade
α-tubulin	Mouse	WB (1:1000)	12G10	*DSHB*

Table 3-6 Secondary antibodies

Antibody	Species	Utilization (Dilution)	Origin/References
Alexa Fluor 488-anti Guinea Pig	Goat	IF (1:200)	Life Technologies
Alexa Fluor 488-anti Rat	Goat	IF (1:200)	Life Technologies
Alexa Fluor 488-anti Rabbit	Goat	IF (1:200)	Life Technologies
Alexa Fluor 488-anti Mouse	Goat	IF (1:200)	Life Technologies
Alexa Fluor 568-anti Guinea Pig	Goat	IF (1:200)	Life Technologies
Alex Fluor a 568-anti Rabbit	Donkey	IF (1:200)	Life Technologies
Alexa Fluor 568-anti Rat	Goat	IF (1:200)	Life Technologies
Alexa Fluor 568-anti Mouse	Donkey	IF (1:200)	Life Technologies
Alexa Fluor 647-anti Guinea Pig	Goat	IF (1:200)	Life Technologies
Alexa Fluor 647-anti Rabbit	Goat	IF (1:200)	Life Technologies
Alexa Fluor 647-anti Rat	Goat	IF (1:200)	Life Technologies
Alexa Fluor 647-anti Mouse	Goat	IF (1:200)	Life Technologies
HRP-anti Guinea Pig	Goat	WB (1:10000)	Roche
HRP-anti Rabbit	Goat	WB (1:10000)	Roche
HRP-anti Rat	Goat	WB (1:10000)	Roche
HRP-anti Mouse	Goat	WB (1:10000)	Roche

3.1.6 Oligonucleotides

Oligonucleotides were designed with DNADynamyo (BlueTractorSoftware, UK) and synthesized by Biotez (Berlin, Germany) or Metabion (Martinsried, Germany). Plasmids were obtained from Amersham Pharmacia Biotech (now GE Healthcare Life Sciences), Invitrogen, Murphy lab (Carnegie Institution for Science, Department of Embryology, Baltimore, USA) and New England Biolabs (Frankfurt am Main, Germany).

Table 3-7: Oligonucleotides

Name	Sequence 5' → 3'	Description
GST for	CAGCAAGTATATAGCATGGC	Sequencing of destination vectors
LKB1-F	CACC ATGCAATGTTCTAGCTCTCGG	
LKB1-N-R	CTACGAAGTTCGGCAGTGG	
LKB1-C-R	CGAAGTTCGGCAGTGGCT	
LKB1-EGFP-C1-F	AAAAGATCTATGCAATGTTCTAGCTCTCGG	
LKB1-EGFP-C1-R	AAAGAATTCG CTACGAAGTTCGGCAGTGG	
LKB1-302-R	TGGCTTGATATCCTTGTGGA	
LKB1Exon5-seq-F	GAACACGACGTAAATC	Sequence primer
LKB1 C564A-F	GTG CGC AAG CTT AGC CAC GCC CGA ACT TCG TAG	Mutagenesis of farnesyl acceptor cysteine

Name	Sequence 5' → 3'	Description
LKB1DeltaNLS-F	ATCATCTATCAGCAGGCCGCGGCGAGCATTAA GATGGTG	Mutagenesis of NLS???
LKB1Delta1-162-F	GCCCCCTTCACCATGATCTATCAGCAGAAA	N-teminal truncation
LKB1Delta1-162-R	TTTCTGCTGATAGATCATGGTGAAGGGGGC	N-teminal truncation
LKB1 K201M-F	AACCTGTGCCGGCTGGCCGTCATGATCCTGAC TAAG	Mutagenesis
LKB1genomic-F	CACC CACTAGCGTAATTTGACGG	2830before Start
LKB1genomic-R	CTC GAG CAGCAGTACGGTCATCTC	+ XhoI 3kbp
LKB1genomic2nd Half-F	CACC CATCTACATCATCCCACGG	
LKB1genomic2nd Half-R	CTC GAG GACATTTCCAGATTGCCCT	+ XhoI 2800bp
LKB1genomic+XbaI-F	GGCTCCGCGGAGGTTT TCT AGA CAATGTTCTAGCTCTC	deletes Start codon
LKB1GFP-F	AAA TCT AGA ATGGTGAGCAAGGGC	???
LKB1genomic+XbaI-F2	GGGGCTCCGCGGAGGTTTTCTAGATGTTCTAG CTCTCGGCCA	Deletes 2aa

Name	Sequence 5’ → 3’	Description
LKB1-552-C-F	CACCATGCTGACGTCCTGCATCTCCGTGCGCA AGCTGAGCCACTGCCGAACTTCGTAG	
LKB1-552-C-R	CTACGAAGTTCGGCAGTGGCTCAGCTTGCGCA CGGAGATGCAGGACGTCAG CAT GGTG	
LKB1K548A-F	TCGGCACTGAAGAGGGCCGCCAAGAAGCTGA CGTCC	
LKB1Delta537-551-F	GGTAGCAGAGAGGAGGCG CTGACGTCCTGCATCTCC	
LKB1R547AK548A-F	TCGGCACTGAAGGCGGCCGCCAAGAAGCTGA CGTCC	
LKB1K539AK540AK541A-F	GAGGAGGCGCCCGTCGCCGCGGCGGGATCG GCA CTG	
LKB1K550AK551A-F	GCA CTG AAG AGG CGC GCT GCG GCG CTG ACG TCC TGC	
LKB1R547AR548AK550AK551A-F	TCG GCA CTG AAG GCG GCC GCC GCG GCG CTG ACG TCC TGC	
LKB1 335-F	CACC ATGACGGGCCAAGGTTCT	
LKB1 512-F	CACC ATG CACACCTACGAACCGCC	
LKB1-536-C-F	CACC ATG GCGCCCGTCAAGAAG	

Name	Sequence 5’ → 3’	Description
LKB1R547AR548AK550AK551A-F	TCG GCA CTG AAG GCG GCC GCC GCG GCG CTG ACG TCC TGC	
LKB1K546AR547AR548AK550AK551A-F	TCG GCA CTG GCG GCG GCC GCC GCG GCG CTG ACG TCC TGC	
LKB1 K205K206K207A-F	GTCAAGATCCTGACTGCCGCGGCGTTGCGCCG GATT	
LKB1 R206A-F	GTCAAGATCCTGACCAAGGCGAAGTTGCGCC GGATT	
LKB1 K205-210A-F	CAAGATCCTGACTGCCGCGGCGTTGGCCGCGA TTCCCAACGGCG	
M13 for	GTAAAACGACGGCCAG	Sequencing of inserts in pENTR vector
M13 rev	CAGGAAACAGCTATGAC	Sequencing of inserts in pENTR vector
MBP for	GCGTGCTGAGCGCAGGTATTAACGCCGC	Sequencing of destination vectors

3.1.7 Plasmids

Plasmids were obtained from Amersham Pharmacia Biotech (now GE Healthcare Life Sciences), Invitrogen, Murphy lab (Carnegie Institution for Science, Department of Embryology, Baltimore, USA) and New England Biolabs (Frankfurt am Main, Germany).

Table 3-8: Plasmids

Plasmid	Description	Source/Reference
pENTR/D-TOPO	Entry vector for Gateway cloning, kanamycin resistance	Invitrogen
pGEX-4T-1	Vector for expression of GST fusion proteins in *E. coli*, ampicilin resistance	Amersham Pharmacia Biotech
pGGWA	Destination vector for expression of GST fusion proteins in *E. coli*, ampicilin resistance	Invitrogen
pMAL-c2X	Expression of Maltose binding protein, ampicilin resistance	New England Biolabs
pMGWA	Destination vector for expression of MBP fusion proteins in *E. coli*, ampicilin resistance	Invitrogen
pPGW	Expression vector for Drosophila cells, UASp promoter, N-terminal GFP tag, ampicilin resistance	Murphy lab
pPWH (attB)	GAL4-driven somatic and female germline expression in vivo	Murphy lab

Plasmid	Description	Source/Reference
pTGW (attB)	Expression vector for Drosophila cells, UASt promoter, N-terminal GFP tag, ampicilin resistance, GAL4-driven somatic expression	Murphy lab, Baltimore, USA

3.2 Molecular biology methods

3.2.1 Polymerase chain reaction (PCR)

DNA fragments were amplified by the PCR method (Mullis and Faloona, 1987) according to standard protocols. PCR reactions were done in 25 µl or 50 µl total reaction volume. Typically 20-100 ng/µl of plasmid DNA were mixed with 200 nM of forward/reverse primer, 250 µM of each dNTP (Bioline) and 0,02 µl polymerase per µl of total volume in the corresponding reaction buffer. For most applications Pfu S polymerase (lab internal) was used, for site-directed mutagenesis Accuzyme (Bioline, London, UK) was utilized.

Table 3-9: Standard PCR program

Step	Temperature	Duration (minutes:seconds)
1. Initial denaturation	**95°C**	**5:00**
2. Denaturation	**95°C**	**0:30**
3. Annealing	**50-70°C, depending on primer pair**	**0:30**
4. Elongation	**72°C**	**Depending on construct length (1 min/kb)**
Repeat steps 2-4 35 times		
5. Final elongation	**72°C**	**5:00**
6. Hold	**12 °C**	∞

The thermocycler "Master Cycler Nexus Gradient"(Eppendorf, Hamburg, Germany) was used for running the PCR programs, a standard program is shown in Table 3.9. PCR products were purified after gel electrophoresis (3.2.2) using the "Nucleo Spin®Gel and PCR Clean-Up" kit (Machery-Nagel) according to manufacturer's instructions and eluted in 30 µl distilled water.

3.2.2 Agarose gel electrophoresis

For the analysis of DNA fragments resulting from enzymatic digestion of DNA or PCR, samples were separated in 1% (or 2% for fragments < 500bp) agarose gels containing TAE buffer and 0.5 µg/ml ethidium bromide. Samples were mixed with the respective amount of 6X loading dye solution (Thermo Scientific). To estimate the size of the separated fragments, 10 µl of GeneRuler 1 kb DNA Ladder (Thermo Scientific) were used in a parallel lane. The gels were run 20-30 minutes at 140 V. To visualize the DNA bands were documented with a UV transilluminator (Intas).

3.2.3 Measurement of DNA concentration

To determine concentration and purity of isolated DNA, the absorption at 260/280 nm was measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific). Since double-stranded DNA has an absorption maximum at 260 nm and protein contaminations show an absorption at 280 nm, an absorption quotient of 1.8 shows a pure DNA solution. Usually the DNA concentration obtained from Midi-preparations has been adjusted to 1 µg/µl.

3.2.4 Gateway cloning

The Gateway™ technology (Invitrogen) has been employed for cloning of genes of interest into diverse destination vectors. Vectors of the "Drosophila Gateway™ Vector Collection" (Murphy Lab, Carnegie Institution for Science, Department of Embryology,

Baltimore) were used for applications in Drosophila cultured cells and to generate transgenic flies (see 3.6.2).

3.2.5 pENTR/D-TOPO cloning

Purified PCR products were introduced into the Entry vector by a topoisomerase-catalyzed reaction (pENTR/D-TOPO Cloning Kit, Invitrogen). This reaction involves a cleavage of the vector DNA by Topoisomerase I, leaving a 5' overhang, which is complementary to the "CACC" sequence at the 5' end of the forward primers used for the amplification of the ORF. The complementary sequences anneal and lead to a correct orientation and in-frame integration of the ORF into the vector. The cloning reaction was used to transform DH5α cells. The success of the ligation and the correct orientation of the integrated ORF were determined by analytical digests with restriction endonuclease (Thermo Scientific) according to manufacturer's instructions. The sequence of the insert was verified by sequencing using M13-Fwd and M13-Rev primers.

3.2.6 Cloning of potential LKB1 interaction partners

alpha-spec was cloned from an EST clone and corrected by PCR-based mutagenesis (Michael Krahn). *β-Spectrin* was obtained from Jan Pielage (Fredrich Mischer Institute for Biomedical Research, Basel).

3.2.7 Cloning of lkb1::gfp-lkb1 genomic

The genomic construct of LKB1 was amplified from a pFlyFos vector (FlyFos025349). In order to express GFP-tagged recombinant transgenes of *lkb1* and several mutant versions of *lkb1* in an expression level and –pattern that resembles the endogenous LKB1 expression, a genomic *lkb1* transgene was created. The upstream region of *lkb1*, starting at 2830 bp upstream of the start codon, was amplified by PCR and *egfp* cloned downstream of the endogenous promoter in a pENTR vector (*lkb1 genomic* pENTR first half). In a second pENTR the genomic *lkb1* was cloned (*lkb1 genomic* pENTR second

half), which was also used for PCR-based mutagenesis for the creation of mutations. Before recombination into a destination vectors, both pENTR were joined by classical cloning.

3.2.8 Gateway LR recombination reaction

Genes of interest were transferred from pENTR/D-TOPO vector into destination vectors via the LR recombination reaction. λ integrase catalyzes the recombination of an ORF, flanked by *attL1* and *attL2* recombination sites, with *attR1* and *attR2* recombination sites of a destination vector. This exchanges the ORF with the *ccd*B gene of the destination vector. The recombination is direction-specific, because of differences between *attL1* and *attL2* as well as between *attR1* and *attR2*.

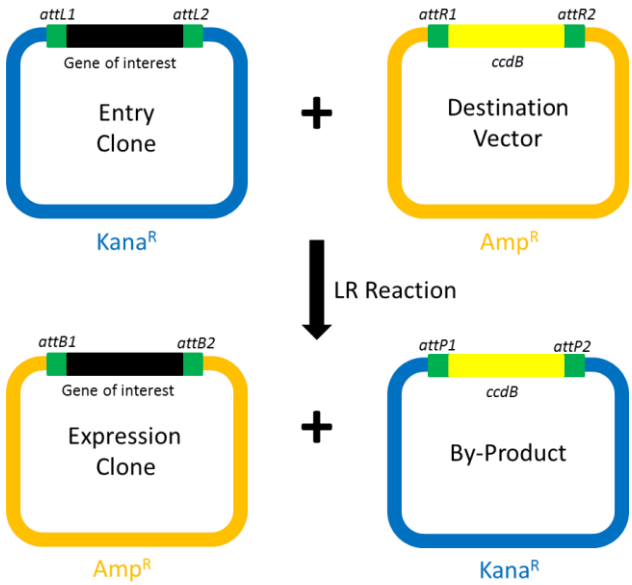


Figure 3-1: Gateway LR recombination reaction. Catalyzed by λ integrase, *attL* and *attR* sites are recombined, yielding the expression clone and a byproduct, carrying the lethal *ccd*B gene (figure drawn according to Liang et. al, 2013).

The recombination was set up by mixing 100 ng of pENTR vector and 90 ng of destination vector and adding 0.4 µl of clonase mix. The mixture was incubated for 25°C for 1 hour, then DH5α chemical competent cells were transformed. Since the *ccd*B gene product is toxic for standard laboratory strains of *E. coli*, it allows a selection of expression clones based on lack of toxicity in addition to the selection by the ampicillin

resistance of the destination vector's backbone (Figure 3-1). Transformants were picked, the success of the recombination was determined by analytical digest with restriction endonuclease (Thermo Scientific) according to manufacturer's instructions.

3.2.9 Transformation of chemically competent *E. coli* cells

Chemically competent *E. coli* cells (Table 3-10) were transformed by the following procedure: First, the cell solution was thawn on ice, 100-1000 ng (3000-5000ng for BL21 Star (DE3) cells) of plasmid DNA were added to 100 µl of competent cell solution and this was incubated for 20-30 minutes on ice. Then the cells were heatshocked at 42°C for 1 minute on a thermoblock (Eppendorf), cooled on ice for 5 minutes, 400 µl LB medium were added and the cells incubated for 1 hour at 37°C with shaking. After that the cells were plated on prewarmed LB agar plates with the appropriate antibiotic for selection.

Table 3-10: Bacterial strains

Strain name	Genotype	**Application**	**Source**
DH5α	φ80*dlacZ*Δ*M15*, Δ(*lacZYAargF*) U169, *deoR*, *recA1*, *endA1*, *hsdR17*(rK-,mK+), *phoA*, *supE44*, λ-, *thi-1*, *gyrA96*, *relA1*	Amplification of plasmid DNA	Invitrogen
BL21	F-, *omp*T, *hsdSB*(rB-, mB-), *dcm*, *gal*, λ(DE3)	Expression of recombinant proteins	Invitrogen
BL21 Star™(DE3)	F– ***omp***T ***hsd***SB(rB–, mB–) ***gal dcm rne***131	Expression of recombinant proteins	Invitrogen

Strain name	**Genotype**	Application	Source
TOP10	F– ***mcr***A Δ(***mrr-hsd***RMS-***mcr***BC) Φ80***lac***ZΔM15 Δ***lac***X74 ***rec***A1 ***ara***D139 Δ(***ara leu***) 7697 ***gal***U ***gal***K ***rps***L (StrR) ***end***A1 ***nup***G	Cloning of PCR fragments in pENTR vector	Invitrogen
XL1-Blue	endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 $proAB^+$ $lacI^q$ Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	Site-directed mutagenesis	Stratagene

3.2.10 Isolation of plasmid DNA by Alkaline Lysis with SDS

3.2.10.1 Mini Preparation

To isolate recombinant plasmid DNA from genetically modified bacteria colonies of transformed *E. coli* were inoculated in 2 ml LB medium and incubated at 37°C at 200 rpm overnight or for 6 hours. The cells were pelleted by centrifugation for 1 minute at 6000 rpm. For resuspension 200 µl of buffer P1 (including 100 µg/ml RNase A) were added. Lysis buffer P2 and after about one minute of incubation neutralization buffer P3 were added. The mixture was inverted several times and centrifuged for 6 minutes at 12000 rpm at 4 °C. The supernatant was then transferred to a new Eppendorf tube filled with 900 µl pure ethanol, mixed and then centrifuged at 12000 rpm for 12 minutes to precipitate the DNA. The supernatant was discarded, the pellet washed with 70% ethanol. After 5 minutes of centrifugation at 12000 rpm, the supernatant was discarded and the pellet was dried at 65°C in a drying cabinet for 10 minutes. The pellet was dissolved in 25 µl ddH_2O.

3.2.10.2 Midi Preparation

For the isolation of larger amounts of DNA plasmid DNA was purified with the NucleoBond ®PC 100 kit (Machery-Nagel), but by the same principle as the mini preparations (alkaline lysis with SDS), according to the manufacturer's protocol. In brief, 50 ml overnight culture were centrifuged, the pellet resuspended in 4 ml S1 resuspension-buffer (provided with the kit), 4 ml S2 lysis-buffer were added, mixed, incubated for 3 minutes at room temperature 4 ml S3 neutralization-buffer were added, the resulting solution inverted several times. After 5 minutes of incubation on ice it was inverted again and centrifuged for 8 minutes at 10000 rpm at 4°C. The columns were prepared by addition of 2.5 ml N2 equilibration-buffer. The supernatant of the solution was filtered with a cellulose filter and loaded onto the column. After the lysate solution passed through the column, it was washed by filling up the column with N3 (washing buffer). The DNA was then eluted with 5 ml N5 elution-buffer. 3.5 ml Isopropanol were added and mixed by inverting. For the precipitation the solution was centrifuged 30 minutes at 12000 rpm at 4°C. The supernatant was discarded; the pellet washed with 5 ml 70% ethanol for 10 minutes at 12000 rpm. The pellet dried for 10 minutes (or until no liquid could be seen) at 65°C in a drying cabinet. To dissolve the pellet 100 µl of sterile distilled water were added, the concentration was measured with a NanoDrop 1000 Spectrophotometer and adjusted to 1 µg/µl.

3.2.11 Site-directed mutagenesis

The site-directed mutagenesis kit QuikChange (Agilent Technologies) was employed for adding point mutations in ORFs cloned into pENTR. The pENTR vector was amplified with a primer containing the desired mutation and a silent mutation that can be used to identify the mutated vector by adding a restriction site to the given vector. Using 20 ng of entry clone and 125 ng of the primer, the site directed mutagenesis was performed in a "Master Cycler nexus gradient" (Eppendorf, cycler program see Table 3-11). The template DNA was removed by digestion with 1 µl DpnI for 1 hour at 37°C. The DH5α cells were transformed with 2 µl of the solution (3.2.9).

