Doctoral Thesis

Identifying downstream components of the small GTPase RHEB

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IDENTIFYING DOWNSTREAM COMPONENTS OF THE SMALL GTPase RHEB

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presented by

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Für meine Familie
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1. Summary
1.1 Zusammenfassung


Im zweiten Screening-Ansatz suchte ich nach Genen, welche heterozygot, also dominant, das durch Überexpression von Rheb im Auge erzielte verstärkte Wachstum entweder verstärkten oder abschwächten. Für diesen Ansatz verwendete ich Fliegenstämme mit molekular definierten, chromosomalen Deletionen. Mit 150 verschiedenen Deletionsstämmen werden 70 Prozent des gesamten Genoms abgedeckt. Deletionen, die zu einer Änderung des Augenphänotyps führten, wurden für zusätzliche Untersuchungen verwendet. Drei Deletionen, die das Auge entweder


Zusammenfassend habe ich einen negativen Wachstumsregulator identifiziert, der auf die Zellanzahl und auf die Zellgrösse wirkt. Dieser Wachstumsphänotyp, die gute Konservierung von Kibra in anderen Species und eine mögliche Involvierung im SWH Signalweg führen uns zur Vermutung, dass Kibra ein neuer Tumour Suppressor im Menschen sein könnte.
1.2 Summary

TOR is the central kinase of a signalling pathway that connects the availability of nutrients and the presence of growth factor signals to the growth rate of organisms. The recently identified small GTPase Rheb acts upstream of TOR and has a positive impact on the kinase activity. We have been interested in the intracellular localization of Rheb, and we have also planned to identify downstream components of Rheb transmitting the signal to TOR.

The intracellular localization of Rheb has been detected with a genomic Rheb-GFP construct and has revealed a ubiquitous intracellular distribution of Rheb. However, other research groups found Rheb localized to endomembranes.

The identification of novel downstream genes of Rheb has been approached with two different genetic modifier screens. Both screens have dealt with a reversion of a Rheb overexpression phenotype. The first screen was a lethality suppression screen. The overexpression of Rheb led to pupal lethality unless TOR signalling was reduced, for example by heterozygosity for TOR. The rescued flies were viable and fertile. With this screening system, two single hits complementing Rheb and TOR have been recovered additionally to the expected mutations in TOR. However, those genes have not been mapped, since the mapping of single hits can be a risky project.

In the second approach, I was screening for genes that dominantly enhance or suppress a Rheb induced growth phenotype in the eye. I used fly stocks with molecularly defined chromosomal deficiencies. 150 deletions stocks cover 70% of the whole genome. Deletions leading to an alteration of the eye size were chosen for further investigation. Three deficiencies were identified either reducing or enhancing the eye size. Mutant alleles of Kibra, the best candidate gene of one of the deletions, were analyzed for an alteration of the gain-of-function phenotype of Rheb. However, mutations of Kibra did not enhance the Rheb overexpression phenotype in the eye and therefore Kibra was not a downstream component of Rheb. Since homozygous Kibra mutant heads clearly displayed a growth phenotype, we have been interested in characterizing Kibra.

Kibra is conserved from Drosophila to humans and encodes a scaffold protein that is involved in protein-protein interactions. Its role in growth has not been documented so
far. Our data suggest that Kibra is a negative regulator of growth. Clones of homozygous *Kibra* mutant cells are larger due to an increase in cell number and cell size. In contrast, clones of cells overexpressing *Kibra* are decreased in size due to smaller and fewer cells. The increase of interommatidial cells in *Kibra* homozygous mutant pupal eye discs is reminiscent of mutations of genes involved in the Salvador-Warts-Hippo (SWH) pathway. This signalling pathway acts on cell proliferation, cell growth and apoptosis to restrict the growth of *Drosophila* imaginal discs. Additionally, Kibra has been found to bind Merlin, a component of the SWH pathway. Heterozygosity for SWH pathway members in a *Kibra* mutant background did not result in dominant interactions. Since the phenotype of *Kibra* is similar to that of *Merlin*, it will be interesting to analyze double mutant clones to reveal whether Kibra is involved in the SWH pathway.

Taken together, we have identified a negative growth regulator acting on cell number and cell size. Its conservation among species, its growth phenotype and the putative involvement in the SWH pathway lead us to the prediction that Kibra is a putative novel tumour suppressor in humans.
2. Introduction
2.1 Organ- and organism size control

The study of organ- and therefore of organism size has been largely neglected in the past decades, although the size difference amongst species is a remarkable feature. Vertebrates include species like the blue whale (*Balaenopera musculus*), which is the largest animal on earth with a length of 30 m and more than 100 tons of weight. On the other hand, vertebrates also include a species with a length of only 8 mm and a weight of 0.001 g. This smallest vertebrate has been published in 2004 as a member of the Schindleriidae family. *S. brevipinguis* is a fish distributed in warm-water reef habitants of the Indian and Pacific Oceans.

Large variations can also occur within species, although those individuals normally suffer from defects in size controlling mechanisms (Figure 1). However, variations in size are not restricted to large differences between and minor differences within species, species itself have been changing during evolution. The evolution of horses is a classic example. The ancestors of modern horses were about the size of a dog, and the smallest was the size of a cat. During the past 60 million years, horses have increased in average body size to become as much as ten times heavier.

These few examples illustrate the size variations amongst species and lead directly to the key question: how do organs and ultimately organisms know how large they should become and when to stop growing? This issue has not been solved yet. However, early transplantation and organ regeneration experiments suggest that intrinsic genetic mechanisms actively control organ size.
Figure 1: Dwarfism and gigantism in human beings. To the left a picture of the wedding of the most famous person with an untreated congenital growth hormone deficiency, Charles Sherwood Stratton (1838-1883). It is very likely that Stratton’s shortness was caused by a malfunction of the pituitary gland. However, at that time nobody was able to determine the medical cause of his growth problems. To the right a picture of Robert Pershing Wadlow (1918-1940), the tallest person who ever lived with a height of 2.72 m. He suffered from a tumour within the pituitary gland resulting in the production and secretion of large amounts of growth hormone (GH).

Comparisons between species display that organ size is largely determined by cell number and cell size. Organ transplantation experiments in different species have revealed that external signals like nutrition and growth signals are not the rate-limiting factors to determine the final organ size. Rat infant hearts or kidneys transplanted into adult rats obtained their correct size, indicating that the signals must be intrinsic. This could also be observed by transplantation experiments with salamanders: limbs or eyes exchanged from a smaller species to a larger species led to an organ size characteristic of the donor species. In Drosophila, immature imaginal discs transplanted into old larvae grew to a mature size as well. This suggests that vertebrates and invertebrates contain similar intrinsic mechanisms to control organ size. Furthermore, it also suggests that this size control is determined by a genetic program within each organ.

However, the results of the transplantation experiments can still be explained by a fixed number of divisions the cells undertake leading to the species-specific organ size. Therefore regeneration experiments have been performed to shed light on this
issue. The liver of a rat grows to their original size after removal of two thirds of its mass\(^9\) and similar results have been found in *Drosophila*. Wing imaginal discs can completely regenerate, even if half of the disc has been removed\(^10\). Organ size control is therefore actively monitored and is not just a result of a fixed number of cell divisions.

Experiments accelerating or slowing down the cell division rates also led to the conclusion that the mechanisms of organ size control can be distinguished from those that directly regulate cell proliferation. Organ size was maintained when cell proliferation was increased or decreased by the overexpression of *E2F* or *Rhf*, respectively. Changes in proliferation were accompanied by changes in the growth rate to keep a constant organ size\(^11\), suggesting that the intrinsic mechanism controlling organ size senses the tissue mass rather than the number of cells.

Various different genes have been examined for their ability to influence the final size of organs to better understand the mechanisms underlying organ size control. Whereas genes regulating the cell cycle cannot overcome this control mechanism, genes involved in the regulation of pattern formation can. Mutations affecting wing imaginal disc patterning alter wing size if removed or ectopically expressed. Mutant clones of *engrailed (en)* or ectopic expression of *hedgehog (hh)* or decapentaplegic protein (*dpp*) can lead to duplicated wings and mutant clones of *supernumerary limbs (slimb)* can result in additional legs\(^12-14\). In contrast, the wing organ cannot grow if the expression of *dpp* or *hedgehog* is lost\(^15\). Therefore, organ size can be severely affected by the activity of patterning genes. However, other experiments with genes involved in patterning have not led to the same conclusions. Mutations in *knot (kn)* can result in the loss of wing structures between L3 and L4 and disrupt the patterning, but they do not alter the wing size\(^16\). Therefore the control of overall size of organs is affected by genes regulating pattern formation, but not in every single case and patterning genes are also not the only class of genes monitoring organ size. In *Drosophila*, genetic screens have been used to isolate growth mutations. Amongst these, several patterning genes, members of the Salvador-Warts-Hippo (SWH) and the insulin/TOR signalling pathways have been isolated\(^17-19\). Whereas core components of the SWH pathway
affect apoptosis, proliferation and growth, the insulin/TOR signalling pathway is dedicated to the control of growth and proliferation. Components involved in pattern formation, in the SWH or the insulin/TOR signalling pathway seem to uncouple the intrinsic signal from the organ size and allow changes in final organ size.

The molecular mechanisms of how organs sense their mass are not known, but a couple of years ago a basic model was postulated by Potter and Xu. The model proposed a mass checkpoint measuring total tissue mass and coordinating cell proliferation and cell growth to lead to the correct organ size. Furthermore, they think that the total mass for an organ might be specified by genes regulating the pattern formation and that altering these genes can lead to a resetting of organ size. After an initial specification conducted by the patterning genes, the organ size control mechanism most likely functions to coordinate cellular growth and proliferation. Mutations disrupting this control mechanism downstream of the patterning genes might deregulate organ size but do not affect pattern formation.

The total mass checkpoint is most likely a major target in evolution to alter the size of organisms. It is likely that changes in this mechanism have led to the mentioned size increase in horses during the last 60 million years. However, the mechanisms of the total mass checkpoint might also be relevant for a completely different issue. Although the study of tumourigenesis has mainly focused on the regulation of cell proliferation, tumourigenesis involves an increase in total tissue mass. It is likely that deregulations of organ size control mechanisms are also important in the process of tumour development. This is supported by the fact that many components of the insulin/TOR and the SWH signalling pathways are deregulated in tumourigenesis. Therefore, the studies of organ size controlling mechanisms are crucial for the understanding of cancer and to develop novel therapeutic molecules.

Cancer is considered worldwide as the second most common cause of mortality after cardiovascular diseases. Millions of people die of cancer every year, but with large regional differences. In the year 2000, there were 10 million new cases of cancer, 6 million deaths, and 22 million people living with cancer (within 5 years of diagnosis). Lung cancer is the most common cancer in the world, both in terms of incidence and
mortality. Breast cancer is the second most frequent cancer in the world, and is by far the most common malignant disease in women. The incidence of childhood cancer remains rare. Less than 1% of all cancers in industrialized countries occur in children younger than 15 years old. Nevertheless, cancer is the second most common cause of death in childhood in developed countries.

A lot of efforts have already been invested in revealing genes of pathways involved in the genesis of cancer, but it is still a challenge to understand the mechanisms of tumourigenesis. Deregulated insulin/TOR signalling components are major players involved in the formation of cancer and many of those genes have been isolated during the last couple of years.

2.2 The insulin/TOR signalling pathway

2.2.1 General impact of insulin signalling

The insulin signalling pathway is conserved in a large variety of organisms including the two model organisms mouse and Drosophila. Whereas Drosophila has only a single insulin receptor (InR), mammals do have at least three separate receptors: the insulin receptor (IR), the insulin-like growth factor-I receptor (IGF1R) and the IRR-related receptor (IRR). The Drosophila InR has almost equivalent sequence identity to the insulin receptor and IGF1R in vertebrates and fulfils functions of both of them: growth control as well as metabolic regulation. The mammalian receptors are activated by at least nine different insulin-like peptides, with insulin and insulin-like growth factor-1 and -2 (IGF-1 and -2) as the best studied examples. In mice, the IGF1R mediates IGF-1 and -2 action on prenatal growth and IGF-1 action on postnatal growth. The IR mediates prenatal growth in response to IGF-2 and postnatal metabolism in response to insulin. Mice lacking IR are born with a slight growth retardation and their metabolic control rapidly deteriorates after birth. Inactivation of the two nonallelic insulin genes (Ins-1 and Ins-2) present in rodents results in a slight
impairment of embryonic growth and therefore to a decrease in birth weight\textsuperscript{24}. These mice die within a few days after birth.

The \textit{Drosophila} receptor also binds mammalian insulin with reasonably high affinity, which suggests that insulin-like ligands should be present as well\textsuperscript{25}. Brogiolo et al.\textsuperscript{26} identified seven conserved peptides named \textit{Drosophila} insulin-like peptides (DILPs). The amino acid sequences of these peptides are most closely related to vertebrate insulin. DILP2 displays the broadest expression pattern of all DILP\textsuperscript{26}. Ubiquitous expression of \textit{dilp2} leads to an increased body mass. The \textit{dilp3} and \textit{dilp5} are expressed in the insulin producing cells (IPCs, also called median-neurosecretory cells (median-NSCs)) and are controlled in a nutrient-dependent fashion\textsuperscript{27}. It was speculated that the DILPs may be released from the corpora cardiaca in a nutrient-dependent manner to activate the InR\textsuperscript{26}.

Flies carrying mutations that reduce insulin signalling have a growth deficiency phenotype similar to that seen in mice with disruptions of genes encoding insulin-like growth factors (IGFs) or the IGF-1 receptor\textsuperscript{25}.

\subsection*{2.2.2 Molecular function of the insulin/TOR signalling}

The IR/IGF1R and InR belong to the family of receptor tyrosine kinases. Upon ligand binding they undergo a conformational change, which enables them to bind ATP and to autophosphorylate\textsuperscript{24}. The insulin receptor substrate adaptor proteins (IRS-1-4/Chico) bind to the phosphorylated residues (Figure 2). The p85/p60 Phosphotidylinositol-3-kinase (PI3K) regulatory subunit binds IRS-1-4/Chico and thereby recruits the p110 catalytic subunit of the PI3K to the plasma membrane\textsuperscript{28}. This localization of PI3K stimulates the synthesis of PIP3 (phosphatidylinositol-3,4,5-triphosphate) out of PIP2 (phosphatidylinositol-4,5-biphosphate). PIP3 is a critical membrane-embedded second messenger able to interact with high affinity with several PH (Pleckstrin homology) domain-containing proteins\textsuperscript{29}. It triggers the translocation of PH domain-containing proteins to the membrane, and this localization enables those proteins to perform their functions in a variety of signalling pathways. Levels of PIP3 in the cell also control the amount of cellular growth\textsuperscript{30}. The amount of PIP3 is balanced by PI3K and PTEN (phosphatase and tensin homologue deleted in chromosome 10), a tumour suppressor
lipid phosphatase with specificity for PIP₃. Partial loss of PTEN function in the fly increases body size and decreases lipid and glycogen stores in the adult, suggesting that the levels of PIP₃ also control metabolism in the adult. The insulin signalling pathway involves PH domain-containing proteins, such as the serine/threonine kinases protein kinase B (PKB) and phosphoinositide-dependent kinase 1 (PDK1). Activation of PKB requires localization to the plasma membrane and phosphorylation by PDK1 and a kinase named PDK2. Sarbassov et al. recently identified PDK2 as the rictor-mTOR complex in mammals, which will be described later in more detail. In Drosophila, the phosphorylation of PKB by the rictor-TOR complex could be confirmed recently. PKB is the focal point for regulating multiple cellular processes such as cell growth and proliferation, glucose metabolism, transcription, apoptosis, angiogenesis and cell motility.

Several signals have to be integrated for a cell to grow. The insulin signalling pathway is thought to sense the uptake of carbohydrates. Another important aspect for growth is the availability of amino acids. Studies revealed a parallel pathway regulated by amino acids. A model is emerging where the full activation of growth targets requires two distinct signals, growth factor-mediated intercellular signals through PI3K and another from the amino acid sensitive pathway, ensuring that individual cells can coordinate their responses to growth factors with nutrient availability. TOR (target of rapamycin), a member of the phosphoinositide kinase (PIK)-related family (serine/threonine protein kinase), is the central component in the amino acid sensing pathway. Yeast and Arabidopsis do not have an insulin signalling system, but TOR exists in both organisms. This leads to the assumption that TOR may be a common ancestral nutritional sensor. In Drosophila, TOR mutations result in a reduction of cell size and recapitulate aspects of both PI3K-dependent signalling and nutritional sensing. In mammals, mTOR activity is also regulated by nutrient availability, and PI3K and Akt have also emerged as candidates for the regulation of mTOR function.
PKB is a putative link between the insulin/IGF and the TOR signalling pathways due to its direct phosphorylation of TSC2 (Tuberous sclerosis complex 2). This is
supposed to lead to the disruption of the TSC1/2 (hamartin and tuberin) complex and enables the activation of TOR\textsuperscript{41-43}. The tumour suppressors TSC1/2 antagonize the TOR signalling pathway, which controls the translation machinery via phosphorylation of S6K and 4E-BP1 (4E-binding protein1) in response to amino acids and growth factors. Phosphorylated 4E-BP1 releases the translation initiation factor eIF4E (eukaryotic initiation factor 4E) and allows the formation of the translation initiation complex\textsuperscript{44, 45}. Activated S6K phosphorylates the 40S ribosomal protein S6, which leads to the preferential translation of 5’ TOP mRNAs (containing an oligopyrimidine tract at the 5’end of their mRNA), which belong to a small family of abundant transcripts that primarily encode ribosomal proteins and components of the translational apparatus\textsuperscript{46}. However, this model of 5’ TOP mRNA translation through S6 has been challenged by analyzing mice double mutant for S6K-1 and -2. Although both kinases are required for the full phosphorylation of S6, the translation of 5’ TOP mRNAs is not altered\textsuperscript{47}. 

Supporting the central role of PKB, Lizcano et al. published a study demonstrating that TOR, PI3K and PKB are required for the activation of S6K by insulin in Drosophila cell lines\textsuperscript{48}. However, other authors have evidence arguing against PKB having a key position in activating one of the major downstream targets of TOR, namely S6K. Whereas S6K activity is abolished in TOR mutants, S6K activity is unchanged in Drosophila larvae lacking PKB\textsuperscript{49}. This discrepancy can be due to the comparison of results obtained in cell culture versus results received in organisms. Organisms are likely to be more complex than isolated cells cultivated in petri dishes.

In mice lacking Akt1 and Akt2, the phosphorylation and activation of S6K1 is not impaired as well\textsuperscript{50}. This leads to the assumption that PKB does not have a crucial influence on TSC2 under normal conditions and that other connections between the two pathways are likely to be discovered. PKB could only display its influence on S6K in an overexpression situation. Supporting this hypothesis, a Drosophila TSC2 protein version lacking the PKB phosphorylation site is still capable of fully rescuing TSC2 mutant animals, suggesting that TSC2 is not a critical substrate of PKB in normal Drosophila development\textsuperscript{51}. Overexpression and cell culture experiments do not always reflect the in vivo situation\textsuperscript{52}, which may have led to the previous model with PKB
being a central component acting on TOR/S6K. However, the influence of TSC1/2 on TOR and TOR’s major role, the coordination of the synthesis of ribosomal proteins as well as translational efficiency with the levels of available amino acids, are widely accepted. As a nutrient- and hormone-sensitive signalling pathway, TOR can be regarded as a central controller of cell growth.

The function of TOR includes the stimulation of ribosome biogenesis, protein synthesis, nutrient import and cell cycle progression, leading to an increased growth rate. On the other hand, TOR also regulates processes like metabolism or autophagy and receives inputs not only about the availability of amino acids and growth factors, but also about oxygen and ATP levels. Diverse signals converge at the level of TOR and if the supply of any of TOR’s inputs drops, the growth rate decreases.

The mammalian TOR is the catalytic component of two different complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The major growth-stimulating functions of mTOR are achieved via the mTORC1, which additionally includes Raptor, PRAS40 and mLST8. The mTORC2 consists of mTOR, mLST8, Rictor, mSIN1 and PROTOR. A major difference between the two complexes is the sensitivity to rapamycin, a molecule capable of inhibiting the function of mTORC1, but not of mTORC2. Other differences concern the function of the two complexes. As described, mTORC1 is mainly involved in the accumulation of biomass, whereas mTORC2 has been shown to modulate the phosphorylation of Protein Kinase C α (PKCα) and the actin cytoskeleton, therefore regulating the organisation of the cytoskeleton with PKCα as a mediator. The signals that activate mTORC2 are not known. Growth factors and nutrients do not regulate PKCα phosphorylation or the association of mTORC2.

2.2.3 Rheb – an upstream factor of the protein kinase TOR

Recently, the small GTPase Rheb (Ras homologue enriched in brain) has been found to be involved in the TOR signalling pathway. Rheb is a member of the Ras superfamily and is highly conserved in a wide range of organisms. These Rheb homologs share several key features. First, the overall sequence of these proteins is conserved. Second, the effector domain sequence is highly conserved as well. Third,
all Rheb proteins maintain a C-terminal CAAX farnesylation motif, which is thought to localize the protein to the plasma membrane. Fourth, the arginine 25 in the G1-box (S. cerevisiae), which is usually a glycine in other Ras-like G proteins, is perfectly conserved as an arginine. Mutating this arginine to a glycine results in a significant decrease in S. cerevisiae Rheb function. A mutant form of Ras with an arginine at this position is inhibited in its ability to hydrolyze GTP and remains in a constitutive active form. Therefore, it is speculated that Rheb proteins are constitutively active, which would be a unique feature among all the members of the Ras superfamily.

Recently, Rheb has been investigated in Drosophila. Clonal analysis of Rheb mutant cells has revealed that the affected cells are tiny, but differentiate properly into adult structures. In the eye, Rheb mutant tissue leads to small photoreceptor cells with normal structure and arrangement without affecting the surrounding non-mutant cells. This indicates that the small GTPase is necessary for growth in a cell autonomous manner. The sufficiency of Rheb in promoting growth has been shown in overexpression experiments. Cells misexpressing Rheb display a larger size. In mitotic tissues, Rheb accelerates the passage through G1-S phase, but the cell doubling time remains unchanged. In endoreplicating tissues, Rheb increases both DNA ploidy and cell size.

Additionally, clonal analysis of Rheb in Drosophila not only reveals a size decrease, but also an elongated shape of clones of mutant cells with thin extensions. Mutant cells may try to minimize contact to other mutant cells. Such a behaviour could not be detected for any other gene involved in growth and could be a hint that Rheb is additionally involved in cell adhesion.

In starved larvae, cells overexpressing Rheb reach a normal size in the fat body, indicating that Rheb may function in the amino acid sensing pathway. Indeed, Rheb has been shown to function downstream of TSC1/2 in the TOR signalling pathway. In both Drosophila and mice there is evidence that TSC2 functions as a highly specific GAP for Rheb. Stocker et al. happened to raise flies lacking the essential gene TSC1, if they simultaneously reduced Rheb function. This genetic experiment and the fact that TSC1/2 is a GAP indicates that the major function of TSC1/2 is the
activation of the GTPase function of Rheb. Inhibitory effects of TSC1/2 on TOR are therefore mediated through inhibition of Rheb, which in turn acts as an activator of TOR. Consistently, epistasis experiments place the small GTPase upstream of TOR\textsuperscript{56, 57}. Meanwhile, several groups could reconstruct a very similar interaction of the key components TSC1/2, Rheb and TOR in mammals\textsuperscript{65}.

The interaction of Rheb and TSC1/2 is clear, but the connection between Rheb and TOR remains unknown. As already mentioned, Rheb is an upstream activator of TOR, but how this function is fulfilled was not known at the time this study was initiated. Additionally it was not possible to find a direct binding of Rheb to TOR. This situation led us to the assumption that there were other genes existing to transmit the activation signal from Rheb to TOR. Therefore we planned to screen for new components involved in signalling downstream of Rheb.

Identifying new components acting in TOR signalling is not only of academic interest. Several diseases are linked to insulin/TOR signalling and the more components of this pathway are known, the better are the chances to find specific molecules to alter the signalling output.

### 2.2.4 Medical relevance of Insulin/TOR signalling

In humans, insulin signalling influences numerous processes, including growth and proliferation, glucose uptake, metabolism and protein synthesis. Impaired insulin signalling can result in type 1 (an autoimmune disease resulting in impaired insulin secretion) and type 2 diabetes (insulin resistance), whereas increased signalling can lead to cancer in various tissues. The next two chapters are focused on a brief summary of the two main insulin signalling diseases.

#### 2.2.4.1 Insulin resistance

Diabetes mellitus is a disease that was described over 3’500 years ago in a compendium of medical diseases acquired in Luxor in 1872\textsuperscript{66}. The disease is a disorder caused by the body’s inability to lower sugar levels in the blood. Untreated
patients exhibit high concentrations of sugar in the blood and urine and therefore the disease was also called “sugar disease”.

In 1857 Claude Bernard suggested that altered glucose metabolism was the cause of diabetes, and 1869 the Islets of Langerhans in the β-cells of the pankreas were discovered by P. Langerhans. Others discovered pancreatic and islet abnormalities in diabetic patients. This led to the assumption that the active substance was found in the islets and hence it was named insulin (from the Latin word for island). Later researchers started to experiment with depancreatized dogs. After many failings, the dogs blood sugar levels could be lowered by giving them extracts from the islets of Langerhans. In 1923, two years after the success of Banting & Best, insulin was produced commercially and used to treat diabetes in most western countries. Diabetic people had hopes to live a normal life, and the Canadian scientists received the Nobel price for one of the most revolutionary discovery in medicine.

