Drosophila Genetics as a Tool in the Search for Novel Components of the S6 Kinase Signaling Pathway

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In memory of Anne and Stanley Bennion

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ABSTRACT

Signaling events that converge on the activation of the TOR/S6K pathway have been shown to be critically related to growth. Recent investigations on the molecular network controlling growth have revealed that *Drosophila melanogaster* constitutes an efficient alternative model to the mammalian system. As the nature of interactions between known components of the pathway is becoming more understood, it is clear that a number of elements are lacking. Recently the Thomas laboratory initiated an EP screen in *Drosophila* utilizing a unique phenotype in the wing, where the yeast Gal-4 promoter was used to drive expression of random genes in the dorsal wing compartment of the flies. An enhancement of growth in this tissue could then be observed by curvature of the wing. When UAS dS6K is driven with apterous- GAL4 in *Drosophila*, a bent-down wing phenotype is observed. By screening of random EP insertions combined with UAS dS6K, a number of enhancers were discovered, two of which are presented in this thesis. We show that both *orb2* and *IP3K1* are genes which have a role in the control of growth, and provide models which can help to explain their role in S6K/TOR signaling.

ABBREVIATIONS

4E-BP1	eIF4E binding protein 1
dS6K	*Drosophila* S6 kinase
dTOR	*Drosophila* Target of Rapamycin
EP	enhancer–promoter
IR	Insulin receptor
IP_3	Inositol (1,4,5)Phosphate
IP_4	Inositol(1,3,4,5)Phosphate
IP3K	Inositol Phosphate -3 Kinase
IRS	Insulin receptor substrate
GAP	GTPase-activating protein
mRNA	messenger RNA
mTOR	Mammalian target of rapamycin
Orb1	oo18 RNA-Binding Protein 1
Orb2	oo18 RNA-Binding Protein 2
PDK1	PtdIns(3,4,5)P_3-dependent protein kinase-1
PI(3)K	phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol(4,5) Phosphate
PIP_3	Phosphatidylinositol (3,4,5) Phosphate
PKB	Protein Kinase B
Rheb	Ras Homologue Enriched in Brain
RNAi	RNA mediated interference
S6	40S ribosomal subunit protein 6
S6K1	S6 kinase 1
S6K2	S6 kinase 2
TOR	Target of Rapamycin
TSC	Tuberous Sclerosis Complex
UTR	Untranslated region

Less frequently used abbreviations are defined upon their first use in the text.

CHAPTER 1: Introduction and Background

1.1 Growth

Cellular growth

The successful development of a multicellular organism relies on fine-tuned events controlling cellular growth, differentiation, proliferation, and death. The number and size of cells ultimately dictates the size of the organism [1]. At the most basic level, final cell size is limited by its volume to surface area ratio, and there comes a point in the growth of a cell when its surface area is insufficient to meet the demands of the volume of the cell. Two daughter cells generated by mitosis, which are initially the half size of the mother cell, must grow before they can undergo the next round of cell division, a process controlled by the cell cycle. For this reason it has been rationalized that progression through cell-cycle is a mediator of growth regulation. To the contrary, it has been shown that proliferation is determined by growth, and not vice versa [2, 3].

Given the coordination of mitosis with growth and cell cycle, it is easy to understand why it has been long proposed that cell size is tightly linked to DNA content [4]. However, there is an obvious limitation to this paradigm, which is that metazoans may have many different cell types of variable size, but with constant DNA content. In fact, the growth rate of a cell must be proportional to its cellular content or 'dry mass'. Since the majority of this dry mass is accounted for by proteins, it is

reasonable to assume that the important factors for increased cell size would be those important for the synthesis of proteins, such as ribosomes. Ribosomes are vital organelles that catalyze protein synthesis, and in eukaryotes are composed of four RNA molecules and one molecule each of 79 different proteins ('ribosomal proteins') [5]. It has been shown in *Drosophila* that the amount of protein in a cell is in fact dependent upon ribosomal RNA content, whose synthesis requires RNA polymerase I [6]. Of course, without a source of the essential amino acids required for protein synthesis, this process would also be limited and therefore nutrient input is also essential for growth.

Cell cycle control

Cell division is an evolutionarily conserved process requiring tight spatial and temporal control of its molecular events. An intricate network of regulatory pathways ensures that each cell cycle event is performed correctly and in proper sequence, leading to the replication of chromosomal DNA and equal distribution of duplicated DNA between two daughter cells [7]. The mammalian cell cycle can be divided into five phases: G_0, G_1, S, G_2 and M. These phases are categorized as 'Gap', 'Synthesis', and 'Mitosis' phases. A cell in the G_0 phase is in a quiescent state, and will remain so until external stimuli trigger signaling events such as the activation of the ras-dependent extracellular signal-regulated kinase 1 (ERK1)/2 mitogen-activated kinase (MAPK) cascade, which plays a central role in cell proliferation [8]. MAPKs are serine-threonine protein kinases that are activated by diverse stimuli ranging from cytokines, growth factors, neurotransmitters, hormones, cellular stress, and cell adherence[9]. In the case of cell cycle regulation, the small GTPase Ras is activated

following cell surface receptor activation, which recruits the MAP kinase kinase kinase Raf to the membrane for subsequent activation by phosphorylation. Raf then activates the MAP kinase kinases MEK1/MEK2, which in turn activate effector MAP kinases ERK1 and ERK2 [9]. ERK1/2 activity is necessary for G1- to S-phase progression and is associated with induction of positive regulators of the cell cycle and inactivation of anti-proliferative genes [8].

Signaling events triggered by the binding of growth factors to cell surface receptors cause the cell to exit G_0 and re-enter the cell cycle at G_1, where synthesis of the mRNAs and proteins required for DNA synthesis will take place before progression into S-phase [10]. Upon entry into G_1 phase, progression of the cell through each phase of the cell cycle is dependent upon the sequential formation, activation, and inactivation of cell cycle control molecules. These include the cyclin (regulatory subunit) and cyclin-dependent kinase (Cdks, catalytic kinase subunit) complexes, which are regulated at the level of transcription, translation, and post-translational modification [10]. The first of these complexes consists of the D-type family of cyclins (D1, D2, and D3) plus Cdk4/6 and allows the cell to exit G_1. Cyclins A and E pair with Cdk2 for the G_1/S transition and progression through S phase, while Cdk1 governs the G_2/M transition in complex with cyclin B1 [11]. Studies in knock out animal models indicate that loss of the catalytic member of these complexes can be compensated for by other Cdk isozymes and cell cycle progression continues. For example, despite their small size and tissue-specific defects, Cdk4 knockout mice are viable [12], as are Cdk2 knockouts [13].

In complex with D-type cyclins, Cdk4 phosphorylates G1-specific substrates, including the retinoblastoma protein (Rb) [14]. Unphosphorylated or hypophosphorylated forms of Rb form transcriptional repressor complexes with E2F-1–3 [15]. Rb phosphorylation in collaboration with cyclin D/Cdk4 and cyclin E/Cdk2 activity results in release of Rb from the E2F complex, leading to transactivation of the E2F target genes important for the S-phase [16]. Highlighting the importance of Rb phosophorylation in the cell cycle whilst illustrating the compensation between Cdk2 and 4, mice deficient in both Cdk2 and Cdk4 are embryonic (E15) lethal. Studies carried out in Cdk2-/-, Cdk4-/- Mouse Embryonic Fibroblasts (MEF) revealed a decreased proliferation rate, impaired S phase entry, and premature senescence due to hypophosphorylation of Rb, which was rescued by inactivation of the protein [17]. Throughout the cell cycle, control mechanisms exist which impose a dependency of a particular event on the completion of the preceding event to ensure correct timing of the cell cycle. Following completion of all steps, the cell can enter mitosis [18]. See Figure 1.

The role of Growth and Proliferation in Drosophila development

In *Drosophila*, two striking alterations to the classical conserved cell cycle exist. Cells in the early fly embryo exhibit simple, rapid and synchronous cell cycles, similar to those seen in *Xenopus* [19], consisting only of alternating S and M phases driven exclusively by maternal factors. This partitions the egg into smaller and smaller cells. This growth-independent strategy used by the fly during embryogenesis to create the emergent first instar larva is in contrast to the growth-linked process used to

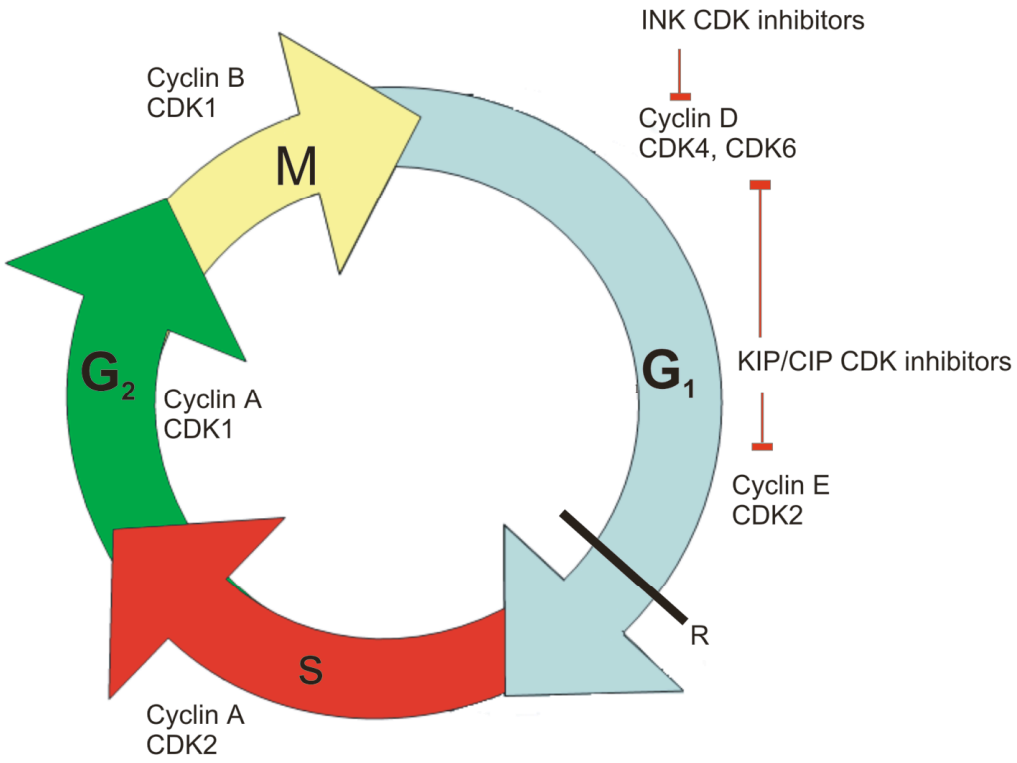

Figure 1: The conserved eukaryotic regulatory pathways that regulate cell-cycle progression.

Two cell-cycle checkpoints control the order and timing of cell-cycle transitions (G1S and G2M), ensuring that critical events such as DNA replication and chromosome segregation are completed correctly before cell cycle progression. A major cell-cycle restriction point (R) is located at the end of the G1 phase, after which, precursors will invariably complete the cycle. Mitotic cycle progression is driven by the actions of CDKs and their activating cyclin subunits, CDK activity is suppressed through the INK4 family (that exhibits selectivity for CDK4 and CDK6) and the CIP/KIP family (a broader range of CDK inhibitory activity). The rate of cell-cycle progression is determined by the relative abundance of positive and negative regulators.

This figure was adapted from; "Cell-cycle control and cortical development", Dehay & Kennedy, Nature Reviews Neuroscience 8, 438-450 (June 2007

generate the adult fly which develops from imaginal cells nurtured by the larva [20]. It is not until later in embryogenesis that the cell cycle incorporates a G1 phase, linking cell cycle progression to extracellular cues and allowing for coordination of growth and division. This phase occurs after the disappearance by proteosomal degradation of maternally transcribed cdk activators, which are zygotically transcribed once the embryo enters a cell-cycle containing a growth phase.

Endoreplication conversely, entails rounds of S- and G- phases exclusively, as a strategy for cellular growth without mitosis in the larval stages. In some endoreplicative cells, levels of G_2-M phase machinery such as cyclins B and A, and Cdk1 have been found to be either absent or present at very low levels. It is clear that cyclin E/Cdk2 kinase plays a major role in endocycling, with induction of cyclin E triggering precocious DNA replication in such tissues. On the other hand, continuous over-expression of cyclin E inhibits endocycle progression, leading to the suggestion that oscillations in cyclin E levels may be important for the process. In flies, the G1 cyclins include one D-type and one E-type cyclin, which allosterically activate Cdk4 and Cdk2, respectively. Unlike cyclin E and Cdk2, neither cyclin D nor Cdk4 is essential for viability in *Drosophila*, and mutant flies lacking the latter genes develop to adulthood, although they exhibit a loss of fertility and small size [21, 22]. Therefore, not all of the cell cycle proteins that contribute to G1-phase regulation are obligate components of the cell division machinery, but rather couple the activity of the core S- and M-phase oscillator to diverse mitogenic signal transduction pathways [23].

In contrast to the unusual cell-cycle alterations outlined above, *Drosophila* imaginal discs, the epithelial sheets which will later form the adult structures, undergo

classical cell division cycles, coupled to an extensive increase in the size of the disc by growth [24]. An example of this is in the imaginal wing disc, whose development is discussed later.

1.2 Intracellular signaling

Intracellular Signal Transduction

The activation of a cell surface receptor by the binding of an extracellular ligand triggers a series of events which elegantly coordinates cell signaling pathways for metabolism, growth and survival with the organism's surroundings. The evolution of such coordination has allowed multicellular organisms to succeed in adapting to a dynamic environment in terms of nutrient availability, temperature change and pathogenic invasion. The extracellular molecules involved in regulation of cell processes can be arranged into diverse classes (Table 1). Binding of ligand to the external portion of an integral plasma membrane receptor evokes a conformational change within the intracellular domain. The conformational change initiates signal transduction by activating the enzymatic activity of the receptor or by exposing binding sites for other proteins. Downstream components of these pathways usually possess enzymatic activity and include, but are not limited to, heterotrimeric G proteins, small GTPases, protein kinases, protein phosphatases, lipid kinases and lipid hydrolases. In addition to modification of existing proteins, second messengers are often produced, which play a powerful role in the amplification of the signal from the cell surface.

Class	*Example*	*Origin*	*Target*	*Major effect*
Amino acid-derived	Serotonin	Platelets	Arterioles Venules	Vasodilation Increased vascular permeability
Peptide hormones	Glucagon	Pancreatic α-cells	Liver Adipose tissue	Glycogenolysis Lipolysis
Growth factors	Epidermal Growth Factor (EGF)	Multiple cell types	Epidermal and other cells	Growth
Eicosanoids	Prostaglandins	Most body cells	Multiple	Inflammation Vasodilation
Membrane permeant hormones	Progesterone	Corpus Luteum Placenta	Uterine endometrium	Preparation of endometrial layer Maintenance of pregnancy

Table 1: A selection of first messengers found in the circulation.

Adapted from "Signal Transduction", Gomperts, Kramer& Tatham, Elsevier 2003.

Protein modification and second messengers

Post-translational modification of proteins ranges from the addition of other proteins or peptides, addition of functional groups, changes in the chemical nature of amino acids, or structural changes. Major second messengers in a cell are cyclic AMP (cAMP), calcium ions (Ca^{2+}), diacylglycerol, and the 3-phosphorylated inositol lipids, all of whom's role is to amplify a signal from the plasma membrane via modification of existing proteins or molecules in the cell [25]. A key regulatory modification in the rapid transduction of a signal in eukaryotes is protein phosophorylation, the addition of a phosphate group (PO_4) from a nucleotide triphosphate (such as ATP) to a serine, tyrosine or threonine residue, which can affect protein structure and polarity thus activating or inactivating a protein, or induce changes in protein structure that alters docking potential for other proteins.

1.3 The PI3K/TOR/S6K signaling pathway

S6 Kinase and Target of Rapamycin proteins

S6 kinase (S6K) was originally described as a kinase responsible for phosphorylation of ribosomal protein S6, isolated from Swiss mouse 3T3 cells [26], with identification of two isoforms shortly after [27, 28]. The shorter form, originally termed $p70^{S6K}$, is largely cytoplasmic, while the longer form, originally termed $p85^{S6K}$, contains a nuclear localization signal, and speculation exists for a role of the nuclear form in phosphorylation of chromatin-bound S6 [29]. Since the identification of S6K2

as a second S6 Kinase [30], the $p70^{S6K}$ and $p85^{S6K}$ isoforms (recently termed S6K1S and S6K1L, respectively) of the original gene are collectively known as S6K1. Both S6K1 and S6K2 display kinase activity which is inhibited by treatment with the macrolide rapamycin (see below), or by phosphatidylinositol 3OH-kinase (PI3K) inhibitors.

The Target of Rapamycin (TOR) is a member of the PI3K-related family of protein kinases, along with ATM, ATR and DNA-dependent kinase, by virtue of similarity in its kinase domain [31]. This family of kinases is largely involved in cell-cycle checkpoint signaling related to DNA damage [32]. A large protein of ~280kDa, mTOR has been documented as having several other domains, important for regulation of the kinase and protein-protein interactions [33]. TOR proteins are so-called due to their specific inhibition by the macrolide rapamycin, a drug which until recently has been used as an immunosuppressant in transplant patients, and derivatives are now in clinic for renal cell carcinomas and clinical trials for use as an anti-cancer treatment [34-36]. When Rapamycin is added to cells, it forms an inhibitory "gain-of-function" complex with the FK-506 Binding Protein 12 (FKBP12) and TOR [37, 38].

During evolution of higher mammals the TOR signaling pathway has gathered complexity, and is considered to be a major point of integration for controlling growth, in relation to nutrient availability [31]. This control is largely mediated through translation, the production of proteins from an mature messenger RNA template [39], and autophagy, a process in which parts of the cytoplasm, including entire organelles, are first sequestered in vacuoles and then destroyed through a lysosomal pathway [40, 41]. TOR signaling has two major extra cellular inputs, growth factors and nutrients,

such as amino acids and glucose. Two distinct signaling complexes exist that are often referred to as the rapamycin-sensitive complex and the rapamycin-insensitive complex, and whose pathways cross-talk at several points.

The two TOR complexes; TOR complex 1 and TOR complex 2

The first hint at the existence of two independent TOR signaling complexes was in *Saccharomyces cerevisiae*, when two genes, TOR1 and TOR2 were identified on the basis of their sensitivity to rapamycin [42]. These genes were later identified as present in two complexes; a rapamycin-sensitive complex (TOR complex 1) formed with either TOR1 or TOR2 along with newly described proteins KOG1 and LST8, and a rapamycin-insensitive complex (TOR complex 2), formed only with TOR2, AVO1, AVO2, AVO3 and LST8 [43]. In other eukaryotes, a single TOR gene exists, and thus the two TOR complexes are formed only with one TOR protein that is the functional homolog of yeast TOR2 [44].

TOR binding partners identified in yeast have now been shown to have conserved counterparts in mammals, and invertebrates. TOR complex 1, which is sensitive to rapamycin, is a complex of mTOR with GβL and Raptor (in yeast; LST8 and KOG1 respectively) [45, 46]. More recently mammalian PRAS40 (proline-rich Akt (PKB) Substrate 40 kDa; for PKB, see below), has been demonstrated to preferentially bind Raptor, thereby inhibiting complex 1 activity [47]. This result has been controversial, with other laboratories claiming that PRAS40 is a substrate for TOR complex 1, and competes with other substrates for binding and phosphorylation

by TOR [48-50]. PRAS40 contains a TOR signaling (TOS) domain [48, 49], a conserved five amino acid sequence that is crucial for their regulation by the mTOR pathway [51]. Other substrates of complex 1 also contain this TOS motif, such as S6K1 and the eIF4E binding protein which inhibits translation, 4E-BP1 [51]. A common readout for the activity of TOR complex 1 is phosphorylation of S6K1 at threonine 389. S6K1 requires this threonine 389 (T389) phosphorylation a C-terminal hydrophobic motif by TOR complex 1, for activity, as it serves as a docking site for the phosphoinositide-dependent protein kinase 1 (PDK1), which phosphorylates T loop of and leads to S6K1 activation [52].

TOR complex 2, which is resistant to short-term rapamycin inhibition, is a complex of mTOR with, GβL, Rictor and SIN1 (in yeast; LST8, AVO3, and AVO1 respectively) [53-55]. GβL, Rictor, and SIN1 are all required for TOR complex 2 activity, as removing one of these components destabilizes the complex [44]. Recently, Protor-1 (protein observed with Rictor-1) and Protor-2 have been identified as binding partners of complex 2 [56], along with PRoline-Rich protein 5 (PRR5) [57], but so far, little is known about these interactors. Importantly, long term rapamycin treatment is reported to have an effect on TOR complex 2 by inhibition of new complex formation [58]. The main readout for TOR complex two activity is phosphorylation of protein kinase B, which is phosphorylated by TOR complex 2 at serine 473 (S473) [59]. PKB was originally reported as a cellular counterpart of the viral oncogene v-Akt, and its over-expression or increased activity has been implicated in a wide range of cancers [60]. Recently, the importance of this gene in cancer was highlighted by the discovery of a somatic mutation in the PH-domain (see below) of

PKB found in human breast, colorectal and ovarian cancers that transforms cells and induces leukemia in mice [61].

Drosophila homologues of most these complex components have been identified (with the exceptions of the most recent discoveries PRR5 and Protor) and current data agrees with observations in mammalian cells [47, 52, 62].

The mammalian PI3K/TOR/S6K signaling pathway

PI3K/TOR/S6K signaling begins at the plasma membrane by ligand binding-induced activation of a cell surface receptor. In the case of insulin to the Insulin Receptor (IR), this results in recruitment of Insulin Receptor Substrates (IRS) to the cell membrane [63] and subsequent recruitment and activation of a class 1 PI3K, a lipid kinase that converts PtdIns(4,5)P2 (PIP_2) to the potent second messenger PtdIns(1,4,5)P3 (PIP_3) at the plasma membrane [64]. The production of PIP_3 by PI3K is antagonized by two phosphases, PTEN (the phosphatase and tensin homolog on chromosome 10), which dephosphorylates PIP_3 at the '3' position, and), SHIP (SH2-domain-containing inositol-5-phosphatase) dephosphorylates PIP_3 at the '5' position, in both cases generating different forms of PIP_2 [65, 66]. PIP_3 binds to the pleckstrin homology domain (PH domain) of several proteins such as PDK1 and Protein Kinase B (PKB), resulting in their recruitment to the cell membrane. Upon localization to the membrane PDK1 phosphorylates the critical site T308 on PKB [67]. PKB is fully activated by further phosphorylation at S473 by TOR complex 2, allowing it to signal to its downstream substrates [59]. One of these substrates is the tumor suppressor

tuberous sclerosis complex 2 protein (TSC2), phosphorylation of which leads to the degradation of the TSC1&2 complex [68]. The TSC1/2 complex acts as a GTPase Activating Protein (GAP) inhibiting the small GTPase Ras Homologue Enriched in Brain (Rheb) by driving it into the inactive GDP bound state [69, 70]. Alone, Rheb has very low intrinsic GTPase activity, thus degradation of the TSC1/2 complex relieves Rheb of the suppressive GAP regulation resulting in the active GTP bound state. GTP bound Rheb is able to signal to TOR complex 1 through direct binding [71]. Thus growth factors such as insulin are able to use this canonical cascade to activate raptor-mTOR through a PI3K dependent mechanism.

The raptor-mTOR complex is also regulated by amino acids and glucose through a PI3K dependent pathway, however this does not appear to be orchestrated by the activity of class I PI3K but rather class III PI3K [72, 73]. The amino acid input to TOR complex 1 is completely dominant to the growth factor input where, in the absence of these nutrients, phosphorylation of TOR complex 1 substrates does not occur even in the presence of insulin [73, 74]. Until only recently the cascade involved in the amino acid stimulation of raptor-mTOR remained largely unknown. Based on the sensitivity to PI3K inhibitors of this arm of the pathway and a number of elegant loss and gain of function experiments, Nobukuni *et al* eliminated a role for the PI3K-Akt-TSC1/2 signaling axis in the activation of Rheb and introduced the class III PI3K hVps34 as the mediator of the amino acid signal to TOR complex 1 [73]. An active TOR complex 1 is required for phosphorylation of several components of the ribosome recruitment machinery, some of which are indirect through S6K activity [33, 75-78]. See Figure 2.

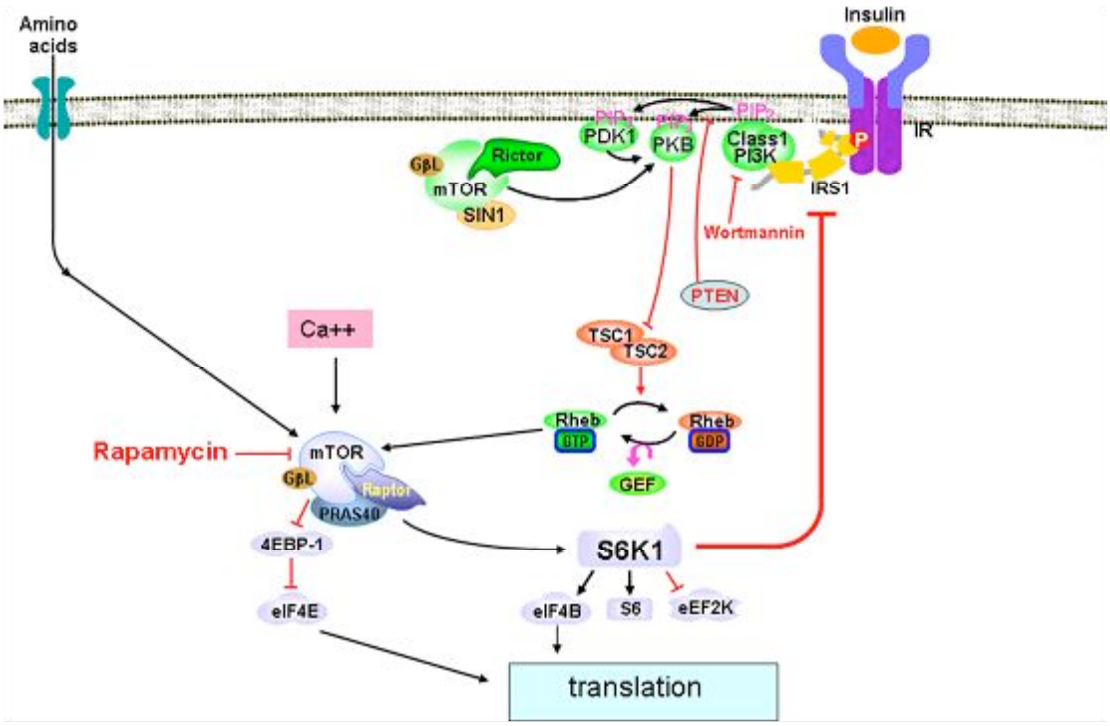

Figure 2: The TOR/S6K Signaling Pathway.

See text for explanation of pathway.

Adapted from "mTOR Complex1–S6K1 signaling: at the crossroads of obesity, diabetes and cancer" Dann,S. G. et al, Trends in Molecular Medicine,Volume 13, Issue 6, June 2007, Pages 252-259

Conservation of the pathway

Although there are differences between mammalian and invertebrate models of S6K/TOR signaling, the pathway is highly conserved in *Drosophila*, and several key regulators were discovered through this model organism; for example, Rheb and TSC1&2 [79-81]. The initial implication of insulin signaling in the control of cell size in *Drosophila* was provided by the laboratories of Sally Leevers and Ernst Hafen, who demonstrated that PI3K (p110) and IRS (*chico*) are required cell-autonomously to promote cell growth (see below) [82]. The *Drosophila* TOR protein (dTOR) is required for normal growth and proliferation during larval development, and for growth factor-induced *Drosophila* S6K (dS6K) phosphorylation [83-85]. *Drosophila* has a single receptor system for signaling though drosophila insulin-like-peptides (DILPs) and insulin-like growth factor (IGF), and upon binding of either ligand, the *Drosophila* Insulin Receptor (InR) tyrosine kinase becomes activated leading to the recruitment of Chico, the *Drosophila* homologue of IRS1-4 [86]. *Chico* mutants are less than half the size of wild-type flies, owing to fewer and smaller cells [87]. The viability of these flies, despite the *InR* null mutant being embryonic lethal, could be due to an extended C-terminal portion of the InR receptor that contains multiple PI3K sites that could contribute to low level insulin/IGF signaling levels [88]. As in mammals, PI3K plays a central role in the *Drosophila* insulin signaling pathway with the generation of second messenger PIP_3 from PIP_2 [89]. The monomer dp60 is the fly orthologue of the mammalian p85 regulatory subunit of PI3K, and upon over-expression, acts to inhibit PI3K in a dominant-negative manner [90, 91]. In

Drosophila, an orthologue of PTEN (dPTEN) antagonizes PI3K activity to suppress growth [92], along with Susi, a protein with a coiled-coiled domain that interacts with the dp60 regulatory subunit of dPI3K, regulating its activity [93]. Over-expression of Susi produced a reduction in organismal, tissue and cell-size, along with a reduction in rate of proliferation, and a starvation-resistance phenotype, while all heteroallelic combinations of *Susi* mutants resulted in adult flies with reduced viability and increased body size. Based on the relatively weak loss-of function phenotype observed, Wittwer *et al* postulated that Susi is involved in fine-tuning the cellular response to insulin [93].

Downstream of PI3K, studies in *Drosophila* revealed that the S6K1 and S6K2 orthologue, dS6K, is a negative effector of dPKB activation, which suggested for the first time that S6K regulates PKB phosphorylation, which was soon replicated in mammalian experiments [85, 94]. DPDK1 action downstream of insulin receptor signaling conforms to the mammalian pathway in terms of phosphorylation of both dS6K and dPKB [95]. In 2001, the importance of two additional suppressors of insulin-receptor mediated growth became apparent; tuberous sclerosis 1 and 2 (dTsc1&2) [96]. These two proteins are binding partners in the tumor suppressor complex TSC, which has a conserved function in regulation of Rheb and the TOR complex 1 [82], as outlined in the mammalian section above. In addition to the above, the *Drosophila* S6K/dTOR pathway is also negatively regulated in an oxygen-sensitive manner by Scylla and Charybdis. Simultaneous loss of *Scylla* and *Charybdis* under normoxic conditions resulted in a slight increase in growth, whereas their absence under reduced oxygen concentrations severely compromised larval development,

indicating that *Scylla* and *Charybdis* have a critical function for survival under hypoxic conditions [97]. Growth inhibition of these mutants was associated with a reduction in dS6K but not dPKB activity. Under conditions of low oxygen, the transcription factor HIF-1 is stabilized as a complex, and binds to short regulatory hypoxia response elements (HRE) in the genomic region of target genes [98]. Reiling *et al.* showed that Scylla is a proven target of the hypoxia-inducible factor-1 (HIF-1), like its mammalian counterpart, REDD1 [99], which has concurrently been proven to inhibit mTOR signaling through a TSC-dependent mechanism [100].

