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Insulin and Nutrients signaling regulate cellular and organismal growth through the *myc* proto oncogene in *Drosophila*

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GENERAL INTRODUCTION

Drosophila melanogaster has from nearly a century a fundamental role in the understanding of the basis of genetic mechanisms, due to the relative semplicity of its genome, to the short life cycle, to the abundance of the progeny and to the great amount of genetic markers of its body wall.

From the pioneering studies of Thomas Hunt Morgan and the members of his laboratory, the fruitfly has early become the best characterized model organism for genetic studies. Moreover, the development of mutagenesis techniques by nobel prizes Christianne Nüsslein-Volhard and Eric Wieschaus has permitted the isolation and characterization of a large number of genes involved in each step of *Drosophila* development. With the advancement of molecular biology techniques and genome sequentiation, this animal represents today an excellent model to understand the genetic mechanisms at the basis of almost all metazoan development.

The fruitfly is a holometabolous organism, characterized by a life cycle that starts from an embryonic stage, made inside the egg, followed by three larval instars (L1, L2, L3), during which the larva increases its dimensions. At the end of the third larval instar, at the pupal stage, a catastrophic metamorphosis occurs characterized by the histolysis of the larval tissues and by imaginal tissues differentiation, responsible for the adult appendages definition.

At the end of this stage the adult insect (also called imago) ecloses.

The life cycle of a wild type organism is about ten days at 25℃.

LARVAL AND PUPAL DEVELOPMENT (M. Ashburner, K. G. Golic, R. Scott Hawley, Drosophila a laboratory Handbook)

After 23h AEL, L1 larva emerges; the three larval instars are spaced out by moults and followed by metamorphosis. Moults and metamorphosis are governed by ecdysone peaks, a steroid hormone produced by the ring gland. Larval body is composed of two cellular types: larval cells, that are polyploid, and imaginal cells, that are diploid.

Imaginal cells segregate precociously from the surrounding larval cells, forming small cell groups at 9-10h AEL and are organized in two fundamental groups, imaginal discs and abdominal histoblasts.

Imaginal discs begin an intense proliferative activity from the second larval instar until pupariation, while abdominal histoblasts proliferate later, during the pupal stage. At this moment the majority of larval cells are eliminated and substituted by imaginal cells that originate the integument and the adult appendages.

Imaginal discs originate the structures of the head, thorax external appendages, genitalia and adult muscles. The histoblats originate the abdomen structures with the exception of the 8th segment that derivates from the genital imaginal disc.

Each imaginal disc at the end of larval development is constituted by a pseudostratified columnar epithelial tissue, that represents the actual imaginal disc, and by a squamous epithelium that forms the peripodial membrane. The first one originates the integument and the appendages, the second one originates the epithelial veil that welds the structures derived from different imaginal discs.

Imaginal discs location in the larval body is peculiar for each one of them (Fig.1).

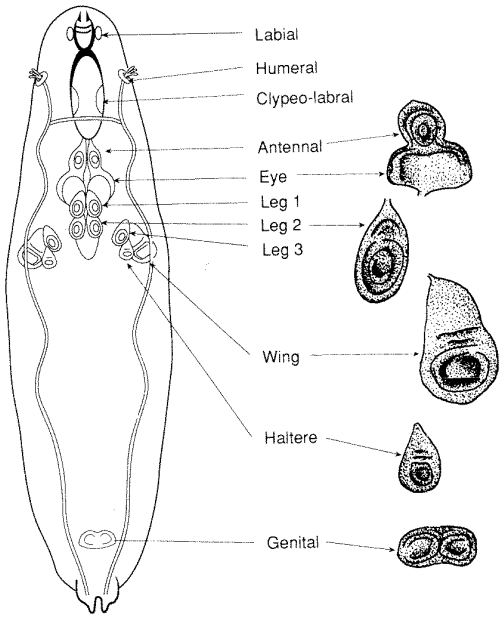


Figure 1 - Localization of the imaginal discs in the larva at the L3 instar (Bate e Martinez-Arias, 1991).

One interesting feature of imaginal discs is their ability to survive, metamorphose and differentiate after transplantion in adult flies' abdomen, (Schubiger *et al*, 1969; Simcox *et al*., 1989). Fragments of imaginal discs

can regenerate the entire disc structure and experiments in this direction allowed the characterization of “fate maps” of regeneration (Bryant, 1978).

The wing imaginal disc is an excellent model for the elucidation of organogenesis and proliferation mechanisms; at the end of L3 it is subdivided into a series of folds (Fig. 2). The centrifugal regions of the imaginal disc originate the thorax structures: notum and pleura. The middle region originates the hinge region while the central region is the presumptive territory from which the wing lamina differentiates. Imaginal tissues are virtually two-dimensional structures, but they originate adult appendages with three axes (AP, DV, PD); this is due to a mechanism of eversion during methamorposis, in which the most central regions of the disc originate the distal structures, while the external regions originate the most proximal structures of the appendage.

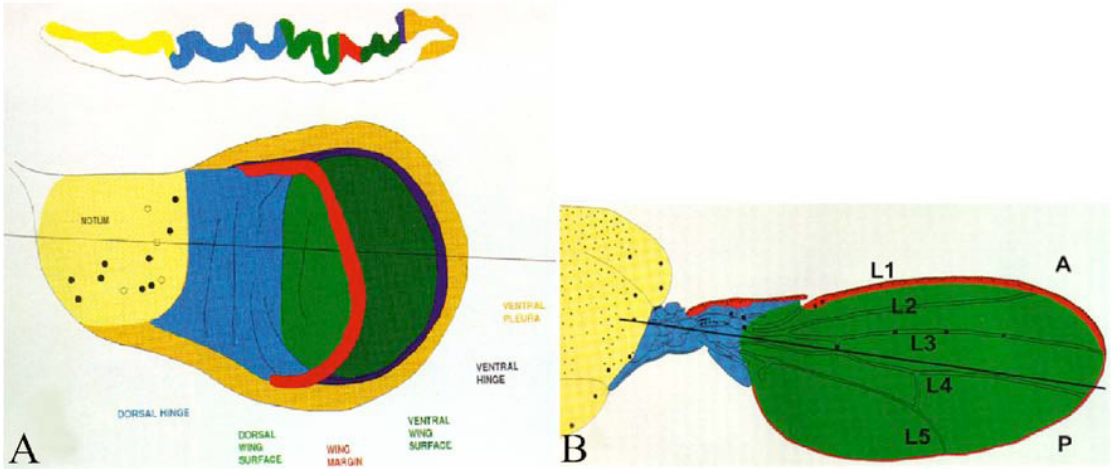


Figure 2 - Schematic representation of the wing imaginal disc at L3 instar (A) and of the adult wing (B). Different colors in the figure A indicate the presumptive territories of the structures visualized in B [from Bate and Martinez-Arias, 1991].

HOW MULTICELLULAR ORGANISMS GET THEIR SIZE: THE IMPORTANCE OF CELL GROWTH

In addition to being the unit of life, the cell is also the unit of growth.

Growth is an increase in mass over time, and the term can describe mass increase of individual cells ("*cell growth*") as well as tissues, organs or entire animal growth ("*growth*"). Understanding what regulates the overall growth of a cell and how cell growth is coordinated with progression through the cell cycle are important problems in biology.

Recently, converging studies of cell growth and proliferation, pattern formation, endocrine regulation and evolution have generated new perspectives to these problems. Progress in the insect model systems has been particularly noteworthy, as genetic studies in *Drosophila melanogaster* have converged with classical endocrinological studies in other insects to generate a hypothesis to explain body size, if not shape and form. The physiology of growth control in insects is, of course, different to that in mammals, but the genes and signalling pathways that are involved are surprisingly similar.

Insects do not grow as adults, and so their final size can be considered, to a first approximation, as a product of their growth rate during the larval phases and the duration of this growth period.

REGULATION OF SIZE: VARIATION IN PLOIDY

The first evidence that animals can monitor dimension came from haploids and polyploids. For a given cell type, cell size is usually proportional to ploidy. Hence haploid cells are about half the volume of diploid cells, diploid cells are about half the volume of tetraploid cells, and so on.

The ploidy of newts and salamanders can be manipulated to produce animals with chromosome complements ranging from haploid to pentaploid (Humphrey *et al*., 1957). In all cases, animals with unusual ploidy grow to the normal (diploid) size but contain very different numbers of cells. Thus mature tetraploid salamanders (*Amblystoma mexicanum*) look little different from diploid ones despite having half the number of cells.

Mammals are not so robust; tetraploid mice usually die *in utero*. However, they compensate for the larger size of their cells by a reduction in cell number (Henery *et al*., 1992). After birth, these mechanisms of compensation seem not to operate in mammals. Knockout mice that lack p27, an inhibitor of the cyclin D- and cyclin E-associated kinases which are required for entry into S phase, are born normally sized but subsequently grow considerably larger than littermates (Fero *et al*., 1996; Kiyokawa *et al*., 1996; Nakayama *et al*., 1996). The increase is a result of increased cell proliferation. In these knockout mice, increased cell proliferation does *not* result in a compensatory decrease in cell size.

In *Drosophila* the growth and final size of diploid/haploid mosaics is about normal (Santamaria, 1983), the haploid regions of such flies containing more numerous but smaller cells. The ploidy of *Drosophila* cells can be increased by loss of function of the cyclin-dependent kinase Cdc2. Cdc2 is required for mitosis in *Drosophila* and when the *Cdc2* gene is inactivated in cells of the wing imaginal disc, cells switch from a mitotic cycle to cycles of endoreduplication without division (Fig 3 A-B) (Weigmann *et al*., 1997).

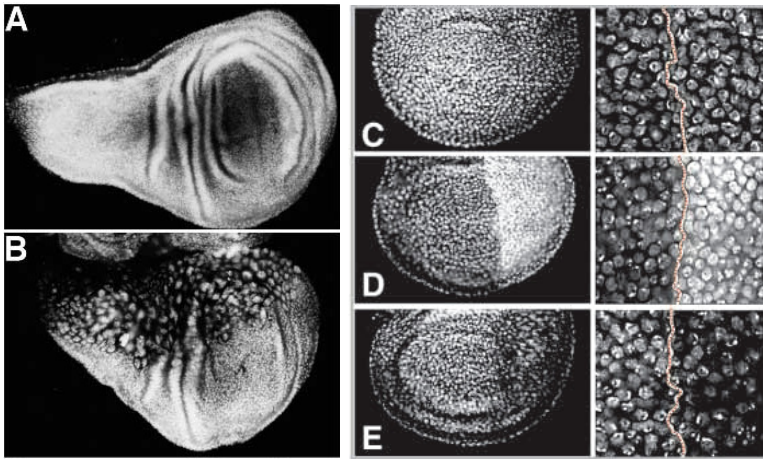


Figure 3. **Pattern can be conserved indipendently of cell numeber or size.** *Left*: Inactivation of Cdc2 by the use of a temperature sensitive allele (*Cdc2*$^{E1\text{-}24}$) specifically in the anterior compartment of the wing disc (upper part) does not change shape and size of the wing, even though the affected region contains fewer but much larger, polyploid cells. Cell size is visualized by DNA staining. Wild-type (A). *Cdc2*$^{E1\text{-}24}$, *en*-Gal4*;* UAS-*Cdc2*$^{+}$ (B) [from Weigmann *et al*., 1997].
Right: Wing imaginal discs in which the cell density of the posterior compartment has been manipulated. There is no change in the shape and size of the compartments or the wing. The interface between A and P cells was drawn with a dotted line. C is entirely wild type. In (D) the P compartment has many more cells; in (E) many fewer cells than normal. The A compartment in (D) and (E) has normal cell densities [from Neufeld *et al*., 1998].

The effects of polyploidy argue that there is a strong positive relashionship between genome size and cell size. However, different cell types within a multicellular organism often vary in size, despite containing identical genomes of the same ploidy. Thus, there is not a strict adherence

to a DNA:Cytoplasm ratio, indicating that amount of DNA is not the sole determinant of cell size.

However these experiments seems to demonstrate that changes in cell number can be compensated for by changes in cell size, both locally in groups of cells, or more globally in the whole compartment.

MEASURING DIMENSIONS: THE STANDING OF NUMBERS

During the growth of most organs, rates of cell growth and cell division are coordinated so that cell size does not change much over time, in other words growth is normally accompanied by an increase in cell number. The mechanisms that match rates of cell growth with cell division are mysterious, and are only recently being addressed in metazoans.

Whereas mitotic cells often coordinate growth with division, in many natural processes the two phenomena can be separated. Early embryogenesis, for example, involves several rounds of division without growth and endoreplication, by the other side, it consists in growth without cell division.

In the developing *Drosophila* wing discs, growth can be uncoupled from cell division (Fig.3 C-E). For example, when cell division is slowed or blocked, cells continue to accumulate mass (cell growth) and hence increase in size. Conversely, when division rates are accelerated by overexpression of specific cell-cycle regulators, cell growth rates are

unaffected and cells divide at smaller size (Neufeld *et al.* 1998). These observations demonstrate that, in metazoans, cell division rates do not drive cell growth. It is still unclear whether the opposite is true and cell growth is sufficient to drive cell division.

EXTRINSIC AND INTRINSIC REGULATION OF SIZE

The identity of a particular organ, the position of a cell within this organ and its interactions with neighboring cells all play important roles in determining cellular growth rates. Although different models have been put forward to explain this *local*, organ-intrinsic control of growth, its molecular basis has remained elusive. In addition to local control, cells also experience more *global* signals that control their growth rates. Temperature and nutrient availability, for example, strongly influence growth rates and can affect the final size of many animals.

It seems that, generally, *extrinsic* mechanisms are concerned with a link between *growth and nutrition*; they are not involved in proportion and pattern, but do affect the rate of growth and also the final size of the fly. Edgar and colleagues (Johnston *et al*., 1999) have argued that nutrition-based and pattern-based regulation of growth operate in distinct ways: nutrition regulates the cell cycle via *myc*-dependent cyclin E regulation, acting at the G1/S checkpoint, and pattern acts through *cdc25*/*string* which intervenes at the G2/M checkpoint.

Our emphasis in this essay is on the nutrition-based regulation of growth and *myc* role in size determination.

NUTRIENTS AVAILABILITY INFLUENCES GROWTH RATES

Body and organ size are related to nutrition: poorly fed *Drosophila* larvae develop more slowly and can produce smaller flies. The wings of such flies are smaller because they contain smaller and fewer cells. Apart from this reduction in size, wing pattern is unaffected (Robertson, 1972; Bryant and Simpson, 1984).

Dietary protein restriction has been shown to extend *Drosophila* development from 5 days to several weeks under constant temperature conditions. Not surprisingly, larval growth can be arrested by removing dietary proteins completely. This treatment rapidly arrests cell growth and DNA replication in most of the differentiated larval-specific tissues, but if the *minimum viable weight* (weight at which larvae can develop into adults if food is completely withdrawn) has been attained, the progenitor cells that will form the adult continue to grow and proliferate (Britton *et al.*, 1998), eventually generating a small but otherwise normal fly.

The fact that cells can grow within a starved animal indicates that the haemolymph (blood fluid) in such animals is not critically depleted of nutrients. Indeed, *D. melanogaster* and other insects are known to maintain haemolymph nutrients when they are starved by mobilizing triglycerides and glycogen stored in the *fat body*, a mesoderm-derived energy-storage organ that fulfills the functions that are assumed by the liver and adipose tissues in mammals (Bradley and Leevers, 2003; Mirth and Riddiford, 2007) .

Nutrient mobilization from the fat body is mediated by the induction of metabolic neuropeptides called adipokinetic hormones (AKH), which are produced by the Corpora Cardiaca, a region of the neuroendocrine Ring Gland (Fig.4). AKHs function analogously to vertebrate glucagons and, together with insulin-like peptides (ILPs), are part of an endocrine signalling system that allows the animal to coordinate rates of cell growth and changes in diet, with minimal disruption of the developmental programme.

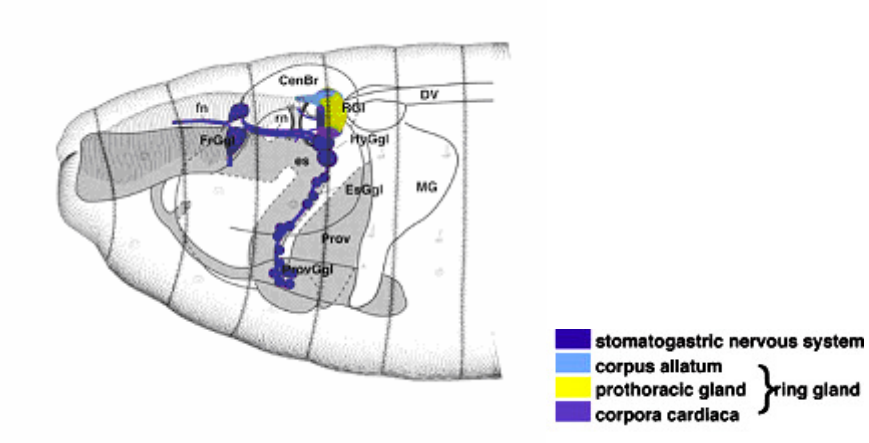


Figure 4. Schematic representation of *Drosophila* stomatogastric Nervous System and Ring Gland**.**

INSULIN/INSULIN-LIKE GROWTH FACTORS SIGNALING

The insect Insulin/Insulin - like growth factors system (IIS) (Fig.5) is highly homologous to that found in mammals. IIS activity promotes glucose import and nutrient storage in the fat body and other organs, fulfilling the homeostatic function of vertebrate insulins (Shingleton *et al.,* 2005). In this capacity, it affects feeding behaviour, lifespan and reproduction. During

development, IIS also regulates cell growth, fulfilling the developmental function of the mammalian insulin-like growth factors (IGFs) (Efstradiatis 1998).

IIS activity has been manipulated in various ways in *D. melanogaster,* using the Gal4–UAS system (Brand and Perrimon, 1993) to overexpress genes in specific tissues, and the Flp–FRT system (Xu and Rubin, 1993) to delete gene functions in specific tissues at defined times.

These manipulations show that many IIS components not only are essential for cell and organ growth, but are also sufficient to *autonomously* increase the growth rate of about any cell type in *D. melanogaster*. In whole animals, increased expression of several of *D. melanogaster's* seven Insulin-like peptides (ILPs), a class of peptide hormones homologous to vertebrate insulins and insulin-like growth factors, can increase both larval growth rates and adult size (Brogiolo *et al.,* 2001; Ikeya *et al*., 2002), whereas ablation of the small cluster of medial neurosecretory cells (mNSC) in the brain, which are the principal source of ILPs, reduces growth rates and final body size (Ikeya *et al*., 2002).

ILPs bind the insulin receptor and promote cellular glucose import, energy storage in the form of glycogen and triglycerides, and cell growth. *Drosophila melanogaster* has seven paralogous genes (*dILPs 1-7)* expressed in different clusters on mNSCs (Ikeya *et al*., 2002).

Activation of the InR/PI3K by dILPs increases macromolecular synthesis and this is most likely responsible for the potent effects of InR/PI3K on cell size. The ability of different cell types to respond to insulin

(or other growth factors) could be regulated by their accessibility to the ligand(s), or by their expression of the receptors or downstream components. Different expression levels of these components could dictate cell-type-specific size control.