Table 3-11: Site directed mutagenesis program

Step	Temperature (°C)	Duration (minutes:seconds)
1) Initial denaturation	95	0:30
2) Denaturation	95	0:20
3) Annealing	55	0:20
4) Elongation	68	3:00
Repeat steps 2-4 25 times		
5) End of the reaction	37	∞

3.2.12 Sequencing of DNA

During the first year of this work sequencing reactions were set up as follows: 300 ng of plasmid DNA were mixed with 8 pmol of sequencing primer, 1.5 µl sequencing buffer and 1.5 µl sequencing mix added and filled up with sterile water to 10µl. The PCR program for sequencing reaction is shown in Table 3-12.

Table 3-12: PCR program for sequencing reactions

Step	Temperature (°C)	Duration (minutes:seconds)
1) Initial denaturation	96°C	2:00
2) Denaturation	96°C	0.20
3) Annealing	55°C	0:30
4) Elongation	60°C	4:00
Repeat steps 2-4 26 times		
5) End of the reaction	12°C	∞

After the PCR reaction the mixture was transferred to a new tube, 1 µl of 125 mM EDTA, 1 µl of 3 M Sodium acetate and 50 µl of 100% ethanol were added, the sample incubated for 5 minutes. For precipitation the sample was centrifuged for 15 min at 13000 rpm, the supernatant removed and the pellet washed with 70% ethanol. Then 5 minutes of centrifugation at 13000 rpm were done, the supernatant removed and the pellet air-dried. The pellet was then dissolved in 15 µl of HiDi (Applied Biosystems).

Analysis of sequencing reactions was done by in-house sequencing service in the Department of Developmental Biochemistry, Ernst-Caspari-Haus, GZMB, Göttingen. For external sequencing reactions were set up using 1.2 µg of the plasmid, 30 pmol of the sequencing primer filled up to 15 µl and sent to Seqlab/Microsynth (Göttingen, Germany).

3.2.13 Isolation of genomic DNA from flies

Adult flies were collected in an 1.5 ml reaction tube and frozen for 10 minutes at -80°C. Then the flies were ground with in 200 µl buffer A with a biovortexer, another 200 µl of buffer A were added and the grinding continued until only cuticles remained visible. Afterwards it was incubated for 30 minutes at 65°C. Then 800 µl of potassium acetate/lithium chloride solution were added and the mixture incubated on ice for 30 minutes and centrifuged for 15 minutes at 13000 rpm. 1 ml of the supernatant was transferred to a new tube and 600 µl Isopropanol mixed to it for precipitation at 13000 rpm for 15 minutes. The supernatant was then discarded, the pellet washed in 200 µl 70% ethanol and centrifuged for 10 minutes at 13000 rpm. The pellet was then dried and resuspended in 150 µl TE buffer.

3.3 *Drosophila* cell culture

3.3.1 Culture and transfection of Schneider 2 cells

Schneider 2 (S2) cells are an immortalized embryonic cell line (Schneider, 1972), the S2R+ cell line is known to express the Wingless receptor Frizzeled-2, unlike other known S2 cell lines and therefore reacts to wingless (Yanagawa *et al*.,1998). Cultured cells were maintained in flasks of either 25 cm² or 75 cm² at 25°C in Complete Schneiders's Medium (PAN biotech). Cells were split once or twice per week. For transfections S2R cells were split to 6 well plates 24 hours before transfection (usually 0.6 – 2 x 10^6 cells in 2 ml). For immunostaining a sterile coverslip was placed in each well before adding the cells to the wells.

S2 cells were transfected with FuGENE HD Transfection Reagent (Roche). For this 2 µg plasmid DNA were diluted in 94 µl sterile water or Schneider's Medium without serum, mixed and 4 µl FuGENE added to the solution and incubated for 15 minutes at room temperature. All reagents were warmed to room temperature before mixing. After 15 minutes of incubation the transfection mixture was added to the cell suspension. After transfection the cells were incubated at 25°C for 2-7 days. For GFP-tagged recombinant proteins the transfection rate was checked by fluorescence microscopy.

Table 3-13: S2 cell lines

Strain	Further Information	References
S2R *Drosophila* Schneider Cells	---	AG Wodarz, Göttingen
S2R^{+} *Drosophila* Schneider Cells	cell culture line established from OregonR embryos (Schneider, 1972)	AG Sprenger, Regensburg

3.3.2 Leptomycin B assay

To inhibit the protein nuclear export factor embargoed (a homolog of human CRM1/ Exportin-1), which mediates the transport of proteins carrying leucine-rich nuclear export signals (NES) from the nucleus to the cytoplasm, cells were incubated with a 1:100 dilution of Leptomycin B solution (Sigma-Alldrich) in S2 cell culture medium.

3.4 Histology

3.4.1 Fixation and immunostaining of Schneider 2 cells

To prepare transfected cells for confocal microscopy (3.4.6) they were fixed with 4% formaldehyde 15 minutes at room temperature in PBS. Prior to fixation, the medium was sucked of and the cells washed with PBS. An incubation with 5% NHS in PBT was performed to block unspecific binding. Afterwards the cells were incubated with the staining solution containing the first and secondary antibody in PBT with 5% NHS for 2 hours at room temperature. To stain DNA a DAPI staining was performed with a solution of 1:1000 DAPI (5 µg/ml) in PBT for 10 minutes, and washed two times with PBT for 10 minutes. Finally the cells were embedded in 35 µl Mowiol.

3.4.2 Fixation and immunostaining of embryos

For the collection of embryos, flies were kept in either small or big cages with apple juice agar plates with yeast paste (made from blocks of baker's yeast with water) at the bottom. To collect the eggs, the plates were first removed from the cage, rinsed with water and the eggs released from the bottom with a brush. After that about the same volume of 5% sodium hypochlorite was added and the embryos incubated for 4-5 minutes, until the chorion was dissolved. The resulting slurry was filtered through a sieve with a vacuum pump and washed extensively with water to collect and clean the embryos.

After the collection and dechorionization the embryos were fixed with 3 ml of 4% formaldehyde in PBS with 3 ml heptane on top in a glass vial and incubated on a rocker for 20 minutes. The lower phase was then removed and 3 ml methanol added. The embryos were washed three times with methanol and stored at -20°C for at least one hour.

Fixed embryos were washed three times for 20 minutes with PBT on a rocker. To block unspecific binding the embryos were incubated with PBT with 5% NHS for 30 minutes. After that the embryos were incubated with the primary antibodies in PBT with 5%

NHS and incubated overnight at 4°C. Then three washing steps with PBT for 20 minutes were done. The secondary antibodies, which were tagged with fluorescent markers, were then applied 1:200 in PBT with 5% NHS for two hours at room temperature on a rocker. Afterwards the embryos were washed again three times for 20 minutes with PBT, the first washing step includes a staining with DAPI (solution applied 1:1000). Finally the embryos were transferred to a microscope slide and embedded in Mowiol.

3.4.3 Fixation and immunostaining of ovaries

Newly hatched flies were put in cages with apple juice agar plates and yeast paste for two days to enhance the development of ovaries in the females. For dissection the females were anesthetized with carbon dioxide, the ovaries dissected with forceps, collected in 900 µl of PBS 1.5 ml eppendorf tubes and fixed with 110 µl of 37% formaldehyde. After three washing steps with PBT, the ovaries were blocked and permeabilized by incubation with PBS with 1% Triton X-100 and 0.5% BSA for 2 hours. To separate the ovarioles, the ovaries were then pipetted up and down with a 1000 µl pipette tip. The primary antibodies were applied in PBS with 0.3% Triton X-100 overnight at 4°C on a rocker. Then the eggchambers were washed again three times with PBT, blocked for 2.5 hours in PBS with 0.1% Triton X-100 and 10% NHS. Afterwards the secondary antibodies were applied in a 1:200 dilution in PBS with 0.1% Triton X-100 and 10% NHS. Subsequently the eggchambers were stained with DAPI for 20 minutes (DAPI-solution 1:1000 in PBT) and washed two times with PBT. Finally the eggchambers were transferred to a microscope slide and embedded in Mowiol.

3.4.4 Fixation and immunostaining of larval brains and imaginal discs

Larvae of the L3 wandering stage were dissected in PBS and the brains fixed for 20 minutes in 4% formaldehyde in PBS on a rocker. After three washing steps with PBS with 0.1% Triton X-100, the brains or imaginal discs were permeabilized and blocked for 1 hour in PBS with 1% Triton X-100 and 5% NHS. The staining procedure was performed as described for the embryos (1.5.2).

3.4.5 Detection of apoptosis in imaginal discs

The hallmark of apoptosis is DNA degradation. In order to detect apoptosis TUNEL (TdT-mediated dUTP-X nick end labeling)-assays were made with the "*In Situ* Cell Death Detection Kit, TMR red" (Roche). This method uses modified nucleotides (in this case TMR-dUTP) to label free 3'-OH termini of DNA breaks (nicks) in an enzymatic reaction. This reaction is catalyzed by terminal deoxynucleotidyl transferase (TdT) and polymerizes the modified nucleotides to the 3'-end of single-and double-stranded DNA.

To detect apoptosis in imaginal discs they were, after staining with primary and secondary antibodies, incubated for one hour at 37°C with a mixture of 45 µl of Label Solution and 5 µl of Enzyme Solution. After this the embryos were washed and stained with DAPI as described above. Since the red fluorescent TMR-dUTP is incorporated into damaged DNA strands it enables detection of apoptotic cells by fluorescence microscopy.

3.4.6 Confocal microscopy

Images were made on a laser scanning confocal microscope (LSM 510 Meta using 45x 0,8 NA Plan Neofluar or 63x 1.4 NA Plan Apochromat objectives or LSM 710 Meta using either 25xNA 0.8 or 63x NA 1.2 water objectives and ZEN 2010 software (Carl Zeiss). Images were processed using Photoshop CS5 (Adobe) and ImageJ (version1.43m, NIH, USA).

3.4.7 Preparation and imaging of wings

Wings were prepared for light microscopy by dissection of adult flies, benumbed by carbon dioxide in ethanol using forceps. The wings were spread on a microscope slide and mounted using DPX Mounting medium (Sigma-Alldrich). The wings were imaged by transmitted light microscopy using a EC Plan-Neofluar 2.5x/0.075 Pol M27 objective (Zeiss).and an AxioCam MRc camera (Zeiss).

3.5 Biochemical methods

3.5.1 Protein extraction from embryos

Embryos were collected, their chorion was removed and they were washed as described in 3.4.2 and collected in 1.5 ml eppendorf tubes in 1000 µl water. Then they were centrifuged at 12000 rpm for 1 minute and the supernatant discarded. After this embryos were homogenized for 30 seconds with a biovortexer (Roth; for small amounts just the tip of the device was used to squash the embryos) in lysis buffer (TNT with 1:500 of PMSF, Aprotinin, Leupeptin and Pepstatin). If the phosphorylation status of the protein of interest had to be preserved, 1:100 of phosphatase inhibitor cocktail (CALBIOCHEM) and 1:500 of cantharidin solution were added. After 20 minutes incubation on ice the lysate was centrifuged for 10 minutes at 12000 rpm, 4°C, the supernatant transferred into a new eppendorf tube and 1 µl taken for measuring the protein content of the supernatant.

3.5.2 Measurement of protein concentration

The total concentration of protein in solutions was estimated according to the Bradford method with 200 µl Roti-Quant reagent (Roth), which was mixed 1:5 with water and 1-10 µl of the protein solution (dependent on the expected concentration). The absorption was measured at 595 nm with a spectrophotometer with an integration time of 2 seconds. For calibration a curve with BSA standard was used.

3.5.3 Co-Immunoprecipitation

For co-immunoprecipitation embryos were lysed in NP-40 buffer with freshly added protease inhibitors (Pefabloc 200 µg/ml, Pepstatin 2 µg/ml, Aprotinin 2 µg/ml, Leupeptin 2µg/ml (Roche) using a biovortexer (Roth). After 20 minutes of incubation at 4°C the sample was centrifuged for 10 minutes at 12000 rpm, 4°C. The supernatant was then transferred to a new tube, a 10 µl aliquot taken for the input sample, which was mixed with 10 µl 2x SDS loading buffer. The concentration of protein was measured and adjusted to the lowest protein concentration by diluting the higher concentrated

samples with lysis buffer. The guinea pig anti-LKB1 antibody was added to the lysates in 1:500 dilution, they were placed on a rocker at 4°C for 30 minutes. Then 20 µl of Protein A sepharose beads were added and the samples shaken for 1.5 hours at 4°C. The beads were then spun down by centrifugation at 6500 rpm for one minute and washed three times with NP-40 buffer. After removing excess liquid, 16 µl of 2X SDS loading buffer were added to the beads and the sample boiled at 95°C for 5 minutes.

3.5.4 SDS-polyacrylamide gel electrophoresis

Denaturing discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein samples electrophoretically. 10% or 7.5% resolving gels were used with a stacking gel according to Table 3-14.

Table 3-14: Acrylamide gel recipe

Gel	**Component**					
Resolving Gel	**Acrylamide**	**1M Tris-HCl pH 8.8**	**Water**	**10% SDS**	**10% APS**	**TEMED**
7.5% Gel	3.75 ml	5.75 ml	4.9 ml	150µl	150µl	6µl
10% Gel	5 ml	5.75 ml	3.65 ml	150µl	150µl	6µl
Stacking Gel	**Acrylamide**	**1M Tris-HCl pH 6.8**	**Water**	**10% SDS**	**10% APS**	**TEMED**
5%	830µl	630µl	3.5 ml	50µl	50µl	5µl

Numbers given for two gels.

Protein samples were mixed 1:1 with 2X SDS loading buffer or 1:5 with 6X SDS loading buffer and boiled for five minutes and centrifuged to spin down the sample. After that the samples were loaded into the pockets of the gel. 3 µl of PageRuler Prestained Protein Ladder (Thermo Scientific) were loaded as molecular weight size marker. Gels were usually run for 1 hour in SDS running buffer.

3.5.5 Western Blot

Protein samples were separated by SDS-PAGE (3.5.4) and transferred to nitrocellulose membrane with the Rotiphorese® PROclamp MINI Tank-Blotting-System (Roth) according to manufacturer's instructions. For this transfer buffer with 20% methanol added was used, the transfer took place at 100 V for 1 hour at 4°C. The success of the transfer could be tested by staining with Ponceau Solution, which was then removed by washing with TBST. Blocking of the membrane to prohibit background signal was done in blocking buffer (1% BSA, 3% skim milk powder in TBST).

3.5.6 Protein purification

For the expression of tagged protein by bacteria 50-300 ml LB medium were inoculated with 1-6 ml overnight culture of BL21 or BL21 Star bacteria carrying the target plasmid. The cultures were shaken at 200-250 rpm at 37°C, until they reached mid-log phase at an OD_{600} of 0.6. To induce expression of the recombinant protein IPTG was added to a final concentration of 0,5 mM, 2% of pure ethanol and 3% of potassium dihydrogenphosphate were added to aid the induction. The cultures were chilled on ice for a short time and incubated for 16-18 hours at 18°C. The bacteria were harvested by centrifugation at 6000 rpm for 5 minutes. The resulting pellet was frozen at -80°C for 30 minutes and resuspended in half to one volume of the culture of LEW buffer containing 1% Triton X-100, proteinase inhibitors, and 10 mM mercaptoethanol. Then the supspension was incubated for 30 minutes at 4°C and in most cases also sonicated (6 cycles of 15 seconds). The lysates were then cleared at 12000 rpm for 15 minutes at 4°C.

GST fusion proteins were purified by adding 10 µl of Protino Glutathione Agarose 4B beads (Machery-Nagel) for each milliliter of supernatant and incubated for two hours at 4°C. For MBP fusion proteins 10 µl of Amlyose Resin beads (New England BioLabs) were added for each milliliter of supernatant and incubated for two hours at 4°C. The column or beads were washed once with LEW, once with LEW with 2 M sodium chloride and then again with LEW. After each washing step the beads were centrifuged for 1 minute at 6000 rpm. GST fusion proteins were eluted with 30 mM Gluthatione in

50 mM TRIS-HCl pH 7.5 with 150 mM NaCl. MBP fusion proteins were eluted with 20 mM Maltose in 50 mM TRIS-HCl pH 7.5 with 150 mM NaCl.

3.5.7 GST-Pulldown assay

To analyze direct interactions between proteins *in vitro*, a GST fusion protein pulldown assay was used. Beads for the pulldown were preincubated overnight with 500 µl of 5% BSA solution in incubation buffer at 4°C to block unspecific binding. Proteins were expressed in BL21 Star cells and purified using respective beads and elution buffers (3.5.6). 1 µM GST-fusion protein solution and 1 µM of MBP-fusion protein solution, which were before dialyzed overnight using incubation buffer buffer (20 mM Tris pH 8.0, 100 mM potassium chloride, 10 mM magnesium dichloride, 1mM DTT (added immediately before use)), were adjusted to a total volume of 60 µl with incubation. in a 1.5 ml reaction tube and incubated for 1 hour at 4°C in ice with occasionally snipping. Then 10 µl of preincubated Protino Glutathione Agarose 4B beads (Machery-Nagel) were added and incubated for 1 hour at 4°C on ice with occasionally snipping. The beads were then collected by centrifugation at 6000 rpm for one minute and the supernatant discarded. Afterwards the beads were washed five times with 500 µl wash-buffer (20 mM Tris pH 8.0, 20 mM potassium chloride, 0.1 % Triton X-100 and 1 mM DTT added immediately before use) and subsequent centrifugation at 6000 rpm for one minute and removal of the supernatant. Finally the supernatant was removed except for about 20 µl of total volume including the volume of the beads. Then 5 µl of 6X SDS loading buffer were added and the sample boiled for 5 minutes at 95°C. The samples were analyzed by Western blot.

3.5.8 Lipid overlay assay

To determine lipid binding specificity of the LKB1 C-terminus PIP Strips (Echelon Biosciences, USA) were used according to the manufacturer's protocol. In brief, the membrane was first blocked in blocking buffer (1x TBS, 0.1% Tween, 3% BSA, the pH of was adjusted to 7.4) for one hour. After that, the membrane was incubated overnight with 1µg/ml of MBP-fusion protein or MBP as control in blocking buffer. The

membrane was washed three times in TBS with 0.1% Tween and incubated with anti-MBP antibody in blocking buffer for one hour. Afterwards it was washed as previously described and incubated for one hour with HRP-coupled secondary antibody. The membrane was again washed three times and bound protein detected by a chemiluminescence reaction using the "Super Signal West Pico Chemiluminescence Substrate" kit (Thermo Scientific).

3.6 Fly genetics

3.6.1 Fly breeding

Fly stocks were kept at 18°C, 21°C or 25° with standard food (Ashburner, 1989) with sprinkles of dry yeast on top. The vials where changed in intervals of three to five week at 21°C. For the collection of embryos, flies were kept in either small or big cages with apple juice agar plates with yeast paste (made from blocks of baker's yeast with water) at the bottom.

The standard medium was made of 712 g cornmeal, 95 g soya flour, 168 g dry yeast, 450 g malt extract, 150 ml 10% Nipagin solution (700 ml 99% ethanol, 300 ml H_2O, 100 g Nipagin), 45 ml propionic acid, 50 g agar, 400 g sugar beet syrup, solved in 9.75 l distilled water.

3.6.2 Generation of transgenic flies

For the generation of transgenic flies the ϕC31 integrase system was utilized. It allows a site-specific integration of transgenes into specific loci on the genome of flies carrying landing sites integrated into their genome. ϕC31 integrase mediates recombination between two 34 bp attachment sites (*attB* in the donor plasmid and *attP* in the landing site). This recombination creates two different sites (*att*R and *att*L), which cannot serve as functional substrates for the integrase, making the reaction unidirectional (Figure 3-2). The used fly lines (P{nos-phiC31\int.NLS}X, P{CaryP}attP25C and P{nos-phiC31\int.NLS}X, P{CaryP}attP68A) express ϕC31 integrase under the control of the *nanos* regulatory elements to aid germline transformation. The integrase further has a C-

terminal nuclear localization signal attached to enhance integration (Bischof et al., 2007). Since the position of the transgene is controlled, all constructs integrated into the same locus can be directly compared (Groth et al., 2004).