The dS6K null phenotype in Drosophila

Loss of *dS6K* in the adult fly (as described by Montagne *et al* [101]), generated by an imprecise excision of a P-element insertion in the 5' non-coding region resulted in partial lethality ($dS6K^{l\text{-}1}$). Homozygous escapers had a five-day developmental delay, and lived no longer than two weeks, with a reduction in body size. The P-element insertion prior to removal was female sterile, with a three-day delay, indicating that an intact *dS6K* gene is required for normal development. Analysis of cell number was carried out using the individual hairs on the adult wing as a guide to cell density. From this, it could be seen that cells were around 30% smaller in the $dS6K^{l\text{-}1}$ flies, while cell number over the entire wing remained the same. As discussed later, imaginal discs give rise to adult structures [102], and because of the mode of development the size of the adult wing is largely predetermined by the final size of the wing disc [103]. Therefore, fluorescence-activated cell-sorter (FACS) analysis of cells from imaginal discs was performed to understand if the cell size reduction was due to

proliferation at a smaller size, and if there was a particular phase of the cell-cycle involved. The results showed that while cell-cycle progression proceeded more slowly than in wild-type cells, no apparent cell-cycle phase was selectively affected. The growth defects observed were confirmed as cell-autonomous by clonal analysis. Genetically marked homozygous mutant cells were generated by the authors in a heterozygous mutant background by somatic recombination in first instar larvae to create a mosaic animals [104]. Direct comparison of neighboring heterozygous and homozygous *dS6K* mutant wing bristles and ommatidia showed a reduction in bristle and photoreceptor size of the mutant clones, indicating that the cell size defect observed in the mutant cells was not due to a humoral effect. Since removal of the *dS6K* gene caused a reduction in cell growth, the authors decided to test if compartment-specific expression of an extra copy of *dS6K* could also enhance growth. They found that by using the GAL4 transcription factor under control of the apterous promoter, over-expression of a UAS dS6K transgene positively effected growth in a cell-autonomous and compartment-dependent manner (see 'Screening for effectors of the pathway' below for details, and [105] for full explanation of UAS-GAL4 system in *Drosophila*).

S6K/TOR signaling and translation

Following transcription, processing and nucleo-cytoplasmic export, eukaryotic mRNAs are competent for translation. Translation initiation is the first step in this process, and requires several polypeptide initiation factors which serve to direct the

sequential assembly and positioning of the ribosome at the AUG initiation codon on the mRNA [106]. The major downstream targets of the S6K/TOR signaling pathway are involved in translation, especially the translational machinery required for ribosomal recruitment to the mRNA [107]. Although no single mechanism controls the translation of all mRNAs, emerging evidence indicates that the regulated binding of translation initiation factors (eIFs) to the 7-methyl guanosine residue that caps the 5' ends of all nuclear-encoded eukaryotic mRNAs is critical [108]. In particular, the interaction of the ribosomal-subunit-associated eIF4G with the cap-bound eIF4E is necessary for cap-dependent translation, and this association can be prevented by eIF4E binding proteins (4E-BPs) [109, 110]. TOR has been shown to phosphorylate and inhibit the 4E-BPs, which promotes their dissociation from eIF4E bound to the mRNA 7- methyl guanosine cap-structure, thereby allowing for eIF4G association with eIF4A recruitment [111, 112]. In addition, eIF4G binds eukaryotic initiation factor 3 (eIF3), which, in turn, recruits the 40S ribosomal subunit along with its associated ternary complex (eIF2/Met-tRNA/GTP) to form the pre-initiation complex [33]. eIF3 is a complex of at least 12 subunits [113], which interacts with mTOR and S6K1 intermittently in a growth-factor and rapamycin-sensitive manner [114]. mTOR associates with the complex upon mitogen or hormone stimulation, whilst S6K1 dissociates, promoting the phosphorylation and activation of S6K1 in a coordinated fashion. Phosphorylation of T389 was found to be critical for this process, and once released, S6K1 would become fully activated before phosphorylating downstream targets ribosomal protein S6, and eIF4B [114, 115]. The consequences of S6 phosphorylation on translation are still unclear [116], while upon phosphorylation,

eIF4B is recruited to the pre-initiation complex in concert with eIF4A [114], a co-recruitment that potentially increases the RNA-helicase activity of eIF4A [117, 118]. The RNA helicase activity of eIF4A would allow unwinding of secondary structures in the 5′UTR of mRNAs and thereby facilitate binding and scanning of the 40S ribosomal subunit [119].

Along with its role in translation initiation, mTOR has downstream targets involved in translation elongation. The elongation phase of mRNA translation is the stage at which the polypeptide is assembled, and requires a set of non-ribosomal proteins; eukaryotic elongation factors (eEFs) [120]. In addition to the S6Ks and 4E-BP1, both of which modulate translation initiation, mTOR signaling regulates the translation elongation process via the phosphorylation of eukaryotic elongation factor 2 (eEF2) [121]. eEF2 is a GTP-binding protein that mediates the translocation step of elongation, and when phosphorylated at Thr56, it loses its ability to bind to ribosomes and is thus inactivated [122]. Insulin and other stimuli induce the dephosphorylation of eEF2, an effect which is is blocked by rapamycin [123]. E2F kinase is a substrate of S6K1, as well as several other kinases [124, 125].

1.4 Screening for effectors of the pathway

Use of Drosophila

The TOR/S6K signaling pathway is well studied in a broad range of organisms, from yeast through to human [126]. Having covered various aspects of growth, cell cycle control, and signaling pathways in earlier paragraphs, it is clear that many

important discoveries were made in model organisms. *Drosophila melanogaster* is an extremely useful tool for investigating the genetic basis of signaling pathways, due to its strong conservation of fundamental signaling pathways, such as the insulin-signaling pathway and the many powerful genetic techniques that have been developed over the last 100 years since *Drosophila* has been used for genetic investigation [127]. The extent to which genetic methods and tools have been developed for this organism far exceeds that for any other complex multi-cellular organism [128].

In addition, *Drosophila* particularly lends itself to experimental investigation, as it is an organism with a short life cycle, and is easy to maintain. Finally, pathways are often simplified in the fly, allowing easier manipulation, for example, whereas two S6 Kinases exist in mammals, in Drosophila, there is one [30, 101].

dS6K modifier screen

The 'UAS GAL-4' system uses the yeast GAL-4 transcription factor to induce expression of a target gene via an upstream activating sequence (UAS) [105]. Gal-4 expression can be driven specifically, both temporally and spatially, depending upon the specific promoter used to bring about its expression. In this screen described in Appendix I, apterous-GAL4 (*ap*-GAL4) was used to over-express random genes in the dorsal wing compartment using an UAS EP (Enhancer-Promoter) construct.

Imaginal discs give rise to adult structures [102]. A *Drosophila* adult wing develops from a wing imaginal disc, which originates as a group of approximately 30 cells attached to the inside of the larval epidermis, corresponding to two adjacent

clonal compartments (anterior and posterior) and after a quiescent period of just over a day, starts dividing [129]. This proliferation continues up to 24 hours after pupariation, and during this period the disc becomes subdivided once more into dorsal and ventral compartments. By the time the animal reaches metamorphosis, the disk has grown in cell number 1000-fold, with only two net cell-size reduction divisions to follow in the early pupal stage [130]. Due to the mode of development, the size of the adult wing is largely predetermined by the final size of the wing disc [103].

If the growth or proliferation process is disrupted in the dorsal or ventral compartment compared to the other, the resulting difference in size will cause a discrepancy leading to a phenotypic change (Fig. 3), This is because the dorsal and ventral components of the wing fold in an apposed manner to generate the flattened wing [131, 132]. For example, in the case of *ap*-GAL4-induced UAS dS6K over-expression, which drives expression of an extra copy of dS6K in the dorsal compartment of the wing blade, a bent-down wing phenotype is observed [85, 101]. Using this sensitive phenotype, differences between cell number and cell size are not readily identifiable. However, the ability of random genes to alter this *ap*-GAL4, UAS dS6K bent-down wing phenotype upon co-over-expression with dS6K was used as a readout to identify possible upstream and downstream effectors of dS6K function (see Appendix I).

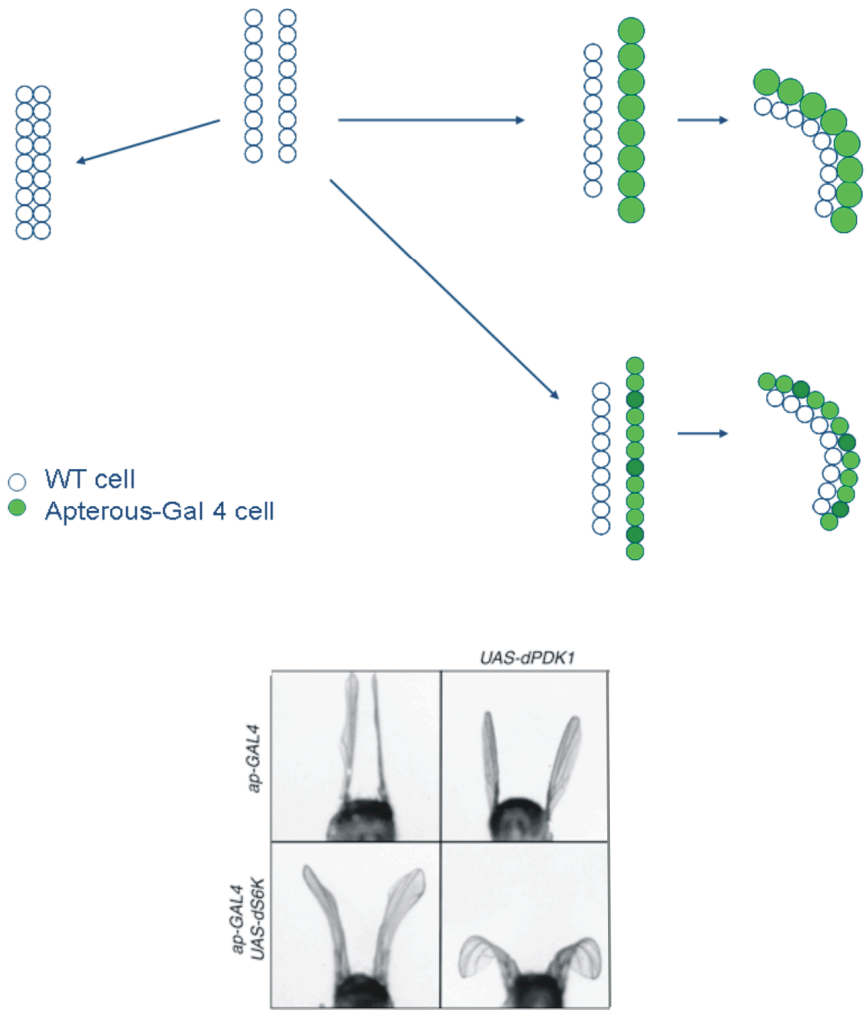

Figure 3: Apterous-GAL4 induced growth causes bending-down of the wing.

Top panel: Apterous-GAL4 is used to drive a growth-promoting agent in the dorsal wing compartment, resulting in a bending down of the wing due to either an increase in cell number, or cell size. Lower panel: an example of *ap*-GAL4; UAS dS6K induced growth, with enhancement by co-over-expression with UAS DPDK1.

Lower panel adapted from; dS6K-regulated cell growth is dPKB/dPI(3)K-independent, but requires dPDK1, Thomas Radimerski et. al. Nature Cell Biology 4, 251 - 255 (2002)

Candidates

Based on their phenotypic enhancement, we decided to look closely at two candidate enhancers of *ap*-GAL4, UAS dS6K mediated growth, and their potential for modification of the pathway. This thesis addresses two candidates, which will be discussed in separate chapters. The first is the *Drosophila* oo18 RNA-binding protein 2 (orb2), which is a homologue of the mammalian CPEB family of proteins, which are known for their role in translation. The second is Inositol Phosphate-3 Kinase 1 (IP3K1), which is an inositide kinase with relevance to second messenger generation in calcium signaling.

1.5 Aims of the Project

As the nature of interactions between known components of the TOR/S6K pathway is becoming more understood, it is clear that a number of elements are lacking. Our aim was to use the model organism *Drosophila melanogaster* as a tool to elucidate novel components of the pathway, and to find a link with their mammalian counterparts in hopes of better understanding a pathway with implications for human disease.

CHAPTER 2: MATERIALS AND METHODS

2.1 Supplies

Ampicillin, *Sigma* Antimycotic: *GIBCO*, Benzamadine hydrochloride: *Sigma*, Bis- acrylamide: *Biorad*, Bradford Reagent: *Biorad*, *Drosophila* Schneider's media: *Gibco,* Foetal bovine serum, *Hyclone*, Insulin: *sigma*, Pepstatin; *sigma*, Phenylmethylsulfonylflouride: *sigma*, Nitrocellulose membranes: *Whatman*, Restriction enzymes; *New England Biolabs*, RNAsin: *Promega*, Taq Polymerase and Buffer A; *Fisher*. IP3 Kinase inhibitor; *Calbiochem.*

2.2 *Drosophila* Experiments

Fly stocks

Fly stocks were maintained in a specially conditioned room at 18°C and 62% relative humidity. Stocks were transferred to fresh fly food (yeast supplemented cornmeal and molasses) every four weeks. Small scale experimental crosses were set with approximately 10 virgin females and 5 males in 30ml plastic vials, and generally kept at 25°C and 70% relative humidity, unless otherwise stated. Experimental crosses were transferred daily into fresh tubes. Fly stocks used are previously published as indicated, except; EP IP3R: Bloomington; $y^{1}w^{67c23}$;P{EPgy2}Itp-r83A^{EY02522}/TM3, $Sb^{1}Ser^{1}$, and EP dFMR1: Bloomington; w^{1118}; P{EP}Fmr1^{EP3517}.

Screening for dS6K modulators

Over-expression of an extra copy of dS6K within the developing dorsal wing compartment using the *ap*-GAL4 driver induced a bent-down wing in the adult (Appendix I, Fig. 1A,B) due to a moderate overgrowth of the dorsal wing blade [101]. Consistent with PDK1 being the S6K T-Loop kinase [133, 134], it was further observed that this bending-down was enhanced by co-expression of the drosophila PDK1 (DPDK1). Over-expression of DPDK1 alone, however, was without noticeable consequence [85]. This epistatic interaction demonstrated that the intensity of the bent-down wing phenotype was modified with respect to the dS6K activation status. Therefore, this sensitized phenotype was used in a gain-of-function genetic strategy to identify new components with the potential ability to regulate dS6K activity Interestingly, over-expression of an active form of the mammalian S6K ($S6K1^{dE/D3E}$) [135] induced a bent-down wing phenotype identical to that induced by dS6K (Appendix I, Fig. 1 B,C). Like dS6K, co-expression of $S6K1^{dE/D3E}$ and DPDK1 led to an enhancement of the bent-down wing, but not to the extent observed with dS6K (data not shown). This differential phenotypic interaction may be a hallmark of specificity, as dS6K, unlike $S6K1^{dE/D3E}$, is the genuine target for DPDK1 [85, 95]. These differential effects were utilized to improve the selectivity of the gain-of-function screen (see below).

Over-expression of About 5000 Enhancer-Promoter (EP) bi-directional insertions [97] were induced in combination with dS6K in the developing dorsal wing compartment, and the dS6K-dependent bent-down wing phenotype was monitored for

enhancement or suppression. To further restrict their specificity, about 1000 EP lines that displayed striking phenotypes possibly unrelated to dS6K function were then retained and analyzed for their effects when induced alone with the *ap*-Gal4 driver. Nonspecific modulators that, alone, induced a phenotype identical to that obtained in concert with dS6K were eliminated. Eventually, 220 lines were tested again to precisely compare their effects when induced alone, in combination with dS6K or with the active $S6K1^{dE/D3E}$. The enhancers identified in the screen could be separated into two subsets with respect to their interaction with dS6K and $S6K1^{dE/D3E}$. One subset enhanced the bent-down wing phenotype to the same extent in combination with either kinase, while the other subset displayed a differential effect that was stronger in combination with dS6K than with $S6K1^{dE/D3E}$ (data not shown). As DPDK1 belongs to the latter group, we reasoned that candidate enhancers that interacted differentially with dS6K and $S6K1^{dE/D3E}$ were more likely to affect dS6K signaling, whereas those with no differential response might be involved in the general process of wing formation.

Plasmid rescue was performed using genomic DNA isolated as described (see below). 1μg DNA was digested with EcoRI using standard restriction digest procedure. Using EcoRI digestion should yield a fragment of DNA containing the antibiotic resistance gene along with immediately flanking genomic *Drosophila* DNA. Digests were purified using a DNA clean-up kit (Qiagen) before ligation of the resultant DNA with standard procedures. Following transformation of the ligation into E. coli, colonies were selected and sequenced using a plasmid directed primers: Internal 3' EP forward primer; ACTATTCCTTTCACTCGCAC, and External 3' EP forward primer ATATCGCTGTCTCACTCAGA.

Removal of one of the two UAS elements present in the EP construct was performed using cre-recombinase [136] expressing line to Cre/loxP site-specific recombination to yield a single-headed EP element .

Generation of transgenic flies

The *Drosophila melanogaster* orb2 short and long isoform cDNAs were obtained from the DGRC (AT03031 and LP05645) in the pOT2 and the pOT7a vectors respectively. The cDNAs were cloned into the pUAST (pCaSpeR3, contains hsp70 TATA box) vector (Brand and Perrimon 1993) and correct orientation of the inserts was checked by restriction digest and sequencing. A single colony of bacteria containing each cDNA was grown overnight in 200ml LB-Ampicillin medium at 37°C in a shaking incubator. Plasmid DNA was extracted with the Qiagen plasmid maxi-kit according to the manufacturer's protocol. The prepared construct was sent to Duke University (see results section) for injection. Animals produced from the injection procedure were received and separated out at the pharate stage to maximize virginity. Upon emergence, these flies of the G0 generation were crossed individually to 3 *yw* flies of the opposite sex, and the resulting F1 generation was screened for transformants. Transformers were crossed to balancer stocks and standard mating schemes were used to genetically map the affected chromosome. Transgenes on the X-chromosome were balanced over the *FM7* chromosome (*In(1)FM7, y^{31d} $sc^{8}w^{a}B$*), second chromosome insertions were balanced over the *CyO* chromosome (*In(2LR)O, Cy dp^{lvI} pr cn^{2}*), and transgenes on the third chromosome were kept over either the

TM3sb (*In(3LR)TM3, ri p_p sep l(3)89 Aa bx^{34e} e sb*), or the TM6B (*In(3LR)TM6B, Hu e Tb*) balancer (Lindsley and Zimm 1992).

Extraction from Drosophila tissues

Heads and bodies, separated: 20-30 flies per genotype were isolated, and placed into an eppendorf tube with a needle puncture in the lid, and flash frozen in liquid nitrogen. After removal from the nitrogen, the tube was rapidly and aggressively shaken for five seconds. The contents were emptied into glass dish chilled over ice, where the still- frozen heads and bodies were separated from other tissues using a fine point paintbrush, and placed into a new eppendorf tube, and replaced in the liquid nitrogen. Samples were processed for protein extraction immediately after removal from liquid nitrogen, by maceration with an eppendorf-sized pestle in 60-80µl of extraction buffer (as above), and followed by centrifugation at 14000XG for 20 minutes at 4°C. Supernatants were transferred to a fresh eppendorf tube and flash frozen in liquid nitrogen before storage at -80°C.

Larvae/pupae/total adult: staged larvae/pupae/total adult were extracted in 10 animals/100µl buffer by maceration with an eppendorf-sized pestle extraction buffer (as above), followed by centrifugation at 14000XG for 20 minutes at 4°C. Supernatants were transferred to a fresh eppendorf tube and flash frozen in liquid nitrogen

Extraction of Genomic DNA from flies

For each genotype, 50 anesthetized flies were transferred to a 1.5ml eppendorf tube on ice and 250µl DNA isolation buffer (100mM Tris-HCl pH9.0, 100mM EDTA, 1% SDS) was added. Flies were crushed in the buffer using a plastic eppendorf pestle. One fiftieth volume of Proteinase K (10mg/ml) was added to the tube, and the extract was incubated for 30 minutes at 65°C. An equal volume of ice-cold K-acetate (3M K-acetate, 8.7% glacial acetic acid (v/v) was added and the mix incubated on ice for 30 minutes. Cell debris and precipitated proteins were removed by two subsequent centrifugation steps at 12000g for 5 minutes at 4°C. The supernatant was transferred to a new tube and one half volume isopropyl alcohol was added, and kept at room temperature for 20 minutes. DNA was pelleted by centrifugation at 12000g for 5 minutes, and the pellet was washed with 1ml 70% ethanol. The pellet was dried on the bench top, and resuspended in 25µl TE (Tris- EDTA buffer; 10mM Tris, 1mM EDTA, pH8) at room temperature for three hours. DNA was extracted with half volume of phenol, and half volume of chloroform/isoamylalcohol, and finally with chloroform/isoamylalcohol. DNA was precipitated with 2.5 volumes of 95% ethanol, and incubated overnight at -20°C. The DNA was pelleted by centrifugation at 12000g for 5 minutes, washed with 1ml iced 70% ethanol, and dried on the bench. DNA was resuspended in 50µ TE at room temperature overnight, and then stored at -20°C.

Orb2 sequencing primers;

CPEBLseq1: AGAAAGTGTTGGTGCGGGCG

CPEBLseq2: AGTGGCGGCGGAGATGCCTC

CPEBsseq1: GATTGTGAGTGTCCGTAAAA

CPEBLseqU1: CCCACAGCTATCTATGGCAA

CPEBLseqU2: CTTAAATGCCCTCAAAATGG

CPEBLseqU3: ACCATGTCACTGGACCCACG

CPEBLseqU4: GACGCGAATATCACAAGCCG

These primers were used for sequencing the exact EP insertion, and for checking the pUAST constructs of orb2-PA and orb2-PB.

Photography and Figure preparation

Material used for preparation of flies; a Nikon Coolpix 995 digital camera mounted to a Leica Binocular microscope. Flies were either killed using ether, or temporarily anesthetized for photography. Scanning electron microscopy preparation: Flies were chilled at -20°C for 30 minutes until <5% viable. Flies were then mounted onto a metal pedestal with a sticky carbon paper, and underwent a timed sputter of gold particles (65 seconds,40% setpoint) at a mTORR of 55-60.

2.3 Mammalian Experiments

HeLa cells, derived from an adenocarcinoma [137], were cultured in high glucose DMEM supplemented with 10% heat inactivated foetal bovine serum (65°C, 30 minutes), and penicillin plus streptomycin (Fisher), in a standard mammalian cell

culture incubator. siRNA was transfected into 6-well dishes at 300,000 cells per well, using oligofectamine (Invitrogen) as per the manufacturer's protocol, using 1.5µl of 20µM solution per well.

Qiagen siRNAs;

Hs_ITPKA_6_HP Validated siRNA (S100605507)

Hs_ITPKA_5_HP Validated siRNA (S100605500)

Hs_ITPKB_6_HP Validated siRNA (S102621927)

Hs_ITPKB_5_HP Validated siRNA (S102621934)

Extraction from mammalian cells

Experiments were performed in 6 well dishes. Following treatments, the dishes were immediately transferred to a metal plate on ice. The experimental media was removed and cells washed twice with 1ml/ well iced PBS. Extraction buffer (120mM NaCl, 50mM Tris-HCl, pH7.0, 20mM NaF, 1mM benzamidine, 1mM EDTA, 6mM EGTA, 15mM $Na_4P_2O_7 \cdot 10H_2O$, 1% Ipegal, 30mM paranitrophenylphosphate, 30mM β-glycerolphosphate, 4µM pepstatin A, 100µM PMSF, 2X promega complete inhibitor, 1x phosphatase inhibitors.) was added on top of cells (75µl/well for HeLa and T98G, 100µl/well for HEK293 and N41), and cells were scraped down with a rubber policeman. Extractions for western blot were briefly vortexed, frozen and stored at -80°C, and centrifuged at 14000XG for 20 minutes to remove cell debris prior to protein determination and use. Extracts for immunopreciptation were lysed by pipetting up and down, and cell debris was removed by centrifugation at 14000XG for 15 minutes

at 4°C. Supernatants were transferred to a fresh eppendorf tube and flash frozen in liquid nitrogen before storage at -80°C.

2.4 General methods

Western blotting

Gels were soaked briefly in transfer buffer (0.01M CAPS PH11, 15% Methanol) following SDS-PAGE. The gel was then arranged on top of a nitrocellulose membrane on top of two sheets of whatman filter paper equilibrated in transfer buffer, set on top of a semi-dry transfer apparatus. Two sheets of equilibrated whatman paper were placed on top, and the lid replaced. Proteins were transferred to the nitrocellulose paper by passing a constant current of 70mA for two hours. Membranes were removed from the apparatus, rinsed with water, and checked for successful transfer and loading by ponceau S stain. Membranes were then blocked in PBS with 3% BSA and 0.1% tween 20 for 30 minutes at room temperature with agitation. Primary antibodies were incubated in the blocking buffer at 4ºC overnight. (antibodies). Following this incubation, the membranes were washed in PBS with 0.1% tween-20 for thirty minutes, with a buffer change every ten minutes. The secondary antibody (HRP conjugated) was then applied in a 1% non-fat milk PBS with 0.1% tween-20, and incubated at room temperature with agitation for two hours. Membranes were then washed in PBS-Tween-20 0.1% for 15 minutes with a buffer change every five minutes. A final wash was performed in PBS alone, before allowing membranes to completely dry on a kimwipe. Once dry, membranes were incubated with ECL

solution for one minute, placed inside a purpose-cut sheet protector, and exposed to photographic film (Kodak BioMax).

Antibodies and concentrations

Anti-IP3KA; *Santa Cruz*, 1:1000 Anti-IP3KB; *Santa Cruz*, 1:1000 Anti-S6k T389; *Cell Signaling Rabbit Monoclonal*, 1:2000, Anti-dS6K 398; *Cell Signaling*, 1:1000 Anti-dPKB505; *Cell Signaling*, 1:1000, Anti-tubulin; *University of Iowa Hybridoma Bank*, 1:5000, Anti-orb2; *gift from E. Kandel*, 1:2000, Anti-Phospho dS6; *Phosphosolutions,* 1:2000, Anti S6; *Cell Signaling*, 1:1000, Anti total S6K; [101].

CHAPTER 3: OO18 RNA-BINDING PROTEIN 2

3.1 Introduction

Conservation and structure

The *Drosophila melanogaster oo18 RNA-binding protein* (*orb*) protein was first described in 1992 as an RNA-binding protein [138] with potential for regulation of mRNA localization. Since then, a number of studies in a wide range of organisms has found a conserved family of similar proteins with a role in regulation of mRNA translation by direct binding to consensus sequences in the 3' UTR of mRNAs [139-142]. This family of genes is referred to as the Cytoplasmic Poly-adenylation Element Binding (CPEB) family of proteins, after the *Xenopus* and mammalian genes. There are often two CPEB genes in invertebrates, whereas there are four present in mammals, and evidence suggests that in both cases there are at least two subfamilies of proteins, the CPEB1 group and the CPEB2-4 group [143]. CPEB family members are identified on the basis of two RNA-recognition motifs (RRMs) and a zinc-finger motif, which CPEB1 requires for the binding of a specific cis-acting element in the 3'UTR known as the Cytoplasmic Poly-adenylation Element (CPE) [144] (Fig. 4). The CPE element has a consensus sequence of UUUUUAU, although several minor variations of this sequence exists [143]. Polyadenylation of dormant mRNAs, for example, *Xenopus* cyclin B1, is controlled by this cis-acting CPE together with the hexanucleotide AAUAAA, through associations with CPEB and the cleavage and polyadenylation

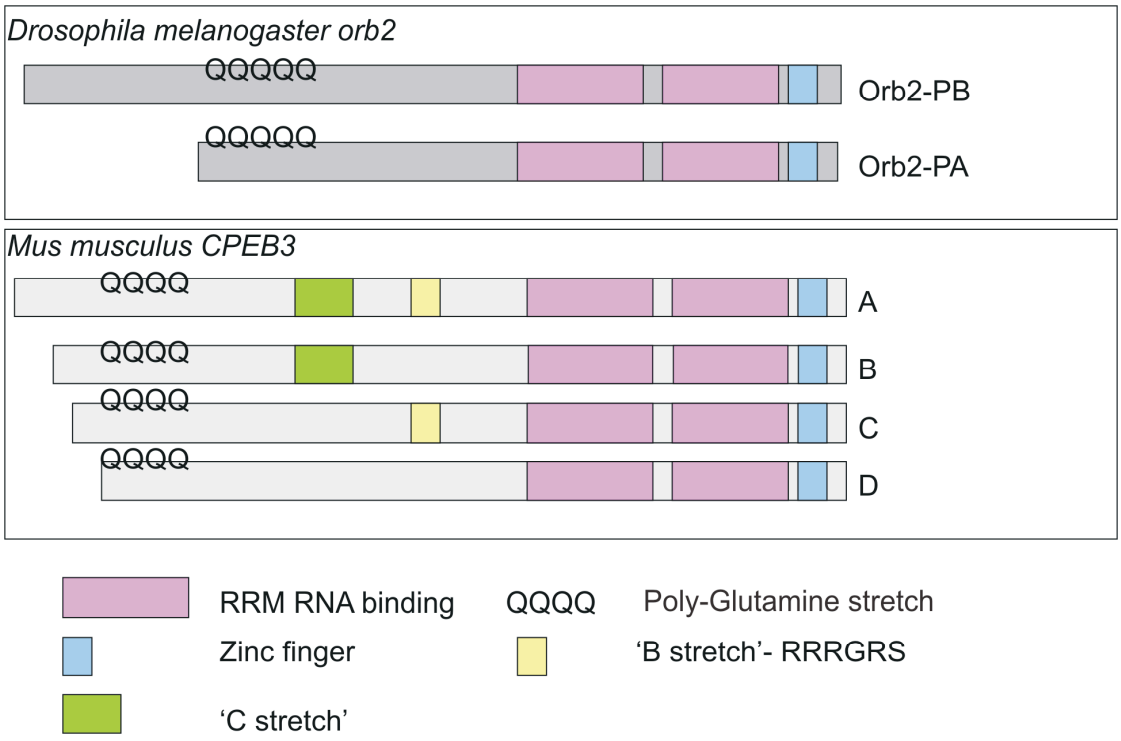

Figure 4: Comparison of orb2 and mCPEB3

Conserved domains are indicated

.Adapted from "Two previously undescribed members of the mouse CPEB family of genes and their inducible expression in the principal cell layers of the hippocampus" Theis M, Si K, Kandel ER. Proc Natl Acad Sci U S A. 2003 Aug 5;100(16):9602-7

specificity factor (CPSF) respectively [145]. The translational activation or repression of eukaryotic mRNAs has been observed as regulated by dynamic changes in the length of their poly(A) tails. This phenomenon, which is widespread among metazoans, is particularly characteristic of early development where poly(A) addition is associated with translational stimulation and poly(A) removal is correlated with translational repression [146, 147]. The interactions involved between CPEB and other proteins know to be involved in this process are discussed below.

CPEB proteins have been implicated in the control of localization and translation of select messenger RNAs such as alpha-CaMKII [148], cyclin B1 [149, 150], and AMPA receptor GluR2 [151]. The *D. melanogaster* CPEB *orb1* is widely studied for its role in oogenesis, and was discovered as a protein with importance in formation of the egg chamber and establishment of polarity by anteroposterior and dorsoventral patterning [152, 153], whereas *orb2* has a newly identified role in long-term courtship memory [154].