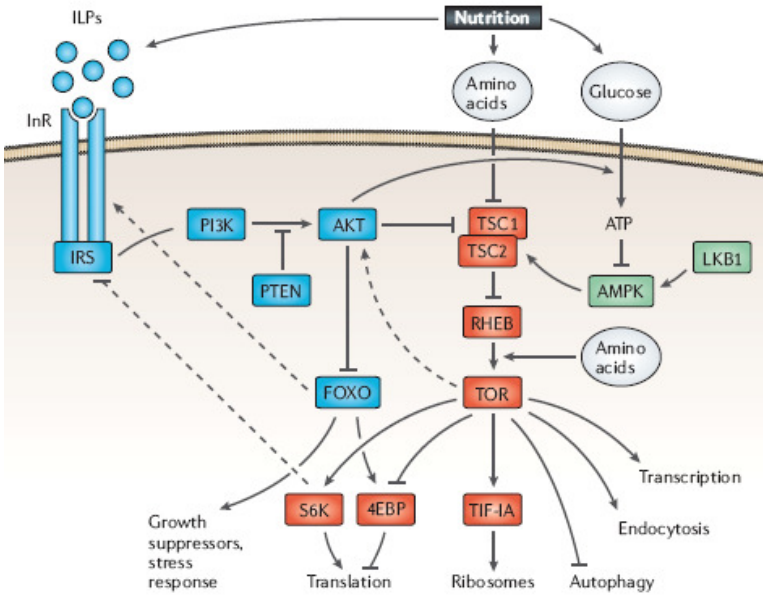


Figure 5. ***Drosophila*** **Insulin pathway.** Upon binding its ligand (ILPs), dInr activates an evolutionarily conserved signal transduction cascade. PI3K is recruited to an activated insulin receptor (InR) through an insulin receptor substrate and an adaptor protein, p60. In turn, the membrane localization of PI3K allows it to phosphorylate phosphatidylinositol-4,5-P2 (PtdIns[4,5]*P*2), producing phosphatidylinositol-3,4,5-P3 (PtdIns[3,4,5]*P*3). This second messenger then recruits and activates protein kinase B (PKB), in cooperation with phosphoinositidedependent kinase (PDK). A negative regulator of this pathway is the phosphatase PTEN, which converts PtdIns(3,4,5)*P*3 back into PtdIns(4,5)*P*2. Together with the pathway acting through dTOR and the ribosomal protein S6 kinase (dS6K), the dInr pathway controls the activity of the protein translation machinery. [modified from Edgar 2006]

TOR (Target of Rapamycin) AND THE NURIENTS SENSOR CASCADE

Given a cue to grow, such as insulin signaling, a cell needs a nutrient-rich environment to execute the command. One proposed

mechanism for a cell to sense its environment involves the target of rapamycin, TOR. TOR was first identified in yeast as a mediator of protein synthesis in response to nutrients (reviewed in Schmelzle and Hall, 2000) and has been shown to regulate translation, ribosome biogenesis and uptake of amino acids.

The first genetic analyses of TOR in a multicellular animal were performed by two laboratories using *Drosophila* (Oldham *et al*., 2000; Zhang *et al*., 2000). The researchers found that cells mutant for *TOR* were reduced in size at all stages of the cell cycle. In addition, *TOR* mutant animals had a growth arrest phenotype reminiscent of amino acid withdrawal (Zhang *et al*., 2000). Genetic epistasis tests demonstrated that in *TOR/Pten* double mutants, the phenotype of the *TOR* mutation was dominant, implying that TOR is required for the increased growth resulting from elevated insulin signaling.

Further observations support the argument that TOR does not function downstream of InR/PI3K in a simple, linear pathway. First of all, careful examination of cell-size defects in endoreplicating cells revealed that cells mutant for *TOR* were phenotypically different from cells mutant in the Insulin Receptor Substrate (IRS)/*chico*: loss of *TOR* demonstrated a more pronounced decrease in cytoplasmic volume relative to the nuclear compartment (Oldham *et al*., 2000). Second, in contrast to the cell-enlargement phenotypes seen following increases in InR/PI3K activity, overexpression of *TOR* reduced cell size. This contraddictory effect of TOR on growth has been repeatedly documented (Hennig *et al*., 2002) but has

no obvious molecular explanation and will be addressed later on in this dissertation.

TOR has been well documented as responsive to nutrients in yeast and tissue culture, but despite intensive study, it is still unclear how it senses environmental conditions and how this pathway relates to the InR/PI3K pathway in multicellular organisms.

Recent work on the two effectors of TOR signaling, S6K and 4EBP has supported the role of TOR in maintenance of cell size. S6 kinase (S6K) is implicated in promoting translation of proteins involved in ribosomal assembly, and requires TOR (Oldham *et al*., 2000) and PDK (Radimerski *et al.,* 2002) for activation. 4E-BP is a negative regulator of the translation initiation factor, eIF4E.

A mutant version of 4E-BP, which binds to eIF4E more strongly than wildtype, was used to perturb eIF4E function in *Drosophila* (Miron *et al*., 2001). Overexpressing the modified 4E-BP in transgenic animals resulted in a cell-size decrease. However, co-overexpression of eIF4E was not sufficient to bypass the effect of 4E-BP. The failure of TOR overexpression to promote cell size and the inability of eIF4E to counter 4E-BP inhibition suggests regulation beyond total levels of TOR and its effectors. Modification by phosphorylation could be this additional level of control. For example, coexpression of the kinase PDK greatly enhances S6K-directed growth (Radimerski *et al.,* 2002) and mutation of phosphorylation sites of eIF4E impedes normal growth (Lachance *et al*., 2002) .

Proteins of the TSC complex (TSC1 and TSC2) have been identified as potential upstream negative regulators of TOR (McManus and Alessi, 2002), and may also play a role in nutrient sensing. In cells lacking TSC1 or TSC2, phosphorylation of S6K is resistant to amino acid deprivation; conversely, increased levels of TSC1 and TSC2 prevent S6K phosphorylation even in the presence of abundant amino acids (Gao *et al.,* 2002).

Recent studies in *Drosophila* have identified the small GTPase Rheb as a link between TSC1/TSC2 and TOR (Saucedo *et al*., 2003; Stocker *et al*., 2003). Rheb, which is inhibited by the GTPase activating protein (GAP) activity of TSC2 (Zhang *et al*., 2003), appears to be a proximal activator of TOR, although its mechanism of action remains to be elucidated.

S. pombe mutants for *rheb* (*rhb1*) arrest in G0/G1 as small, rounded cells (Mach *et al*., 2000; Yang *et al*., 2001), suggesting a role for *Rheb* in cell cycle progression and cell growth.

Highly conserved *Rheb* genes have been described throughout the metazoa (Yamagata *et al*., 1994; Reuther and Der, 2000; Urano *et al*., 2000; Urano *et al*., 2001; Im et al., 2002; Panepinto *et al*., 2002). *Drosophila* Rheb (dRheb) has both GTP binding and GTPase activities (Patel *et al*., 2003).

Overexpression of *dRheb* results in tissue overgrowth and increased cell size in the whole organism, and transition into S phase and cell growth in culture. Conversely, reduction of *dRheb* activity results in reduced tissue growth and smaller cell size in the whole organism, as well as G1 arrest and smaller cell size in culture (Patel *et al*., 2003; Stocker *et al*., 2003).

dRheb overexpression is sufficient to counteract the effects of aminoacid deprivation, since, despite the lack of aminoacids, larval cells expressing *dRheb* reach normal size (Stocker *et al*., 2003).

The results of treating S2 cells and flies with Rapamycin, an inhibitor of TOR, suggest that the effects of dRheb are probably mediated by dTOR.

At this regard, our data (see Results) show for the first time a growth effect, enhanced by Rheb, which seems to be, at least in part, indipendent from TOR, opening the possibility for the existence of another level of size regulation in response to nutrients availability.

Finally, TOR has recently been shown to form a nutrient-dependent complex with a WD40 and HEAT repeat-containing protein, raptor, which appears to have both positive and negative effects on TOR activity, perhaps depending on nutrient levels (Hara *et al*., 2002; Kim *et al*., 2002; Loewith *et al*., 2002).

Although the dissection of the activity of TOR presents some complexities, TOR is clearly required for maintaining normal cell size and permitting growth-factor-mediated cell hypertrophy.

CROSSTALKS BETWEEN InR AND TOR SIGNALING

Apparently cells are instructed to grow according to two different extracellular inputs: growth factors on one hand, activating a PI3K-mediated signaling, and nutritional cues on the other triggering a TOR-mediated

signaling response. These two signaling pathways are known to send branches to each other.

Inhibition of TOR with rapamycin can potently block activation of S6K in response to growth factors, and thus TOR function is required for effective growth factor signaling (Jacinto and Hall, 2003). However, immunokinase assays have found that TOR activity increases only slightly in response to growth factors (Burnett *et al*., 1998). Furthermore, mutant forms of S6K have been isolated that are resistant to rapamycin and amino acid withdrawal, but remain sensitive to wortmannin, a PI3K inhibitor (Hara *et al*., 1998; Weng *et al*., 1995b).

Thus TOR and PI3K appear to regulate essentially parallel signaling pathways which may ultimately converge on common targets. In this sense, TOR signaling can be thought of as a nutrient-dependent checkpoint on growth factor signaling. The modest increase in TOR activity that does occur in response to growth factors was initially thought to be mediated through phosphorylation of TOR by Akt, which can directly phosphorylate TOR *in vitro* on sites whose phosphorylation is serum sensitive *in vivo* (Scott *et al*., 1998). However, mutation of these sites to alanine does not diminuish signaling downstream of TOR (Sekulic *et al*., 2000); furthermore, these sites are not conserved in the fly, worm or plant TOR homologs.

Recent experiments suggest that a link between PI3K and TOR may occur through Akt-mediated phosphorylation of TSC2, which was found to disrupt and inactivate the TSC1/TSC2 complex (Dan *et al*., 2002; Inoki *et al*., 2002; Potter *et al*., 2002). It will be important to determine whether

phosphorylation by Akt is a critical factor regulating TSC function during normal development.

An additional level of cross-talk between the PI3K and TOR pathways occurs through a negative feedback loop involving TOR-mediated inhibition of IRS1/*chico*, an adapter protein required for PI3K activation by the insulin receptor. Activation of TOR results in phosphorylation and subsequent proteasomal degradation of IRS1, leading to reduced PI3K signaling (Haruta et al., 2000). Similarly, mutations in S6K have been found to increase the kinase activity of Akt in *Drosophila* (Radimerski *et al*., 2002).

These trans-pathway interactions are likely to play an important role in coordinating cell growth with other metabolic programs.

THE WELL DOCUMENTED ROLE OF *myc* IN GROWTH CONTROL

Myc proteins are members of the basic-helix-loop-helix-zipper class of transcription factors and are deregulated in several human cancers (Nesbit *et al*. 1999). c-Myc expression is quickly induced in response to many mitogens but the promoter elements directing induction are not well characterized.

Numerous reports have indicated that Myc family members activate transcription of genes involved in protein synthesis and cellular metabolism (Coller *et al*., 2000; Guo *et al*., 2000; Boon *et al*., 2001). These functions

correlate well with Myc's ability to promote cell size increases in tissue culture and *in vivo* (Johnston *et al*., 1999) when overexpressed.

A recent publication of an allelic series of c-myc-hypomorph/null mice demonstrated that Myc regulates body size in mice by controlling cell number without detectable changes in cell size (Trumpp *et al*., 2001).

Animals with reduced levels of c-Myc weighed less than normal counterparts and the weight loss was distributed among all organs. The researchers performed flow cytometry on dissociated cells from several organs and found no decrease in cell sizes as measured by forward scatter. Additionally, no differences in cell size were seen in haematopoietic cells or primary embryo fibroblasts. However, cell number was lower in the spleen, lymph nodes, and bone marrow of mutant mice.

This observation, along with the discovery that primary fibroblasts of embryos with decreased *c-myc* have an elongated cell cycle, led the authors to conclude that c-Myc is not required for cellular growth but rather that c-Myc controls the decision to divide or not divide. Another interpretation of these data is that cell growth and proliferation are coupled in these animals. In this case, a defect in cell growth would, by default, result in fewer cells.

Two other groups used tissue-specific deletion of c-*myc* to examine its role in haematopoetic cells (de Alboran *et al*., 2001; Douglas *et al*., 2001). In these studies, absence of *myc* prevented activation of B- and T-cells, which involves a growth transition from small to large cells. This result differs from Trumpp *et al*., who reported normal T-cell activation in the

absence of *myc*, leaving the necessity of Myc for growth-coupled maturation of B and T-cells uncertain.

There are three *myc* family members in mammals, and it remains to be examined whether L- or N-Myc has a role in maintaining cell size.

Years of research on c-Myc in mammalian systems have led to a dauntingly diverse range of potential genetic and functional targets. Yet it has remained difficult to evaluate Myc's function in its entirety due to redundancy among family members, tissue specificity, and complex phenotypes.

These problems spurred the search for Myc in genetically tractable animal models, and led to the identification of Myc family members in zebrafish, in the nematode *Caenorhabditis elegans*, and in *Drosophila*.

The relatively simple genome and the highly developed genetics that *Drosophila* offers have made it the model of choice for the study of Myc family members. In fact, the very first *myc* mutant of any was identified as a spontaneous mutation in *Drosophila* in the 1930s by Eleanor Nichols-Skoog and Calvin Bridges, which they called *diminutive* (*dm*), for its smaller body size. *dm* was identified as a mutation in the *Drosophila myc* (*dmyc*) gene in 1996, and since then more than a dozen mutations of *dmyc* have been characterized (Gallant *et al.*, 1996; Schreiber *et al.*, 1990).

In the last several years, work on dMyc has clarified some of the protein's more enigmatic functions and also led to the identification of new functional roles.

The single *myc* gene of *Drosophila* can partially rescue the proliferation defect of fibroblasts from c-*myc* mutant mice, suggesting shared functional targets (Trumpp *et al*., 2001).

One of the greatest advantages *Drosophila* provides to Myc biology is the ease with which growth can be studied in a living animal. All *dmyc* mutations show profound growth defects. Null mutant embryos hatch into larvae at the same time as wildtype animals, but fail to grow and die early in the second larval instar (Pierce *et al*., 2004).

Hypomorphic alleles are lethal at progressively later stages of development, depending upon their severity (Pierce *et al*., 2004; Maines *et al*., 2004). In animals bearing the weakest alleles, such as the original *dm1* mutation and $dmyc^{P0}$, development is delayed and yields small flies – the result of smaller cells – with short, thin bristles (Johnston *et al*., 1999). Animals carrying the slightly stronger $dmyc^{P1}$ allele also have a significant reduction in cell number.

Interestingly, although the hypomorphic mutant flies are smaller than normal, they appear normally proportioned (Fig. 6) (Johnston *et al*., 1999; Moberg *et al*., 2004). Many of these same defects are characteristic of flies with growth deficits, and also appear in flies with mutations in genes encoding ribosomal proteins (the *Minute* class of mutations) and other components of ribosome biogenesis (Lambertsson, 1998).

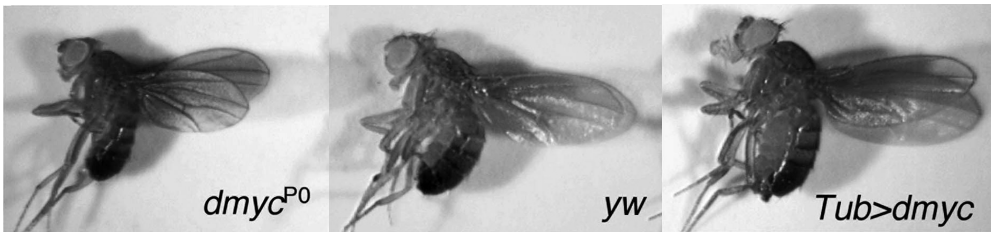


Figure 6. dMyc controls animal growth. Flies expressing less *dmyc*, such as a viable *dmyc* hypomorph (*dmyc*^{P0}), are smaller in overall body size than wildtype flies (*yw*), and wing size is reduced approximately 15%. Conversely, flies with increased *dmyc* expression, from a *Tubulin promoter-dmyc* transgene (*Tub>dmyc*), are approximately 30% larger in overall body size than wildtype flies. [Modified from: de La Cova and Johnston, 2006]

The existence of a broad *dmyc* allelic series with a range of phenotypes suggests that some developmental processes in the fly require *dmyc* more than others. *dmyc* is expressed in numerous tissues throughout fly development, and its expression occurs in a dynamic pattern (Gallant *et al*., 1996). It is expressed in both endoreplicating cells, which oscillate between G1 and S phase but do not divide, and in mitotic tissues (Pierce *et al*., 2004; Johnston *et al*., 1999).

Use of the FLP/FRT technique of mitotic recombination to generate somatic *dmyc* mutant cells in an otherwise normal animal demonstrated that in all tissues examined, clones of *dmyc* mutant cells result in pronounced defects in cellular growth. This defect is manifested by a small cell size and by impaired progress through the cell cycle (Johnston *et al*., 1999).

In endoreplicating larval cells that can normally achieve a DNA content upwards of 2000C, *dmyc* null cells fail to increase in size as development progresses, a defect that is directly related to the number of endocycles a cell undergoes (Pierce *et al*., 2004). Since the dramatic growth

of these cells is required to sustain the overall growth of a fly larva, the impaired growth of *dmyc* mutant endoreplicating tissue probably accounts for the growth arrest and subsequent death of these null mutant larvae (Pierce *et al*., 2004). *dmyc* is also required for growth of polyploid cells within the female germline, and in the diploid imaginal cells that give rise to the adult body structures of the fly (Maines *et al*., 2004; Johnston *et al*., 1999).

Proliferating *dmyc* mutant imaginal cells are markedly reduced in size and spend a disproportionate amount of the cell cycle in the G1 phase; these cells are also smaller in S, G2, and M phases (Johnston *et al*., 1999).

Conversely, overexpression of dMyc in imaginal cells increases cell size by accelerating cellular growth, and when expressed throughout the animal the size of the fly is increased by nearly 30% (Fig. 6) (Johnston *et al.,* 1999; de la Cova *et al*., 2004). Collectively, these mutant phenotypes provide solid evidence that dMyc is required *in vivo* for cellular growth, and that it acts in a dose-sensitive manner.

The vast majority of *dmyc* targets encode factors used in ribosome biogenesis (Orian *et al*., 2003, Hulf *et al*., 2005; Grewal *et al*., 2005). These targets include RNA Polymerase I and II transcribed genes, and many appear to be directly responsive to dMyc activity. dMyc overexpression is accompanied by a dramatic increase in nucleolar size and in pre-rRNA expression. Bigger nucleoli are usually a good indicator of increased ribosome activity (Grewal *et al*., 2005). This effect is not observed when

other growth regulating factors, such as the PI3K, Dp110, or Cyclin D/Cdk4 are overexpressed (Grewal *et al*., 2005).

Fibrillarin expression is also increased in these larger nucleoli, and the cytoplasm of larval salivary gland cells expressing dMyc are packed with ribosomes and polysomes, with a dense network of rough endoplasmic reticulum (Fig. 7).

By contrast, in *dmyc* mutant larvae, pre-rRNA levels are low compared to controls. Levels of rDNA and nucleolar size are reduced (Grewal *et al*., 2005). As a whole, the data indicate that modulation of ribosome biogenesis is an important effector of dMyc during normal growth, with dMyc-dependent transcriptional regulation of ribosome biogenesis resulting in greater translational activity within the cell.

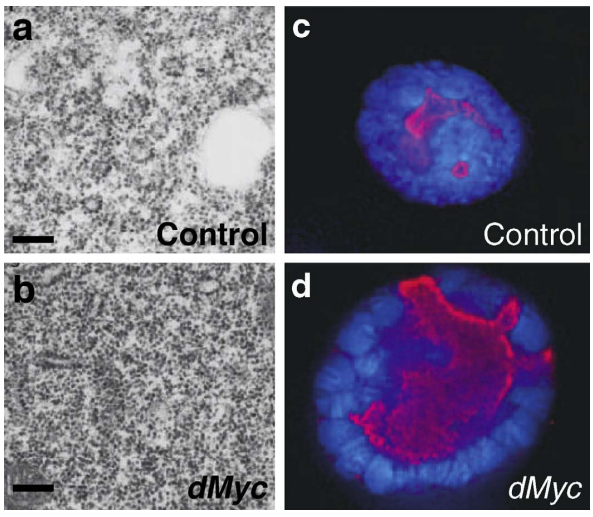


Figure 7. dMyc regulates nucleolar size and ribosome biogenesis *in vivo*. Cells of the fly salivary gland overexpressing dMyc have increased ribosome content (dark dots in a and b), and larger nucleolar size (c and d) as visualized by Fibrillarin (red), and nuclear size (c and d) as seen by DAPI (blue). (Modified from: Grewal *et al*., 2005)

dMYC AND CELL CYCLE REGULATION

Given its pivotal role in human cancer and in developmental control of growth, understanding how Myc regulates the cell cycle has historically been of great interest. Like vertebrate Myc, dMyc is required for efficient transit through G1 into S phase, as *dmyc* mutations stall cells in G1, and overexpression of dMyc accelerates G1 (Pierce *et al*., 2004; Maines *et al*., 2004; Johnston *et al*., 1999).