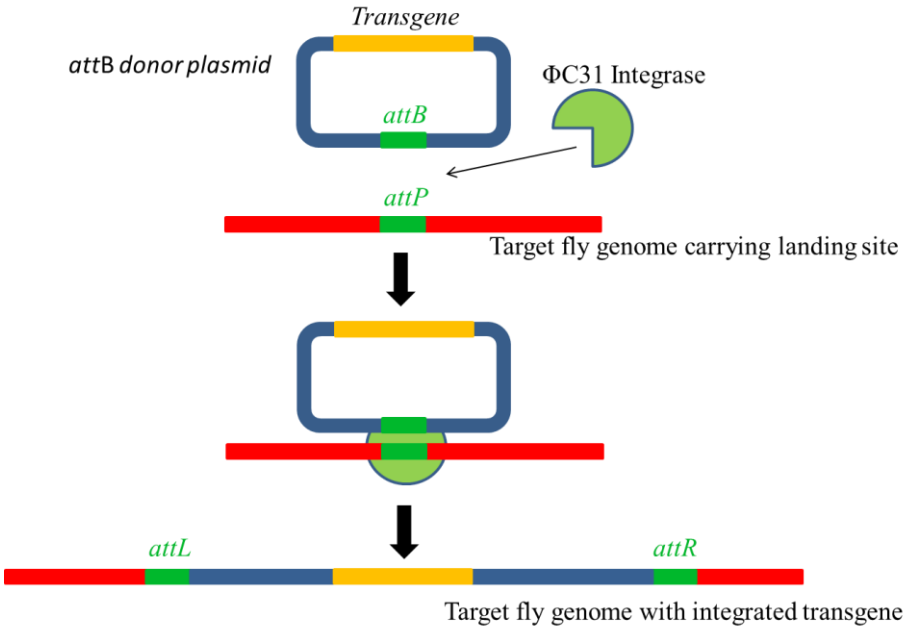


Figure 3-2: Integration mediated by ϕC31 integrase. ϕC31 integrase mediates the unidirectional recombination between the attB site of the donor plasmid and attP site of the landing site, creating an attL site and an attR site while integrating the transgene (figure drawn according to *http://www.systembio.com/phic31*).

Transgenes were introduced into the germline by microinjection of integration vector DNA into the posterior of preblastoderm embryos like described by Bachmann and Knust, 2008. In brief, 20 µg of plasmid DNA were added to 10X-injection buffer (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8) and water to a final volume of 50 µl. Before use the solution was centrifuged for 30 minutes. Dechorionated embryos of the fly line carrying the desired landing site were lined up on a block of apple juice agar and transferred to a coverslip coated with "embryo glue". The DNA solution was injected into the posterior end of the embryos with an Femptotips®II microinjection capillary (Eppendorf) by micromanipulator InjectMan NI2 (Eppendorf, Hamburg, Germany). Afterwards the injected embryos were covered by 10S Voltalef oil at 18°C for 48 hours before the hatched larvae were collected into Drosophila vials. Hatched adults were crossed to *w⁻; Gla/CyO* flies for the transgenic fly selection. Vectors of the "Drosophila

Gateway™ Vector Collection" (Murphy Lab, Carnegie Institution for Science, Department of Embryology, Baltimore) were used to generate transgenic flies (Table 3-8).

3.6.3 The UAS-GAL4 system

The UAS-GAL4 system is a commonly used binary expression system in *Drosophila* research. It utilizes the yeast transcription factor GAL4, expressed under control of a known promoter that binds to an upstream activating sequence (UAS). This binding activates gene expression in a region downstream of this sequence (Brand and Perrimon, 1993). In this study this system was used to ectopically overexpress genes of interest in time-, tissue- and cell-specific patterns.

3.6.4 FLP/FRT- mediated recombination

FLP recombinase mediates recombination between two FRT sites on homologous chromosomes. It has been transferred from yeast into the *Drosophila* genome to catalyze site-specific recombination (Golic and Lindquist, 1989). The FLP (Flipase)/ FRT (FLP recombinase-target) technique can be utilized to create mutant clones in both germline and somatic *Drosophila* cells by mitotic recombination (Theodosiou and Xu, 1998). In this study it was used to create clones of homozygous mutant cells in otherwise heterozygous follicle cell epithelia (Figure 3-3). The FLP-recombinase was expressed under control of a heatshock promoter, which allows a temporal control of the mitotic recombination by inducing a heatshock of 1.5-2 hours length at 37°C at specific time points. To create clones of different sizes, the heatshock was induced during larval stages or during pupal stages.

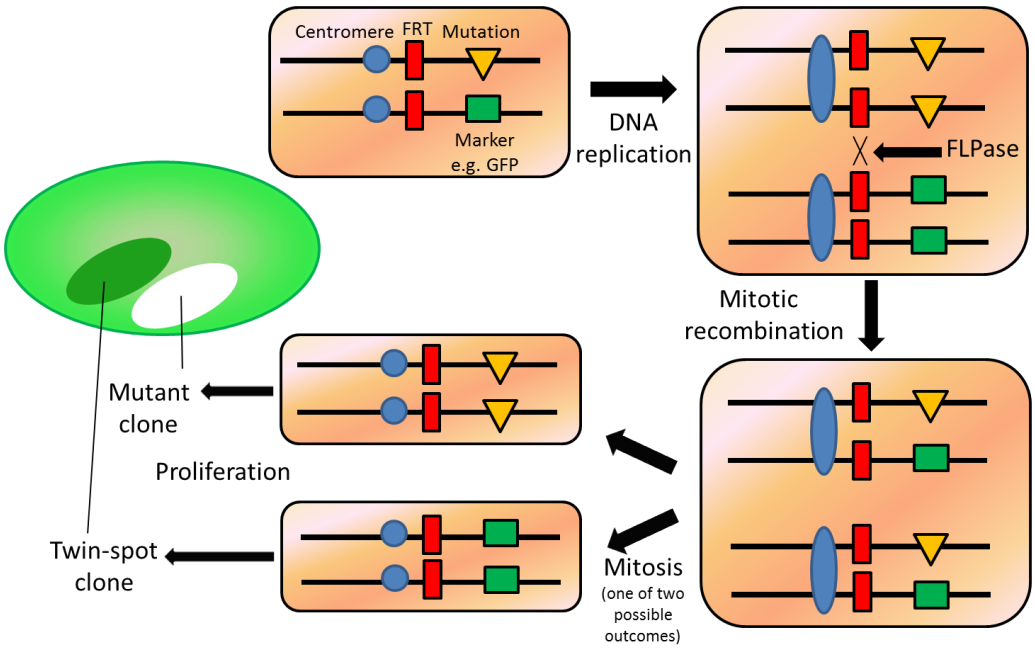


Figure 3-3: Generation of mutant clones by mitotic recombination. A mutant allele of the gene of interest and a wild type version of the chromosome can, catalyzed by FLP, recombine during mitosis, which can give rise to homozygous daughter cells (figure drawn according to Tabata, 2001).

3.6.5 Fly lines

Fly lines were either created by FLP/FRT-mediated germline transformation or taken from various sources (reference in Table 3-15).

Table 3-15: Fly lines

Stock	Description	Reference
arm-Gal4	GAL4 driver line	Bloomington #1560
Cu2-Gal4	Gal4 driver line, expression in follicle cells from stage 8, 2nd chromosome	Trudi Schüpbach
da-Gal4	Gal4 driver line, ubiquitous expression	Bloomington #5460

Stock	Description	Reference
	in *daughterless* gene pattern, 3rd chromosome	
Gla/CyO	Second chromosome balancer line, curly wings	Krahn lab stock collection
hsFlp;; α-Spec FRT80A/TM3	Mutant	
If/F; LKB1^{x5}/TM6	Mutant, double balanced	
If/F; lkb1::GFP-LKB1 genomic @99F/TM6	LKB1 transgene; double balance	
LKB1 RNAi VALIUM22	RNAi	#35151 Made by Transgenic RNAi Project, works in germline
lkb1::GFP-LKB1 C564A genomic @99F/TM6	LKB1 transgene	
lkb1::GFP-LKB1 C564A genomic/CyO; MKRS/TM6	LKB1 transgene; double balance	

Stock	Description	Reference
lkb1::GFP-LKB1 genomic @25C/F	LKB1 transgene	
lkb1::GFP-LKB1 genomic @25C/F; LKB1x5/TM6	LKB1 transgene	
lkb1::GFP-LKB1 genomic @99F/TM6	LKB1 transgene	
lkb1::GFP-LKB1 genomic 25C/CyO	LKB1 transgene	
lkb1::GFP-LKB1 ΔLB genomic 25C/CyO	LKB1 transgene	
$LKB1^{x5}$ FRT82B /TM3-tw-GFP	Mutant	Allele from Lee et al., 2006
MKRS/TM6b	Third chromosome balancer line	Krahn lab stock collection
MTD gal-4		
UASp::LKB1		
UASt::GFP-LKB1 @25C C564A/F	LKB1 transgene	
UASt::GFP-LKB1 @25C D317A "KD"/CyO	LKB1 transgene	

Stock	Description	Reference
UASt::GFP-LKB1 @25C K201M "KD"/CyO	LKB1 transgene	
UASt::GFP-LKB1 @25C/CyO	LKB1 transgene	
UASt::GFP-LKB1 ΔLB/CyO	LKB1 transgene	
Ubi::GFP-KB1 512-C @25C/Gla CyO	LKB1 transgene	
Ubi::GFP-LKB1 @25C/CyO	LKB1 transgene	
Ubi::GFP-LKB1 C564A @25C/(CyO)	LKB1 transgene	
Ubi::GFP-LKB1 C564A @99F /(TM6)	LKB1 transgene	
Ubi::LKB1 C564A @25C/CyO	LKB1 transgene	
white-(w1118)	White eyes	Bloomington #5905
β-Spec^{0} FRT19A/FM7;	Mutant, double balanced	

Stock	Description	Reference
Tft/CyO		
β -Spec^{0}FRT/FM7-GFP; lkb1::GFP-LKB1 genomic @25C/F	LKB1 transgene; β-spec mutant	

3.6.6 Lethality assay

The lethality of flies was tested by collecting 100 embryos of the given fly line on an apple juice plate with yeast paste and observing them daily to check in which stage of their development they died. To avoid drying of the plate, small amounts of tap water were added occasionally. Those flies that developed until adulthood were counted as survivors. Each experiment was performed three times.

4 Results

4.1 Subcellular localization of LKB1

4.1.1 LKB1 localizes to the cortex of epithelial cells and embryonic neuroblasts

GFP-tagged LKB1 has been observed to localize to the cortex of female germ line cells and to the lateral membrane in follicle epithelia cells of *Drosophila* (Martin and St Johnston, 2003). In embryonic epithelial cells and embryonic neuronal stem cells (neuroblasts, NBs) overexpressed GFP-LKB1 was found along the cell cortex (Yamamoto et al., 2008), while the localization of endogenous LKB1 has not yet been described in these cell types.

To examine the expression and subcellular localization of endogenous LKB1 during development of *Drosophila* we raised an antibody against the N-terminus of LKB1 and immuno-stained embryos. A ubiquitous expression of LKB1 in the *Drosophila* embryo was observed (Figure 4-1, A). Immunostaining of the early embryo during cellularization indicates a maternal contribution of LKB1 (Figure 4-1, B). The antibody showed a lack of staining in embryos in which LKB1 expression has been knocked down by RNAi, showing the specificity of the antibody (Figure 4-1 D compared to C). For this RNAi knockdown experiment an UAS-LKB1 RNAi fly line (carrying the vector VALIUM22 P{TRiP.GL00019}attP2) was crossed with a maternal triple driver GAL4 fly line (*MTD-Gal4*) to drive expression of shRNA during oogenesis in the germarium, leading to a knockdown of the maternal component of LKB1 and ongoing knockdown in the embryo (Staller et al., 2012). Most of the resulting eggs show no sign of embryonic development, probably due to defect in oogenesis like described for germ line clones (Martin and St Johnston, 2003).

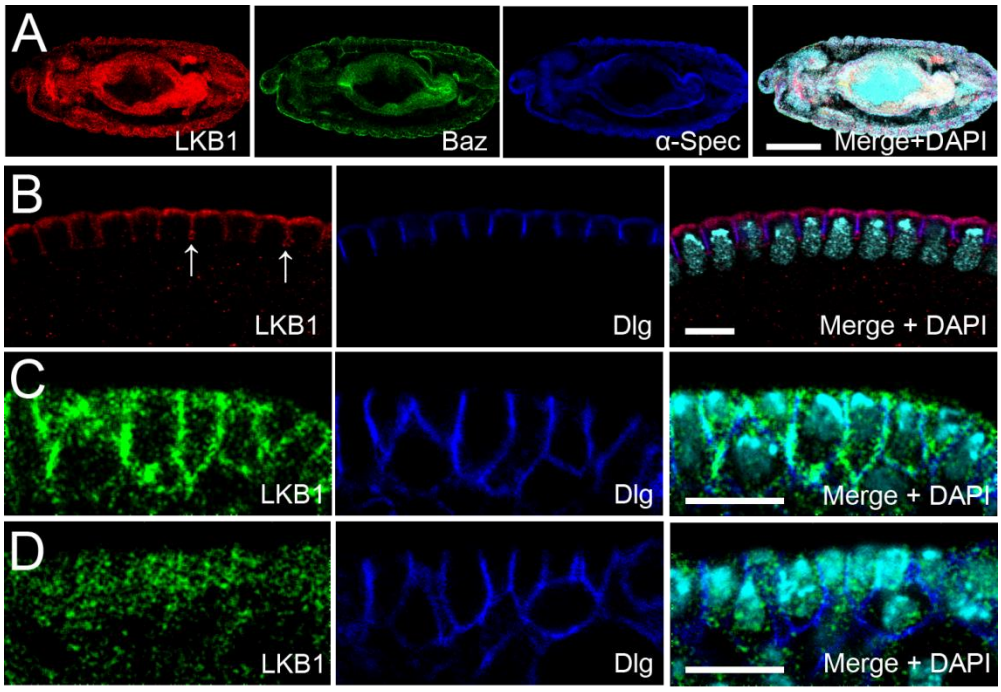


Figure 4-1: Characterization of the LKB1 antibody. A-C – Wild type embryos stained with an antibody raised against the N-terminus of LKB1. A - Stage 14 embryo displays ubiquitous expression of LKB1. B – LKB1 staining in a stage 5 embryo revealing a maternal contribution of LKB1, arrows indicate location of furrow cannels. C, D – Stage 11 embryos. D – *MTD-Gal4* driven RNAi knockdown of LKB1 in embryonic epithelium, stage 11. Scale bar in A, 100 µm; scale bart in B-D, 10 µm.

Subcellular localization of LKB1 in the embryonic epithelium was analyzed by co-staining of LKB1, the adherens junction marker DE-Cadherin and the membrane skeleton protein α-Spectrin (Figure 4-2, A). LKB1 shows a co-localization with α-Spectrin in the basolateral cortex of epithelial cells (Figure 4-2, A). In embryonic NBs, a cortical localization was observed throughout different phases of their cell cycle (Figure 4-2, B-E). While other regulators of asymmetric division in NBs, like the apically localized protein Bazooka (Baz) and the adaptor protein Miranda (Mir) exhibit a polarized distribution, which is most prominent during metaphase (Figure 4-2, C; Wodarz and Huttner, 2003), LKB1 was found symmetrically along the cortex of embryonic NBs during different phases of mitosis (Figure 4-2, B-D). This cortical localization of endogenous LKB1 in epithelia and NBs is in line with observations from overexpressed GFP-LKB1 (Yamamoto et al, 2008). In contrast to this, the localization

of endogenous LKB1 in larval NB differs remarkably and displays a diffuse cytoplasmic localization (Figure 4-2, E), like previously observed (Bonaccorsi et al., 2008).

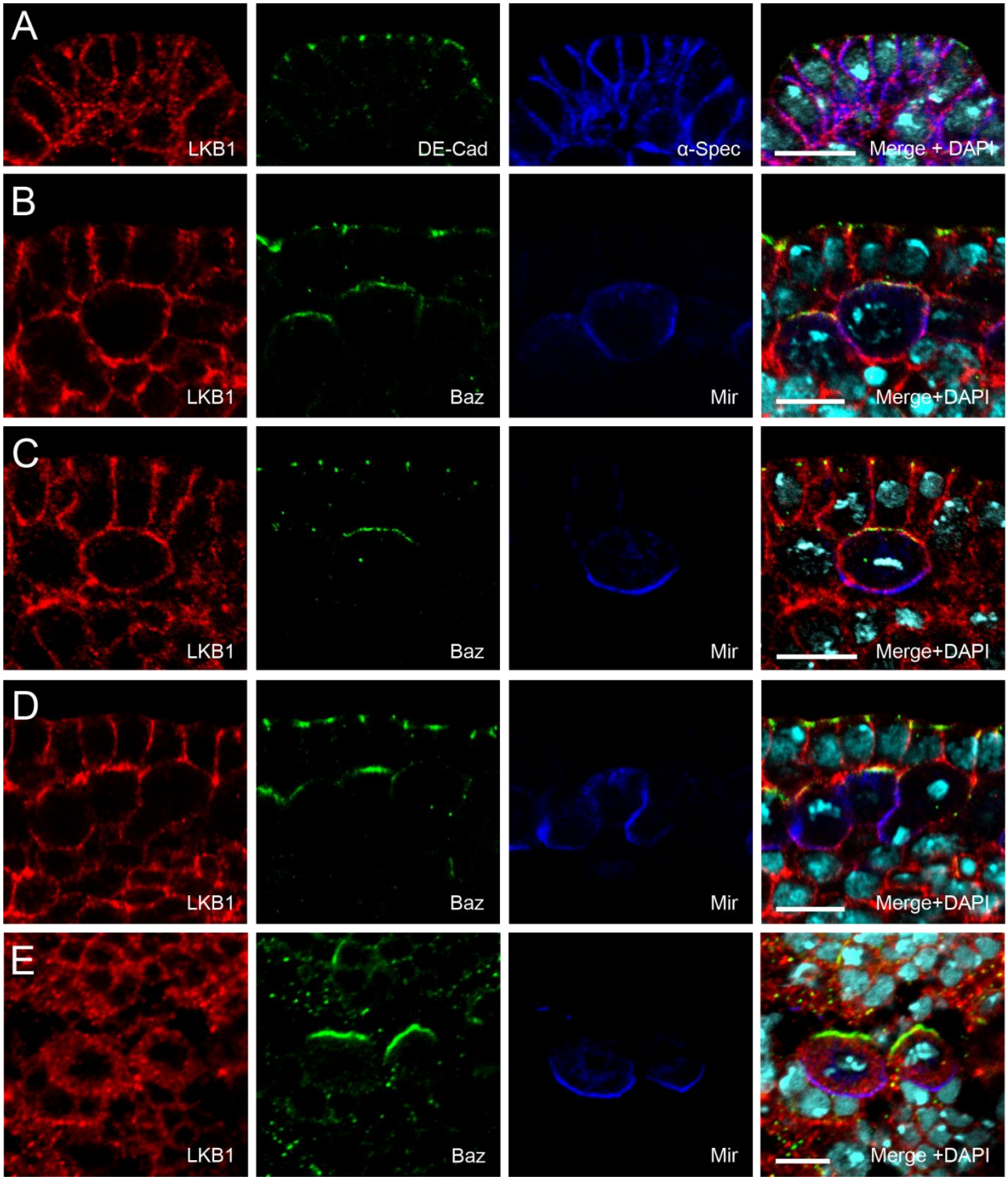


Figure 4-2: LKB1 is localized to the cortex of embryonic epithelial cells and embryonic NBs and displays a diffuse cytoplasmic localization in larval NBs. A – Embryonic epithelium, stage 12 embryo, LKB1 staining at the basolateral membrane.

B, C , D – Neuroblasts on the basal side of the embryonic epidermis in different phases of mitosis in stage 9-10 embryos display cortical localization of LKB1. B - Prophase. C – Metaphase D – Telophase (asymmetrically dividing cell). E – LKB1 staining is diffuse cytoplasmic in NBs of third instar larval brains (both NBs in metaphase). All pictures were rotated to display the apical side on top. Scale bars, 10 µm.