CPEB interactors and regulation

Although CPEB is a proven translational regulator in several species, it interacts with other proteins forming complexes which appear to vary depending on the species. The most well characterized interactor, *Xenopus* Maskin, has been shown to be an eIF4E-interacting protein, whose binding prevents recruitment of the translational machinery to bound mRNA (Fig. 5) [155]. The site on eIF4E that interacts with Maskin is the same as that which is normally occupied by ribosomal-

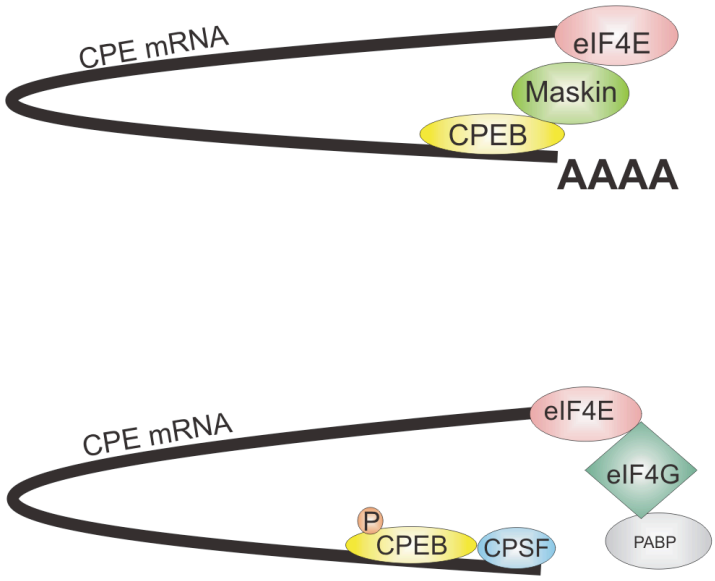

Figure 5: Translational control by Maskin and CPEB.

CPEB associates with both CPE-containing mRNAs and Maskin. Maskin, in turn, interacts with the cap (7mG)-binding factor eIF4E. In this configuration, Maskin binding to eIF4E precludes eIF4G from binding eIF4E, thus, inhibiting translation. Following CPEB phosphorylation and polyadenylation, PABP binds the newly elongated poly(A) tail; PABP also binds eIF4G and helps it displace Maskin from eIF4E. Because eIF4G is indirectly associated with the 40S ribosomal subunit (not shown), translation initiation proceeds. For clarity, other polyadenylation and translation factors are omitted.

Adapted from "CPEB: a life in translation" Joel D. Richter, Trends in Biochemical Sciences, Volume 32, Issue 6, June 2007, Pages 279-285

subunit-associated eIF4G, whose interaction with the cap-bound eIF4E is necessary for cap-dependent translation (see general introduction) [110]. Thus, Maskin is functionally similar to the 4E-BPs, as an inhibitor of translation, which precludes the interaction of eIF4G and eIF4E. Another *Xenopus* 4E-binding-protein, neuroguidin, has also been identified as an interactor of CPEB, demonstrating the ability of CPEB to interact with multiple inhibitory 4E-binding proteins [156]. Although an association with CPEB has not been demonstrated, human orthologues of Maskin exist, termed the Transforming Acidic Coiled-Coil (TACC) family of proteins, are suspected to play a role in oncogenesis [157]. The *Drosophila* homologue D-TACC is important for proper dynamics of spindle pole microtubules during cell [158] via TACC-family interaction with the Aurora-A kinase, an interaction which is highly conserved [159].

During *Xenopus* oocyte maturation, progesterone induces the inactivation of glycogen synthase kinase 3, which in turn is necessary for the activation of Aurora A kinase, a serine/threonine kinase which phosphorylates CPEB at serine 174 [160]. This event enhances the interaction between CPEB and CPSF, which is thought to aid cleavage and polyadenylation specificity factor stably associate with a second important *cis* element, AAUAAA [156]. The scaffold protein Symplekin contacts CPEB and CPSF, and aids them in interacting with Gld2, a poly(A) polymerase. This event is required for CPEB-mediated polyadenylation [161]. More recently, additional interactors have been identified that add further complexity to the mechanism of translational control by CPEBs. It was previously thought that negative regulation of translation by CPEB was due to a repression of polyA tail elongation. More recently, however, the poly(A)ribonuclease (PARN) has been discovered as important factor in

the de-adenylation of mRNAs bound to CPEB. Both PARN and Gld2 are present in complex with CPEB, and because PARN is the more active of the two, the poly(A) tail is short. When oocytes mature, CPEB phosphorylation causes PARN to be expelled from the ribonucleoprotein complex, allowing Gld2 to elongate poly(A) by default [145].

The role of CPEB in the Brain

Chemical synapses are specialized junctions through which the cells of the nervous system signal to one another and to non-neuronal cells via neurotransmitters. These synapses allow the neurons of the central nervous system to form interconnected neural circuits, and provide the means through which the nervous system connects to and controls the other systems of the body. Synapses are asymmetric both in structure and in operation. Presynaptic neurons secrete neurotransmitter, which binds to receptors facing into the synapse on the postsynaptic cell [162].

'Neuronal plasticity' is the term used to describe the specialized structural and physiological events orchestrated by the nervous system to mediate the adaptive response of the organism to environmental changes or changes in the organism itself. Part of this plasticity is localized to synapses, and is dependent on the capability of synapses to modify their function, to be replaced, and to increase or decrease in number when required. Neuronal plasticity is maximal during development and is expressed after maturity in response to external or internal perturbations, such as changes in hormonal levels, environmental modification, and injury [163]. The giant sea slug,

Aplysia californica is used extensively for research into synaptic plasticity, as it is an organism with a simple nervous system, consisting of just a few thousand large, easily-identified neurons which are straightforwardly visualized in vivo, and is capable of a variety of learning tasks. It has been shown that growth factors, such as transforming growth factor β (TGF- β) can enhance synaptic communication which is associated with plasticity [164]. While short-term changes in synaptic efficacy probably involve only posttranslational modifications, long-term changes require protein synthesis. For example, the production of new proteins is necessary for the acquisition of long-term memory, and for the long-lasting phases of hippocampal long-term potentiation (L-LTP) in mammals, and long-term facilitation (LTF) in *Aplysia*. During LTP the strength of synapses between neurons in the central nervous system is potentiated for prolonged periods following brief but intense synaptic activation, and there is correlative evidence that the mechanisms behind LTP are the same as those responsible for learning and memory. In Aplysia and some other invertebrate synapses, this is referred to as LTF. The converse mechanism to LTP is long term depression (LTD). Once synapses are potentiated, the level of synaptic efficacy can be reversed by prolonged low-frequency stimulation in a process known as depotentiation [165]. Both LTP and LTD are implicated in learning and memory processes [166].

The CPEB family has been identified from *Aplysia* nervous tissue [167], and its role in the selective translation of mRNAs during L-LTP and LTF has since been investigated by several laboratories [167-169]. Work by Kandel's group has demonstrated that a neuron-specific isoform of apCPEB (similar to the CPEB 2-4 family) regulates LTF-associated growth [169]. It appears that apCPEB is required not

for the initiation, but for the stable maintenance of LTF. Interestingly, the TOR signaling pathway has been implicated in translation-dependent L-LTP in the rat hippocampus, where several components of the pathway (mTOR, eukaryotic initiation factor-4E-binding proteins 1 and 2, and eukaryotic initiation factor-4E) can be detected [170, 171]. Kandel's laboratory showed that the CPEB-dependent translation they witnessed during LTF was rapamycin sensitive [169]. They also discovered that apCPEB, has prion-like properties, meaning it has the unusual capacity to fold into two functionally distinct conformations, one of which is self-perpetuating. The cellular prion protein PrPc is a glycosylphosphatidylinositol-anchored cell-surface protein whose biological function is unclear, but some studies indicate that it may have a role in signal transduction [172]. When yeast prion proteins switch state, they produce heritable phenotypes. A high glutamine content and predicted conformational flexibility are observed in N-terminal portion of the neuron-specific apCPEB, which is most similar in structure to orb2 in *Drosophila*. Kandel and his colleagues suggest that conversion of CPEB to a prion-like state in stimulated synapses helps to maintain long-term synaptic changes associated with memory storage [173].

Recently, the role of Orb2 in long-term behavioral memory has been investigated by Keleman *et al.* using a male-courtship behavioral readout [154]. Courtship conditioning is thought to be a form of associative learning, involving male response to female pheromones [174, 175]. While the CPEB1 knockout mice have defects in memory extinction, they have no obvious phenotype associated with long-term memory [176]. Orb2, on the other hand, is required for long-term behavioral memory, and is dispensable for learning and short-term memory [154]. Keleman *et al*

suggest that Orb2 is a regulator of component of the “synaptic tag”, an event postulated to mark specific synapses for the protein synthesis-dependent changes underlying long-term behavioral memory.

3.2 Results: o*rb2*

The Drosophila orb2 gene effects S6K signaling.

The *Drosophila* S6 kinase gene (dS6K) is a known regulator of organismal and cellular growth [101]. While the small size of tissues and cells lacking dS6K can be scored for, over-expression of the gene using the UAS GAL4 system *in vivo* has a much more subtle effect on growth. Apterous-GAL4 can drive expression of UAS target genes specifically in the dorsal wing compartment during development, and in the case of UAS dS6K over-expression, the resulting difference in size between dorsal and ventral compartments leads to a bent-down wing phenotype (see "Introduction"). This phenotype has been used as a readout for enhancers of dS6K mediated growth [85].

From an Enhancer-Promoter (EP) screen outlined in Montagne et al (Appendix I), 57 lines containing randomly inserted 'double-headed' EP elements giving a clear enhancement of the dS6K induced bent-down wing phenotype were retained, and 45 were molecularly mapped. Males from balanced EP lines were crossed at a constant temperature of 25 ºC with double-transgenic female flies containing enhancer-trapped apterous-GAL4 (*ap*-GAL4) on the second chromosome recombined with UAS dS6K, and screened for modification of the previously observed bent down wing phenotype. Because so many EP insertions affecting a modification of this phenotype were isolated, a secondary screen was performed to compare their effects with the active $S6K1^{dE/D3E}$ (Montagne *et al.*, Appendix I). The subset of lines that displayed a weaker

effect with S6K1$^{dE/D3E}$ than by combination with dS6K were considered more likely to be involved with dS6K signaling, as this was the effect observed with co-over-expression of DPDK1, a known effector of the pathway [95]. This differential phenotypic interaction may be a hallmark of specificity, as dS6K, unlike S6K1$^{dE/D3E}$, is the genuine target for DPDK1 (Montagne *et al.*, Appendix I).

One of these lines, EP24.061, was found to be a strong enhancer of dS6K mediated growth, and produced a mild effect on its own when driven with the *ap*-GAL4 driver (Fig. 6). The genomic region of insertion EP24.061 was discovered by plasmid rescue as described [97]. After identification of the insertion cytological region 66E5, this location was verified by sequencing of EP24.061 homozygous males. This insertion was found to be located in the second intron of the gene oo18 RNA-binding protein 2 (orb2). The genes in the immediate vicinity in either direction of the insertion included the Gram-negative bacteria binding protein 3 (GNBP3), and the mitochondrial ribosomal protein L12 (mRpL12). Because the mobilized EP element from the screen contained two independent UAS sequences for GAL4 to bind to in opposing directions, loxP sites were included to allow removal of one site, thus narrowing the search for the most likely gene involved . The UAS sites at the 5' end of EP24.061 were excised by crossing the line with a cre-recombinase-expressing lines. Cre-loxP-mediated recombination yielded flies containing a single-headed EP element, removing the UAS sequence in a position capable of driving genes GNBP3 and mRpL12. Upon removal of this UAS sequence, the *ap*-GAL4 phenotype persisted, indicating that this phenotype was caused by either an overexpressed gene product of

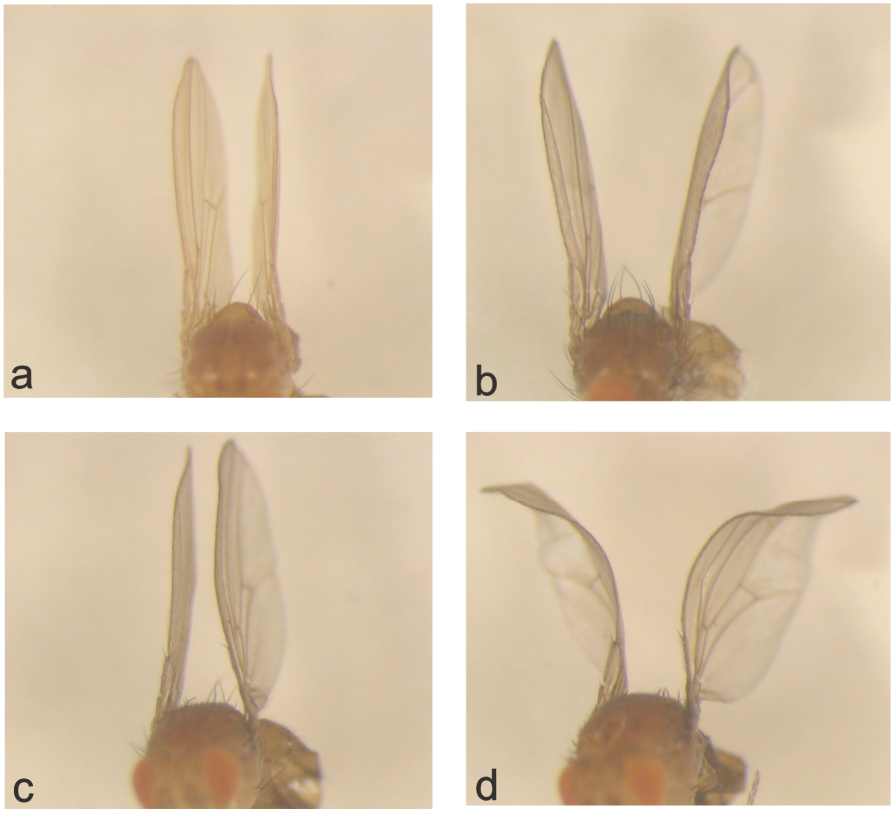

Figure 6: Modification of a dS6K induced growth phenotype by an EP insertion in the drosophila dorsal wing blade.

ap-GAL4 was combined the following genotypes; (a) yw (b) UAS dS6K (c) EP 24.061 (d) UAS dS6K; EP24.061, and reared at 25 ºC. The dS6K transgene resulted in a bent down wing phenotype, enhanced by the EP 24.061.

the orb2 gene, or a disruptive influence of the insertion on the orb2 gene or one of its close neighbors. To determine which gene was responsible, we made two pUAST [177] constructs bearing cDNA variants of orb2. The annotated gene from flybase [178] has 4 annotated transcripts (named -RA, -RB, -RC and -RD) and 4 corresponding polypeptides (-PA, -PB, -PC, -PD), three of which are identical. This essentially results in two differential forms of the protein; orb2-PA which has a polypeptide of 551 amino acids, and orb2-PB (and the identical -PC and -PD) with a polypeptide of 704 amino acids (Fig. 7). We obtained two clones from the Drosophila Genomics Resource Center (DGRC), the verified 'DGC gold clone' LP05645 (pOT2 vector) from the transcript orb2-RB, and AT03031(pOTB7 vector), which had a 5' sequencing data file showing full sequence homology with the 5' UTR from transcript orb2-RA. To determine if AT03031 was indeed a full-length clone of orb2-RA, we performed a PCR using primers corresponding to 5' and 3' of the expected coding sequence of the orb2-PA reading frame. After obtaining a product of the expected size, the amplicon was sequenced and found to indeed contain the coding sequence for orb2-PA. Both clones were digested out of their original vectors and directionally cloned into the pUAST vector using XhoI and EcoRI, present only in the multiple cloning site of the plasmids. The resulting constructs, pUAST orb2-PA and pUAST orb2-PB were purified and injected into *Drosophila melanogaster w1118* embryos by Model System Genomics at Duke University [179]. Post-injection larvae were received from Model System Genomics and separated, survivors backcrossed with a yw- line, and their progeny screened for transformants. Eight and eleven independent lines were obtained for UAS orb2-PA and UAS orb2-PB respectively, and these were balanced into stable lines.

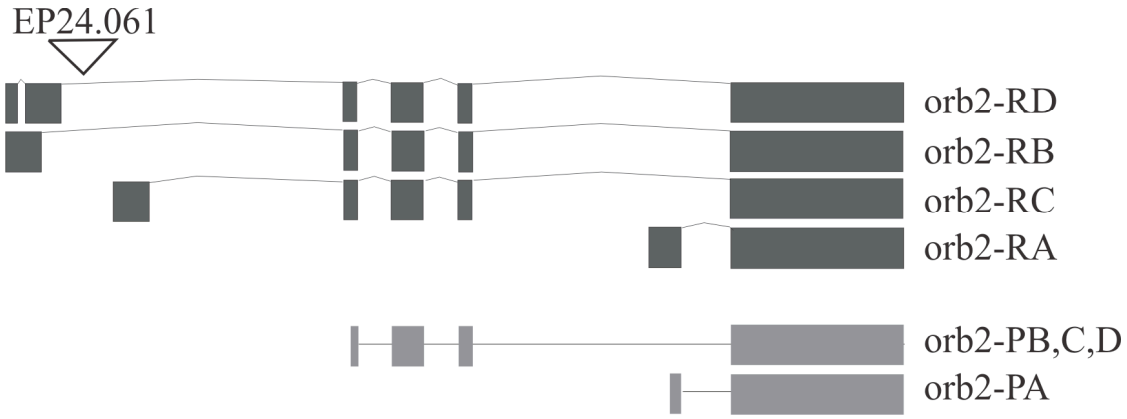

Figure 7: orb2 transcripts and proteins, and position of EP24.061 insertion.

Four mRNAs exist encoding two proteins, designated by Flybase as follows; RA, RB, RC, RD. RB, RC and RD encode a 704 amino acid protein (from here referred to as orb2-PB) and RA encodes a truncated version at 551 amino acids (from here referred to as orb2-PA).

UAS orb2-PA and UAS orb2-PB transgenics were then crossed into the original *ap*-GAL4, UAS dS6K setting from the screen to test whether the enhancement on the bent-down wing phenotype originally observed with EP24.061 could be reproduced with either of the *orb2* isoforms. Unfortunately, the result was unclear for many of the lines because of lethality, possibly be due to the nature of the endogenous expression pattern of apterous, present not only in the dorsal wing compartment, but also in the nerve cords, eyes, olfactory organs, and brain [180]. Several of the lines for UAS orb2-PB did yield survivors (lines CL3, CL5, CL6), which surprisingly had a phenotype the opposite to the expected; a bent-up wing (Fig. 8). This phenotype was present to the same extent whether the UAS dS6K transgene was present or not, and was observed in more than one independent line. The natural properties of the yeast GAL4 transcription factor make it partially temperature sensitive [181], with the *Drosophila* window of use ranging from the less active 18 ºC to the more active 29 ºC, which is closer to the yeast's natural optimum temperature. This allows some manipulation of expression of UAS transgenes through raising or lowering the environmental temperature. Therefore, we decided to test the effect of reducing the experimental temperature from 25 ºC to 18 ºC on the bent up wing phenotype of the UAS orb2-PB lines. The resulting flies phenocopied those of the EP24.061 insertion from the screen, both in the presence and absence of the UAS S6K transgene (Fig. 9). Following this result, we were confident that *orb2* gene product was responsible for the enhancement of the *ap*-GAL4 induced S6K phenotype observed with EP24.061. The UAS-orb2-PA transgenic lines produced very few surviving flies when combined with

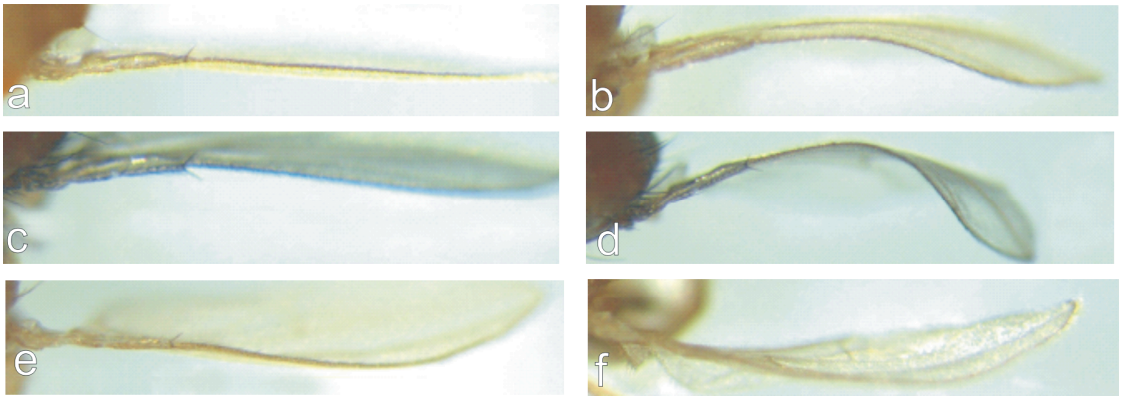

Figure 8: UAS orb2-PB suppresses a dS6K-induced growth phenotype in the drosophila wing.

Apterous GAL4-expressing flies combined with (a) yw (b) UAS dS6K (c) EP24.061 (d) UAS dS6K; EP24.061 (e) UAS orb2-PB (f) UAS dS6K; UAS orb2-PB at 25 ºC

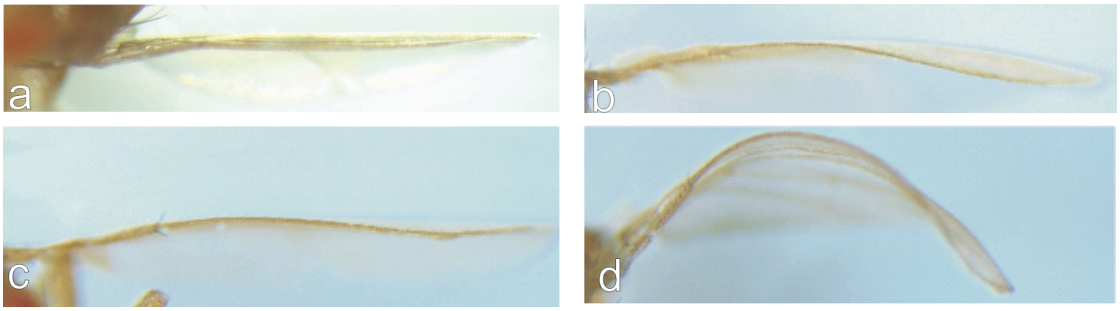

Figure 9: UAS orb2-PB enhances a dS6K-induced growth phenotype in the drosophila wing.

Apterous GAL4-expressing flies were combined with (a) yw (b) UAS dS6K (c) UAS orb2-PB (d) UAS dS6K; UAS orb2-PB at 18 ºC.

ap-GAL4 or *ap*-GAL4, UASdS6K. The survivors had deformed wings, with signs of extensive necrosis, which improved enough with decreased experimental temperature to detect an extreme curling up of the wing. However, the necrosis was too advanced to draw conclusions about the effect of orb2-PA.

orb2-PB over-expression does not rescue a TSC1&2 or PTEN growth defect

Drosophila S6K resides downstream of dTOR complex 1, in a nutrient pathway parallel to the insulin-signaling TOR complex 2 pathway [85, 94]. These pathways are linked by a negative feedback loop, and although dPKB activity is not required for dS6K signaling in vivo [85, 182], cross talk between the two has been shown in cell culture [183]. The ectopic over-expression of tumor suppressors TSC1&2 and PTEN using the *eyeless*-GAL4 (ey-GAL4) driver results in a small-eye phenotype [184, 185]. The rescue of either of these phenotypes by co-over-expression of a candidate gene could be indicative of a role in the nutrient-responsive dS6K arm (TSC1&2) or the insulin receptor-mediated dPKB arm (PTEN) of the pathway (Montagne *et al*., Appendix I). Unknown elements can therefore be tested in this manner to discern which arm they belong to. Given that the over-expression of orb2-PB not only enhances dS6K mediated growth (see above), but also produces a growth phenotype by its mis-expression alone, we decided to test it in this system. Combinations of UAS TSC1, UAS TSC2 and UAS PTEN were established with either the EP24.061, or UAS orb2-PB (line CL6), and crossed with ey-GAL4. The *eyeless*-induced over-expression of UAS TSC1&2 and UAS PTEN affected a small-eye phenotype as expected [184, 185]. The combination of either the EP24.061 or the UAS orb2-PB with these

transgenes did not result in a rescue (Fig. 10); indicating that *orb2* over-expression is unable to compensate for the phenotype provided by tumor suppressors in either arm of the pathway. This experiment was also repeated at a lower temperature (18 ºC), with the same result (data not shown).

Over-expression of orb2 in the eye suppresses growth and dS6K signaling

One observation resulting from the *eyeless*-GAL4 experiment outlined above was that the eye size of *eyeless*-GAL4; UAS orb2-PB flies appeared to be reduced. To gain a better understanding of the underlying cause of the size defects observed both in this system and also hinted at earlier in the wing, the *eyeless*-GAL4;UAS orb2-PB and GAL4;UAS orb2-PA flies were prepared for scanning electron microscopy along with controls (as described in "materials and methods" section). Images were recorded of the flies at 180x and 1000x magnification (Fig 11, a-f), and later assessed for number of ommatidia and size of ommatidia (using Image J to quantify a pixels per ommatidia value). Changes in both size and number of ommatidia were observed (Fig 11 g, h).

The UAS orb2-PA over-expression decreased the number of ommatidia reflected in compensation of ommatidial size. UAS orb2-PB had no effect on the number of ommatidia, but did appear to reduce the size. These differences were statistically significant using an unpaired two-tailed t-test, with p-values of <0.01 in each case. We concluded that the reduced eye size observed in this and the previous experiment was significant, and due to two different underlying reasons for each construct; fewer ommatidia for orb2-PA and smaller ommatidia for orb2-PB.

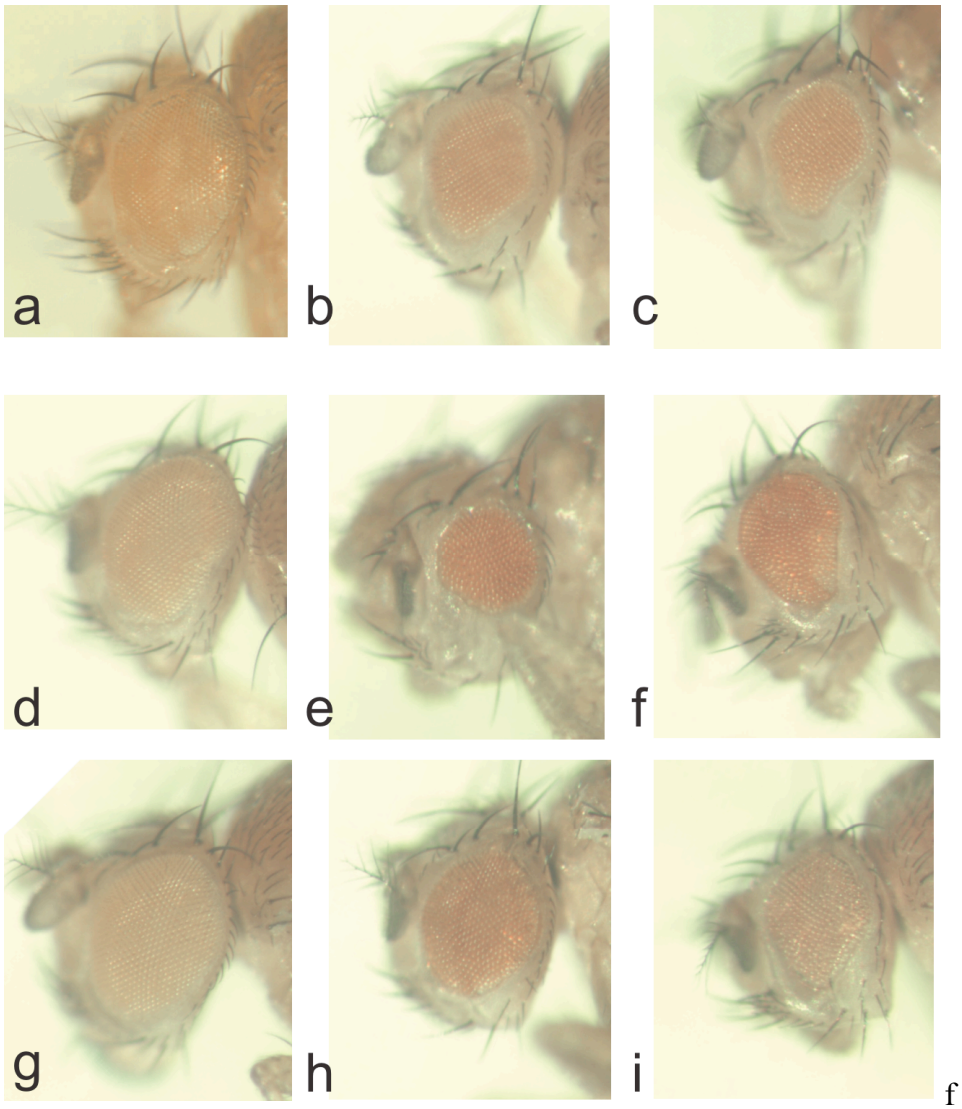

Figure 10: orb2-PB over-expression does not rescue TSC1&2 or PTEN induced small eye phenotype.

Eyeless-GAL4 was combined with the following genotypes: (a)yw (b) UAS TSC1, UAS TSC2 (c) UAS PTEN (d) UAS orb2-PB (e) UAS TSC1, UAS TSC2; UAS orb2-PB (f) UAS PTEN; UAS orb2-PB (g) EP24.061 (h) UAS TSC1, UAS TSC2; EP24.061 (I) UAS PTEN; EP24.061.