However, dMyc is not essential for cell cycle progression. This is clearly demonstrated in the female fly germline, where mitotically dividing cytoblast cell clones carrying a strong hypomorphic allele of *dmyc* still undergo all the normal divisions, producing the expected 16 cells of a normal germline cyst (Maines *et al*., 2004). On the other hand, as mentioned above, endoreplicating cells mutant for *dmyc* undergo significantly fewer rounds of S phase. The molecular nature of the defect in endoreplication is unclear, as *dmyc* cells still periodically express the G1 regulators Cdk2, CyclinE and Dacapo, the fly $p27^{cip/kip}$ homolog, and are able to complete the entire endocycle (Maines *et al*., 2004).

One possibility is that dMyc regulates the frequency of S phase entry in these cells. Consistent with this idea, overexpression of dMyc increases the rate of endoreplication in cells of the *Drosophila* fat body, and this effect is accompanied by (and dependent upon) oscillating activity of Cyclin E/Cdk2 (Pierce *et al*., 2004).

Moreover, dMyc expression in endocycling cells can partially reverse a growth arrest imposed by expression of the phosphoinositol-3-kinase

(PI3K) adaptor p60, rescuing both endoreplication and cellular growth (Pierce *et al*., 2004; Maines *et al.,* 2004).

By controlling cellular growth and cell proliferation, dMyc has a major impact on the regulation of animal size. In contrast to the growth-regulating network of Insulin/PI3K and dTOR, *dmyc* expression is regulated by at least two of the major developmental signaling pathways that regulate pattern in *Drosophila*, Wingless (Wg)/Wnt, and Decapentaplegic (Dpp)/BMP/TGFβ. While Insulin/PI3K/dTOR signaling controls growth in response to nutrients, the responsiveness of dMyc to Wg and Dpp, which are also required for growth of many fly organs, suggests a model wherein developmental signals contribute to tissue growth, and ultimately body size and proportion, by regulating dMyc activity.

dMYC AND CELL COMPETITION

Recently, work in *Drosophila* has revealed a new aspect of dMyc function: high levels of dMyc provide cells with a competitive edge that allows them to kill nearby cells that have less dMyc. In the fly, cell competition is a process that is operationally defined by the progressive elimination of normally viable (but less “competitive”) cell types.

When cell clones overexpressing dMyc are generated in the developing wing, such clones grow faster than the surrounding wildtype

cells, which in turn actually grow less than expected and die more frequently (de la Cova *et al*., 2004; Moreno *et al*., 2004).

The ability of dMyc to induce cell competition is a remarkable property that is not shared by all growth regulators. Competition can be induced whenever neighboring cells differ in levels of dMyc. Wildtype cells, containing endogenous *dmyc*, are only killed when they reside near cells overexpressing dMyc.

Likewise, although *dmyc* hypomorphic cells are viable when surrounded by each other, when they exist in somatic clones surrounded by wildtype cells, they are eliminated from the wing (Johnston *et al*., 1999).

Similar competitive outcomes result from other manipulations that allow some cells to have higher dMyc levels than their neighbors. For example, loss of *archipelago* (*ago*), which encodes a *Drosophila* F box protein homologous to human Fbw7, results in elevated dMyc protein levels and a competitive advantage that allows *ago* mutant cells to overtake whole body structures while wildtype cells are eliminated (Moberg *et al*., 2004).

Cells with mutations in receptors for patterning factors, such as Dpp, Wg, or EGF, or those deficient in ribosome biogenesis, such as *Minutes*, a large class of mutations in genes encoding ribosomal proteins, are also subject to competitive elimination (Burke *et al*., 1996; Johnston *et al*., 2003; Baker and Yu, 2001; Morata and Ripoll, 1975).

Cells lost in competition die by apoptosis, but how the apoptotic program is initiated is not clear (de la Cova *et al*., 2004; Moreno *et al*., 2004; Moreno *et al*., 2002).

Two models have been proposed to explain how cell competition occurs (Fig. 8). In one model, less competitive cells are deprived of growth factors due to the capture of such factors by their more competitive neighbors (Milan *et al*., 2002). The elimination of cells unable to receive Dpp, Wg, or EGF is certainly consistent with this idea, as is a report that some *Minute* cells are deficient in responding to Dpp (Moreno *et al*., 2002). However, the predictions that this model makes do not always hold. For example, dMyc expression neither enhances a cell's response to signals such as Dpp or Wg, nor alters the response of neighboring cells, as would be predicted from a ligand-capture model (de la Cova *et al*., 2004).

A second model posits that competition is due to secretion of a factor that initiates an apoptotic program in neighboring cells. This model comes from the observation that although physical contact is not necessary for wildtype cells to be killed, they must be in close proximity to dMyc-expressing cells (de la Cova *et al*., 2004). Thus, dMyc may allow cells to sense each other's presence, and induce competition via a short-range signal.

Interestingly, many of the *Minute* genes, as part of the large number of genes involved in ribosomal biogenesis, are dMyc transcriptional targets. Also, as mentioned above, dMyc expression is influenced by Wg and Dpp. These observations might imply that cell competition involving dMyc, a reduction in ribosome biogenesis, or lack of patterning factors, operate by the same mechanism. However, this has not been demonstrated, and the molecular mechanism of cell competition remains to be established.

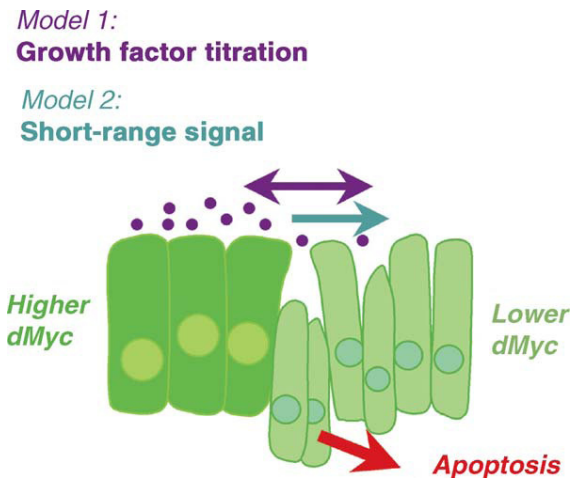


Figure 8. Two models to explain dMyc induced cell competition. (1) cells expressing more dMyc deprive their neighbors of growth and/or survival factors and (2) dMyc expressing cells induce a short-range signal that kills nearby cells that express less dMyc [Modified from: de la Cova and Johnston 2006].

dMYC PROTEIN STABILITY AND DEGRADATION

Given *myc* importance in the regulation of many different aspects of a cell's life, like growth, proliferation and apoptosis, its expression is tightly regulated by multiple signaling events acting at transcriptional, translational and post-translational levels (Bernard and Eiles, 2006; Vervoorts *et al.*, 2006). Phosphorylation is one of the most relevant post-translational mechanisms by which Myc stability is regulated and has been described in depth for the mammalian c-Myc isoform (Fig. 9)

c-Myc protein is rapidly stabilized in response to Growth Factors, since Ras-dependent MAPK activation is associated with c-Myc phosphorylation at Serine 62 in mammalian cell lines (Sears, 2004). At the

same time, this first event is ment to keep c-Myc presence in the cell under strict control because it triggers a second phosphorylation at Threonine 58, mediated by the Glycogen-Synthase Kinase 3β (GSK3β) (Sears *et al.*, 2000) and subsequently the Serine 62 de-phosphorylation by the phosphatase PP2A. This mono-phosphorylated form of Myc is then recognized and bound by the ubiquitin-ligase Fbw7 that promotes its degradation by the proteasome pathway (Yeh *et al.*, 2004). It must be noticed that GSK3β phosphorylation requires a priming event (Dajani *et al.*, 2001) which, for some proteins such as β-catenin/Armadillo or Cubitus interruptus (Ci), is carried out by members of the CKI family (Jia *et al.*, 2002; Liu *et al.*, 2002; Price *et al.*, 2002; Yanagawa *et al.*, 2002).

Mutations of the *Drosophila* ubiquitin-ligase *archipelago* (*ago*), homologous to the mammalian Fbw7, result in dMyc protein accumulation suggesting that coordination between phosphorylation and ubiquitination is a conserved feature between species (Moberg *et al.*, 2004).

In addition to phosphorylation at its N-terminus, c-Myc protein stability is regulated by phosphorylation in the PEST domain (amino acids 253-266), located near the conserved Acidic Box. Deletion of the Myc PEST domain enhances its protein stability (Gregory *et al.*, 2000).

Although the *Drosophila* Myc protein (dMyc) is only 26% identical to human c-Myc (the most represented form of the family) over its length, the homology increases up to 57% in critical functional regions such as the Acidic Box and the PEST domain (Gallant *et al.*, 1996). Moreover, dMyc and c-Myc proteins share similar functions; human c-Myc can rescue lethal

dmyc mutations to viability **(**Benassayag *et al.*, 2005) and dMyc is able to partially substitute for the proliferation defect of *c-myc*-deficient mouse embryo fibroblasts (Trumpp *et al.*, 2001).

Taken together all these data promped us to question if Growth Factors and Nutrients ability to control size could be mediated by Myc regulation either at its transciptional or at its protein level.

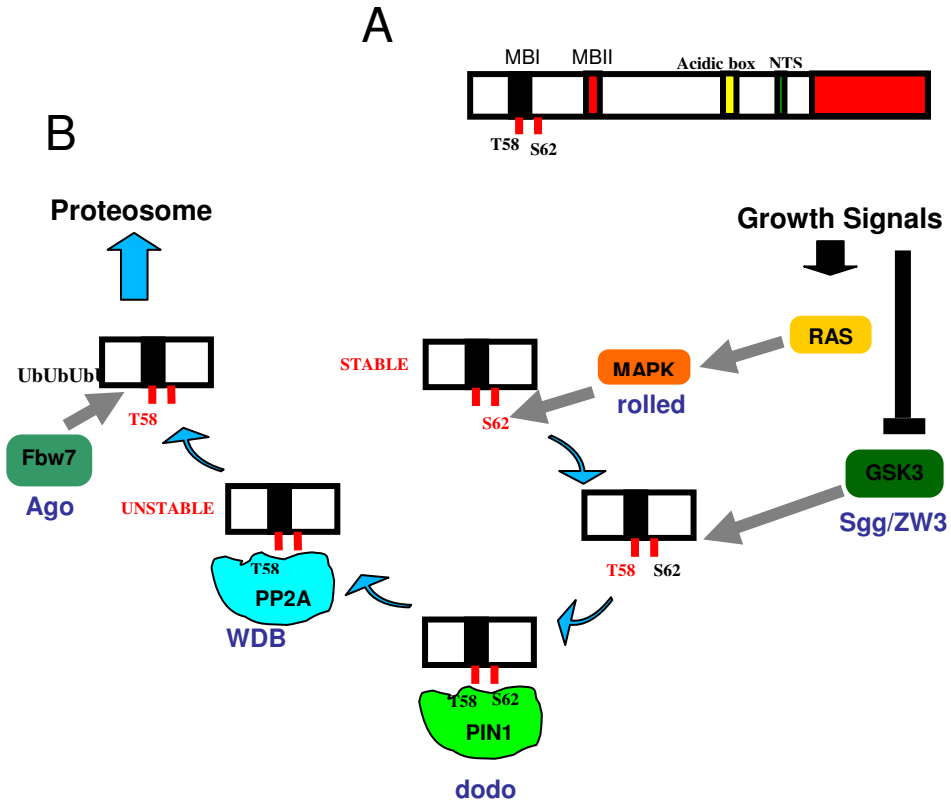


Figure 8. Regulation of Myc protein stability by phosphorylation. Schematic representation of Myc protein domains important for its stability (A). RAS/MAPK signaling activated by Growth Factors triggers Myc phosphorylation at Ser62 which results in its stabilization. At the same time the presence of this phosphate allows GSK3β binding and phosphorylation at Thr58. This second event targets Myc for degradation through the proteasome since PP2A is now able to remove the phosphate group on Ser62. Thr58 mono-phosphorylated Myc protein bound to the ubiquitine ligase Ago is sent to proteasome for degradation.

Thesis outline

Throughout development cellular and organ growth are regulated in a coordinated manner. Each cell and organ integrates hormonal signals, nutrients availability and intrinsic developmental programs to gain the correct final size.

Description of pathways involved in these processes and the way they are integrated is important to understand how the growth control is achieved during development and provides new insights into mechanisms that cause aberrant growth in diseases as cancer.

In recent years, biochemical analysis in cell culture and genetic dissection of growth control in model organisms allowed the identification of two evolutionarily conserved signaling pathways devoted to size determination: TOR signaling that responds to nutrients availability, and Insulin signaling involved in growth control in response to hormonal stimuli. Genetic studies suggest that a fine coordination between these two cascades exist that prevent a cell to divide prior it has reached a critical weight.

Molecular mechanisms involved in this coordination are still poorly understood, nevertheless TOR and Insulin signalings are well conserved between *Drosophila* and vertebrates making the fruit fly an excellent model for studying the relative role of these two pathways in growth regulation.

With regard to Myc protein, its involvement in size control has long been known. Mice and flies carrying a hypomorphic mutation of the *myc*

gene are smaller in size. Moreover, genomic and functional analysis suggests an involvement of a great percentage of genes transactivated by dMyc in metabolism regulation, ribosome biogenesis, protein synthesis and cell cycle progression.

Growth Factors have been shown to rapidly stabilize Myc protein in mammalian cells, subsequent to activation of the Ras-ERK/MAPK cascade (Sears *et al.*, 1999). At the same time our previous data clearly demonstrated dMyc protein up-regulation subsequent to Insulin and EGF subministration in *Drosophila* S2 cells.

These results prompted us to further investigate, in an *in vivo* model, growth controlling mechanisms that show a dependence on Myc. Our analysis ranges from the characterization of dMyc protein stability to the dissection of InR/TORC1 pathway in order to understand Myc contribution to Growth Factors and Nutrients mediated growth.

In fact, although a direct modulation of *myc* expression and/or protein stability from signaling controlling cellular and organismal growth could be predicted, until now it has never been observed *in vivo* due to the instability of Myc protein. To overcome this issue it is now available a new method of inducible gene expression that renders the UAS-Gal4 system conditional to the presence of a steroid in the culture medium (Rogulja and Irvine, 2005). This way it became possible to induce the transgene expression when the clone reached the appropriate size and, at that time, to check for the gene of interest.

In this work we were able to highlight the mechanism that leads to dMyc protein stabilization and to demonstrate a synergic behaviour of Insulin/TORC1 pathways on Myc proto-oncogene both *in vitro* and *in vivo.* Moreover we observed for the first time a genetic interaction between Myc and Rheb that seems to be TOR independent, opening new interesting perspectives toward the understanding of growth regulation.

Materials and Methods

Cell culture and Western blot

Drosophila Schneider S2 cells were grown at 25° C using Schneider medium (GIBCO) supplemented with 10% heat-inactivated FCS and 100 U.I. of penicillin/streptomycin (GIBCO).

Treatment with Insulin and inhibitors: S2 cells were serum starved in 0.1 % serum for 12 hrs then various chemical inhibitors were added to the medium together with 1 um/ml of Insulin (from porcine Sigma) for 4 hrs at 25 °C. Cells were washed in PBS and lysed using a buffer containing 50 mM HEPES pH 7.4, 250 mM NaCl, 1mM EDTA, 1 % Triton and protease inhibitors. After measurement of the protein concentration, using the Biorad-kit, proteins were separated by SDS-PAGE, electrotransferred to a nitrocellulose membrane, subjected to Western blot analysis with specific antibodies, and visualized by ECL-Plus (Amersham Pharmacia Biotech). Chemical Inhibitors were 10 µM for Wortmannin, 100 nM for Rapamycin 10 µM for LiCl, 50 µM for MG132, 10 mM for Okadaic Acid, 10 µM for CHX (Sigma), 10 µM CKI-7 (US-Biological).

Primary antibodies were: anti-dMyc mAb (Prober *et al.,* 2004 1:5); anti-actin mAb and anti vinculin mAb (1:3000 Sigma), anti HA (1:1000 Sigma).

Quantitative real-time PCR

Drosophila S2 cells were reared at the indicated condition (see Results) and total RNA was extracted using the RNeasy kit (Qiagen) and on-column DNase digestion to eliminate genomic DNA contamination. Reverse transcription was carried out with oligo-dT primers on 1 µg of RNA per sample using the Omniscript RT kit (Qiagen). For quantitative real-time PCR we used SYBR green PCR mastermix (Applied Biosystems) on a ABI 7900HT machine, and the following primer pairs:

rp49 (ref): 5'- AAACGCGGTTCTGCATGAG -3' (reverse),

5'- GACGCTTCAAGGGACAGTATCTG -3' (forward);

dmyc: 5'- CATAACGTCGACTTGCGTG-3' (reverse),

5'- GAAGCTCCCTGCTGATTTGC -3' (forward);

fibrillarin (ref): 5'- ATGCGGTACTTGTGTGGATG -3'(reverse),

All PCR reactions were carried out in triplicate, and control PCR-reactions on "no RT" templates (i.e. templates which had been mock reverse-transcribed) confirmed that genomic DNA had been efficiently removed.

Microarray analysis

S2 cells were seeded in 6 wells plates, washed, and 15 µg of dsRNA against *dmyc* or *lacZ* in 3 biologically independent replicates were added in each well in serum free medium. After for 12 hrs cells were treated with

Insulin for 4 hrs, then washed and lysated as described (Hulf *et al.*, MCB 2005). Total RNA was harvested from the different samples and processed for analysis on Affymetrix whole-genome microarrays v 2.0

Fly lines

Fly stocks were obtained from the Bloomington stock center, with the exception of:

- *yw; UAS-Dp110*
- *yw; UAS-PTEN*
- *UAS-Rheb*
- *tor^{2l1}/TM3*
- *w; FRT82>$Rheb^{7A1}$/TM6b*

kindly provided from H. Stocker ETH-Zurich;

- *yw, $dmyc^{PL35}$*, *tub*>FRT>(*dmyc*-cDNA)>FRT>GAL4, *ey*-Flp / Y
- *w, dm^{P0} tub*-FRT>(*dmyc*-cDNA)>FRT GAL4 *ey*-Flp / Y
- *yw, dm^{+} tub*-FRT>(*dmyc*-cDNA)>FRT GAL4 *ey*-Flp / Y

characterized in Bellosta *et al.,* PNAS 2005

- *hs-Flp; tub-Gal4, UAS-GFP; FRT82>tub-Gal80 [Hs-CD2]*

were a gift from M. Zecca, Columbia Medical Center, NYC;

- *UAS-dco^3*
- *FRT82B dco^3/TM6B*

stocks were a gift from M. Noll;

- *sgg^{D127} hsFLP; FRT82B Dp(1;3)w^{+}67K, sgg^{+} / FRT 82B Hs-GFP*

from our collaborator J. Jiang, UT Southwestern Med Center, Dallas, TX, USA

Clone Generation and Transgene Induction

For generation of Flp-out clones, flies of the genotypes

- *w, hs-Flp;UAS-dp110*
- *yw, hs-Flp;UAS-PTEN*,
- *yw, hs-Flp; UAS-Rheb/CyO*,
- *yw, hs-Flp; UAS-Tor*
- *yw, hs-Flp; UAS-Tor^{TED}*

were crossed to *UAS-GFP; Act-Gal4:PR/TM6b* flies (Rogulja and Irvine, 2005). Flp-out clones were then generated by heat shock for 30 min at 37°C. Heat shocks were performed 48h AEL.