4.1.2 Farnesylation is not crucial for the cortical localization of LKB1 and its physiological function

Farnesylation is a posttranslational modification that adds a 15-carbon isoprenoid group to the cysteine of a CAAX motif at the C-terminus of a protein. This process is important for protein-protein and membrane-protein interactions and regulates localization and function of many proteins (Novelli and D'Apice, 2012). LKB1 has a farnesylation motif at the C-terminus that is conserved in *Drosophila* (Martin and St Johnston, 2003). A farnesylation deficient version of GFP-LKB1 has been reported to show a weaker cortical localization in the *Drosophila* germ line than the wild type version (Martin and St Johnston, 2003). To examine the role of the farnesylation of LKB1 for its subcellular localization in different developmental contexts a point mutation of its farnesyl acceptor cysteine (C564) to alanine was performed to create a farnesylation deficient mutant. We used the endogenous *lkb1* promoter to express GFP-tagged farnesylation-deficient and *wild type* genomic LKB1 and created transgenic flies with the ΦC31 integrase system, so that both constructs are in the same genomic background and should be expressed in equal amounts.

In the embryonic epidermis GFP-LKB1 localizes to the basolateral cortex of epithelial cells (Figure 4-3, F). The cortical localization of GFP-LKB1 in NBs also matches the localization of endogenous LKB1 (Figure 4-3, C). The localization of farnesylation deficient GFP-LKB1^{C564A} is astonishingly similar to its wild-type counterpart in both epithelia and NBs (Figure 4-3 A-D). In the oocyte and the follicle cells surrounding the oocyte, the farnesylation-deficient is decisively more cytoplasmic (Figure 4-3, E and F). Although the staining of egg chambers suggests an apical localization of GFP-LKB1 in follicle cells, expression of GFP-LKB1 utilizing the UASp-promoter in a tissue-specific manner reveals a lateral localization in follicle cells (driven by Cu2-GAL4, Figure 4-4 B) and a cortical localization in the oocyte (Figure 4-5, A).

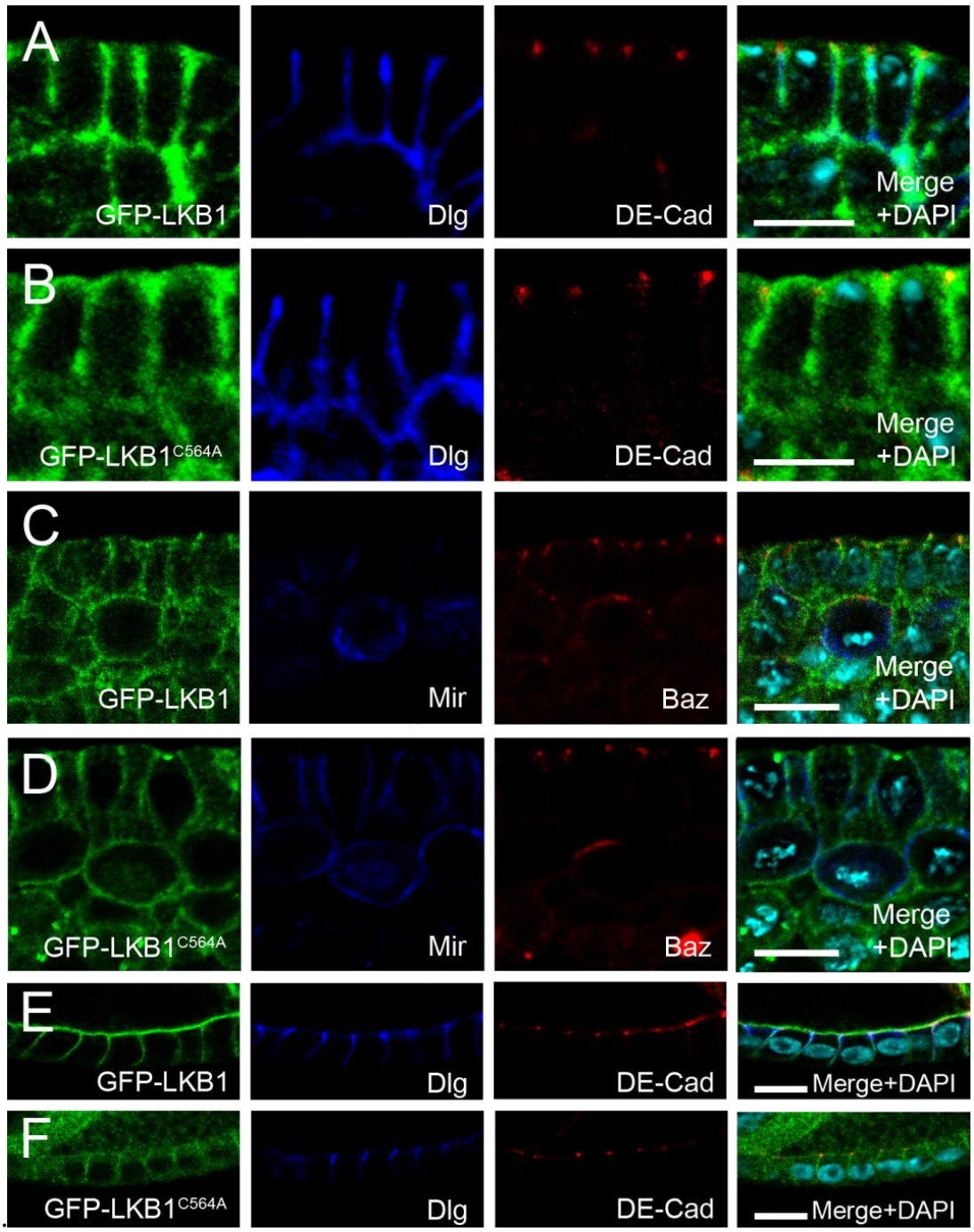


Figure 4-3: Farnesylation is not crucial for cortical localization of LKB1. A-F Rescued embryos expressing GFP-LKB1 or GFP-LKB1^{C564A} under control of the *lkb1* promoter. A, B – Embryonic epithelium of stage 12 embryos. C,D – NBs on the basal side of embryonic epithelium, stage 10 embryos. E, F – Follicle cells surrounding the oocyte. Top is apical in all figures. Scale bars in A and B, 5 µm; in C-F, 10 µm.

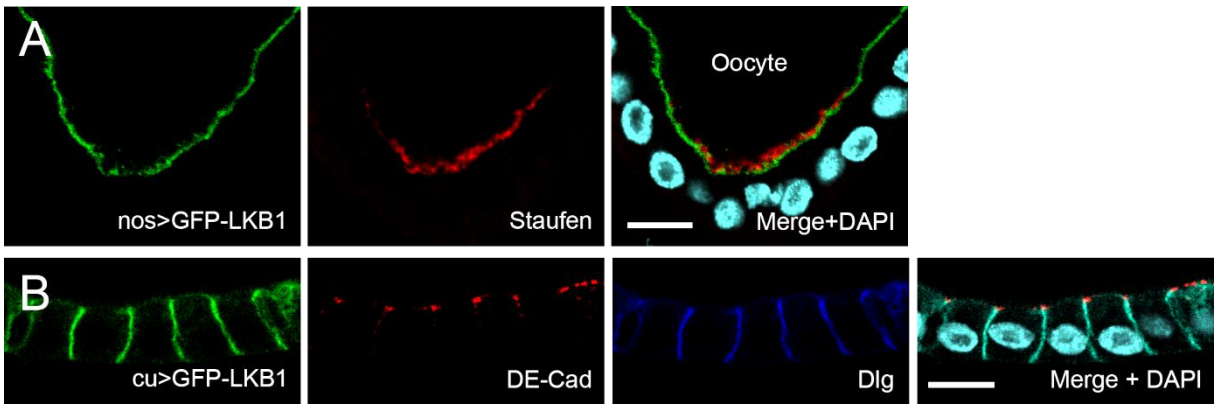


Figure 4-4: Localization of GFP-LKB1 is strictly lateral in the follicle cell epithelium. A – Expression of GFP-LKB1 driven by *nos::GAL4* shows strong cortical localization in the oocyte. B – Expression of GFP-LKB1 in the follicle cell epithelium shows clear lateral localization. Top is apical in both figures. Scale bars, 10 µm.

4.1.3 Farnesylation of LKB1 is necessary for cortical localization of GFP-LKB1 in S2R+ cells, but not sufficient

To investigate the regions of LKB1 that are involved in its cortical localization, we utilized transient expression of GFP-LKB1 and mutated counterparts using FuGENE (Promega) as a transfection reagent in Schneider S2R+ cells. Expressed under control of a ubiquitous promoter, GFP-LKB1 is localized almost exclusively to the plasma membrane/cell cortex region of transfected S2R+ cells (Figure 4-5, A). A farnesylation deficient mutant version (GFP-LKB1^{C564A}) is localized mostly cytoplasmic; only a fraction is found at the plasma membrane/cell cortex (Figure 4-5, B). To analyze if the farnesylation motif is sufficient for targeting GFP to the plasma membrane/cell cortex, a fusion protein consisting of GFP and the last 16 amino acids of the C-terminus of LKB1 (GFP-LKB1$^{552\text{-}C}$) was expressed, which was not able to target GFP to the plasma membrane (Figure 4-5, C). Farnesylation is generally not sufficient for a stable membrane localization of a protein; this usually requires a second signal, which is often located at the C-terminus of CAAX proteins (Zhang and Casey, 1996). In accordance, a longer C-terminal fragment of the last 55 amino acids of LKB1 (LKB1$^{512\text{-}C}$) is able to localize GFP to the plasma membrane, albeit showing a nuclear localization that is not observed in the full length construct (Figure 4-5, D and A). In the embryonic epithelium LKB1$^{512\text{-}C}$ is able to localize a GFP fusion protein to the lateral membrane (Figure 4-5, J).

4.1.4 A polybasic motif at the C-terminus targets LKB1 to the plasma membrane

Since GFP-LKB1$^{512\text{-}C}$ was able to localize GFP to the cortex of S2R+ cells and the embryonic epithelium (Figure 4-5 D and J), we investigated which region might be responsible for supporting the farnesylation motif in localizing LKB1 to the cortex. CAAX proteins usually need a second signal in addition to the farnesylation for plasma membrane targeting, which is usually either a palmitoylation or the presence of a polybasic domain (Zhang and Casey, 1996).

To test the hypothesis that a polybasic region (amino acids 539-551, Figure 4-5, K) at the C-terminus is involved in cortical targeting of GFP-LKB1 in transfected S2R+ cells, the effect of a deletion of this region (GFP-LKB1$^{\Delta 537\text{-}551}$) was analyzed, which lead to a complete loss of LKB1 cortical localization (Figure 4-5, F). This could also be observed in a version of GFP-LKB1, where the positively charged amino acids (lysines and arginines) of this region have been mutated to alanines (LKB1$^{K539A,\ K540A,\ K541A,\ K546A,\ R547A,\ R548A,\ K550A,\ K551A}$, termed LKB1$^{\Delta LB}$). A mutation of just on side of this region (LKB1^{K539A,K540A,K541A}, termed LKB1$^{K539\text{-}541A}$ or LKB1$^{K546A,\ R547A,\ R548A,\ K550A,\ K551A}$, termed LKB1$^{546A\text{-}551A}$) did not alter the localization remarkably (Figure 4-5, G and H).

For a biochemical characterization of the polybasic region, C-terminal constructs of LKB1 (all amino acids from amino acid 335 until the C-terminus) were N-terminally tagged with MBP, expressed in *E. coli* and analyzed in a lipid overlay assay using PIP-Strips^{TM} (Echelon). PIP-Strips are hydrophobic nitrocellulose membranes spotted with 15 different lipids. A binding to several phoshpolipids could be detected (PtdIns(5)P, PtdIns(4,5)P2, PtdIns(3,4,5)P2 and phosphatidic acid), which was reduced in a mutant version, where the polybasic region has been mutated to alanine (Figure 4-6, L). This is why we consider the polybasic motif to be a lipid binding domain and termed the constructs in which all the basic amino acids (arginine with the symbol "R" and lysine with the symbol "K") of this region were mutated to alanine (symbol "A") LKB1$^{\Delta LB}$ (Fig 4-3, K). A phosphodeficient mutation of the PKA phosphorylation site S562, which has been reported to be less functional than its wild type counterpart (Martin and St Johnston, 2003), did not alter the localization of GFP-LKB1 in S2R+ cells (Fig 4-3, I).

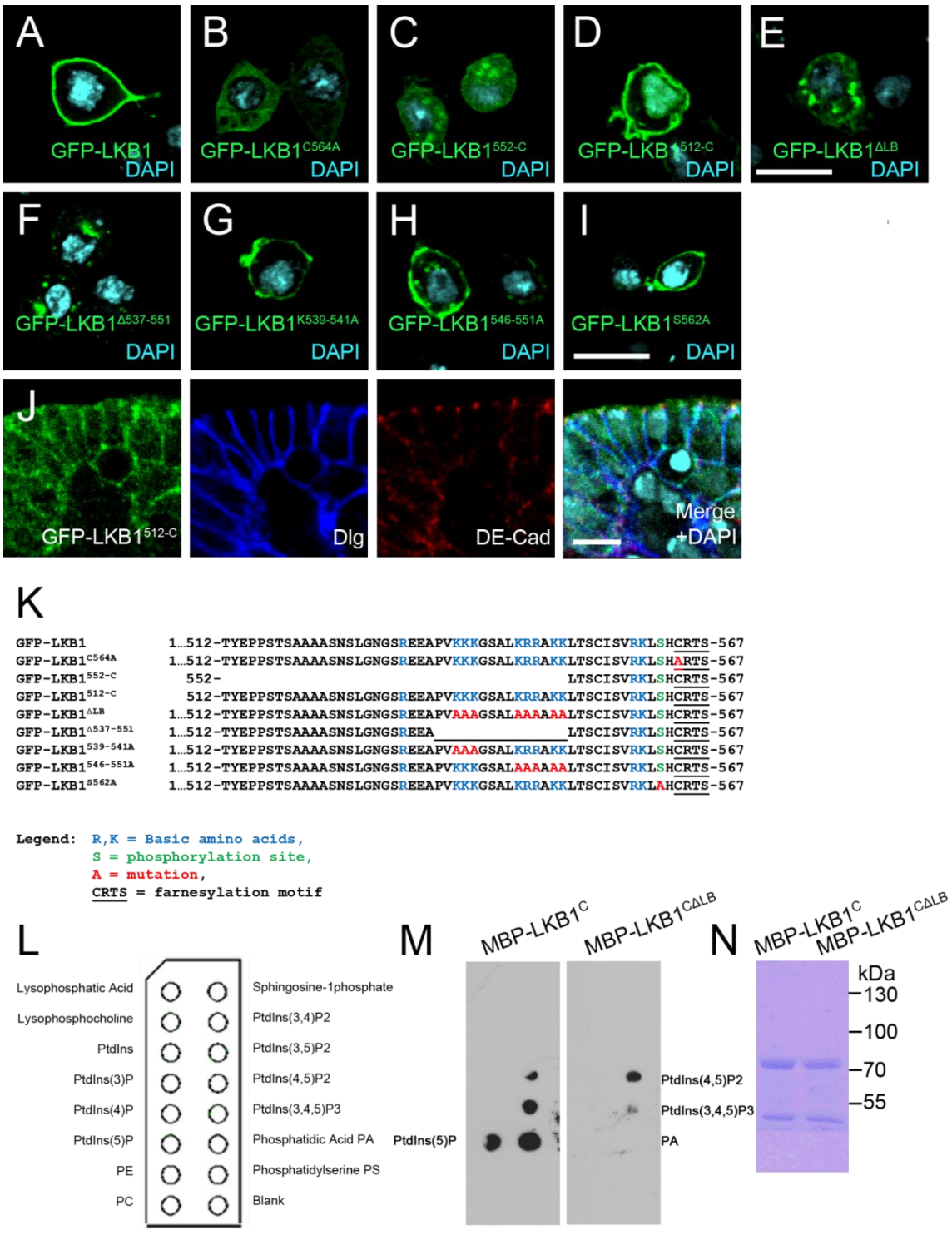
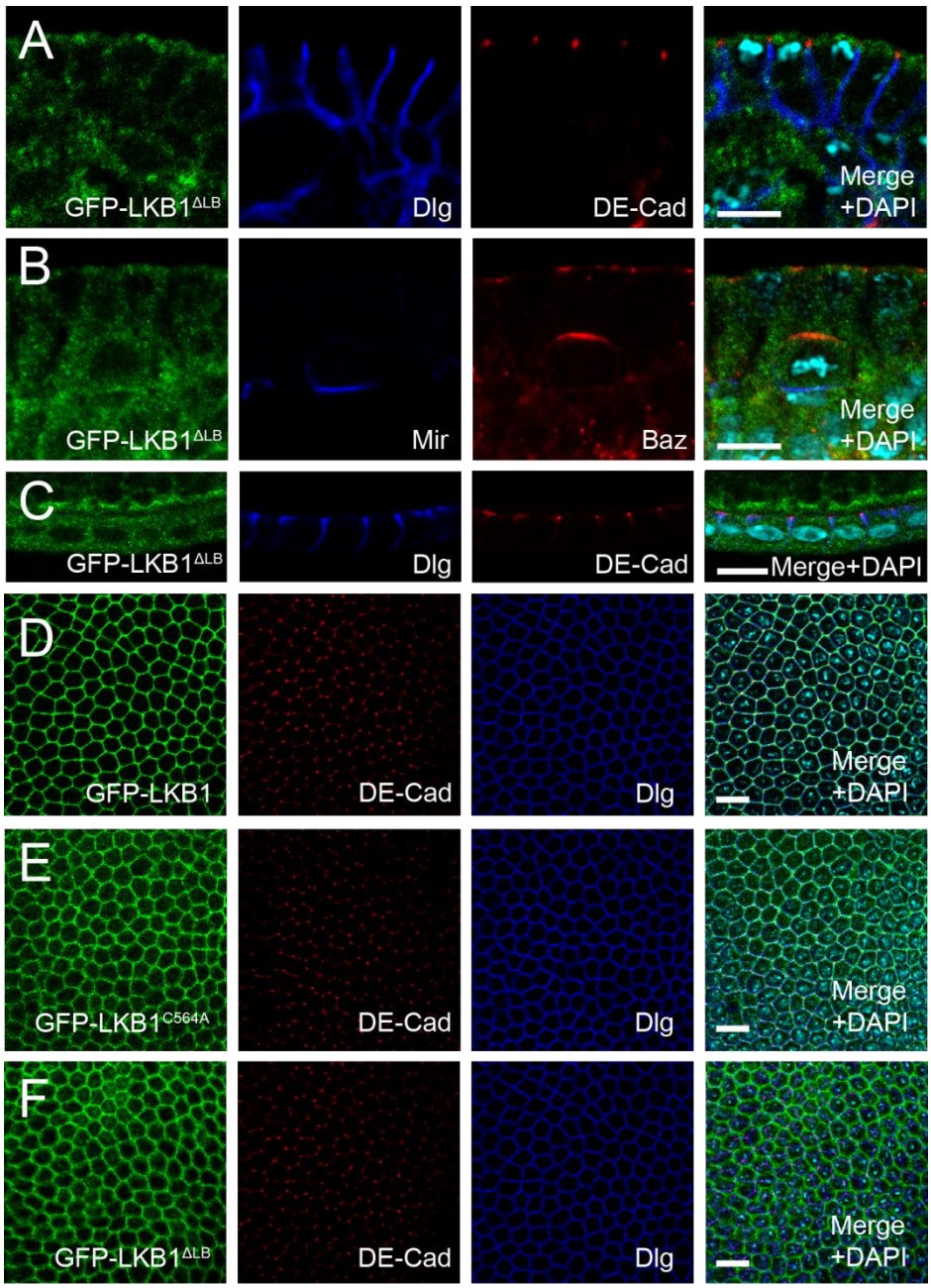


Figure 4-5: A polybasic motif at the C-terminus targets LKB1 to the plasma membrane. A-I localization of GFP-LKB1 constructs in transfected S2R+ cells. J – LKB1$^{512\text{-}C}$ is able to target GFP to the lateral membrane of transgenic embryos. Top is apical in this figure. K – Alignment of the C-termini of the constructs used in A-I. L – Schematic of the arrangement of lipids on a PIP-strip. M - Indication of bound lipids on the PIP-strips. N - Coomassie-stained acrylamide gel depicting the purified C-terminal

constructs tagged with MBP. PA – phosphatidic acid, PC – phosphatidylcholine. PE – phosphatidylethanolamine, PtdInsP – phosphatidylinositol phosphate. Scale bars, 10 µm.