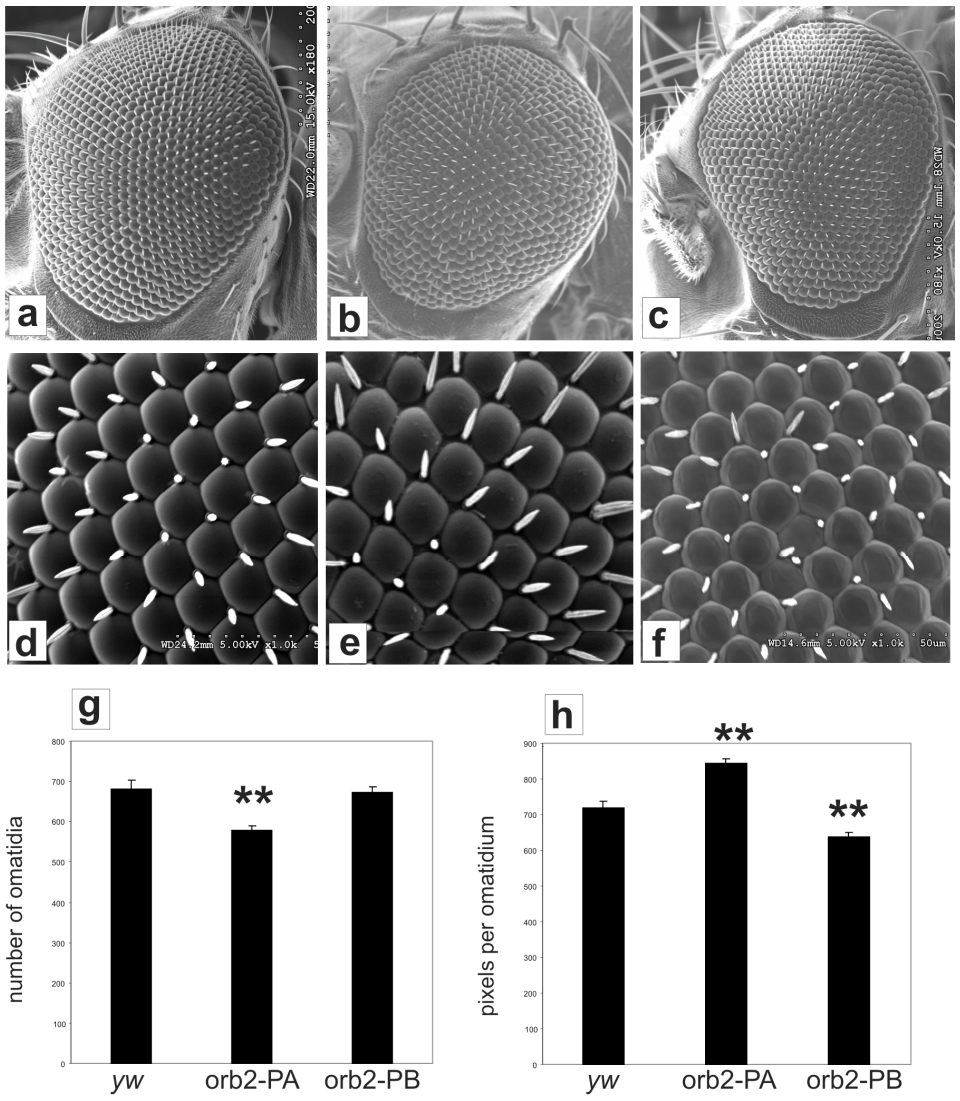

Figure 11: *Eyeless* over-expression of orb2 induces ommatidial number and size defects.

Eyeless GAL4-expressing flies were combined with (a) yw (b) UAS orb2-PA (c) UAS orb2-PB, and pictures were recorded at x180 magnification and for the same genotypes at x1000; (d)yw (e) UAS orb2-PA (f) UAS orb2-PB (g). These effects on number (g) and size (h) of ommatidia are quantified in the charts, with ** denoting $p<0.01$

To gain a better understanding of the signaling involved in the growth phenotypes observed to this point, heads from 1-day-old flies overexpressing UAS orb-PA and -PB, as well as over-expression from EP24.061 were removed and extracted for proteins. Using an antibody raised against orb2-RA [169] (gift from Eric Kandel), we were able to visualize all forms of the overexpressed orb2 proteins, and indeed orb2-PB was found to run at the same molecular weight as both the induced EP24.061 and what appears to be an endogenous band, ~70 kDa (Fig. 12). UAS orb2-PA ran faster, and indicated a lower molecular weight of ~60 kDa. Next, we used two phospho-specific antibodies raised against phosphorylated dS6K T398 and dPKB S505 (analogous to mammalian S6K T389 and PKB S473 respectively) to check if signaling was intact in these animals. We found that over-expression of EP24.061 showed a slight and variable effect on dS6K phosphorylation, while UAS orb2-PA and -PB strongly suppressed the native phosphorylation status of dS6K. dPKB appeared largely unaffected, suggesting that this effect was restricted to the dS6K arm of the pathway.

With this knowledge, we attempted to rescue the more obvious growth defects caused by the eyeless induced orb2-PA induction. We employed co-over-expression of UAS orb2-PA with UAS dS6K, since increasing expression levels of dS6K alone can increase the amount of phosphorylated dS6K present (Appendix II). This co-over-expression had no effect on the phenotype of the flies (Fig. 13), indicating that the small eye phenotype observed is not due to the reduced capacity for dS6K signaling; that the increased levels of dS6K was insufficient to maintain activity; or that the *orb2* effect is simply dominant to dS6K over-expression.

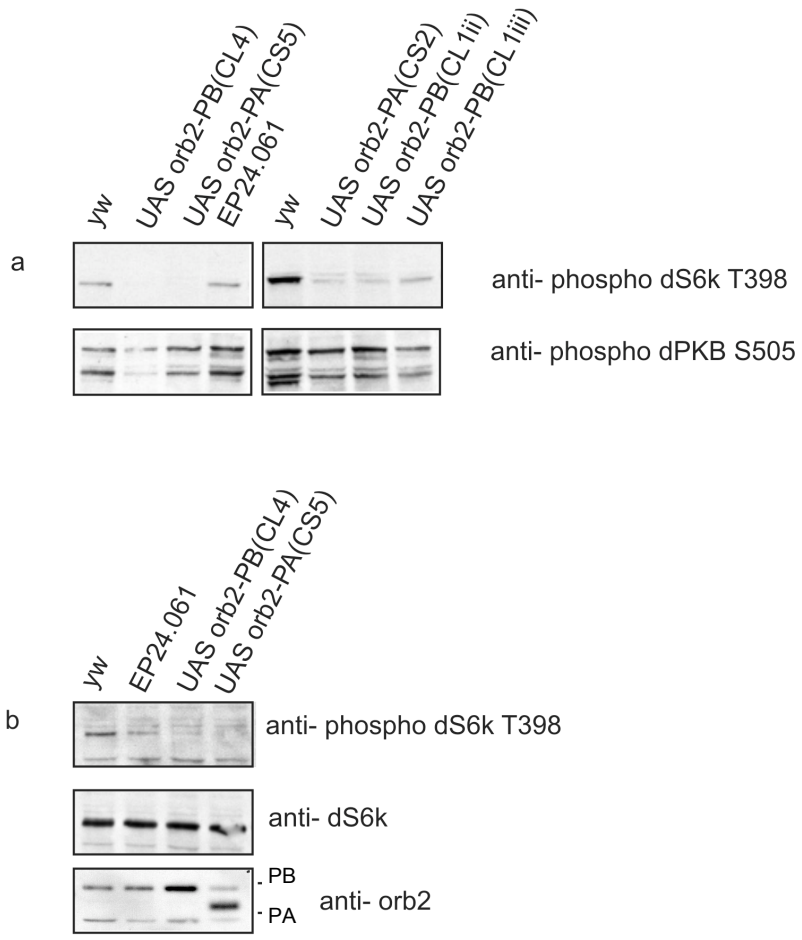

Figure 12: Over-expression of orb2-PA and orb2-PB with eyeless-GAL4 suppresses dS6K phosphorylation at T389, but not dPKB S505 in *Drosophila* heads.

All experiments shown are in presence of *eyeless* gal4. (a) Over-expression of either -PA or -PB form of orb2 reduces a signal as visualized by a phospho-specific dS6K antibody. This effect is reproduced by several independent lines, whereas no consistent significant effect occurs by detection with dPKB phospho-specific antibody. (b) The reduction in phosphorylation status of dS6K is not due to a reduction of protein levels, since expression of dS6K is not affected. Orb2 -PA and -PB isoforms can be differentiated based on size, with the -PA (shorter) form running just lower than the -PB form, which runs around 70 kDa.

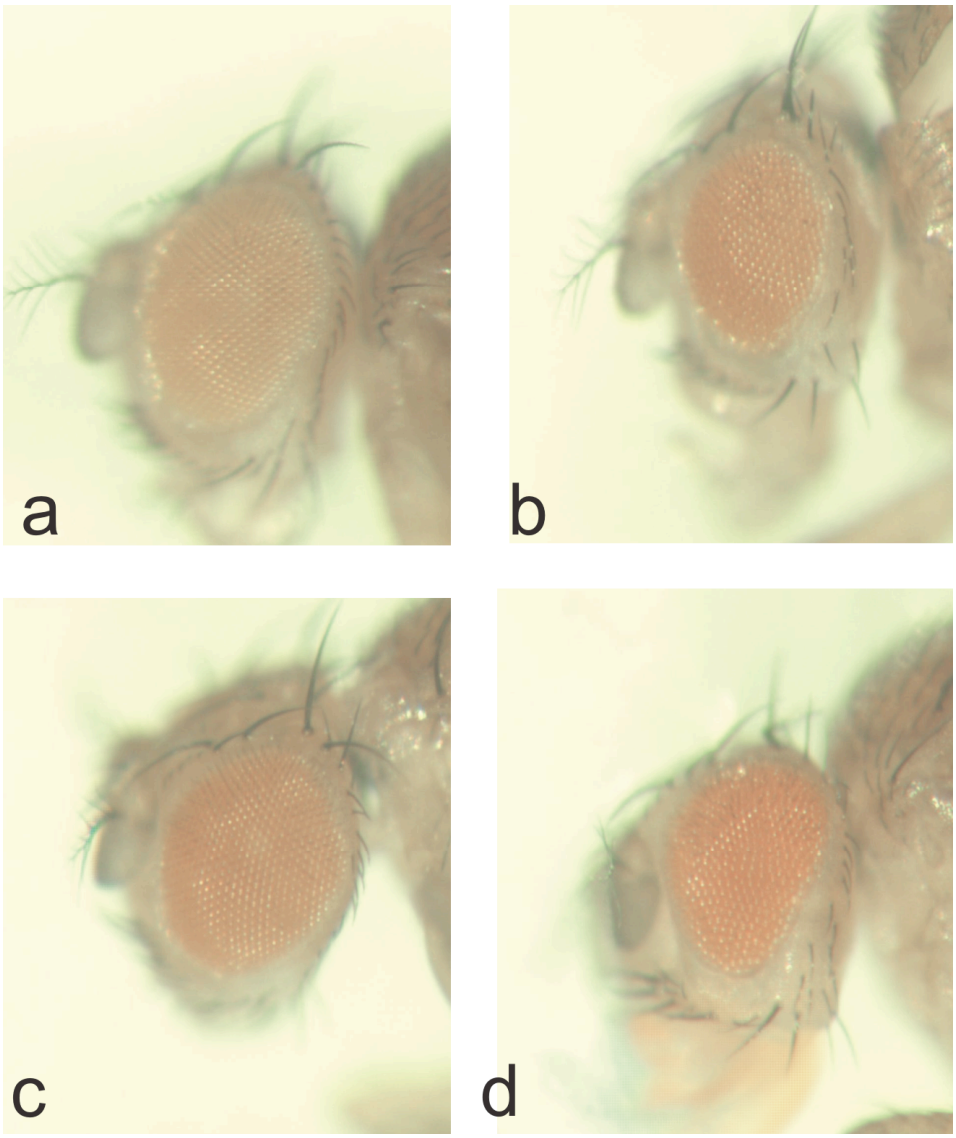

Figure 13: Over-expression of dS6K does not rescue an orb2-PA induced small eye phenotype.

Eyeless-GAL4 females were crossed to males of the following genotype; (a) yw (b) UAS orb2-PA (c) UAS dS6K (d) UAS dS6K; UAS orb2-PA. Males shown

Since there is a defined component of this pathway which is known to give increased activity of dS6K by over-expression alone; the small GTPase Rheb [81], we decided to examine the effect of orb2-PA co-over-expression with this protein. We tested the EP Rheb line by using the *eyeless*-GAL4 system in order to allow comparison with our previous experiments. Since over-expression of Rheb with this driver gave ambiguous results in terms of eye size we employed the published phenotype observed with GMR-GAL4. The EP Rheb insertion driven by GMR-GAL4 resulted in flies with increased eye size, ommatidial bulging, roughness, and sensory hair irregularities. GMR-GAL4; UAS orb2-PB also generated a rough eye, with apparently elongated and often duplicated sensory hairs and bulging ommatidia, but without an increase in overall eye size. The combinatorial effect of these two transgenes by GMR over-expression was increased roughness of the eye, without a clear effect on the size of the eye (Fig 14).

Ubiquitous over-expression of orb2 suppresses growth and dS6K signaling

The data above hinted at a role for *orb2* as a suppressor of growth in certain conditions. Although the fly heads developed smaller eyes following developmental expression of *orb2* isoforms, and the apterous GAL4 driven over-expression bent the wings up in some circumstances, this was potentially indicative of some other phenomenon, such as loss of cell number by apoptosis. To address this question of lethality at the level of the organism, we used ubiquitous drivers daughterless-GAL4 (da-GAL4) and actin-GAL4 (act-GAL4) to test the viability of the flies. Over-

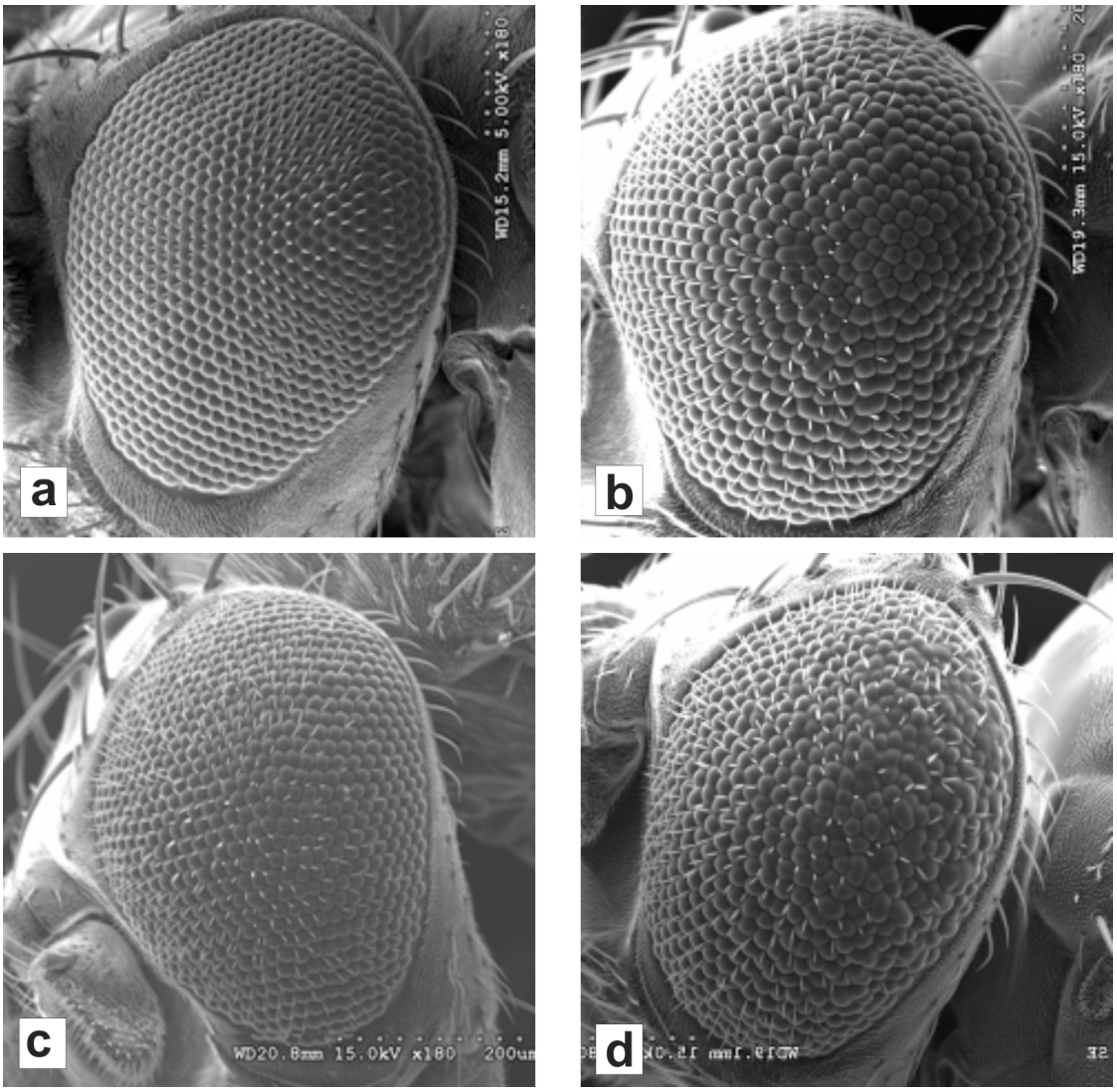

Figure 14: Modification of a GMR-Gal4 -EP Rheb-induced growth phenotype in the eye by co-over-expression of UAS orb2-PB.

GMR Gal4-expressing flies were combined with (a) yw (b) EP Rheb (c) UAS orb2-PB (d) UAS orb2-PB; EP Rheb. Females shown

expression of all orb2-PA lines proved to be embryonic lethal with both of these drivers. Several of the orb2-PB lines, however, produced adult emergers, albeit fewer than expected for wild type. The majority of resulting flies were female with a small minority of male survivors (Fig. 15 a). Pupae from the experimental tubes were compared for size, and it was found that those overexpressing orb2-PB were smaller than their wild type counterparts (Fig. 15b). The fact that functioning adult flies could emerge from these smaller sized pupae adds credence to the notion that the previous size-related effects were not simply due to cell-lethality.

Western blot analysis of wild-type and homozygous EP24.061 adults showed that, in general, female flies of both genotypes expressed higher levels of dS6K, and that EP24.061 homozygous females had increased phosphorylation of both dS6K and dS6 (Fig. 16). Interestingly, when induced with the actin-GAL4 driver, EP24.061 and UAS orb2-PB (line CL5) females had reduced levels of dS6K T398 phosphorylation compared to the controls, suggesting that the EP insertion itself may have an effect on *orb2* endogenous levels.

Ectopic dFMR1 driven by ap-GAL4 phenocopies the induced orb2 bent up wing

There is evidence for the binding and translational inhibition of *Orb* by the *Drosophila* Fragile X Mental Retardation (dFMR1) protein [186]. This information compelled us to test an EP insertion in dFMR1 (Bloomington) in the *ap*-GAL4 over-expression system. This EP, either alone or in concert with UAS dS6K produced a bent up wing (Fig. 17). This was a clear indication that the bent up wing can be

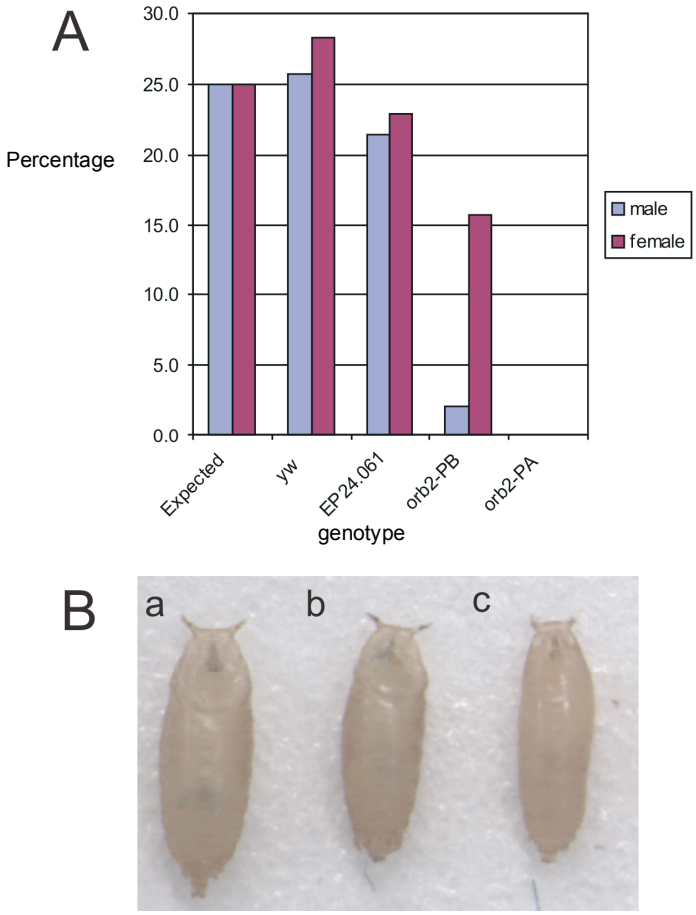

Figure 15: Size and viability defects of orb2-PA and orb2-PB over-expression.

The daughterless-GAL4 driver was used to induce expression of target genes. Top panel (A) shows percentages of unbalanced flies progressing to adult by comparison to their in-tube balancer control. An expected Mendelian value is also shown for reference, and total is 50% due to selection of correct markers. The lower panel (B) shows size defects in pupae overexpressing orb2-PB by daughterless-GAL4 combined with EP24.061 (b) or UAS orb2-PB (c) compared to yw; da-GAL4.

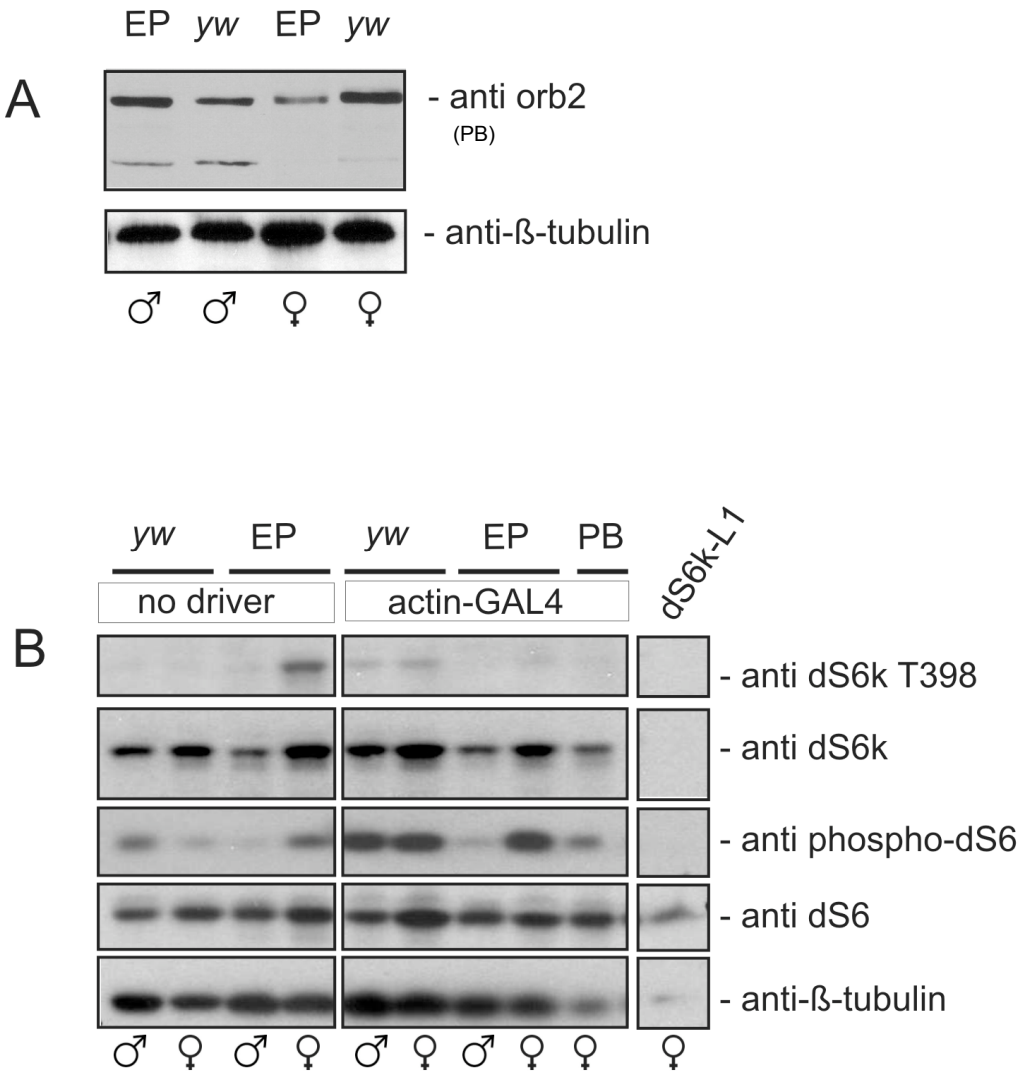

Figure 16: orb2 expression effects dS6K signaling.

In this figure 'EP' refers to the EP24.061 line, and 'PB' refers to the UAS orb2-PB line. (A) Extraction of protein from adult heads reveals a reduction of orb2 expression in female flies homozygous for the EP24.061 insertion. (B) Total protein extracts from adult flies, shows female flies homozygous for the EP insertion have increase dS6K signaling. Over-expression of orb2 by actin-GAL4 reduces dS6 phosphorylation in males. *dS6K* null extracts shown as a control.

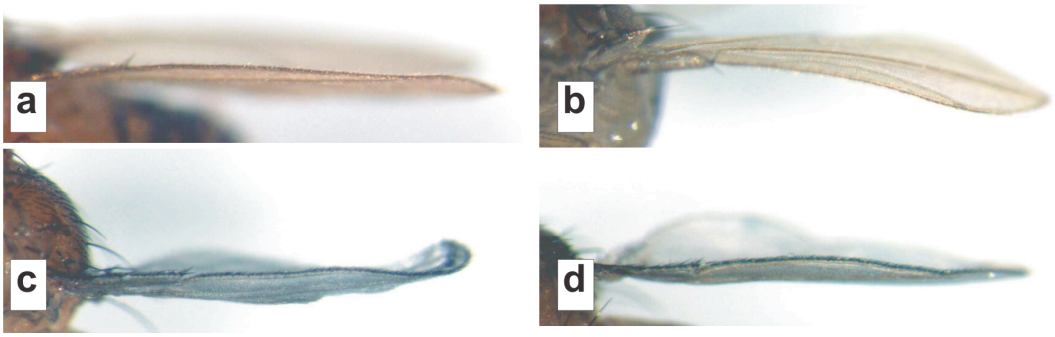

Figure 17: Over-expression of dFMR1 causes a suppression of growth phenotype.

a) *ap*-GAL4, b) *ap*-GAL4, UAS dS6K, c) *ap*-GAL4; EP dFMR1 d) *ap*-GAL4, UAS dS6K; EP dFMR1. Females shown.

produced by a retardation of growth by a known translational repressor, and that this inhibition is sufficient to overcome the enhancement of growth provided by over-expression of dS6K.

3.3 Discussion

Orb2 has a role in growth control

The *Drosophila*-EP screen initiated by the laboratory was aimed at finding novel components of the dS6K pathway, using the subtle growth phenotype observed by the over-expression of dS6K to screen for modifiers. Because so many EP insertions affecting a modification of this phenotype were isolated, a secondary screen was performed to compare their effects with the active $S6K1^{dE/D3E}$ (Montagne *et al.*, Appendix I). The subset of lines that displayed a weaker effect with $S6K1^{dE/D3E}$ than by combination with dS6K were considered more likely to be involved with dS6K signaling, as this was the effect observed with co-over-expression of DPDK1. This differential phenotypic interaction may be a hallmark of specificity, as dS6K, unlike $S6K1^{dE/D3E}$, is the genuine target for DPDK1 (Montagne *et al.*, Appendix I).

In the identification of the *orb2* gene as an enhancer of the pathway, we appear to have also discovered a repressor. Far from being a straightforward titration effect of the over-expression, it seems that the growth inhibition observed with increased levels of orb2-PB expression is due to a threshold effect. Potentially, the level of orb2 protein required by the cell for efficient growth is fine-tuned, and once this level is overcome, effects become inhibitory. This theory is supported by the fact that although both enhancement and repression of the dS6K-induced bent-down wing phenotype was observed, the effect was always an extreme modification of the phenotype; it never simply resulted in a straight wing, and the suppression of growth observed was always

dominant to the dS6K bent-down wing phenotype (Fig. 6). We also observed that over-expression with various other drivers consistently resulted in growth defects rather than enhancements (for example, *eyeless*-GAL4 resulted in smaller heads, and daughterless-GAL4 resulted in smaller pupae; Figs. 11 and 15), which could be a result of temporal or tissue-specific threshold effects, or promoter-related expression levels.

The CPEB family of proteins first discovered in *Drosophila* by the Schedl laboratory [138] and soon after studied extensively by the Richter laboratory [187] has been shown to influence gametogenesis and early development, synaptic plasticity and cellular senescence through regulation of mRNA translation [143]. While CPEB1 has been implicated in the growth of mouse oocytes [188], and the role in control of translation and embryonic cell division of the CPEB family is clear from the literature mentioned above, a role in tissue or organismal growth has not yet been demonstrated. The overespression studies presented in this thesis strongly argue that orb2-PB can indeed enhance growth in the developing tissue of the wing imaginal disc, resulting in the *ap*-GAL4; UAS orb2-PB bent-down wing phenotype observed.

The bent-up wing phenotype observed with the *ap*-GAL4; EP dFMR1 experiment shows that this system can also be used to assess growth inhibition, since it is a known suppressor of tissue size (Fig. 17) [189]. With this knowledge, and knowing that lower levels of orb2-PB expression can enhance growth in this tissue, we hypothesize that the bent-up wing observed at higher temperatures is a clear indication that at a certain point, high levels of this protein become inhibitory.

However, over-expression studies have the caveat that even when using wild-type gene products, mis-expression can cause both “dominant-negative” type effects,

as well as artifactual ones. As mentioned in the introduction, dPI3K function can be inhibited by over-expression of its adapter subunit, dp60 [90, 91]. If interacting proteins are required at equimolar amounts in the cell (or at specific ratios), disruption of this balance would possibly cause deleterious effects, opposing the endogenous role of the protein. To ascertain the true role of this gene *in vivo*, a mutant must be generated. In the case of *orb2*, this could be achieved by an imprecise excision by remobilization of the EP element in the 5' UTR of the gene, leading to a disruption of the gene [190]. Alternatively, ethyl-methanesulfonate (EMS) mutagenesis of EP24.061 male flies, followed by a re-screening of the progeny with the *ap*-GAL4; UAS S6K flies could result in revertants, and help to identify a point mutation causing either a loss-of-function allele, or a null. This has already proved a successful strategy for another EP candidate from the screen (Appendix I). This would be a useful tool, because if a point mutation could be isolated occurring at a residue critical for function of the gene, it could be used for both loss-of-function and dominant negative over-expression studies due to the EP insertion upstream.

Orb2 over-expression produces large amounts of protein

Using larval extracts with a one-hour recovery from heat-shock-GAL4 induction of the transgenes (appendix III), we noticed that orb2 proteins accumulate very soon after induction. This means that extracts observed in Figure 12 (b) are slightly misleading, because although only a moderate induction of the proteins was observed, we have noticed that it is difficult to visualize other overexpressed proteins using this driver (*eye*-GAL4), possibly due to the timing of expression compared to

extraction of proteins from adult heads. One explanation for the strong induction of orb2 proteins is that they were cloned into the pUAST vector including the full-length 3'UTR, which could potentially contain auto-regulatory sequences, as has been demonstrated with orb mRNA [191]. Although there does not appear to be the required hexanucleotide or classical CPE usually required for CPEB1 regulation (AAUAAA and UUUUAU respectively; see orb2 introduction and [192] for full explanation), the possibility remains that orb2 mRNA is translationally auto-regulated. For example, CPEB proteins 2–4 do not bind the CPE to the same extent as CPEB1 in mammals, but instead strongly interact with a U-rich loop within a stem–loop structure in the 3' UTR of mRNAs [151]. Engineering a transgene containing only the coding sequence of the orb2-PB would be one way to circumnavigate this particular issue.

In addition, since *orb1* and *orb2* are auto-regulated [186, 193], and Rapamycin blocks induction of the Aplysia homologue of CPEB3 [194], it would be interesting to see if this rapid accumulation of orb2 protein is rapamycin-sensitive, by performing the heat-shock experiment on larvae pre-fed with rapamycin.