Gal4:PR was activated by transferring larvae to instant food (Instant *Drosophila* Medium, Connecticut Valley Biological) containing RU486 (Mifepristone, Sigma). Two grams of instant food was mixed with 7 ml of water previously supplemented with RU486, resulting in a final concentration of 20 µg/ml of Mifepristone.

For generation of MARCM clones, flies of the genotype:

- *w; UAS-dMyc; FRT82>Rheb^{7E1}/TM6b*
- *w; FRT82>Rheb^{7E1}/TM6b*

were crossed to a *hs-Flp; tub-Gal4, UAS-GFP; FRT82>tub-Gal80 [Hs-CD2]*. Mitotic clones were then generated by heat shock for 1 hr at 37 ℃, 48 and 72hrs AEL and detected with an α-CD2 staining (M. Zecca) and GFP visualization.

Analysis of cell size and number in the adult eyes

Flies were reared at 25 ℃ under reproducible growth conditions and were age matched (3 days old males) before determin the ommatidial size and number. Ommatidia were counted from scanning electron micrographs (SEM) pictures. From the same photographs, the size of the ommatidia was calculated by measuring the area of 20 ommatidia located in the center of the eye using Adobe Photoshop as previously reported (Bellosta *et al*, PNAS 2005).

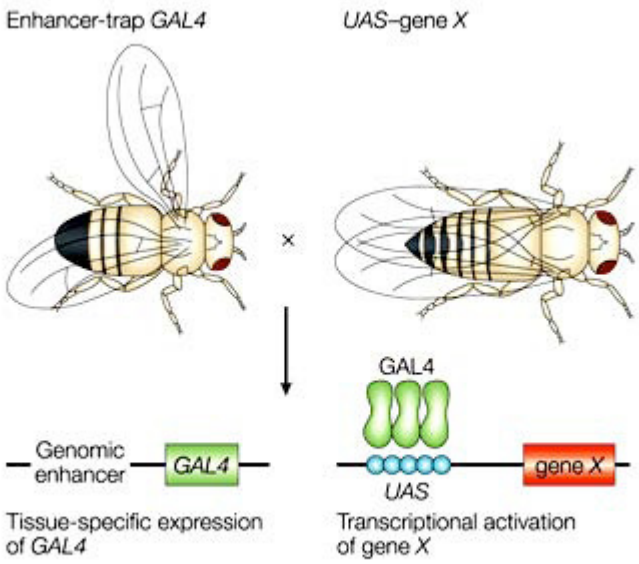
Immunofluorescence analysis

Third instar larvae were dissected in PBS 1X. Imaginal wing or eye discs collected were fixed in 4% paraformaldehyde/PBS for 20 min at room temperature, washed twice in PBS 1X then permeabilized by repeated washes in 0.3% Triton X-100/PBS prior to incubation with specific antibodies.

All antibodies incubations were performed in 3% BSA/PBT. Primary antibodies used were mouse anti-dMyc (Probe and Edgar 1:5); mouse anti-CD2 (a gift from M. Zecca 1:1000) rat anti-caspase-3 (DHSB 1:200); rat anti-ELAV (DHSB 1:1000). Secondary antibodies were anti-mouse Alexa Fluor 555 (Invitrogen 1:200); anti-rat FITC (Invitrogen 1:200). After washing, discs were mounted in Mowiol and analyzed in a confocal microscope. Images were processed using LSM Image browser (from Zeiss) and Adobe PhotoShop software at 20X and 40X magnification.

GENETICS

UAS-Gal4 system (Brand and Perrimon, 1993)



The UAS-Gal4 is a binary system that allows, in the fly, to study the effects of the ectopic expression of a gene of interest.

Gal4 is a powerful transcriptional activator of *Saccharomyces cerevisiae*, that recognizes and binds to specific regulatory sequences called UAS (Upstream Activating Sequences).

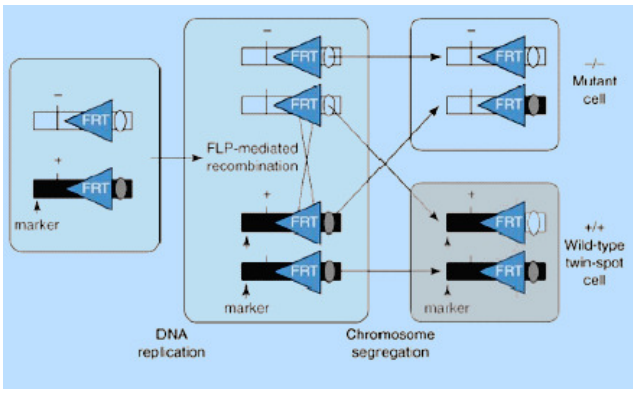
In *Drosophila* it is possible to construct two separate transgenic lines. A first line contains the Gal4 sequence under the control of a regulatory element of a *Drosophila*'s gene, that can induce the expression in specific moments of development and in specific body districts. The second line contains a construct in which the *cDNA* sequence of the transgene of interest is put downstream one or more UAS elements.

By crossing those 2 lines it is possible to originate a progeny containing both constructs, and this allows the expression of the Gal4 transactivator, that in turn activates the gene put under the UAS control in a pattern-specific manner (Brand and Perrimon, 1993).

A large series of promoters exists stored in stock centers (the most important is the Bloomington Stock Center) that can drive Gal4 expression in defined types of cells or tissues; it is also possible to drive Gal4 expression by a heat shock promoter, that permits a temporal control of induction.

The use of UAS-hairpin constructs further allows to silence post-transcriptionally the expression of certain genes (UAS-RNAi).

FLP-FRT (Xu and Rubin, 1993)



In *Drosophila* the yeast binary sistem FRT-Flp that allows site-specific recombination is broadly used.

The Flippase (Flp) recombinase induces recombination at the level of specific sequences called FRT (Flippase Recognition Targets) (Golic and Linquist, 1989).

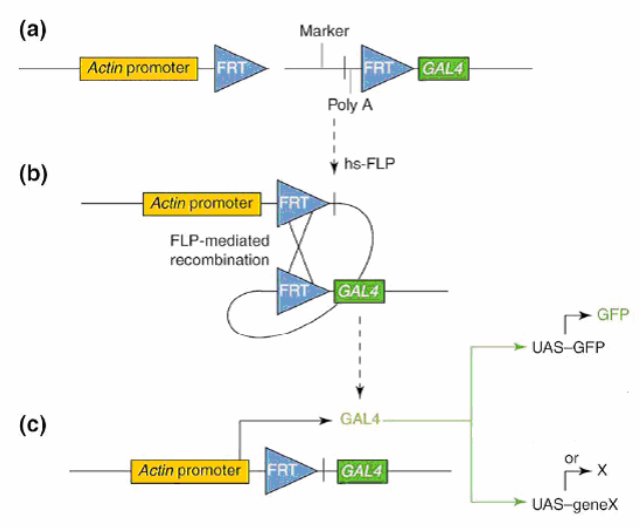
This system can be used to generate genetic mosaics to study LOF mutations.

Stocks are available with the FRT sequence in the pericentromeric region of all the chromosome arms, this allows the exchange of the entire chromosome arm by mitotic recombination.

Mitotic recombination by Flp activation can be induced in heterozygous mutants for a gene of interest; as a result it is possible to obtain a clone homozygous for the mutation and a wild type twin clone (Xu and Rubin, 1993).

The possibility to generate homozygous mutant clones is very useful in the study of lethal recessive conditions and to investigate cell-cell specific interactions. Most commonly, Flp is expressed under the control of a heat-shock promoter (hsFLP) as it ensures the possibility to control either the developmental stage in which to induce recombination or the clone number that is related to heat pulse length; the distribution of clones is instead random within the animal.

Flp - Out (Neufeld, 1998)

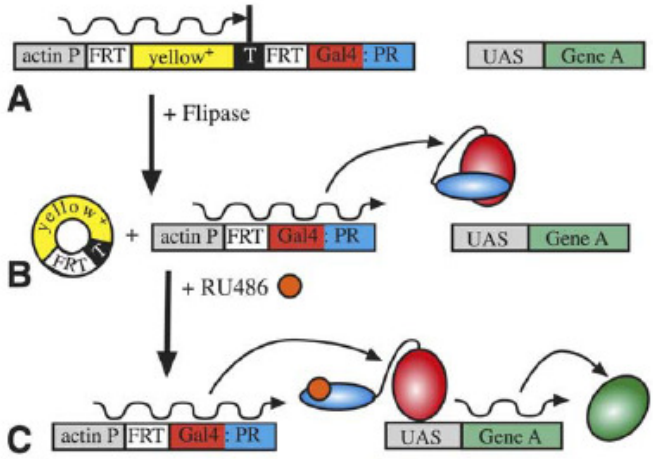


The FLP-Out technique permits the overexpression of a UAS controlled sequences in a clone of cells. A FLP-Out construct consists of a constitutive promoter (i.e. actin or tubulin) followed by an FRT sequence, a marker with a polyA terminator, a second FRT sequence and a Gal4 sequence.

The Flippase expression can induce the excision of the DNA fragment between two FRT sites, and the constitutive promoter can thus transcribe the Gal4 sequences. This, in turn, permits the expression of all the UAS-transgenes present in the line. Most of the time, a FLPout line also carries a cell marker (GFP or LacZ) under the UAS control.

In this essay (see results) we made wide use of this technique. In particular we worked with a FLP-Out line, in which the Gal4 transcriptional activity is rendered conditional to the presence of an hormone (Mifepristone)

in the medium. This allowed us to limit the UAS-transgene expression to a shorter window of time late in development in order to analize its effect on dMyc protein right away (Rogulja and Irvine, 2005)



MARCM system (Lee and Luo, 1999)

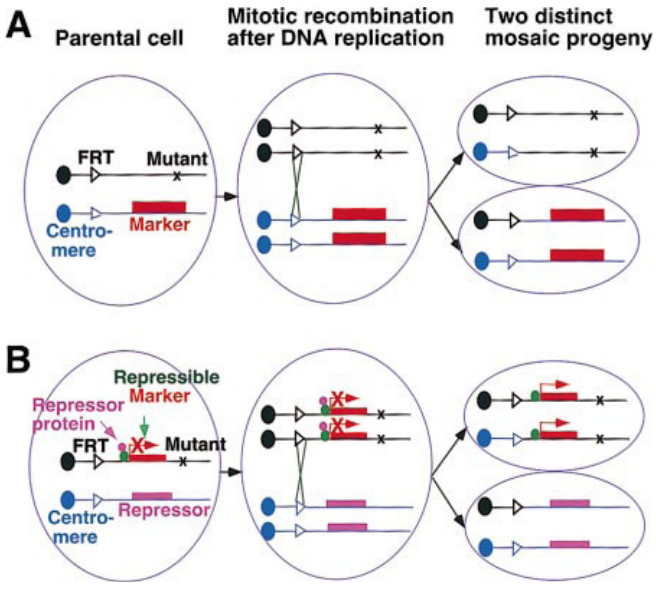
The MARCM system (Mosaic Analysis with a Repressible Cell Marker) (Lee and Luo, 1999) allows to generate homozygous mutant cells uniquely in mosaic tissues.

To achieve this goal, the yeast GAL80 protein was introduced into the GAL4-UAS binary expression system in *Drosophila* (Brand and Perrimon, 1993). The MARCM system initially contains cells that are heterozygous for

a transgene encoding the GAL80 protein, which inhibits the activity of the GAL4 transcription factor.

Following FLP/FRT-mediated mitotic recombination, the GAL80 transgene is removed from one of of the two daughter cells, thus allowing expression of a GAL4-driven reporter gene specifically in this daughter cell and its progeny (see figure). If a mutation is present on the chromosome arm *in trans* respect to the GAL80 transgene, the uniquely labeled GAL80-negative ($GAL80^{-/-}$), Gal4 expressing cells should be at the same time homozygous for this mutation. Therefore, using the MARCM system, it is possible to specifically label clones of cells homozygous for any mutation in a mosaic tissue.

In particular the MARCM system has shown to be useful in the study of genetic interactions because of its ability to generate LOF clones for recessive mutations in which it is possible to overexpress any other gene under the control of a UAS element.



RESULTS

Chapter 1:

dMyc phosphorylation regulates its stability

CKIα and GSK3β induce dMyc ubiquitination and degradation through the proteasome pathway

Our characterization of dMyc role in growth regulation downstream of Growth Factors/Nutrients availability began with the analysis of its protein stability since in mammals Growth Factors stabilized c-Myc protein through MAPK-mediated phosphorylation at Serine 62 (Sears, 2004). At the same time this mono-phosphorylated c-Myc form is recognized as a target by Glycogen-Synthase Kinase 3β (GSK3β) that add a phosphate group on c-Myc Threonine 58 (Sears *et al*., 2002).

At the same time, GSK3β in some cases requires a priming event of phosphorylation in order to recognize and bind its substrates (Dajani *et al.,* 2001). The priming phosphorylation in these case is carried out by members of the Casein Kinase I (CKI) family (Jia *et al*., 2002; Liu *et al*., 2002; Price *et al*., 2002; Yanagawa *et al*., 2002).

Given these preliminary evidences, in order to determine how a cell regulates dMyc activity in response to growth promoting cues, we first decided to investigate if the mechanisms that target Myc proteins for degradation are conserved between species and more interestingly if CKI kinases are required in *Drosophila* as priming kinases for GSK3β taking part to its protein regulation.

We first assessed if dMyc amino acid sequence could contains specific domains potential targets for both kinases (CKI = S/T-XX-S/T; GSK3β = S/T-XXX-S/T as described in Obenauer *et al*., 2003) and indeed

we found several of them (Figure 1A and Table 1).

Once identified these domains are putative targets for phosphorylation, we next analyzed the effect of S2 cells transfection with HA-tagged constructs for CK1α and GSK3β or their Kinase Dead inactive (KD) variants on dMyc protein stability. As shown in Fig. 1B dMyc protein was considerably reduced in presence of both kinases (Fig. 1B), while the expression of their KD mutants caused the opposite effect.

Cycloheximide (CHX), a protein synthesis inhibitor, was used to further confirm these first results. S2 cells were treated with CHX and dMyc protein turnover over time in presence of GSK3β or CKIα or their KD was analyzed at different time points. The intensity of each band obtained in western blot and corresponding to different treatments was quantified and reported in graph (Figure 1C). CKIα or GSK3β activity decreased endogenous dMyc half-life from 35 minutes to approximately 15 minutes. Conversely, expression of their kinase-dead forms increased dMyc half-life up to 80 and 75 minutes, respectively.

We further confirmed this regulation to occur on dMyc protein by analyzing *dmyc*-mRNA and, as expected, we were not able to detect any significant change either in presence of the kinases or of their inactive forms (Figure 2).

Moreover, since it was demonstrated that phosphorylation of c-Myc by GSK3β allows Myc ubiquitination by the ligase Fbw7, we tested whether the presence of GSK3β or CKIα kinase would affect dMyc protein ubiquitination. We used a stable S2 cell line trasfected with HA-dMyc,

expressed under a hsp70 promoter (Bellosta *et al.*, 2005) and, at the same time, we blocked the proteasome activity with MG132 to avoid rapid dMyc degradation. S2-HA-dMyc cells were transfected with different kinases or their inactive mutants whose expression was induced with $CuSO_4$. Subsequently, a one-hour heat shock was applied to induce hs>HA-dMyc expression. Cells were lysed, dMyc was immunoprecipitated with anti-dMyc monoclonal antibodies and ubiquitination levels were analyzed by western blot.

As depicted in Fig. 1D, CKIα or GSK3β expression promotes dMyc ubiquitination, while expression of the kinase-dead mutants do not interfere with this process.

Taken together our data indicate that CKI family members represent yet uncharacterized components of Myc degradation pathways, which, similarly to GSK3β, regulate Myc protein stability through the ubiquitination and proteasome degradation.

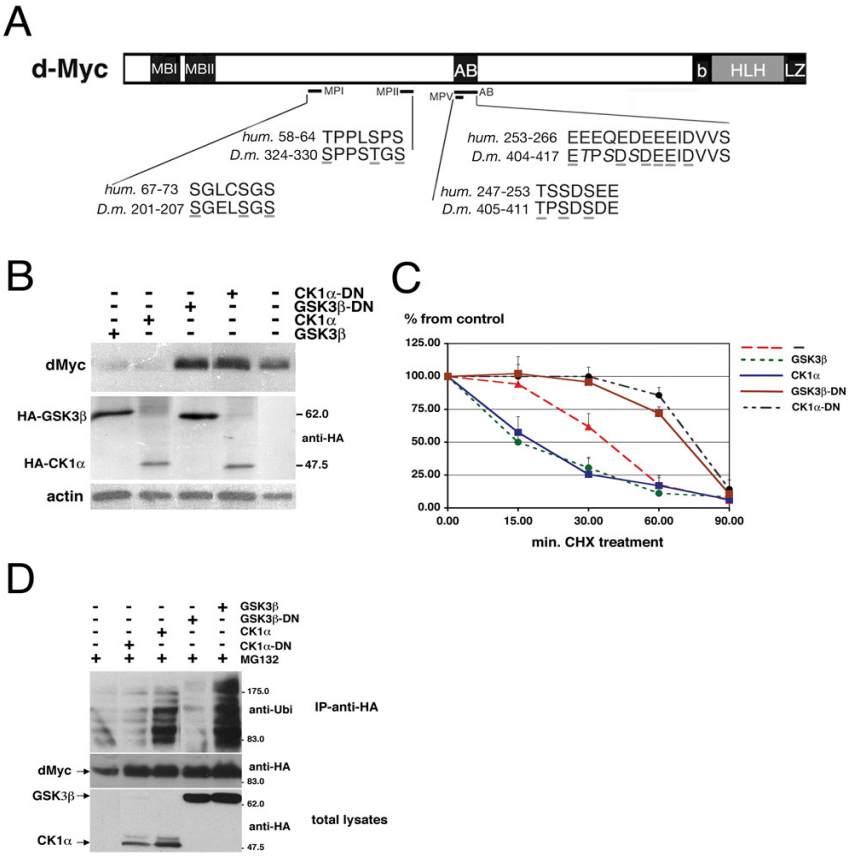


Figure 1.1: CKIα and GSK3β kinases induce ubiquitin-dependent degradation of dMyc.

Schematic representation of dMyc protein. The amino acid sequences of dMyc phosphorylation mutants and homologous c-Myc sequences are shown. Amino acids changed by site-directed mutagenesis have been underlined (A).

CKIα and GSK3β regulate endogenous dMyc protein stability. S2 cells were transfected with the indicated HA-tagged kinases mutants and protein expression was induced using copper sulfate. Their level of expression was quantified by immunoblotting using anti-HA. Endogenous dMyc protein was quantified using anti-dMyc antiserum. Actin was used as loading control. Molecular markers are shown to the right (B).

dMyc Half-life measurement in presence of CKIα and GSK3β or their KD mutants. The graph represents the quantification of endogenous dMyc levels after CHX supplementation. Cells were lysed at the indicated time points after CHX treatment and dMyc expression was visualized by western blot using anti-dMyc antibodies. Intensity of dMyc bands was quantified in pixels from the scanned X-Ray film using AdobePhotoshop. Background was calculated from an equivalent area in each lane and subtracted from the value for dMyc in the respective lane. Time 0 was set at 100. Data are plotted as percentages over control for each point. These experiments were repeated three times (C).