When expressed under control of the *lkb1* promoter, GFP-LKB1$^{\Delta LB}$ localization was predominantly cytoplasmic. This was observed in the embryonic epithelium, embryonic neuroblasts and the follicle cell epithelium (Figure 4-6, A-C). But with expression controlled by the ubiquitin promoter, we see a weakened but still existent cortical staining of GFP-LKB1$^{\Delta LB}$ compared to GFP-LKB1 and GFP-LKB1^{C564A} at the level of adherens junctions displayed by DE-Cadherin (Figure 4-6 D-F). The ubiquitin promoter is considered to lead to a lower expression level than many promoters used in expression studies (Akbari et al., 2009), but the observed GFP-intensity is considerably higher than the expression controlled by the *lkb1* promoter.

Rescue experiments with flies homozygous for a *lkb1*-mutant allele (*lkb1*^{x5}, Lee et al., 2006) with genomic GFP-LKB1 expressed under control of the *lkb1* promoter show a reduced viability of GFP-LKB1$^{\Delta LB}$ (8% +/-1% survival until adulthood) compared to GFP-LKB1 (69% +/- 2% survival until adulthood) and GFP-LKB1^{C564A} (52% +/- 9% survival until adulthood) expressing flies at 25°C (Figure 4-6, G; data were averaged from three different experiment with 100 embryos each). Remarkably, GFP-LKB1^{C564A} is able to rescue the *lkb1*-KO-mutant to almost the same extent as the wild type version. This suggests that farnesylation is not essential for LKB1's physiological function and indicates that the lipid binding motif is more important for the function of LKB1 than farnesylation, but also not essential.


A
GFP-LKB1ΔLB
Dlg
DE-Cad
Merge
+DAPI
B
GFP-LKB1ΔLB
Mir
Baz
Merge
+DAPI
C
GFP-LKB1ΔLB
Dlg
DE-Cad
Merge+DAPI
D
GFP-LKB1
DE-Cad
Dlg
Merge
+DAPI
E
GFP-LKB1C564A
DE-Cad
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F
GFP-LKB1ΔLB
DE-Cad
Dlg
Merge
+DAPI

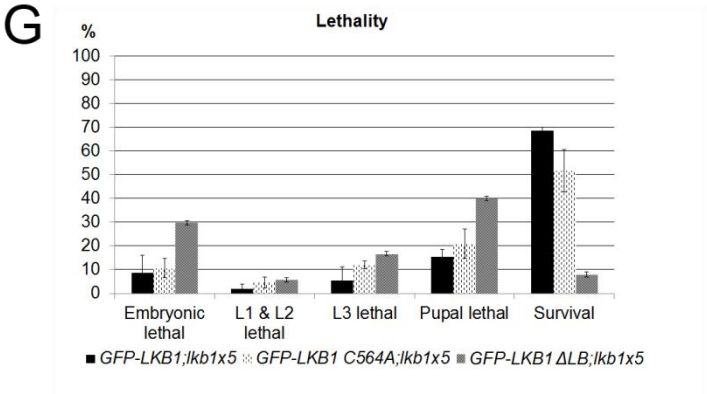
G
Lethality
%
100
90
80
70
60
50
40
30
20
10
0
Embryonic lethal
L1 & L2 lethal
L3 lethal
Pupal lethal
Survival
GFP-LKB1;lkb1x5
GFP-LKB1 C564A;lkb1x5
GFP-LKB1 ΔLB;lkb1x5

Figure 4-6 (page 70): The lipid binding domain is important, but not essential for the cortical localization and physiological function of LKB1. A-C – Rescued flies expressing GFP-LKB1$^{\Delta LB}$ under control of the *lkb1* promoter. A - Embryonic epithelium of a stage 12 embryo. B – NB of stage 10 embryo. C – Follicle cells surrounding the oocyte. D-F – Sagittal plane of embryonic epithelia expressing GFP-LKB1 constructs under control of the ubiquitin promoter, stage 9 (anterior region), lateral view. The wild type version is strictly localized to the cell cortex. G – Wild type GFP-LKB1 and farnesylation deficient GFP-LKB1^{C564A} rescue the lethality of *lkb1*^{x5} at 25°C to a large extent, while only a small percentage of flies expressing the lipid binding mutant GFP-LKB1$^{\Delta LB}$ survive until adulthood. Error bars indicate SDs. Scale bars, 10 µm.

4.2 Investigation of α-Spectrin and β-Spectrin as potential interaction partners of LKB1

In a previous study (Krahn et al., so far unpublished) co-immunoprecipitation with GFP-LKB1 from embryonic lysates with subsequent identification of associated proteins by mass spectrometry has been used to identify potential interaction partners of LKB1. Among the candidate proteins that co-immunoprecipitated with GFP-LKB1 but not with GFP alone were α-Spectrin and β-Spectrin, which are components of the membrane skeleton in *Drosophila* (Dubreuil et al., 1997, Betschinger et al., 2005). Interestingly they are found at the basolateral cortex of epithelial cells, like LKB1.

4.2.1 The lipid binding domain of LKB1 is also involved in binding of α/β-Spectrin

To verify the finding that GFP-LKB1 can co-immunoprecipitate α-Spectrin and β-Spectrin, as identified by mass spectrometry in a previous work (Krahn et al., so far unpublished), embryonic lysates from rescued *lkb1*^{x5} fly lines were prepared and GFP-LKB1 immunoprecipitated with the LKB1 antibody and Protein A sepharose beads. Both α-Spectrin and β-Spectrin were coimmunoprecipitated with GFP-LKB1 (Figure 4-7, A). The same associations can be seen with the farnesylation deficient rescue construct, indicating that Spectrins could play a role in recruiting LKB1 independent of farnesylation. But with GFP-LKB1$^{\Delta LB}$ the association with the Spectrins appeared almost completely lost. This suggests that the lipid binding domain could also interact with Spectrins.

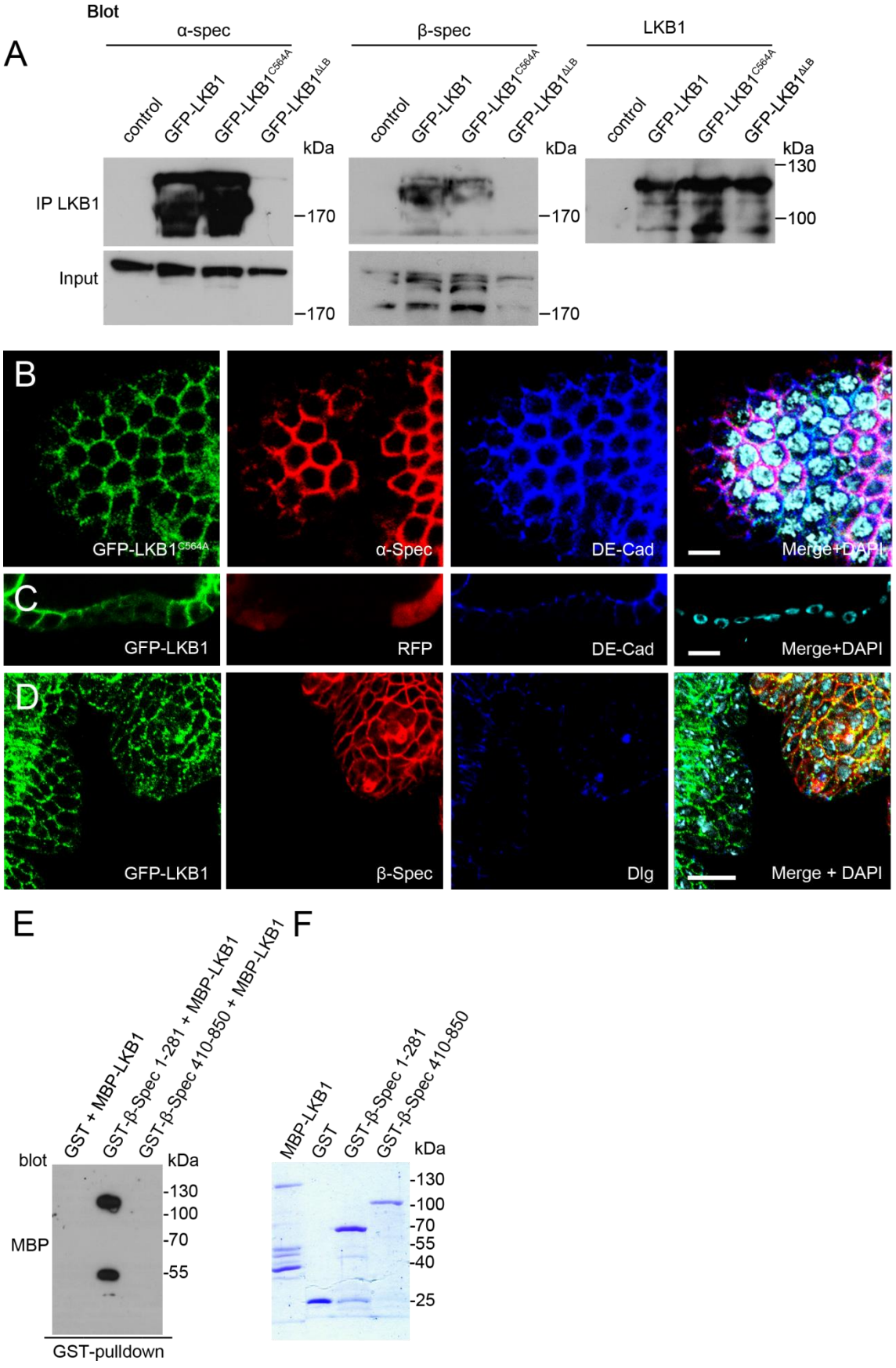
A
Blot
α-spec
β-spec
LKB1
control
GFP-LKB1
GFP-LKB1C564A
GFP-LKB1ΔLB
kDa
IP LKB1
Input
170
130
100
B
GFP-LKB1C564A
α-Spec
DE-Cad
Merge+DAPI
C
GFP-LKB1
RFP
DE-Cad
Merge+DAPI
D
GFP-LKB1
β-Spec
Dlg
Merge + DAPI
E
GST + MBP-LKB1
GST-β-Spec 1-281 + MBP-LKB1
GST-β-Spec 410-850 + MBP-LKB1
blot
MBP
GST-pulldown
F
MBP-LKB1
GST
GST-β-Spec 1-281
GST-β-Spec 410-850
70
55
40
25

Figure 4-7: LKB1 interacts with Spectrins. A – GFP-LKB1 fusion proteins were immunoprecipitated from embryonic lysates of rescue *lkb1*^{x5} flies, bound proteins identified by Western blot analysis. B – Top view on follicle cell clone of α-Spectrin, which is identifiable by loss of α-Spectrin staining. GFP-LKB1^{C564A} is still cortical. C – Cytoplasmic mislocalization of GFP-LKB1 in a *β-Spectrin* follicle cell clone, marked by loss of RFP. Top is apical. D – Embryonic epidermis of two embryos. The one in the top right corner has clearly detectable amount of β-Spectrin, the one on the left lacks a detectable β-Spectrin staining, but shows no loss of cortical localization of genomic GFP-LKB1. E – MBP-LKB1 binds directly to the N-terminus of β-Spectrin. MBP-LKB1, GST-β-Spectrin amino acids 1-281 and GST-β-Spectrin amino acids 410-850 were expressed in *E. coli* and purified. GST alone served as a negative control. F – Inputs for E are shown on a Coomassie-stained acrylamide gel. Scale bars, 10 µm.

4.2.2 β-Spectrin is involved in lateral localization of LKB1 in follicle cells

A potential role of Spectrin in targeting LKB1 to the cortex of polarized cell was investigated by genetic studies. Because of difficulties in staining the endogenous LKB1 in follicle cells, we used GFP-LKB1 expressing flies. In *α-Spectrin* mutant follicle cell clones, we did not observe a mislocalization of GFP-LKB1^{C564A}, indicating that neither the farnesylation nor a potential binding to α-Spectrin are essential for targeting LKB1 to the cortex (Figure 4-7, A). In contrast, *β-Spectrin* follicle cell clones, marked by the loss of RFP, show a cytoplasmic mislocalization of GFP-LKB1 (Figure 4-7, C). In embryos with a zygotic mutation of *β-Spectrin*, the epidermis of later embryonic stages (stage15-17) displays no detectable amount of β-Spectrin (Figure 4-7, D). Surprisingly, GFP-LKB1 is still correctly localized to the lateral membrane of these embryos.

4.2.3 The N-terminus of β-Spectrin interacts with LKB1

To identify which region of β-Spectrin binds to LKB1, pulldown experiments with purified recombinant LKB1 and recombinant fragments of β-Spectrin were performed. GST protein was used as a negative control. The β-Spectrin fragment containing amino acids 1-281, which includes the Calponin-homology domain, was able to bind MBP-LKB1. The β-Spectrin fragment containing amino acids 410-850, which includes the first two Spectrin domains was not able to bind MBP-LKB1 (Figure 4-7, E).

4.3 Three NLS regulate nuclear localization of LKB1

Although a GFP-LKB1$^{\Delta LB}$ did drastically reduce the cortical localization and viability of the flies compared to a wild type rescue construct (Figure 4-6), there were still adult survivors. This indicates that the cortical localization of LKB1 might not be essential for its function. Since many studies in mammalian cells did observe a nuclear localization and nuclear-cytoplasmic shuttling of LKB1 (Table 2-2), the nucleus might be the subcellular compartment in which LKB1 might perform its essential function for survival. This function might be executed by only a minor fraction of the total amount of LKB1, which is not observable by immunostaining.

For an analysis of nuclear shuttling of LKB1 in *Drosophila* a Leptomycin B assay was performed on transiently transfected S2R+ cells expressing GFP-LKB1 constructs. Leptomycin B is an inhibitor for Exportin-1 (CRM1) and its *Drosophila* homolog embargoed, which are required for nuclear export of proteins containing a nuclear export signal (NES). The export of mammalian LKB1 by CRM1 has been described (Dorfman and Macara, 2008). As expected, Leptomycin B treatment of cells expressing a wild type version of GFP-LKB1 leads to a nuclear localization, indicating the presence of one or more nuclear localization signals (NLS) in the *Drosophila* LKB1 (Figure 4-8, A). Predictions of potential NLS, for example with the "NLStradamus" program using a Hidden Markov Model for nuclear localization signal (NLS) prediction (Nguyen et al, 2009), yield three potential NLS in the sequence of *Drosophila* LKB1 (Figure 4-8, E). Mutation of just the homolog region of the described NLS of mammalian LKB1 (Smith et al., 1999), termed GFP-LKB1$^{\Delta NLS1}$ did reduce, but not abolish the nuclear localization of LKB1 when treated with Leptomycin B (Figure 4-8, B). A construct with a combination of this mutation and one of one side of the polybasic motif at the C-terminus, which we have identified before to be important for nuclear localization of small C-terminal GFP-LKB1 constructs, did also have a nuclear localization (Figure 4-8, C). Finally, the mutation of all three predicted NLS lead to a complete loss of detectable nuclear GFP-LKB1 (Figure 4-8. D). The subcellular localization of GFP-LKB1$^{\Delta NLS1-3}$ expressed in wild type background showed an ectopic localization to apical cortex of the embryonic epithelium (Figure 4-9, A). In NBs, the cortical localization of this construct is not affected (Figure 4-9, A).

4.3.1 Nuclear localization supports fertility and embryonic survival

To investigate the relevance of the three identified NLS *in vivo*, transgenic flies were created expressing mutant versions of GFP-LKB1 under control of the endogenous promoter. The single-NLS mutations of the first (GFP-LKB1$^{\Delta NLS1)}$, the second (GFP-LKB1$^{\Delta NLS2}$) and a double mutant of the first and third NLS (GFP-LKB1$^{\Delta NLS1,3}$) could all rescue the lethality of the *lkb1*-KO allele *lkb1*^{x5} (three independent experiments with 100 eggs for each genotype, resulting in a total number of 258, 243 and 254 fertilized eggs respectively, Figure 4-8, F).

Young rescued flies expressing the triple-NLS mutant (GFP-LKB1$^{\Delta NLS1-3}$) displayed a high number of unfertilized eggs, identified by a white and soft appearance (~52% for GFP-LKB1$^{\Delta NLS1-3}$ compared to ~7% for GFP-LKB1$^{\Delta NLS2}$ and ~8% for GFP-LKB1$^{\Delta NLS1,3}$, three independent experiments with 100 eggs for each genotype, Figure 4-8, H). The fertilized eggs of the triple NLS mutant survived to a larger extent then the double mutant. Unlike in the previously mentioned set of experiments, all three displayed a L1 and L2 larval lethality (43%, 28% and 17% Figure 4-8, G). With collections from older flies however, there were still a number of unfertilized eggs of the triple mutant (Figure 4-8, J), but a remarkably high number of lethal embryos, as observed by a brown staining of the aged egg or a developed but not hatching embryo (~47 %), that was not observed in the embryos from collections of younger flies.

4.3.2 Nuclear localization signals are involved in the activation of AMPK

We performed a Western blot assay of embryonic lysates from recued *lkb1*^{x5} embryos using a phospho-specific antibody that detects phosphorylation of the LKB1 phosphorylation site T172 on human AMPK and its counterpart in *Drosophila* (Cell Signaling Technology). The signal of phospho-AMPK in GFP-LKB1$^{\Delta NLS1-3}$ expressing embryos is remarkably lower than in embryos rescued by GFP-LKB1, GFP-LKB1^{C564A} or GFP-LKB1$^{\Delta LB}$ (Figure 4-9 C), indicating a lower activity of GFP-LKB1$^{\Delta NLS1-3}$ towards AMPK.