At the molecular level, the insulin receptor is responsible for mediating the first step in insulin action and it has received the greatest attention in the pathogenesis of insulin resistance. Besides diabetes mellitus, insulin resistance can have other consequences. Humans lacking the IR gene are severely growth retarded at birth and gain little if any weight thereafter. Leprechaunism and the Rabson-Mendenhall’s syndrome belong to the most severe forms of this disease. They are characterized by insulin resistance and growth retardation, and both are caused by mutations in both alleles of the IR gene.

### 2.2.4.2 Tumourigenesis

The insulin/IGF signalling system takes a central role in many aspects of tumourigenesis. Deregulated IGF signalling can lead to the development and progression of human cancer. High levels of circulating IGF-1 and -2 constitute a risk factor for the development of breast, prostate, colon and lung cancer. IGF-2 is also commonly expressed by tumour cells and may act as an autocrine growth factor. One of the clearest examples of how disrupting insulin/IGF signalling leads to severe consequences in humans was provided by the discovery of the tumour suppressor gene PTEN. Germ-line mutations of PTEN are associated with three rare autosomal
dominant inherited cancer syndromes with overlapping features: Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndrome. All syndromes are characterized by hamartomas and benign tumours. It has also been reported that Cowden patients have a high risk of breast cancer. Somatic mutations of *PTEN* are frequently found in a large variety of human cancers. *PTEN* is one of the most common targets of mutations, with a frequency approaching that of p53. In prostate cells, the extent of PTEN inactivation correlates directly with the progression and latency of prostate cancer. The loss of *PTEN* in humans is thought to result in tumour development primarily through defects in Akt-mediated cell-cycle control and apoptosis. Therefore *Akt* is another well-studied gene involved in cancer development. Analysis of the PI3K/Akt pathway in 450 tumours from eight different tumour types has shown that Akt was activated in 55% of these samples. Recently, a direct role of Akt1 in human cancer has been described. The authors have discovered a somatic mutation of the human Akt1 in breast, colorectal and ovarian cancer. The mutation in the PH domain localizes Akt1 constitutively to the membrane and results in deregulated downstream signalling.

Metastasis is the most common cause of death by cancer and has attracted a lot of interest. Metastasis occurs when genetically unstable cancer cells adapt to a tissue microenvironment that is distant from the primary tumours. In human breast cancer tissues, elevated levels of phosphorylated Akt are found in metastatic tissue compared to the primary tumour. Akt phosphorylation is more sensitive to mTORC2 inhibition in metastatic tumour cells than in primary tumour cells. This is due to the fact that mTORC2 acts positively on Akt via phosphorylation.

Another study focused on the invasion of prostate cancer cells in human prostate tumours. Treatments with PI3K inhibitors suppressed the invasive properties of the cells. Expression of a dominant-negative Akt gene could also inhibit the invasion. The authors also found increased protein levels of PI3K and constitutive Akt activation in high-grade tumours compared to low-grade tumours and benign tissues. Deregulation of the PI3K/Akt signal transduction pathway may therefore contribute to a large...
fraction of human cancers. Furthermore, activation of PI3K/Akt signalling may contribute to increased cell invasiveness and facilitate cancer progression.

2.2.5 Open questions

Many questions remain unsolved with respect to the TOR signalling branch of the insulin/TOR signalling pathway. For example, it is known that the kinase TOR is activated by nutrients, namely amino acids. Whether this is a direct or indirect activation is still unclear. In addition, it is not likely that all the pathway members have been found and described yet. Since genes of the TOR branch can be involved in the genesis of cancer, it is crucial to understand their function and to identify all the genes involved. This is the basis to produce therapeutic molecules to inhibit increased signalling in cancer cells.

Rheb is a recently identified member of the TOR signalling branch and we decided to proceed the investigation of the small GTPase\(^{50}\). This protein might be predominantly in the GTP bound form and may therefore not be mainly regulated via a guanine exchange factor (GEF) protein\(^{54, 55}\). Another mechanism is likely to exist. A possibility would be to control the localization of Rheb. It is important to understand the regulation of the small GTPase. Since the activity of Rheb can be deregulated in cancer cells, Rheb is a good target for therapeutic interventions. Molecules delocalizing Rheb from the membrane are known and in case Rheb’s activity is regulated via its localization, those molecules would be potentially of therapeutic use\(^{81, 82}\).

Several groups could show that the TSC1/2 complex is a direct upstream component and functions as a Rheb GAP\(^{58, 60, 61}\). On the other hand, Rheb had a positive impact on the activity of TOR, but nothing was known about the mechanism. Therefore we planned to focus on this issue. Since at the time when I started my PhD no binding of Rheb and TOR could be detected, we expected other genetic components to be downstream of Rheb and upstream of TOR. Those unidentified proteins are putative drug targets in cancer cells with upregulated insulin/TOR signalling and thus it was a promising project to screen for novel downstream genes of Rheb.
2.3 Insulin and TOR signalling in the model organism

*Drosophila*

2.3.1 Why using *Drosophila* as a model organism?

Disturbed insulin signalling in mice has been instrumental in dissecting the pathogenesis of diabetes and cancer in humans. Mouse embryos deficient for the tumour suppressor gene *PTEN* exhibit embryonic lethality. Heterozygous *PTEN* mice are viable but develop spontaneous tumours. They display changes in the prostate, skin and colon, which are also observed in Cowden disease patients. Cell lines derived from patients contain elevated levels of PIP3, which correlates with increased activation of Akt. Another success was the reversal of hyperglycemia in diabetic mice via inhibition of *PTEN* mRNA expression. These data indicate that PTEN is a relevant target for modulating mammalian insulin signalling diseases.

Akt knock-out mice are of great value as well. Mice do have three isoforms of Akt (Akt1-3) with similar biochemical characteristics. Both Akt1-null and Akt2-null mice are viable. Akt1-deficient mice exhibit a growth deficiency and a normal glucose metabolism, whereas Akt2-deficient mice exhibit insulin resistance, mild diabetes and a mild growth deficiency that persists throughout life. Alterations in Akt2 mimic some important features of type 2 diabetes mellitus in humans, which demonstrates the reliability of the mouse model. Double-knockout (DKO) mice (Akt1, Akt2) display the additive contributions of these two distinct genes. However, these DKO mice have a more severe growth retardation and die soon after birth. It is possible that this phenotype can only be revealed if Akt activity is reduced below a certain threshold. The phenotypes of these animals are similar to those of *IGF1R*-deficient mice. DKO mice have impaired skin development due to a proliferation defect, a decrease in individual muscle cell size and impaired bone development, demonstrating roles for Akt in cell proliferation, growth and differentiation. For more information, we have to await the triple-knockout mice.
Achievements in mice raise the question why people do research in *Drosophila*, which is not as closely related to humans as the mouse and lacks genes or regulatory mechanisms mice and humans do have in common. However, approximately three-quarters of human disease loci have counterparts in the fruitfly. Metabolic pathways change little during evolution, and the resulting conservation of genes enables us to experimentally approach a large range of human diseases in the fly. *Drosophila* has a lot of advantages based on its completely sequenced genome, the huge collection of already obtained mutations, and the large variety of useful techniques to silence or overexpress genes in target tissues. Other related advantages are the compact genome of *Drosophila* with a far smaller extent of redundancy compared to mammals, and the ability to screen individual chromosomes for mutations at a distant site that enhance or suppress the phenotype of an existing mutation. In vertebrates, no comparable system is available for this approach. The generation time is too long, and the costs would be exorbitant. The lack of a large collection of mapped mutations is an obstacle as well.

Most excitingly, novel components of signal transduction pathways can be discovered in the fly by genetic means. For example, *TSC1* and -2 have been characterized in mammalian cell culture, but there were no data linking these genes to the insulin signalling pathway, until genetic analysis in *Drosophila* revealed that TSC1/2 were involved in TOR signalling. The identification of novel proteins that play a role in conserved signalling pathways belongs to the most important tasks, because members of those signalling pathways may represent new therapeutic targets for human diseases.

### 2.3.2 Planned projects in the model organism *Drosophila*

My PhD was focused on two main aspects connected to the small GTPase Rheb. First, we planned to elucidate the mechanism of how Rheb is regulated. Because it was suspected that Rheb is constitutively active, a possible regulatory mechanism could involve a controlled localization of the protein. Therefore we generated a tagged Rheb fusion construct and examined its localization in vivo and in vitro to clarify the aspect of Rheb regulation via controlled localization.
The second project was the major part of my PhD. The aim was to identify new downstream components of Rheb, which could help us to understand how Rheb signals to TOR. We established two different screening approaches, both of them relying on the overexpression of Rheb. The first screen was a lethality suppression screen. Overexpression of Rheb can lead to lethality depending on the strength of expression. We established conditions in which animals overexpressing Rheb died during the pupal stage. Such pupae could be rescued and were fertile when they were heterozygous for TOR. We expected to find mutations in novel genes that were also able to rescue the lethality. Those genes could act in the TOR signalling pathway.

The second screening system was established in the eye of Drosophila. A construct overexpressing Rheb specifically in the eye was used to generate big-eyed flies. Those flies were crossed against two different sets of isogenic deletions and their progeny was screened for growth alterations in the eye. As a control, the Rheb overexpressing flies were crossed to TOR, Raptor and TSCI mutants to test for growth alterations. These TOR signalling mutations were able to decrease or increase the size of the eye, respectively. Therefore we expected to identify deficiencies deleting genes involved in TOR signalling.

We expected that these two screens would lead to an overlapping set of new TOR signalling components. Since the mapping of EMS mutations can be time consuming, we hoped that the mapping would be more rapidly by complementation analysis between mutants found in the lethality suppression screen and positive deficiencies identified in the deletion screen.

### 2.4 Summary of new findings

The localization experiments of Rheb led us to unexpected findings. In vivo and in vitro Rheb is uniformly distributed in the cells. Since Rheb contains a membrane-localization signal at the C-terminal end (CAAX domain), we expected the protein to be localized at membranes. This discrepancy will be discussed in 3.1.5.

In these screens, we identified several new mutations in Rheb and TOR, demonstrating that the screening paradigm was well chosen. Two mutations on the X and on the 2\textsuperscript{nd}
chromosome, respectively, were isolated in the lethality suppression screen. Both mutations were able to suppress the lethality caused by the overexpression of Rheb. Additionally, they could dominantly decrease the head size of flies overexpressing Rheb in the eye. Therefore both of them are good candidates for novel genes involved in TOR signalling. Since we did not find a complementation group, we did not start to map the mutations and rather focused on a promising candidate gene found in the deletion screen.

The deletion screen led to the identification of three deficiencies altering the eye size of Rheb overexpressing flies. One of these deficiencies, which enhanced eye size, deleted the gene Kibra, which was previously found in a screen for negative growth regulators. We investigated its role in TOR signalling. Mutants of Kibra were not able to enhance the eye phenotype of Rheb overexpressing flies and the phenotype of Kibra mutant cells did not display the characteristics of TOR signalling mutants. Therefore Kibra appears not to be involved in TOR signalling, but it showed a growth phenotype that we wished to characterize further. Due to its unique mutant phenotype we were not able to define its involvement in one of the known signalling pathways. However, regarding the behaviour of the Kibra mutant cells and the conservation of the gene in humans, we predict that Kibra could be a gene deregulated in cancer cells.
3. Results and Discussion

We planned to investigate the function of Rheb in the TOR signalling pathway. On one hand, we established two different genetic screens to identify novel downstream genes of Rheb. The screening procedures differed strongly from each other. One of the screens was an EMS screen, whereas the other screen involved screening a set of deletions for interactions with a sensitized system based on the overexpression of Rheb. We expected the two screens to lead to an overlapping set of new candidate genes. Complementation analysis between candidate deletions and the EMS mutations should eventually lead to cases of non-complementation. This would give us a confirmation that the screening setups were well chosen. Additionally, the EMS mutation would already have been mapped to relatively small candidate intervals.

On the other hand, we were interested in the regulation of Rheb. Rheb is a small GTPase predominantly bound by GTP and might therefore not be regulated by a GEF (guanine exchange factor). Therefore the control of the localization of Rheb could contribute to its regulation. We generated a Rheb-GFP fusion protein to test this hypothesis.

3.1 Subcellular localization of Rheb

A crucial attribute is the intracellular residence of a protein, for example to obtain clues about the connection to other proteins displaying a similar localization pattern. In the case of Rheb, we wished to better understand the mechanism of regulation. Small GTPases are normally regulated by a GAP (GTPase activating protein) and a GEF. GEFs turn on signalling by catalyzing the exchange from GDP to GTP and GAPs terminate the signalling by inducing GTP hydrolysis.

Rheb GTPases are thought to be predominantly bound by GTP and might not need a GEF protein. Therefore it would be possible that the activation of Rheb is controlled via the expression or the localization of the GTPase.
Rheb contains a farnesylated CAAX domain at its C-terminus. Due to the farnesylation the protein can be localized to membranes and therefore we expect Rheb to be at the plasma membrane or to colocalize with intracellular organelles. Since Rheb is negatively regulated by TSC1/2, they have to colocalize at least partially. TSC2 was shown to display a punctuated, mostly perinuclear staining in human cell lines. This staining could be restricted to the Golgi apparatus. It is therefore likely that Rheb might localize to the Golgi membranes.

3.1.1 Strategy to determine the subcellular localization of Rheb

There are several ways to investigate the subcellular localization of a protein. The two main strategies are to generate antibodies against the protein of interest or to construct a tagged fusion protein. Previous experiences from other research groups argue against the antibodies. At least two antibodies against Rheb exist and none of them was able to recognize the endogenous protein (B. Edgar, G. Thomas, personal communication). Instead of generating another antibody with low chances of success, we decided to generate a tagged fusion protein. We used the green fluorescent protein (GFP) as tag for our fusion protein, although it is a rather large tag. There are obvious advantages: no staining has to be done to see the signal and it also allows in vivo imaging on living tissue.

We were not interested in an overexpression state of the tagged Rheb. We wanted to observe the localization of the endogenous protein. Therefore we planned to insert the GFP tag into the endogenous sequence, but at first we had to be sure that the Rheb sequence on its own was able to fully rescue Rheb mutant flies.

3.1.2. Genomic rescue of Rheb mutant flies

It can be tricky to choose the adequate sequence containing all the enhancer and promoter sequences necessary for an endogenous function of a gene. For large genes it can be very difficult to obtain a genomic rescue. The genomic region of Rheb has a length of approximately 2.5 kb, and this small size of genomic DNA was promising for a rescue. We decided to amplify the whole stretch ranging from neighbouring gene
to neighbouring gene (CG1411) (Figure 3). Several transgenic fly lines were established and examined for their abilities to rescue Rheb mutant animals. Heteroallelic null mutants of Rheb die during the first instar and a successful rescue cannot be missed.

![Figure 3: Genomic environment of Drosophila Rheb. Red arrow: genomic region between Rheb's neighbouring genes (2.5kb), which was amplified for the genomic rescue construct.](image)

Four independent lines could be established (line 2, 19, 26 and 44). Whereas all of them were able to rescue several different combinations of Rheb null alleles to adulthood, only one of them led to a complete rescue. Table 1 represents a brief overview of the different lines, which will be described in detail later.

Crossing scheme for flies containing one rescue construct:

\[ \text{LineX/LineX} ; \text{Rheb}^{\text{mut1}}/\text{TM6B} \times \text{Rheb}^{\text{mut2}}/\text{TM6B} \]

Crossing scheme for flies containing two rescue constructs:

\[ \text{LineX/LineX} ; \text{Rheb}^{\text{mut1}}/\text{TM6B} \times \text{LineY/LineY} ; \text{Rheb}^{\text{mut2}}/\text{TM6B} \]

<table>
<thead>
<tr>
<th>Rescue line</th>
<th>Delay</th>
<th>Ratio</th>
<th>Phenotype</th>
<th>Weight</th>
<th>Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 2</td>
<td>no delay</td>
<td>0.65</td>
<td>wt</td>
<td>normal</td>
<td>Fertile</td>
</tr>
<tr>
<td>Line 19</td>
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<td>normal</td>
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<tr>
<td>Line 26</td>
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<td>0.26</td>
<td>wt</td>
<td>-</td>
<td>Fertile</td>
</tr>
<tr>
<td>Line 44</td>
<td>&lt;1 day</td>
<td>0.44</td>
<td>non-wt</td>
<td>-</td>
<td>Fertile</td>
</tr>
</tbody>
</table>

Table 1: Summary of rescue abilities of four independent genomic Rheb fly lines. The rescued flies contain one insertion of the rescue construct. The ratio indicates the ratio of rescued flies to TM6B-control flies, which is expected to be 0.5. For the descriptions of the phenotypes see text section. Abbreviation: wt: wild type.
I) Delay of rescued flies

*Rheb* mutant flies rescued with line 2 did not have a delay, the rescued flies eclosed at the same time as the *TM6B*-balanced control flies. For the other three lines a delay was observed: the rescued flies never eclosed on the same day as the control. However, they were present the day after and displayed therefore a maximum delay of one day.

To use two genomic rescue insertions (19; 26 and 26; 44, respectively) did not change the pattern. The rescued flies still had a delay in eclosion of one day.

II) Ratio of rescued to control flies

For each genomic rescue insertion, we analyzed the ratio of rescued flies to control flies. We prepared fly lines containing the homozygous genomic rescue construct on the 2nd chromosome and a balanced *Rheb* mutation on the 3rd chromosome. We crossed this genotype to flies carrying a balanced *Rheb* mutation on the 3rd chromosome. The progeny consisted of rescued flies (*LineX/+; Rheb\textsuperscript{mut1}/Rheb\textsuperscript{mut2}* ) and of control animals containing the balancer chromosome (*LineX/+; Rheb\textsuperscript{mut1/2}/TM6B*) (Table 2). The rescued animals are expected to eclose in a ratio of 1:2 compared to the control.

The ratio of line 2 of rescued to control flies was slightly larger than expected. For all other lines the ratio of 0.5 was not reached, showing that the flies’ genetic composition was in some lines not ideal.

The various combinations of two rescue insertions (19; 26, 26; 44 and 19; 44, respectively) partially led to the expected ratio of 0.5.

<table>
<thead>
<tr>
<th>Rescue line</th>
<th>Control females</th>
<th>Control males</th>
<th>Rescued females</th>
<th>Rescued males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 2</td>
<td>21</td>
<td>19</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
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<td>56</td>
<td>73</td>
<td>16</td>
<td>22</td>
</tr>
<tr>
<td>Line 26</td>
<td>80</td>
<td>96</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>Line 44</td>
<td>58</td>
<td>73</td>
<td>19</td>
<td>38</td>
</tr>
</tbody>
</table>

*Table 2: Number of control and rescued flies of the different genomic Rheb rescue constructs.* Genotypes: control flies: *LineX/+; Rheb\textsuperscript{mut1/2}/TM6B*; rescued flies: *LineX/+; Rheb\textsuperscript{mut1}/Rheb\textsuperscript{mut2}*
III) Phenotype of rescued flies

*Rheb* mutant flies rescued with line 2 and line 26 did not exhibit any abnormalities compared to the control (Figure 4). The remaining two lines led to a remarkable phenotype: line 19 and 44 resulted in undersized heads compared to the abdomens. The wings showed defects as well; they were crimped, but without any vein defects. This time the use of two genomic rescue insertions that individually resulted in smaller heads (19; 44) rescued the head size and the flies displayed weaker wing phenotypes.

IV) Weight of rescued flies

Adequate weight analysis was only done for *Rheb* mutant animals rescued with lines 2 and 19, respectively. Apart from an expected normal biological variation the weight of males and females was not altered in rescued flies (Figure 4).

V) Fertility

All rescued males and females displayed a normal fertility.

Taken together, we could claim a complete rescue. Although only one of the four tested lines exhibited a complete rescue, the rescuing capabilities of the other lines were also substantial. It was possible to obtain a complete rescue depending on the position of the insertion and therefore we were encouraged to generate the GFP fusion construct.

3.1.3 Evaluation of the Rheb fusion constructs

We decided to use PCR cloning to generate the construct (for details see Materials and Methods). No restriction sites were needed and the GFP sequence could be inserted without any spacer sequence. When we started this project no crystal structures were available and the question whether to do an N-terminal or a C-terminal fusion protein was not easy to answer. Therefore we planned two different constructs (Figure 5). The first one (*Rheb-GFP*) contains the GFP sequence in front of the CAAX domain at the C-terminus. The CAAX domain is important for the proper localization of Rheb to membranes. Therefore to put the GFP sequence at the very end would most likely lead
to a non-functional protein. The second fusion construct (*GFP-Rheb*) has the tag sequence at the N-terminus.

Figure 4: Rescued animals display a wild-type appearance and a normal weight

A, B: The phenotype of a rescued *Rheb* mutant fly (A) does not differ from a control fly (B). C, D, E, F: The weight of rescued *Rheb* mutant females (C, E) and males (D, F) is similar to the weight of control flies. Genotypes are: A: y w; (Rheb-genomic)*2/*+: Rheb*11/*: Rheb*12
B: y w; (Rheb-genomic)*2/*+: Rheb*11/*+: C, D: 1) y w; (Rheb-genomic)*2/*+: Rheb*11/*: Rheb*22
2) y w; (Rheb-genomic)*2/*+: Rheb*11/*: 3) y w; (Rheb-genomic)*2/*+: Rheb*11/*: Rheb*22
FRT82
4) y w; (Rheb-genomic)*2/*+: Rheb*11/*: Rheb*22/FRT82
5) y w; (Rheb-genomic)*2/*+: Rheb*11/*: Rheb*22/FRT82

FRT82 is the parental chromosome of the *Rheb* alleles.
3.1.4 Rescue of the mutant flies with the tagged rescue constructs

Several transgenic lines were generated for both constructs and tested for their rescuing abilities as it was done for the genomic construct (see chapter 3.1.2). Whereas the Rheb-GFP construct did not rescue Rheb mutant animals, the GFP-Rheb construct rescued well. Homozygous mutant animals were able to develop into adult flies. Without the rescue construct they would have died as first instar larvae (Figure 6).

Females as well as males eclosed, but they exhibited variable phenotypes. Some of the flies looked almost like the control, but others had crumpled and/or cleft wings and they looked unhealthy.

Either way the rescue from first instar to adult flies – even with unhealthy flies – was convincing. For a rescue of that degree the tagged Rheb protein must be localized at its correct place and therefore we proceeded with our analysis.
3.1.5 Analysis of rescued Rheb mutant flies

First of all, we had a look at embryos and were able to distinguish unfixed GFP expressing embryos from the control, but the ubiquitous signal was very weak. We proceeded with fixed imaginal discs and salivary glands, but there the signal was not visible. We supposed that the autofluorescence of the tissue and the paraformaldehyde treatment resulted in strong fluorescence. We decided to work with unfixed tissues and under these conditions we obtained a signal clearly distinguishable from the control (Figure 7).

![Figure 7: GFP-Rheb is ubiquitously expressed in the cytoplasm of salivary gland cells. A, B: Unfixed salivary glands of third instar larvae. A: Control, salivary gland of OregonR larvae. B: Salivary gland of Rheb mutant rescued larvae. Genotype is: GFP-Rheb2/++; Rheb1/++/Rheb2/++ (insertion 34).](image)

The GFP pattern was not as we expected. The signal was ubiquitous in the cytoplasm and did not localize to a specific membrane structure in the cell. Stably transfected S2 cells revealed the same pattern. We were puzzled by these results, and although the situation was irrevocable, we did not take it for granted that Rheb was not localized to specific structures within a cell. Indeed, at later dates two papers were published both claiming Rheb to be localized at endomembranes.

3.1.6 Discussion

It is important to elucidate the intracellular localization of a protein to draw connections to other proteins displaying the same localization pattern. The significance of this observation can be tested via genetic interactions and biochemical binding studies. Additionally the localization pattern could reveal a location-dependent regulatory mechanism, for example if a certain protein alters its intracellular
localization due to changing conditions in a cell. Since Rheb is likely to be predominantly in the GTP-bound state, we have tried to find its regulatory mechanism and have tested whether the localization of Rheb is controlled.

Antibodies are good tools to reveal the pattern of a protein during development and also to examine its intracellular localization. However, they may not work for several reasons. In vivo, the protein may not be abundant enough or it may be hidden in a complex and thus cannot be recognized. In the case of Drosophila Rheb more than one antibody was produced, but none of them works properly. Therefore we decided to generate a tagged Rheb protein.

The GFP tag allows to visualize a tagged protein without previous fixation and staining treatments and therefore permits in vivo imaging. As a disadvantage, the large tag might disturb the function or localization of Rheb, but in the case of an in vivo testing system, this can be investigated with a rescue experiment of Rheb mutant animals.