*Raising orb2 to inhibitory levels reduces dS6K signali*ng

The fact that the *ap*-GAL4;orb2-PB wings bent-upwards to the same extent whether overexpressed dS6K was present or not (Fig. 8), showed that the suppression of growth caused by orb-PB was dominant to dS6K mediated growth. While *ap*-GAL4; UAS orb2-PA flies were often lethal, or survived and had wing necrosis, we demonstrated that a suppression of growth in the eye caused by *eye*-GAL4; UAS orb2-

PA could not be reversed with co-over-expression of dS6K (Fig. 13). Following extraction of heads from the over-expression of orb2-PA and orb2-PB using the eyeless driver, it was clear that over-expression of orb2 could have an inhibitory effect on dS6K phosphorylation status (Fig 12). As mentioned in the first section of this discussion, over-expression can produce deleterious side effects which are not necessarily representative of the proteins' endogenous action. However, the effect observed still indicated to us that *orb2* is involved in the dS6K signaling pathway, because there was no effect on the phosphorylation status of dPKB.

Sex-specific effects of orb2 and dS6K

The original phenotype observed from the P-element insertion in the dS6K gene, later used to generate the published excision-mutant $dS6K^{l-1}$ [101] was observed to be female sterile, along with having some of the other traits of the excision mutant; partial lethality, a developmental delay, and reduced body size. From western blots, a difference in total levels of dS6K can be seen between male and female flies (Fig 17). This difference does not appear to translate into a difference in activity of the kinase, as determined by anti dS6K-T398 and anti phospho- dS6 antibodies. Indeed, the only evidence of a sex-specific increase of dS6K T398 phosphorylation is an increase in the homozygous EP insertion for orb2. Meanwhile, orb2 levels are reduced in the female flies homozygous for EP24.061 where this increase occurs (Fig. 17), while driving the EP or UAS orb2-PB with actin-GAL4 causes a reduction in T398 dS6K (and phospho-dS6 in the case of orb2-PB), this would suggest that orb2 has a repressive effect on dS6K phosphorylation. One possible explanation for this is that orb2 is a downstream

target of dTOR, rather than dS6K, and while a small amount of over-expression can enhance growth by orb2-mediated translation, larger doses can titrate out activity of dTOR towards dS6K and other substrates, resulting in growth inhibition (see below). TOR proteins interact with their substrates through conserved TOS motifs [51]. While no clear motif is present in *orb2*, it is possible that the interaction is though an alternative motif, or through a mediator.

Interestingly, it is the homozygous EP24.061 males who appear to have a phenotype. The EP24.061 retained the TM6b balancer with which it was originally maintained, despite the presence of homozygous viable flies in the tube. When working with *Drosophila* lines, this is often an indication that the mutation on the balanced chromosome is either weak or infertile. After setting several tubes of crosses, we discovered that homozygous males were sterile, while females were not. Recently, the first paper on *orb2* was published [154]. This study used homologous recombination to generate two targeted mutations of the gene, one a full deletion of the coding sequences ($orb2^{\Delta}$), and the other an in-frame deletion removing the conserved polyglutamine region in the N-terminus of the protein ($orb2^{\Delta Q}$), (refer to Fig. 4 for conserved region). While $orb2^{\Delta}$ was homozygous lethal, the $orb2^{\Delta Q}$ homozygotes and $orb2^{\Delta}$/ $orb2^{\Delta Q}$ trans-heterozygotes were viable with no gross morphological defects. Using a courtship-based memory assay, the authors found that long term, but not short term memory was impaired in these animals. It is tempting to think, therefore, that the EP homozygous flies do indeed have a disruption of normal *orb2* function, and this results in the homozygous sterility phenotype of the males.

In addition to the potential effect on fertility, when driving the EP insertion with the actin promoter, we found that phosphorylation of the dS6K substrate, dS6 was abolished by comparison to a *yw*- control specifically in males (Fig 17). Driving any of the UAS orb2-PB lines with actin did not produce adult males, although driving with another ubiquitous driver, daughterless-GAL4, did produce a small number of male survivors (Fig 15, graph). These data suggest that orb2 protein levels play an important role in the development of male flies.

Relationship of orb2 and TSC1&2

Taken against the backdrop of the *ap*-GAL4 enhancement of the UAS dS6K-induced bent-down wing phenotype, our initial reaction to the *eye*-GAL4, UAS TSC1&2 and UAS PTEN experiments (Fig 10), where UAS orb2-PB did not rescue the small eye phenotype, was that orb2 is hypostatic to the TSC complex. However, it was soon apparent that the action of UAS orb2 in this case was potentially different to the enhancement phenotype observed in the wing. While over-expression of the tumor suppressor complex dTSC1&2 or dPTEN in the eye resulted in an obvious small-eye phenotype, over-expression of orb2-PB also reduced eye size, albeit in a much more subtle manner. With this knowledge, the argument that dPTEN or dTSC1&2 are epistatic to orb2 is lost, since the over-expression may be inducing an inhibitory phenotype, as indicated in the above paragraphs. In the case of co-over-expression of UAS TSC1&2 and UAS orb2-PB, there was even what appeared to be an enhancement of the phenotype in approximately half the flies. The *eye*-GAL4, UAS dTSC1&2 phenotype has been attributed largely to cell cycle disruption, rescued by co-over-

expression of cyclins D or E, or dS6K [185] and since the CPEB proteins play an important role in the control of cell cycle [195], this could be a potential link between these genes. However, unlike TSC, orb2-PA induced suppression of growth could not be rescued by over-expression of dS6K. This would argue that orb2 has a greater effect on the suppression of growth than merely inhibiting dS6K phosphorylation. There is a strong possibility that over-expression of orb2 could result in a phenotype stronger to that of the loss of orb2. This hypothesis is based on CPEB3 in mammals, where whilst translation of certain messages are enhanced by its activation of CPEB3, the expression of these proteins is not completely suppressed upon loss of CPEB, and RNAi depletion of CPEB can even enhance the expression of these messages [151].

Models of orb2 influence on dTOR/dS6K signaling

Bearing in mind the above observations that *orb2* can both enhance and suppress dS6K mediated growth, four possibilities stand out as theories for the action of orb2 on dS6K activity (Fig. 18); (1) *orb2* is an upstream activator of dS6K, which, when overexpressed at high levels causes an inhibitory effect on the pathway through a "dominant negative" effect of mis-expression, potentially titrating out necessary upstream components. (2) *orb2* regulates translation of an upstream component of the pathway, and over-expression or the component behaves as described in (1). (3) *orb2* is a downstream target and binding partner of dS6K, and can interfere with its the normal localization, sequestering it from phosphorylation, or bring it into contact with a regulatory phosphatase. Work from John Blenis [114] showed that mTOR and S6K1 undergo dynamic interchange by interaction with the pre-initiation complex on mRNA,

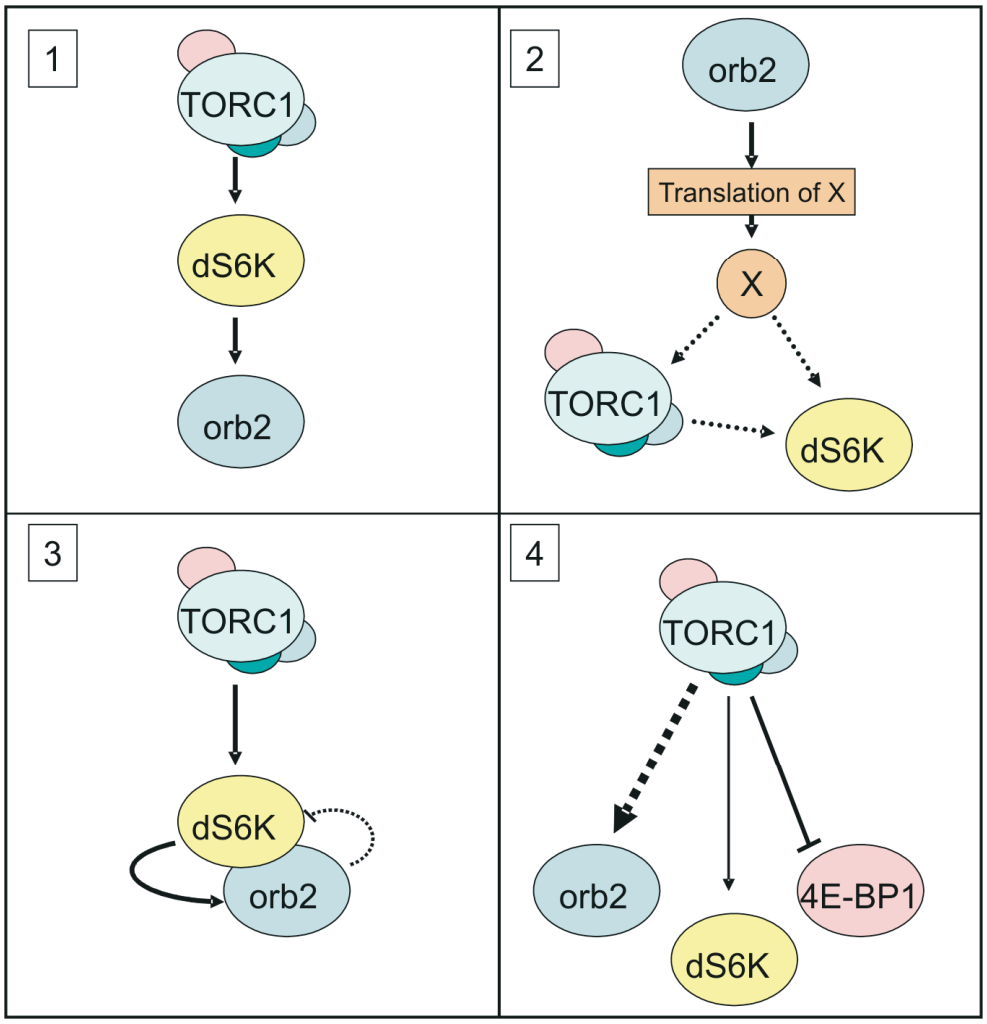

Figure 18: **Models of orb2 involvement in the dTOR/dS6K signaling pathway**.

See text for details

and since CPEB family members interact with eIF4E-binding proteins, it is a possibility that over-expression of *orb2* interferes with this process. (4) *orb2* is downstream target of dTOR, parallel to dS6K, and its over-expression titrates out TOR activity away from dS6K. In this scenario, size defects observed by over-expression of orb2 could be due to high levels causing a reduction of phosphorylation of other substrates, such as dS6K. This model is supported by work by Sonenberg's group earlier this year, which showed that the double knockout mouse for 4E-BP1 and 4E-BP2 showed an elevated sensitivity to diet-induced obesity and insulin resistance in mice [196]. This was an unexpected result, considering that the single knockout mice for 4E-BP1 had a lean phenotype [197]. The increased adiposity of the double knockout mice was concurrent with increased activity of S6K, and reduction of PKB activity, indicating that by removing one substrate of TOR, another may benefit [196]. This indicates that an imbalance of signaling can be brought about by changing the levels of TOR substrate expression.

Future work with orb2

The potential interaction between dS6K or dTOR and orb2 is the most tempting of avenues to proceed with. The next steps with this project should be heavily weighted towards identifying any interaction by direct association, or by phosphorylation. A potential S6K consensus motif exists in both the mammalian CPEB3 and 4 [194], and the drosophila orb2 (by analysis using MIT Scansite [198]. Because mCPEB3 has been shown to regulate translation of GluR2, it would interesting to find out if GluR2 translation is also affected by Rapamycin, either in the

fly or in mammals. dFMR1, the *Drosophila* Fragile X gene, has been shown to negatively regulate both orb and orb2 translation [186, 193]. The potential role of this gene in our orb2/S6K story is of great interest, since the role of both CPEB and FMR1 is well documented in the brain, and area of widening research for TOR signaling. In addition, imprecise excision crosses are currently being screened for a loss-of–function allele of orb2, following which, it will be possible to address the question of whether orb2 is essential for growth in drosophila, and if lethal (as expected from [154]), to check if any growth effects observed are cell-autonomous events using clonal analysis.

CHAPTER 4: Inositol(1,4,5) Phosphate Kinase 1

4.1 Introduction

Inositides

Crystallization of a novel carbohydrate compound, 'inositol' from muscle extracts more than one hundred and fifty years ago by the German chemist Josef Scherer[199] began what the widely studied field of inositol second messenger research. The structural basis of the most naturally abundant form of this inositol (also known as 'myo-inositol') is a carbocyclic polyol where the 1st, 3rd, 4th, 5th and 6th hydroxyls are equatorial, while the 2nd hydroxyl group is axial. As well as being readily available in many food sources, inositol is synthesized *de* novo from glucose in a number of organs, including brain, testis, kidney and liver[200]. The general term 'inositides' refers to the group of cellular components whose commonality is the inclusion of *myo*-inositol as part of their chemical structure. This group can be divided into two very different subgroups: insoluble inositol lipids that contain a phosphatidyl group, and the soluble inositol phosphates that do not. Inositol phospholipids are found concentrated at the cytosolic surface of membranes, after delivery of the precursor phosphatidylinositol (PI) by vesicular transport or specialized transfer proteins from the major site of synthesis, the endoplasmic reticulum. Reversible phosphorylation of positions 3, 4, or 5 of the inositol ring in various combinations gives rise to several species of phosphoinositide with different signaling capabilities, such as

phosphatidylinositol-4,5-bisphosphate (PIP_2), which is both a precursor of intracellular messengers generated by phospholipases and also a direct signaling molecule [201]. Phospholipase C (PLC) induced PIP_2 cleavage as a result of agonist occupation of cell surface receptors generates a second type of inositide, in this case specifically inositol (1,4,5)-phosphate (IP_3), which can be metabolized into various other forms of inositol phosphate [202, 203]. In the same reaction, diacylglycerol is produced, which is also a second messenger, and provides positive feedback stimulation of PLC to amplify agonist-induced Ca^{2+} signaling in response to weak extracellular stimuli [204]. Very little is known about the higher inositol phosphates such as Ins(1,3,4,5,6)P_5 (IP_5), Ins(1,2,3,4,5,6)P_6 (IP_6) and the diphosphoinositol polyphosphates, but it is thought that they could have extremely diverse roles in the cell, such as aiding efficient messenger RNA export [205], or acting as primary phosphate donors to acceptors other than ADP, such as proteins [206, 207].

Inositol signaling

Activation of phosphoinositide-specific phospholipase C (PLC) generates an ensemble of intracellular inositol phosphate (IP) second messengers involved in diverse cellular processes [208]. Among these are IP_3, which functions as a regulator of calcium release [209] and as a precursor to more highly phosphorylated IP molecules, including inositol (1,3,4,5)-phosphate (IP_4), IP_5, IP_6, and inositol pyrophosphates (PP-IPs) [210]. The PP-IPs have more phosphates than IP_6 [211, 212], functional analyses of the IP_6 kinase that produces such higher phosphates is required for maintaining cellular integrity, temperature-dependent growth, and rod-shape morphology in yeast

[208]. Work on IP_3 and IP_4 went hand in hand, following the discovery of inositols IP_3 [213] and IP_4 [214] upon Carbachol stimulation of rat cortical slices and parotid gland fragments, when it was thought that IP_4 could be the precursor. Since then, it has been found that Ins(1,4,5)P_3 3-kinase (IP3K), which converts IP_3 to IP_4, is the most active inositol phosphate kinase detectable in mammals, having a prominent role in rapidly metabolizing the pool of IP_3 that is generated when phospholipase-C-coupled receptors are activated [215]. As a consequence of IP_4 formation, IP_3 is protected from dephosphorylation by 5-phosphate phosphatase [216]. Together, IP_3 and IP_4 aid in the mobilization of calcium within the cell.

Inositol phosphates IP_3 and IP_4 regulate calcium mobilization via the inositol 3-phosphate receptor (IP_3R), of which there are three isoforms in mammals, and one in drosophila. These intracellular calcium ion ($Ca^{2+)}$ channels that play a critical role in generating Ca^{2+} signals that accompany the stimulation of cells with many different types of agonists [217], and are regulated by Ca^{2+} and IP_3 binding. The IP_3R exists physiologically as a tetramer and, despite some tissue specificity, there is evidence that it may exist as a heterotetramer [218]. The Ca^{2+} regulation of IP_3 receptors is biphasic, the Ca^{2+} binding site which binds to two distinct sites, and IP_3 promotes channel opening by controlling whether it binds to the stimulatory or inhibitory sites [219]. When Ca^{2+} levels begin to rise, the ER-based IP_3R channel is activated, in a positive feedback loop, which is known as 'Ca^{2+}-induced Ca^{2+}-release' (CICR). At high levels of Ca^{2+} concentration, the channels close again, which is required for replenishment of the ER calcium stores. This results in oscillating levels of Ca^{2+}. In addition, depletion

of ER-calcium stores can cause extracellular calcium influx, via the store-operated calcium channels [220].

IP3K regulation

Three different IP3K genes exist in mammals, IP3KA, B and C (sometimes referred to as ITPKA, B and C), and they differ in molecular mass, Ca^{2+}/calmodulin sensitivity, intracellular distribution, and tissue expression [221, 222]. Many of the cellular effects of calcium ions are mediated by the Ca^{2+} binding protein, calmodulin (CaM). Upon binding up to four ions, CaM undergoes a conformational change, which enables it to bind to specific proteins such as Calmodulin kinase II (CaMKII) and Calcineurin [223]. When bound by Ca^{2+}/CaM, up to a 20-fold of increase in IP3Ks enzymatic activities can be observed in a *in vitro* assay using purified IP3Ks from rat.

Calcium sensitivity of the slightly larger IP3KB isoform is greater that that of the IP3KA form, and rat IP3 kinase isoforms have been found to show a tissue-specific expression pattern, with RnIP3KA present in brain and testes, and RnIP3KB expressed predominantly in lung, thymus, heart, testes and brain [224]. Mammalian IP3Ks are substrates of cAMP-dependent kinase (PKA), protein kinase C (PKC) and Ca^{2+}/CaMdenpendent kinase II (CaMKII). PKA can stimulate IP3K activity . In contrast, PKC is a negative regulator of IP3K by phosphorylation [225] at Ser-175 of rat IP33K-A. Ser-109 serves as a site for both PKC and PKA, and while simultaneous phosphorylation of Ser-109 and Ser-175 leads to inactivation of the enzyme, a single phosphorylation at Ser-109 activates it, suggesting that Ser-175 is probably the

inhibitory phosphorylation site [226]. CaMKII is also a positive regulator of IP3K, by phosphorylation of Thr-311 on human IP3KA, which stimulates enzyme activity by 8~10-fold [225, 227].

There are several downstream consequences of IP3K activity (Fig. 19). A complete inhibition of IP3K activity in Hela cells by adriomycin (an IP3K inhibitor) or by an IP3K specific antibody blocked Ca^{2+} oscillations, whereas a partial inhibition caused a significant reduction in oscillation frequency [228]. Rat and human IP3Ks may be involved in brain development, as rat IP3K activity is low at birth (~50% of the adult expression), while spatial learning training leads to an increase of rat IP33K-A level [229, 230]. Meanwhile, ubiquitous over-expression of *D-IP3K1*, the *Drosophila* homologue of IP3KA, confers specific resistance to hydrogen peroxide induced oxidative stress, and the authors claim that the protective effect of *D-IP3K1* is mainly due to reduced IP3 levels, and thus reduced calcium release from internal stores [231].

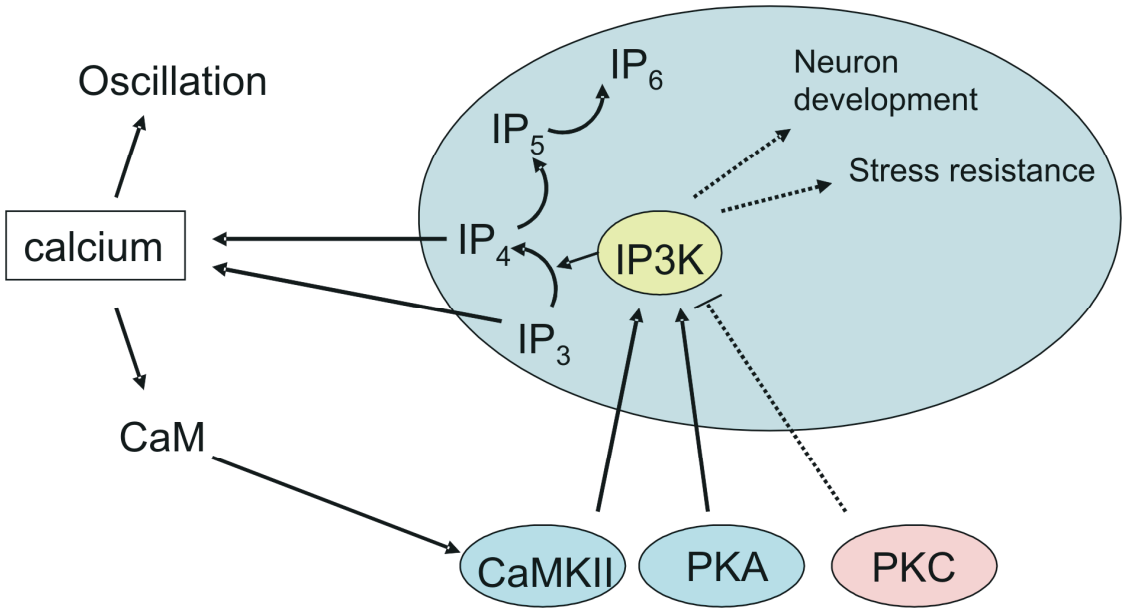

Figure 19: The network of IP3K functions.

IP3Ks are involved in inositol signaling pathway, calcium signal transduction, brain development, stress responses and gene transcription. They are activated by the Ca(2+)/calmodulin-dependent protein kinase II (CaMKII) and protein kinase A (PKA). Evidence suggests that phosphorylation by protein kinase C (PKC) is inhibitory, although this is still debated.

Adapted from "Inositol 1,4,5-trisphosphate 3-kinases: functions and regulations", Xia HJ, Yang G. Cell Res. 2005 Feb;15(2):83-91

4.2 IP3K1 results

IP3K1 enhances dS6K induced growth

IP3K1 was identified as a candidate enhancer of dS6K mediated growth from the screen outlined in the previous chapter, where the bent-down wing phenotype of *ap*-GAL4, UAS dS6K was used to screen an EP collection in *Drosophila* for potential novel components of the pathway. Because so many EP insertions affecting a modification of this phenotype were isolated, a secondary screen was performed to compare their effects with the active $S6K1^{dE/D3E}$ (Montagne *et al.*, Appendix I). The subset of lines that displayed a weaker effect with $S6K1^{dE/D3E}$ than by combination with dS6K were considered more likely to be involved with dS6K signaling, as this was the effect observed with co-over-expression of DPDK1. This differential phenotypic interaction was used to identify enhancers similar to DPDK1, a proven upstream component of the pathway (Montagne *et al.*, Appendix I and [85]).

Fifty-seven lines containing randomly inserted 'double-headed' EP elements giving a clear enhancement of the dS6K induced bent-down wing phenotype were retained, and 45 were molecularly mapped using plasmid rescue. Males from balanced EP lines were crossed at a constant temperature of 25ºC with double-transgenic female flies containing enhancer-trapped apterous-GAL4 (*ap*-GAL4) on the second chromosome recombined with UAS dS6K, and progeny were screened for enhancement of the bent-down wing phenotype. One line, EP13.148, was found to be a moderate enhancer of dS6K-mediated growth, and had no effect on its own when driven with the *ap*-GAL4 driver (Fig. 20). The genomic region of insertion EP13.148

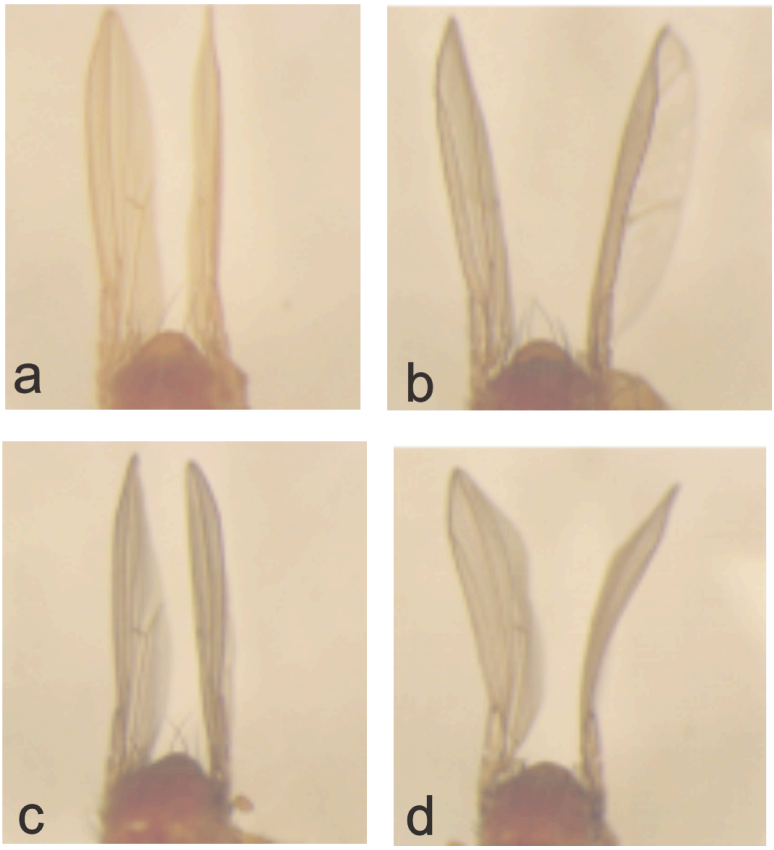

Figure 20: Modification of a dS6K induced growth phenotype by an EP insertion in the drosophila dorsal wing blade.

ap-GAL4 was combined with the following genotypes.; (a) yw (b) UAS dS6K (c) EP13.148 (d) UAS dS6K; EP13.148 flies, and reared at 25 ºC. The dS6K transgene resulted in a bent down wing phenotype, enhanced by the EP 13.148 insertion

was identified by plasmid rescue (Materials and Methods), located at cytological location 30C9. This was found to just upstream of the first exon of the Inositol-1,4,5-triphosphate kinase 1 (IP3K1) gene. The other genes in the immediate vicinity of the insertion included the sodium transport activity genes *pickpocket 11* and *pickpocket 16*. The EP element used in the screen contained two independent UAS sequences for GAL4 to bind to in opposing directions, but unfortunately, unlike the case with *orb2*, removal of one of these UAS sites was not possible. This is because the first EP lines generated for the screens did not contain 'LoxP' sites, and hence could not be recombined in this manner. Since the closest gene to the insertion was IP3K1, and two other published EP insertions in the immediate vicinity had been shown to drive over-expression of this gene [231], we decided to first assess whether this gene could indeed be responsible for the effect. The IP3K1 gene in *Drosophila* has only one described transcript and protein (IP3K1-RA and IP3K1-PA respectively), and will be referred to simply as IP3K1 (Fig. 21). Fly lines containing a p(UAS)IP3K1 insert in the second and third chromosomes in a w1118 background [231] were provided by H. Tricoire (University of Paris, France).

The UAS IP3K1 lines were introduced into the sensitized *ap*-GAL4, UAS dS6K system, and the resulting flies displayed a moderate enhancement of the bent-down wing phenotype similar to that observed in the screen, with no effect on its own (Fig. 22). This indicated that the result from the screen could be attributed to an over-expression of IP3K1, rather than to disruption of the gene, or over-expression of either of the *pickpocket* genes.

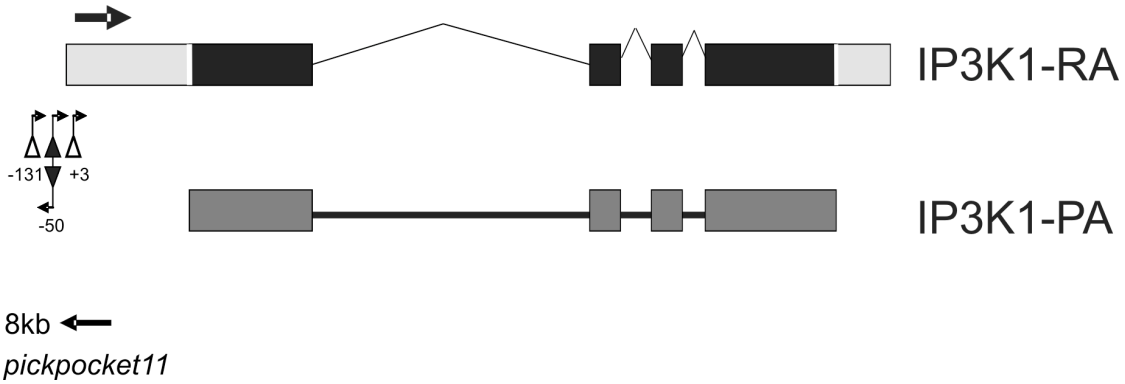

Figure 21: IP3K1 locus and insertion point.

The IP3K1gene has a single transcript and protein annotated in Flybase. The EP13.148 insertion point of the EP at 50 bp upstream of the first exon, and in-between two previously described EP insertions, and 8 Kb from the next closest gene, pickpocket11. The two UAS lines obtained from Hervé Tricoire contain the coding frame associated with IP3K1-RA, predicted to make a protein of around 50kDa.

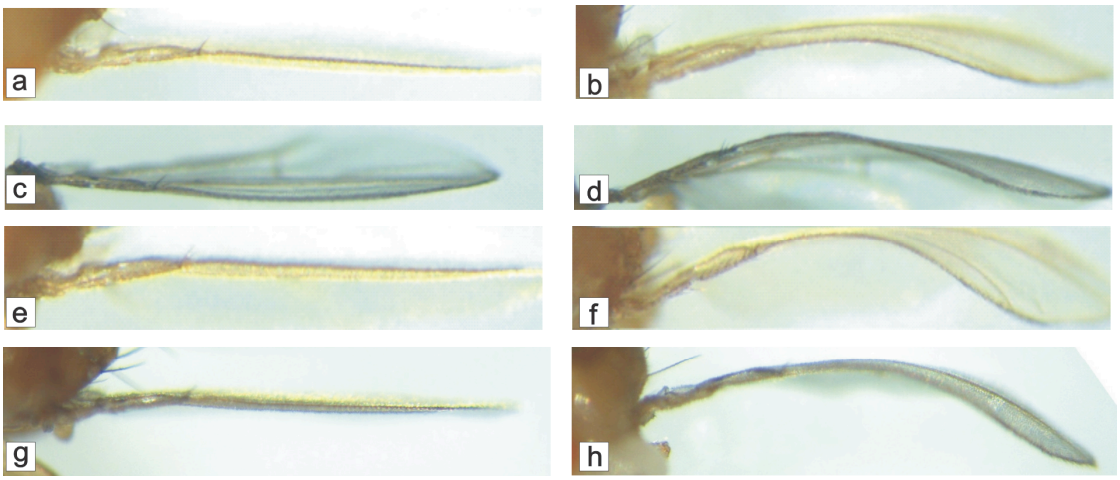

Figure 22: Modification of a dS6K-induced growth phenotype in the wing by IP3K1 and an EP insertion in the IP3 Receptor (ITPR).

ap-GAL4-expressing flies were combined with (a) yw (b) UAS dS6K (c) EP IP3K1 (d) EP IP3K1/ UAS dS6K (e) UAS IP3K1 (f) UAS dS6K; UAS IP3K1(g) EP IP3R, (h) UAS dS6K; EP IP3R at 25° C

As mentioned earlier, manipulation of GAL4 expression by changing the ambient temperature is a characteristic of the UAS-GAL4 system. Therefore, the level of IP3K1 gene expression was raised with increased temperature to test the dose-responsiveness of the bent-down wing phenotype. The effect of increasing the experimental temperature from 25ºC to 29ºC was indeed an increase in the enhancement of the *ap*-GAL4, UAS dS6K bent-down wing phenotype by both the UAS IP3K and EP13.148 from the screen (Fig. 23). This confirmed that over-expression of IP3K1 was responsible for the enhancement of the *ap*-GAL4-induced S6K phenotype, and an indication that the enhancement was sensitive to levels of IP3K1 produced in the developing fly. We also obtained a stock from Bloomington, which has an EP-element inserted in the 5'UTR of the IP3 Receptor gene (IP3R), to test in our system. We were interested to see if another component of the same pathway could have an effect on dS6K-mediated growth. The EP insertion in IP3R did also enhance the bent-down wing phenotype, but we do not know if this is due to a disruption of the IP3R gene, or over-expression, since successful visualization of a gene product has yet to be achieved.