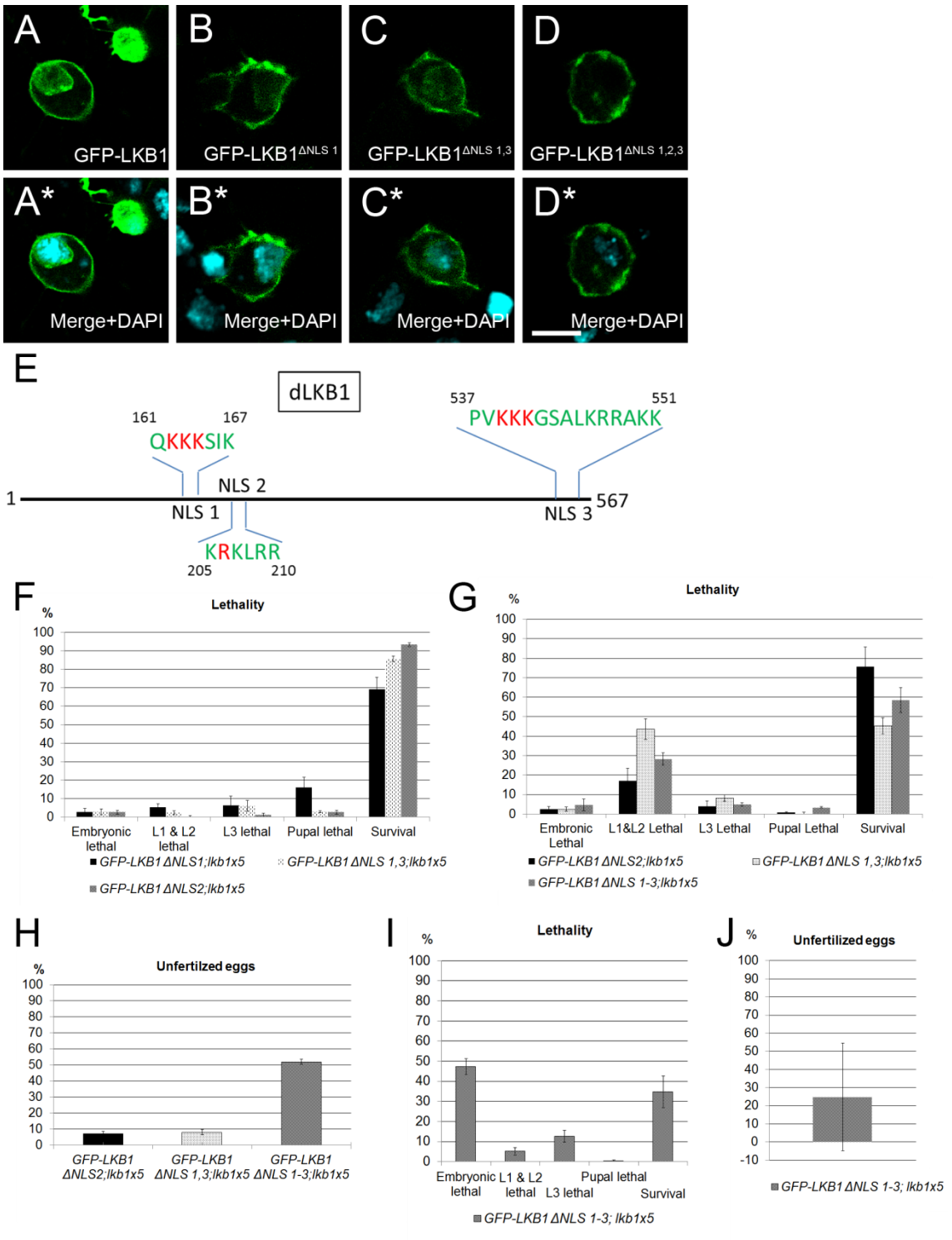

Figure 4-8: Three NLS control the nuclear localization of LKB1. A-D Transfected S2R+ expressing GFP-LKB1 constructs treated for 90 minutes with Leptomycin B. Note that all three NLS have to be mutated to lose the nuclear accumulation of LKB1. E – Schematic of the predicted NLS of LKB1, the amino acids which were mutated to disrupt the nuclear localization signal are indicated in red. F - GFP-LKB1$^{\Delta NLS1}$, GFP-LKB1$^{\Delta NLS2}$ and GFP-LKB1$^{\Delta NLS1,3}$ can rescue the lethality of *lkb1*^{x5}. G - GFP-LKB1$^{\Delta NLS2}$,

GFP-LKB1$^{\Delta NLS1,3}$ and GFP-LKB1$^{\Delta NLS1-3}$ can rescue the lethality of *lkb1*^{x5}. H - In the same set of experiments that the diagram G displays, GFP-LKB1$^{\Delta NLS1-3}$ expressing flies show a high number of unfertilized eggs in comparison to GFP-LKB1$^{\Delta NLS2}$ and GFP-LKB1$^{\Delta NLS1,3}$ expressing flies. I - GFP-LKB1$^{\Delta NLS1-3}$ expressing flies show a high number of lethal embryos. J - In the same set of experiments that the diagram I displays, the number of unfertilized eggs is highly variable. Error bars indicate SDs. Scale bars, 10 µm.

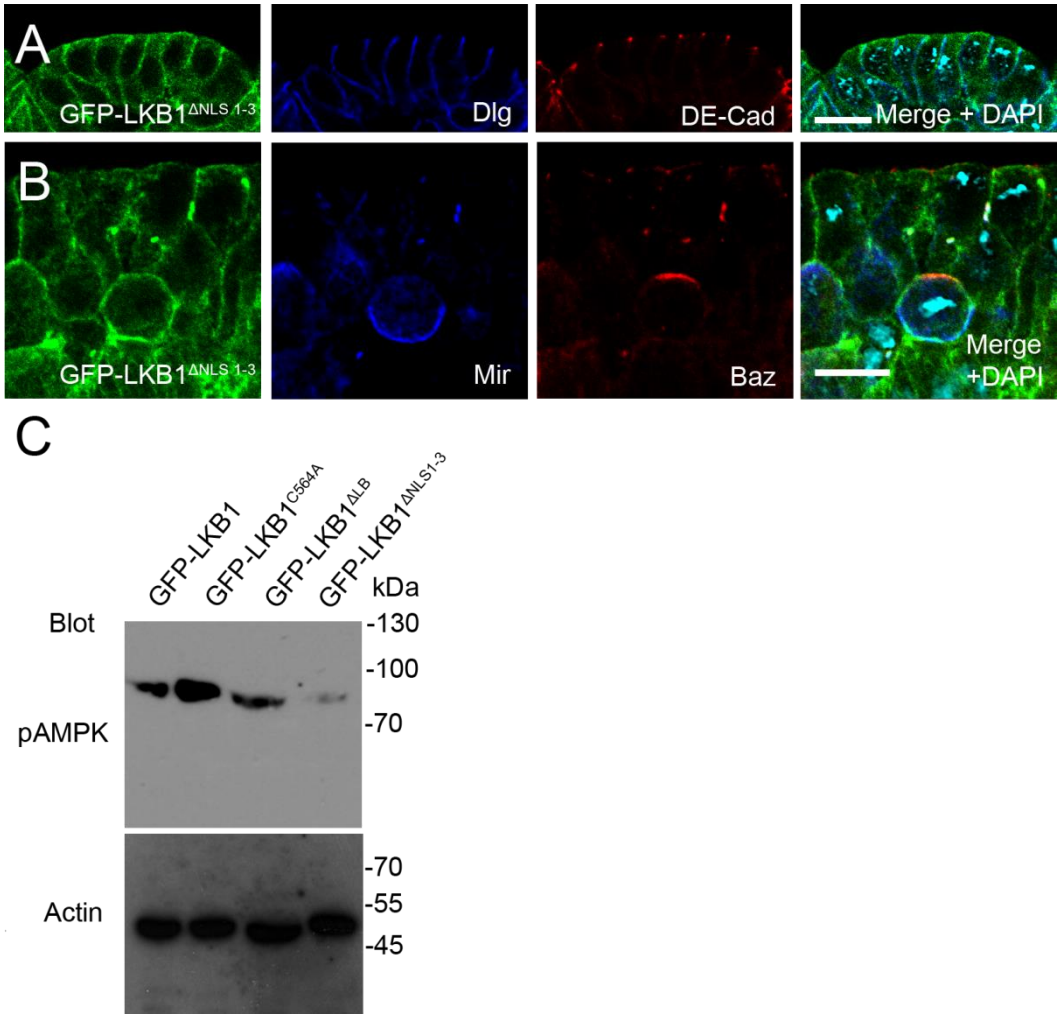

Figure 4-9: GFP-LKB1$^{\Delta NLS1-3}$ localizes to the apical surface of epithelial cells and displays a reduced activity towards AMPK. A,B – localization of genomic GFP-LKB1$^{\Delta NLS1-3}$ expressed in wild type background. A - GFP-LKB1$^{\Delta NLS1-3}$ localization in embryonic epithelium is found on the basolateral, but also on the apical cortex and cytoplasmic, embryonic stage 12. B - GFP-LKB1$^{\Delta NLS1-3}$ localization in embryonic NB is predominantly cortical, embryonic stage 10. C – The comparison of GFP-LKB1, GFP-LKB1^{C564A}, GFP-LKB1$^{\Delta LB}$ and GFP-LKB1$^{\Delta NLS1-3}$ activity estimated from phosphorylation status of the downstream kinase AMPK (anti-pAMPK T172 antibody) in a Western blot assay of lysates of rescued embryos displays a reduction of phospho-AMPK in GFP-LKB1$^{\Delta NLS1-3}$ expressing flies. 80 µg lysate loaded for pAMPK blot, 15 µg loaded for Actin blot. Scale bars, 10 µm.

4.4 Effects of LKB1 overexpression

4.4.1 LKB1 overexpression in embryonic neuroblasts and ubiquitous expression in the embryo lead to embryonic lethality independent of farnesylation, the lipid binding motif and kinase activity

LKB1 has been described to be important for asymmetric cell division and spindle formation in larval NBs, were LKB1 is localization is diffuse cytoplasmic (Bonaccorsi et al., 2007 and Figure 4-2 E). To investigate whether a neuroblast-specific overexpression of GFP-LKB1 affects viability the UAS-GAL4 system was utilized.

Furthermore we wanted to analyze, if the farnesyl-acceptor, the lipid binding site or the kinase activity are required for an effect. For this GFP-LKB1, GFP-LKB1^{C564A}, GFP-LKB1$^{\Delta LB}$ and the kinase dead GFP-LKB1^{K201M} were expressed driven by *wor-GAL4*. When expression was driven at 25°C, GFP-LKB1 increased the embryonic lethality, though the effect was not strong (33% +/- 22 % compared to 14% +/- 14% for GFP-LKB1^{C564A} and 7% +/- 2% GFP-LKB1^{K201M}, averaged from three independent experiments with a total number of 242, 270 and 262 embryos respectively; Figure 4-10, A). Expression at 29°C, which increases the activity of the UAS-GAL4 system and thereby expression of the transgenes, lead to a significant embryonic lethality in all GFP-LKB1 construct investigated (42% +/- 13% for wild type GFP-LKB1, 29% +/-7% for GFP-LKB1^{C564A}, 51% +/- 12% for GFP-LKB1$^{\Delta LB}$ and 49% +/- 8% for GFP-LKB1^{K201M}; Figure 4-10, B; three independent experiment with 100 embryos each were performed) suggesting that neither of the before mentioned mutations affect this phenotype significantly (p-value of a two tailed t-test assuming equal variances comparing the embryonic lethality of mutant and wild type counterpart is 0.27 for GFP-LKB1^{C564A} , 0.54 for GFP-LKB1$^{\Delta LB}$ and 0.70 for GFP-LKB1^{K201M}).

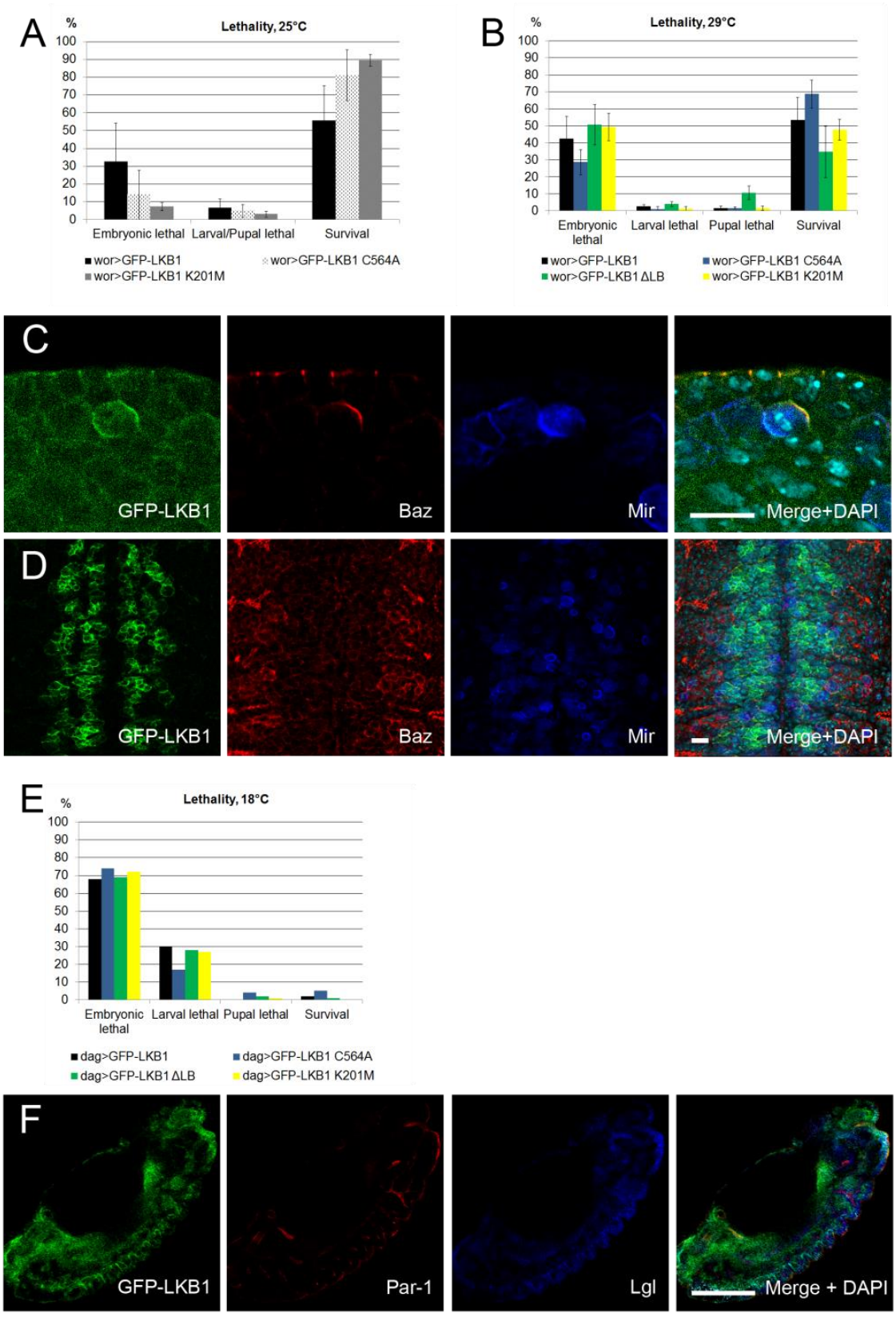
A
Lethality, 25°C
%
Embryonic lethal
Larval/Pupal lethal
Survival
wor>GFP-LKB1
wor>GFP-LKB1 C564A
wor>GFP-LKB1 K201M
B
Lethality, 29°C
%
Embryonic lethal
Larval lethal
Pupal lethal
Survival
wor>GFP-LKB1
wor>GFP-LKB1 C564A
wor>GFP-LKB1 ΔLB
wor>GFP-LKB1 K201M
C
GFP-LKB1
Baz
Mir
Merge+DAPI
D
GFP-LKB1
Baz
Mir
Merge+DAPI
E
Lethality, 18°C
%
Embryonic lethal
Larval lethal
Pupal lethal
Survival
dag>GFP-LKB1
dag>GFP-LKB1 C564A
dag>GFP-LKB1 ΔLB
dag>GFP-LKB1 K201M
F
GFP-LKB1
Par-1
Lgl
Merge + DAPI

Figure 4-10: Overexpression of GFP-LKB1 in embryonic neuroblasts and ubiquitous expression in the embryo lead to embryonic lethality. A – At 25°C overexpression of wild type GFP-LKB1 increases embryonic lethality, but GFP-LKB1^{C564A} and GFP-LKB1^{K201M} do not (data were averaged from three different experiment with 100 embryos each). B – At 29°C overexpression of GFP-LKB1, GFP-LKB1^{C564A}, GFP-LKB1^{K201M} and GFP-LKB1$^{\Delta LB}$ increase embryonic lethality (data were averaged from three different experiment with 100 embryos each). C – Expression of GFP-LKB1 under control of *wor-GAL4* is hardly detectable in earlier stages of embryonic development (epithelium of stage 9 embryo). D – During late embryogenesis expression of GFP-LKB1 is clearly visible (dorsal view of frontal plane). E – GFP-LKB1, GFP-LKB1^{C564A}, GFP-LKB1^{K201M} and GFP-LKB1$^{\Delta LB}$ expressed ubiquitously under control of *dag-GAL4* lead to a high embryonic lethality. Almost all surviving larvae die (one experiment with 100 embryos for each genotype). F – Ubiquitous expression of GFP-LKB1 driven by *daugtherless-GAL4* at 18°C. Error bars indicate SDs. Scale bars in C and D, 10 µm. Scale bar in F, 100 µm.

The effect of LKB1 overexpression driven by the ubiquitous embryonic driver *daughterless-GAL4* on the lethality was first examined at 25°C. Overexpression of GFP-LKB1 and the kinase dead GFP-LKB1^{D317A} at this temperature resulted in high embryonic lethality (4 of 280 GFP-LKB1 expressing embryos reached and died during larval stage 1, 271 embryos expressing GFP-LKB1 ^{D317A} were embryonic lethal), almost all developed until late stages of embryonic development (as seen by the formation of jaws, not counted). To exclude non-kinase effects that might result from drastic overexpression, expression at 18°C has been analyzed for GFP-LKB1, GFP-LKB1^{C564A}, GFP-LKB1$^{\Delta LB}$ and the kinase dead GFP-LKB1^{K201M}. GFP-LKB1 driven by *daughterless-GAL4* was still expressed at 18°C (Figure 4-10, F). In one experiment with 100 embryos for each phenotype some embryos survived, but most were still embryonic lethal regardless of the mutation of LKB1 (68% of GFP-LKB1 expressing flies, 74% for GFP-LKB1^{C564A}, 69% for GFP-LKB1$^{\Delta LB}$ and 72% for GFP-LKB1^{K201M}), only sporadic animals survived larval stages (Figure 4-10, E).

4.4.2 LKB1 overexpression leads to a slight reduction of eye size dependent on kinase activity

LKB1 overexpression results in slight reduction of eye size and a weak rough eye phenotype (Wang et al, 2007), that is dramatically increased when it is co-overexpressed with PAR-1 or Silnoon (Wang et al, 2007; Jang et al., 2008). The

peculiarity of this phenotype differs from an earlier study, were the overexpression of LKB1 alone has been reported to be sufficient for a significant reduction of eye size (Lee et al., 2006).

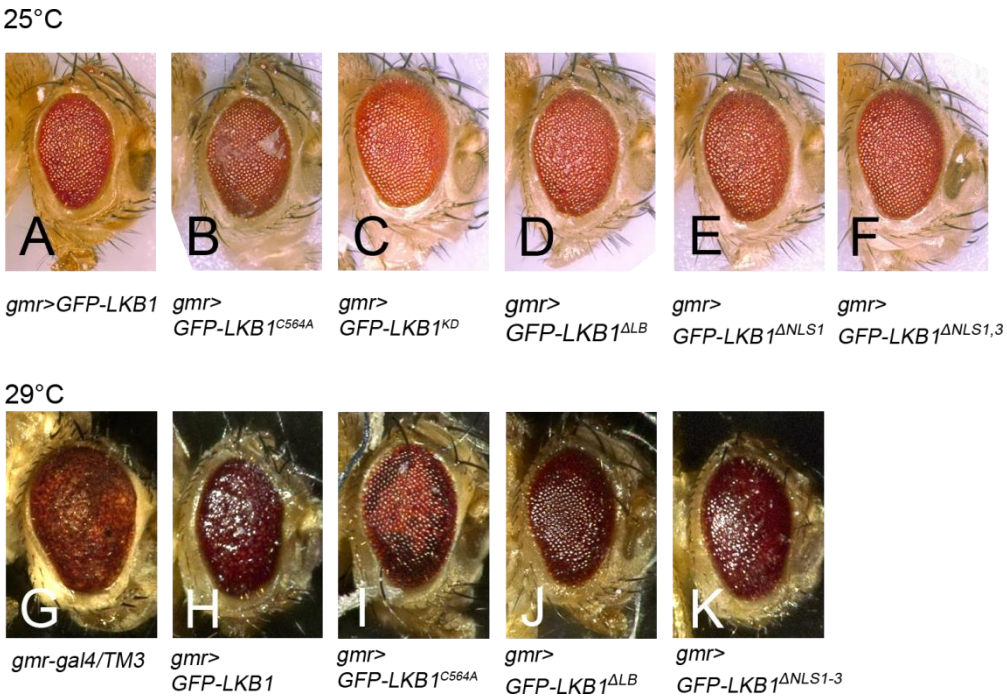

Figure 4-11: LKB1 overexpression reduces eye size depending on kinase activity. A-F Adult male flies expressing GFP-LKB1 constructs under control *gmr-GAL4* at 25°C. G-K Adult male flies expressing GFP-LKB1 constructs driven by *gmr-fal4* at 29°C.