GSK3β and CKIα kinases mediates dMyc ubiquitination. The stable S2-HA-dMyc cell line was transfected with various kinases and their expression was induced using copper ions (Bellosta *et al.*, 1995). Upper panel: HA-dMyc was immunoprecipitated from cell extracts using anti-HA antiserum and its ubiquitinated forms were visualized using anti-ubiquitin antiserum. Lower panel: HA-dMyc and HA-kinases epression was visualized using anti-HA antiserum. MG132 was added to the medium at heat-shock to avoid Myc degradation.

mutants	Sequence Homology	% identity[(a)]	stability[(b)]
dMyc-PI	*D.m.* mutation **A---A-A** *D.m.* 201-207 **S**GEL**S**G**S** *hum* 67-73 SGLCSPS	50	< 30 min
dMyc-PII	*D.m.* mutation **A---A-A** *D.m.* 324-330 **S**PPT**T**G**S** *hum* 58-64 TPPLSPS	43	60 min
dMyc-PV	*D.m.* mutation **A-A-A--** *D.m.* 405-411 **T**P**S**D**S**DE *hum* 247-253 TSSDSEE	75	180 min
Acidic Box	*D.m.* mutation **Q---N-NQQ-N** *D.m.* 404-417 **E***TP**S***D**S***DEE**IDVVS *hum* 253-266 EEEQEDEEEIDVVS	54	180 min

Table 1.1. (a) Percentage of identity between *Drosophila melanogaster (D.m.) and human* aminoacidic Myc protein sequences, calculated using DNA-Strider TM 1.3. Amino acids substitutions in *D. m*. Myc protein are represented in bold. Underlined are the conserved substituted amino acids in the human Myc sequence. The residues in dMyc-PII and PV, putative substrates for GSK3β are represented in shadow and for CKIα in engrave .
(b) The stability of dMyc mutants was compared to that of dMyc-WT, which, in our biochemical experiments, was estimated to be of 30 minutes.

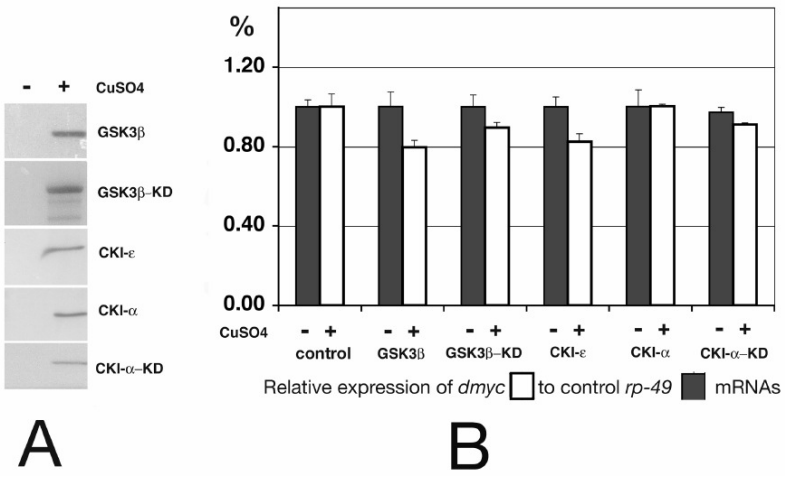


Figure 2.1. Relative *dmyc*-mRNAs level after over-expression of CKIα, CKIε, GSK3β kinases or their kinase-dead (KD) mutants analyzed by qRT-PCRs in S2 cells.
The kinases, or kinase-dead mutants, do not affect endogenous *dmyc*-RNAs level.
S2 cells were transfected with the indicated kinases and their expression was induced by $CuSO_4$ supplemented medium for 12 hrs. Cells were harvested and lysed for protein or RNAs extraction. Kinases presence in the lysates was quantified by western blot using anti-HA antiserum (A). Relative *dmyc*-mRNAs level was measured by qRT-PCR, *ribosomal protein* 49 (*rp49*) was used as control (B).

***In vivo* reduction of GSK3β, CKIα or CKIε results in dMyc protein accumulation**

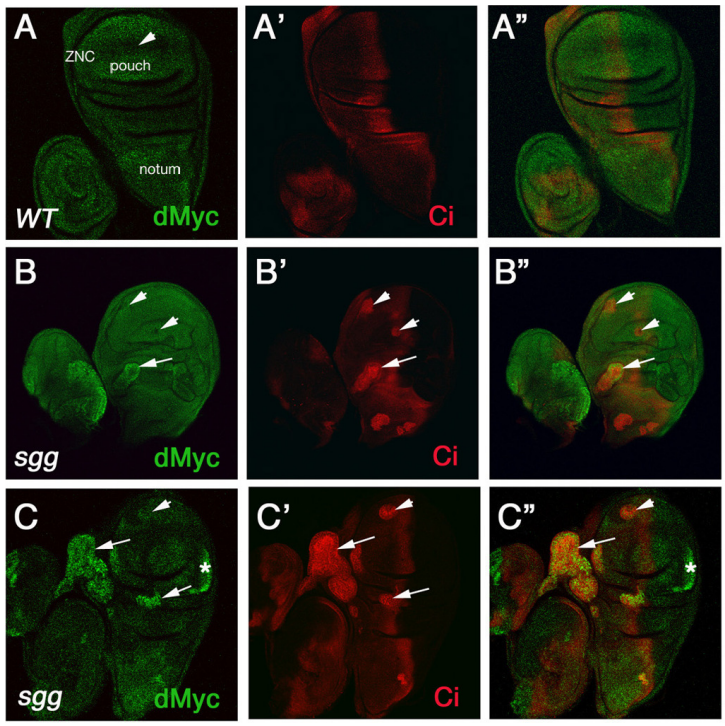


Figure 3.1: dMyc is up-regulated in *sgg/Gskβ* mutant cells. (A-A") Immunostaining of third instar wing imaginal disc for endogenous dMyc (green) or for the protein Ci155 (red), which is expressed in the anterior compartment of the disc and is up-regulated in *sgg* mutant cells. Endogenous dMyc levels are reduced along the zone of non-proliferative cells (ZNC) of the Dorsal/Ventral region and in the hinge, but are higher in the wing pouch and notum. (B-C") expression of dMyc increases in clones with reduced level of *sgg,* marked by the expression of Ci155 in the anterior as well as in the posterior compartment of the discs where Ci155 is not expressed (asterisk). Discs are oriented A/P with Anterior to the left and D/V with Ventral to the top.

The *Drosophila* homologue of mammalian GSK3β is known as *shaggy* (*sgg*) or *zeste-white3* (*zw3*) (herein referred to as *sgg*). It acts as a negative regulator of the Wnt/Wingless (Wg) signaling and is involved in a

range of developmental processes, from pattern formation to cell fate determination and proliferation (Cohen and Frame, 2001). We examined *in vivo* the effect of reducing *sgg* expression on dMyc protein level in third instar wing imaginal discs. At this stage of development, dMyc is primarily expressed in the notum and wing pouch (Figure 3A-A" arrowhead) except for a stripe of cells along the dorsal-ventral boundary known as Zone of Non-proliferative Cells (ZNC). Here *dmyc* is transcriptionally repressed by Wg signaling (Duman-Scheel *et a*l., 2004; Herranz *et al*., 2008). *sgg* mutant clones were induced by mitotic recombination and marked by Ci155 staining. Ci155 is normally expressed in the anterior compartment of the wing imaginal disc in absence of *sgg*.

dMyc accumulation was visible in *sgg* mutant clones falling in the peripheral region of the wing disc and in the hinge (Figure 3B-C" arrows), while clones in the wing pouch failed to show dMyc accumulation (arrowhead).

To explain this positional effect we considered that Wg signaling is known to downregulate *dmyc*-mRNA in the ZNC (a defined stripe of cells at the dorsal-ventral boundary) while its effect slopes down while moving away from this boundary (Johnston *et al*., 2003; Strigini *et al*., 2000). At the same time *sgg* silences Wg signaling during development. For this reason *sgg* mutant clones are supposed to activate Wg signaling and, as a consequence, downregulate *dmyc* at its mRNA level (Duman-Scheel *et al*., 2004; Herranz *et al*., 2008). These experiments indirectly excluded a role for Hh signaling in the regulation of dMyc in the wing disc since dMyc

protein also accumulated in clones located in the posterior compartment, where Ci normally is not expressed (Figure 3C asterisk).

A more straightforward way to show the same results is depicted in Fig 4 where *sgg* mutant clones were induced randomly in the wing disc and directly detected by GFP coexpression. dMyc protein was analized by immunohistochemistry. Clones falling outside of the wing pouch were considered. They show overgrowth features and dMyc staining enhancement suggesting an important role for Myc in control of shape and size.

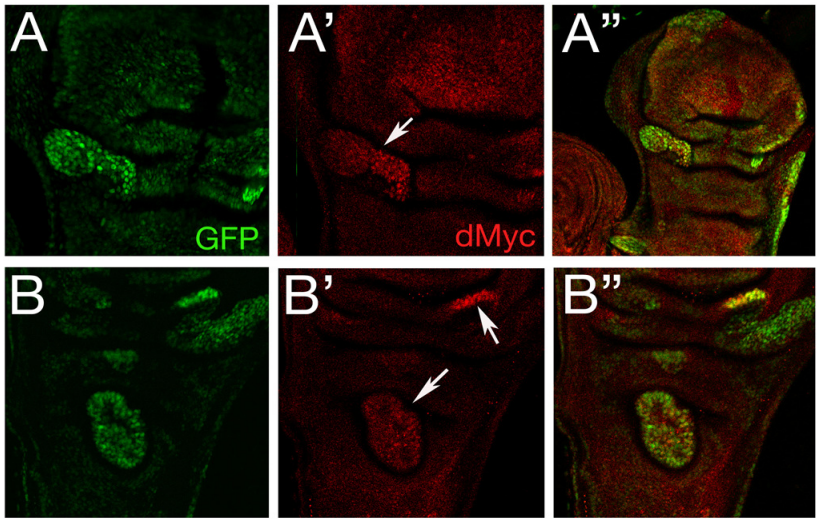


Figure 4.1. *sgg* mutant clones show dMyc upregulation.

We next analyzed how CKIs regulate dMyc expression *in vivo*. CKIs are believed to modulate the Wingless signaling cascade (Knippschild *et al.*, 2005) however their effect has been subject of controversy. In this work we considered two different CK1 isoforms: CK1α was shown to act as a

negative regulator of Wg signaling because it induces the degradation of its main transducer (β-catenin/armadillo). The interesting feature of this degradation mechanism consists in the requirement of CKIα as a primer kinase for a subsequent phosphorylation event carried out by GSK3β (Amit *et al.*, 2002). In contrast, CKIε was identified as a positive regulator of Wg in mammals and Xenopus because it stabilizes the β-catenin (Peters *et al.*, 1999).

In *Drosophila*, Zhang and co-workers demonstrated that hypomorphic CKIα mutations co-operate with CKIε-RNAi to induce ectopic Wg signaling during limb formation, suggesting a synergistic effect for these kinases (Zhang *et al.*, 2006). Using genetic recombination and dsRNAi techniques, we knocked down CKIα or CKIε kinases *in vivo* and we analyzed whether this would affect dMyc protein.

Mutant clones for dco^3 allele (Zilian *et al.*, 1999), the *Drosophila* homologue of mammalian CKIε, were induced by mitotic recombination and marked by the absence of GFP (Fig. 5). At high magnification it was possible to appreciate dMyc protein accumulation in homozygous dco^3 mutant clones falling in the hinge domain (Figure 5A).

Using a combination of Flp-out, UAS/Gal4 and RNAi techniques, we next assessed the CKIα contribution to dMyc stability in CKIα-RNAi clones of cells of the wing imaginal disc. Silencing CKIα by the use of a RNAi construct, results in different outcomes according to the position of the clone inside the wing disc. CKIα-RNAi clones in the hinge, visualized by GFP coexpression, showed dMyc protein accumulation (Figure 5B , arrow),

while silencing CKIα in the ZNC region cells resulted in dMyc protein down-regulation (arrowhead). Because CKIα, together with GSK3β, is known to downregulate Wg signaling *in vivo* (Liu *et al*., 2002), which in turn down regulates *dmyc* at its transcriptional level (Duman-Scheel *et al*., 2004; Herranz *et al*., 2008), the reduced dMyc expression in the ZNC region might be the result of Wg activation as already noticed for *sgg* clones.

Taken together, our data suggest that both CKIε and CKIα are able to down-regulate dMyc protein *in vivo*, however this effect is dependent on patterning cues like Wg signaling that seem to be epistatic on the kinases control of dMyc stability.

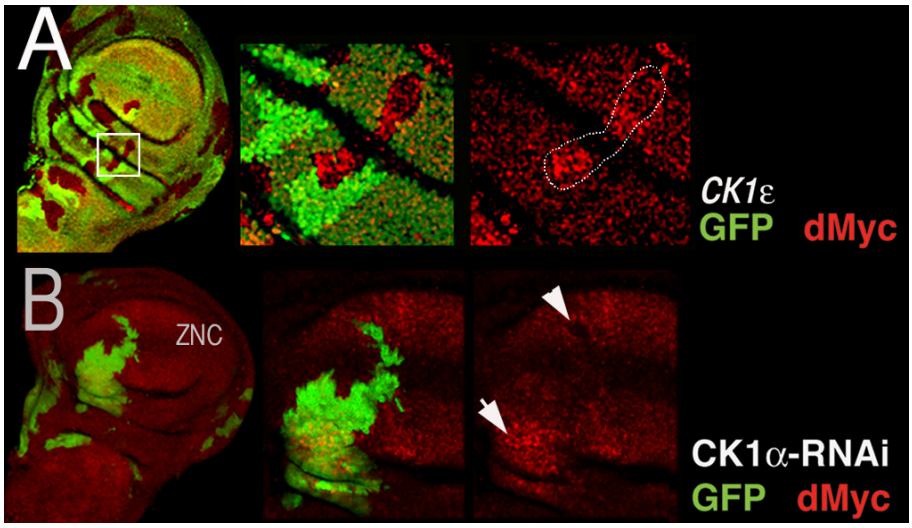


Figure 5.1: dMyc is up-regulated in cells with reduced levels of *dco3/CkIε* and *CKIα* dMyc protein expression (red) in *dco3/CKIε* mitotic clones. Clones were induced at 65±2 hours after egg laying (AEL) by mitotic recombination. dMyc protein level was analyzed in *dco3/CkIε* mutant clones (unstained) and compared to sibling clones (marked with GFP). Higher magnification of the area (right) shows an increase in dMyc protein level in clones *dco3* (marked with a white line) (A). Flip-out clone expressing *UAS-CKIα-RNAi* in the wing imaginal discs induced at 52 ±2 hours AEL. Endogenous dMyc expression in *UAS-CK1α-RNAi* clones (marked by GFP) is visualized by immunostaining with anti-dMyc antiserum (red). Expression of dMyc is higher in clones located in the hinge region (arrow) and lower in the ZNC (arrowhead) (B).

***In vitro* characterization of dMyc phosphorylation mutants reveals that dMyc-PII and Acidic Box mutants are resistant to CKIα and GSK3β degradation**

Analysis of the dMyc amino acid sequence reveals the presence of conserved motifs that are potential substrates for phosphorylation by CKIs and GSK3β (Figure 1A). In order to understand the contribution of these domains to dMyc protein stability, we performed site-directed mutagenesis and substituted Serine and Threonine residues within the dMyc-PI, PII, and PV boxes into Alanine. We also converted the Acidic Box (AB) Glutamic Acid and Aspartate Residues into glycine and asparagine, respectively (Table 1.1). All dMyc mutants were tagged with the hemagglutinin (HA)-epitope at their N-terminus and expressed in S2 cells using the UAS/Gal4 system.

We then analyzed if the newly identified dMyc domains would render dMyc protein sensitive to CKIα and GSK3β kinases-induced degradation.

dMyc-WT, dMyc-PI, dMyc-PII, dMyc-PV and dMyc-AB were co-expressed in S2 cells together with increasing concentrations of CKIα or GSK3β kinases. The kinases expression was induced with cupper sulfate and dMyc protein level was analyzed by western blotting using anti-HA antiserum.

As shown in Figure 6, dMyc-PII and dMyc-AB were resistant to degradation induced by the two kinases, while dMyc-PI and dMyc-PV presented degradation kinetics similar to dMyc-WT.

These data suggest that dMyc-PII and the Acidic Box contain functional domains necessary for the regulation of dMyc protein stability, and this effect is regulated by CKIα and GSK3β kinases.

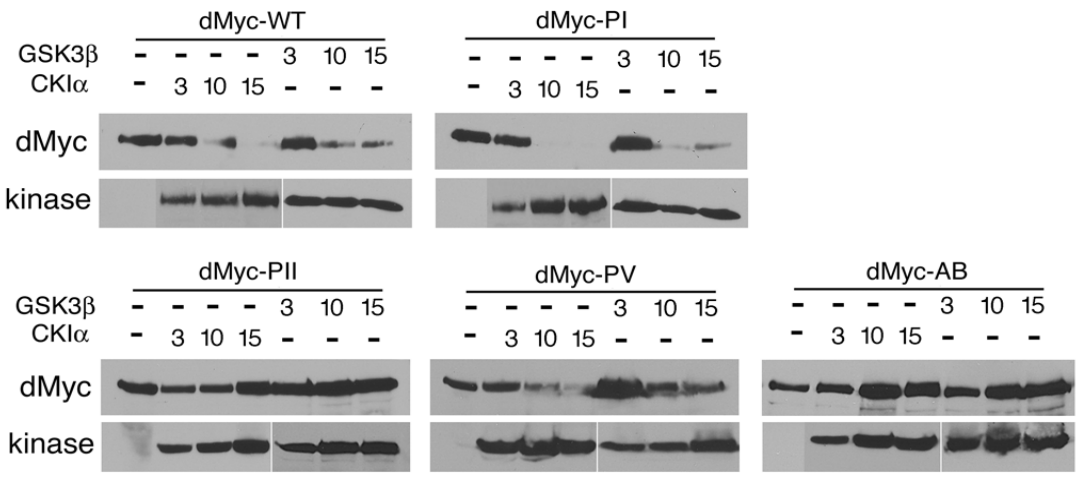


Figure 6.1: *In vitro* stability of dMyc-WT and mutants in presence of GSK3β and CKIα kinases. HA-dMyc-WT and dMyc phosphorylation mutants were transfected in S2 cells together with the indicated concentrations (µg) of plasmids encoding HA-CKIα or HA-GSK3β kinases. Proteins expression was analyzed in the cell extracts by western blot using anti HA antiserum.

Increased dMyc stability inhibits ommatidial differentiation and induces cell death during eye development

Once performed a detailed *in vitro* analysis on dMyc protein stability we next switched to an *in vivo* approach in order to characterize the physiological relevance of our dMyc phosporylation mutants.

We took advantage of FLP-out technique and of the UAS-Gal4 system to screen for enhancers or attenuators of the *dmyc*^{P0} phenotype.

dm^{P0} hypomorphic mutation results from a P-element insertion in the *dmyc* promoter. *dmyc*^{P0} flies are less viable and fertile than *wt* (Johnston *et al*., 1999). To overcome this problem we made use of a mosaic *dmyc*^{P0} line (*dmyc*^{P0} *tub>FRT>[dmyc-cDNA]>FRT>GAL4 ey-FLP*, hereafter called *ey>dmyc*^{P0}) designed to rescue *dmyc*^{P0} level of *myc* to *wt* through an exogenous *dmyc-cDNA*. The same line is also carrying a Flp (Flippase) enzyme, expressed under the *ey* (*eyeless*) promoter, which is thought to cut *dmyc-cDNA* off in eyes and cephalic capsula and bring these tissues back to the *dmyc*^{P0} genetic background (Bellosta *et al*., 2005). Moreover the recombination event allows the expression of a GAL4, specifically in the *ey* territories and as a consequence, to overexpress any transgene, under a UAS-promoter, in a *dmyc*^{P0} mutant background.

Since dMyc expression modulates growth and ommatidial number of during compound eye development (Johnston et al., 1999), we performed genetic epistasis experiments to assess the contribution of our various dMyc phosphorylation mutants.

dMyc-PI expression increased ommatidial size, but to a lower extent than dMyc-WT (317 µm^2 in *ey>dmyc*^{P0}*/Y*; *dMyc-PI/+* and 341 µm^2 in *ey>dmyc*^{P0}*/Y; dMyc-WT/+*). In addition, the total ommatidial number in *ey>dmyc*^{P0}*/Y*; *dMyc-PI/+* flies (756) was higher than that of *ey>dmyc*^{P0}*/Y*; *dMyc-WT/+* animals (633), prompting us to investigate whether dMyc-PI could be less effective than dMyc-WT in inducing apoptosis (Montero *et al*.,

2008).