As a further confirmation, GMR-GAL4 was used to drive both the UAS IP3K and EP13.148 in the developing eye, and heads were removed for western blot analysis. While no *Drosophila*-specific IP3K1 antibody is available, several antibodies raised against areas of strong homology with the human homologue, IP3KA, were tested using western blotting techniques as outlined in "Materials and Methods". One

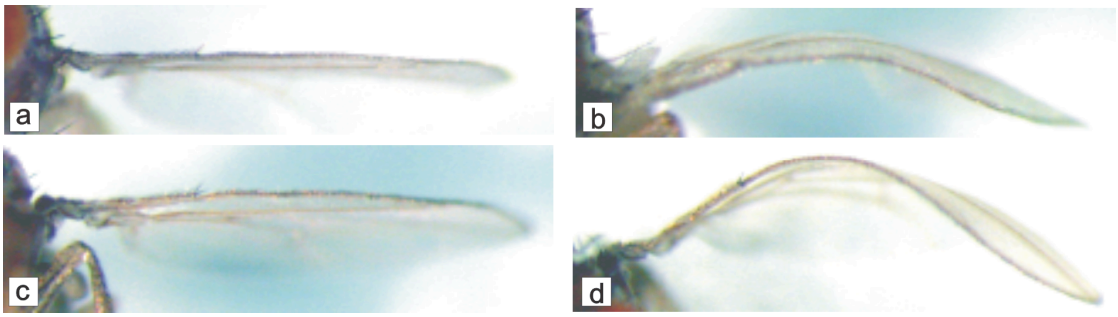

Figure 23: Temperature modification of the IP3K1-enhanced dS6K-induced bent-down wing phenotype.

ap-GAL4-expressing flies were combined with (a) yw (b) UAS dS6K (c) EP IP3K1 (d) EP IP3K1/ UAS dS6K (e) UAS IP3K1 (f) UAS dS6K; UAS IP3K1 at 29°C.

of these antibodies (anti-IP3KA, Santa Cruz) showed no signal in the yw; GMR-GAL4 flies, but detected a protein of the approximate estimated molecular weight of IP3K1 (~50kDa) in both the GMR-induced UAS IP3K and EP13.148 (Fig. 24). This implies that the antibody can detect *Drosophila* IP3K1 when overexpressed, but not at endogenous levels, and suggests that IP3K1 protein levels were responsible for the enhancement effect witnessed.

IP3K1 rescues a TSC1&2 over-expression-induced phenotype

The tumor suppressor complex containing TSC1 and TSC2 has been well studied in the *Drosophila* model. Co-over-expression of TSC1&2 proteins using the *eyeless* promoter results in a retarded growth phenotype [184, 185]. Known components of the dS6K signaling pathway have been shown to rescue this phenotype [94]. To test if IP3K1 over-expression can help to overcome the growth suppression caused by this complex, we tested both the UAS and EP13.148. We found that, in both cases, the small-eye phenotype was completely reverted by over-expression of IP3K1 (Fig. 25). This indicates that not only can IP3K1 enhance dS6K-induced growth, it can also compensate for upstream inhibition of the dS6K signaling pathway by a well-defined negative effector.

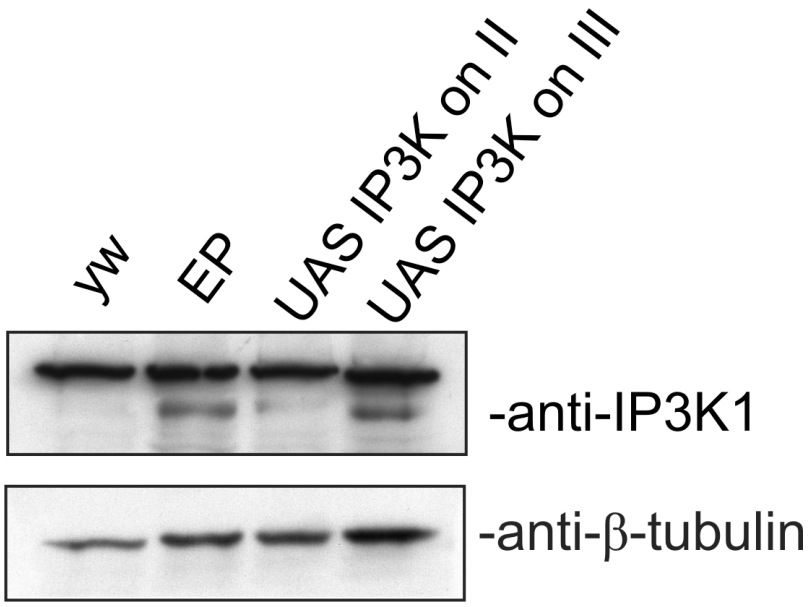

Figure 24: Identification of IP3K1 gene product from fly heads.

GMR-GAL4 was used to drive UAS IP3K1 from two independent lines, along with the EP13.148 (as annotated).

Figure 25: IP3K1 rescues a small eye phenotype induced by TSC1&2 over-expression.

Eyeless-GAL4 combined with (a) yw (b) EP13.148 (c) UAS IP3K1 (d) UAS TSC1, UAS TSC2 (e) EP13.148; UAS TSC1, UAS TSC2 (f) UAS TSC1, UAS TSC2; UAS IP3K1. Males shown.

The mammalian IP3KA gene is implicated in the TOR/S6K signaling pathway

Following the indication from *Drosophila* over-expression studies that IP3K1 is an upstream enhancer of S6K signaling, we decided to investigate if this effect was conserved in humans. Small interfering RNAs (siRNAs), designed with IP3KA and IP3KB mRNA as target sequences were obtained, along with a non-silencing control (Qiagen). HeLa cells were transfected with the siRNAs 48 hours prior to stimulation using oligofectamine (Invitrogen) and deprived of serum for 16 hours, followed by deprivation of amino acids for an additional two hours. Cells were stimulated by amino acids (2X concentration compared to cultivation media) for 30 minutes. Cells were harvested and extracts prepared as described in the "Materials and Methods" section. Standard western blotting techniques were applied to check phosphorylation status of S6K at threonine 389, along with total levels of IP3KA, IP3KB, and an actin-loading control. The amino acid stimulation resulted in a robust phosphorylation of S6K in the non-silencing siRNA control compared to amino acid-depleted conditions, however, when IP3KA or IP3KB levels were reduced by siRNA this activation was completely inhibited (Fig. 26), indicating that IP3KA is required for the amino acid sensing arm of the pathway.

Next, we wanted to understand if the reduction in TOR complex 1 signaling was due the absence of the IP3KA protein itself, or a side effect due to changes in concentrations of specific inositol phosphates. To this end, we applied the IP3 kinase inhibitor N2-(m-Trifluorobenzyl), N6-(p-nitrobenzyl)purine (Calbiochem) to cells as an acute treatment at a 50μM concentration, both in the absence and presence of amino acids. Surprisingly, rather than blocking the amino acid response, the inhibitor

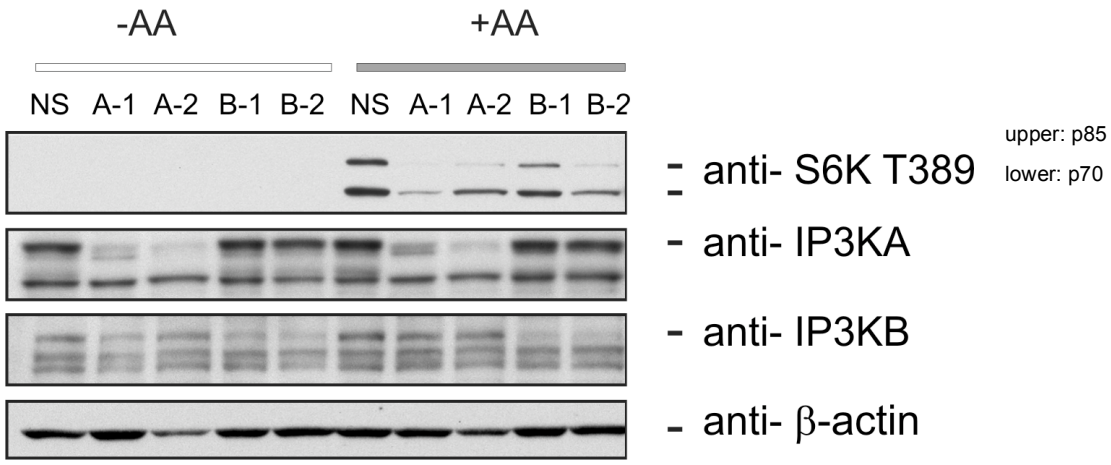

Figure 26: siRNA against IP3KA or IP3KB suppresses phosphorylation of S6K T389.

siRNA against targets; non-silencing (NS), IP3KA (A-1, A-2) and IP3KB (B-1,B-2). Amino acid deprivation (-AA) suppresses S6K T389 phosphorylation, and add- back (+AA) provides a robust stimulation, which is blocked by the siRNA treatment against either IP3KA or IP3KB.

stimulated phosphorylation of S6K-389 even in the absence of amino acids (Fig. 27). Furthermore, when added in concert with amino acids, the phosphorylation of this site was stronger than by re-addition alone. This unexpected result led us to consider the effect of long-term inhibition of the kinase, a situation more analogous to siRNA knockdown of the gene product in terms of time.

Upon long-term (12 hour) treatment with the inhibitor, the stimulation of the pathway by starvation and re-addition of amino acids was almost completely suppressed, with no phosphorylation observed after starvation alone (Fig. 27). This result demonstrates that the long-term inhibition of IP3K suppresses S6K signaling, so it is most likely that it is the loss of kinase activity that causes the effect seen during knockdown of IP3KA. This finding suggests that levels of the inositol phosphates IP3 and IP4 are important for the S6K signaling pathway.

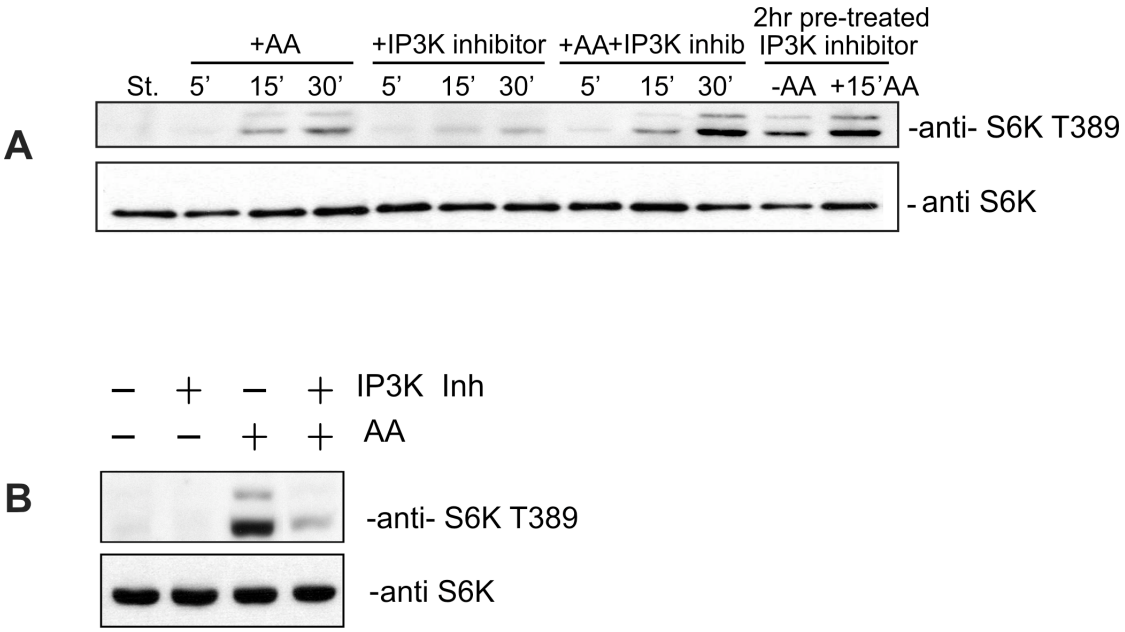

Figure 27: Treatment of HeLa cells with an IP3K inhibitor.

(A) Short term treatment of HeLa cells with 50μM of an IP3K inhibitor increases phosphorylation of S6K T389 at 15 and 30 minutes post treatment, and with a two hour pretreatment of inhibitor, while long term inhibition of IP3K (B) inhibits amino-acid induced phosphorylation of S6K

4.3 Discussion: IP3K1

IP3K1 has a role in growth control

As with orb2 in chapter one of this thesis, the *Drosophila*-EP screen initiated by the laboratory was aimed at finding novel components of the dS6K pathway, using the subtle growth phenotype observed by the over-expression of dS6K to screen for modifiers. Because many EP insertions that caused a modification of this phenotype were isolated, it was decided to keep only enhancers producing a similar phenotype to DPDK1 upon co-over-expression with the active $S6K1^{dE/D3E}$ (Montagne *et al.*, Appendix I, and the chapter on *orb2*), since these were more likely to be involved in the pathway. The possibility that the *Drosophila IP3K1* gene enhances the dTOR/dS6K signaling pathway was especially appealing, since the family of IP3 kinases is very well conserved. We hoped to show that the modulation of S6K activity by IP3K1 was also conserved.

As the EP13.148 insertion was found to be located in an area with existing insertions proven to drive expression of the gene (Fig. 20), we were confident that this insertion would be capable of driving IP3K1 expression, rather than disrupting it. The enhancement of the phenotype observed with *ap*-GAL4, UAS dS6K model was only moderate, but appeared to be fully reproducible with a UAS IP3K1 transgene and sensitive to titration (Figs. 22 and 23). Western blotting confirmed an exogenous product of the expected size was present in GMR-GAL4 flies with either the EP13.148 or the UAS IP3K1 (Fig. 24). After obtaining these results, we hypothesized that the

most likely explanation was that the phenotype was related to the catalysis of Inositol 1, 4, 5-triphosphate (IP_3) to Inositol 1, 3,4, 5-triphosphate (IP_4), since this is the main function of the IP3-kinases [226]. Studies on the IP3K1 gene over-expression had previously shown that oxidative stress resistance due to both an EP insertion in the gene, and the same UAS IP3K1 construct used in the experiments of this thesis was probably due to a decrease in IP_3, rather than an increase in IP_4 [231].

IP3R involvement in IP_3-mediated calcium response

IP_3 is an important second messenger in the cell, mediating calcium release from the internal endoplasmic reticulum stores to the cytosol by Inositol 1, 3,4, 5-triphosphate receptor (IP3R) binding [232]. More recently, IP3Rs have been discovered as having an important role in calcium entry into the cell, being unusual as endoplasmic reticulum proteins because they are also functionally expressed at the plasma membrane. In B-cells, as few as two or three receptors per cell at the plasma membrane contribute substantially to the Ca2+ entry; accounting for as much as half of the influx, with the rest attributable to through the several thousand low-conductance store-operated channels [233, 234]. The IP3R has been described as a macro signaling complex, functioning as a center for signaling cascades, after identification of binding partners homer, protein 4.1N, Huntington-associated protein-1A, protein phosphatases (PPI and PP2A), RACK1, ankaryin, chromogranin, carbonic anhydrase-related protein, IRBIT, Na, K-ATPase, and ERp44 [235]. Suprisingly, an EP insertion in the IP3R locus also enhanced the *ap*-GAL4 dS6K bent-down wing phenotype. This was unexpected because we had considered that the over-expression of IP3K would reduce

the IP_3 pool and as the IP3R requires IP_3 binding for its activity, the effect of over-expression would be the opposite from the IP3K1 phenotype. With this result, and with the knowledge that IP_3 regulates calcium signaling, on which S6K activation has been shown to be dependent in mammalian cells [236], the question of over-expression causing negative effects on the endogenous signaling abilities of IP3K1 was raised. We therefore decided to move into cell culture to assess the effect of RNAi on the signaling ability of the TOR signaling pathway.

IP3K1 enhances dS6K signaling; the IP_3 / IP_4 enhancer model

Considering that reduction of S6K T389 phosphorylation is observed in amino-acid stimulated cells in both the RNAi knockdown of IP3KA (the mammalian homologue of IP3K1, Fig. 26), and the long-term treatment with an IP3 kinase inhibitor (Fig 27), it is clear that it should be the over-expression of a functional IP3K1 that is responsible for the growth enhancement observed in the fly, rather than a negative effect. All the evidence for IP_3 mediated-events point to a positive role in calcium mobilization via the IP3R, either by external entry via the plasma membrane or from ER stores the cytosol, thereby amplifying the signal via store-operated calcium channels (see above). Since it has been shown that in IP3KB knock out mouse, IP_3 levels are not significantly increased, [237], we could assume that the effect of reduced S6K signaling by knockdown of the IP3K genes in mammalian cells was due to a deficiency in IP_4, rather than an increase of IP_3. Although other IP3 kinases still exist in the cell, and the inositol polyphosphate kinase 2 (IPK2) can also produce the same isomer of IP_4 [238], the specificity of spatial or temporal regulation of these proteins is

unknown. Our results indicate that even if there is a competent compensatory mechanism for IP_3 catabolism in the cell, it is not able to make up for loss of an IP3K in terms of S6K1 signaling.

Although the role of IP_3 in calcium oscillations of a cell is well documented, IP_4 was long considered merely a product of IP_3 metabolism. The complicated and intriguing question of what function this inositide performs is still highly controversial. In a dispatch from Current Biology in 2001 [239], Robin Irvine surmised that it can have several roles based on recent evidence; firstly, protection of IP_3 from hydrolysis through its 10-fold-greater affinity for inositol 5-phosphate phosphatase, whilst maintaining relatively high levels in the cells due to a V_{max} 100-fold lower than that of IP_3. The combination of these factors mean that after IP_4 has been produced by calcium-signaling pathways, it will be present as a protector of the next episode of IP_3 production. Secondly, IP_4 can behave as an antagonist to the IP3R, if the ratio of IP_4:IP_3 becomes greater than 10:1, enabling re-loading of the ER calcium pools. Thirdly, IP_4 can specifically and directly activate epithelial and neuronal plasma-membrane calcium channels, and finally; IP_4 is an important precursor for higher inositol phosphates such as IP_5, IP_6, IP_7, IP_8, and also for regulation of an alternatively phosphorylated form of IP_4, (inositol 3,4,5,6-tetrakisphosphate) which acts as a second messenger controlling chloride efflux.

By applying this knowledge to our results, one model which emerges is one in which over-expression of IP3K1 protein may allow the “priming” step alluded to in the above paragraph to occur more readily than at endogenous levels. This would then protect IP_3 levels, and the feedback response of calcium signaling would replenish IP_3

stocks by activation of phospholipase C (PLC), with the end result of normal IP_3 and elevated IP_4 levels in the cell. Although the increase in IP_4 could inhibit the calcium activation of IP3Rs, this would also allow accumulation of calcium for future effective oscillations. In this context, TOR/S6K signaling could be enhanced, as it is already known that this pathway is regulated by, and dependent on, calcium flux. Interestingly, it could also form part of a positive feedback loop, itself regulating calcium oscillations- since mTOR has been shown to potentate calcium release from the IP3R in smooth muscle [240].

IP3K1 enhances dS6K signaling; the IP_4 enhancer model

More recently, a new role for IP_4 has been discovered in regulation of protein tyrosine kinase Itk by enhancement of an important mechanism which is generally required for full activation of a subset of proteins [241]. Pleckstrin homology (PH) domain-containing protein recruitment to cellular membranes is mediated in many cases through phosphatidylinositol 3,4,5-trisphosphate (PIP_3), and regulation of such proteins is based on production and turnover of this ligand. The authors demonstrated that phosphorylation of IP_3 to IP_4 establishes another mode of PH domain regulation, through a soluble ligand, which at physiological concentrations promotes the interaction between PIP_3 and the PH domain of Itk. This discovery could have revealed a general model for the optimization of many PIP_3-PH interactions, and therefore a requirement for IP_4 for the full activation of protein kinases. If this is indeed the case, a new model for the enhancement of S6K/TOR signaling based on the interaction between PIP_3 and IP_4 can be envisaged (see Figure 28). In this model

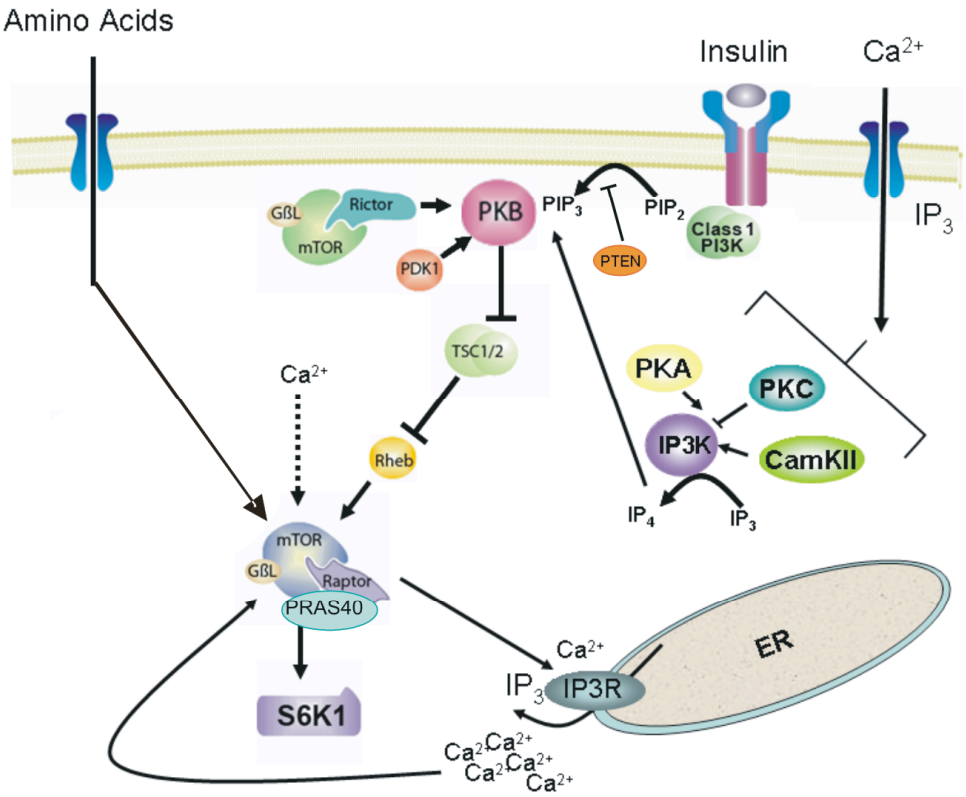

Figure 28: Proposed model for action of IP_4 on S6K/TOR pathway.

Enhanced signaling through PIP_3-IP_4 interaction has downstream consequences resulting in amplification of PIP_3 and calcium-activated signaling pathways, including S6K/TOR signaling.

mTOR Complex1–S6K1 signaling: at the crossroads of obesity, diabetes and cancer

Dann, S.G. et al, Trends in Molecular Medicine, Volume 13, Issue 6, June 2007, Pages 252-259.

upstream growth factor signaling events activate class 1 PI3K, whose action is this to convert PIP_2 to PIP_3 at the plasma membrane. PIP_3 is required for the proximal localization of PKB and PDK1, and this results in the phosphorylation of critical residues on PKB. Meanwhile, active calcium release from the ER, mediated by activated TOR would result in IP3K activity towards IP_3, and IP_4 intracellular concentrations would increase. The IP_4 produced could then enhance pH domain-PIP_3 interaction, therefore amplifying the signal from the plasma membrane to components of the TOR signaling pathway. This model is supported by preliminary data (Appendix IV) that the EP13.148 also rescues the *eyeless*-GAL4; UAS PTEN-induced small-eye phenotype.

In the case of the IP3KA and B knockdowns, even though the suppressed S6K signaling observed is in an amino acid-stimulated context (and therefore separate from the growth factor input) this model cannot be excluded. The reduction in basal levels of IP3KA and B prior to stimulation may already have had an effect on the pathway, for example, by alleviating the inhibitory effect of PKB on the TSC1&2 complex, due to the reduced potential of PIP_3 signaling upstream.

Future work with Inositides and their regulators

More work is needed to understand the consequences of reducing or increasing IP3K activity in the cell. While there are three IP3 kinases in mammalian cells, it appears that removal of a single kinase at one time is enough to cause a perturbation of

TOR/S6K signaling. As IP_3 is important for calcium oscillations, we would like to know if the S6K/TOR pathway relies on oscillations for their activity, and if the frequency of calcium oscillations within a cell can alter the effectiveness of TOR signaling, or vice versa. Over-expression of a kinase-active and kinase-dead form of these IP3 Kinases in mammalian cell culture would help us to understand more about the role of their second messengers in calcium-induced TOR/S6K signaling.

In addition, because both IP_3 and IP_4 appear important the role of IP3K, by virtue of the long-term IP3K inhibitor and RNAi treatments (Figs. 26 and 27), along with the enhancement of dS6K-mediated growth caused by IP3K1 over-expression, it would be interesting to see what effect increased Phospholipase C (PLC) activity has on the pathway, as it cleaves PIP_2 in order to generate IP3 and DAG.

CHAPTER 5: General Discussion

5.1 Overview

Using a genetic approach in *Drosophila*, we were able to identify two conserved genes important for S6K-mediated growth. We found that the orb2 protein can not only enhance dS6K-mediated growth, but above a certain threshold, it can also suppress it. By including orb2 in the TOR/S6K signaling pathway, we add another level of complexity to this model of translational regulation.

The IP3K1 gene from *Drosophila* is highly homologous to its mammalian counterpart, IP3KA. IP3KA and IP3KB activity both appear to be important for maintaining the signaling events that result in phosphorylation of S6K, and demonstrate the importance of IP_3, IP_4, and calcium signaling in growth-factor and amino-acid signaling through mTOR.

By pursuing the mechanism by which the gene products exert their effect on the S6K/TOR pathway, we hope to understand more about the conservation of this pathway and ultimately new clues for the treatment of disease. For example, both of these candidate genes have a role in the brain, and potentially in pathological conditions of the brain, such as Alzheimer's disease [242-245], and prion diseases [169]. Both are present in dendritic spines, morphological specializations that protrude from the main shaft of dendrites. Most excitatory synapses in the mature mammalian brain occur on spines [148, 246-249]. It is entirely possible that by understanding more about the interactions of these proteins with the TOR/S6K pathway, which is

gaining importance in the field of neuroscience [250-253], we may open new avenues for the treatment of neurological conditions.

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APPENDIX I:

MONTAGNE *ET AL* ; MANUSCRIPT IN PREPARATION

The Ligand-Binding Domain of the Nuclear Receptor DHR3 Modulates dS6K-Dependent Growth

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ABSTRACT

The nutrient and insulin signaling pathways play a central role in orchestrating cellular growth. These pathways also integrate extrinsic and intrinsic inputs, including activity mediated by nuclear receptors and their cognate ligands. To identify new regulators involved in the S6Kinase nutrient response we have used
a sensitized phenotype for a gain-of-function genetic screen in Drosophila. We found that the nuclear receptor DHR3, known to coordinate metamorphosis, is also involved in the control of cell growth. We have demonstrated that this effect
is, at least in part, mediated by regulating dS6K activity but not expression. Strikingly, we observed that the ligand-binding domain of DHR3 is required and sufficient to control this growth process. We also obtained data supporting the existence of a natural DHR3 protein lacking its DNA-binding-domain. Our study in *Drosophila* provides the first molecular evidence linking the dS6K nutrient response and a nuclear receptor signal.

INTRODUCTION

During development, the growth process (i.e. increase in cell mass) arrests when organs reach their appropriate size {Conlon, 1999 #99}. However, to maintain homeostasis in adults, some cells are stimulated to grow. Also, perturbation of cell growth can occur in pathologies such as cancers or obesity. This indicates that the regulatory system controlling growth can potentially be activated in quiescent cells of several adult organs.

The *Drosophila* genetic system has been successfully used to investigate the molecular mechanisms underlying growth {Edgar, 2006 #74}. These studies emphasized the central role of the insulin and nutrition signaling network that is conserved throughout evolution {Montagne, 2001 #5}. The identification of regulators that affect growth, either in a negative or a positive manner, significantly contributed to the understanding of this network both in *Drosophila* and in mammal. Binding of insulin or of a related peptide to its cognate receptor induces a kinase cascade that results in the recruitment of the protein kinase B (PKB) at the membrane and thereby its subsequent activation {Manning, 2007 #233}. In this process, the phospho-inositide-3-kinase (PI3K) class I converts phosphatidylinositol 4,5-bisphosphate (PIP2) in phosphatidylinositol 3,4,5-triphosphate (PIP3) constituting a docking site for PKB. Conversely, the tumor suppressor PTEN (Phosphatase and Tensing homolog on chromosome 10) catalyzes the opposite reaction. In *Drosophila*, activation of PKB provokes translocation of the transcriptional repressor, foxo, to the cytoplasm {Puig, 2006 #116}, and degradation of the tuberous sclerosis complex 2 (TSC2), {Gao, 2002 #27; Potter, 2002 #40}. TSC2 heterodimerizes with TSC1 {Potter, 2001 #37; Tapon, 2001 #144; Gao, 2001 #26} to inhibit the protein kinase target of rapamycin (TOR) through inactivation of the GTPase Rheb {Saucedo, 2003 #128; Zhang, 2003 #62; Garami, 2003 #185}. Downstream of TOR, insulin stimulation provokes activation of S6K, which in turn phosphorylates the ribosomal protein Rps6 {Jeno, 1988 #236}{Jefferies, 1997 #242}. Nonetheless, TSC2 inactivation mediated by PKB is controversial, as we and others demonstrated that the nutrient response and insulin signaling operate on parallel pathways to govern drosophila growth {Radimerski, 2002 #4; Radimerski, 2002 #3; Colombani, 2003 #1; Dong, 2004 #132}. That insulin signaling and PKB play a role in the S6K activation pathway mostly relies on over-expression experiments and utilization of inhibitors such as wortmanin that blocks PI3K activity. However, the discovery that Vps34, a class III PI3K, can regulate S6K in a nutrient-dependant manner and is also wortmanin sensitive {Nobukini, 2004 #31}{Byfield, 2005 #232}, may at least in part explain this controversy.