To test whether the mutation of the farnesylation site, the lipid binding motif at the C-termnius or the nuclear localization signals are critical for the regulation of eye development by LKB1, male flies expressing the recombinant proteins GFP-LKB1, GFP-LKB1^{C564A}, GFP-LKB1$^{\Delta LB}$, GFP-LKB1$^{\Delta NLS1}$, GFP-LKB1$^{\Delta NLS1,3,}$ and GFP-LKB1$^{\Delta NLS1-3}$ were generated using the UAS-GAL4 system. GFP-LKB1^{KD} (mutation K201M, kinase dead) was used as a negative control. As previously described, GFP-LKB1 overexpression lead to a reduction of eye size and a rough eye phenotype (Figure 4-11 A), while GFP-LKB1^{KD} expressing flies had a wild type appearance (Figure 4-11 C). The phenotype of male flies that developed at 25°C appears almost identical in all investigated mutant versions except GFP-LKB1^{KD}, indicating that the kinase activity of LKB1 is necessary for this phenotype (Figure 4-11 A-F). To increase the expression level flies were kept at 29°C. Since *gmr-gal4* flies have an irregular eye development at

29°C, they were used as a negative control in this experiment. Since no obvious difference to flies expressing the recombinant GFP-LKB1 has been observed, the successive generation of flies carrying GFP-LKB1, GFP-LKB1^{C564A}, GFP-LKB1$^{\Delta LB}$ and GFP-LKB1$^{\Delta NLS1-3}$ heterozygously driven by *gmr-gal4* were again kept at 29°C and male flies with the most severe phenotype (assumed to carry to copies of the UAS-transgene) were imaged, but the peculiarity of the phenotype was not much stronger than at 25°C (Figure 4-11 G-K).

4.4.3 The reduction of wing size is minimized in the triple NLS mutant

Another organ in which LKB1 has been reported to negatively regulate size by inducing apoptosis is the *Drosophila* wing (Lee et al., 2006). To test whether nuclear localization of LKB1 is involved in this process, GFP-LKB1 and GFP-LKB1$^{\Delta NLS1-3}$ were expressed in the posterior of the wing disc driven by *engrailed-GAL4*. The homozygous driver line was thought as a negative control. Wings of adult male flies were imaged by transmitted light microscopy (Figure 4-12, A-D).

The ratio of anterior to posterior area of the adult wings of males expressing GAL4, GFP-LKB1 or GFP-LKB1$^{\Delta NLS1-3}$ was calculated (Figure 4-12, E). As expected, this ratio was higher in flies expressing GFP-LKB1 constructs (anterior area/posterior area ratio of *engrailed-GAL4* homozygous flies was 0.83 +/- 0.03, in GFP-LKB1 expressing flies 1.02 +/- 0.09 and 0.88 +/- 0.03 in GFP-LKB1$^{\Delta NLS1-3}$ expressing flies), indicating a reduction of the posterior compartment by overexpression of LKB1. Since this ratio is significantly reduced in GFP-LKB1$^{\Delta NLS1-3}$ compared to GFP-LKB1 expressing flies, the negative effect of LKB1 on organ size in the posterior compartment is reduced in GFP-LKB1$^{\Delta NLS1-3}$ expressing flies, suggesting a role of the nuclear localization of LKB1 in apoptosis. These differences were significant (p value of two tailed t-test assuming equal variances *engrailed-GAL4*/GFP-LKB1: 9.6×10^{-11}, *engrailed-GAL4*/GFP-LKB1$^{\Delta NLS1-3}$: 4.4×10^{-6} and GFP-LKB1/GFP-LKB1$^{\Delta NLS1-3}$: 5.8×10^{-7}).

The homozygous driver line displayed a reduced total wing area compared to the other fly strains (Figure 4-12 D) and displayed no or two rudimentary cross veins instead of

one connecting longitudinal veins 3 and 4 (Figure 4-12, A), indicating a non-wild type phenotype.

Imaginal discs of L3 larvae were dissected, stained with immunofluorescence and subjected to a TUNEL assay to detect apoptotic cells (Figure 4-12, F). The TUNEL staining had a high background signal; in most cases the whole wing disc was stained.

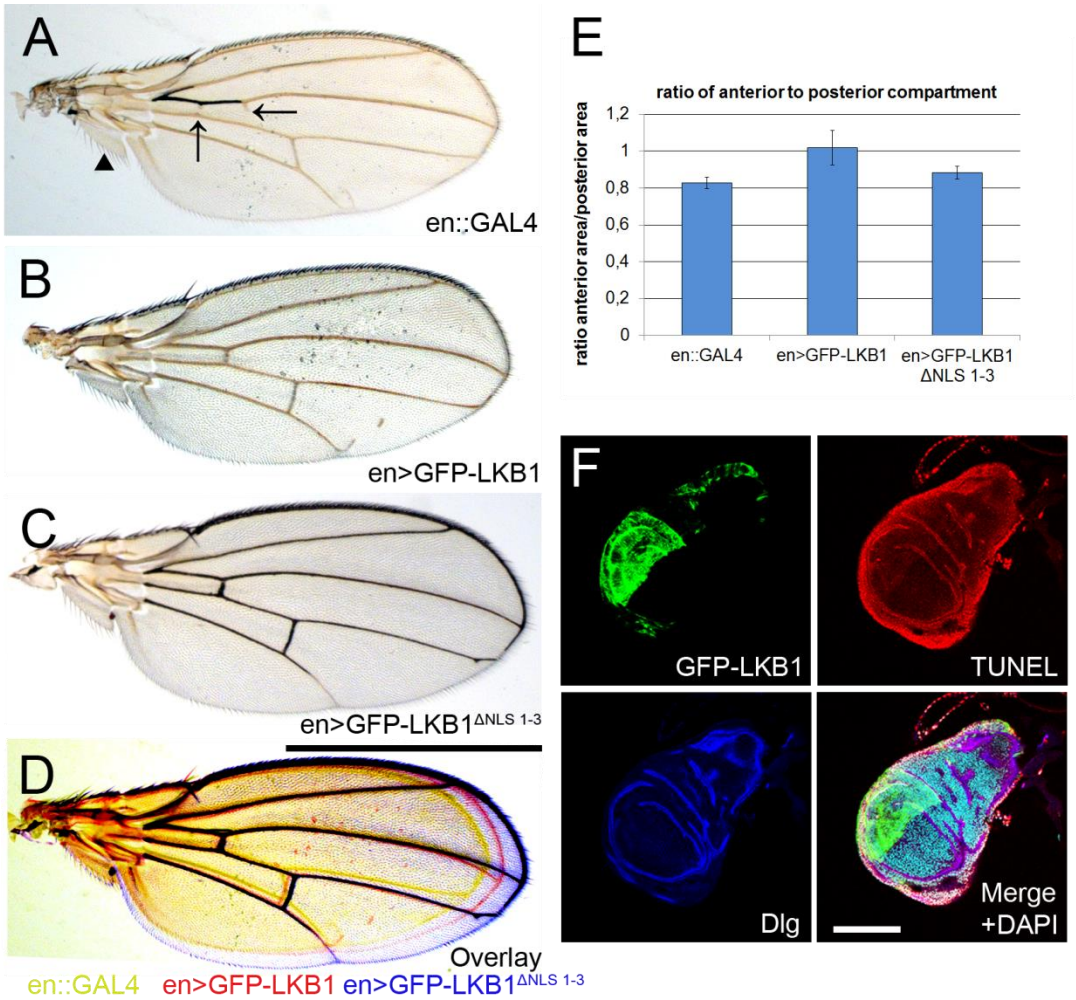

Figure 4-12: The "reduction of wing size"-phenotype by LKB1 overexpression is reduced in GFP-LKB1$^{\Delta NLS1-3}$. A-C Transmitted light microscopic images of representative wings of male flies with the indicated genotype. A – Expression of GAL4 under control of the *engrailed* promoter in the homozygous driver line leads to defect of the anterior cross vein. Arrows indicate the position of two rudimentary cross veins instead of one connecting the longitudinal veins 3 and 4. D – Overlay of the representative wings of A-C indicated by different colors. E – The posterior compartment of flies expressing GFP-LKB1$^{\Delta NLS1-3}$ driven by *engrailed-GAL4* is larger than in flies expressing its wild type counterpart, as indicated by a reduced ratio of anterior to posterior compartment (n= 21 for *en::GAL4* (homozygous), n=20 for GFP-LKB1ΔNLS1-3, n=21 for GFP-LKB1). F – A TUNEL assay of an imaginal disc expressing GFP-LKB1 driven by *engrailed-GAL4* indicates a weak increase in the

number of apoptotic cells in the posterior compartment but display a strong background signal. The arrowhead in A indicates the part of the wing that was not measured for the calculation of areas for E and F. Error bars indicate SDs. Scale bar in D, 1 mm. Scale bar in F, 100 µm.

4.5 Examination of phosphospecific antibodies against LKB1

The ability of mammalian LKB1 to autophosphorylate can be used to estimate its activity (Baas et al., 2003).To investigate the activity of *Drosophila* LKB1 in tissues by immunofluorescence and for use in Western blot analysis phosphospecific antibodies were produced, which should detect the phosphorylated autophosphorylation sites T312 or T460 of *Drosophila* LKB1. However, both investigated pairs of antibodies did show a strong staining even in embryos which lack detectable amounts of LKB1 because of *MTD-GAL4* driven RNAi knockdown of LKB1 (like described in 4.1.1).

In a Western blot analysis of embryonic lysates of flies rescued by different GFP-LKB1 constructs and a wild type control, the antibody against LKB1 phosphorylated at T460 displays at least three bands (Figure 4-14). Since these bands are also present in the negative control, only the band below 70 kDa might be specific for LKB1 (the size of *Drosophila* LKB1 is 67 kDa). This would mean that GFP-LKB1 is degraded and LKB1 can be detected in the rescued flies. A potential band of GFP-LKB1 at about 104 kDa, however, is not seen.

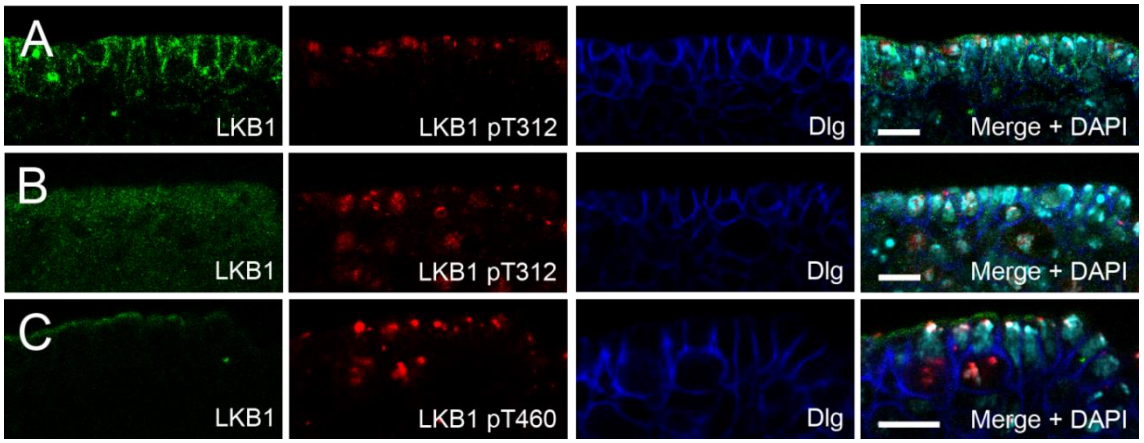

Figure 4-13: The analyzed phosphospecific antibodies are not specific for LKB1 in an immunofluorescence assay. A – Embryonic epidermis of wild type embryo expressing LKB1. Endogenous LKB1 is stained by the anti-LKB1 antibody described in 4.1.1. B, C Both phosphorylation-specific antibodies against LKB1 display a strong staining even in the absence of detectable amounts of LKB1 in the epithelium of embryos in which LKB1 expression is knocked down by MTD-GAL4 driven RNAi.

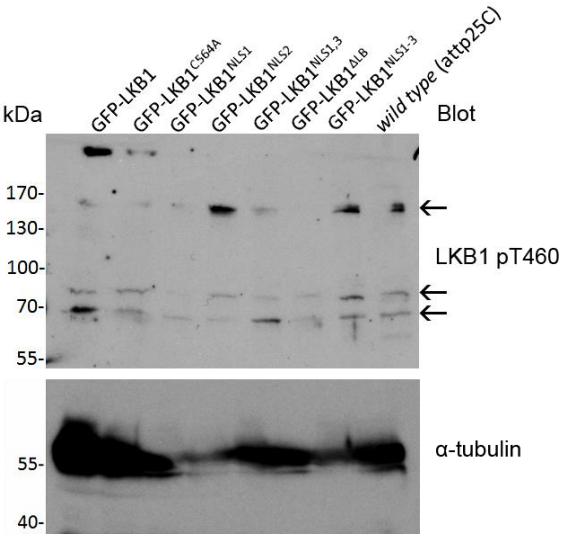

Figure 4-14: The phosphospecific antibody against LKB1 pT460 is not specific. Western Blot of embryonic lysates of flies rescued by the expression of the indicated GFP-LKB1 construct and wild type flies (right lane). Arrows indicate the position of three bands present in the rescued fly lines as well as in the wild type.

5 Discussion

LKB1 has caught a lot of attention since its discovery as the gene mutated in most cases of Peutz-Jeghers syndrome and its function as a tumor suppressor gene mutated in various types of cancer. While the role of LKB1 in energy sensing as part of the LKB1-AMPK/mTOR pathway has been studied extensively, its role as a polarity regulator has been investigated less often. This might be caused by the fact that the involvement of LKB1 in epithelial cell polarity is often not obvious in vertebrate cell lines and in mutant mice (Sebbagh et al., 2011). A study on *Drosophila* embryos, on the other hand, indicates that loss of either AMPK or LKB1 causes similar defects in epithelial cells and a constitutively active form of AMPK is able to decrease the epithelial polarity defect of *lkb-1* null mutant embryos (Lee et al., 2007). AMPK has also been suggested to regulate cell polarity in response to LKB1 signaling in mammalian cells (Nakano and Takashima, 2012). Remarkably, the activation of AMPK can occur by multiple pathways. For example, it can be activated by alternative kinases like Ca^{2+}/calmodulin-dependent protein kinase β (Hurley et al., 2005) or TGFβ-acivated kinase-1 (Xie et al., 2006), which probably contribute to some effects attributed to the LKB1 complex. Though all members of the AMPK-like kinase family and some other proteins have been identified to interact with LKB1, functional consequences of these interactions remain unclear.

In this work, the interactions of two new potential interaction partners of LKB1, α-Spectrin and β-Spectrin, have been investigated. β-Spectrin was identified as a regulator of cortical localization of LKB1 in the follicle cell epithelium. Additionally, several other factors involved in subcellular localization of LKB1 were analyzed. These are of interest because the regulation of the specific activity of LKB1 towards downstream targets is probably mediated by its localization rather than activation by upstream regulators, which could not yet be identified (Sebbagh et al., 2011). Furthermore, the physiological relevance of the farnesylation motif, a lipid binding motif, nuclear localization signals and the kinase activity of LKB1 were investigated.

5.1 Cortical localization of LKB1

5.1.1 LKB1 localizes to the cortex of epithelial cells and embryonic neuroblasts

The specific activity of a kinase inside a cell can be regulated either by directly influencing its enzymatic activity or by targeting it to certain subcellular compartments. Most overexpression studies on mammalian cell lines describe a nuclear localization of LKB1, while endogenous levels of mammalian LKB1 have been studied in just a small number of studies. Surprisingly, in these studies LKB1 was not found in the nucleus, but in the cytosol and membrane fractions (Denison et al., 2009: Sebbagh et al., 2009). GFP-tagged LKB1 has been observed to localize to the cortex of female germ line cells and to the lateral membrane in follicle epithelia cells of *Drosophila* (Martin and St Johnston, 2003). In embryonic epithelial cells and embryonic neuronal stem cells (neuroblasts, NBs) overexpressed GFP-LKB1 was found along the cell cortex (Yamamoto et al., 2008), while the localization of endogenous LKB1 has not yet been described in these cell types. Endogenous *Drosophila* LKB1 has barely been studied, due to lack of an obtainable antibody (Yamamoto et al., 2008). In NBs of *Drosophila* larvae, immunostaining for LKB1 was found diffuse in the cytoplasm (Bonaccorsi et al., 2007). It has also been observed at the subapical and basolateral membrane of the wing disc epithelium (Jang et al., 2008).

To investigate the subcellular localization of LKB1 in *Drosophila* we raised an antibody that could detect endogenous LKB1 at the basolateral cortex of the embryonic epithelium and in the cortex of embryonic NBs and therefore displayed a localization that is in accordance with the localization of overexpressed GFP-LKB1 (Figure 4-2, A-D and Yamamoto et al, 2008). In polarized MDCK cells endogenous mammalian LKB1 has been described to colocalize with the adherens junction marker E-Cadherin instead (Sebbagh et al., 2009). LKB1 is present in the early stages of embryonic development, indicating a maternal distribution, which has been assumed from genetic studies (Martin and St Johnston, 2003). Surprisingly, but in accordance with the previously described localization in larval NBs (Bonaccorsi et al., 2007), a cytoplasmic localization of LKB1 in this cell type was observed (Figure 4-2, E). This difference between larval and

embryonic NBs might reflect differences in their polarity regulation and mechanisms of asymmetric division. A similar localization is described for the transcription factor Prospero, which is localized to the cortex of embryonic but not larval NBs (Ceron et al., 2001).

5.1.2 Farnesylation is not crucial for the cortical localization of LKB1 and its physiological function

Since the upstream regulation of LKB1 remains unknown, it has been proposed that LKB1 activity is mediated by its localization rather than activation by an upstream regulator (Sebbagh et al., 2011). Posttranslational modifications mediate the localization of many proteins. One such modification is farnesylation of a C-terminal CAAX motif. A CAAX motif consists of an invariant farnesyl acceptor cysteine (C), A is usually, but not always, an aliphatic amino acid and X is a variable amino acid. The C-terminal CAAX motif of LKB1, which is conserved in most model animals except *C. elegans*, has been reported to be involved in the localization of LKB1 in the *Drosophila* oocyte (Martin and St Johnston, 2003). A recently published study investigated farnesylation of endogenous LKB1 in mice using a farnesylation specific antibody reports that the majority of LKB1 is farnesylated (Houde et al., 2014). The farnesylation has not been proven directly in *Drosophila*, but conservation of the CAAX motif and the effect on cortical targeting in the oocyte (Martin and St Johnston, 2003) strongly suggests its occurrence.

A farnesylation deficient mutation of LKB1 (GFP-LKB1^{C564A}) had surprisingly little effect on the cortical localization in all tissues investigated, although the localization to the cortex appears weaker (Figure 4-3, A-F). This suggests that there are other factors apart from farnesylation that contribute to the cortical localization of LKB1. Furthermore, the fact that lethality of flies can be rescued to almost the same extent by the farnesylation deficient GFP-LKB1^{C564A} compared to its wild type counterpart indicates that farnesylation is not essential for its physiological function (Figure 4-6, G). It might however be essential in certain physiological conditions that have not been

investigated. Similarly, mice with a knockin of farnesylation deficient LKB1 did not display an overt phenotype (Houde et al., 2014).

5.1.3 Both farnesylation and a polybasic motif target LKB1 to the plasma membrane

S2R+ cells were used as a model system for plasma membrane targeting. These cells are not polarized and do not express transmembrane proteins like DE-Cadherin and Crumbs, making them an ideal model for analysis of plasma membrane targeting (Krahn et al., 2010). Since S2R+ cell also express abundant amounts of Spectrin (Dubreuil and Yu, 1994), they have also been used extensively to study interactions with the membrane skeleton. Most studies on LKB1 focused on overexpressed of LKB1 in mammalian cell cultures, where it is mostly localized to the nucleus if no cofactors are co-overexpressed (Table 2-2). GFP-tagged *Drosophila* LKB1 however displayed a clear cortical localization in S2R+ cells. If the nuclear export mechanism for LKB1 is conserved in *Drosophila*, this suggesting that these cells express enough cofactors for the nuclear export of LKB1.