The α-caspase3 staining confirmed our hypothesis and enforces our previous biochemical observations on dMyc-PI phosphorylation mutant stability underlining the deleterious effect these mutations have *in vivo*. (Fig. 6).

The same kind of analysis was conducted for dMyc-PII, dMyc-PV and dMyc-AB, showing their ability to increase ommatidial size of *ey>dmyc*^{P0}*/Y* eyes (Fig. 7B), but at the same time resulting in a dramatical decrease of ommatidial number and a strong reduction of the head capsule tissue (Figure 7A). We ascribed these defects to Myc-dependent induction of apoptosis (Moreno *et al*., 2008) and we monitored caspase-3 staining to confirm our assumption.

As shown in Figure 8, ectopic expression of dMyc mutant forms correlates with intense caspase-3 but at the same time with loss of ELAV neuronal marker, suggesting that, beside an intensification of cell death, the strongest phosphorylation mutant forms render cells unable to undergo differentiation. According to its lower stability, compared to dMyc-WT, dMyc-PI affected neither photoreceptor differentiation nor apoptosis, further suggesting a significative correlation between stability and the correct development of proliferating tissues.

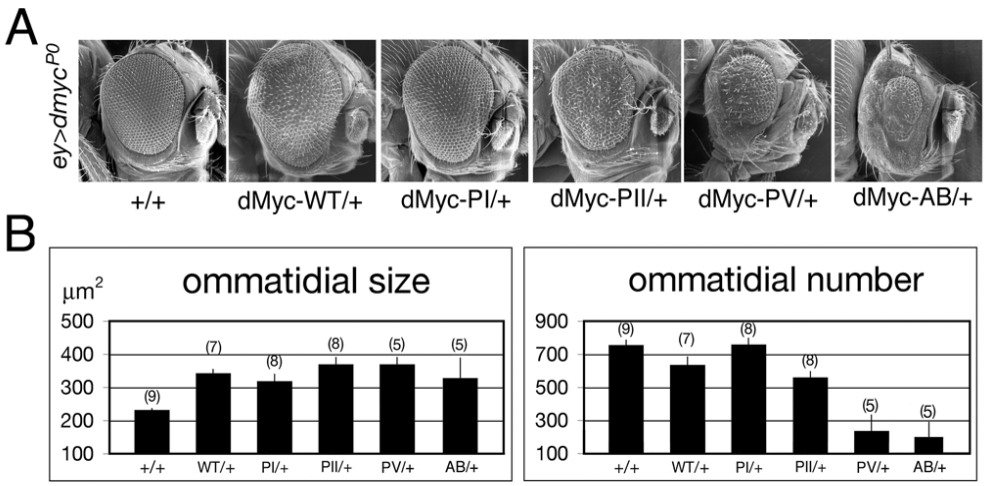


Figure 7.1: Expression of dMyc mutants *in vivo* affects ommatidial size and number.
Representative scanning electron micrographs (SEM) of adult eyes from *ey>dmycP0/Y* flies expressing *UAS-dMyc-WT*, *UAS-dMyc-PI*, *UAS-dMyc-PII*, *UAS-dMyc-PV*, and *UAS-dMyc-AB* transgenes (A). Graphics show the quantification of ommatidia size and number of the indicated genotypes. Standard deviations were calculated based on the number of animals analyzed and is indicated in parentheses (B).

Figure 8.1 (next page): Expression of dMyc-PV and dMyc-AB inhibits ommatidia differentiation and increases apoptosis in eye imaginal discs. Photographs of eye imaginal discs from third instar larvae of the indicated genotypes were stained for dMyc (red) and ELAV (green) expression (A-F) or for apoptosis using anti-active-caspase 3 antibodies (blue) (A"-F"). Photographs were taken at 20x magnification. The morphogenetic furrow is indicated by arrowhead.

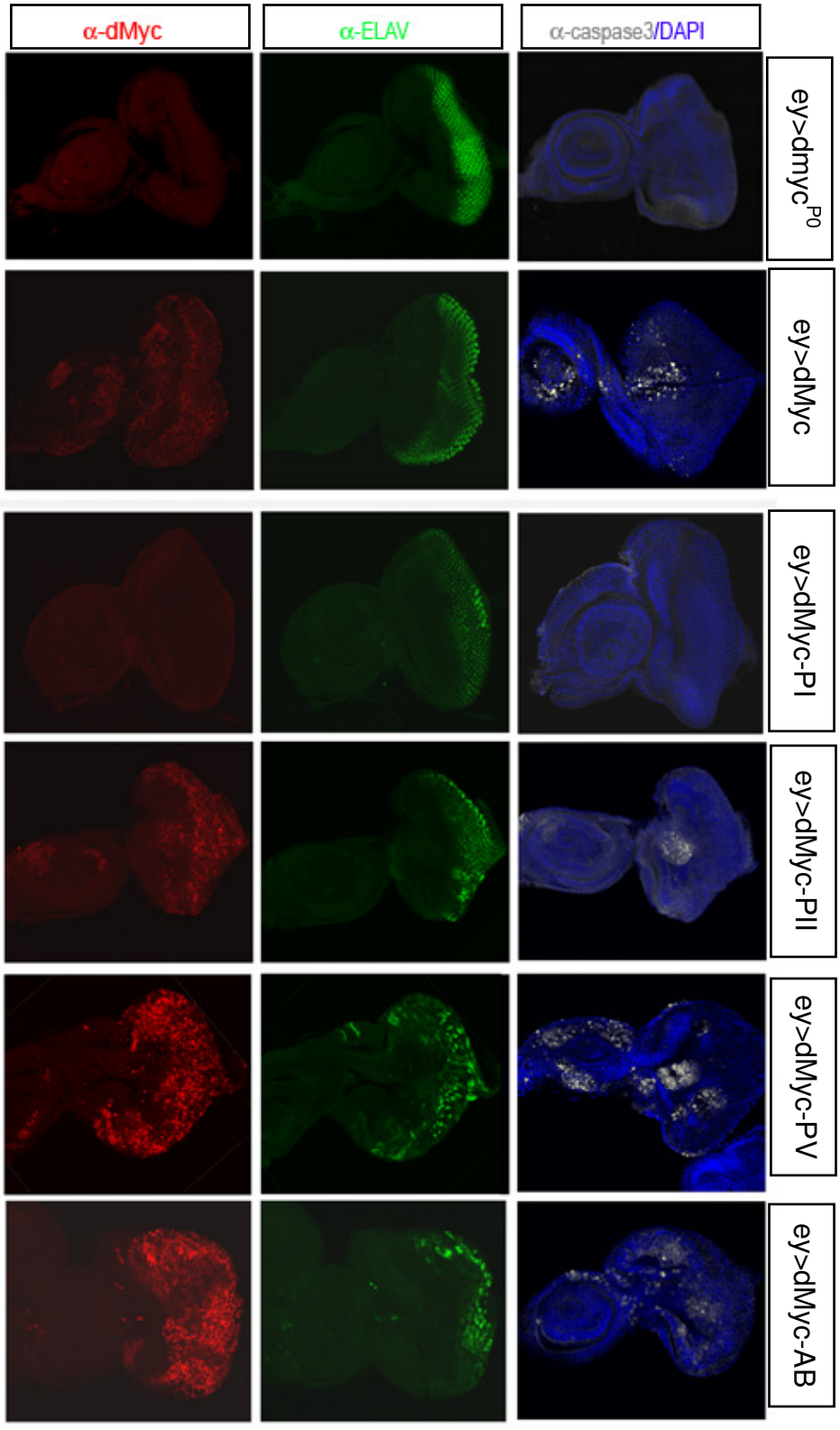


Figure 8.1. see previous page for legend

Chapter 2:

dMyc protein expression is sensitive to Insulin and TOR activity

In vitro and *in vivo* treatments with Insulin increase dMyc protein level

Using biochemical and *in vivo* approaches we previously demonstrated that *dGSK3β/sgg* and members of the CKI family of protein kinases negatively affect dMyc protein stability (Galletti *et al*., 2009). By phosphorylating dMyc on specific residues these two kinases are able to target dMyc protein for degradation through the proteasomal pathway. Moreover we showed to which extent dMyc protein stability correlates with cell growth and how detrimental dMyc overexpression can be in an *in vivo* model.

Since GSK3β lays downstream of pathways activated by growth factors availability (Sears *et al*., 2002) and CKIs are known to prime many of GSK3β targets for phosphorylation (Jia *et al*., 2002; Liu *et al*., 2002; Price *et al*., 2002; Yanagawa *et al*., 2002), our first observations are particularly interesting because they suggest that growth promoting signalings might impinge on dMyc to adjust cell size and that they would do that regulating its persistence into the cell.

Drosophila Insulin/Insulin-like Growth factors system (IIS) is highly homologous to that found in mammals and is able to fulfill the homeostatic function of vertebrate Insulins (Broughton *et al.,* 2005; Rulifson *et al*., 2002; Shingleton *et al*., 2005), mediating glucose import and nutrient storage. At the same time it is able to control cell growth during development, performing the developmental function of the mammalian Insulin-like growth

factors (IGFs) (Esfradiatis A., 1998; Baserga R., in *Cell growth Cold Spring Harbor press*, 2004).

Downstream of Insulin Receptor (InR) activation, the PKB/Akt kinase is able to phosphorylate and inactivate GSK3β/sgg (Zhang *et al*., 2006).

This notion, together with the observation that dMyc and Insulin both act as growth inducers in a cell autonomous fashion (Edgar B., in *Cell Growth,* Cold Spring Harbor press, 2004), prompted us to further investigate the effect of Insulin signaling activation on dMyc protein.

We performed a microarray analysis on *Drosophila* S2 cells, an embryo-derived cell line, trasfected either with a dMyc-RNAi or a LacZ-RNAi constructs, the latter as control (Table 1.2). We first verified whether we could obtain a stable dMyc downregulation with our dMyc-RNAi construct (4 folds *dmyc*-mRNA downregulation respect to Lac-Z-RNAi trasfected cells; Table 1.2), then we treated dMyc-RNA-interfered and Lac-Z-RNA-interfered cells with Insulin and we scored for genes, induced by Insulin but no longer upregulated in the absence of dMyc.

Most of the genes insensitive to Insulin treatment in dMyc-RNAi transfected cells encode ribosomal proteins or factors involved in RNA processing/binding, suggesting that Insulin stimulated cells augment their mass, through a Myc-dependent intensification of their biosynthetic activities (Table 1.2).

Gene	Function	E-box	Ratio*	Gene	Function	E-box	Ratio*
Metabolism				Ribosomal biogenesis			
Prat	Monosaccharide metab	yes	- 1.61	Nopp140	rRNA processing	yes	- 2.80
CG9243	Lipid metabolism	ND	- 1.78	Fib	rRNA processing	yes	- 3.65
CG33174	Lipid metabolism	ND	- 6.20	Nnp-1	rRNA processing	yes	- 2.65
PEPCK		no	+ 3.20	Nop56	rRNA processing	yes	- 3.56
4EBP	Inhibitor of translation	no	+ 2.46	Tif-1A	Pol I dep transcription	yes	- 1.53
Transport amino acids				Rpl135	Pol I dep transcription	yes	- 3.70
mnd	amino acid transporter	yes	- 2.15	mRNA processing and binding			
Signal transduction				hoip	processing	yes	- 3.44
dm	dmyc trascrition factor	no	- 4.57	mod	binding	yes	- 2.68
tkv	Dpp, BMP signaling	no	- 1.60	pit	binding	no	- 2.23
cycD	Cell cycle	no	- 1.54	Nop5	binding	yes	- 4.15
cycJ	Cell cycle	ND	- 2.42	Translational control			
Ribosomal Biogenesis				elf-6		yes	- 1.61
Rpl1	Ribosomal biogenesis	yes	- 3.16	eF1		yes	- 2.10
Nop60B	rRNA processing	yes	- 2.37	efTuM		yes	- 3.10

Table1.2: dMyc targets in S2 cells. After dMyc-RNAi or Lac-Z-RNAi transfection cells have been treated with Insulin. The list of genes whose expression resulted up- or down-regulated is reported. E-box: presence of the E-box relative to the transcription start site between +1 and +500. * Ratio: determined by a comparison of *dmyc* RNAi microarrays with the corresponding Lac-Z RNAi control. All down-regulated genes listed were found in two replicates with a significative ratio [higher then 1.5 fold (p-value 0.01)]. Negative and positive numbers indicate folds of down- or up- regulation relative to control. *diminutive* (*dmyc*) resulted down-regulated 4.57 times, indicating the good efficiency of the *dmyc*-RNAi treatment.

In vitro and *in vivo* assays we performed in S2 cells clearly evidenced dMyc protein upregulation after Insulin stimulation. Figures 1A and B summarize the first result obtained in tissue culture, showing an intensification of dMyc staining after 4 hours incubation with Insulin. This effect is further enhanced in presence of a proteasome inhibitor (MG132).

These results derived from cell culture were confirmed *in vivo* by the analysis of protein extracts from *yw* larvae provided with 1µg/ml of Insulin supplemented food compared with control larvae fed on a regular medium. As shown in Fig. 1C, Insulin fed *yw* larvae show dMyc protein upregulation, detected by western blot on total larval lysates.

To further evaluate the mechanism that leads to dMyc protein accumulation we made use of specific chemical inhibitors known to interfere

with the Insulin pathway in *Drosophila* S2 cells. We then analyzed dMyc by western blot. As shown in Fig. 1D, dMyc protein upregulation after Insulin stimulation depends on PI3K activity, since the presence of its inhibitor (Wortmannin) is able to revert the Insulin effect. More interestigly treatment with LiCl, a specific inhibitor of GSK3β, results in an additional increase of dMyc protein compared to Insulin treatment alone (Fig. 1D), indicating that even in presence of growth factors, GSK3β inhibition is required for dMyc to accumulate.

We next asked if the increase in dMyc protein detection observed in all treatments could be associated with a corresponding increment of its mRNA. Total mRNA was extracted from Insulin treated S2 cells and endogenous *dmyc*-mRNA was isolated by RT-PCR with appropriate primers (see Materials and Methods). Cells were kept in 0.1% Serum prior to stimulation. Rp48-mRNA was used as internal control while Fibrillarin, being a nucleolar protein, target of *dmyc* (Grewal *et al*., 2004), was adopted as a dMyc activity marker. As the graph in Fig. 2 demonstrates, Insulin treatment in S2 cells increased endogenous *dmyc*-mRNA, compared with 0,1% serum treatment, without affecting rp48-mRNA. At the same time Fibrillarin-mRNA raised more than 1,5 times respect to control, indicating a significative enhancement of nucleolar activity in Insulin stimulated cells that correlates with dMyc protein and mRNA increments.

Taken together these data suggest that growth factors impinge on dMyc with two mechanisms, first through post-translational modification "*via"*

dGSK3/sgg phosphorylation (Galletti *et al.*, 2009), secondly at its post-transcriptional level.

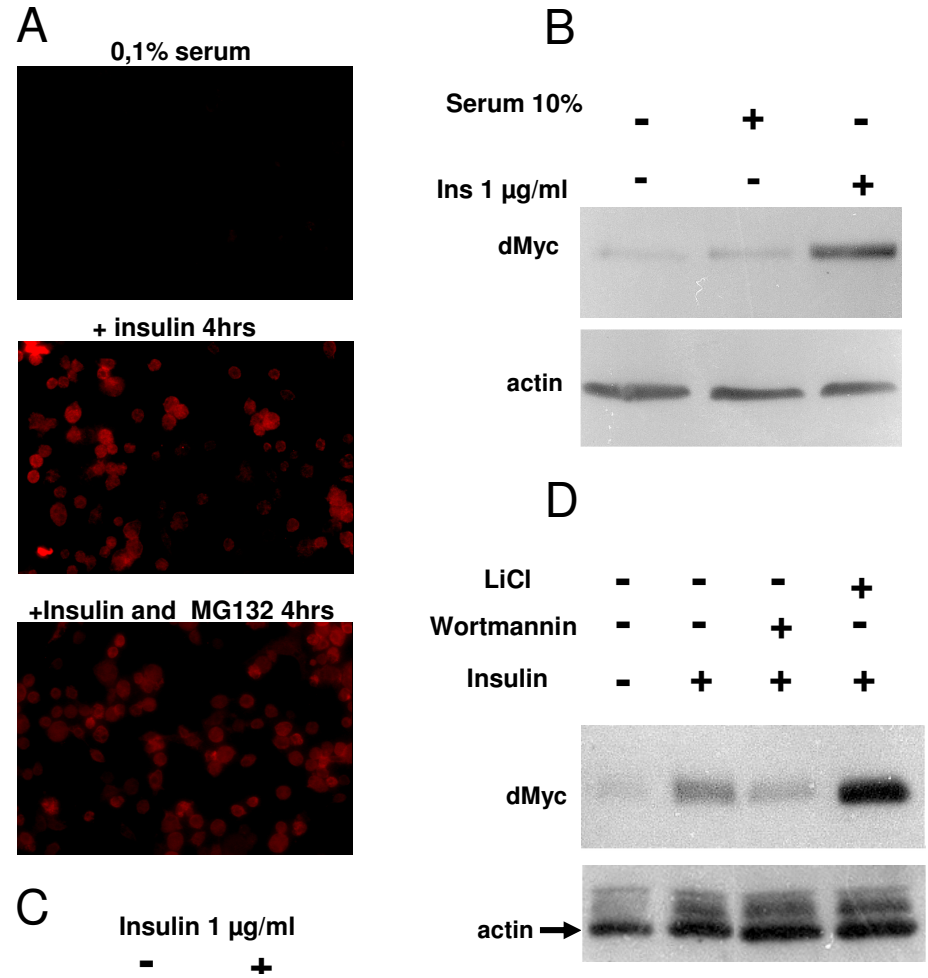
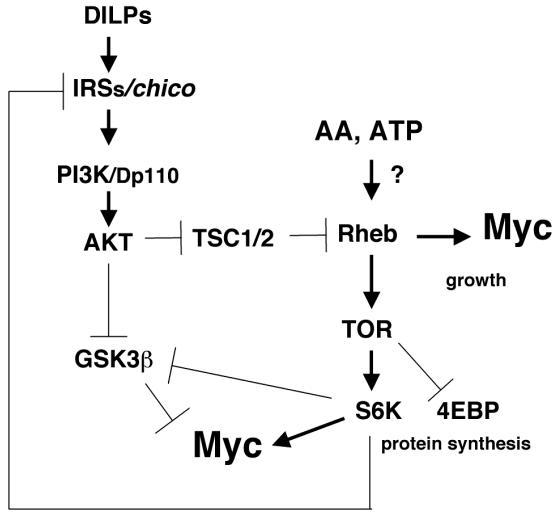


Figure 1.2. Insulin stimulation induces dMyc protein accumulation both *in vitro* and *in vivo*. dMyc protein accumulates in S2 cells after 4 hrs Insulin exposure. MG132 was used to inhibit the proteasome and further enhance Insulin effect on dMyc protein accumulation (A-B). Larval extract from *yw* larvae fed with 1µg/ml Insulin. dMyc accumulates *in vivo* in presence of Insulin (C). PI3K inhibition (Wortmannin) attenuates dMyc accumulation after Insulin treatment, whereas silencing GSK3β/sgg activity (LiCl) enhances dMyc increase (D).



Schematic representation of InR/Nutrients signaling converging on dMyc

TORC1 modulates dMYC post-transcriptionally

Insulin pathway activation is known to regulate translation through TOR-mediated phosphorylation of S6K and 4EBP (Hay and Sonenberg, 2004; Manning and Cantley, 2003).