The molecular mechanisms that integrate growth, nutrition and morphogenesis remain largely unexplained. Indeed, regulators distinct from components of the nutrition and insulin signaling networks have been implicated in the growth process that underlies development and cancer progression {Vidal, 2006 #162}.

In mammals, these include intermediates in morphogenetic signaling, such as TGFβ, hedgehog or Wnt family members {Sancho, 2004 #163}, as well as nuclear receptors (NRs) whose activity is regulated upon binding of their cognate ligands {Singh, 2005 #164}. Almost 50 NRs are present in human. The ligand is known for more than half of them, whereas the others are referred to as orphan receptors. *Drosophila* contains 18 NRs {King-Jones, 2005 #80}, but the ligand is identified for only the ecdysone receptor {Koelle, 1991 #214}; the RXR homologue, Ultraspiracle, which binds to juvenile hormone JH III {Jones, 2006 #166} ; and E75, which has recently been shown to interact strongly with a heme prosthetic group {Reinking, 2005 #90}. This latter interaction is necessary to stabilize the E75 protein and is critical to counteract, in a gas-dependent manner, the transcriptional activity of DHR3, another *Drosophila* NR. Together, these NRs orchestrate developmental processes that occur during embryonic, larval and pupal life. Although unlikely, it is possible that these regulatory modules do not require the insulin/nutrition signaling network. More likely is the possibility that the molecular links between these systems have simply not been identified yet. In favor of the latter idea, recent publications revealed that ecdysone antagonizes insulin signaling {Colombani, 2003 #1}{Mirth, 2005 #168}{Caldwell, 2005 #167}. The negative input provoked by ecdysone is relayed at the fat body (FB, diffuse organ with storage and humoral functions) to protect against cytoplasmic inactivation of foxo. The Drosophila life cycle comprises larval stages where endoreplicative tissues assume the physiological functions. Within larvae and pupae, imaginal discs grow and proliferate prior to the differentiation into adult structures that occurs at late metamorphosis. Discs are subdivided into compartments that constitute the growth units {Garcia-Bellido, 1976 #172}. In this context, overexpression of the *Drosophila* S6K homologue (dS6K) within the developing dorsal wing compartment induced a bending down of the adult wing {Montagne, 1999 #8}. Consistent with dS6K playing a positive role in cellular growth, this phenotype is due to an increase of the size of the dorsal wing blade. As this phenotype can vary with respect to the level of dS6K activity {Radimerski, 2002 #4} {Barcelo, 2002 #220}, it has been retained for a gain-of-function genetic strategy to identify new components that potentially regulate dS6K activity. In this way, we have found that the NR DHR3 acts as a positive regulator of dS6K activity.

RESULTS

Screening for dS6K modulators

Overexpression of dS6K within the developing dorsal wing compartment (using an *apterous*-Gal4 [*ap*-Gal4] driver), induced a bent-down wing in the adult (Figure 1A,B) due to a moderate overgrowth of the dorsal wing blade {Montagne,

1999 #8}. Congruent with PDK1 being the S6K T-Loop kinase {Alessi, 1998 #178; Pullen, 1998 #177}, we further observed that this bending-down was enhanced by co-expression of the drosophila PDK1 (DPDK1), whereas over-expression of DPDK1 alone was without noticeable consequence {Radimerski, 2002 #4}. This epistatic interaction demonstrated that the intensity of the bent-down wing phenotype was modified with respect to the dS6K activation status. Therefore, we made use of this sensitized phenotype in a gain-of-function genetic strategy to identify new components with the potential ability to regulate dS6K activity Interestingly, overexpression of an active form of the mammalian S6K ($S6K1_{dE/D3E}$) {Dennis, 1996 #181} induced a bent-down wing phenotype identical to that induced by dS6K (Figure 1 B,C). Like dS6K, co-expression of $S6K1_{dE/D3E}$ and DPDK1 led to an enhancement of the bent-down wing, but not to the extent observed with dS6K (data not shown). This differential phenotypic interaction may be a hallmark of specificity, as dS6K, unlike $S6K1_{dE/D3E}$, is the genuine target for DPDK1 {Radimerski, 2002 #4; Rintelen, 2001 #16}. These differential effects were utilized to improve the selectivity of the gain-of-function screen (see below).

Overexpression of About 5000 Enhancer-Promoter (EP) bi-directional insertions {Reiling, 2004 #32} were induced together with dS6K in the developing dorsal wing compartment, and the dS6K-dependent bent-down wing phenotype was monitored for enhancement or suppression. To further restrict their specificity, about 1000 EP lines that displayed striking phenotypes possibly unrelated to dS6K function were then retained and analyzed for their effects when induced alone with the *ap*-Gal4 driver. This subsequent screen was designed to eliminate nonspecific modulators that, alone, induced a phenotype identical to that obtained in combination with dS6K (data not shown). Eventually, 220 lines were tested again to precisely compare their effects when induced alone, in combination with dS6K or with the active $S6K1_{dE/D3E}$. The enhancers identified in the screen could be separated into two subsets with respect to their interaction with dS6K and $S6K1_{dE/D3E}$. One subset enhanced the bent-down wing phenotype to the same extent in combination with either kinase, while the other subset displayed a differential effect that was stronger in combination with dS6K than with $S6K1_{dE/D3E}$ (data not shown). As DPDK1 belongs to the latter group, we reasoned that candidate enhancers that interacted differentially with dS6K and $S6K1_{dE/D3E}$ were more likely to affect dS6K signaling, whereas those with no differential response might be involved in the general process of wing formation.

Identification of DHR3 as a genetic modulator of dS6K-dependent growth

As a result of the screen for dS6K modulators, 57 clear enhancer lines were retained and 45 of them were molecularly mapped. Ten lines corresponded to a “hot spot” for insertions within the regulatory region of DPDK1, which confirms the relevance of the genetic screen. In addition, two strong enhancer lines (EP12.218, EP23.014) were shown to be inserted within the locus of the

NR DHR3 {Koelle, 1992 #186}; these lines are hereafter referred to as EP-DHR3. Induction of an EP-DHR3 alone using the *ap*-Gal4 driver was without visible consequence, while the combination with dS6K induced a strong enhancement of the bent-down wing phenotype (Figure 1A,B,D,E). The EP-element used in the screen contained two UAS promoters that can direct transcription in opposite directions {Reiling, 2004 #32}. Therefore, these two EP-DHR3s could potentially induce transcription of either DHR3 or a histidine-decarboxylase (CG3454). As the UAS promoter driving the latter was flanked by lox sequences, the Cre recombinase was induced to excise this promoter. When co-induced with dS6K by the *ap*-Gal4 driver, this novel unidirectional EP-DHR3 element retained the ability to enhance the bent-down wing phenotype (Figure 1J), which rules out the possibility that the histidine-decarboxylase is responsible for this genetic interaction.

Both EP elements were inserted within the first intron (see below) of the DHR3 gene and, when induced, could potentially either interfere with, or trigger DHR3 expression. To address this issue, inducible RNA-interference lines (DHR3-dsRNAi) were generated to counteract DHR3 expression. Gal4 induction of this DHR3-dsRNAi within the developing dorsal wing compartment caused a bent-up wing phenotype (Figure 1G), indicating that normal growth is affected when DHR3 expression is suppressed. Also, when co-induced with EP-DHR3 and UAS-dS6K, DHR3-dsRNAi suppressed the bent-down wing phenotype (Figure 1H,K). Therefore, the genetic interaction with dS6K is due to DHR3 overexpression, and not to interference with its normal expression.

EP-DHR3 also enhanced the bent-down wing phenotype when co-expressed with the $S6K1_{dE/D3E}$ kinase (Figure 1C,F), but to a lesser degree than when co-expressed with dS6K (Figure 1, compare E to F). This suggests that DHR3 may, like DPDK1, act on dS6K activation. Consistent with this interpretation, DHR3- dsRNAi suppressed the bent-down wing phenotype induced by either dS6K or $S6K1_{dE/D3E}$. This suppression was, however, always stronger for dS6K than for $S6K1_{dE/D3E}$ (Figure 1, compare H to I). Altogether, these differential effects at the bent-down wing indicate that $S6K1_{dE/D3E}$ is less sensitive than dS6K to increases or decreases in the dosage of DHR3. As determined for the DPDK1 interaction, this argues in favor of DHR3 playing a specific role in dS6K-dependent growth. dS6K resides in the nutrient response pathway, in parallel to the insulin signaling pathway {Radimerski, 2002 #3; Radimerski, 2002 #4}. This was demonstrated previously by the finding that dTSC1/2 is the dS6K-specific growth suppressor that acts in parallel to the dPTEN/dPI3K module. Both dPTEN and the heterodimer dTSC1/2 induced a growth defect in the adult eye when overexpressed in the developing eye. As another indicator of specificity, co- expression of dS6K counteracted the growth defect due to dTSC1/2, but not the one due to dPTEN (data not shown). To further determine in which of these pathways DHR3 resides, the EP-DHR3 was induced together with either dPTEN or the heterodimer dTSC1/2 in the developing eye. EP-DHR3 induction alone did not modify eye development (Figure 2A,B). In

contrast, induction of EP-DHR3 could suppress the growth defect due to over-expression of the heterodimer dTSC1/2 (Figure 2, compare D to C). Consistent with DHR3 acting in the nutrient response pathway in parallel to the insulin receptor pathway, the EP-DHR3 did not counteract the growth defect due to DPTEN over-expression (Figure 2, compare F to E). This further argues for a tight link between DHR3 and the dTSC/dS6K pathway.
DHR3 has previously been reported to be an NR that plays a central role in coordinating metamorphosis {Lam, 1997 #191; White, 1997 #189}. In addition, the bent-up wing phenotype due to DHR3-dsRNAi in the dorsal wing compartment indicates that DHR3 is also involved in regulating cellular growth. Both EP elements are inserted within the huge first intron of DHR3 (Figure 3A and data not shown), and therefore in the absence of Gal4 induction, might interfere with DHR3 expression. Indeed, these EP lines failed to complement previously reported DHR3 mutants (data not shown). In contrast to these previously described DHR3 mutants that are lethal during early development, homozygous and trans-heterozygous EP-DHR3 insertions were semi-lethal, indicating that they correspond to hypomorphic DHR3 mutants. The few adult escapers emerged with about 2 days delay, and displayed female sterility. These phenotypic features have also been reported for other mutants that affect growth, which confirms that, in addition to coordinate metamorphosis, DHR3 also plays a role in controlling growth. Moreover, ubiquitous suppression of DHR3 through dsRNAi can provoked either larval death or developmental delay (data not shown). Interestingly, suppression of DHR3 targeted to the FB, using the pumpless-Gal4 driver, result in a 2 days delay at metamorphosis onset (data not shown). As the FB is central in coordinating growth with nutrient availability {Britton, 1998 #45; Britton, 2002 #56; Colombani, 2005 #93}, this latter result further argues for DHR3 acting in the nutrient response pathway and thus close to dS6K.

The DHR3 protein that interacts with dSK6 lacks a DNA-binding domain
DHR3 is an NR that typically comprises an N-terminal DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) separated by a linker domain {King-Jones, 2005 #80}. Both EP elements are inserted within the first intron of the DHR3 gene upstream of the second exon that contains the reading frame for the DBD (Figure 3A). All of the DHR3 products reported to date are translated from AUGs located in various alternative upstream first exons. To identify the DHR3 gene product responsible for the interaction with dS6K, RACE PCR has been performed using wild-type and EP-DHR3 larvae ubiquitously induced by a *daughterless*-Gal4 driver (*da*-Gal4). The transcript identified for the latter was a splice variant from the EP to the DHR3 second exon, lacking an AUG initiator codon upstream of the sequences encoding the DBD (R-EP in Figure 3A and supplementary data). In addition to previously described mRNAs, RT-PCR experiments using wild-type larvae allowed the identification of a novel DHR3 transcript (RS in Figure 3A and supplementary

data). This transcript does not contain a first alternative exon, and most likely expresses a DHR3 protein that is devoid of DBD, as the most proximal AUG was located beyond the DBD- coding sequence (Figure 3A and supplementary data).
To identify which DHR3 protein is most likely to be responsible for the genetic interaction with dS6K, three different UAS constructs were generated, allowing the translation of various DHR3 CDS that differ in their N-terminal polypeptide sequence. Two of them contain the DHR3 DBD and correspond to the previously described PA and PB gene products expressed from the RA and RB transcripts respectively (Figure 3A). The other one lacks an upstream translational initiator codon, but retains the AUG that potentially allows the translation of a DBD- lacking DHR3 protein, hereafter called as *S*DHR3 (Figure 3A and supplementary data). When induced by the *ap*-Gal4 driver, both lines expressing a DBD- containing DHR3 protein (PA or PB) result in lethality (data not shown). The lethality is certainly not due to the expression in the developing dorsal wing compartment, but rather to expression in other tissues; for example, the *apterous* promoter is known to be active in the brain {Herzig, 2001 #213}. Nonetheless, all other Gal4 drivers tested induced some lethality (data not shown). Conversely, in agreement with the RACE PCR results, induction of the UAS-*S*DHR3 line with the *ap*-Gal4 driver was not lethal and phenocopied the enhancement observed with EP-DHR3 (Figure 3C, E). Moreover, co-induction of DHR3-dsRNAi led to a loss of the phenotypic interaction (Figure 3, compare G to E), which definitively confirmed that *S*DHR3 was the gene product responsible for the genetic interaction with dS6K. Interestingly, induction of UAS-*S*DHR3 alone was sufficient to induce a bent-down wing (Figure 3B,D), and this phenotype could be almost completely reverted when DHR3-dsRNAi was co-induced (Figure 3, compare F to D). Hence, a DHR3 gene product lacking its DNA-binding domain is able to induce growth and to cooperate with dS6K in that process.

DHR3 regulates dS6K activity

The wing bent-down due to *S*DHR3 driven by *ap*-Gal4 (Figure 3D) indicates that *S*DHR3 is sufficient to drive compartment growth. Therefore, an experiment on a nil $dS6K_{l-1}$ mutant background (some of these flies survive to adulthood {Montagne, 1999 #8}) was performed to determine whether dS6K is required for induction of the bent-down wing due to *S*DHR3 driven by *ap*-Gal4. Interestingly, a clear suppression of the bent-down wing phenotype was observed in the absence of dS6K (Figure 4, compare B to A), which indicates that overgrowth induced by *S*DHR3 is dependent on the presence of dS6K.
To further investigate the relationship between DHR3 and dS6K, kinase activity was monitored in protein extracts from larvae where DHR3 expression was ubiquitously suppressed through dsRNAi, using either a daughterless-Gal4 or an actin-Gal4 driver. As depicted in Figure 4C, interfering with DHR3 expression induced a severe reduction in dS6K activity. The dS6K protein level

was not affected (Figure 4D), ruling out a transcriptional effect of DHR3 on dS6K expression. Together these results indicate that *S*DHR3 is able to induce growth by modulating the kinase activity of dS6K but not its expression.

In search of a genuine DBD-lacking DHR3 protein

To determine whether a DBD-lacking DHR3 protein exists naturally, a rabbit antiserum to DHR3 was produced using peptides that correspond to sequences downstream to the first AUG following the DBD coding sequence (supplementary data). Both EP-DHR3 and the UAS-DHR3 lines (Figure 3A) were induced by a heat-shock-Gal4 driver (HS-Gal4) during larval life. Although larvae expressing a DBD-containing DHR3 protein were dead within a day after heat shock, it was possible to prepare larval extracts a few hours after heat shock and to analyze them by Western blotting. The UAS-*S*DHR3 and both EP-DHR3 produce a protein that migrates to the same position, corresponding to the expected size for a DHR3 polypeptide lacking its DBD (Figure 5A). In agreement with the genetic data showing that, by itself, UAS-*S*DHR3 driven by *ap*-Gal4 is sufficient to induce a bending down of the wing, the amount of protein was much higher with UAS- *S*DHR3 than with EP-DHR3 (Figure 5A). Therefore, the expression level of *S*DHR3 induced by HS-Gal4 correlates with the wing phenotype observed with *ap*-Gal4 driver.

The larvae that expressed DBD-containing DHR3 constructs (RA and RB) displayed differential results (Figure 5A). DHR3-RA produced a single protein that migrated at the expected molecular weight for a DBD-containing DHR3. In contrast, DHR3-RB allowed the translation of 2 polypeptides; one of them migrating roughly to the position of a DBD-containing DHR3, while the other polypeptide migrated to the position of *S*DHR3 (Figure 5A). Therefore, the DHR3-RB transcript permitted the translation of 2 proteins that most likely differ in whether or not they contain the DBD of DHR3. The levels of expression of these two different DHR3 polypeptides are very high and reproducible, which rules out that the lower one might be a degradation product. However, we can not conclude whether it corresponds to alternative translational initiation or post- translational processing of a DBD-containing DHR3 protein.

To better detect the genuine proteins, extracts were then prepared during prepupal stages, as DHR3 has previously been described to be highly expressed at the onset of metamorphosis in response to ecdysone signaling {Koelle, 1992 #186}. Although purified, the antibody might recognize some unrelated DHR3 protein. To unambiguously identify the bona fide DHR3 protein, extracts were also made from prepupae that ubiquitously expressed a DHR3-dsRNAi using a *da*-Gal4 driver. When induced, the DHR3-dsRNAi often resulted in larval lethality, although development sometimes proceeded with some delay (data not shown).

Western blot analysis revealed 2 bands that decreased upon DHR3-dsRNAi expression (Figure 5B), suggesting that 2 different DHR3 products are expressed in the prepupa. The higher band was at the position of the DBD-

containing DHR3, whereas the lower band was at the position of the DBD-lacking DHR3. It is unlikely that the lower band is a degradation product of a full-length DHR3 protein, since both proteins were observed in 5 distinct experiments with always the same ratio. This protein is most likely produced from the RB transcript that allows the translation of 2 distinct DHR3 polypeptides (Figure 5A). Alternatively, the *S*DHR3 protein might be translated from the messenger species devoid of upstream AUG, which has been identified by RACE-PCR experiments (see above and Figure 3A), if this mRNA is not a degradation product.

The antibody has then been used to detect over-expressed *S*DHR3 protein directed by the engrailed-Gal4(en-Gal4) and ap-Gal4 drivers, which are active in the posterior and anterior wing-disc compartments respectively. Activation of UAS-*S*DHR3 by the en-Gal4 driver was associated with a UAS-GFP, allowing double immuno-staining that revealed a perfect match of GFP and *S*DHR3 expression (Figure 5C, D, E). When induced by the ap-Gal4 driver, both the UAS-*S*DHR3 and the EP-DHR3 provoke an increased of the immuno-staining to DHR3 restricted to the dorsal compartment (Figure 5C, G, H, I). However, the signal was much higher with UAS-*S*DHR3 than with EP-DHR3 (Figure 5 compare G to I). Since UAS-*S*DHR3 but not EP-DHR3 provokes a bent-down of the wing when induced alone by ap-Gal4 (compare Figure 1D to 3D), these results indicate that *S*DHR3 can induce growth in a dosage-dependent manner.

Finally, the antibody was used for detection of the genuine DHR3 protein. To guarantee the specificity of the immuno-staining, flip-out clones directing DHR3- dsRNAi were generated, and a UAS-GFP was used to positively label these clones. A very weak but uniform staining could be detected in both imaginal discs and FB from mid-third instar larvae (data not shown). This staining may reflect some unrelated background since we could not observe a significant decrease in GFP-positive flip out clones that direct DHR3-dsRNAi (data not shown). In contrast, the staining observed in prepupal discs was stronger and was decreased in flip-out clones, although not totally abolished (Figure 5J,K). Again, the remnant staining may reflect unspecific background or the moderate effect of dsRNAi in repressing DHR3 expression (Figure 5B). Clones displaying a decrease of this specific staining could be detected in all imaginal discs from prepupae (data not shown), indicating that DHR3 is widely expressed at this stage. In summary, the antibody is able to detect the genuine DHR3 protein in prepupae but not in larval tissues. This might suggest that DHR3 is not expressed in larvae. However, the developmental delay at metamorphosis onset due to either ubiquitous or FB restricted DHR3-dsRNAi suggests that a DHR3 product is required for larval development. Nonetheless, this expression might be very low, stage specific or restricted to certain tissue, since we were unable to detect DHR3-specific staining in endoreplicative or imaginal tissues of third instar larvae.

Generation of LBD-specific DHR3 mutants

An EMS revertant screen was performed to gain further insights into the protein domain of DHR3 required for its growth function with respect to dS6K. EP-DHR3 males were fed EMS and crossed to females bearing the UAS-dS6K and the *ap*- Gal4 driver. In the offspring, flies that had lost the ability to enhance the bent- down wing phenotype were selected for further analysis. About 50,000 individual flies were screened to eventually establish 8 lines that had clearly lost their ability to cooperate with dS6K in producing the bent-down wing phenotype (Figure 6A,B,C). After remobilization of EP-element, only 2 of them displayed homozygous lethality and did not complement previously described DHR3 mutants (Figure 6G). Nonetheless, both homozygous viable and lethal revertants were used to prepare DNA and to sequence the DHR3 coding region. None of the homozygous viable revertants displayed DNA mutations. In contrast, the two DHR3-noncomplementation lines contained a stop codon at position 243 and 284, hereafter referred to as $DHR3^{K243X}$ and $DHR3^{W284X}$ (Figure 6D and supplementary data).
Remobilization of the EP element may provoke imprecise excision, creating a putative deficiency within the DHR3 locus. Hence, for a deeper phenotypic analysis, in order to ascertain that the phenotype could not be due to an accidental deficiency, several lines for each DHR3 mutation were generated from independent remobilization events. Therefore, eight and ten independent lines for $DHR3^{K243X}$ and $DHR3^{W284X}$, respectively, were used for additional genetic tests. All of them were homozygous lethal, failed to complement each other, and neither complemented the previously described $DHR3^{G60S}$ and $DHR3^{R107G}$ mutants that affect the DBD (data not shown). All of these mutants combination die as embryo indicating that the LBD is essentially required for the transcriptional function of DHR3. However, searching among thousands of larva, it has been possible to find very few $DHR3^{K243X}$/$DHR3^{W284X}$ mutants that survive until second larval instar. These larva were then use to perform kinase assays for dS6K. Congruent with the assay from DHR3-dsRNAi extracts (Figure 4C,D) a significant drop of activity but not of dS6K expression was observed (Figure 6E, F). This result indicates that the LBD of DHR3 is required to regulate dS6K activity, and further suggests that a specific ligand binds DHR3 to regulate its functions.

The ligand-binding-domain of DHR3 is required for cell-autonomous growth

To deeply analyze the function of DHR3 with respect to cellular growth, both LDB mutants were fused to an FRT and analyzed for cell autonomous function. First, these FRT-associated mutations were recombined in the eye disc using the eyeless promoter to drive the flipase recombinase during the entire process of eye development. As the FRT chromosome arm in front of the DHR3 mutation contained a homozygous cell-lethal mutation {Oldham, 2000 #6}, the recombined sister cells, wild type for DHR3, were eliminated during development leading to eyes mostly made up of homozygous DHR3 mutant

cells. With both DHR3$_{K243X}$ and DHR3$_{W284X}$ mutations, a moderate but significant reduction of the eye size was observed (Figure 7A-C) indicating that DHR3 controls growth in a compartment autonomous manner. The flipase recombinase was also induced by heat shock. Various homozygous markers were used to identify homozygous DHR3 mutant cells. Within third instar imaginal discs, clones analyzed 3 days after heat shock did not show visible growth defect as compared with the wild-type sister clone (data not shown). As well it was not possible to observe significant cell size reduction by FACS analysis (data not shown). In contrast, using a yellow marker to look at adult tissues, homozygous DHR3 mutant clones, identified by their yellow color, displayed a significant reduction in size (Figure 7D). As the hair corresponds to a single cell, we can conclude that this mutant cell was reduced in size. These DHR3 homozygous mutant bristles were also affected in their orientation, as compare to the surrounding bristles. This phenotypic missorientation was also observed for the ommatidia-associated bristles (insets in Figure 7A-C) and might reflect one of the pleiotropic functions of DHR3. Together, these observations indicate that DHR3 acts in a cell-autonomous manner for various cellular functions. In particular, we have demonstrated here that the control of growth requires the ligand-binding domain, suggesting that DHR3 activity depends on binding of a specific ligand.

DISCUSSION

How DHR3 does regulate growth and dS6K activity?

Using the powerful drosophila genetic system in a gain-of-function strategy, we have fond that the NR DHR3 acts positively on dS6K activity. DHR3 was previously known to coordinate metamorphosis {Lam, 1997 #191; White, 1997 #189} and we demonstrated here that it also controls cellular growth in a cell autonomous manner. Precisely, we have found a new DHR3 polypeptide devoid of DBD (*S*DHR3), and demonstrated that this polypeptide is sufficient to genetically interact with dS6K. When strongly over-expressed, *S*DHR3 can drive cellular growth alone. This process requires dS6K, and we further demonstrated that disruption of DHR3 provokes a drop of dS6K activation but not expression.

The DBD of NRs is typically made off two Zing-finger, with the first one being critical for the specificity of DNA binding {Umesono, 1989 #215}. NRs lacking DBD have been previously reported. In drosophila, the E75A an E75B polypeptides, which differ in their amino-terminal region, may physically interact with DHR3 to repress transcription {White, 1997 #189}{Reinking, 2005 #90}. In contrast to E75A, E75B lacks one of the 2 zing-fingers that together are necessary to form a functional domain to bind DNA {Bialecki, 2002 #83}. E75B however, retains the property to interact with DHR3, thereby modulating its transcriptional activity in a gas responsive manner {Reinking, 2005 #90}. As

well
in mammals, the NR, SHP, is totally devoid of DBD and interacts with other NRs to inhibit their transcriptional activity {Seol, 1996 #205}. As NRs often make homo- or hetero-dimer to regulate transcription {McKenna, 2002 #206}, it is conceivable that *S*DHR3 physically interacts with another NR that provides the DNA-binding function. In that case, *S*DHR3 protein would modulate transcription, either to repress a dS6K-repressor or to activate a dS6K-activator. If they exist, extensive analysis through micro-array would help to identify these potential relaying regulators. Nonetheless, we can exclude that *S*DHR3 behaves as a dominant-negative component to the full-length DHR3, since *S*DHR3 over- expression provokes growth, while dsRNAi to DHR3, in the opposite, inhibits growth. Two nuclear receptor family members have been reported to heterodimerize with DHR3; and this could at least in part explain the growth related function of DHR3. As mentioned above, E75 polypeptides negatively modulate DHR3 activity {Reinking, 2005 #90}. Therefore, *S*DHR3 might titter out these polypeptides, thereby allowing an increase of the transcription induced by the endogenous DHR3 bound to its DNA targets. The second nuclear receptor know to dimerize with DHR3 is the ecdysone receptor (EcR). Both DBD-lacking and full length DHR3 proteins have been show to dimerize with the Ecdysone receptor to repress transcription {White, 1997 #189} and thus, could counteract growth-negative regulation of Ecdysone signaling {Colombani, 2005 #93}. However, this is in contradiction with the observation that dsRNAi disruption of DHR3 results in a growth defect. Alternatively, it is tempting to speculate that *S*DHR3 activates dS6K through a nongenomic process. Such effects do not require DNA-binding and were first described as processes for which, the delay of the specific response was not compatible with a transcriptional mediation {Losel, 2003 #210}. Nongenomic effects typically occur in the few minute following addition of the cognate ligand and are resistant to transcriptional inhibitors. Experimentally, we are unable to challenge this issue, not only because we use genetic events early induced to eventually observe the consequence once development is achieved, but because the DHR3 ligand is yet unknown. This hypothesis is however attractive, since in mammals, the Vitamin D Receptor (VDR) has been shown to modulate S6K1 phosphorylation in a none- genomic manner {Bettoun, 2002 #209}{Bettoun, 2004 #207}{Lal, 2005 #244}.
Identification of all the potential partners for *S*DHR3 will be required to precisely determine in what extend, these molecular processes may explain how *S*DHR3 regulates growth.

Expression and function of DHR3 during larval life

We have shown that beside essential function during early development, DHR3 is required for larval growth, since ubiquitous dsRNAi to DHR3 induces either lethality or delay at metamorphosis onset. We also clearly demonstrated that the lack of the DHR3 LBD results in autonomous cell size reduction of adult

tissues. This effect is likely to result on the decrease of dS6K activity, as we previously demonstrated that dS6K controls cellular growth in a cell-autonomous manner {Montagne, 1999 #8}. The size reduction was however stronger in previously described dS6K mutant clones {Montagne, 1999 #8}, possibly because dS6K activity is partially suppressed but not abolished in DHR3 LBD-mutant larvae. Likewise, the undetectable protein level (unpublished results) in a dS6K P- element induced mutation P{PZ}S6K[07084]) is sufficient to partially restore the functional activity, since this dS6K mutation induced a moderate phenotype as compare to the nil mutation {Montagne, 1999 #8}. Nor FACS analysis of cells dissociated from wing discs, neither observation of imaginal discs, led to visible size or growth defect of DHR3 homozygous mutant clones analyzed in third instar larva (data not shown). This may suggest that DHR3 is not involved in cell- autonomous growth during larval life. Noteworthy, growth defects could neither be observed in dS6K clones of third instar larvae, unless they were induced 4 days before, during embryogenesis (unpublished results). Such a strong delay indicates that strong dS6K extinction is necessary to generate a visible phenotype in imaginal tissues during larval life. Hence the remnant dS6K activity retained in DHR3 mutant clones might be over a certain threshold that is sufficient to support a roughly normal growth in larva. Alternatively, since *S*DHR3 is almost undetectable in mid third instar larva, it is possible that it is not required for dS6K activation in imaginal tissues during the period of larval growth. This is however unlikely, since we have shown that dS6K activation drops in second instar DHR3 mutant larvae. Beside, DHR3 may also retain some systemic function, since DHR3-dsRNAi targeted to the FB also provokes a delay at metamorphosis, although we were unable to detect significant DHR3 protein expression in mid-third instar FB. As well, dS6K is involved in the systemic control of growth, as the defect due to FB disruption of the amino-acid transporter *slif* is override by overexpression of dS6K within the FB {Colombani, 2005 #93}.

In summary, both dS6K and DHR3 have a systemic and a cell-autonomous function in growth, the latter being difficult to distinguish before metamorphosis. Hence, it is possible that larval defect is mostly due to a systemic function of DHR3, whereas the phenotype observed in adults revealed a cell autonomous function occurring at metamorphosis.

Is there a ligand for DHR3?