Another concern is the expression of the tagged protein. To overexpress a protein can lead to artefacts and the obtained data are questionable. This can be avoided by expressing the tagged protein under the control of its endogenous promoter. Rheb mutant animals can be rescued with a genomic rescue construct, indicating that all promoter and enhancer sequences necessary for the correct spatial and temporal expression of Rheb are included in the 2.5 kb stretch of genomic Rheb DNA. All four independent lines rescue mutant animals to adulthood, which is satisfying regarding the fact that Rheb null mutants die during the first larval instar. However, the fine-tuning of Rheb seems to be more sophisticated and depends on the integration and the expression of the rescue construct. Only one of four lines displays a complete rescue regarding the delay of eclosion, the ratio of rescued to control flies, the overall phenotype, the weight and the fertility. In the other three lines, Rheb might not be provided in the required amount in all tissues of the animals. This is observed with two of the lines individually leading to rescued flies with undersized heads. In those cases, the amount of Rheb is probably not sufficient in the head compared to the rest of the body. The use of two genomic constructs can rescue this size defect. However, the
delay in eclosion is not altered by the use of two insertions, which suggests that the expression pattern of \textit{Rheb} is not uniform in the fly. Earlier observations led to similar conclusions. A rescue of \textit{Rheb} mutant flies with a \textit{UAS-Rheb} construct did not work, probably because the complex expression pattern cannot be copied by ubiquitous overexpression (H. Stocker, unpublished results).

These experiments show that a complete rescue with a genomic construct is possible. The rescue abilities depend on the integration site of the construct and therefore several independent insertions should be tested.

A rescue of \textit{Rheh} mutant flies is only possible with one of the two fusion proteins. For the C-terminal \textit{Rheh-GFP} construct, the GFP tag probably disturbs the correct localization of the CAAX domain. The localization of Rheb depends on the farnesylation of the CAAX domain. These four terminal amino acids of Rheb might be partially hidden and the farnesylation signal is not recognized any more. By contrast, the animals rescued by the N-terminal \textit{GFP-Rheb} construct can proceed their development to adults, although the degree of rescue varies and the flies have several abnormalities. Some of them look almost like the control, but others display wing defects and their general appearance is unhealthy. However, the rescue is convincing and we conclude that the tagged Rheb is at least partially localized at its correct intracellular position. If not, the animals could probably not have proceeded their development to adults.

Fixed salivary glands and wing discs do not display a signal compared to the control. By contrast, in unfixed embryos and third instar larvae (salivary glands and wing discs), the signal is clearly distinguishable from the background. This is probably due to the fixation with paraformaldehyde, which might enhance the autofluorescence of the tissue. However, the GFP signal is distributed all over the cell except within the nucleus instead of localizing to a distinct membrane structure. In stably transfected S2 cells the situation is similar: the GFP signal also does not localize to a specific cellular structure. These findings are unexpected and we do not have an accurate explanation. A CAAX-domain localizes proteins to membranes and we expected therefore a distinct membrane localization of the fusion protein. Partially, the GFP-Rheb fusion
protein must be localized at its designated compartment in the cell, otherwise the rescue of *Rheb* mutant animals would not have worked. Takahashi et al. examined the intracellular localization of Rheb in the cell. They used an enhanced green fluorescence protein (EGFP)-tagged Rheb in mouse embryonic fibroblasts (MEFs). The EGFP signal was detected in granule-like fluorescence pattern in the cytoplasm, namely endomembranes, but not at the plasma membrane or in the nucleus. When the coding sequence for the CAAX domain of the fusion protein was mutated, it resulted in a diffuse localization of the EGFP signal. Treatment with farnesyltransferase inhibitors led to the same diffuse localization of the tagged Rheb.

In the second paper, the authors performed timed imaging of live cells expressing EGFP-Rheb. They could show that after a brief association with the ER, Rheb localized to distinct vesicular structures within the cytoplasm resembling Golgi membranes, maybe derived by budding off from the endomembrane. Farnesyltransferase inhibitors impaired the localization of Rheb to endomembranes and the activation of downstream components. Additionally, the inhibitor treatment redistributed EGFP-Rheb uniformly throughout the cytoplasm and the nucleus.

We only observed a diffuse staining in tissues and in cell culture and no clear localization to intracellular compartments despite using very similar constructs. This can be due to the restricted time windows both research groups had in their cell culture experiments. We examined the signal in living tissue or in stably transfected cells. In both cases the tagged protein has been in the cells for a long time and this might be the reason for the diffuse localization pattern. The signal might be specifically localized directly after a transfection, but any other modifications of Rheb in the cell might lead to partially delocalized proteins and therefore to diffuser signals in a natural environment.

Buerger et al. also tried to detect the subcellular localization of Rheb with a new antibody. Their results showed that the decision against a new antibody was correct. The authors did not observe the same pattern with the antibody and the EGFP tagged Rheb construct. They claimed that the treatment of the cells with permeabilization detergents caused these differences. Such conditions destroyed the pattern and led to a
diffuse Rheb staining. Therefore, antibody stainings can be insufficient to answer questions about the intracellular localization of proteins.

Two independent research papers claim Rheb to be localized to endomembranes\textsuperscript{93, 94}. Therefore the data is less questionable, although in both studies they worked with overexpression of Rheb. TSC2 has also been shown to be expressed in intracellular structures and not at the plasma membrane, namely in the Golgi apparatus\textsuperscript{92}. TOR has been shown to be predominantly cytoplasmic but associates with membranes including those of the mitochondria, the ER and Golgi\textsuperscript{95-98}. Therefore it is very likely that Rheb is also localized to intracellular membranes and that the signalling takes place at those membranes. However, it does not rule out that Rheb is cytoplasmic as we could observe it and only transiently localizes to intracellular membranes.

3.2 Lethality suppression screen

3.2.1 Genetic screens in \textit{Drosophila}

The primary goal of my PhD was to identify new downstream genes of Rheb. Binding studies or genetic screens are the major approaches to detect new components in a defined signalling pathway. Genetic screens in a genetically sensitized system are based on the reversion or enhancement of a specific phenotype, whereas biochemically novel interacting proteins can be isolated via binding to known proteins. Both approaches have advantages and disadvantages; the biochemical approach is more rapid and the binding partners can be directly identified. However, as long as there is no in vivo evidence, the relevance of the data is unclear. To design and conduct a genetic screen takes a lot more time, and afterwards the mapping and identification of the responsible gene or mutation can be difficult as well. Different mutations in a gene can be obtained, which results in an allelic series. Such alleles are useful genetic tools for in vivo studies to provide the functional importance of a gene product.

Taking advantage of the strong genetic background of the laboratory, we decided to establish genetic screens to identify new downstream components of Rheb. The idea
was to create a *Rheb* gain-of-function phenotype and to search for mutations to revert or suppress the phenotype.

### 3.2.2 Introduction lethality suppression screen

The first screen dealt with the suppression of a lethality caused by the overexpression of *Rheb*. The goal and big advantage of this screen was to set up a system where all the animals but the ones containing the appropriate mutations died. No extensive screening of flies would be necessary, only the survivors would be focused on. For this reason it should be possible to screen a large number of mutagenized chromosomes. As a disadvantage, we would only find genes positively involved in growth control, but no negative factors.

The principle of the screen is represented in Figure 8. To establish this kind of screen we made the following assumptions. Rheb should be expressed in a tissue-specific manner, because the examined F1 animals derived from the mutagen-treated males would be genetic mosaics and the restricted overexpression increased the chances that the region of enhanced Rheb signalling was heterozygously mutant. A second criterion was the time point of the lethal phase. The animals should die late in development, ideally shortly before hatching, to enable mutant animals to reach adulthood. The third criterion was the expected survival of animals heterozygous for TOR (Figure 8). This used to be an important control for the screen. If heterozygous *TOR* animals were able to eclose, mutations in other downstream genes of Rheb were also expected to lead to adult flies. Of course those flies should also be fertile, if not the whole attempt would be pointless.
Figure 8: Overview of the principle of the screen (left). Overexpression of Rheb leads to lethality, whereas animals heterozygous for TOR develop into adult flies. To the right, a rudimentary section of the genetic interaction of the core components of the TOR signalling pathway can be seen. Abbreviations: wt: wild type; mut: mutant.

3.2.3 Establishing conditions for the lethality suppression screen

Various tissue-specific Gal4 drivers, UAS- and EP-Rheb lines were tested under different temperature conditions to elucidate the optimal conditions for a screen. Finally a suitable set-up was found:

\[ \text{ppl-Gal4/ppl-Gal4} \times \text{P(y')}/\text{P(y')}; \text{GsjE2(w')}/\text{GsjE2(w')} \]

\text{ppl-Gal4: fat body specific driver line (2nd chromosome)}
\text{P(y'): marker for the second chromosome (BL: 10191)}
\text{GsjE2(w'): Rheb EP (3rd chromosome)}^{27}

Temperature: 19.5°C
Relative humidity: 70%

Drosophila containing the genotype \text{ppl-Gal4/P(y')}; \text{GsjE2(w')/+} and brought up at 19.5°C died during the larval or pupal stage. There was a certain variability, but most importantly there were no escapers, which would be false positives. Rearing the animals at 18°C already led to escapers, showing that the temperature window was relatively small. Drosophila heterozygous for \text{TOR (ppl-Gal4/TOR}^{\text{w}}; \text{GsjE2(w')/+})
eclosed, looked mostly healthy and behaved normally at first glance. Both males and females reached the adulthood and their fertility was not reduced. The \( \text{TOR}^{2L} \) allele contains a mutation in the kinase domain and is most likely a dominant-negative. Tests with \( \text{TOR} \) null alleles also led to adult flies, but the frequency and fertility were reduced compared to the dominant-negative allele.

The output of the screen was expected to bring up new \( \text{Rheb} \) and \( \text{TOR} \) alleles besides the desired mutations in novel genes. From previous EMS screens we knew that \( \text{Rheb} \) was hit with a frequency of 1 in \( 5'000^{56} \). For \( \text{TOR} \) we had an estimation of 1 in \( 25'000 \), because we expected to receive predominantly dominant-negative versions (H. Stocker). It was not possible to make a prediction about the frequency of the novel genes.

3.2.4 Screening procedure and mapping of the mutants

\[
\begin{array}{c}
\text{♀} \text{ppl-Gal4/ppl-Gal4 x ♂ P(y+)/P(y+)(*)}; \text{GsjE2(w+)}/\text{GsjE2(w+)(*)} \\
(\text{(*)}: \text{EMS treated}) \\
\text{Genotype of survivors:} \quad P(y+)(*)/\text{ppl-Gal4}; \text{GsjE2(w+)(*)}/+ \\
\end{array}
\]

Treatment of the male survivors (only 2\textsuperscript{nd} and 3\textsuperscript{rd} chromosomes are mutagenized):

Retest and mapping of the chromosome:

1. \( \text{CyO}/\text{Sp}; \text{TM6B}/\text{MKRS} \times P(y+)(*)/\text{ppl-Gal4}; \text{GsjE2(w+)(*)}/+ \\
2. \text{ppl-Gal4/ppl-Gal4} \times P(y+)(*)/\text{CyO}; \text{GsjE2(w+)(*)}/\text{TM6B} \)
The second cross was done at 19.5°C (screening temperature) to test whether animals carrying the mutagenized chromosomes survive again (Retest).

Analyzing non-TM6B progeny from the second cross allows the allocation to the 2nd or 3rd chromosome:

- only y flies survived: 2nd chromosome
- y and CyO flies survived: 3rd chromosome

**Treatment of the female survivors (X, 2nd and 3rd chromosomes are mutagenized):**

\[ \frac{X}{X^C}; P(y^+)^{(-)}; Ppl-Gal4; GsjE2(w^+)^{(-)}; w^+ / CyO; \frac{Y^+}{TM6B} \times w^+/ Binsinscy \]

Establish individual lines for the X chromosome (not marked) with four females eclosed from the first cross (surviving female):

\[ X^{(\times)} / Binsinscy; P(y^+)^{(-)}; GsjE2(w^+)^{(-)}; w^+ / Binsinscy \]

If males eclosed in all four individual lines, the X chromosome contained no lethal hit and the lines were removed.

In case the X chromosome contained a lethal hit (no viable male flies), this line was crossed to GMR-Rheb (big eye phenotype, Figure 10) and tested for suppression of the eye size. Such a reduction in size was expected for a positive growth regulator. This was the only test made for the X chromosome, everything else would have been too time consuming.

In parallel, four males were taken that emerged from the female survivor and individual lines were established for the 2nd (y') and the 3rd (w') chromosome (\( P(y^+)^{(-)}/CyO; GsjE2(w^+)^{(+)} / TM6B \)). The retesting and the mapping of the lines took place at 19.5°C as described above (Treatment of the male survivors).
Further testing of the positive candidates on the 2nd and 3rd chromosome, respectively:

Besides to mutations in novel downstream candidates of Rheb, new Rheb alleles themselves, TOR alleles and also mutations reducing the Gal4 signalling were expected.

Test for new Rheb and TOR alleles:
- Cross against RhebTT or TOR2L, respectively, if mapped to the appropriate chromosome.

Test for mutations reducing Gal4 signalling:
- All established lines (P(y′)(3z)/CyO; GsjE2(w+)(3z)/TM6B) were crossed to GMR-Gal4 (eye-specific driver line) and examined for a reduction of the eye size (Overexpression of GsjE2 (Rheb EP) with GMR-Gal4 leads to increased eyes). This would most likely be due to a mutation reducing Gal4 signalling, a mutation of the UAS-sequences of the EP construct was far less likely. However, mutation in downstream components of Rheb would also lead to a suppression of the eye phenotype. This could be elucidated with the following steps.

Test for downstream candidates:
- Test mutations by crossing to a GMR-Rheb line for suppression of the phenotype. Downstream components of Rheb should influence the GMR-Rheb phenotype (direct construct). This big-eye phenotype is not depending on Gal4 and only mutations in downstream components of Rheb should lead to a size decrease, but not mutations reducing Gal4 signalling.

- Test mutations in TOR106/TOR152 hypomorphic background
Downstream components of Rheb should influence a hypomorphic TOR combination. Those hypomorphic flies are viable, but smaller. TOR signalling components are expected to lead to lethality or reduce the viability.
3.2.5 Mutagenesis rounds and results

Mutagenized males \( P(y^+)/P(y^-)(*) \); \( GsjE2(w^+)/GsjE2(w^-)(*) \) were crossed to virgin females \( ppl-Gal4/ppl-Gal4 \) in bottles and screened for survivors. For the first mutagenesis round all the dead larvae and pupae were counted, as well as the survivors. For this reason it was possible to determine the ratio of the survivors to the whole population. Afterwards it was possible to estimate the total number of screened flies out of the number of survivors. Because the ratio was very small and the recovery of the bottles was rather poor, another mutagenesis round was performed at 19°C (Table 3). The second mutagenesis round resulted in a larger number of surviving flies.

Screen at 19.5°C
6 survivors versus 4096 dead larvae/pupae: 0.15%

Screen at 19.0°C
15 survivors versus 3444 dead larvae/pupae: 0.44%

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Independently eclosed flies</th>
<th>Minimum of screened animals</th>
<th>All eclosed flies</th>
<th>Maximum of screened animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>19.5°C</td>
<td>80</td>
<td>53'000</td>
<td>98</td>
<td>65'000</td>
</tr>
<tr>
<td>19°C</td>
<td>75</td>
<td>17'000</td>
<td>94</td>
<td>21'000</td>
</tr>
</tbody>
</table>

Table 3: Numbers of eclosed survivors and estimated numbers of screened flies. For the minimum number of screened animals only one survivor per bottle was counted. For all eclosed flies a maximum of two flies per bottle was counted. Bottles containing more than two survivors were taken as clonal events and counted as one survivor only.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Independent fertile flies</th>
<th>Rheb alleles</th>
<th>TOR alleles</th>
<th>Passed the retest</th>
<th>Did not pass the retest</th>
</tr>
</thead>
<tbody>
<tr>
<td>19.5°C</td>
<td>41</td>
<td>12</td>
<td>4</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>19°C</td>
<td>20</td>
<td>6</td>
<td>0</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 4: Numbers of independently eclosed and fertile flies. At 19.5°C the 41 lines contained 16 Rheb or TOR alleles and 25 others without mutations in those genes. Out of them, 11 passed the retest at 19.5°C. At 19°C 20 independent lines could be tested. 6 lines contained Rheb alleles, no TOR alleles were found. From the other 14 lines only 9 were retested and 3 passed.
The newly identified Rheb alleles were sequenced to reveal a connection between the structure and the function of Rheb (Table 5).

<table>
<thead>
<tr>
<th>Stop codons (4)</th>
<th>Rheb allele bp exchange</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhei 335</td>
<td>C to T</td>
<td>Gln 24 Stop</td>
</tr>
<tr>
<td>Rhei 170</td>
<td>C to T</td>
<td>Gln 51 Stop</td>
</tr>
<tr>
<td>Rhei 300</td>
<td>C to T</td>
<td>Gln 51 Stop</td>
</tr>
<tr>
<td>Rhei 91</td>
<td>G to A</td>
<td>Gln 161 Stop</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peptide exchanges (6)</th>
<th>Rheb allele bp exchange</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhei 101</td>
<td>G to A</td>
<td>Ala 61 Thr</td>
</tr>
<tr>
<td>Rhei 140</td>
<td>G to A</td>
<td>Met 75 lle</td>
</tr>
<tr>
<td>Rhei 16</td>
<td>G to A</td>
<td>Gly 79 Asp</td>
</tr>
<tr>
<td>Rhei 373</td>
<td>G to A</td>
<td>Gly 79 Asp</td>
</tr>
<tr>
<td>Rhei 6</td>
<td>G to A</td>
<td>Gly 117 Asp</td>
</tr>
<tr>
<td>Rhei 302</td>
<td>G to A</td>
<td>Glu 146 Lys</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Splicing mutations (6)</th>
<th>Rheb allele bp exchange</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhei 198</td>
<td>G to A</td>
<td>3' splice site exon 2</td>
</tr>
<tr>
<td>Rhei 169</td>
<td>G to A</td>
<td>5' splice site exon 2</td>
</tr>
<tr>
<td>Rhei 232</td>
<td>G to A</td>
<td>5' splice site exon 4</td>
</tr>
<tr>
<td>Rhei 346</td>
<td>G to A</td>
<td>5' splice site exon 4</td>
</tr>
<tr>
<td>Rhei 20</td>
<td>G to A</td>
<td>3' splice site exon 5</td>
</tr>
<tr>
<td>Rhei 133</td>
<td>G to T</td>
<td>3' splice site exon 5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Frameshift mutations (1)</th>
<th>Rheb allele bp exchange</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhei 34</td>
<td>A insertion</td>
<td>Exon 3, aa 99</td>
</tr>
</tbody>
</table>

| Mutation not found (1) | Rheb 295 | - |

Table 5: Summary of isolated Rheb alleles in the lethality suppression screen. Peptide exchanges: Rhei 101: amino acid conserved from yeast to humans, G3 box; Rhei 140: no conserved amino acid or domain; Rhei 16 and Rhei 373: amino acid conserved from yeast to humans, no conserved domain; Rhei 6: amino acid conserved from yeast to humans, G4 box; Rhei 302: amino acid conserved from yeast to humans, G5 box (G1-G5 boxes: involved in the recognition of guanine nucleotides). Nucleotide exchanges in the splicing mutations of Rheb: Rhei 198: last nucleotide in the 1st intron; Rhei 169: first nucleotide in the 2nd intron; Rhei 232: 5th nucleotide in the 4th intron (like Rhei 346); Rhei 346: 5th nucleotide in the 4th intron (like Rhei 232); Rhei 20: last nucleotide in the 4th intron; Rhei 133: mutation 31 bp downstream of 3' splice site in the 4th intron.

Finally 14 lines passed the retests at 19.5°C or 19°C, respectively. As already discussed, those lines were tested by crossing them against GMR-Gal4 and GMR-Rheb. 13 lines did not show an alteration of the big eye phenotype compared to the control and were therefore not interesting for us. We also did not find any complementation groups by crossing the lines against each other. At first glance, we could not give an explanation of why those lines passed the retests. Therefore only one line tested for the 2nd and 3rd chromosome led to the expected phenotype (131.2). For
the X chromosome, which was treated differentially, we also found only one candidate. Line 232.1 was established from a female survivor and was found to carry a lethal hit on the X chromosome.

<table>
<thead>
<tr>
<th>Line</th>
<th>Chromosome</th>
<th>x Rheb$_{11}$</th>
<th>x TOR$_{11}$</th>
<th>x GMR-Gal4</th>
<th>x GMR-Rheb</th>
</tr>
</thead>
<tbody>
<tr>
<td>131.2</td>
<td>2$^{nd}$</td>
<td>Complementation</td>
<td>Complementation</td>
<td>Suppression</td>
<td>Suppression</td>
</tr>
<tr>
<td>232.1</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Suppression</td>
</tr>
</tbody>
</table>

Table 6: Summary of complementation analysis and functional tests of line 131.2 and 232.1.

Both mutations on the X and 2$^{nd}$ chromosome, respectively, could dominantly suppress the size of fly eyes overexpressing Rheb (Table 6) and were good candidates for new genes involved in TOR signalling. Since we found no complementation group, we did not map those two single hits. To map a single hit was a risky project. Single hits might never be found due to mutations in the promoter or enhancer regions. Additionally, two genes might be mutated concomitantly leading to the observed phenotype. In such a case it would also be very difficult to map those genes.

In parallel, I was carrying out a second screen, which seemed to be more promising at this time. Therefore we decided to focus on the output of the second screen only and did not perform substantial work with any of the two single hits found in the lethality suppression screen.

### 3.2.6 Discussion

Identifying and understanding the network of genes involved in growth is amongst the most crucial steps in developing therapeutical tools against cancer. Depending on their various other functions, their structure and abundance, not all proteins might be useful targets to interfere. The broader the knowledge about the involved molecules, the higher the chances to find a drug repressing the malfunction of a certain pathway.

Although we are not involved in finding drugs against proto-oncogenes, we are able to identify new components involved in growth control. Rheb is amongst those candidates and we exploited the fact that overexpression of Rheb results in lethality to identify new downstream candidates.
The idea behind the lethality suppression screen is simple to understand. Strong overexpression of Rheb leads to lethality and can be used to screen for mutations suppressing this Rheb induced death. Animals with elevated Rheb levels, which normally die during the larval/pupal stage, survive and are fertile when heterozygous for TOR. Thus, it can be expected to identify other downstream genes capable of suppressing the lethality caused by the overexpression of Rheb. The proteins encoded by those identified genes could be involved in TOR signalling. Although by this approach we are only able to find genes positively involved in growth control, the advantages of this type of screen overbalance this disadvantage. Due to the fact that all animals but the ones containing mutations influencing TOR signalling die, no extensive visual screening of flies is necessary. Therefore it is possible to screen a large number of chromosomes without the huge work load normally associated with screening flies.

After testing various conditions for the screen a suitable setup fulfilling the requirements has been found. Overexpression of Rheb does not lead to any escapers. Such escapers would be false positives and result in extra work. Conversely, the control flies (heterozygous for TOR) survive well and - very importantly - are fertile. The first screen at 19.5°C led to a survival rate of 0.15%, whereas the second screen at 19°C gave three times more surviving flies (0.44%). At 18°C the flies survived and were fertile, indicating that already slight changes in the temperature have a large impact on the survival. However, despite the fact that a lot more flies survived at 19°C, only one quarter of them could be used for further crossings, because many of them died or were sterile. In these flies, the level of TOR signalling was reduced enough to enable the animals to eclose, but the signalling was probably still too high and negatively interfered with fertility. At 19.5°C about half of all surviving flies could be taken for further crosses.

It is not possible to count the exact number of screened animals, but it can be estimated. The minimal number of screened animals is 70'000, whereas the maximum number is more than 85'000. For the minimal number only one fly per bottle was taken for the calculation. We cannot exclude the possibility that all surviving flies in
one bottle contain the same clonal event. Thus, can therefore not be taken as individual incidents. However, it is very likely that not all the flies emerging from the same bottle are clonal events. To calculate the upper limit we took a maximum of two flies per bottle. Bottles containing more than two surviving flies were regarded as clonal events and counted as one survivor only.

These calculations seem to be reliable, regarding the expected frequencies of Rheb and TOR alleles, although the maximum number of screened flies appears to be more precise. This is conceivable, because not all flies in one bottle are clonal events and even three flies from the same bottle could be independent events. In the ey-Flp screen, one Rheb allele was found in 5'000 flies\textsuperscript{56}. The number of Rheb alleles established in our screen is 18, which leads to a total number of screened flies of 90'000, slightly more than the calculated maximum number (85'000). This confirms that our estimation is reasonable. Regarding the TOR alleles, the estimation is not as simple as for Rheb. For TOR, null alleles are not strong enough to lead to fertile adults. However, the rescue works much better with a dominant-negative allele and therefore we expect to obtain mainly special TOR alleles at the low frequency of about 1 in 25'000 flies. This estimation is probably the weakest one, but it is still in range with the other evaluations. The recovery of four TOR alleles suggests a number of 100'000 flies screened. Having in mind all the different estimations we reckon that we have screened about 90'000 flies.