TOR is an aminoacid responsive kinase that can be found in two complexes named TORC1 and TORC2 (Inoki *et al.*, 2005). TORC1 is the only complex responsive to Rapamycin inhibition also known to phosphorylate S6K and 4EBP. TORC1 activation depends upon the small GTPase Rheb (Ras Homolog enriched in Brain) which in turn for its growth promoting activity requires the TSC1/TSC2 complexes to be inactive (reviewed by Neufeld, 2003)

Since we found dMyc protein and mRNA both to be responsive to Insulin stimulation, we next tried to verify to what extent TOR-dependent mRNAs translation could contribute to *dmyc*-mRNA and protein accumulation.

We tested whether Rapamycin inhibition of TOR would affect Insulin-dependent *dmyc*-mRNA upregulation. We double treated S2 cells with Insulin and Rapamycin and analyzed dMyc protein by western blot and mRNA by RT-PCR.

Arresting TORC1 activity prevents Insulin-dependent dMyc accumulation both at the mRNA (Fig. 2A) and protein level (Fig. 2B). However Rapamycin seemed to be able to negatively act only on the *newly* synthesized mRNA with *no alteration* of the endogenous *dmyc*-mRNA level. This suggests that TORC1 somehow regulates the "*de novo"* synthesis of *dmyc*-mRNA but it does not change its "steady state".

While endogenous *dmyc*-mRNA doesn't vary much in presence or absence of Rapamycin, dMyc protein seems to decrease below the endogenous amount (confront Fig. 2 A-B). These data are in agreement with recent observations from Teleman and co-workers (Teleman *et al*., 2008), which demonstrate that TOR has only a modest impact on dMyc transcription regulation, while it seems to regulate dMyc mostly *post-transcriptionally* with a mechanism not yet identified.

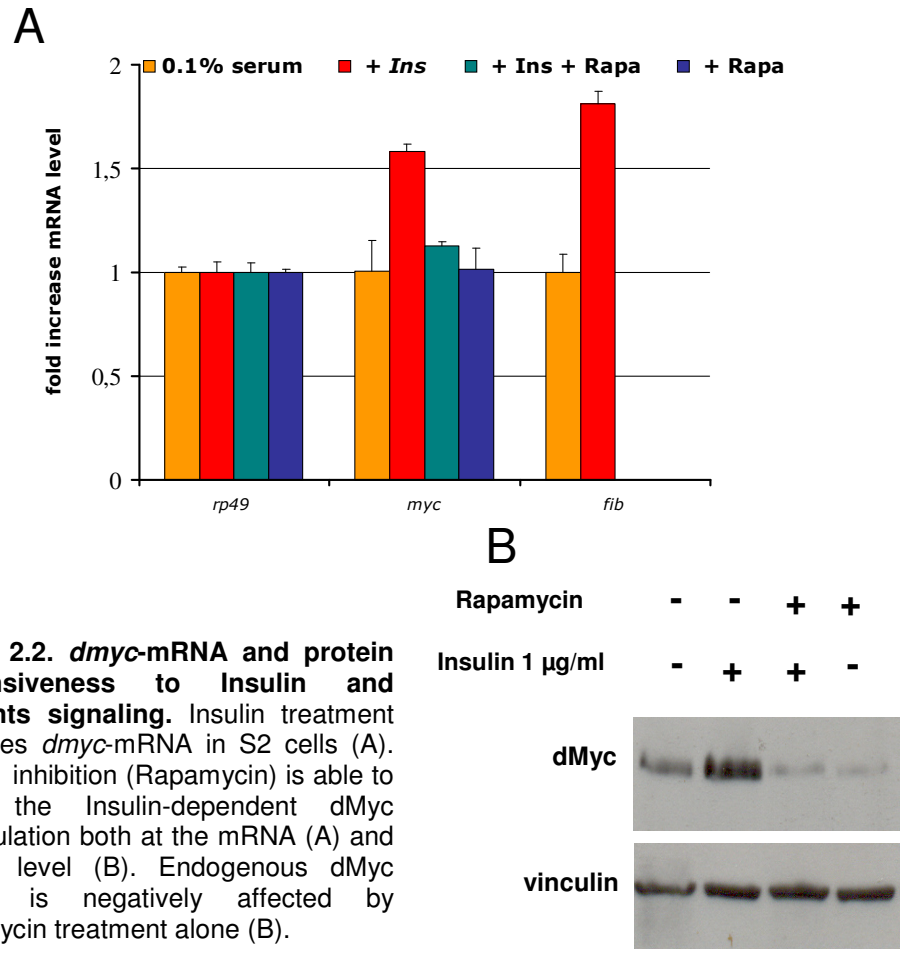


Figure 2.2. *dmyc*-mRNA and protein responsiveness to Insulin and Nutrients signaling. Insulin treatment increases *dmyc*-mRNA in S2 cells (A). TORC1 inhibition (Rapamycin) is able to revert the Insulin-dependent dMyc accumulation both at the mRNA (A) and protein level (B). Endogenous dMyc protein is negatively affected by Rapamycin treatment alone (B).

dRheb expression increases dMyc protein level through GSK3β inactivation

As already metioned in the Introduction of this dissertation the Ras-like gene *Rheb* was shown to be required for growth throughout metazoa

(Yamagata *et al*., 1994; Reuther and Der, 2000; Urano *et al*., 2000; Urano *et al*., 2001; Im *et al*., 2002; Panepinto *et al*., 2002).

This effect of Rheb-dependent growth enhancement is thought to rely on TOR activity (Patel *et al*., 2003; Stocker *et al*., 2003). Moreover TOR signaling is known to negatively regulate GSK3β/sgg through direct phosphorylation by S6K in mammals (Zhang *et al*., 2006). According to our previous data showing a dependence of dMyc protein stablity on GSK3β activity (Galletti *et al*., 2009), GSK3 inhibition by S6K phosphorylation should result in dMyc protein accumulation.

In order to validate this model, we first assessed dMyc responsiveness to aminoacids availability. We provided S2 cells with an aminoacids rich culture medium and we monitored dMyc protein by western blot after increasing periods of exposure (Fig. 3A). Already after 30 minutes of exposure we were able to observe dMyc protein induction above its endogenous level. This correspondence between aminoacids presence and dMyc accumulation persisted and reached its plateau after 120 minutes. On the other hand the levels of actin protein as loading control didn't change in our assay, proving the specificity of dMyc response to Nutrient and suggest that, given a nutrients rich environment, cells engage in biosynthetic activities upregulating growth promoting factors.

We next verified wheter GSK3β/sgg phosphorylation status would be affected by TORC1 activation and at the same time we monitored dMyc protein expression, by western blot. A stable tub-Gal4 cell line, constitutively expressing the Gal4 trancriptional activator, was co-transfected either with

UAS-Rheb and UAS-Myc or with UAS-S6K and UAS-Myc constructs. All the UAS constructs were HA tagged, they were detected with a α-HA antibody and recognized according to their molecular weight. The requirement of a double transfection came from technical difficulties to detect subtle changes in the endogenous dMyc protein in these kind of experiments. GSK3β/sgg phosphorylation at Ser9 (as a marker of its inactivation) was clearly visible after *Rheb* overexpression, whereas S6K by itself failed to strongly reproduce this effect, although a moderate dMyc induction was detectable. We argue the S6K defeat might be due to the fact that it needs to be activated in order to manifest its activity . At the same time, our data clearly demonstrate a requirement for TOR signaling on dMyc protein stability through S6K-dependent GSK3β/sgg inactivation, demostrating a functional conservation of this pathway between *Drosophila* and mammals (Zhang *et al*., 2006).

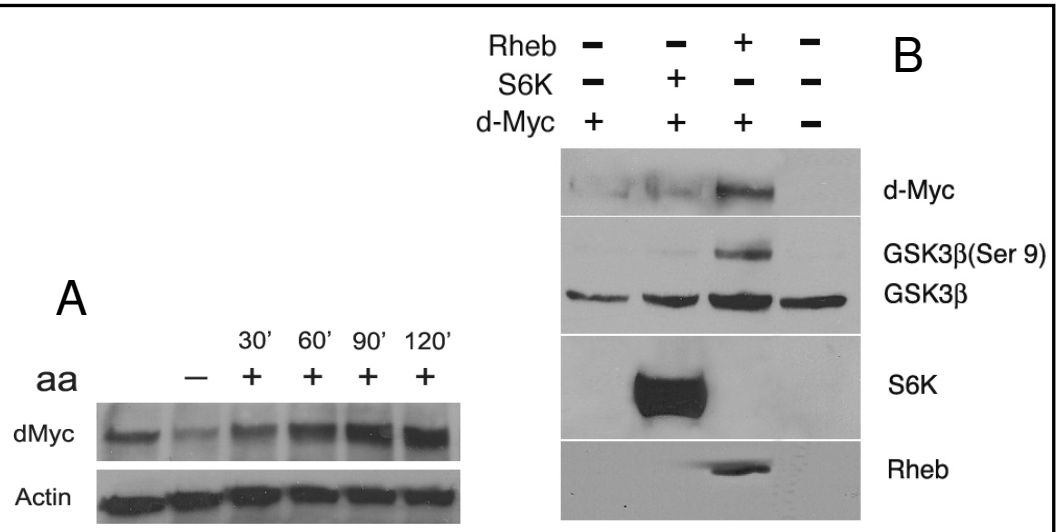


Figure 3.2. TORC1 contributes to dMyc protein stabilization. S2 cells were provided with aminoacids and dMyc protein was detected at the indicated time point. Longer aminoacid exposure correlates with a proportional intensification of dMyc signal (A). This effect might be the result of the TORC1-dependent GSK3β/sgg inhibition through its phosphorylation at Ser9 (B).

PTEN, TOR and Rheb show significative epistatic interactions with *dmyc*

Given the biochemical results so far presented and the similarity of phenotypes between *dmyc* hypomorphic flies and mutans of Insulin and TOR cascades (Johnston *et al*., 1999; Montagne et al., 1999; Zhang *et al*., 2006; Stocker *et al*., 2003), we next wished to assess the possibility that dMyc could act *in vivo* downstream of one or both of these growth controlling pathways. To this purpose a detailed epistasis analysis was performed using the adult eyes.

We took advantage of the same system described in Chapter 1 of the Results section (Bellosta et al., 2005) to screen for enhancers or attenuators of the *dmyc*^{P0} phenotype.

Using the *ey>dmyc*^{P0} line and *ey>dm*$^{+}$ as a control (see Materials and Methods), we analyzed whether ectopic expression of Dp110 and PTEN, respectively positive and negative regulators of Insulin signaling would significantly modulate the *dmyc* contribution to the adult eye development.

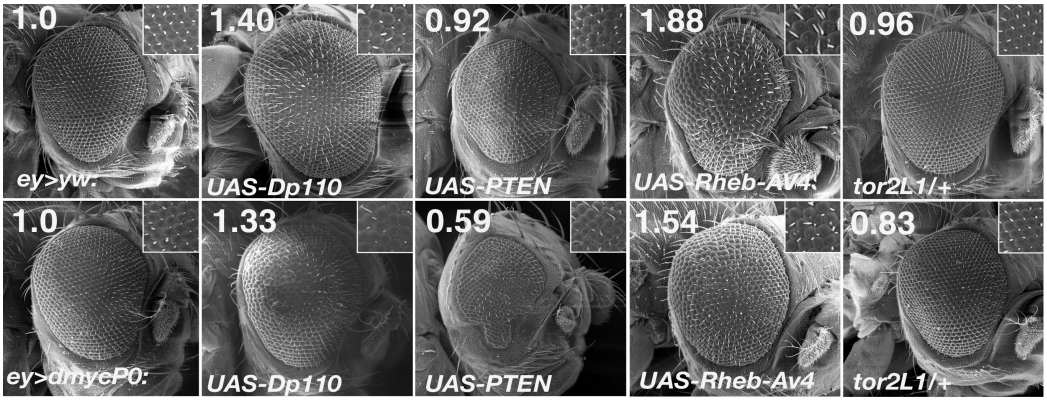


Figure 4.2. Epistatic interaction of InR and TORC1 signaling with *dmyc.* SEM analysis of ey>dm+ and *ey>dmyc*^{P0} eyes overexpressing different modulators of InR or TORC1 pathways.

genotypes	*ey>wt/Y*	Size (μm^2)	Number	*ey>dmyc*^{P0}*/Y*	Size (μm^2)	Number
	+/+	237 ± 10	727 ± 16 (9)	+/+	217 ± 9	735 ± 16 (9)
	UAS-Dp110	332 ± 10	811 ± 30 (6)	UAS-Dp110	293 ± 12	692 ± 36 (6)
	UAS-PTEN	220 ± 11	538 ± 25 (9)	UAS-PTEN	128 ± 14	420 ± 42 (7)
	UAS-S6K	251 ± 12	692 ± 37 (11)	UAS-S6K	216 ± 12	683 ± 35 (13)
	UAS-S6K-SDTE	283 ± 10	629 ± 38 (11)	UAS-S6K-SDTE	248 ± 7	637 ± 33 (8)
	UAS-RHEB	289 ± 16	699 ± 20 (11)	UAS-RHEB	253 ± 6	730 ± 14 (15)
	UAS-RHEB-AV4	452 ± 19	554 ± 18 (6)	UAS-RHEB-AV4	334 ± 9	658 ± 38 (9)
	UAS-dMyc *	346 ± 15	609 ± 35 (8)	UAS-dMyc *	326 ± 14	629 ± 22 (8)
	tor l1	233 ± 5	741 ± 35 (10)	*tor l1*	181 ± 2	739 ± 17 (6)

TABLE 2.2. cell size and cell number of *ey>dm*$^{+}$ and *ey>dmyc*^{P0} eyes overexpressing various transgenes.

As shown in Fig. 4 and reported in Table 2, ectopic *Dp110* expression totally rescued the morphological defect of *dmyc*^{P0} eyes resulting in 31% increase of ommatidial size. These effect is slightly more pronounced (40%) in the *ey>dm*$^{+}$ background. Ommatidial number of dp110 overexpressing eyes was greatly increased in both genetic backgrounds, in line with dp110 role in proliferation control. In contrast, overexpression of *PTEN* showed an 8% reduction of the ommaditial size of *ey>dm*$^{+}$*/Y* flies, which is dramatically increased to 43% in the *ey>dmyc*^{P0}*/Y* background. In addition to its effect on cell size, PTEN overexpression reduces the ommatidial number of *ey>dm*$^{+}$*/Y* from 710 to 538 (Table 2.2). This reduction is further enhanced in *ey>dmyc*^{P0}*/Y* males where 98% of flies show very small and slightly misshaped eyes (Fig. 4), suggesting that inhibition of Insulin signaling by PTEN dominantly affect the role of dMyc in growth and proliferation during eye development.

With a similar approach we subsequently analyzed the contribution of TORC1 to *myc*-dependent growth during eye development. Since a controversial effect of TOR kinase overexpression on growth has been reported both in eye and wing discs (Hennig and Neufeld, 2002) and will be further discussed in our clonal analysis, we decided to avoid *TOR* direct overexpression and make use of *dRheb* and *dS6K*, both normally activated by aminoacid availability and to make use of a null mutant form for TOR (*tor*^{2l1}).

Ectopic expression of *dRheb* increased the ommatidial size by 22% in the *ey>dm*$^{+}$ background (from 237 ± 10 to 289 ± 16) and of about 16%

(from 217 ± 9 to 253 ± 6) in the *dmyc*^{P0} eyes (Fig 4 and Table 1). More compelling results were obtained with *UAS-dRheb*^{AV4} overexpression. *dRheb*^{AV4} derives from a P-element insertion within the 5' UTR of *dRheb* and it has been shown to act as a null allele in absence of a Gal4 transcriptional activator (Patel *et al.,* 2003).

dRheb^{AV4} overexpression results in ommatidial size increase of 88% in *ey>dm*$^{+}$ eyes, while the size increment was only about a 54% in *dmyc*^{P0} background, indicating a significative contribution of *dmyc* in the growth phenotype induced by *dRheb*. The strong size increment of *ey>dm*$^{+}$ ommatidia seems to be counteracted by a decrease of their number. This mechanism of compensation has been extensively described in the Introduction section of this thesis and appears to be conserved even when *dRheb*^{AV4} is overexpressed in a *dmyc*^{P0} background. *ey>dmyc*^{P0}*/Y; UAS-Rheb*^{AV4} eyes are still bigger than *dm*$^{+}$ and *dmyc*^{P0} one, but their ommatidia never reach the size of *ey>dm*$^{+}$*/Y;UAS-Rheb*^{AV4} counterparts. At the same time their number is remarkably decreased even if to a lower extent than in *ey>dm*$^{+}$*/Y;UAS-Rheb*^{AV4} eyes.

Moreover we were able to demonstrate that *Rheb*^{AV4} overexpression triggers apoptosis in *ey>dm*$^{+}$ eyes (Fig. 5), which no longer takes place in *ey>dmyc*^{P0}*; UAS-Rheb*^{AV4}, probably accounting for the more pronounced reduction in cell number of the former. This effect of *dRheb* on apoptosis during development has never been observed before and more interestingly it shows to be *dmyc* dependent.

With regards to the investigation about a direct TOR contribution to the *dmyc*-mediated effect on growth we used the tor^{2l1} null allele (Oldham *et al*., 2000) in our epistasis screening. As shown in Fig. 5, heterozigosity for tor^{2l1}, did not result in ommatidial size reduction in the *ey>dm+/Y* animals. However a 14% reduction in ommatidial size was observed when tor^{2l1} was in trans-heterozygosis with $dmyc^{P0}$ allele. Moreover no changes in the ommatidial number were found between the $ey{>}dm^{+}$; $tor^{2l1}/+$ and $ey{>}dmyc^{P0}$; $tor^{2l1}/+$ lines, (Fig 4 and Table 2.2) confirming that TOR plays a major role in cell growth rather than in cell division.

Similarly, ectopic expression of *dS6K* or its activated form showed only a 19% increase of ommatidial size in the $ey{>}dm^{+}$ eyes and 11% in the $ey{>}dmyc^{P0}$ eyes indicating a minor requirement for S6K during eye development.

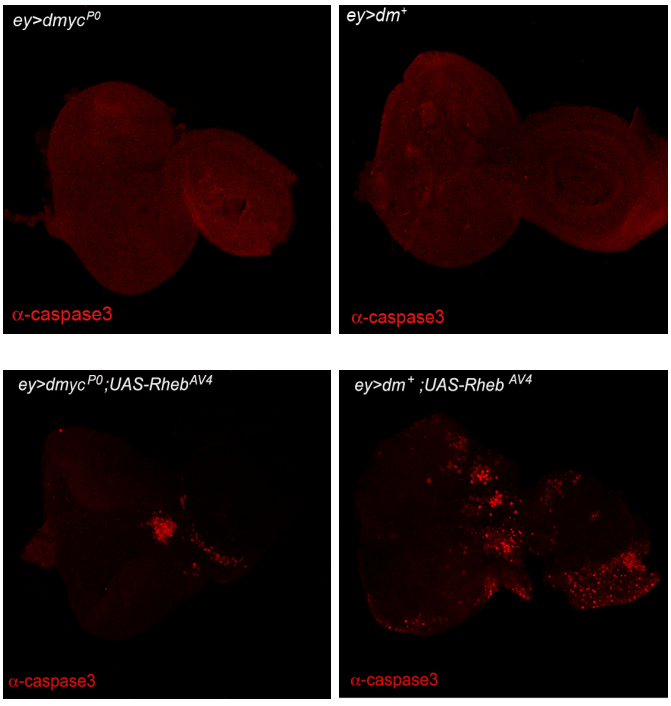


Figure 5.2. $Rheb^{AV4}$ overexpressing eyes buffer cell size variation with cell number modulation. The endogenous level of apoptosis occurring during eye discs development is shown in the upper panel both for $ey{>}dmyc^{P0}$ and $ey{>}dm^{+}$ 3rd instar discs with an α-caspase3 staining. $Rheb^{AV4}$ overexpression enhances apoptosis (lower panel). The cell death intensification appears to be *dmyc*-dependent because it is attenuated in a $dMyc^{P0}$ background (left).