Our data provide the first evidence that the LBD of DHR3 is required to achieve a function related to growth, although DHR3 participates in several additional functions as homozygous mutation of the DBD provokes embryonic death {Carney, 1997 #193}. Interestingly, the none-complementation between LBD and DBD mutants indicates that the LDB domain is also required for the essential function of DHR3 related to transcription. This strongly supports the notions that a ligand exists for DHR3 and that this ligand is required for DHR3

pleiotropic activities. Nuclear receptors that bind steroid hormones are high affinity receptors, whereas low affinity receptors bind ligands present in high concentration as food- provided lipids {Berkenstam, 2005 #234}. In light of a recent report, this ligand must be widely present since a fusion of the DHR3 LBD with the DBD of Gal4 is functionally active in transcription in many embryonic and larval tissues {Palanker, 2006 #76}. This heterologous Gal4-DBD/DHR3-LBD transcription factor is active
in tissues that provide basal nutrients in particular in a group of cells of the larval midgut that is essential for the transfer of nutrients into the hemolymph {Palanker, 2006 #76}. We previously demonstrated that dS6K activity is regulated by food- provided amino acids {Oldham, 2000 #6}. Therefore it is tempting to speculate that dS6K receive another nutrient input through the DHR3 ligand. The ligands for the mammalian homologues RORα and RORβ have been shown to be the cholesterol {Kallen, 2002 #212} and the long chain stearic fatty acid {Stehlin, 2001 #216} respectively. However, predicted models of structure indicate that the size of the ligand-binding pocket of DHR3 is smaller {Stehlin, 2001 #216}. Thus, DHR3 is likely to be a low affinity receptor for an abundant food-provided ligand. Nonetheless, it will be of fundament interest to further investigate this issue, as lipid metabolism and the TOR/S6K nutrient responsive pathway play a critical role in disease such as diabetes and cancers in humans.

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EXPERIEMENTAL PROCEDURES

Genetics

The following fly strains were used: *dS6K$_{l1}$* and *UAS-dS6K*, *ap-Gal4* {Montagne, 1999 #8}, *UAS-dTsc1/2* {Tapon, 2001 #144}, *UAS-dPTEN* {Huang, 1999 #39}, *pumpless-Gal4* {Colombani, 2003 #1}, ; *DHR3$_{G60S}$*, *DHR3$_{R107G}$* {Carney, 1997 #193}; *eyeless-Gal4* {Hauck, 1999 #221}; Cre-lox (**Hafen**); *daughterless-Gal4*, *actin-Gal4*, *engrailed-Gal4,UAS-GFP*, *HR46(K10308), FRT-42D,M(2)53, FRT-42D,P(y+)44B* (Bloomington stock center). Since *y+* and *w+* markers were used, all the experiments were performed in a *y,w* genetic background. About 5000 EPy+ independent insertion {Reiling, 2004 #32} were mated to ap- Gal4>UAS-dS6K virgin females and screened for enhancement and suppression of the bent-down wing phenotype.
For the EMS revertant screen, about 500 EP-DHR3 males were starved over night and then transferred on wet paper containing a mM EMS solution in 10 mg/ml sucrose. After one day these males were mated to about 1500 ap-

Gal4>UAS-dS6K virgin females. Flies were then transferred every day for egg laying. An estimation of 150.000 F1 flies were obtained; as both parental lines were balanced over a CyO chromosome, about 50.000 flies were screened for the reversion of the bent-down wing phenotype.

Molecular Biology

Location of the EP-insertions was performed as described {Reiling, 2004 #32}. To generate UAS-DHR3-dsRNAi, a PCR fragment spanning the DHR3 reading frame from Leu_{114} to Lys_{265} was cloned as described {Reichhart, 2002 #203}. Congruent results were obtained, by repeating the experiments with 2 other distinct UAS-DHR3-dsRNAi strains provided by H. Tricoire and the National Institute of Genetics (http://www.nig.ac.jp/). For RACE-PCR, poly A+ cDNA were obtained using RNAeasy kit and Oligotex mRNA purification (both from Qiagen) and then amplified with SMART TM RACE cDNA Amplification Kit (Clontech). 5' RACE to obtain endogenous cDNAs and the chimeric EP-DHR3 cDNAs followed a 2-step process : first using a DHR3-specific primer (catggtctgctgtggcgtcacggaggc) and universal primer mix, then by nested PCR using a combination of nested universal primer mix/ DHR3-specific primer (cggttgcgattaacacggtccaccac). UAS-dE/D3E and DHR3 cDNAs were clones in pUAST vector and injected as previously described {Montagne, 1999 #8}. the RA-cDNA was kindly provide by Carl Thummel; the RB-cDNA was obtained from DHRC; the RS transcript was artificially generated by truncation of the RA-cDNA, lacking AUG initiator codon upstream of the DBD coding sequences. To identify EMS point mutations, DHR3 coding sequences were PCR-amplified from genomic DNA of revertant flies. Fragments were then sequenced and search for double picks as compared to wild type genomic DNA. Identified point mutations were confirmed by independent repetition of the entire procedure.

Observation

Larval tissues were dissected, stained as previously described {Montagne, 1999 #8}, and observed on a Leica Sp2 confocal microscope. For SEM, flies were fixed by successive baths of increased ethanol concentrated solution up to 90%, and directly observed on an S-3000N HITACHI scanning-electron microscope.

Biochemistry

Protein extracts and Western blotting were performed as previously described {Oldham, 2000 #6}. For timing determination of prepupal stages, wondering larvae of the corresponding phenotype were collected and transferred to a new tube. Newly formed prepupae and late third instar larvae were collected to make protein extracts. In vitro dS6K kinase activity assay from second instar larval extracts were performed essentially as described {Oldham, 2000 #6} using histone H2B as substrate {Reinking, 2005 #90}. The antiserum to DHR3 has been produced by Eurogenetec Company.

The peptides $_{144}$ QMRAQSDAAPDSSYYD$_{159}$ and $_{209}$SADYVDSTTYEPRSTI$_{224}$ were used to immunize a rabbit. The specific antibodies were then affinity purified as previously described {Stewart, 1996 #219}.

FIGURE LEGENDS

FIGURE 1: DHR3 is a specific dS6K interactor. The *ap* promoter directs Gal4 expression within the dorsal compartment of the wing imaginal disc (A) to induce UAS-dS6K (B, E, H,J,K), UAS-mS6K$_{dE\text{-}D3E}$ (C,F,I), EP-DHR3 (D, E, F), UAS- DHR3-dsRNAi (G,H,I,K) and a unidirectional EP-DHR3 (J). The bending-down of the wing indicates a slight overgrowth of the dorsal compartment, whereas a bending-up reveals a slight growth defect of this compartment.

FIGURE 2: The EP-DHR3 counteracts dTsc-dependent growth suppression. The eyeless promoter directs Gal4 in the developing eye (A) to induce EP-DHR3 (B,D,E), UAS-dTsc1 and UASdTsc2 (C, D) and UAS-dPTEN (E, F).

FIGURE 3: The DHR3 protein that genetically interacts with dS6K does not contain DNA binding-domain. (A) The locus of DHR3 is represented at the top with the 2 EP insertions (arrows); at least 4 different transcripts have been reported (RA, RB; RC and RD are not represented here). RS is a putative novel transcript whose first AUG is located beyond the DBD coding sequences (grey box). The various polypeptides (black and grey boxes) mostly differ in their N- terminus; RS and R-EP potentially allow the translation of the *S*DHR3 product. (B-G) Gal4 is expressed in the developping dorsal wing imaginal discs (B), to induce dS6K (C), *S*DHR3 (D) or both (E). Induction of a dsRNAi to DHR3 suppresses the bending down wing phenotype due to *S*DHR3 alone (F) or in combination with dS6K (G).

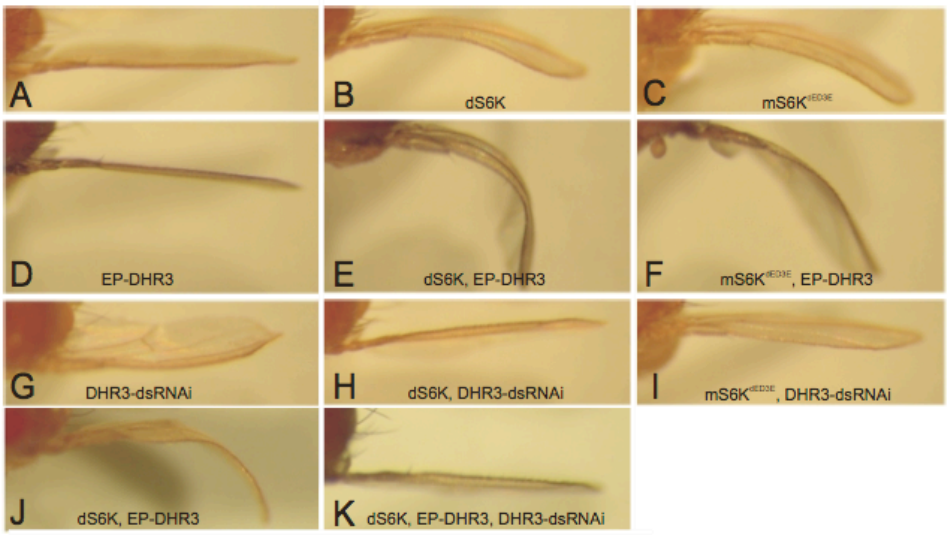
FIGURE 4: DHR3 is a positive regulator of dS6K activity. (A, B) *S*DHR3 is over-exppressed in the developing dorsal wing compartment; this induces a bent-down of the adult wing (A) that is suppressed in dS6K mutant escaper (B). (C) Ubiquitous induction of a double-stranded interfering RNA to DHR3 (RNAi) using either a daughterless-Gal4 (da>) or an actin-Gal4 (ac>) drivers, provokes a drop of dS6K activity, as compared to control drivers alone (da, ac); (D) Western-blot to dS6K indicating that dsRNAi to DHR3 has no effect on dS6K expression; (E) Western-blot to α-tubulin as a loading control.

FIGURE 5: Immuno-detection of DHR3 proteins. (A) western-blotting using a rabbit antibody to DHR3 with protein extracts from heat shock induced UAS lines expressing the RA, RB transcripts, or the EPs (E1 and E2); *S*DHR3 is produced from a UAS construct whose most proximal AUG is located beyond the DBD (see Figure 3 for the various transcripts). The arrows at the left indicate the position of the DBD-containning (high) and DBD-lacking (low)

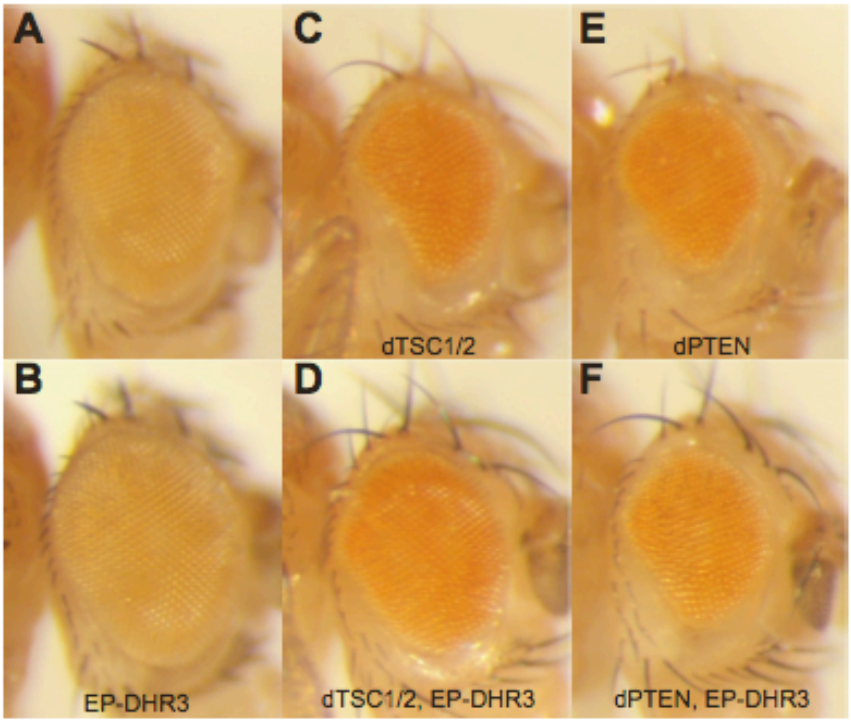
DHR3 proteins. (B) western- blotting to the endogenous DHR3 protein in late third instar larvae (L3), prepupae (pp), and prepupae expressing a dsRNAi to DHR3 (RNAipp); *S*DHR3 is as in (A) but the membrane is over-exposed to detect genuine DHR3 proteins. (C-K) Wing
imaginal discs stained with DAPI (C, F, H), antibody to DHR3 (D, G, I, K) and antibody to GFP (E, J). (C-E) Over expression of UAS-*S*DHR3 and USA-GFP in
the posterior compartment, using an engrailed-Gal4 driver. (F, G) Over expression of UAS-*S*DHR3 in the dorsal compartment using the ap-Gal4 driver. (H, I) Induction of the EP-DHR3 in the dorsal compartment using the ap-Gal4 driver. (J, K) Flip-out clone in prepupal wing imaginal disc expressing a dsRNAi to DHR3 and a UAS-GFP; the clone was induced 3 days prior dissection; note the visible decrease of DHR3 staining (K) in the clone labeled by GFP (J), indicating that DHR3 is expressed in this tissue.

FIGURE 6: Generation of EMS mutations in the LBD of DHR3. (A-C) The ap-Gal4 driver induces both the UAS-dS6K and the EP-DHR3 transgenes; this provokes a bent down of the wing (A) that is lost when the EP directs the expression of the EMS DHR3 mutations (B, C) generated in the revertant screen. (D) Structure of the full-length DHR3 protein showing previously described DBD mutants (G60S, R107G) and the EMS-mutants that disrupt the LBD (K243X, W284X); the light and dark grey boxes represent the DBD and the LBD respectively. Measurement of dS6K activity (E) and corresponding western-blot to dS6K (F) in the trans-heterozygote LBD-mutant combination (LBD), as compare to heterozygous control (Co).

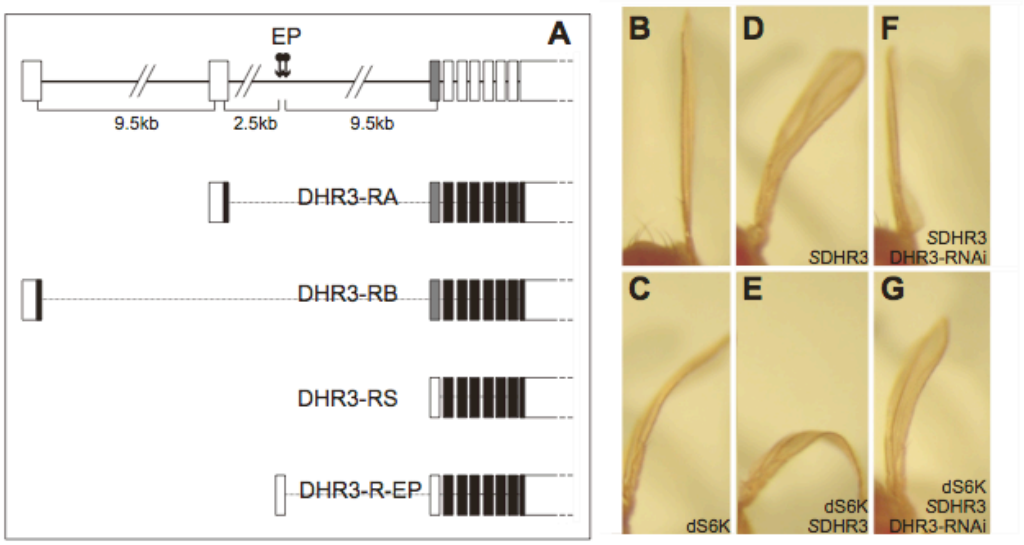
FIGURE 7: DHR3 regulates growth in a cell-autonomous manner.(A-C) The eyeless promoter directs the flipase recombinase during eye development. The flipase allows recombination on the right arm of the second chromosome and eventually leads to an adult eye that is homozygous for the wild type allele of DHR3 (A); homozygocity for each LBD-DHR3 mutation (B, C) results in a decrease of the size of the adult eye; a doted yellow line surrounding the control eye (A) has been copied and pasted on the mutant eyes (B,C); insets are higher magnification of ommatidies showing misorientation of bristles. (D) An heat shock induced LBD-DHR3 mutant bristle (arrow) that is visible with respect to its yellow genetic marker (arrow) has smaller size as compare to the surrounding bristles.



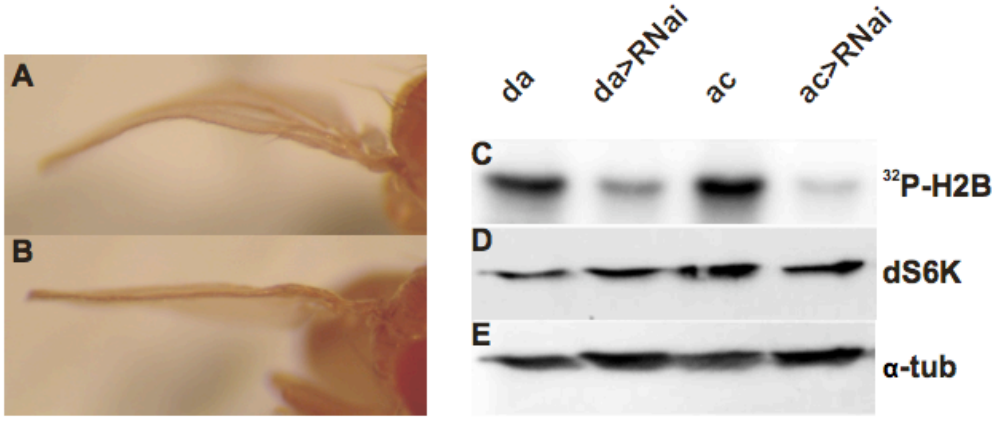
Montagne et al., FIGURE 1



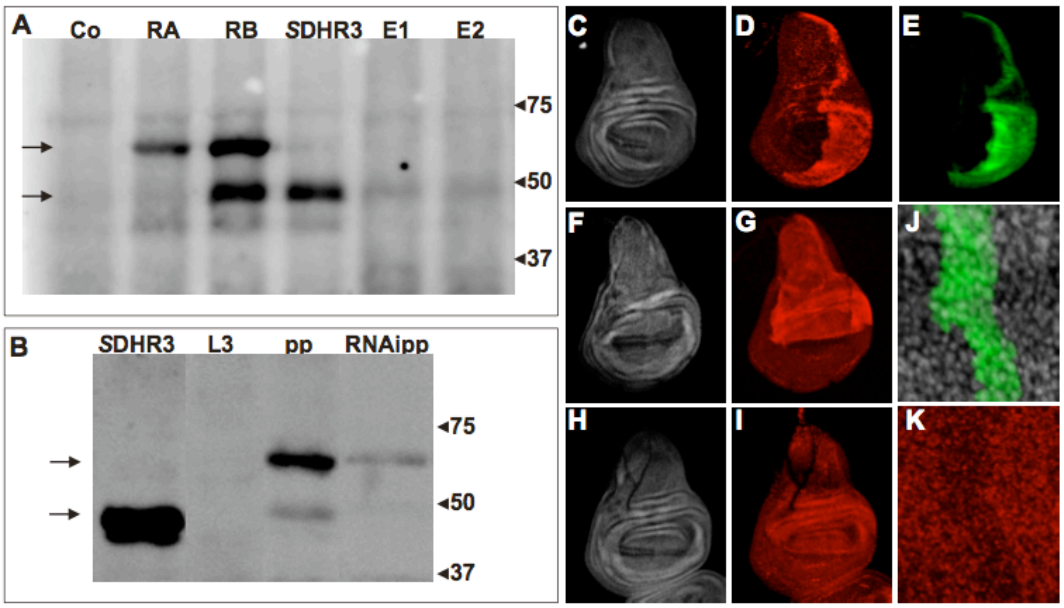
Montagne et al., FIGURE 2



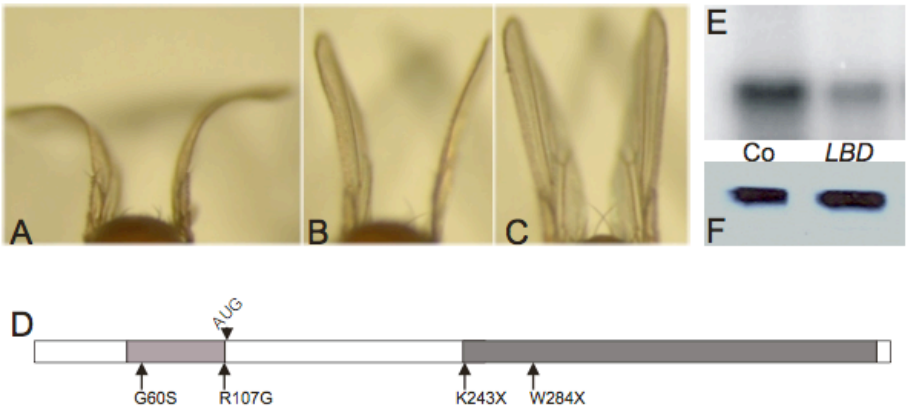
Montagne et ., FIGURE 3



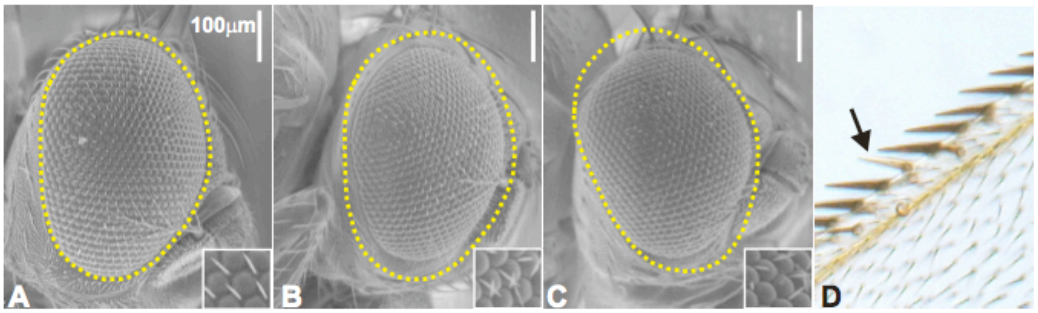
Montagne et al., FIGURE 4



Montagne et al., FIGURE 5



Montagne et al., FIGURE 6



Montagne et al., FIGURE 7

Supplementary material:

Supplementary Data 1: The EP12.218 (**E1**) and EP23.014 (**E2**) are inserted into chromosome 2R at nucleotides 6107302 and 6107230 respectively.

cacacacgcactttccgatagagcaaatg**E1**gggaagagcgagcgctggcgggagtggg
agagcgggagagagtgcgtgagagtggagcgttcgttgactgtta**E2**tgcgggagcgtg

Supplementary Data 2: (A) 5'end of a chimeric mRNA produced upon EP induction by Gal4; EP sequences are italicized. (B) 5'end of a novel DHR3 transcript starting at nucleotide 6097546 of chromosome 2R. The nucleotide sequence corresponding to the classically referred DHR3 2nd exon is in normal character; the first AUG codon is boxed for each transcript.

A *AATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAATA*
AACAAGCGCAGCTGAACAAGCTAAACAATCTGCAGTAAAGTGCAAGTTAAAGTGAATCAA
*TTAAAAGTAACCAGCAACCAAGTAAATCAACTGCAACTACTGAAATCTGCCAAGAA*CTCA
AATTGAGATAATTCCATGCAAAGTCTGCGGCGACAAGTCATCCGGCGTGCATTACGGAGT
GATCACCTGCGAGGGCTGCAAGGGATTCTTTCGAAGATCGCAAAGCTCCGTGGTCAACTA
CCAGTGTCCGCGCAACAAGCAATGTGTGGTGGACCGTGTTAATCGCAACCGATGTCAATA
TTGTAGACTGCAAAAGTGCCTAAAACTGGGA**ATG**AGCCGTGATG.....
M S R D . . . .

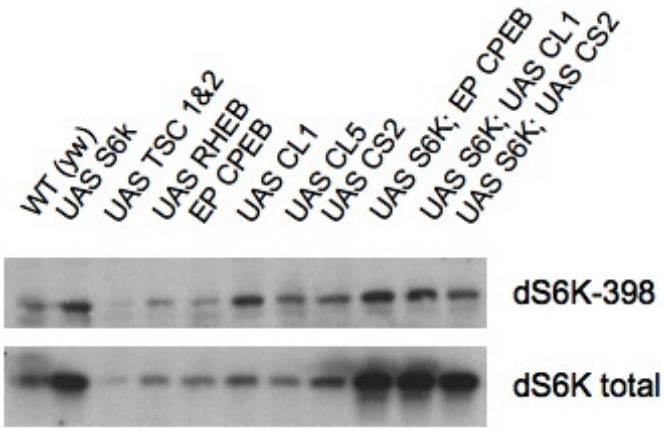
B TCACCTGCGAGGGCTGCAAGGGATTCTTTCGAAGATCGCAGAGCTCCGTGGTCAA
CTACCAGTGTCCGCGCAACAAGCAATGTGTGGTGGACCGTGTTAATCGCAACCGATGTCA
ATATTGTAGACTGCAAAAGTGCCTAAAACTGGGA**ATG**AGCCGTGATG.....
M S R D . . . .

Supplementary Data 3: EMS point mutations (boxed letters) of the DHR3 polypeptide PA; $G^{60}S$ (G) and $R^{107}G$ (R) affect the DBD, whereas $K^{243}X$ (K) and $W^{284}X$ (W) are early stop codons within the LBD (underlined). Methionines are in bold; sequences of the 2 peptides used for rabbit immunization are highlighted.

MYTQRMFDMWSSVTSKLEAHANNLGQSNVQSPAGQNNSSGSIKAQIEIIPCKVCGDKSS
GVHYGVITCEGCKGFFRRSQSSVVNYQCPRNKQCVVDRVNRNRCQYCRLQKCLKLG**M**SR
DAVKFGR**M**SKKQREKVEDEVRFHRAQ**M**RAQSDAAPDSSVYDTQTPSSSDQLHHNNYNSY
SGGYSNNEVGYGSPYGYSASVTPQQT**M**QYDISADYVDSTTYEPRSTIIDPEFISHADGD
INDVLIKTLAEAHANTNTKLEAVHDMFRKQPDVSRILYYKNLGQEELWLDCAEKLTQMI
QNIIEFAKLIPGFMRLSQDDQILLLKTGSFELAIVRMSRLLDLSQNAVLYGDVMLPQEA
FYTSDSEEMRLVSRIFQTAKSIAELKLTETELALYQSLVLLWPERNGVRGNTEIQRLFN
LSMNAIRQELETNHAPLKGDVTVLDTLLNNIPNFRDISILHMESLSKFKLQHPNVVFPA
LYKELFSIDSQQDLT

APPENDIX II:

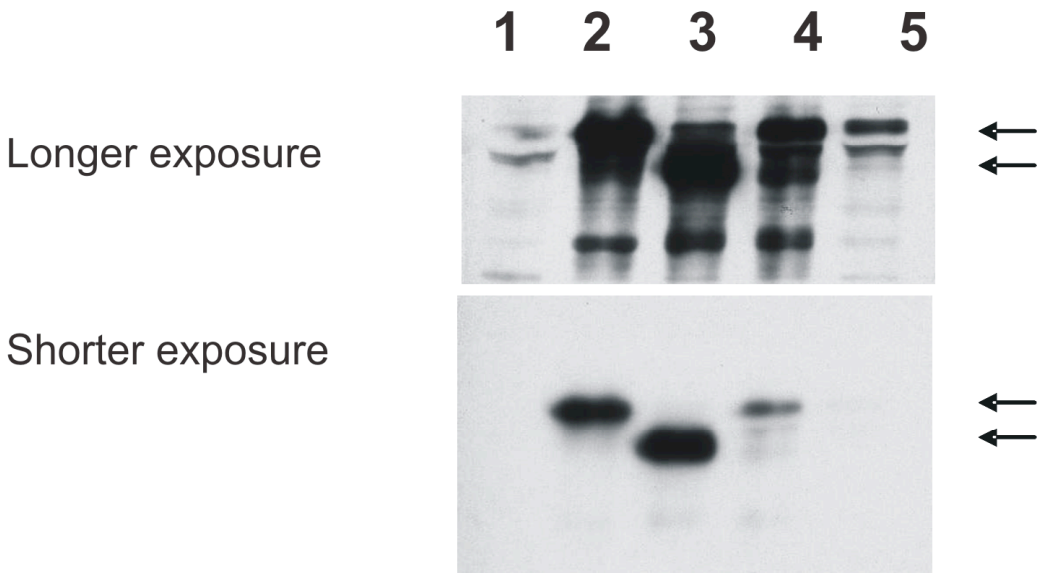
OVER-EXPRESSION OF DS6K RESULTS IN INCREASED LEVELS OF PHOSPHORYLATED DS6K



Overexpression of a dS6K transgene alone and with various UAS orb2 transgenic flies using Elav-GAL4. In all cases, where there is overexpression of dS6K, phosphorylation of dS6K serine 398 follows suit.

APPENDIX III:

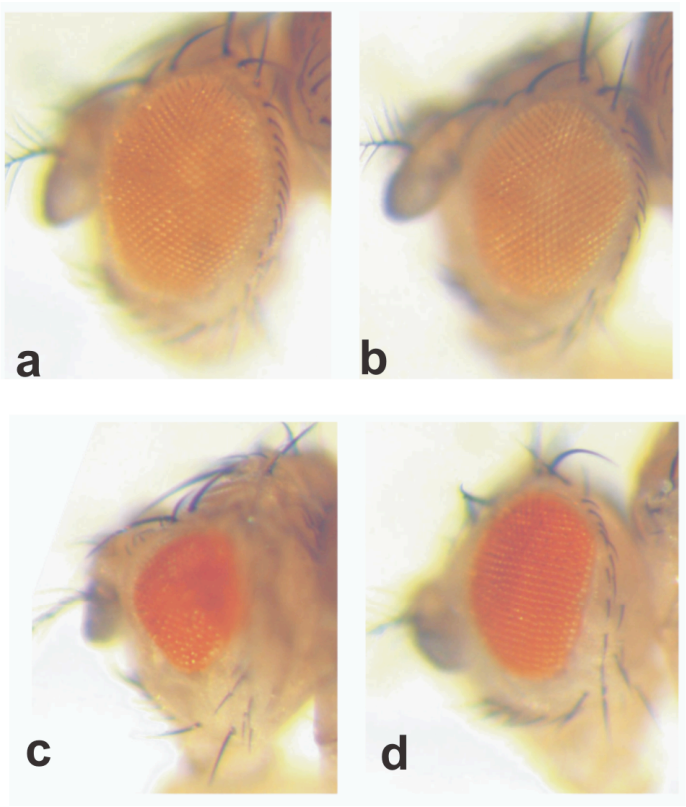
HEAT-SHOCK INDUCTION OF ORB2



Overexpression of orb2 transgenes using Heat-Shock-GAL4 (HS-GAL4). Lane 1= HS-GAL4, 2= HS-GAL4, UAS orb2-PB (CL2), 3= HS-GAL4, UAS orb2-PA (CS7), 4= HS-GAL4, UAS orb2-PB (CL5), 5= HS-GAL4, EP orb2. Note that the UAS orb2-PB (CL5) line was the line which reproduced the bent-down wing phenotype of *ap*-GAL4, UAS dS6K, EP orb2.

APPENDIX IV:

EYELESS-GAL4, UAS PTEN; EP IP3K PHENOTYPE



a) *Eyeless*-GAL4 b) Eye-GAL4, EP IP3K1 c) Eye-GAL4, UAS PTEN d) Eye-GAL4, UAS PTEN; EP IP3K1