We sequenced all 18 Rheb alleles found in the screen, because we were interested in the connection between the structure and function of Rheb. In 17 cases we were able to identify a mutation. However, most mutations cannot be taken for this analysis, since many of them lead to a stop codon (4), to a splicing mutation (6) or to a frame shift (1). Mutations resulting in amino acid exchanges were found in six cases only (Table 5). Three of them alter a conserved amino acid in the G3, G4 or G5 box, respectively, and they are all conserved from yeast to humans. Those boxes (G1-G5) are involved in the recognition of the guanine nucleotides. Two mutations resulted in exactly the identical nucleotide exchange. This amino acid is also conserved from yeast to humans, but it does not affect one of the domains of Rheb. Since it is well
conserved, it might have an important function in defining the structure of the native protein. We presume that most of the mutations are important for the function of Rheb, because they code for conserved amino acids. Additionally, they all enable the *Rheb* overexpressing animals to survive. However, one of the mutations leads to an amino acid exchange, which is not conserved and does not affect a conserved domain of Rheb. We suppose that this might be a hypomorphic allele, but strong enough to enable animals to survive.

61 independent mutations were isolated from this lethality reversion screen. 14 mutations were left after retesting the mutations under screening conditions and testing for complementation with *Rheb* and *TOR*, respectively. Out of those established 14 lines, only one displayed an involvement in *TOR* signalling and is the type of mutation we were screening for (131.2, 2nd chromosome). All other lines were neither Gal4 signalling mutations nor did they influence the eye size of flies overexpressing *Rheb* (*GMR-Rheb*). It is possible that a reduction of gene function by 50% does not lead to a suppression of the eye size. However, since mutations in *TOR* and *Raptor* (see Figure 10) do decrease the eye size of *GMR-Rheb* flies, we think that other pathway members may display a reduction as well. Exactly this screening system was used for the second screen we performed (chapter 3.3).

We do not know why most of the lines passing the retests did not influence a *Rheb* overexpression situation. In most cases the mutations of those lines could be mapped to either the 2nd or the 3rd chromosome and something must be on those chromosomes to enable the flies to survive the enhanced Rheb signalling. We do not know the reason for the lethal effect of elevated Rheb signalling and therefore we are unable to determine, in which process the mutations suppressing the lethality of *Rheb* overexpression could be involved. Since we have not found any complementation groups, those mutations might not even be involved in the same process. However, all the chromosomes contained lethal hits, but we do not know whether the lethality was due to the mutation leading to the survival of *Rheb* overexpressing animals. In the case those mutations were homozygous viable, no complementation groups could be found.
For the X chromosome, only chromosomes carrying a lethal hit were analyzed. Non-essential genes were lost, but to test every single X chromosome would have been too laborious. Most of the known genes involved in TOR signalling are essential, therefore we expect most of our candidate genes to be essential. We crossed the lethal X chromosomes to *GMR-Rheb* flies as the only test for an involvement in TOR signalling. A second single hit has emerged from the screen. The mutation is homozygous lethal and it is able to suppress the *GMR-Rheb* big eye phenotype. Therefore, two single hits on the 2nd and X chromosome were the output of the screen. The screening process has been laborious, because the yield of the crosses has not been as large as expected and a lot more initial crosses have been required. Additionally, a large part of the surviving animals have not been able to reproduce and could not be examined.

To find these two single hits is far less than we have hoped for. Especially the lack of complementation groups has been disappointing. To map a single hit can be risky and is not recommended. A mutation in a single hit might never be found due to mutations outside of the coding region, in promoter or enhancer sequences. If mutations in two genes lead to a phenotype screened for, the genes might also never been identified.

Of course we have wondered about the genes underlying the two isolated mutations, but finally we have decided against the mapping of these single hits. The chances of success have been assessed as too small and the mapping of X chromosomal mutations is even more complicated than on the autosomes. For the mutation on the 2nd chromosome, we do not have an idea about the involved gene. However, *Raptor* and *LST8* are good candidates for the X chromosome. Both genes are located on the X chromosome. They have not been described yet in *Drosophila* and no mutants exist, but their function in TOR signalling is most likely conserved. Both genes encode proteins associated with TOR and are essential for the function of TOR in growth control\textsuperscript{19}. However, we were not able to test our mutation on the X chromosome for complementation due to the lack of mutations in *Raptor* or *LST8*.

A second screen has been conducted in parallel, being more promising at this time. Therefore we have decided to focus on the output of this second screen only and have
not started to do substantial work with any of the two single hits found in the lethality suppression screen. However, regarding their phenotypes both single hits would be worthwhile to be mapped and characterized.

3.3 Deletion screen

3.3.1 Introduction

This kind of screen used a completely different genetic method in addition to the utilization of a gain-of-function situation of Rheb. Furthermore, Rheb was specifically expressed in a certain tissue. Deletion mutants in downstream genes were identified by their ability to alter the Rheb gain-of-function phenotype. Rather than searching for rescue of lethality this second screen focused on the alteration of eye size since Rheb was specifically overexpressed in the developing eye.

![Diagram of signal strength and thresholds for gof and wt phenotypes]

1) Heterozygosity: no effect 2) Heterozygosity: suppression of the gain-of-function phenotype

Figure 9: Schematic view of the function of a sensitized system. In a wild-type situation (green), heterozygosity does not influence the wild-type phenotype. The threshold is still reached and one copy of a gene is sufficient for its function. In a gain-of-function situation (red), heterozygosity is not sufficient any more (gene function is below the threshold) to transmit the amplified signal and leads to a weaker phenotype. Abbreviations: gof: gain-of-function; wt: wild type.
Most deletions can only be used when heterozygous, because they uncover essential genes and are homozygous lethal. To detect a dominant effect by reducing only one copy of a gene, a sensitized system was needed. This system renders a recessive mutation dominant (haplo-insufficient). Figure 9 displays in a simple way the elegance of a sensitized system.

### 3.3.2 Establishing and testing of the screen setup

For our approach we used a sensitized system to screen a set of molecularly defined and isogenized deletions. Those deletions were produced by a European consortium to overcome the limitations of the Bloomington core deficiency kit\(^9\). This kit contained fly stocks with different genetic backgrounds and the break points of the deletions were not molecularly mapped. Most deletions were generated by irradiation or chemical mutagenesis, which resulted in secondary mutations. Additionally, the parental chromosomes were not available. Geneticists used this collection for classical genetic mapping and also for modifier screens. However, for screens that require a homogeneous genetic background those core deletions did not fulfil the requirements. Therefore a new set of deletions was generated called the DrosDel Collection\(^9\). This collection was produced in an isogenic background and all deletions were molecularly mapped. The deletions were produced with special \(P\)-elements containing a non-functional 5'- or 3' part of the \textit{white} gene and an \textit{FRT} site. A new \(P\)-element with a functional \textit{white} gene was produced by FLP-mediated recombination between two \(P\)-elements arranged in \textit{trans} on homologous chromosomes. The intervening DNA was deleted and marked by \(w^+\), whereas the corresponding chromosome contained a non-marked tandem duplication of the deleted segment.

In parallel, a second kit of molecularly defined and isogenic deletions was generated (Exelixis deletions\(^{10}\)). We used both collections to screen for modifiers of a \textit{Rheb} gain-of-function phenotype.

A \textit{GMR-Rheb} construct was generated that allowed the overexpression of \textit{Rheb} specifically in the eye (Figure 10). Those transgenic flies contained large eyes compared to their controls. We planned to cross those transgenic \textit{GMR-Rheb} lines to
flies containing deletions and to screen for a decrease or increase in the eye size in the progeny. In the case of an alteration of eye size the deletion could contain a gene involved in the downstream or upstream signalling of Rheb.

Figure 10: TOR signalling mutants alter the eye size of flies overexpressing Rheb. Overexpression of Rheb in the eye with a direct GMR-Rheb construct leads to a sensitized system. A) To the left, a control eye is visible and to the right a GMR-Rheb overexpressing eye. B, C: Removal of one copy of Raptor reduced the ommatidial size of GMR-Rheb overexpressing flies (C) compared to the control (B). D, E: In contrast, heterozygosity for TSCI results in larger ommatidia in GMR-Rheb overexpressing flies (E) compared to the control (D). Genotypes are: A: y w (left); y w; GMR Rheb+/+ (right); B: w; GMR-Rheb7+/+; C: w, ED6882/+; GMR-Rheb7+/+; D: w, GMR-Rheb39/+; E: y w, GMR Rheb39/+; TSCI/+

As a control we tested the screen setup with two obvious candidates of the TOR signalling pathway, namely with TSCI and Raptor. In the case of TSCI, we found an increase of the GMR-Rheb phenotype, whereas for Raptor, we could observe a decrease in eye size. These results confirmed us that the screen setup was well chosen. For the screen, we planned to use two different GMR-Rheb lines. The strong insertion was expected to lead mainly to the discovery of deleted genes suppressing the growth phenotype. A weaker GMR-Rheb line was anticipated to lead to the identification of deletions enhancing the growth phenotype. Both lines were tested in advance with the appropriate mutations or deletions (Figure 10).

Once a promising deletion was uncovered, the next step was to identify the responsible gene in the deficient region. First of all, we had to exclude genes using overlapping deletions. A second point was to test existing loss-of-function mutations in candidate genes for their suppression or enhancement of the GMR-Rheb phenotype. Third, lines
producing RNAi against candidate genes could be tested for growth alterations in the eye\textsuperscript{101}. If the deletion was huge or if there were no mutants available, it was difficult or almost impossible to identify the responsible gene.

### 3.3.3 Results of the deletion screen

When we initiated this work the creation of the DrosDel lines was still ongoing and not all currently available deletions could be tested. Nevertheless a large fraction of the fly genome could be analyzed. For the strong insertion (\textit{GMR-Rheb7}), we tested 564 lines and for the weaker insertion (\textit{GMR-Rheb39}) we tested 588 lines. Interactions were found in about 6.5\% of all crosses, but most of them displayed rough or necrotic eyes. We found only three reasonable modifiers with normal eye structure.

<table>
<thead>
<tr>
<th>I) Suppressor, 3R, 84F-85C</th>
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<tr>
<td>II) Enhancer, 3L, 69C-69D</td>
</tr>
<tr>
<td>III) Enhancer, 3R, 88C-88D</td>
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**I) Suppressor, 3R, 84F-85C**

This suppression was caused by the DrosDel deletion \textit{ED5296} and led to a reduction of eye size of both the strong and the weaker \textit{GMR-Rheb} insertion. The suppression of \textit{GMR-Rheb39} resulted in a smaller eye with wild-type structure, whereas the suppression of \textit{GMR-Rheb7} did not lead to a wild-type structure. The structure was more irregular. All the same we decided to follow up this suppressor. \textit{ED5296} deletes more than 100 genes. Other smaller DrosDel and Exelixis deficiencies completely spanned the region of \textit{ED5296}. We expected therefore to find at least one additional deletion that suppressed the phenotype. This was not the case: none of the other tested lines led to a suppression of \textit{GMR-Rheb7} or \textit{GMR-Rheb39}, respectively. We explained this fact with the presence of an interacting second hit on the \textit{ED5296} chromosome. Two deleted genes could also be responsible for the effect. In any case the identification of the responsible gene(s) would have been difficult. Therefore we focused primarily on the other two promising deletions.
II) Enhancer, 3L, 69C-69D

This enhancement of GMR-Rheb39 was found with two overlapping DrosDel deletions, ED4483 and ED4486, and it was only clearly seen with GMR-Rheb39. Their common overlap contained 13 genes only, and there was another Exelixis deletion in the same region as well. This deletion also showed an enhancement and the common region could be restricted to a region of 20 kb situated in the Iroquois gene complex (establishes the dorsoventral midline (equator) and is expressed in the dorsal half of the eye). Within this stretch there was only one predicted gene, CG32111. Evidence for the existence of this gene was the presence of two ESTs (expressed sequence tag) published in Flybase (http://rail.bio.indiana.edu/). There were no mutants available and the gene displayed no homology to genes in higher organisms. Therefore we suspected that the gene might be a non-coding RNA. A P-element was used to generate mutants removing the first exon of the gene. However, homozygous mutant flies were viable, showed no obvious defects and displayed no enhancement of GMR-Rheb39. This indicated that CG32111 was not the gene responsible for the enhancement of the GMR-Rheb phenotype. Therefore we went back to the 13 genes overlap of the two DrosDel deletions. We ordered all available mutants of the deleted genes and crossed them to GMR-Rheb39. None of the lines led to a significant enhancement.

For other reasons we launched a collaboration with Fried Zwartkruis from Utrecht, Netherlands. His group was working on the same signalling pathway, but using a biochemical approach. We exchanged our data from the deletion screen and they tried to identify the candidate genes of the interacting deletions with a biochemical approach. They produced double stranded RNA (RNAi) against the candidate genes. S2 cells were incubated with RNAi for several days to lead to a knock-down of the corresponding gene. Afterwards the RNAi-treated cells were examined for the phosphorylation of S6K in Western blots. The phosphorylation of S6K is a frequently used readout of TOR signalling activity and interacting genes can alter the phosphorylation of S6K. Indeed, the elongation initiation factor eIF-2β showed a very clear pattern of elevated S6K phosphorylation upon insulin stimulation, when its gene function was decreased in S2 cells[102]. This phosphorylation could be blocked with the
addition of rapamycin, a suppressor of TOR. Those promising experiments in cell
culture made us focusing on the gene in *Drosophila*.

The gene *eIF-2β* is a subunit of *eIF-2*, which binds Met-tRNA and GTP to form the
first exon and oriented in the opposite direction as *eIF-2β* was ordered. The line was
homozygous lethal. Crossed to *GMR-Rheb39*, *EP745* led to an increase in eye size.

From a different source a second *EP* element was obtained (*G4/64*), which was also
inserted in the first exon of *eIF-2β*, but oriented in the same direction as the gene.
Overexpression of *G4/64* in the eye with *GMR-Gal4* and *ey-Gal4* showed no
phenotypical alterations. The line was also homozygous lethal and heteroallelic
combinations with *EP745* led to viable flies. However, they were more than two
weeks delayed and contained thin bristles. This resembled a Minute phenotype, as
expected for a gene involved in protein synthesis. Minute mutations are characterized
by thin bristles and a developmental delay and mostly encode ribosomal proteins.
Additionally, the heteroallelic flies displayed very bright eyes. This bright eye colour
might reflect a defect in pigment synthesis. *G4/64* did not dominantly enhance the
*GMR-Rheb* phenotype; there was no size increase of the ommatidia. Since this could
be explained by different backgrounds, we wanted to test null mutants.

The genomic region of *eIF-2β* spans only 1.65 kb and both *EP*-elements (*P*-elements
with UAS-sites) are inserted less than 100 bp upstream of the ATG. We generated
precise excisions of both *EP* lines by crossing the *P* elements to a fly stock containing
a transposase source. The *P*-element transposase leads to the excision of *P*-elements in
the genome. *P* elements tend to insert in the 5' region of a gene and can result in the
reduction of gene function. The function of such hypomorphic or lethal alleles can be
reverted by a precise excision of the *EP* element. A reversion of the lethality indicates
that the insertion was responsible for the mutant phenotype and additionally it shows
that there is no unrelated second lethal hit on the chromosome. For our *EP* insertions
in the *eIF-2β* locus, only the lethality of *G4/64* could be reverted with a precise
excision. Therefore we used *G4/64* to generate mutant alleles of *eIF-2β*. The excision
of *EP* elements can lead to imprecise excisions caused by the transposase and this
failure is used to screen for mutant alleles. If the *EP* element is close to the ATG, the
start codon can be removed and this leads to a mutant allele. We generated a large number of male flies with excised EP elements (w'). PCR reactions were established to screen for deletions. Male animals were allowed to mate to balancer virgins. Afterwards those males were PCR screened in groups of ten animals. The primers were set upstream of the EP-element insertion (primer 1) and downstream of the ATG (primer 2). As long as the deletion did not remove the binding site for primer 2, the deletion could be detected by the presence of a shorter amplified PCR product by these two primers. Once a group of tested males displayed a shorter fragment, the progenies of all group members were individually screened for the deletion.

Three imprecise jump outs were obtained and established. Two of them removed more than half of the genomic region and were regarded as null mutations. Those alleles did not lead to an increase of the eye size by crossing them to GMR-Rheb39. This clearly demonstrated that eIF-2β was not the gene responsible for the observed enhancement by the two DrosDel deletions. Because the cell culture data were very convincing and we decided to continue the characterization of eIF-2β.

The idea behind our second strategy was to reduce the gene function in vivo by more than half as it was probably the case for the RNAi cell culture experiments. Heterozygosity might not have reduced the eIF-2β levels enough to enhance the GMR-Rheb phenotype. We had no dominant-negative alleles of eIF-2β and therefore the only way to reduce the gene function by more than 50% was to use RNAi. We wanted to test whether RNAi against eIF-2β led to an enhancement of the GMR-Rheb phenotype. We received a RNAi line against eIF-2β (15G11T2)101. However, first trials with various Gal4 driver lines displayed a fairly strong action of the construct. Overexpression in the eye with GMR-Gal4 led to pupal lethality and the few escapers had small, glassy eyes with black spots (25°C). Overexpression with GMR-Gal4 in a GMR-Rheb background also led to only few escapers with small, glassy eyes and black spots. Crosses with ey-Gal4, C10, MS1096, ppl-Gal4, arm-Gal4 and Act-Gal4 did not result in adult flies at all (25°C). The cells were not able to survive properly due to the strong reduction of gene function. Therefore we decided to establish weaker inducible RNAi lines by mobilizing the RNAi construct with transposase. The aim was to isolate weaker lines compared to the original line 15C11T2. However, the lines
should lead to a reduction of gene function by more than 50% to have a stronger effect than heterozygosity for eIF-2B.

The RNAi construct was w+ marked and we selected and established new lines containing a very faint w+. There was not always a correlation between the strength of expression of the w+ and the corresponding transgene, but the correlation was good enough to obtain very weak lines based on this selection criterion. At least five independent lines were established and tested for their phenotypes with different GMR-Gal4 insertions and various other Gal4 driver lines at temperatures ranging from 16 to 29°C.

There were two patterns observable up to temperatures of 25°C. Either the new RNAi insertion lines were still too strong and the progeny did not survive at 25°C, or the flies displayed a wild-type phenotype. Because we did not know whether these weaker lines were functional, we tested them with various Gal4 drivers at 29°C. Overexpression with GMR-Gal4, C10, MS1096, da-Gal4 and Act-Gal4 led to viable flies, and certain lines displayed an altered phenotype. Strong overexpression with Act-Gal4 and da-Gal4 resulted in blisters in the wing centre, slight vein 2 defects or crumpled wings. The phenotypes of the GMR-Gal4 driven expression ranged from a slight to a clear enlargement of the eyes. This was a very promising finding and to test its relevance we used animals heterozygous for TOR as a control. Those heterozygous animals were expected to display smaller eyes, if the cell culture data were also valid in vivo. However, this experiment revealed no difference in eye size and therefore we decided not to invest more time to find more suitable RNAi insertions. It was also not possible to identify the responsible gene in the deletions leading to the observed phenotype and we focused therefore on the last candidate deletion.

III) Enhancer, 3R, 88C-88D

This enhancement was found with one deletion only, and much to our surprise it was better seen with GMR-Rheb7 that with GMR-Rheb39. The deletion was from the Exelixis collection (Exel8160) and partially overlapped with Exel6275, which did not show a growth alteration. Therefore we could restrict the candidate region to nine predicted genes. A first inspection of the deleted genes led us to CG7552, a gene
which was previously found in a screen for negative growth regulators in the eye of
*Drosophila*103. We identified this gene as the best candidate of all the deleted genes.
*CG7552* is very small, codes for a WW domain and has no obvious homologs in other
organisms. We realized later that the Flybase annotation was not correct. We found an
EST spanning the whole region from *CG7552* to and including the neighbouring gene
*CG12600*. Thus the gene - recently updated and renamed as *CG33967* - became even
more interesting for us (Figure 11).

![Figure 11: Genomic region of CG33967. CG7552 (Exon 1) and CG12600 (Exon 2-9) encode different parts of
the same protein and are separated by a huge intron. The whole genomic regions spans about 25.7kb.](image)

*CG33967* codes for a protein conserved from *Drosophila* to humans. To confirm the
published growth phenotype data we ordered EP lines inserted in this locus as well as
RNAi lines101. Tables 7 and 8 and Figure 12 display a summary of the phenotypes that
resulted from the initial crosses.

<table>
<thead>
<tr>
<th>Gal4 driver line</th>
<th>x EP Kibra (19909)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMR-Gal4</td>
<td>smaller and rougher eyes</td>
</tr>
<tr>
<td>ey-Gal4</td>
<td>wild-type phenotype</td>
</tr>
<tr>
<td>MS1096</td>
<td>slightly bent up and smaller wings</td>
</tr>
<tr>
<td>C10</td>
<td>wild-type phenotype</td>
</tr>
<tr>
<td>ppl-Gal4</td>
<td>wild-type phenotype</td>
</tr>
<tr>
<td>Act-Gal4</td>
<td>pupal lethal, few escapers have wild-type appearance</td>
</tr>
</tbody>
</table>

Table 7: Overexpression of the EP Kibra (19909) results in smaller organs or lethality. 19909 is inserted in
the 5'UTR of Kibra. All crosses were made at 25°C.
Overexpression of the Kibra EP line (19909) led to smaller eyes as published in Tseng et al.\textsuperscript{103}. With one of the inducible RNAi lines (81D12), we received the opposite phenotype, namely enlarged eyes and wings. The second inducible RNAi line (143C11) led to lethality with all tested Gal4 driver lines, which could be due to off targets of this RNAi line. This issue will be discussed later in chapter 3.3.4. We co-overexpressed the RNAi line against CG12600 together with the Kibra EP line 19909 in the eye. Depending on the different potency of both lines it should have been possible to find conditions where the two lines compensate for each other. Indeed, such conditions existed at 25°C (Figure 13).
Figure 13: Co-overexpression of a *Kibra* EP line and RNAi against *CG12600* leads to an intermediate growth phenotype. Whereas overexpression of the *EP Kibra* (19909) leads to small eyes (1), overexpression of RNAi against *CG12600* results in large eyes (2). Co-overexpression of the *EP Kibra* (19909) and of RNAi against *CG12600* leads to an intermediate eye size (3). The values display the area of a rosette of seven ommatidia located in the centre of the eye. Genotypes are: 1) *yw; GMR-Gal4/+; 19909/+*; 2) *yw; GMR-Gal4/+; UAS-RNAi (CG12600)/+*; 3) *yw; GMR-Gal4/+; 19909/+ UAS-RNAi (CG12600).*

The data obtained with *Kibra* were promising. Overexpression of *Kibra* resulted in smaller eyes, whereas overexpression of RNAi displayed the opposite phenotype. Since the deficiency deleting *Kibra* led to an enhancement of the *GMR-Rheb* phenotype, we expected a negative growth regulatory gene to be our candidate. *Kibra* fulfilled this requirement and we planned to create imprecise excision alleles of *Kibra* to continue its characterization.

### 3.3.4 Discussion

The overexpression of *Rheb* has been used to find new downstream components in the TOR signalling pathway, similar to the lethality suppression screen. However, this time the goal was to establish a sensitized system in the eye of *Drosophila*. This system has enabled us to reveal the effects of heterozygosity of a certain gene. Heterozygosity normally does not lead to an effect, because in most cases a sufficient function is reached with one copy of a gene. However, if a signalling pathway of interest is enhanced, one copy of an involved gene might not be sufficient any more to transmit the elevated signal. This leads to an alteration of the phenotype. Therefore, a
sensitized system renders certain genes haploinsufficient. We have created a sensitized system in the eye of *Drosophila* by overexpressing *Rheb* specifically in this organ.

For the first time, isogenic deletion libraries have been generated and this has been very useful, since the background itself can have a large impact on the size of organs. Furthermore, all generated deletions are molecularly defined. In many other cases deletions we do not have clearly defined break points. For genes situated in these regions it is not clear whether they are deleted by the deficiency or not.

To screen for alterations of a phenotype it would have been an alternative to induce mutations with EMS instead of using deletions. However, to take deletions has several advantages. With the deficiencies, the range of candidate genes is already strongly restricted to a defined genetic region. The deletion screen itself and the mapping takes far less time than the mapping of the EMS mutations. On the other hand, a complementation group can be mapped in nearly every case, whereas the identification of a gene in a deletion can be almost impossible if the deletion is large and no mutants of the candidates are available. However, there is another advantage of the deletion screens. In a deletion screen, the progeny of an entire cross of flies can be examined for size alterations. In an EMS screen, a size alteration has to be assessed in individual animals, which can be difficult if the effect is mild.

Ideally, both types of screens would be performed. A deletion screen quickly shows whether there are dominant interactions at all. A sensitized system does not lead to haploinsufficiency in every case. If no suitable tests can be done prior to an EMS mutagenesis, a deletion screen would be a good alternative. Additionally, the two screens should lead to a similar set of candidate genes. Therefore, complementation analysis between EMS mutations and positive deletions should quickly narrow down the set of candidate genes for the EMS mutants.

As a proof of principle for the screen setup, we have crossed the *GMR-Rheb* flies to animals carrying mutations or deletions for *TSC1*, *TOR* and *Raptor*, respectively. We have found the expected size alterations, indicating that the system was reliable enough to proceed with the crossing of the deletions.
6.5% of all tested deletions led to an interaction with the *GMR-Rheb* phenotype, but only three deficiencies (one suppressor and two enhancers) did not display additional patterning defects or necrosis.