Genetic interaction of *dmyc* with members of the InR-TORC1 signaling in wing imaginal discs

Our attempts to analyze *in vivo,* at the cellular level the effect on dMyc protein regulation by Insulin and TOR signaling, have been initially inconclusive for technical reasons. As most of cell cycle regulators, dMyc protein has a short half-life (about 30' in S2 cells) that needs to be tightly regulated, as its misexpression is detrimental for the cell.

Therefore cells have evolved mechanisms to buffer changes in *dmyc* expression rapidly modulating its protein stability, in order to mantain its physiological steady state (Galletti *et al*., 2009).

For this reason the use of conventional techniques for clonal analysis, requesting at least 48 hrs between clones induction and protein detection, might not be useful to detect subtle changes in protein expression, especially when such a protein will be degraded shortly after its synthesis.

Moreover, ectopic expression of genes that would reduce *dmyc* level will result in *cell competition* between the two populations of cells with different *myc* levels, (de la Cova *et al*., 2004; Moreno *et al*., 2004) finally leading to the elimination of lower-expressing-*myc* cells and negatively affecting the analysis.

For these reasons we took advantage of a new method for conditional gene expression in genetic mosaics that represents a modification of the standard UAS-Gal4/Flp-out technique.

This new developed system renders the expression of the UAS-transgene conditional to the presence of a hormone (RU486, Mifepristone) in the medium, allowing the experimenter to obtain a temporal control on transgene expression (Rogulja and Irvine, 2005).

Using this technique we were still able to induce the clone formation 48hrs AEL, this set of cells expressing the Gal4 as a fusion protein with the hormone receptor (PR) that prevents its binding to UAS-sequences on DNA (see Materials and Methods).

We then let clones grow for additional 72hrs, then we provided larvae with RU486 to set the Gal4 free from the PR inhibition and allow the binding to its target genes.

After 5 hours exposition to the hormone, we analyzed dMyc protein by immunohistochemistry (Fig 6).

Clones expressing *Dp110,* the catalytic subunit of PI3K, clearly showed dMyc protein accumulation while inhibition of Insulin pathway, by the use of *PTEN* phosphatase overexpression, resulted in dMyc protein downregulation. Interestingly, dMyc autonomous modulation inside the clone also triggers a strong non-autonomous effect in the nearby cells which respond adjusting their endogenous Myc level in the opposite way (arrow) (Gallant, 2005).

Our observations provide for the first time evidence that cells can adjust their endogenous dMyc levels in response to Insulin stimulation indicating that Myc acts downstream of this signaling.

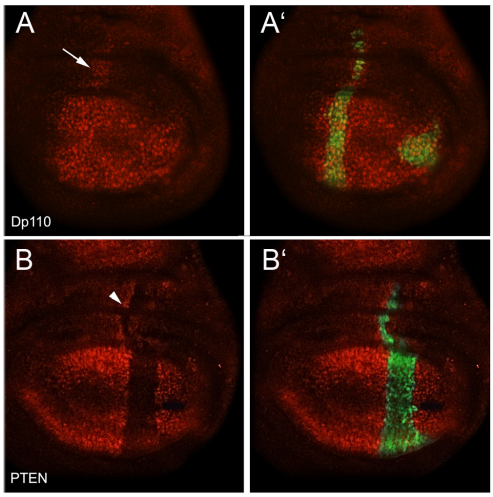
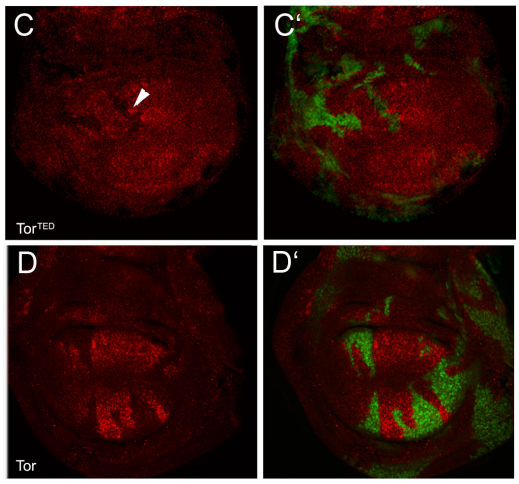


Figure 6.2. PTEN and dp110 overexpression results in dMyc protein modulation. Clones of cells, generated with a flp-out event are marked with the use of a GFP. Overexpression of a positive regulator of InR signaling (Dp110) in these cells associates with an increased dMyc staining (A-A' arrow). On the other hand PTEN overexpression turns off the Insulin signal and downregulates dMyc protein (B-B'). A non autonomous effect on dMyc protein is visible in cell nearby the PTEN overexpressing clone (B – arrowhead)

Overexpression of a dominant negative form of the TOR kinase (TOR^{TED} in C-C') results, as exprected, in dMyc attenuation. Surprisingly TOR^{wt} overexpression produced the same outcome (D-D').



Having demostrated a genetic interaction between Insulin signaling and *dmyc*, we next turned to confirm our results involving TORC1, derived from the epistatic analysis in the adult eye.

With the same approach described for Dp110 and PTEN we generated random flp-out clones overexpressig either a *wt* form of the TOR kinase or a modified form known as TOR^{TED} (*toxic effector domain*) whose kinase domain has been mutated in order to be inactive (Hennig and Neufeld, 2002). A controversial effect of TOR overexpression on growth in

Drosophila was already reported. In fact, while the TOR family of protein kinases are known to be conserved regulators of eukaryotic cell growth, proliferation and metabolism (Schmelzle and Hall, 2000; Raught *et al.,* 2001) it is unclear whether incremental increases in TOR activity or expression can potentiate cell growth rates, or whether TOR activation sends a permissive signal necessary but not sufficient for growth.

Surprisingly, dTOR overexpression caused phenotypes remarkably similar to those of *dTOR* loss of function mutations, including reductions in cell size and proliferation rate, accumulation of cells in the G1 phase of the cell cycle, and specific genetic interactions (Hennig *et al*., 2002).

Having implemented the RU486 method of conditional gene expression in our lab, we decided to verify whether a transient *TOR* or TOR^{TED} overexpression could unmask their effect on growth. Surprisingly both transgenes, when ectopically driven in clones of cells, resulted in dMyc downregulation (Fig. 6 C-C' and D-D') suggesting again a dualty in TOR behaviour.

In light of the potential scaffolding function of TOR proteins, we suggest that abnormally high TOR expression may reduce signaling output by titrating and diluting essential cofactors, thereby inhibiting formation of functional signaling complexes. Furthermore data so far presented underlined a significative dependence on Myc for dRheb mediated growth, opening the possibility that Rheb could regulate cell size in a TOR-S6K indipendent fashion.

TORC1 AND dMYC REGULATION

Teleman and coworkers in a recent publication demonstrated the *in vivo* requirement for Myc in TORC1-mediated growth control, since knock-down of TSC1 failed to induce tissue overgrowth in the absence of dMyc (Teleman *et al*., 2008). Nevertheless, dMyc overexpression did not show to be able to rescue the impaired growth caused by low *TOR* activity in $TOR^{\Delta P}$ mutant clones (Teleman *et al*., 2008). The authors argued that although Myc is essential in the growth-promoting signaling network downstream of TORC1, *myc* expression is not sufficient to fulfill the requirement for TOR activity.

Considering these results, the well known controversy about the ambiguous effect exerted on growth by TOR overexpression (Hennig and Neufeld 2002), our epistatic analysis in the adult eye and our clonal data, we decided to obtain a more straightforward result by overexpressing *UAS-dmyc* transgene in clones of cell homozigous for *Rheb* mutation, using the MARCM technique.

$Rheb^{7A1}$ is a strong hypomorphic allele (Stocker *et al*., 2003), homozigous lethal whose growth features can only be charachterized in a clonal assay.

Clones of cells $Rheb^{7A1}/Rheb^{7A1}$, or bearing even stronger null *dRheb* mutations, are prone to disappear from the epithelial layer, outcompeted from their *wild type* neighbours and have the tendency to form elongated, thin clones that often fuse together maybe in the attempt to

minimize the contact with the surrounding more competitive cells (Stocker *et al.*, 2003).

We generated Rheb^{7A1}/Rheb^{7A1} mutant clones, marked with a UAS-GFP, by mitotic recombination and we compared their growth rate with their siblings generated in the same recombination event and supposed to grow at the same extent (Fig. 7).

Although these data are still very preliminar and lack of a substantial statistical analysis, the genetic mutant background of Rheb^{7A1}/Rheb^{7A1} mutant clones, negatively affected their growth rate respect to the sibling clone. When mutant clones were induced 48hrs AEL a very little amount of them survived to the L3 stage (72 hrs later), when they are detected by immunofluorescence. Mutant clones induced 72 hrs AEL have more chances to survive but they still grow poorly respect to siblings (Fig. 7 A-B).

dMyc overexpression in the Rheb^{7A1}/Rheb^{7A1} background partially rescued the growth defect of mutant clones. They still never reach the sibling dimensions, being in the average 1/4 of their size, but they show to be able to survive competition from *wt* cells when induced 48 hrs AEL (Fig 7 A'-B'). Moreover Rheb^{7A1}/Rheb^{7A1}*;UAS-dmyc* clones, induced 72 hrs AEL show a small but significative increase in size respect to Rheb^{7A1}/Rheb^{7A1} clones induced at the same time indicating a genetic interaction between Rheb and Myc in growth regulation that we suppose to be TOR independent.

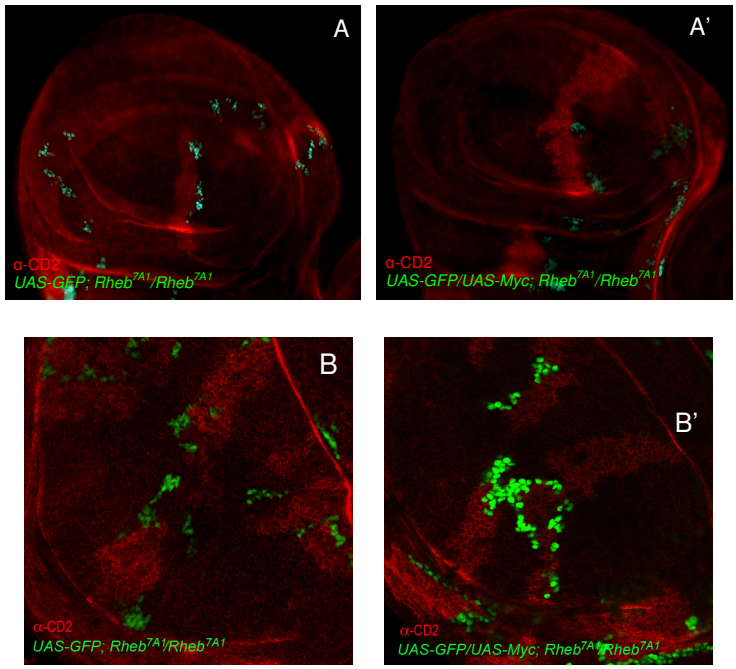


Figure 7.2. dMyc overexpression rescues *Rheb* mutant growth defect. *Rheb*^{7A1} homozygous mutant clones suffer of growth disadvantage. Clones induced 48hrs AEL are rarely detectable and significativey smaller than wt siblings (A). dMyc overexpression in *Rheb*^{7A1} mutant background is able to rescue their growth disadvantage allowing them to survive throughout development (A' see Table 2). An even more pronounced effect is visible in clones induced 72hrs AEL. *UAS-dMyc; Rheb*^{7A1} expressing clones (B') appear to be slightly bigger than *Rheb*^{7A1} clones (B) although they never reach the siblings size.
Picture were taken at two different magnifications (A-A': 20X; B-B' 40X)

Discussion

A large body of literature documents in both mice and *Drosophila* the involvement of Insulin pathway in growth regulation, probably due to its role in glucose and lipids import, nutrient storage, and translation of RNAs associated with ribosome biogenesis.

Studies in cell culture have implicated the Myc family of transcription factors as regulators of the cell cycle that are rapidly induced in response to growth factors.

Myc is a potent proto-oncogene that is rearranged and overexpressed in a wide variety of human tumors. Evidences from *in vivo* studies on *Drosophila* and mammals suggest a critical function of *myc* in regulating cell growth (Iritani and Eisenman 1999; Johnston *et al*. 1999; Kim *et al*. 2000; de Alboran *et al*. 2001; Douglas *et al*. 2001). This role is also supported by our analysis of Myc targets in *Drosophila*, which include genes involved in RNA binding, processing, ribosome biogenesis and nucleolar function (Orian et *al* 2003, Bellosta *et al*, 2005, Hulf *et al*, 2005).

The fact that Insulin signaling and Myc both seem to be involved in cell growth regulation suggests that they may interact with each other. However, genetic evidences indicating that Insulin signaling regulates Myc *in vivo* were lacking.

Here we show for the first time a direct modulation of dMyc in response to Insulin stimulation/silencing both *in vitro* and *in vivo*. In presence of growth factors a cell needs to engage in macromolecular biosynthesis and, in the case of Myc, Insulin stimulation intensifies its

expression and protein stability, resulting in enhanced ribosomal biogenesis and protein translation.

dp110 overexpression in clones of cells of the wing disc leads to dMyc protein accumulation (Fig. 6.2 A-A') . As shown by Zhang and coworkers (Zhang *et al*., 2006) PI3K elicits GSK3β inactivation in mammals. For this reason our analysis on *dmyc* contribution to InR/TOR signaling mediated growth begins with the characterization of dMyc protein stability.

Our biochemical data demonstrated that CKIα and GSK3β induce dMyc ubiquitination and degradation through the proteasome pathway, while dMyc protein results more stable in presence of their KD mutants (Fig. 1.1). Neither the *wt* form of the two kinases nor their KDs were able to alter dmyc-mRNA, confirming they act at post-transcriptional level (Figure 2.1).

In vivo down-regulation of GSK3β and CKIα or CKIε kinases in wing imaginal discs resulted in dMyc protein accumulation, particularly in the hinge and notum regions. This effect is masked in cells adjacent to the ZNC (Figure 3.1-4.1-5.1). Reduction of GSK3β and CKIα activates Wingless (Wg) signaling (Liu *et al*., 2002), which in turns is known to negatively regulate *dmyc*-RNA specifically in the ZNC (Duman-Scheel *et al*., 2004; Herranz *et al*., 2008; Johnston *et al*., 1999). This functional relationship might explain the failure to detect dMyc protein in clones crossing ZNC. Moreover, this positional effect suggests that dMyc activity is regulated by patterning signals active during development.

Our analysis of dMyc amino acid sequence uncovered novel conserved domains, which serve as potential phosphorylation substrates

for CKIs or GSK3β. Aminoacid substitution inside these domains rendered dMyc insensitive to phosphorylation and increases its protein stability. The *in vivo* effects associated with this prolonged perduration of dMyc protein inside the cell are in some cases detrimental, as shown in overexpression experiments in the eye imaginal disc. Ectopic expression of the stable mutants dMyc-PII, dMyc-PV and dMyc-AB results in a visible eye defect (Figure 7.1), accompanied by a reduction of the head capsule and a decrease of ommatidial number. This is particularly visible in dMyc-PV and AB overexpressing eyes.

A closer analysis on third instar larvae eye imaginal discs revealed that expression of these mutants induced apoptosis during disc development. Cell death is not only limited within the dMyc expressing territories (cell autonomous) but also detectable in the neighboring cells (non-cell autonomous; Figure 8). This phenomenon illustrates the role of dMyc in cell competition, a well-documented mechanism of size regulation in which high expressing dMyc cells kill slower proliferating neighbours (de la Cova *et al*., 2004).

These first results demonstrate the physiological importance of dMyc protein titration during development and allowed us to speculate that pathways such as Ras or Insulin signaling responding to Growth Factors, may control growth and survival through the activity of different kinases that in turn, phosphorylating Myc on different residues, determine its half life. Being Myc a transcription factor involved in cellular mass regulation, proliferation and cell death its phophorylation status could directly correlate

with the decision wheter or not to engage in biosinthetic activity or rather stop proliferating and die.

In support to this hypothesis, we produced data underlining that activation of the DILPs (Drosophila Insulin-like peptides) pathway increases dMyc protein stability both *in vitro* and *in vivo* (Fig. 1.2)

Moreover our work showed that dMyc up-regulation in response to DILPs signaling requires activation of the TOR pathway. Our *in vitro* assays demonstrate that dMyc protein accumulates in response to amino acid stimulation and that TOR inhibits GSK3β activity, since Rheb overexpression in S2 cells promotes its phosphorylation at Ser9 (Fig.3.2).

At the same time, while InR pathway is able to increase *dmyc*-mRNA in cells, TORC1 signaling mostly appears to impinge on *myc* post-trascriptionally. In fact, the use of Rapamycin as a TORC1 inhibitor do not seem to affect *dmyc*-mRNA in S2 cells but visibly lowers dMyc protein under its endogenous level (Fig. 2.2).

Furthermore our data prove that Rheb but not S6K, both downstream of the TOR signaling cascade, contributes to the dMyc induced growth phenothype in the eye, suggesting that Rheb might control growth independently of TOR/S6K (Fig . 4.2)

To better understand the complex interplay between Myc and the Nutrients cascade and given the controversial effect of TOR overexpression on growth (Hennig and Neufeld, 2002) we decided to further investigate the cell response to Rheb modulation.

Rheb^{AV4} overexpression under the *eyless* promoter resulted in a dramatic overgrowth of the eye that is attenuated in a *dmyc* hypomorphic background. Moreover the ommatidial size increase is balanced in *Rheb*^{AV4} overexpressing eyes with a reduction of their number that is achived through apoptosis. Cell death is strongly abated in absence of *myc* suggesting that Rheb is able to regulate organ size during development inducing cell death in a *dmyc* dependent fashion. Moreover these results infer that in order to reach its correct proportions during development, every organ needs to finely tune cell growth and apoptosis. Both these processes show a dependence on *dmyc* since *dmyc* overexpressing eyes significatively overgrow. Moreover we characterized the behaviour of highly stable dMyc protein isoforms, *in vivo* in the eye imaginal discs and once again we were able to demonstrate how *dmyc* overexpression can be detrimental leading to massive apoptosis and impaired development (Galletti *et al.*, 2009).

In a recent paper Teleman and coworkers addressed the issue of dMyc dependent growth control downstream of TORC1 signaling (Teleman *et al,* 2008). Although they provided evidence of an interplay between TOR and dMyc in size regulation they couldn't rescue the growth defect of *Tor* mutants clones with *dmyc* overexpression coming to the conclusion that the contribution of TOR on cell growth is only partially dependent on Myc. Our results with *Rheb*^{AV4} overexpression and *Rheb*^{7A1} mutant rescue might provide a different explanation. In our MARCM analysis we were able to rescue the *Rheb*^{7A1} growth disadvantage by overexpressing Myc. Clones of

cells mutant for Rheb are small in size and are prone to be eliminated by the neighbor more competitive cells. *Rheb*^{7A1}/*Rheb*^{7A1} mutant clones induced early during development are very rarely detectable at the end of larval development while *dmyc* expression in this genetic background is able to rescue their viability. Moreover *Rheb*^{7A1}*;UAS-dMyc* clones induced later in development appear bigger in size respect to *Rheb*^{7A1}/*Rheb*^{7A1} clones induced at the same time but lacking dMyc expression. These results suggest the possibility that dMyc lays downstream of Rheb in the growth promoting pathways activated by Aminoacids.

Taken together our data raise the intriguing possibility of the existence of a yet uncharacterized branch of the Nutrients pathway that impinge on Myc to promote growth, downstream of Rheb activation but in a TOR-independent fashion.

The present work addresses for the first time the role of *dmyc* in cellular and organismal growth downstream of Growth factors and Nutrients availability. These signaling pathways are often deregulated in many diseases like diabetes or cancer. Moreover *myc* mutation impairs development while its misexpression has been found associated to the vast majority of cancer forms.

For these reasons, we believe the dissection of the signaling used by cells to grow or restrain growth as well as the characterization of the mechanisms that control cell, organ and body size during development, beside their speculative interest, have a great applicative potential.

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