Farnesylation was observed to be necessary for membrane localization of LKB1 in S2R+ cells, but the farnesylation motif was not sufficient to target GFP to the membrane (Figure 4-5 B and C). It is known that farnesylation is generally not sufficient for stable membrane targeting; this requires a second signal, usually a palmitoylation or the presence of a polybasic domain (Zhang and Casey, 1996). In this work, a polybasic motif at the C-terminus, which is involved in membrane targeting of LKB1, was identified. A lipid overlay assay revealed a direct binding of this motif to certain phospholipids *in vitro*. Of these, phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2, PIP_2) has been reported to localize to the apical membrane of polarizing MDCK cells and proposed to be a determinant of apical identity (Martin-Belmonte and Mostov, 2007), which is surprising given the observed localization of LKB1. The binding of PIP_2 was apparently not weaker in the lipid binding mutant, suggesting the presence of other lipid interaction motifs. Another bound lipid was phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3, PIP_3), which has been

reported to be restricted to and to regulate formation of the basolateral plasma membrane of polarized epithelia (Gassam-Diagne et al., 2006). Furthermore, phosphatidic acid (PA) was bound, which is known to localize cytosolic proteins to membranes (Jang et al., 2009). Finally, phosphatidylinositol-5-phosphate (PtdIns(5)P) was identified, which is known as a signaling molecule in both the nucleus and the cytoplasm (Shisheva, 2013). As PIP-strips provide a two dimensional surface, they might not reflect lipid binding *in vivo*. A more precise model for protein-lipid interactions are membrane floatation assays utilizing liposomes (Krahn et al., 2010). The interaction of LKB1 with phospholipids has not been described before. A deficit of PIP_2 in the plasma membrane created by the absence of the phosphatidylinositol-4-phophate 5-kinase (PIP5K) Skittles, does not remove GFP-LKB1 from the oocyte cortex of *Drosophila* but delocalizes Bazooka (Gervais et al., 2008). In a similar manner the role of the other phospholipids in cortical localization could be investigated. Which of the potential interactions are relevant for LKB1 localization or signaling remains to be answered, but the membrane localization properties of phosphatidic acid and the basolateral localization of PIP_3 match with the observed localization of LKB1. GFP-LKB1$^{\Delta LB}$ could not rescue *lkb1*-KO flies to the same extent as its wild type or its farnesylation deficient counterpart, indicating a physiological relevance of this motif, though it is not absolutely essential under the given circumstances.

Farnesylation might facilitate the initial targeting of LKB1 to the plasma membrane, but for a more stable localization a polar interaction with the head groups of phospholipids in the polybasic motif could be necessary. Though GFP-LKB1$^{\Delta LB}$ is mislocalized in overexpressing S2R+ cells and in other tissues when expressed under the control of the endogenous promoter, it can still localize to the cortex of the embryonic epithelium in a weak overexpression setting (Figure 4-6, F). The question remains open whether both the farnesylation motif and the lipid binding motif are sufficient for the cortical localization of LKB1. It will be necessary to investigate if a combination of both the farnesylation- and the lipid binding motif mutations abolishes cortical localization completely.

5.1.4 β-Spectrin is involved in lateral localization of LKB1 in follicle cells

The fact that farnesylation-deficient LKB1 localizes remarkably similar in most polarized cells investigated indicates that a redundant mechanism for targeting LKB1 to the cortex exists. One possibility would be a direct binding of the lipid binding domain to phospholipids; another possibility would be an interaction with other cortically localized proteins or membrane-associated proteins.

Spectrins are known to be important for crosslinking multiple proteins and membrane lipids. A colocalization of LKB1 with Spectrin in the basolateral cortex of embryonic epithelial cells was observed. Furthermore a co-immunoprecipitation assay verified the interaction of LKB1 and α-Spectrin and β-Spectrin. Whether LKB1 binds to either one of them or to a complex of both in embryonic lysates is not known. Other factors, like other proteins and lipids could also influence binding under these conditions. The interaction was depended on the presence of the lipid binding motif at the C-terminus of LKB1. This positively charged motif could thus function both in targeting LKB1 to the membrane, by interacting with the negatively charged head groups of phospholipids, as well as to Spectrin in the membrane skeleton. Notably, this interaction could not be observed when using a Triton X-100 containing buffer, which is likely disrupting polar interactions (Koley and Bard, 2010).

Furthermore, in *β-Spectrin* mutant follicle cells we observed a loss of lateral localization of GFP-LKB1. It should be noted though that there is also a loss of the GFP-LKB1 staining apically of the follicle cells in this picture (Figure 4-7, C), which is normally also located at the cortex of the oocyte and should not be affected by follicle cell mutants. This could indicate a so-called “false clone” (Haack et al., 2013), resulting from damage of the follicle cell epithelium, but we also observed a mislocalization of GFP-LKB1 in several other follicle cell epithelia, which were not positioned for imaging in median optical sections. Since the fixation took place before disruption of the ovarioles (like recommended for avoiding these artifacts in Haack et al., 2013), it seems unlikely to be an artifact. A safer approach is the direct staining of the product of the homozygous mutant gene, like performed for α-Spectrin follicle cell clones, which

did not affect localization of LKB1. α-Spectrin and β-Spectrin are generally thought to form heterotetrameric complexes, but β-Spectrin can accumulate independently of α-Spectrin in S2 cells (Dubreuil et al., 1994). It has also been shown, that loss of β-Spectrin in imaginal discs also reduces the level of α-Spectrin expression, but not vice versa (Hülsmeier et al., 2007). It is thus not astonishing that β-Spectrin can localize in the absence of α-Spectrin and interacts with LKB1 independent of α-Spectrin. Remarkably, in embryonic epithelia lacking detectable amount of β-Spectrin GFP-LKB1 still localized cortically. The follicle cell epithelium and the embryonic epidermis might have different compositions of their lateral membrane domains, which could cause this difference. While the follicle cell epithelium is a secondary epithelium derived from mesodermal cells of the mother, the embryonic epithelium is a primary epithelium and thus they differ in some aspects of their cell polarity (Johnston and Ahringer, 2010). Maybe the embryonic epithelium has a higher content of phospholipids targeting LKB1 to the cortex independent of the presence of β-Spectrin.

Furthermore, the N-terminus of β-Spectrin (containing the actin-binding Calponin-homology domain) interacts with LKB1 *in vitro*. Because of technical difficulties only two fragments of β-Spectrin were purified and tested. There could therefore be other domains of β-Spectrin involved in binding of LKB1. The spectrin repeats are known to be involved in binding of peripheral membrane proteins. Human βI-Spectrin, for example, is known to interact with the basic motifs of the peripheral membrane ankyrin through electrostatic and hydrophobic interactions in the Spectrin repeats 14 and 15 (Ipsaro and Mondragón, 2010). Whether the interaction of LKB1 and β-Spectrin is also caused by a polar interaction, possibly with the polybasic motif of LKB1 at the C-terminus, remains to be investigated.

5.1.5 A functional role of LKB1 membrane localization

The lipid binding mutant GFP-LKB1 could not rescue the lethality of the *lkb1*^{x5} allele to the same extent as a farnesylation deficient or its wild type counterpart and had a reduced but probably not a complete loss of membrane localization. Under certain physiological conditions the membrane binding might be essential for the function

LKB1. Genetic and *in vitro* experiments suggest a phosphorylation and possible activation of LKB1 by PAR-1 (Martin and St Johnston, 2003), which is, like LKB1, present at the basolateral cortex (Shulman et al., 2000; Cox et al., 2000). The membrane localization of LKB1 might thus be necessary for a localized activation of LKB1. This could be investigated by finding the specific phosphorylation site of this kinase and investigating the localization of a phosphorylation-specific antibody. Another kinase that could regulate LKB1 is aPKC, the *Drosophila* homolog of PKCζ, which has been reported to phosphorylate LKB1 resulting in an export of nuclear LKB1 and hence AMPK activation (Xie et al., 2008). aPKC has been described to localize to the apical cortex (Chabu and Doe, 2008), where it might phosphorylate and thereby exclude LKB1 from the apical cortex, like it does exclude Lgl (Betschinger et al., 2003). Another role of LKB1 membrane targeting might be the co-localization with its substrates. AMPK has been described to be myristoylated (Mitchelhill et al., 1997; Oakhill et al., 2010), which also leads to membrane localization. Another cortical protein that has been shown to be phosphorylated by LKB1 is PAR-1 (Lizcano et al., 2004), they might thus phosphorylate each other. Furthermore, interactions with phospholipids of the membrane might target LKB1 to certain membrane microdomains and control its activity. Elucidation of this potential regulation might explain how LKB1 can be a context and tissue-specific kinase (Hermann et al., 2011).

5.2 Three NLS regulate nuclear localization of LKB1

The fact that GFP-LKB1 expressed in S2R+ cells is not localized to the nucleus suggests, if the nuclear export mechanism of LKB1 is conserved in *Drosophila*, that these cells express enough of the STRAD homolog Ste20-like kinase (Stlk) to export LKB1 from the nucleus. Since Stlk lacks key residues for kinase activity (Anamika et al., 2009), it can be assumed that it is a pseudokinase with no enzymatic activity but a regulatory function. The fact that Stlk co-iummoprecipitates with LKB1 (Krahn et al., so far unpublished) further indicates a conservation of its interaction with LKB1. Remarkably, a GFP-LKB1 with a phosphodeficient mutation of the conserved PKA phosphorylation site (S562) did not alter the localization, contradicting an importance of

this site for the nuclear export of LKB1 that has been proposed for mammalian LKB1 (Xie et al., 2008).

Nuclear-cytoplasmic shuttling of mammalian LKB1 has been studied intensively (Table 2-2), the nuclear accumulation of LKB1 is thought to be driven by a NLS in the N-teminal noncatalytic region (Nezu et al., 1999; Smith et al., 1999; Tianinen et al., 2002). Three NLS of *Drosophila* LKB1 have been identified in this study, using a Leptomycin B assay in S2R+ cells. Mutation of all three nuclear localization signals lead to a high number of unfertilized eggs and embryonic lethality in rescued flies, but the phenotype appeared to be dependent on the age of the parents. Since LKB1 is described to be essential for spermatogenesis in mice (Denison et al., 2011) the high number of unfertilized eggs might be caused by infertile males. In line with this, we had problems creating this fly stock because of infertile males and suspected a dominant negative phenotype. The triple NLS mutant protein GFP-LKB1$^{\Delta NLS1-3}$ does ectopically localize to the apical membrane in the embryonic epithelium. This might reflect a different interaction with other components involved in localization of LKB1 apart from the nuclear import machinery.

5.3 Effect on mutations of LKB1 on AMPK activity

AMPK has been described to regulate cell polarity under energetic stress conditions in response to LKB1 (Lee et al., 2007). In gastrointestinal mouse tissues, an antibody against AMPK phosphorylated at T172 (the site of phosphorylation by LKB1) displayed a localization to mitotic spindle poles, but in *LKB1* mutant intestinal tissues phospho-AMPK was mislocalized to the cell cortex of mitotic cells (Wei et al., 2012). LKB1 might thus be responsible for the localization of AMPK in mitotic cells.

Neither the farnesylation motif, the lipid binding motif nor the NLSs are essential for the function for LKB1 under the investigated circumstances, since mutant GFP-LKB1 construct of either one could rescue the lethality of the *lkb1*^{x5} allele to some extent. Nevertheless, each might be essential under certain physiological conditions, like energetic stress.

In this study the basal activity of AMPK in embryos rescued by GFP-LKB1 constructs was analyzed. The basal level of AMPK phosphorylation does not seem to be affected in the farnesylation-deficient mutant, which differs from recent observations in mouse tissues (Houde et al., 2014). In all examines tissues and cells taken from homozygous $LKB1^{C433S/C433S}$ mice the basal as well as the induced level of AMPK activation was significantly reduced, while the activity of several AMPK-related kinases was not affected. This indicates that the farnesylation of LKB1 is required for the selective activation of AMPK *in vivo*. Interestingly, the endogenous immunoprecipitated mutant $LKB1^{C433S}$ protein was able to phosphorylate a recombinant AMPK complex to the same extent as wild-type LKB1 (Houde et al., 2014), indicating that neither the kinase activity nor the direct interaction with AMPK are affected. It has therefore been proposed that the membrane association of LKB1 might promote activation of AMPK. AMPKβ1 and AMPKβ2 are known to be myristoylated (Mitchelhill et al., 1997; Oakhill et al., 2010) and might thus function to co-localize the AMPK complex and farnesylated LKB1 on a membrane surface. The C-terminus of LKB1 has been observed to be crucial for cell polarity and the AMPK pathway in mammals (Forcet et al., 2005), but for the lipid binding mutant of GFP-LKB1 we did not observe a reduction of LKB1 kinase activity towards AMPK. The triple NLS mutant however did show a significantly reduced activity towards AMPK. Since one of the NLS (NLS2) is localized in the kinase domain of LKB1 it is questionable if the kinase activity of the triple NLS mutant is generally reduced. Therefore, an assay analyzing the kinase activity of GFP-$LKB1^{\Delta NLS2}$ will be necessary.

For the phospho-AMPK blot the total amount of protein in the lysate was used to adjust protein concentrations. Since the mutations might affect protein levels (for example by increasing degradation), a blot to assess the GFP-LKB1 concentration more directly would be favorably. If this reduction of kinase activity of $LKB1^{\Delta NLS1-3}$ is specific for AMPK could probably be analyzed by using LKBtide, a synthetic peptide derived from human NUAK2 protein commonly used to measure LKB1 activity and mass spectrometrical analysis. Furthermore, recombinant AMPK and immunoprecipitated GFP-LKB1 could be used to assess if the interaction of LKB1 and AMPK is directly affected, because the *in vivo* interaction might also depend on membrane targeting, like

proposed for LKB1 and AMPK in mice (Houde et al., 2014). Possibly we would see a greater difference in the effect of mutant and wild-type LKB1 on AMPK phosphorylation in embryos under energetic stress, where LKB1 and AMPK have been shown to interact to regulate epithelial polarity (Lee et al., 2007),

5.4 Kinase dependent and independent effects of overexpression of LKB1

In this work, an increase in embryonic lethality induced by overexpression of LKB1 in the embryonic nervous system was not significantly affected by mutation of the farnesylation motif or the lipid binding motif. Furthermore, even a kinase dead version had the same effect, indicating that this phenotype is independent of kinase activity of LKB1. It has been described, that LKB1 can recruit the GC kinase Fray and Mo25 to the cortex of embryonic NBs to regulate asymmetric divisions independent of kinase activity (Yamamoto et al., 2008). This presumably affects the development of the nervous system, leading to the observed lethality phenotype. If LKB1 and Mo25 or Fray interact at the cortex, it is surprising that even the lipid binding mutation, which results in low cortical localization levels of LKB1, has the same phenotype. Since the effect appeared at 29°C but apparently not at 25°C, it is probably dependent on a high expression rate of LKB1. The effect on asymmetric cell division was not further investigated because of difficulties in imaging the GFP-LKB1 expression in the NBs of early embryos. Ubiquitous overexpression in the embryo had a more dramatic effect, resulting in high embryonic lethality. Even at 18°C, a temperature at which the activity of the UAS-GAL4 transcription machinery is decreased, most animals die at embryonic or early larval stages independent of farnesylation, the C-terminal lipid binding motif and kinase activity. High maternal expression of GFP-LKB1 has been reported to result in abnormally shrunk embryos (Yamamotao et al., 2008), which I also observed at higher temperatures (not imaged) but not at 18°C.

An effect of LKB1 overexpression that was found to be dependent of the kinase activity is a slight reduction of eye size and a weak rough eye phenotype (Wang et al, 2007), which is dramatically increased when LKB1 is co-overexpressed with PAR-1 or Silnoon (Wang et al, 2007; Jang et al., 2008). In an earlier study, the overexpression of

LKB1 alone did significantly reduce eye size (Lee et. 2006). I used the same combination of driver and promoter that was used in these three studies (*gmr-GAL4 and UAST* promoter) and observed the weak phenotype described in the Wang et al, 2007 and Jang et al., 2008. These differences might be caused by different expression levels or differences in the LKB1 construct used causing a different activity of the protein (Martin and St Johnston 2003 for example used a 540 amino acid construct of LKB1 compared to 567 amino acids in this study). The rough eye/reduced eye size phenotype of GFP-LKB1 overexpression was not significantly reduced when the farnesylation motif, the lipid binding motif or the three NLS were mutated. Since the triple NLS mutant had a probably reduced activity towards AMPK in embryonic lysates, it could be assumed that it would have a decreased eye phenotype as well or even appear like the kinase dead version. Maybe a difference of wild type and triple a mutant overexpression of LKB1 would be visible when PAR-1 or Silnoon would be co-overexpressed, increasing the severity of the phenotype. Scanning electron microscopy could be utilized to identify more subtle differences. Furthermore, the lack of a significant difference between wild type and triple NLS mutant LKB1 on eye development might be a result of reduction of LKB1 activity that is specific for AMPK. Neither knockdown of AMPK nor its co-overexpression with LKB1 affect the development of the eye significantly (Amin et al., 2009; Wang et al., 2007). Instead, polarity establishment and remodeling probably dependent on an array of targets including PAR-1 and other AMPK-like kinases (Amin et al., 2009). If just the phosphorylation of AMPK is affected by the triple NLS mutation it would thus probably not affect the eye phenotype.

Wing size can be negatively regulated by overexpression of LKB1, which induces apoptosis in wing discs (Lee et al., 2006). The overexpression of wild type GFP-LKB1 resulted in significantly smaller posterior wing compartments (as indicated by a higher ratio of anterior to posterior compartment), compared to its triple NLS counterpart, indicating a reduced induction of apoptosis by the latter. The apoptosis induced by LKB1 might thus be, at least partly, regulated by nuclear LKB1. The reduced phenotype could also be caused by a reduced catalytic activity of the triple NLS mutant, like the possibly reduced activity towards AMPK shown in embryonic lysates. The fact that the homozygous driver line displayed a reduced wing size and the loss of a crossvein

connecting to longitudinal veins compared to the LKB1 overexpressing flies is most likely an artifact resulting from GAL4 overexpression. An actual negative control would be the overexpression of GFP or a GFP-RNAi construct driven by *engrailed-GAL4*.

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7 Appendix

7.1 Table of Figures

7.2 List of tables

7.3 Abbreviations

ADP	adenosine diphosphate
AICAR	AICA-riboside
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
aPKC	atypical protein kinase C
ATM	Ataxia telangiectasia mutated
ATP	adenosine triphosphate
Baz	Bazooka
BSA	bovine serum albumin
Cdc37	Cell division cycle 37
C. elegans	*Caenorhabditis elegans*
CHIP	carboxy terminus of Hsp70p-interacting protein
CLIP-170	cytoplasmtic linker protein 170
CREB	cAMP response element-binding protein
CRTC2	CREB regulated transcription coactivator 2
DAPI	4', 6-diamide-2'-phenylindole dihydrochloride
Dbl	diffuse B-cell lymphoma
DE-Cad	DE-Cadherin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphsophate
Dlg	Discs large
Drosophila	*Drosophila melanogaster*
DTT	dithiothreitol
E. coli	*Escherichia coli*
FCC	Follicle cell clones
FLP	Flipase
FRT	Flipase recognition target sequence
GAL4	GAL4 transcription factor
GFP	green fluorescent protein
GMC	ganglion mother cell
GST	Gluthathione-S-transferase
HRP	horse radish peroxidase
Hsp90	heat shock protein 90
Hsc70	heat shock cognate 70
KD	kinase dead
kDa	kilodalton
KO	knockout
LB	Lysogeny broth
Lgl	Lethal (2) giant larvae
LKB1	liver kinase B 1
MBP	Maltose binding protein
MDCK	Madin-Darby canine kidney (cell line)
Mir	Miranda
Mo25	Mouse protein 25
MRLC	myosin II regulatory light chain

Mst4	mammalian STE20-like protein kinase 4
mTOR	mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NB	neuroblast
NES	nuclear export signal
NHS	normal horse serum
NLS	nuclear localization signal
ORF	open reading frame
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PAR	Partioning defective
PC	phosphatidylcholine
PE	phosphatidyl-ethanolamine
PCR	polymerase chain reaction
PIP	phosphatidylinositol phosphate
PIP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-triphosphate
PJS	Peutz-Jeghers-Syndrome
PKA	Protein kinase A
PtdInsP	phosphatidylinositol-phosphate
rpm	rotations per minute
Scrib	Scribble
S2	Schneider 2
SD	standard deviation
SDS	sodium dodecyl sulfate
STRAD	STE-20-related adaptor
STK11	Serine/threonine kinase 11
Stlk	Ste20-like kinase
TEMED	tetramethylethylen-diamide
TGFβ	Transforming growth factor β
Tris	Trishydroxymethyl-aminomethane
TUNEL	TdT-mediated dUTP-biotin nick end labeling
UAS	upstream activating sequence
w	white