The suppressor was caused by a deficiency on the third chromosome (3R, 84F-85C), deleting more than 100 genes. Other smaller DrosDel and Exelixis deficiencies completely span this region, but none of them led to a suppression of the *GMR-Rheb* eye phenotype. This can have several reasons. There might be a second hit on the deletion chromosome causing the reduction of eye size, or two genes in the deletion cause the reduction together. In both cases, the identification of the responsible gene(s) is a large work load with uncertain success. A third explanation for this phenomenon is the altered neighbourhood of the genes. Deletions bring the flanking genes in close proximity to each other and the affected genes could be influenced such that they lead to a suppression of the *GMR-Rheb* phenotype. This would be an artificial situation in which we are not interested. For all those reasons we have not further tried to identify the responsible gene(s).

The first enhancer is caused by two overlapping DrosDel deficiencies on the third chromosome (3L, 69C-69D). The overlap of all three deletions contained only one gene, *CG32111*. No homolog is known in higher organisms and it might be a non-coding RNA. Mutant alleles of *CG32111* are homozygous viable and the flies show no visible phenotypes. Those alleles also do not enhance the *GMR-Rheb* phenotype, indicating that *CG32111* is not the gene responsible for the enhancement. We suspected that there was something wrong with the Exelixis deletion. The two DrosDel deletions have been generated in exactly the same way and contain the same isogenic background. It is known about the Exelixis deficiencies that around 8% (http://flystocks.bio.indiana.edu/) of them do not delete the genes they are supposed to delete. It might be that another deleted gene, which we do not know, led to the observed enhancement. Another possibility would be a second hit on the chromosome. However, we decided to focus on the 13 genes in the overlap of the two Dresden deletions.
We used a collaboration with Fried Zwartkruis to reveal whether any of the deleted candidate genes had an effect on the phosphorylation of S6K in cell culture. Our collaborators found eIF-2β as a candidate. RNAi against this gene shows a clear pattern of elevated S6K phosphorylation upon insulin stimulation. This increase in phosphorylation can be blocked by the addition of rapamycin, a compound potently suppressing TOR.

We have not been able to clarify the role of eIF-2β in the fly despite the availability of two EP-element insertions leading to mutant alleles of eIF-2β. Imprecise excision alleles did not lead to an enhancement of GMR-Rheb. Therefore eIF-2β is definitively not the gene causing the enhancer effect of the two DrosDel deletions. We have not been able to identify another candidate gene in the overlap of the deletions. Instead we have decided to proceed the work with eIF-2β, because the cell culture data were very convincing.

Heterozygosity for eIF-2β might not reduce the gene function to levels enhancing the GMR-Rheb phenotype. Therefore we tested whether we find an enhancement of the GMR-Rheb phenotype by reducing the gene function of eIF-2β by more than 50%. An RNAi line against eIF-2β was used for this experiment, but the overexpression has been too strong and the cells have not been able to survive. We mobilized the w+ marked construct and established weaker RNAi lines. At 29°C, the weak lines crossed with all Gal4 diver lines led to viable flies, and some of the lines displayed a weak mutant wing or eye phenotype. A GMR-Gal4 dependent eye size increase was not expected. Since the cell culture data have positioned eIF-2β upstream of TOR, we planned to test the significance of the eye size increase by removing one copy of TOR. Heterozygosity should reduce the RNAi induced eye size, if the cell culture model was true. However, removing a functional copy of TOR has not altered the eye size.

Despite the phenotypes we observe at 29°C, we are not convinced that our newly established weak RNAi lines reduce the gene function by more than 50%. Especially the mild wing phenotypes caused by the ubiquitous Gal4 drivers is not convincing. In light of the very strong effect caused by the original RNAi line, we expected a stronger phenotype of the weak lines when ubiquitously overexpressed at 29°C.
It might be that whole organisms are more sensitive to a reduction of eIF-2β levels than individual cells in cultures. We might have received two classes of new insertion lines. The first class appears to be as strong as the original line and overexpression leads to lethality. The second class of lines seems to be non-functional or only weakly functioning. It might be that we have been unlucky and have not received a suitable insertion line. But maybe even slightly larger decreases of gene function below 50% lead to lethality. In this case it would not be possible to obtain the lines we have screened for. If this is the case, we would not be able to test our hypothesis whether a reduction of more than 50% of the eIF-2β gene function leads to a size increase. On the other hand, it is also possible that the cell culture data are just an artefact and a reduction of eIF-2β is not supposed to enhance the GMR-Rheb phenotype.

The second enhancer was found with an Exelixis deletion on the third chromosome (3R, 88C-88D). Due to another overlapping Exelixis line, which did not display an enhancement, the candidate region could be restricted to nine predicted genes. We focused on a gene that has already been found in a screen for negative growth regulators in the eye of Drosophila. This gene has homologs from Drosophila to humans and is named Kibra. The overexpression of this protein in the eye of Drosophila by means of an EP insertion in the 5’UTR led to smaller eyes. In contrast, weak overexpression of RNAi against CG12600 led to increased eyes and wings. However, RNAi against CG7552 always resulted in dead animals, even with weak Gal4 driver lines. We assume that the differences in the On and OFF target rates of the two RNAi lines could cause these discrepancies. The ON- and OFF target rates of all generated RNAi lines are published in the Web.

Whereas the RNAi line against CG12600 is very specific, the RNAi line against CG7552 is directed against 128 OFF targets. Its most important OFF target is CG4013. This gene is involved in the negative regulation of transcription and in the regulation of transcription from the RNA polymerase II promoter. A downregulation of CG4013 might cause the lethality observed by overexpressing RNAi against CG7552.
Since the RNAi line against CG12600 leads to the opposite phenotype of the Kibra EP overexpression, we are convinced that the data obtained with the RNAi line against CG12600 are more likely to be relevant than the data received with the RNAi line against CG7552. Additionally, co-overexpression of the Kibra EP and the RNAi line against CG12600 led to an intermediate growth phenotype. This could still be additive, but regarding all the data it looks promising that the EP and the RNAi lines affect the same gene. Therefore we have ignored the results received with the RNAi line against CG7552. Due to the promising data, we have decided to generate null alleles of Kibra and to further characterize its putative involvement in TOR signalling.

3.4 Characterization of Kibra

3.4.1 Introduction

The mammalian Kibra has been found in a yeast two hybrid screen with dendrin, a putative modulator of the postsynaptic cytoskeleton\textsuperscript{104}. The protein consists of 1113 amino acids and is predominantly expressed in kidney and brain, hence the name. Kibra contains several protein-protein interaction domains. At its N-terminus, two WW domains are present and more to the C-terminal end a C2-like domain (similar to the C2 domain of synaptotagmin\textsuperscript{104}) followed by a glutamic acid-rich stretch (Figure 14). Additionally several putative coiled-coil domains exist. Therefore, Kibra appears to function as a scaffold protein and has no catalytic activity on its own. Protein-protein interactions are important for a precise transmission of the cellular signals. The WW motif mediates the interactions through binding to proline-rich sequences\textsuperscript{105}. Those domains contain approximately 40 amino acids (aa) and have two conserved tryptophane residues separated by 20-22 aa. WW domains are found in cytoplasmic as well as in nuclear proteins and are involved in various cellular processes like protein degradation, regulation of transcription, RNA splicing and protein clustering\textsuperscript{106,107}. 


The C2 domain was first discovered in the protein kinase C\textsuperscript{108}. The domain is conserved, consists of about 120 aa and binds phospholipids in a calcium-dependent manner. Most proteins with C2 domains are involved in signal transduction or membrane trafficking\textsuperscript{109}, but not all of them are calcium- and phospholipid-binding domains. Some of them are calcium-independent and recently it has been shown that the C2 domain of the protein kinase C\textdelta is a phosphotyrosine binding domain\textsuperscript{110}.

Coiled-coil domain-containing proteins exhibit a broad range of different functions related to the specific design of their domains. Coiled-coil assembly can be modulated by phosphorylation or by interaction with ions and this makes this domain a highly versatile protein folding motif adaptable to many biological processes\textsuperscript{111}. Coiled-coil domains are involved in signal transduction, but they can also provide mechanical stability to cells or can be involved in movement processes.

![Figure 14: Schematic view of the mammalian Kibra domains. 1, 2) WW domains 3) C2-like domain (similar to the C2 domain of Synaptotagmin) 4) Glutamic acid-rich stretch. Additionally there are 7-8 putative coiled-coil domains present.](image)

The \textit{Drosophila Kibra} genomic locus has a length of 25.7kb. The gene contains nine exons and the transcript length is 4.9kb. The protein is composed of more residues than its mammalian counterpart (1228 instead of 1113 amino acids) and has a molecular weight of 144kDa. All domains are conserved and the overall conservation between the \textit{Drosophila} and the human protein is 51% similarity and 32% identity.

Kibra has been found in a screen for new downstream components of the small GTPase Rheb (chapter 3.3). Rheb is involved in TOR signalling and displays a positive effect on the kinase TOR. However, this regulatory mechanism was not known when this study was initiated. Since no binding studies of Rheb and TOR were published at this time, we expected additional proteins to transmit the signal from Rheb to TOR and initiated two different screens to find downstream components of
Rheb. Kibra has been the most promising candidate gene. The aim of the further studies was to analyze Kibra’s putative function in the TOR signalling pathway and to characterize this gene. For that, mutant alleles were generated to test whether Kibra was the responsible gene uncovered by the deficiency leading to an enhancement of the Rheb overexpression and to further characterize Kibra.

3.4.2 Results

Mutants were generated by imprecise excision of the Kibra EP 19909 (for procedure see chapter 3.3.3). In the mutant alleles, only the first exon containing the ATG could be removed, since the first two exons are separated by a large intron (Figure 11). EP 19909 was inserted in the 5’ UTR of Kibra and less than 1kb away from the ATG of the gene. We screened around 330 individual w flies and were able to recover three imprecise excision alleles (Figure 15).

![Figure 15: Two imprecise deletions remove the ATG of CG7552. The EP insertion is in the 5’ UTR of CG7552 and imprecise excisions were made by mobilizing EP 19909. Kibra<sup>54</sup> is a homozygous viable allele and flies display a wild-type phenotype. Kibra<sup>71</sup> and Kibra<sup>123</sup> are homozygous lethal alleles (1<sup>st</sup> larval instar). Deletions are indicated as lines.](image)

In both Kibra<sup>71</sup> (deletion of 891bp) and Kibra<sup>123</sup> (deletion of 1016bp), the ATG was removed and homozygous animals died during the first larval instar. Those alleles did not complement each other. In contrast, in the allele Kibra<sup>54</sup> (deletion of 536bp) the ATG was not removed and homozygous animals were viable and displayed a wild-type phenotype.

We wanted to test whether Kibra was the gene responsible for the enhancement of the GMR-Rheb phenotype. By crossing the putative Kibra null alleles to GMR-Rheb and
analyzing the progeny, we did not find an enhancement of the phenotype and excluded *Kibra* as candidate gene. At the same time we realized that the original deletion line, which caused the increased eyes of *GMR-Rheb* flies, contained very few homozygous mutant adults. Since *Kibra* is an essential gene, the deletion was supposed to be homozygous lethal as well. Around 8% of all Exelixis deletions are incorrectly assigned (http://flystocks.bio.indiana.edu/). We suspected that the defined break points were wrong, although a mutation in the essential gene (*eff*) deleted by this deficiency did not complement the deletion (tested in Bloomington), showing that the indicated deficiency was present. We ordered the line from Bloomington a second time and observed homozygous mutant flies again. However, *Kibra* had been shown to have a growth phenotype and we were interested in proceeding its characterization.

In parallel to the characterization of the *Kibra* imprecise excision alleles we tried to obtain different loss-of-function mutations of the gene. The first two exons are separated by a large intron and other ATGs were present in *CG12600*. The largest part of the protein could still be translated by deleting the ATG of *CG7552*. For that reason we started an EMS reversion mutagenesis and hoped to receive stop mutations in *CG12600*. We crossed *GMR-Gal4* flies to mutagenized *EP 19909* flies, which leads to smaller eyes, and screened the progeny for wild-type eyes to obtain mutations in the *Kibra* locus. In a first mutagenesis round we screened 18'000 flies and found six revertants. Although none of them displayed a complete wild-type phenotype, they differed from the average *Kibra* overexpression eyes. We established individual lines from the isolated revertants and realized later that we did not get the expected alleles. All six reversion mutant lines were homozygous viable and there were no mutations found in the coding region of *Kibra*. Therefore we started a second mutagenesis round and screened 15'000 flies. We isolated three revertants and for one of them we found a mutation in the coding region. This base pair exchange led to the mutation of the initial ATG (ATA) in *CG7552* and was therefore similar to the imprecise excision alleles. In the remaining two lines no mutation was found. We did not perform a third mutagenesis round and decided to use the alleles we had. More precisely, we made all experiments with an imprecise excision allele and the reversion mutagenesis allele in
parallel. Since these alleles were generated in different backgrounds, this was a useful strategy to recognize the contributions of the background. The mutant alleles were used to examine the behaviour of Kibra mutant cells. Homozygous mutant animals die early in development, therefore we examined mutant cells surrounded by wild-type tissue or restricted the loss-of-function of Kibra to distinct organs to enable the animals to survive.

A first step of the growth analysis of Kibra was to generate homozygous mutant heads by means of the ey-Flp technique. Mutant alleles were recombined onto FRT-containing chromosomes. When the flipase is present, recombination between homologous chromosome arms (at the FRT sites) can lead to homozygous mutant cells. The ey-Flp construct leads to the expression of the flipase in the progenitor cells of the eye during development. Therefore a large proportion of tissue consists of homozygous mutant cells. Kibra mutant heads were enlarged (Figure 16), as it was observed for the overexpression of RNAi against CG12600 (Table 8 & Figure 12). The size increase was not as large as it was for the inducible RNAi line, but the similarity of the two phenotypes was obvious. By measuring the size and number of the mutant ommatidia, we found that the mutant ommatidida were enlarged in size and that the number of the ommatidia remained constant (Figure 16). Interestingly, the imprecise allele Kibra^35.1, which does not remove the ATG, displays a wild-type size of ommatidia and is therefore not a mutant allele.
To investigate why *Kibra* mutant ommatidia were enlarged we generated tangential eye sections of mosaic heads (Figure 17). The *hs-Flp* technique leads to a controlled expression of the flipase by the induction of a heat shock. Therefore not all the cells undergo a flipase-mediated recombination and the mutant cells are surrounded by wild-type tissue.

**Figure 16:** Homozygous mutant *Kibra* heads contain a wild-type number of ommatidia, but the ommatidia are increased in size. A, B: Electron microscopy pictures show that the *Kibra* mutant head is clearly increased in size (B) compared to the control (A). C, D: The number of ommatidia is not significantly increased (C), whereas the size of ommatidia is clearly enlarged in *Kibra* mutant heads (D). *Kibra*\textsuperscript{351}, which does not remove the ATG, displays a wild-type size of ommatidia. Genotypes are: A: *yw, ey-Flp; FRT82, w\textsuperscript{+}, cl/FRT82, Kibra\textsuperscript{351}; B: *yw, ey-Flp; FRT82, w\textsuperscript{+}, cl/FRT82, Kibra\textsuperscript{1231}. C, D: 1) *yw, ey-Flp; FRT82, w\textsuperscript{+}, cl/FRT82, Kibra\textsuperscript{351}; 2) *yw, ey-Flp; FRT82, w\textsuperscript{+}, cl/FRT82, Kibra\textsuperscript{351}; 3) *yw, ey-Flp; FRT82, w\textsuperscript{+}, cl/FRT82, Kibra\textsuperscript{714}; 4) *yw, ey-Flp; FRT82, w\textsuperscript{+}, cl/FRT82, Kibra\textsuperscript{1231}; 5) *yw, ey-Flp; FRT82, w\textsuperscript{+}, cl/FRT82, Kibra\textsuperscript{R441}. Alleles are: *Kibra*\textsuperscript{351}: precise excision allele; *Kibra*\textsuperscript{1231}: imprecise excision allele containing the ATG; *Kibra*\textsuperscript{714} and *Kibra*\textsuperscript{1231}: imprecise excision alleles removing the ATG. *Kibra*\textsuperscript{R441}: Reversion mutagenesis allele (ATG mutated in CG7552). Statistical analyses: C: 1,2: \(p>0.06\); 1,3: \(p>0.7\); 1,4: \(p<0.014\); 1,5: \(p>0.97\); D: 1,2: \(p>0.08\); 1,3: \(p<0.0000012\); 1,4: \(p<0.0003\); 1,5: \(p<0.0001\). Analyses were done with a Students T-test (two-tailed). Error bars indicate the standard deviation.
Figure 17: Homozygous mutant Kibra tissue displays an increased distance between the ommatidia. A,B: Tangential sections of mosaic eyes consist of Kibra mutant tissue (absence of w+) and wild-type tissue (marked by the presence of w+). The distance between the mutant ommatidia (w-) is clearly enlarged compared to the distance of the wild-type ommatidia (w+). B: Higher magnification of A. C, D: Statistical analyses of the distances from R7 to R7 photoreceptor cells of three independent eye sections per genotype (1-3). The white bar of every section reflects the distance between the control ommatidia (w+), whereas the black bar of every section reflects the distance between the Kibra123/ mutant (C) or the KibraB.M.11/ mutant (D) ommatidia (w-), respectively. C: section 1: p>0.11; section 2: p<0.0035; section 3: p<0.038. D: section 1: p<0.006; section 2: p<0.0005; section 3: p<0.001. Genotypes are: A, B: hs-flp; FRT82, Kibra71/FRTR2, w+; C: hs-flp; FRT82, Kibra123/FRTR2, w+; D: hs-flp; FRT82, KibraB.M.11/FRTR2, w+. Alleles are: Kibra123/ impersive excision allele removing the ATG, KibraB.M.11/ Reversion mutagenesis allele (ATG mutated in CG7552). Analyses were done with a Students T-test (two-tailed). Error bars indicate the standard deviation.

The tangential eye sections revealed a normal structure and differentiation of the ommatidia. The rhabdomeres, the black light sensing organelles of the ommatidia, also seemed to be of equal size. However, the distance between the mutant ommatidia (w-) was enlarged compared to the control (w+), which was most likely the reason for the increased head size of the mutants (Figure 16). The size increase could be explained in two ways. There could be more interommatidial cells or the cell bodies of the ommatidia could be enlarged. This issue could not be solved with the tangential eye sections. Therefore we performed two different approaches: we prepared eye sections of mosaic Kibra heads to examine them with a transmission electron microscope (TEM) and we made phalloidin stainings of pupal eye discs. The TEM allowed us not only to detect the structures of the cell membranes, but also to observe the content of
the examined cells. If the cell bodies were larger, the content of the mutant cells compared to the control cells could give us a clue about the function of Kibra. The phalloidin staining was made to detect the outline of the cells to see whether there were additional interommatidial cells present in the mutant clones, which could account for the increased ommatidial distance.

Figure 18: Sections of Kibra mutant ommatidia reveal partially larger cell bodies and slightly smaller rhabdomeres. A, A', B, B': Transmission electron microscopy (TEM) pictures of two control (w') ommatidia (A, A') and two Kibra\textsuperscript{RM,11} mutant (w) ommatidia (B, B') that display enlarged cell bodies (CBs). C, D: The sizes of CB1-CB6 have been measured in the same eye sections of Kibra\textsuperscript{RM,11} (C) and Kibra\textsuperscript{RM,11} (D) mosaic eyes and the mutant CBs tend to be larger or are significantly increased in size. The white bars represent the control, whereas the black bars represent the mutant areas. E, F: The sizes of rhabdomer 1 – rhabdomer 7 have been analyzed and Kibra\textsuperscript{RM,11} (E) and Kibra\textsuperscript{123,1} (F) and the mutant rhabdomeres (black bars) tend to be slightly smaller than the control. Genotypes are: A, B, C, E: hs-flp; FRT82, Kibra\textsuperscript{RM,11}/FRT82, w'; D, F: hs-flp; FRT82, Kibra\textsuperscript{123,1}/FRT82, w'. Alleles are: Kibra\textsuperscript{123,1}: imprecise excision allele removing the ATG. Kibra\textsuperscript{RM,11}: reversion mutagenesis allele (ATG mutated in CG7552). Statistical analyses: C: CB1: p<0.003; CB2: p<0.006; CB3: p<0.022; CB4: p<0.006; CB5: p>0.35; CB6: p<0.002; D: CB1: p<0.05; CB2: p>0.06; CB3: p>0.6; CB4: p<0.025; CB5: p>0.05; CB6: p<0.015. Analyses were done with a Students T-test (two-tailed). Error bars indicate the standard deviation.
The TEM pictures of two different alleles were examined for their size of the cell bodies and the rhabdomeres (Figure 18). Whereas the rhabdomeres showed tendency towards a smaller size, the cell bodies were slightly increased in size. Although not all the cell bodies were significantly enlarged, we concluded that the increased distance between the ommatidia in the mutant tissue was at least partially due to the increased size of cell bodies.

The phalloidin stainings of mutant pupal eye discs revealed more interommatidial cells, and this could also partially lead to the observed size increase of Kibra mutant heads (Figure 19). The outline of the ommatidia lacking GPF was thicker due to the presence of extra cells. Therefore the increased distance of the ommatidia is probably due to a combination of more and larger cells.

![Figure 19: Homozygous mutant Kibra ommatidia of pupal eye discs contain more interommatidial cells.](image)

A clonal analysis of Kibra mutant cells was done with two different loss-of-function alleles. We decided to perform this analysis in the wing disc of third instar larvae, because we also wanted to examine the growth phenotype in another organ than the eye. The clones in the wing discs were obtained by heat shocking hs-flp; FRT82, KibraR.M1125.1/FRT82, UbiGFP animals. The heat shock conditions had to be evaluated first. Depending on the length and temperature, the number and size of the Kibra mutant clones alters. Once a reasonable number of unfused clones per wing disc
was found, several of them were measured for their size and the number of cells. The system used led to a non-mutant twin spot of the mutant clone and therefore the consequences of *Kibra* loss-of-function could be directly compared to an internal control (Figure 20).

![Figure 20: Clones of homozygous mutant *Kibra* cells display more and larger cells compared to their twin spot clones.](image)

**A-C:** The hs-*Fli* induced twin spot clone of *Kibra* mutant cells (absence of GFP) is larger than the control twin spot clone (marked by two copies of GFP). **B:** DAPI staining of the wing disc; **C:** merge of A and B. **D, E:** Not only the clone size is increased, but also the cell number is increased in the *Kibra* mutant clones. The white bar indicates the clone sizes of the control (twin spot) and the black bar indicates the sizes of the *Kibra* mutant clones. Analyses of clone sizes and cell numbers: The average number of pixels are 5910 and 12050 for the control clones and the *Kibra* mutant clones, respectively (p<0.000001). The average numbers of cells are 14.8 (control clone) and 22.2, respectively (p<0.0002). For *Kibra*12/+4, the average number of pixels are 7060 (control clone) and 12530, respectively (p<0.000001), whereas the average numbers of cells are 16.5 (control clone) and 21.3, respectively (p<0.0008). The average size of control cells is 400 (control twin spot of *Kibra*12/+4 clones) and 430 (control twin spot of *Kibra*12/+4 clones) pixels, respectively. The average size of *Kibra* mutant cells is 545 (*Kibra*12/+4) and 590 (Kibra12/+4) pixels, respectively. Therefore *Kibra* mutant clones contain more and larger cells compared to their twin spot clones. The clones were allowed to grow for 48h. In this time the control cells divided four times (16 cells on average), whereas the mutant cells divided 4.375 times (22 cells on average). Therefore the cell doubling time for the control cells was 12 h, whereas the mutant cells only used 11 h. Genotypes are: **A-C:** hs-*Fli*; FRT82, *Kibra*12/+; FRT82, UbiGFP; **D, E:** hs-*Fli*; FRT82, *Kibra*12/+; FRT82, UbiGFP and hs-*Fli*; FRT82, *Kibra*12/+; FRT82, UbiGFP, respectively. Alleles are: *Kibra*12/+4: imprecise excision allele removing the ATG. *Kibra*12/+4: reversion mutagenesis allele (ATG mutated in CG7552). Statistical analyses were done with a Students T-test (two-tailed). Error bars indicate the standard deviation.
Kibra mutants display a very clear growth effect. Similar results were obtained with two independent Kibra alleles. On average the clones of Kibra loss-of-function cells had around twice the size of their associated twin spots after 48h of clonal growth. The number of cells is also increased, but not to the same extent as the clone size. Therefore it was evident that the increase in clone size is due to more and larger cells. Additionally we calculated the cell doubling time for the control and the Kibra mutant cells. Whereas the control cells used approximately 12h for a round of cell divisions, the mutant cells only needed 11h for a cell division round.

We established rescue constructs to prove that the loss-of-function phenotypes of our Kibra alleles were indeed due to the partial deletion of CG33967. Due to the size of the genomic region (25.7kb) we were not able to perform a genomic rescue and had to use UAS overexpression constructs based on Kibra ESTs. Two different constructs were obtained, one only including the genomic region coding for the first WW domain (CG7552), and a second including the whole EST. We did not expect a rescue of Kibra mutant animals with the short construct, but we wondered about its overexpression phenotype. If the intron between the first and the second exon was not spliced, CG7552 would be directly followed by a stop codon (TAA). Therefore CG7552 could have a function on its own. However, overexpression of the short construct with various Gal4 drivers at 18 and 25°C always produced a wild-type phenotype. Overexpression of both constructs together always resembled the overexpression phenotype of the long construct alone. A summary of the overexpression of the Kibra full length transcript can be found in Table 9.
<table>
<thead>
<tr>
<th>Gal4 driver line</th>
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<tr>
<td>GMR-Gal4</td>
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</tr>
<tr>
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<td>large variability, wild type, rough eyes, smaller eyes</td>
</tr>
<tr>
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<td>smaller and bent up wings, some with vein defects</td>
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<td>few females and fewer males, smaller, some with wing and leg defects</td>
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<tr>
<td>Act-Gal4</td>
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<table>
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<tr>
<td>GMR-Gal4</td>
<td>small and rough eyes</td>
</tr>
<tr>
<td>ey-Gal4</td>
<td>large variability, some with smaller eyes or even deformed heads</td>
</tr>
<tr>
<td>MS1096</td>
<td>smaller and bent up wings</td>
</tr>
<tr>
<td>C10</td>
<td>smaller flies, females with blunt abdomen</td>
</tr>
<tr>
<td>daGal4</td>
<td>lethal</td>
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<tr>
<td>Act-Gal4</td>
<td>lethal</td>
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Table 9: Summary of the overexpression phenotypes of UAS-Kibra 18 and UAS-Kibra 9. The phenotypes are similar to the overexpression phenotypes of the Kibra EP line (Table 7). Crosses were performed at 25°C.

Overexpression of Kibra with GMR-Gal4 led to the same phenotype as the Kibra EP line (Table 7 and Figure 21). Ubiquitous expression with daGal4 led to viable flies with the weaker insertion line (UAS-Kibra 18). The size of those flies was smaller compared to the control flies, which fit well to the smaller eyes caused by GMR-Gal4 mediated overexpression. At 18°C ubiquitous expression of Kibra led to more adult flies than at 25°C, but most of the survivors were females as well (Figure 21).
There was a significant difference between the control and the experimental group in terms of weight.

Those female flies were significantly lighter than the control and they looked proportionally smaller. Some of the escapers appeared normal; others displayed bent wings, deformed legs or missing legs.

A next important step was the rescue of homozygous mutant Kibra animals, once the functionality of the UAS-Kibra constructs was proven. For this issue, we generated two different rescue experiments. The first was done by overexpressing Kibra with daGal4, the second was done without any Gal4 driver line (Figure 22).
Figure 22: *Kibra* mutant animals can be rescued by overexpressing *UAS-Kibra*. A, B: Homozygous mutant Kibra female (A) and male (B) flies can be rescued by the presence of two copies of *UAS-Kibra* (no Gal4 driver present). C, D: Heterozygous Kibra mutant control female (C) and male (D) flies, respectively. E, F: Weight analyses of homozygous mutant Kibra female (E) and male (F) flies rescued by ubiquitous overexpression of *UAS-Kibra* display a similar weight of rescued and control flies. G, H: Weight analyses of homozygous mutant Kibra females (G) and males (H) rescued by two copies of *UAS-Kibra* (no Gal4 driver present) show a similar result as for E, F. Genotypes are: A, B: y w; *UAS-Kibra*+/*UAS-Kibra* 9; *Kibra*^2x+/*Kibra*^7+/. C, D: y w; *Kibra*^m+/*Kibra*^7++; E, F: 1) y w; *daGal4/UAS-Kibra* 9; *Kibra*^+/*Kibra*^2+/ 2) y w; *UAS-Kibra* 9+/+. *Kibra*^2+/*Kibra*^m+/ 3) y w; *UAS-Kibra* 9+/+; *Kibra*^m+/; *Kibra*^2+/ 4) y w; *daGal4+/+; Kibra*^+/*Kibra*^2+/ 5) y w; *daGal4+/+; Kibra*^+/*Kibra*^m+/. G, H: 1) y w; *UAS-Kibra* 9/*UAS-Kibra* 9; *Kibra*^2+/+ 2) y w; *Kibra*^m+/+; *Kibra*^+/+. Alleles are: *Kibra*^m+/: precise excision allele; *Kibra*^+/+ and *Kibra*^2+/: imprecise excision alleles removing the ATG. *Kibra*^m+/: reversion mutagenesis allele (ATG mutated in CG7552). All crosses were done at 25°C. Error bars indicate the standard deviation.

Ubiquitous overexpression of *UAS-Kibra* with *daGal4* led to a rescue of heteroallelic Kibra mutant animals, but a rescue was also possible with two *UAS-Kibra* insertions (Figure 22). The leakiness of the *UAS-Kibra* constructs produced enough Kibra protein. With two *UAS-Kibra* insertions, the flies looked essentially wild-type (Figure...
Later we realized that the males seemed to be fine, but not the females. The males lived longer than the females and were fertile, whereas the fertility of the females could not be tested, because they died too early. A rescue was also possible with one insertion only. However, those flies looked rather sick, probably because the levels of Kibra were not sufficient.

The rescued flies were delayed by one day, but they eclosed in the expected ratio. Although the rescue was not complete, the result could be taken as an argument that the mutant phenotype was due to the loss of Kibra or a truncated and therefore not fully functional Kibra version.

Next we examined the structure of ommatidia overexpressing Kibra in clones (Figure 23). Overexpression with the weaker line UAS-Kibra 18 basically led to a wild-type phenotype. On the other hand, overexpression of UAS-Kibra9 led to a slightly disturbed structure of the ommatidia. However, the normal number of photoreceptor cells was present, but their structure was slightly irregular.

We compared the distances between the R7 cells in control and mutant tissue of several different eye sections (Figure 23). There was a tendency towards smaller distances between the ommatidia of mutant tissue compared to the control tissue. However, the reduced distances were not in all of the analyzed sections significant, especially in the case of UAS-Kibra 18.
Figure 23: *Kihra* overexpressing eyes display slightly decreased interommatidial distances.

A, B: tangential sections of *Kihra* overexpressing eyes. The control tissue is marked by *w*+, the tissue overexpressing *UAS-Kihra* 9 (A) or *UAS Kihra* 18 (B), respectively, is marked by the absence of *w*+. C, D: Analyses of R7-R7 distances in *Kihra* overexpressing and control tissue reveal a tendency of shorter distances in the mutant tissue. C, D: Three independent sections were measured (1-3). The white bar of a section represents the R7-R7 distances of the control ommatidia, whereas the black bar represents the R7-R7 distances of the *UAS-Kihra* 9 (C) or *UAS Kihra* 18 (D) overexpressing ommatidia. Genotypes are: A, C: *y w; UAS-Kihra* 9/+; *GRM> w > Gal4+/+. B, D: *y w; GRM> w > Gal4/UAS-Kihra* 18. All crosses were done at 25°C. Statistical analyses: C: 1) p>0.18; 2) p<0.011; 3) p<0.0013. D: 1) p>0.09; 2) p<0.08; 3) p<0.0035. Analyses were done with a Student's t-test (two-tailed). Error bars indicate standard deviation. Abbreviation: o/e: overexpression.

*Kihra* overexpression in third larval instar wing discs led to a more severe phenotype (Figure 24). Two different *UAS-Kihra* constructs were tested by means of the Flp-out technique. This technique leads to overexpression of *UAS* constructs in clones in different part of the animal body. As a marker, *UAS-GFP* is co-overexpressed. Overexpression of *Kihra* led to smaller clones compared to the *UAS-GFP* overexpressing control clones. *UAS-Kihra* 9 was the stronger insertion than *UAS-Kihra* 18 and those clones were about five times smaller than control clones. However, the cell number did not show a similar reduction. Overexpression of *UAS-Kihra* 9 only led to a reduction of about 50% in cell number and therefore *Kihra* overexpressing clones are smaller due to fewer and smaller cells. This strong size decrease could be
due to apoptosis in the *Kihra* overexpressing cells and therefore we made an anti-cleaved Caspase-3 staining. As a control we stained eye discs of third instar larvae overexpressing *GMR-grim*. However, *Kihra* expressing and surrounding cells displayed no cleaved Caspase-3 staining (data not shown). Thus, the small size of cells overexpressing *Kihra* is not due to apoptosis and another phenomenon must account for this observation.

**Figure 24:** Overexpression of *Kihra* in clones in wing discs leads to fewer and smaller cells. A-D: Co-overexpression of *UAS-GFP* and *UAS-Kihra* 9 (C) or *UAS-Kihra* 18 (D), respectively, leads to smaller clones compared to the control (overexpression of GFP) (A, B). E, F: Overexpression of *UAS-Kihra* 9 or *UAS-Kihra* 18, respectively, leads to a reduction in clone size (E) and a reduction in cell number (F). 1) Clones overexpressing *UAS-GFP* 2) Clones co-overexpressing *UAS-GFP* and *UAS-Kihra* 18 3) Clones overexpressing *UAS-GFP* and *UAS-Kihra* 9. E: The average number of pixels are 11410 (1), 3850 (2) and 2390 (3), respectively. 1.2) p<0.00001; 1.3) p<0.00001. F: The average number of cells are 15.5 (1), 9.6 (2) and 7.2 (3), respectively. 1.2) p<0.006; 1.3) p<0.0001. The average size of control cells is 735 pixels whereas the average size of cells overexpressing *UAS-Kihra* 18 is 400 pixels and 330 pixels for cells overexpressing *UAS-Kihra* 9. The cell number is not reduced to the same extent as the clone size, therefore *Kihra* overexpressing clones consist of smaller and fewer cells. Genotypes are: A, B: *y w; Act>CD2>Gal4 UAS-GFP/+*  C: *y w; UAS-Kihra 9/+; Act>CD2>Gal4 UAS-GPF/+*  D: *y w; UAS-Kihra 18/Act>CD2>Gal4 UAS-GPF*  E, F: 1) *y w; Act>CD2>Gal4 UAS-GFP/+*  2) *y w; UAS-Kihra 18/Act>CD2>Gal4 UAS-GFP*  3) *y w; UAS-Kihra 9/+; Act>CD2>Gal4 UAS-GFP/+*. Analyses were done with a Students T-test (two-tailed). Error bars indicate the standard deviations.
Another important aspect is the intracellular localization and the general distribution of Kibra during development. We ordered a peptide antibody against Kibra. We chose a nonconserved peptide sequence of Kibra N-terminally of the C2-like domain. Another group produced a antibody against the human Kibra and they chose a stretch of 336 amino acids starting N-terminally of the C2-like domain and including the C2-like domain. This antibody was functional and therefore this amino acid stretch is at least partially exposed and might be a good region for our peptide. We did not want to include conserved sequences in the peptide to avoid cross-reactions. So we only included the sequences N-terminally of the C2-like domain. 93 amino acids (aa 665-757) were chosen and the company in charge (Eurogentec, Seraing, Belgium) computationally defined the most promising 14 amino acid peptide (aa 668-681) as an epitope to produce the peptide antibody.

We tested the functionality of the Kibra peptide antibody in different approaches. First, we wanted to confirm that the antibody could recognize the peptide it was designed against. Therefore we performed dot blots with the antibody against Kibra and could see that this antibody was able to specifically detect the Kibra epitope. This was the case for the Kibra antibody, the binding to its epitope was very specific (data not shown). To test the antibody in vivo, we induced clones of Kibra mutant cells in wing imaginal discs by using the same conditions as for the clonal analysis (Figure 20). As a positive control for the staining, we used an antibody directed against Boss (data not shown).

A functional Kibra antibody was not expected to stain the cells lacking Kibra, but only the heterozygous cells surrounding the mutant clones.
Figure 25: The peptide antibody against Kihra does not recognize the protein in vivo. A, B: Clones of Kihra mutant cells were induced in wing discs and display the same staining as the surrounding wild-type tissue. C, D: UAS-Kihra 9 (C) and UAS-Kihra 18 (D) are overexpressed posterior to the morphogenetic furrow, but no stronger antibody staining can be detected in the posterior part. E: Western blot of extracts of cells treated with RNAi against Kihra and extracts of homozygous mutant Kihra larvae reveal no functionality of the antibody. 1) Extracts of S2 cells incubated with RNAi against GFP (control) 2) Extracts of S2 cells incubated with RNAi against Kihra 3) Extracts of heterozygous Kihra third instar larvae 4) Extracts of homozygous mutant Kihra third instar larvae. Molecular weight of Kihra: 144kDa. The dilutions of the 1° (Kihra) antibody are indicated in the figure. Genotypes are: A: hs-flp; FRT82, Kihra124/FRT82, UbiGFP B: hs-flp; FRT82, KihraR3-1/FRT82, UbiGFP C: y w; GMR-Gal4/+; UAS-Kihra 9/+ D: y w; GMR-Gal4/+; UAS-Kihra 18/+ E: 3) y w; Kihra13-1/TM6B y; Kihra14-1.

The staining of the Kihra mutant clones did not differ from the staining of the surrounding wild-type tissue (Figure 25). As another test, we overexpressed Kihra in the developing fly eye in cells posterior of the morphogenetic furrow. This should lead to a difference in staining posterior to anterior of the morphogenetic furrow (Figure 25).
As in the previous experiment the peptide antibody did not work. There was no difference in staining posterior and anterior of the morphogenetic furrow.

A last test was the use of the antibody in a Western blot. Due to the unfolding of the proteins the chances of recognizing a certain peptide stretch was higher than in a native protein. We prepared extracts of cell culture treated with RNAi against Kibra, extracts of homozygous mutant Kibra larvae and the appropriate controls (Figure 25). Two different dilutions of the Kibra antibody were used for the Western blot and in both cases the same bands were recognized by the Kibra antibody. In extracts of cell culture treated with RNAi against Kihra and a control against GFP, the antibody did not recognize a protein of the correct size. However, there was a band at the correct length in the larval extracts, but it was present in homozygous mutant and control larvae. Regarding all the in vivo and Western blot experiments we concluded that the peptide antibody against Kibra is, at least in vivo, not functional.

The phalloidin staining of Kibra mutant pupal eye discs (Figure 19) led to a result characteristic for genes involved in the Salvador-Warts-Hippo (SWH) pathway\(^1\) (Figure 30). The core components of this pathway act negatively on cell proliferation and cell growth, and positively on apoptosis. Therewith they restrict the growth of imaginal discs in Drosophila. Loss of function in the head leads to a large increase in size due to more cells. Mutant ommatidia display additional interommatidial cells as we observed it for Kibra mutant heads. Additionally, a yeast-two-hybrid protein interaction map revealed a binding between Kibra and Merlin\(^1\)\(^2\). Merlin is a component involved in the SWH pathway and therefore we thought that Kibra might play a role in this signalling pathway as well. We tested several mutant SWH genes for interactions with Kibra (Table 10).
<table>
<thead>
<tr>
<th>tester line</th>
<th>x GMR-Gal4/CyO; UAS-RNAi CG12600 (81D12)/TM6B</th>
</tr>
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<tbody>
<tr>
<td>w</td>
<td>control</td>
</tr>
<tr>
<td>y w; sav4Q2/TM6B</td>
<td>no size alteration</td>
</tr>
<tr>
<td>y w; wts3x1/TM6B</td>
<td>no size alteration</td>
</tr>
<tr>
<td>y w; hpo297/TM6B</td>
<td>no size alteration</td>
</tr>
<tr>
<td>y w; yki35/TM6B</td>
<td>no size alteration</td>
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<tr>
<td>y w; mer3/FM6</td>
<td>no size alteration</td>
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<tr>
<td>y w; cycEAR95/CyO</td>
<td>no size alteration</td>
</tr>
<tr>
<td>y w; UAS-p35/TM6B</td>
<td>larger eyes</td>
</tr>
<tr>
<td>y w; UAS-cycE/TM6B</td>
<td>rough, slightly necrotic eyes, no size alteration</td>
</tr>
<tr>
<td>y w; UAS-RNAi yki (117C7)</td>
<td>smaller eyes, slightly rough</td>
</tr>
<tr>
<td>y w; UAS-p35/TM6B</td>
<td>slightly larger eyes compared to wt</td>
</tr>
<tr>
<td>y w; UAS-cycE/TM6B</td>
<td>smaller and more irregular eyes</td>
</tr>
<tr>
<td>y w; UAS-RNAi yki (117C7)</td>
<td>mostly wt eyes, some slightly smaller, few glassy eyes</td>
</tr>
</tbody>
</table>

Table 10: Summary of crosses for interactions between Kibra and SWH components. Heads enlarged due to overexpression of RNAi against CG12600 are not reduced if heterozygous for genes involved in the SWH pathway. Co-overexpression of RNAi against yki leads to smaller eyes, co-overexpression of UAS-p35 leads to heads enlarged in size. Co-overexpression of cyclin E does not alter the eye size.

Heterozygosity for SWH signalling components did not reduce the size of heads overexpressing RNAi against Kibra. However, interactions were observed by inhibiting apoptosis. Co-overexpression of UAS-p35 resulted in eyes enlarged in size. However, overexpression of p35 on its own also led to a slight increase of eye size. On the other hand, reducing the function of yki by overexpression of RNAi led to a decrease in the size of heads overexpressing RNAi against Kibra. yki is a SWH pathway component positively acting on growth, and overexpression of RNAi against yki led to slightly smaller eyes in a part of the progeny. Therefore, the reduction of eye size could be additive.

3.4.3 Discussion

Kibra has been identified in a deletion screen for downstream components of Rheb (chapter 3.3). A sensitized system in the eye of Drosophila has been used to screen for deletions modifying a Rheb overexpression phenotype. We have identified three
deficiencies that alter this phenotype. Within one of these deficiencies, Kihra has been one of the candidates. Kibra had already been found in a screen for negative growth regulators and has therefore been a good candidate. Overexpression and RNAi experiments of Kibra confirmed the growth phenotype and we decided to examine whether Kibra is the gene responsible for the enhancement of the Rheb overexpression phenotype. We have generated mutant alleles of Kibra to test this issue. However, the mutant Kibra alleles did not enhance the Rheb overexpression phenotype, indicating that Kibra was not the gene we were looking for. In the meantime, we also realized that the deletion that enhanced the phenotype was not entirely lethal. Since Kibra is an essential gene, a deficiency deleting Kibra is expected to be homozygous lethal as well. Therefore the enhancement by this deletion is probably an artefact and Kibra has most likely never been tested in our deletion screen. All the same, the growth phenotype of Kibra was interesting and we decided to continue its characterization, although it was not the kind of gene I was originally screening for.

The first two exons of Kihra are separated by a large intron and other ATGs are present downstream of this intron. The imprecise excision alleles that we generated could potently encode a truncated protein. There exists an in frame ATG upstream of CG12600, which could lead to an N-terminally truncated protein missing the first WW domain. Alternatively, the codon coding for the second amino acid (M105) of the second WW domain might be used as an ATG. Other start codons are present downstream of the sequences coding for the second WW domain and an encoded truncated protein would only contain the C2-like domain. Since no other suitable EP-element insertions for an imprecise excision of CG12600 were available, we performed a reversion mutagenesis screen to obtain mutations in CG12600. Those alleles should reveal based on the phenotypes, if CG7552 and CG12600 have the same function.

However, we have obtained only one mutant allele in this screen and it contains a mutagenized ATG in CG7552. The low frequency of obtained reversion mutagenesis alleles and the absence of any mutations in CG12600 are unexpected. An undirected EMS screen (ey-Flp) for various growth genes has also not revealed any Kibra alleles. ey-Flp screens have been performed for every autosomal chromosome arm. The
screened flies contained homozygous mutant heads, whereas their bodies were heterozygous for the EMS induced mutations. Mutagenized negative growth regulators resulted in flies with larger heads. Since homozygous mutant Kibra heads show a bighead phenotype, Kibra should have been found in the ey-Flp screen. Growth genes were hit several times, indicating that the ey-Flp screens were saturated. For reasons we do not understand, mutations in the coding region of CG12600 do not seem to be well tolerated. We cannot exclude that it is a coincidence that we have not obtained mutations in CG12600, but this is rather unlikely. Haploinsufficiency is a rather rare phenomenon, but it exists and Kibra might partially be such a locus. However, mutations in CG7552 do not behave in the same way, such mutants are heterozygous viable. If haploinsufficiency is the explanation, it might be that a truncated protein is sufficient for the animals to survive, but a more severely truncated protein (mutations in CG12600) is not sufficient any more. When we generated the imprecise excision alleles of Kibra, there was no deficiency for this genomic region available. However, recently a new DrosDel deletion uncovering Kibra amongst other genes has been published. This deletion uncovers a haploinsufficient male sterile gene and is not publicly available. If this deletion exists and we could prove by complementation analysis that this deficiency deletes Kibra, the theory about the haploinsufficiency would be disproven.

Homozygous mutant Kibra heads are enlarged, as it can be observed for the overexpression of RNAi against CG12600. Homozygous mutant Kibra heads consist of larger ommatidia, but their number remains unchanged. Although we have already thought that Kibra is not involved in the insulin/TOR signalling, we have compared the Kibra mutant phenotype with mutants of this signalling pathway. An ommatidial increase in size but not number argues rather against an involvement in the insulin/TOR signalling pathway, although members of this pathway act predominantly on cell size.

Tangential eye sections of hs-Flp mosaic heads reveal a normal structure and differentiation of the Kibra mutant ommatidia, but the distance between the ommatidia is enlarged. This is most likely the reason for the increased head size caused by the
loss of *Kibra*. However, the light sensing organelles of the ommatidia, the rhabdomeres, seem to be of equal size as the control. This observation also argues against an involvement in the insulin/TOR signalling pathway. In those mutants, the cell bodies and the rhabdomeres are always proportionally increased or decreased in size.

There are two different explanations for the size increase of *Kibra* mutant heads. The cell bodies of the ommatidia could be enlarged or there could be more interommatidial cells. For both *Kibra* alleles certain cell bodies are significantly increased in size. In the case of *Kibra*\textsuperscript{R.M.1}, CB1, 2, 3, and 6 are increased, whereas for *Kibra*\textsuperscript{23.1} CB1, 4 and 6 are significantly increased in size. This discrepancy depends probably on the small number of sections we analyzed. We have only a two dimensional picture of the cellular arrangements. Ommatidia consist of cells elongated in the apical-basal axis and it is possible that the diameter of the different cell bodies varies, depending on the depth of the sections. The limited size of our analysis does not allow to draw conclusions about the size differences between the different cell types. In contrast to the CBs, the mutant rhabdomeres tend to be slightly smaller. Although not all cell bodies are increased in size and the rhabdomeres are slightly smaller, we think that the increased distance between the ommatidia in *Kibra* mutant tissue is partially due to enlarged cell bodies.

Additionally, phalloidin stainings in pupal eye discs revealed that there are clearly more interommatidial cells in the mutant tissue. The outline of the loss-of-function ommatidia is increased due to the loss of the single cell architecture.

We postulate that the increased distance of the ommatidia in homozygous mutant *Kibra* heads is caused by a combination of enlarged cell bodies and the presence of extra interommatidial cells. Although we have no possibilities to detect those additional cells in the adult fly eye, we do not think that the additional interommatidial cells are eliminated during the development from the pupal to the adult stage. A reason for that speculation is the fact that the examination took place after the initiation of the apoptotic wave removing all supernumerary cells. However, we cannot exclude that those additional cells die during a later stage.
In wing discs, *Kibra* mutant clones have on average twice the size of the twin spot clones. In contrast, clones of cells homozygous for a precise *EP*-element excision of *Kibra* do not show a size increase compared to the control twin spot. Therefore we conclude that the twofold increase in size is due to the loss of *Kibra* in the mutant clone. This increase can be achieved in different ways. Either the cells could be larger, there could be more cells instead or it could be a combination of both. In the case of *Kibra* there are more cells in the mutant clone, but the increase in cell number is not twofold as for the clone size increase. Therefore, the increase in size is due to larger and more cells. The analysis has been done with two different *Kibra* alleles and in both cases the same result was obtained.

Overexpression of a full length transcript of *Kibra* in the eye during development leads to the same result as the overexpression of the *Kibra* *EP* line (19909). This shows us that the *EP* line overexpresses *Kibra* and not another gene situated in the intron of *Kibra*. Ubiquitous *Kibra* overexpression can lead to a limited number of flies, predominantly females. All escapers are proportionally smaller, indicating that the reduction of organ size is not restricted to the eye or the wing of *Drosophila*.

Homozygous mutant *Kibra* animals can be rescued to adulthood by means of the *UAS-Kibra* construct. Although we do not obtain a complete rescue, the rescue proves that the mutant phenotype we observe is due to a reduction of *Kibra* function. The rescued flies have a normal appearance, but whereas the rescued males are fertile, the females do not live long enough to test them for their fertility. We do not have an explanation for the females' early death, and we also do not understand the mechanism of the sex-specific tendency. The strength or pattern of expression might slightly differ in males and females and therefore a rescue with *UAS*-constructs might not lead to the same result in the two sexes. A genomic rescue could lead to a complete rescue, but since the *Kibra* locus has a size of almost 26kb, a genomic rescue is difficult to perform.

The first exon of *Kibra* would be followed by a stop codon, if the intron between the first and the second exon was not spliced. Therefore another overexpression construct
coding for the first exon has been generated to reveal a putative function of CG7552. However, overexpression with various Gal4 driver lines leads to wild-type phenotypes. From that we conclude that the first exon of Kihra does probably not have a function on its own. Since the first exon codes for a WW domain, it could have acted in a dominant-negative manner by titrating away binding partners. In this case, it would have been difficult to distinguish between a wild-type function and a dominant-negative function. The lack of any phenotypic alterations can have several reasons: the mRNA might not be stable and no protein could be produced. If a small protein was produced, the protein itself could be unstable and hence shows no effect. Even if a stable protein was produced, its WW domain might not be able to bind the binding proteins without the second WW domain.

Overexpression of Kibra in clones during eye development results in slightly disturbed structure of the ommatidia with the stronger UAS-Kibra insertion, but the photoreceptor cells are present in the accurate number and mostly in the correct orientation. Overexpression with the weaker construct does not lead to any visible differences at all. The distance from adjacent R7 to R7 rhabdomeres is slightly smaller in the tissue overexpressing Kibra. The differences have not always been significant, but there is a tendency towards decreased ommatidial distances. This decrease is not unexpected, since the overexpression in the developing eye leads to smaller eyes. However, the resolution of our analysis was not high enough and we do not know what happens at the level of the individual cells. In contrast, the overexpression of Kibra in clones of third instar larval wing disc cells gives us the possibility to analyze the behaviour of single cells. The effects in wing discs are more dramatic than in adult eyes, but this could be explained by the different efficiencies of the Gal4 driver lines. Kibra overexpressing cells are reduced in size by 50% on average. Additionally, the number of cells in Kibra overexpressing clones is also reduced by about 50%. These are very strong effects and might be due to unhealthy cells. However, no apoptotic signals have been detected in the Kibra overexpressing cells and their adjacent wild type cells. To further analyze this phenotype, p35 or cyclinE could be co-overexpressed with Kibra to investigate a putative rescue of the number of cells in the
overexpression clones. It will be interesting to see whether the cell size would also be rescued or whether the function of Kibra on cell number and cell size could be separated.

We have ordered a peptide antibody to reveal the expression pattern of Kibra during development and to examine its intracellular localization. Several different experiments have been performed with our peptide antibody, in vivo and in tissue culture. In Kibra mutant clones in wing discs, a functional Kibra antibody should not detect anything. The anti-Kibra staining of the mutant clone has been similar to the staining of the wild-type tissue. The perdurance of a protein could lead to such a result and we have no possibilities to measure whether Kibra protein is still present in the mutant cells after two days. However, the differences in size between the mutant clones and their twin spots argue against this assumption. Another test has been the overexpression of Kibra posterior to the morphogenetic furrow. In this case, the staining should be more intense posterior to the furrow. This is also not the case and we assume that the Kibra antibody does not work in vivo. We have tried to recognize Kibra in Western blots. In Western blots, the proteins are unfolded and the epitopes should be recognized more easily. In extracts of Kibra RNAi treated cells, there is no signal at the appropriate position in the Western blot. However, it is possible that Kibra is not expressed in S2 cells. For the larval extracts we find a band at the correct position, but it is present to the same extent in Kibra heterozygous and homozygous mutant larvae. The majority of the Kibra protein of the imprecise excision alleles could still be translated as already discussed. The 88 missing amino acids would probably not make a huge difference in the position of the band. Therefore we cannot conclude that the antibody does not work in Western blots.

The phalloidin staining of Kibra mutant pupal eye discs revealed a striking phenotype reminiscent of mutations in components of the Salvador-Warts-Hippo (SWH) pathway members. One of their characteristics is an increase in the number of interommatidial cells when homozygous mutant and Kibra displays the same phenotype. In a yeast-two-hybrid screen Kibra has been found as a binding partner of Merlin\textsuperscript{112}, a component
involved in SWH signalling. Therefore we have started to investigate a putative role of Kibra in this signalling pathway. Although there are no dominant interactions amongst Kibra and the tested SWH mutants, this does not rule out an involvement of Kibra. It might be that the tested genes are not rate-limiting and the gene function of the SWH pathway member has to be reduced by more than 50%. yki mutants do not show a dominant interaction, but co-overexpressing RNAi in heads against yki and CG12600 leads to a decrease in size. However, this could also be additive, since overexpression of the RNAi line against yki on its own slightly decreases eye size. Blocking apoptosis in heads overexpressing RNAi against CG12600 leads to an increase in the size, demonstrating that the increased size of Kibra mutant heads is not (or not only) due to the loss of apoptosis. Two major targets of the SWH pathway are DIAP1 and cyclin E\(^1\). DIAP1 inhibits apoptosis and cyclin E drives cell proliferation. Cells of mutant SWH core components display an upregulation of both DIAP1 and cyclin E levels\(^1\). It would be worthwhile to analyze the levels of these two genes in Kibra mutant clones. Additionally, double mutant clones of Kibra and Merlin and expanded, respectively, could be tested. Merlin and expanded are redundant genes and only display the typical SWH phenotype in double mutants. Epistasis experiments with Kibra and downstream components of Merlin like Hippo, Warts and Salvador could be performed. Biochemical binding studies should also be performed to confirm the binding of Kibra and Merlin and to find new interacting proteins.

### 3.5 Kibra in Learning & Memory

A completely different approach was taken with our Kibra alleles. Kibra was found to be a novel substrate of PKCzeta, which is involved in synaptic plasticity and memory formation\(^1\). By chance, we met the authors of an at that time unpublished paper dealing with a SNP chip approach to find genes involved in good memory performance in humans\(^1\). They analyzed 500'000 SNPs of genomes of good learners versus bad learners of a verbal memory test and revealed that the Kibra\(^T\)-allele was associated with good memory performance in both tested cases, after 5 min and after 24 hours. This SNP could be confirmed in three other tested groups as well. With
fMRI (functional magnetic resonance imaging) they could show that bad memory performers (non-carriers of the Kibra\textsuperscript{T}-allele) needed greater hippocampus activation for the same memory performance. Kibra was the best candidate found in this study. Thus, we became interested in testing a possible role of Kibra in learning and memory in the fly and agreed on a collaboration to test our Kibra alleles in \textit{Drosophila}.

3.5.1 Background

Several learning and memory mutants were isolated during early chemical mutagenesis screens decades ago, but some of them still remain unidentified\textsuperscript{115}. Other mutations could be mapped and assigned to a certain gene. Those genes could be divided into two different classes: first, genes essential for the development of the structures involved in learning and memory; second, genes involved in the biochemistry of formation, storage and supply of the conditioned responses within the appropriate neurons. Many mutations were found to be members of the first class of genes, and for a rescue they had to be expressed with a transgene throughout development. By contrast, transgene expression for the rescues for the second class of genes was sufficient prior to conditioning. The focus for overexpression effects could be restricted to specific parts of the brain. For olfactory learning, the mushroom bodies (MB) were found to have a pivotal role.

The mushroom bodies (MB) in the brain of \textit{Drosophila} have a central role in olfactory learning and memory\textsuperscript{116}. The MBs are derived from four neuroblasts and are bilaterally symmetric structures consisting of about 2500 intrinsic neurons (Kenyon cells) per brain hemisphere. Flies devoid of their MBs show normal behaviour but are deficient in olfactory learning. \textit{Drosophila} senses odorants primarily with sensory hairs on the third antennal segment, and the odors are detected by a family of G-protein coupled receptors (\textit{Drosophila} odorant receptors, DOR). The information about odors comes from about 1500 olfactory sensory neurons on each antenna via the antennal nerves to the antennal lobe. From there projection neurons transmit olfactory information to the dendrites of the MB cells.
As in humans, there are different types of memories in the fly (for reviews see T. Tully et al.\textsuperscript{117} (Figure 26)). There are two ways to train the flies, and they have different consequences. A massed training is a repeated training session with no rest interval in between. A spaced training is a repeated training session with a rest interval between each training. Spaced trainings produce a stronger and longer-lasting memory than the massed trainings and only spaced training leads to the formation of the stable long-time memory.

\textit{dunce}: cAMP phosphodiesterase \hfill \\
\textit{rutabaga}: type I adenylyl cyclase \hfill \\
\textit{amnesiac}: pituitary adenylyl cyclase activating peptide \hfill \\
\textit{CREB}: transcription factor, PKA target \hfill \\
\textit{radish}: ?

\textbf{Figure 26: Memory scheme\textsuperscript{117} in adult \textit{Drosophila}.} For abbreviations see text section.

\textbf{LRN:} Learning: memory immediately after training

\textbf{STM:} Short-term memory: reaches maximum levels after one-cycle training, decays to zero within 2h. Independent of transcription and translation, disrupted by depolarizing drugs like LiCl (disrupted in \textit{dunce} and \textit{rutabaga} mutants).

\textbf{MTM:} Middle-term memory: reaches maximal levels after 1h (one-cycle training) and decays to zero within 7h, is sensitive to disruption by cold-shock. Requires new protein synthesis from pre-existing messages (disrupted in \textit{amnesiac}, \textit{dunce} and \textit{rutabaga} mutants).

\textbf{ARM:} Anesthesia-resistant memory: induced after one-cycle training, massed training or ten-cycle spaced training. Reaches maximal levels within 2h, decays to zero within 4 days, is resistant to cold shock, independent of protein synthesis and independent of \textit{LTM} (disrupted in \textit{radish} mutants).

\textbf{LTM:} Long-term memory: only induced after ten-cycle spaced training, reaches maximal levels within 1 day, shows no large decay over 1 week, is independent of
ARM and requires de novo transcription and translation. This leads to the establishment of long-lasting structural changes in synaptic morphology.

Many of the genes involved in learning and memory in Drosophila are members of the cAMP-dependent pathway. For example, homologs of rutabaga and creb are also essential for mammalian learning and memory\textsuperscript{115}, which demonstrates the conservation of the underlying mechanisms. Therefore genes discovered to function in learning and memory in humans are likely to perform the same function in other organisms, for example Drosophila, and it has not been too far-fetched to analyze the function of Kibra in Drosophila based on the data of Papassotiropoulos et al\textsuperscript{114}.

\subsection*{3.5.2 Results}

To test our mutants in Drosophila we were looking for a simple system, which did not need a long introduction time and also no special equipment. Hence no experiments with adult flies could be arranged, but we found an established system dealing with Drosophila third instar larvae\textsuperscript{118}. This protocol was simple and all necessary tools could be produced by ourselves. The system was based on larvae trained to correlate a given odor with the availability of food (fructose). The larvae were allowed several training rounds. On agar plates containing fructose, they were exposed to odor 1, on plain agar plates they were exposed to odor 2. Afterwards the larvae had the choice to crawl to odor 1 or to the opposite direction (odor 2). Therewith a learning index (LI) could be calculated, which differed between mutant and control larvae, if they displayed different performances during the assay. The larvae were able to remember for more than 60 min, but after 90 min the training effect decreased to zero. The background of the tested fly strains should be identical, if possible, because it can have a large influence on the LI.

We first tried to obtain positive LIs for a wild-type strain. Once this was achieved (data not shown), we tested Kibra heterozygous animals versus wild-type animals containing exactly the same background (Figure 27). Some of the genes involved in learning and memory already displayed an effect if heterozygous.
Figure 27: Heterozygosity for Kibra has no effect on the performance in a learning & memory assay. n=12, \( p>0.12 \) (Mann-Whitney test). Genotypes are: 1) Kibra\(^{6.2}/\)Kibra\(^{8.2}\) 2) Kibra\(^{6.2}/\)Kibra\(^{7.1}\). Alleles are: Kibra\(^{6.2,9.2,1.2}\): precise excision allele; Kibra\(^{7.1}\): imprecise excision allele.

There was a decrease in the LI of the heterozygous larvae, but not enough to be significant. We wanted to more strongly reduce the Kibra gene function, but still paid special attention to keep identical backgrounds. As a second test, we used a \( P \)-element insertion in the Kibra locus (11567). Heteroallelic animals (11567/Kibra\(^{7.1}\)) died during the pupal stage. We expected Kibra to be reduced in those larvae. Nothing is known about the different memories in larvae and proteins involved in learning & memory might not be needed directly after learning, but rather at a later time point. Therefore, we tested the trained larvae under three different conditions (Figure 28).

Figure 28: Kibra mutant larvae tested after different periods do not display a learning and memory failure. A: n=10, \( p>0.38 \). Test immediately after training. B: n=9, \( p>0.56 \). Test 10 min after training. C: n=11, \( p>0.4 \). Test 30 min after training. Genotypes are: 1) Kibra\(^{6.2}/11567\) 2) Kibra\(^{7.1}/11567\). Alleles are: Kibra\(^{6.2}\): precise excision allele; Kibra\(^{7.1}\): imprecise excision allele; 11567: mutant \( P \)-element insertion in the Kibra locus.

None of those different conditions led to a difference in the LI. As a last try we used the transgene producing RNAi against Kibra. Although the problem of different backgrounds could not be avoided, the data would be convincing if the difference in the LI was large enough.

As the MBs are the responsible organ for olfactory learning and memory, we overexpressed our transgene with a mushroom body-specific driver line (Figure 29).
Figure 29: Overexpression of RNAi against Kibra in the MB does not alter memory performance. n= 5, p>0.69. Test 30 min after training. Genotypes are: 1) w; 81D12/+ 2) Gal4^{247} /81D12. Constructs: 81D12: RNAi line against Kibra; Gal4^{247}: mushroom body-specific Gal4 driver line.

The last test also showed no differences in the LI of the tested genotypes. Thus, we concluded that Kibra has no effect on learning and memory in this assay.

3.5.3 Discussion

Kibra has been found to be involved in good memory performance in humans. A SNP chip approach has been performed to find genes involved in memory function, and Kibra has been amongst the best candidates. The authors have tested their other candidates in C. elegans to see if the function of those genes in memory performance is conserved in lower organisms. Genes involved in learning and memory are conserved throughout the animal kingdom and therefore experiments in other organisms with the same genes are not far-fetched.

Kibra has also been found to be a novel substrate of PKCzeta, which is involved in synaptic plasticity and memory formation. Therefore, performing behavioural studies was reasonable and further encouraged by this binding studies.

Kibra is not conserved in C. elegans and the authors of the study asked us to test our mutant Kibra flies for learning and memory defects. We have decided to perform the experiments ourselves and have been looking for a simple system to perform learning and memory tests. To work with adult flies has not been an option for us. This requires a special machine to prevent the flies from flying away. We have found a simple learning and memory test in third instar larvae, which does not require special
equipment and is easy to perform\textsuperscript{118}. The system is based on larvae trained to correlate a given odor with the availability of food (fructose). Two different odors are used and one of them is coupled to the presence (odor 1) of fructose, the second is coupled to the absence (odor 2) of food (fructose). After several training rounds exposed to odor 1 or odor 2, the larvae are given the choice to crawl to odor 1, which has been coupled to the presence of fructose, or to the opposite direction (odor 2). Therewith a learning index (LI) can be calculated.

Because our mutants are homozygous lethal, we have faced different problems compared to dealing with other genes involved in learning and memory. Many of those examined genes are non-essential, and homozygous mutant animals can be tested. However, it has been shown that heterozygosity can already have an influence on the memories of the flies\textsuperscript{119} and therefore we have tested heterozygous larvae as a first experiment. We have not found a decrease in the LI for heterozygous mutant \textit{Kibra} larvae. Animals with heteroallelic combinations (\textit{Kibra}\textsuperscript{71/11567}) can proceed their development to the pupal stage, then they die as pharate adults. We have examined the performances of third instar larvae in different time windows. The LIs decrease over a time period of 30 min, but to a similar extent in the mutant and in the control larvae. As an additional test, we have decided to test RNAi against \textit{Kibra}, although in this setting the genetic background would not be identical any more. However, larvae overexpressing RNAi against \textit{CG12600} in the MBs did not differ in their performance compared to the control.

These results cannot exclude an involvement of \textit{Kibra} in learning and memory in \textit{Drosophila}. \textit{Kibra} can still be involved in memory processes. There are several explanations why we have not received differences in the LIs. The amount of \textit{Kibra} protein in the larvae might still be large enough for a proper memory function. \textit{Kibra} could also be used in adult \textit{Drosophila} only to maintain the memory function. In this case we would never find positive results in a larval assay.

The overexpression of the RNAi line against \textit{Kibra} might not have worked for similar reasons. Maybe the protein is not expressed in larvae or the downregulation of \textit{Kibra} function has not been sufficient. Another reason might be the location of its
overexpression. We have used a MB-specific *Gal4* driver, but maybe the RNAi should have been expressed in a small region of the MBs not covered by this *Gal4* driver line. Our data suggest that Kibra is not involved in larval learning and memory. However, a function of Kibra in memory formation in adult *Drosophila* cannot be excluded.
4. General discussion

We have been interested in the intracellular localization of Rheb and in the identification of novel TOR signalling components downstream of Rheb. For these reasons, we generated a Rheb-GFP fusion protein to examine the intracellular localization of Rheb. Additionally, two complementary screens were performed to isolate novel downstream candidates of Rheb. With one of the isolated candidate genes a learning and memory assay was performed.

4.1 Intracellular localization of Rheb

Our Rheb localization studies led to different results than those found in two independent publications\(^93,\,94\). We observed a ubiquitous intracellular staining, whereas both research groups found Rheb to be at endomembranes. Rheb is supposed to be localized at membranes due to its farnesylation motif. Since two independent research groups could detect Rheb at the same membranes, we think that those data are relevant. Rheb should be in proximity to its upstream and downstream components. For TSC2 it was shown that it is also localized to intracellular structures and not to the plasma membrane. It localizes to the Golgi apparatus\(^92\). A similar localization has been observed for TOR. TOR is predominantly cytoplasmic, but associates with membranes including those of the mitochondria, the ER and Golgi\(^95-98\). This supports the localization data obtained for Rheb and also suggests that TOR signalling takes place at intracellular membranes and not at the plasma membrane. However, in contrast to Takahashi et al.\(^93\) and Buerger et al.\(^94\) we did not work with overexpression of Rheb in cell culture. We used a tagged Rheb construct that was expressed under its endogenous promoter. A rescue of Rheb mutant animals was possible and proved the functionality of the tagged protein. It might be that Rheb is ubiquitously expressed in the cell and only transiently interacts with the endomembranes.
4.2 Novel genes interacting with Rheb and TORC1

When I started my PhD, no physical interactions between Rheb and TOR have been documented and we expected new genes downstream of Rheb and upstream of TOR to exist. However, in the meantime Rheb has been shown to be associated with the TORC1\textsuperscript{120, 121} and TOR is likely to be activated by Rheb via direct binding to TOR. Nevertheless our two genetic modifier screens were worthwhile to be performed. It could be shown that Rheb binds to the kinase domain of TOR\textsuperscript{120}, but whether this is a direct binding or whether it requires an unidentified binding partner in between is not known. Additionally, not all TOR-associated proteins were found at this time and if a Rheb GEF existed was also not clear. Therefore we expected that a Rheb modifier screen could reveal new components.

In our lethality reversion screen we could establish two single hits on the 2\textsuperscript{nd} and on the X chromosome, respectively. Both of them suppress a Rheb overexpression phenotype and are good candidates for downstream components or genes acting in parallel to Rheb. Candidates for the mutation on the X chromosome are raptor and LST8, respectively. Both single hits have not been mapped and no mutations of raptor and LST8 in Drosophila exist. Therefore complementation analysis was not feasible. For the mutation on the 2\textsuperscript{nd} chromosome we do not have a candidate. A putative Rheb GEF has been described in Drosophila called Translationally controlled tumour protein (Tctp)\textsuperscript{122}, but this gene is located on the 3\textsuperscript{rd} chromosome and cannot be our candidate.

The function of Tctp it is not validated in mammals yet. In our hands, mutations of Tctp did not suppress the Rheb overexpression phenotype. Tctp might not be rate-limiting and therefore not lead to an alteration of the phenotype. However, our collaborators (Fried Zwartkruis and Marta Roccio, University of Utrecht) tested Tctp in their cell culture assay and did not find evidence for an involvement of Tctp in the TOR signalling pathway.

The isolation of two single hits in a non-saturated genetic screen points to additional target genes. One of those novel components in the TOR signalling pathway is PRAS40, an inhibitor of the TORC1 kinase regulated by insulin\textsuperscript{123, 124}. PRAS40 interacts with raptor and inhibits cell growth, the phosphorylation of S6K and the
Rheb-induced activation of TORC1. The loss of this novel protein increases the in vitro activity of TORC1 from insulin-deprived cells\textsuperscript{123}. This negative regulation of TORC1 could be confirmed in mammalian and \textit{Drosophila} cell lines. Upon insulin stimulation, Akt phosphorylates PRAS40, which relieves the inhibition of TORC1. The homolog in \textit{Drosophila} is called Lobe and has already been known to be involved in the regulation of imaginal disc growth\textsuperscript{125}. Akt has been thought to influence TORC1 via phosphorylation of TSC2. However, this notion is controversial, as conflicting results were obtained depending on the experimental setup\textsuperscript{19,41,42,51}. PRAS40 raises the possibility that Akt could bypass TSC2 and signal directly to TORC1\textsuperscript{123,124}. This finding is a step towards the understanding of the mechanism of TORC1 activation. Both PRAS40 and Rheb have a strong influence on TORC1 activity and either protein is capable of overcoming the effects of the other\textsuperscript{123}. Phosphorylated Akt promotes the GTP loading of Rheb and also hinders PRAS40 from inhibiting TORC1. It is not clear whether Akt performs these dual regulatory functions in all cell types. The inputs of both regulators on TORC1 may also vary in different cell types depending on the activity and abundance of each protein. PRAS40 might have an important role in the regulation of a negative feedback regulation. The inhibitory function of PRAS40 is relieved by the phosphorylation of Akt and this enables TORC1 to phosphorylate S6K amongst other target proteins. The feedback inhibition of activated S6K negatively acts on the PI3K branch and decreases the phosphorylation of Akt, which leads to increased repression of TORC1 through PRAS40 and to a relief of the negative feedback inhibition by S6K\textsuperscript{19}.

### 4.3 Novel downstream components of TORC1

Our genetic modifier screens did not allow the identification of downstream components of TOR, since the pathway is branching and heterozygosity for any of the various downstream genes may not be sufficient to alter the Rheb gain-of-function phenotype. However, since we have been interested in identifying novel components upstream of or in parallel to TOR, we have not tested S6K for its ability to influence the Rheb overexpression phenotype.
The two best-described TORC1 substrates are S6K and 4E-BP, both involved in the regulation of protein synthesis\textsuperscript{126, 127}. TORC1 phosphorylates 4E-BP, which leads to the release of the elongation initiation factor 4E (eIF4E). A downstream target of activated S6K is eIF4B. Its phosphorylation stimulates the interaction between eIF4B and eIF3\textsuperscript{128}. This interaction correlates with increased rates of translation. Additionally, it was found that the stimulation of protein synthesis by S6K is achieved by phosphorylating the tumour suppressor programmed cell death protein 4 (PDCD4)\textsuperscript{129}. PDCD4 inhibits translation by binding to and preventing the eIF4A RNA helicase from unwinding the secondary structure in the 5' UTR of mRNAs. PDCD4 is lost in certain aggressive human carcinomas\textsuperscript{130} and its expression inhibits transformation in cultured cells and in tumourigenesis in mice\textsuperscript{131, 132}. In Drosophila, a homolog of PDCD4 exists (CG10990), but its function downstream of S6K has not been analyzed yet. Activated S6K also phosphorylates the ribosomal protein S6, but the function of S6 remains elusive.

4.4 Novel components in TORC2

Novel components have also been isolated in TORC2. The function of TORC2 is conserved from yeast to mammals. It is involved in the regulation of the actin cytoskeleton\textsuperscript{53, 133}. Furthermore, TORC2 directly phosphorylates Akt and facilitates the phosphorylation and complete activation of Akt by PDK1\textsuperscript{34}. However, to identify novel components in TORC2 was not possible with our screen setup. Although TORC2 acts on Akt and Akt can stimulate the function of Rheb in certain situations, the phenotype of the artificial overexpression of Rheb would most likely not have been altered due to a reduced Akt phosphorylation. We have however not tested this issue.

TORC2 consists of TOR, LST8 and Rictor. Additionally, Protor has been isolated as a member of the TORC2, but its function is not known\textsuperscript{134}. It is only found in higher eukaryotes and lacks any functional domains. Sin1 is another subunit of TORC2\textsuperscript{135, 136}. Human Sin1 is necessary for the assembly of TORC2 and for TOR’s capacity to phosphorylate Akt. It might be that the PH-like domain of SIN1 localizes TORC2 at membranes in proximity to Akt\textsuperscript{137}. In Drosophila Kc cells, RNAi against Sin1
completely abolished the phosphorylation of Akt, suggesting that this mechanism is conserved\textsuperscript{136}. Interestingly, \textit{Sin1} is alternatively spliced and at least five isoforms exist\textsuperscript{138}. Three of them assemble with TORC2 and generate three distinct TORC2s. All of them are capable of phoshporylating Akt, but only two of them are regulated by insulin. This diversity could contribute to the differential responses of various cell types with regard to Akt phosphorylation\textsuperscript{136}. \textit{Drosophila Sin1} mutants are viable, fertile and normal in appearance and have a modest delay of development\textsuperscript{35}. The phosphorylation of Akt is also reduced in these mutants, but they display only minor growth impairments. The authors can show that the influences on Akt phosphorylation by TORC2 are more important in situations of elevated PI3K activity\textsuperscript{35}.

The phosphorylation of Akt by TORC2 is counteracted by the recently described PH domain leucine-rich repeat protein phosphatases 1 and 2 (PHLPP)\textsuperscript{139, 140}. They can trigger apoptosis and suppress tumour growth. PHLPP levels are reduced in several cancer cell lines displaying elevated Akt phosphorylation. Although PHLPP1 and PHLPP2 dephosphorylate the same amino acid, they regulate distinct Akt isoforms and therefore influence different downstream targets. In \textit{Drosophila}, a PHLPP homolog has been identified and experiments have revealed a conserved function in dephosphorylating Akt in \textit{Drosophila} cell culture\textsuperscript{139}.

**4.5 Open questions in the TOR signalling pathway**

An important issue is the function of the drug rapamycin. Rapamycin has been thought to specifically inhibit TORC1 and is currently in clinical development\textsuperscript{141-143}. However, recent data have revealed that prolonged exposure to this drug decreases the phosphorylation of Akt in some cancer cell lines due to the disruption of TORC2\textsuperscript{144}. There is a high variability of cancer cells in response to rapamycin and this might partially be due to the dual function of the drug. Additionally, treatment with rapamycin might lead to losing the negative feedback loop of activated S6K on the PI3K signalling branch, which is not wanted and might also contribute to the high variability of reactions of cancer cells to rapamycin. Therefore, a next step will be to elucidate the types of cancer that react in a favourable way to the drug rapamycin.
Other important unsolved issues include the mechanism of how amino acids regulate TOR. Although TOR’s dependency on amino acids has been known for many years, the molecular mechanism has remained elusive. Additionally, the phosphorylation of TSC2 by Akt in the regulation of TORC1 has also led to conflictive data. The answer might be complex, as the importance of TSC2 phosphorylation by Akt could be cell-type specific. Nevertheless it will be important to understand the underlying mechanism in the different types of cancer to predict the effects of therapeutical molecules.

TOR might function both upstream (TORC2) and downstream (TORC1) of Akt. It will be important to define the connection of TORC1 and TORC2 in respect to Akt. This might also be cell-type specific and is very important for the treatment of the different cancers. Furthermore the function of TORC1- and TORC2-associated proteins like LST8 and protor has not been revealed yet. Their function and the additional identification of novel interacting proteins might provide new bases for developing drugs specifically directed against the two distinct TOR complexes.

4.6 Kibra and the Salvador-Warts-Hippo (SWH) pathway

The focus of my work has been changing as it progressed. We have not been able to identify and characterize a new component of the TOR signalling pathway, but we have found another growth regulating gene called Kibra. Kibra encodes a scaffold protein and leads to more and larger cells when mutated and to fewer and smaller cells when overexpressed. One of the phenotypical features of Kibra is the additional interommatidial cells in Kibra mutant pupal eye discs. This phenotype is reminiscent of the phenotype of mutated genes involved in the Salvador-Warts-Hippo (SWH) pathway (Figure 30). Additionally, Kibra has been found to bind Merlin, an upstream component of the SWH pathway. Therefore we have started to investigate a role of Kibra in the SWH pathway.
Salvador is the first gene described in *Drosophila* to be a member of the SWH pathway\textsuperscript{145}. Interestingly, the SWH pathway controls cell proliferation, cell growth and apoptosis in a coordinated way. The involved genes are conserved from yeast to humans and deregulations of pathway members occur in human tumours\textsuperscript{17}.

In *Drosophila*, the SWH pathway is mainly active in the columnar epithelia of the imaginal discs\textsuperscript{17}. Its main purpose is to restrict the size of the discs, since a loss of SWH activity leads to tremendous tissue outgrowth due to increased proliferation, growth and missing apoptosis\textsuperscript{146}. The kinase cascade including Salvador, Warts, Hippo and Yorkie, constitutes the core of the signal transduction pathway. Hippo is responsible for the phosphorylation of Salvador and Warts, whereas Warts itself phosphorylates and inactivates Yorkie, a transcriptional co-activator. *DIAP1* and *cycE*
have been the first identified targets of the SWH pathway, they inhibit apoptosis and promote cell proliferation, respectively.

The tumour suppressor Neurofibromatosis 2 (NF2), also called Merlin in *Drosophila*, is an upstream component of the SWH pathway. Merlin provides a link between membrane-associated proteins and the actin cytoskeleton. It is expected to function in receiving signals from the extracellular milieu and might coordinate processes of growth-factor receptor signalling and cell adhesion. The key consequence of cultured *Merlin* mutant cells of several cell types is the failure to undergo contact-dependent inhibition of proliferation. In *Drosophila*, Merlin has been shown to function together with another member of the Protein 4.1 superfamily called expanded, a related FERM domain protein. Animals homozygous for hypomorphic mutations in either *expanded* or *Merlin* survive and display slight overgrowth of adult structures like wings. In contrast, the phenotypes of *Merlin; expanded* double-mutant cells are very similar to those of mutations in *hpo, sav* and *wts*. Merlin and Expanded act together to transduce a growth-regulatory signal from an unknown receptor to Hippo and Warts, which in turn regulate the expression of target genes to promote proliferation arrest and apoptosis.

Similar to *Merlin* and *expanded* mutations, *Kibra* mutant heads do not display a SWH-like bighead but they share some phenotypic similarities (additional interommatidial cells). It might be that Kibra also functions in a redundant way like Merlin and expanded, although preliminary genetic interaction experiments have not confirmed this hypothesis. Double mutant heads for *Kibra; Merlin* or *Kibra; expanded* might be more telling. Additionally, the Y2H binding data of Merlin and Kibra has to be confirmed. Since Y2H data might be artefacts, it would be reasonable to use a different method. A tagged Kibra protein will be expressed in Kc cells and Kibra containing complexes will be isolated and analyzed by mass spectrometry.

Merlin and expanded synergistically induce Warts phosphorylation. Therefore extracts of cells with downregulated *Kibra* could also be used to investigate the phosphorylation of Warts. A decrease in phosphorylation would be another strong hint for the involvement of Kibra in the SWH pathway.
In summary, we have isolated a gene named Kibra involved in the negative regulation of growth. Loss of Kibra leads to more and larger cells, whereas the overexpression leads to fewer and smaller cells. Kibra is likely to be involved in the SWH pathway and is a putative new tumour suppressor in humans. It will be important to further investigate a potential role of Kibra in the SWH pathway. In parallel, it should be determined whether Kibra mRNA is downregulated in human tumours. If this were the case, Kibra could be used for diagnostic purposes.

4.7 Review and outlook of genetic screens in Drosophila

We have performed two complementary genetic screens in Drosophila to find new downstream components of Rheb. The lethality suppression screen has enabled us to screen a large amount of mutagenized chromosomes in a short time and without the time-consuming screening of flies normally attributed with genetic screens. Due to the screen setup we were only able to find genes positively involved in growth, which was certainly a limitation of this screen. However, no screen leads to the identification of all genes involved in a process of interest and the advantages overwhelmed this disadvantage.

In addition to mutations in already known genes that served as a positive control of the screen, we identified two single hits. For that we screened around 90'000 flies, which took us more than half a year. On one hand, this showed that it was possible to identify new components with this type of screen. On the other hand, it also showed us unexpected constraints that can lead to the unexpectedly low recovery rate of meaningful new candidates. One of the major problems was the fact that the test of the screen setup was done in small vials, whereas the actual screen was performed in large bottles. Whereas the crosses worked well in the vials, the number of progeny was drastically reduced in the bottles for reasons we do not know. Additionally, a large part of the surviving flies were either sterile or died shortly after eclosion. All these facts together played a major role in the unsuccessfulness of this screen and were not expected. Nevertheless, the screen setup was well chosen and led to new components, but not in a reasonable time due to the reduced number of progeny.
The second screen was a deletion screen and we were able to identify three deficiencies that led to an alteration of the induced growth phenotype in the eye of *Drosophila*. We used newly generated deletion kits that contained isogenic backgrounds, which was a great advantage compared to the core deficiency kit at Bloomington. However, this kind of screen also had its limitations, namely the coverage of the deletion kits. These kits do not cover the whole genome, but only approximately 70% and therefore candidate genes can be missed. There might also be cases, in which a reduction of gene function by 50% is not enough to alter the growth phenotype in the eye of *Drosophila*. Although heterozygosity of the core components of the TOR signalling pathway does alter the eye size, this does not have to be the case for all genes involved in this signalling pathway and those genes would be missed. Once the deletions altering the eye size are identified, other limitations can occur. Large deficiencies can delete more than 100 genes and to find the locus responsible for the alteration of the eye phenotype can be an obstacle, especially if no mutants of the deleted genes exist. Additionally, a certain percentage of the deletions are incorrectly assigned, which can also lead to a falsified set of candidates.

We have not been able to identify a novel downstream component of Rheb with this deletion screen, but we have identified another gene involved in growth control due to an incorrectly assigned deletion. However, this gene has also been worthwhile to be characterized.

Although we have not been very successful with our complementary genetic screens, several other successful screens have been performed in the fly. *Drosophila* is a great model organism to identify genes that are involved in biological processes. It is cheap and easy to rear in the laboratory, has a short generation time and produces many offspring. Traditional forward genetic screens led to the discovery of genes involved in various different biological processes. The screen for embryonic-patterning mutants was the first large-scale screen where the genome was saturated for mutations that affect a particular process\(^\text{169}\). The large set of different techniques in the fly allows not only to identify the earliest phenotype of a mutation, as it is the case for the traditional screens, but gives the possibilities to perform more sophisticated screens. Enhancer
and suppressor screens use a sensitized system to render heterozygous mutations haploinsufficient. The progeny of the mutagenized flies can be screened directly, there is no need to make the mutations homozygous. Additionally, lethal mutations in essential genes that function at many stages of development can be isolated, because the sensitized background only affects an organ that is not required for viability. Many such screens have been carried out in Drosophila and they are very effective in finding new components in almost any process\textsuperscript{153, 154}. Clonal screens are another way to avoid the problem of only being able to analyze the first phenotype of a mutation. Mitotic clones are induced\textsuperscript{155}, in which only the cells of interest are homozygous for the mutagenized chromosome, and the rest of the organism is heterozygous\textsuperscript{38, 56, 156, 157}. Misexpression screens (gain-of-function screens) are used to find genes that might be missed in other screens because they have redundant functions. EP-elements are P-elements that carry UAS sites at one end, in a way that any gene that it inserts next to can be activated by Gal4\textsuperscript{158}. Like that a large number of EP insertions can be screened for their phenotypes by simply crossing them to a Gal4 driver that is expressed in the appropriate tissue\textsuperscript{159-161}.

Genetic screens in Drosophila do not only lead to a better understanding of biological processes in the fly. Due to the high degree of evolutionary conservation among genes that control the basic developmental processes, approximately three-quarters of the human disease loci have counterparts in the fruit fly\textsuperscript{162-165}. In many cases, genes from invertebrate can functionally replace their counterparts in vertebrates. Insights gained from model genetic systems can be applied to the vertebrate system. Therefore Drosophila is one of the most effective tools for analyzing the function of human disease genes, including genes involved in development, neurological disorders, cancer, cardiovascular disease, metabolic and storage diseases, and genes required for the function of the visual, auditory and immune system\textsuperscript{166}. Drosophila can be used to address specific questions in human genetics that have been difficult to resolve using a vertebrate model system due to the greater genetic redundancy in vertebrates. Of course there are also limitations to the invertebrate system, particularly due to biological processes that have evolved only within the vertebrate lineage. However,
the fly has been a very good tool for identifying and analyzing a broad range of human disease genes and this will most likely also be the case in the future. An important focus of human genetics will probably be to determine the genes that contribute to complex disorders, and flies might again provide a powerful system to identify interacting partners and to understand the mechanisms that underlie such interactions.\textsuperscript{166}.
5. Materials and Methods

Fly work
All experiments were done in a y w genetic background unless otherwise stated. The following fly stocks and transgenes have been used for this study: DrosDel deletions were received by collaborators of the DrosDel project, X chromosomal deletions were generated in the lab. Exelixis deletions were received from K. Basler. RNAi lines against Kibra (81D12, 143C11) eIF-2ß (15G11) and yorkie (117C7) were ordered from B. Dickson. EP745 and G4164 were obtained from Szeged and GenExel, respectively. The Kibra EP insertion (19909) was obtained from Bloomington. y w; ey-Flp; FRT82, cl, w+/TM6By+: Newsome et al.157; y w; hs-Flp; FRT82, w+: Xu and Rubin156; y w; hs-Flp; FRT82, UbiGFP: Bloomington; y w; GMR>wt>Gal4: Brogiolo et al.26; y w; Act>CD2>Gal4, UbiGFP/TM6B: Neufeld et al.11; y w; TOR21/CyOy+: Oldham et al.38; y w; TSC1/TM3: generated in the lab; y w; GMR-Rheb 7/39; y w; UAS-Kibra 9/18; Kibra+/TM6B: generated in the lab.

Weight measurements
All weight measurements were done with a precision scale from Mettler Toledo (Greifensee, Switzerland). Flies were separated by sex and individually balanced. 3 days old flies were anaesthetized by ether for one minute prior to weighting.

Jump out screens
Kibra: isogenized virgin flies (EP 19909, w+) were crossed to male A2-3 flies and the progeny jump starter males were mated to virgin MKRS/TM6B females. w males were selected and individually mated to virgin MKRS/TM6B females. After mating, the males were removed and 10 flies each were pooled and examined with PCR for imprecise excisions of the EP element. The primers used were CGAGTGTATCGATATGCAATGCG as forward primer and CCTTTTCTTTCGGTTCAAGAGC as reverse primer. A positive pool was analyzed
by PCR analysis of all 10 individual fly lines. Around 330 individual crosses were screened for deletions.

*eIF-2β*: The procedure was the same as for the imprecise excision of *Kibra*. An isogenized *G4164* line was used to generate the excisions. The following primers were used for the detection of the imprecise excision alleles: forward primer: `CTCGTCTAATAAGCTCACGG`; reverse primer: `CGGTTCTGCAAAATATGTGACC`.

*CG32111*: The procedure was the same as for the imprecise excision of *Kibra*. An isogenized *P*-element situated 5' to *CG32111* was used to generate the excisions. The following primers were used for the detection of the imprecise excision alleles: forward primer: `CCACCTGGAAAGAGCAGTCACG`; reverse primer: `CGCAAGTCTATTCTTTGCGC`.

**Reversion mutagenesis screen**

This screen was generated by crossing *GMR-Gal4* virgins to mutagenized 19909 (*Kibra EP*) males. Prior to mutagenesis the males were starved for approximately seven hours and then fed 25mM EMS (ethylmethanesulfonate) in 1% sucrose solution overnight. After one day of recovery the males were mated to the corresponding virgins and their progeny was screened for wild-type eyes. Positive animals were taken to establish lines for the 3rd chromosome containing the *Kibra EP* and examined for mutations in the *Kibra* locus.

**3rd instar disc fixation**

The larvae were stored and dissected in Ringers on ice followed by an incubation in 4% paraformaldehyde in PBS on ice for 40min or at room temperature (RT) for 20-30min. The dissected larvae were washed 3 x 30min in PBT (0.3% Triton-X in PBS) and washed twice with PBS at RT. Afterwards imaginal discs were mounted in Vectashield.
Clonal analysis in 3\textsuperscript{rd} instar wing discs

Virgin y w hs-Flp; FRT82, UbiGFP/TM6B flies were crossed to male y w FRT82, Kibra\textsuperscript{z}/TM6B flies. The progeny received a 37°C heat shock 48-72h after egg deposition for 5min. 3\textsuperscript{rd} instar larvae were dissected 48h after the heat shock. Fixation and DAPI staining were done as described in antibody staining. The discs were analyzed with a Leica confocal microscope (DM5500Q). The size of the clones and the number of cells were determined with Photoshop software.

Overexpression analysis in 3\textsuperscript{rd} instar wing discs

Virgin y w; Act>CD2>Gal4 UAS-GFP/TM6B flies were crossed to male yw; UAS-Kibra9/UAS-Kibra9 or yw; UAS-Kibra18/TM6B flies, respectively. The progeny received a 34°C heat shock 48-72h after egg deposition for 15min. 3\textsuperscript{rd} instar larvae were dissected 48h after the heat shock. Fixation and DAPI staining were done as described in antibody staining. The discs were analyzed with a Leica confocal microscope (DM5500Q). The size of the clones and the number of cells were determined with Photoshop software.

Overexpression analysis in adult fly eyes

Virgin yw; GMR>w+>Gal4/TM6B flies were crossed to male y w; UAS-Kibra9/UAS-Kibra9 or y w; UAS-Kibra18/TM6B flies, respectively. The progeny received a 37°C heat shock 24-48h after egg deposition for 1h. Tangential eye sections of mosaic eyes were generated and examined with a standard Zeiss light microscope and a Leica colour camera. Distances between ommatidia were determined with Photoshop software.

Phalloidin staining of pupal eye discs

Virgin y w, ey-Flp; FRT82, UbiGFP/TM6B flies were crossed to male y w, FRT82, Kibra\textsuperscript{z}/TM6B flies. The progeny received a 37°C heat shock 24-48 h after egg deposition for 30min. Wandering larvae were put to a fresh vial and two to five hours
later white prepupae were marked. Exactly 46h later the pupae were prepared. The pupal eye discs were fixed in 4% PFA in PBS for 20min at RT, washed three times for 30min in PBT and afterwards incubated for two hours in PTF (200µl) containing 5µl Phalloidin. After washing for 1h in PBT the eye discs were mounted in Vectashield (for abbreviations see antibody staining protocol).

**Antibody staining**

The larvae were stored and dissected in Ringers on ice followed by an incubation in 4% paraformaldehyde in PBS on ice for 40min or at room temperature (RT) for 20-30min. The tissue was permeabilized and blocked with PTF (1.5ml 10% Triton-X, 0.5g BSA Sigma, 50ml 1xPBS) at RT for 15min followed by overnight incubation with the 1° antibody diluted with PTF at 4°C. The tissue was washed six times with PTF at RT for 1 hour (total) followed by an incubation with the 2° antibody diluted with PTF in the dark at RT for 90min. The tissue was washed six times with PTF at RT for 1 hour (total) and washed twice with PBS at RT (10min each). For DAPI staining the tissue was incubated with DAPI solution (1:200 in 1xPBS) at RT for 20min and washed twice with PBS followed by mounting in Vectashield medium.

Kibra antibody stainings:

1° antibody diluted in glycerol: diluted 1:30

2° antibody α-rabbit Texas red: diluted 1:100

Boss antibody stainings:

1° antibody: diluted 1:100

2° antibody α-rabbit Texas red: diluted 1:100

**Tangential eye sections of adult fly heads**

For every genotype, glass tubes were labelled and filled with 1ml Ringers solution (kept on ice). The heads were pulled off with forceps and cut in half between the eyes with a razor blade. Then the eyes were transferred into the labelled glass tubes (kept
on ice). The Ringer solution was replaced with 250μl of fixative for each line and the eyes were incubated for 30min on ice.

For 1 ml of fixative (prepare in glass and on ice in the fume hood):

210μl H₂O
250μl Cacodylate buffer (4°C)
40μl 50% Glutaraldehyde in H₂O (4°C)
500μl 2% OsO₄ (4°C)

After 30min, the fixative was replaced with 250μl 2% OsO₄ and incubated for 2h on ice. Then the eyes were given in 30%, 50%, 70%, 90% and 100% acetone at RT for at least 5min each, and in 100% acetone (+ dessicating beads) for 10min. The Spurr solution was removed from -20°C, a 1:1 solution with absolute acetone was made and eyes were incubated in this solution o/n at RT. In the next morning, Spurr/Acetone solution was replaced with Spurr and incubated for at least 4h. Embedding trays were filled with Spurr and into each embedding tray one eye was pipetted using a Pasteur pipette and orientated that it was facing down. Once the eyes were arranged, the embedding trays were carefully moved to 65°C for at least 18h. Afterwards, embedded eyes were cut with a Zeiss microtome (2μm) and examined with a standard Zeiss light microscope and a Leica colour camera.

**Transmission electron microscopy of eye sections**

The embedding procedure of the eyes was the same as for the tangential eye sections. The cutting of the eyes (90nm) was done with an ultracut microtome (LEICA Ultracut UCT) and the sections were contrasted with a 3% Uranylacetat solution for 30min. Analysis of sections was done with a ZEISS EM 902 A transmission electron microscope.
Learning and memory

All experiments were done as described in Michels et al., except that we made all trainings and tests in daylight\textsuperscript{118}.

Generation of transgenes

The genomic \textit{Rheb} construct was generated by amplifying genomic DNA from \textit{yw} flies with the following primers: Forward primer, \texttt{GATTTCGTTCCCAATCGATTGC}; reverse primer, \texttt{GGTGGATTGGCTATTTTCGTGG}. The PCR product was cloned into the pCAP\textsuperscript{5}-cloning vector and afterwards excised with NotI and Xhol to clone the \textit{Rheb} fragment into pCaSpeR4.

The generation of the \textit{GFP-Rheb} constructs was done with PCR reactions. C-terminal fusion protein (GFP-Rheb\textsubscript{1}: GFP in front of CAAX domain): GFP sequence was PCR amplified using pEGFP-C1 (Clontech) as a template with the following primers: Forward primer, \texttt{GAGAAGAGCGGTATGGTGAGCAAGGGCGAGGAGCTG}; reverse primer, \texttt{CGATACAAGACATCTAGATCCGGTGGATCCCGGGC}. The amplification of the \textit{Rheb} locus excluding the CAAX domain and the 3'UTR (Rheb – (CAAX + 3'UTR)) was done with PCR reactions with genomic DNA from \textit{yw} flies and the following primers: Forward primer, \texttt{CGAAAGTGCTGCAGAAAACTCGGAGTAC}; reverse primer, \texttt{CCTTGCTCACATACCCGCTCTTTCTGCGGGGATTGCCG}. The amplification of the CAAX domain and the 3'UTR (CAAX + 3'UTR) was done with the forward primer \texttt{CACCCGATCTAGATGTCTTGTATCGTAGGGCGGCTGAG} and the reverse primer \texttt{CGCGTTGTGGTGGATTTGCTATTTTCG}. The first fusion PCR (GFP\textsubscript{1} + (CAAX + 3'UTR)) was done with the following primers: Forward primer, \texttt{CGCGTTGTGGTGGATTTGCTATTTTCG}; reverse primer, \texttt{GAGAAGAGCGGTATGGTGAGCAAGGGCGAGGAGCTG}. The second fusion PCR ((Rheb – (CAAX + 3'UTR)) + (GFP\textsubscript{1} + (CAAX + 3'UTR))) was done with the forward primer \texttt{CGAAAGTGCTGCAGAAAAACTCGGAGTAC} and the reverse primer \texttt{CGCGTTGTGGTGGATTTGCTATTTTCG}. 
N-terminal fusion protein (GFP-Rheb2: N-terminal GFP): PCR of the GFP sequence was done with the pEGFP-C1 vector from Clontech and the following primers: Forward primer, CCCAGGAAATGGTGAGCAAGGGCGAGGAGCTG; reverse primer: CTTTGGTTGGCCATTCTAGATCCCGGTGGATCCCGGGC. The amplification of the Rheb 5'UTR was done with genomic DNA of \( yw \) flies and the following primers: Forward primer, CGAAAAGTGCAGAGGAACACTCGGAGTAC; reverse primer, CGCCCTTGCTACCATTTTCTGTGGGCCCAGTGGTATTTTG. The amplification of the second part of the \( Rheb \) locus (Rheb – 5'UTR) was done with the forward primer ACCGGATCTAGAATGCCAACCAAGGGAGCGCCACATAG and the reverse primer CCGTGGTGGCTGATCCCGGT. The first fusion PCR (Rheb 5'UTR + GFP2) was done with the following primers: Forward primer, CGAAAGTGCTGCAAAACACTCGGAGTAC; reverse primer, CTTTGGTTGGCCATTCTAGATCCCGGT. The second fusion PCR ((Rheb 5'UTR + GFP2) + (Rheb – 5'UTR)) was done with the forward primer CGAAAGTGCTGCAGAAACACTCGGAGTAC and the reverse primer CCGTGGTGGCTGATCCCGGT.

The \( GMR-Rheb \) construct was generated with a cDNA sequence of \( Rheb \) in the TOPO cloning vector. A tubulin SV40 trailer insert was excised with XhoI and XbaI and inserted into the TOPO-Rheb vector. This vector was digested with Asp718 and XbaI and the insert was cloned into a GMR vector with the same restriction sites.

The \( UAS-Kibra \) construct was generated with \( Kibra \) ESTs. SD2076 was digested with BglII and SacI, RE26350 was digested with SacI and KpnI. These fragments were inserted into a pUAST vector cut with BglII and KpnI. The \( UAS-WW \) construct was generated from a PCR reaction of SD2076 with the following primers: forward primer, CGAGAAACAAATGGTTAATAATGCCG; reverse primer, ACACTTATTAACTCTCAGATCC. The PCR product was inserted into the TOPO-vector, digested with KpnI and XbaI and cloned into the pUAST vector.
6. References


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7. Curriculum Vitae

Last name: Buser
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Date of birth: 9.12.1975
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Education
1996:
Matura (Typus C, Kantonsschule Wohlen)

1996 – 2000:
Studies in Biology at the University of Zurich
(specialized in developmental biology, genetics, molecular biology and evolutionary biology)

2001 – 2002:
Diploma Thesis
"Comparison of sex-determination in Drosophila and Musca", supervised by Dr. D. Bopp, Institute of Zoology, University of Zurich

Oct 2002:
Diploma in Zoology

2003 – 2007:
PhD Thesis at the Institute of Zoology, University of Zurich and at the Institute of Molecular Systems Biology (IMSB), ETH Zurich, supervised by Prof. Dr. E. Hafen and Dr. H. Stocker
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