# Tuberous Sclerosis Complex 1 (Tsc1) regulates dE2F1 protein expression during development and cooperates with Rbf1 to control cell proliferation and survival in *Drosophila melanogaster*

Ting-Chiu Hsieh
Biology Department
McGill University, Montreal
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ABSTRACT

Retinoblastoma tumour suppressor Rb is a cell cycle regulator that is active during early G1 preventing the transition from G1 to S-phase. This is achieved by Rb inhibiting E2F transcription factors from activating expression of genes required for G1 to S-phase progression and DNA synthesis. In our initial genetic test searching for genes that interact with mutations of *rbf1*, the homologue of *rb* in *Drosophila melanogaster*, one of the genes identified was *tsc1*, which is also a tumour suppressor gene that regulates translation and cell growth. We found that in *Drosophila* eye imaginal disc cells, *tsc1* and *rbf1* mutations have a synergistic effect on increasing the level of cell death and promoting ectopic S-phase entry. In addition, I found that dE2F1 protein level increased in *tsc1* mutant eye disc cells, which implies that Tsc1 is a negative regulator of dE2F1 expression. The goal of my thesis study was to characterize the synergistic relation between Rbf1 and Tsc1 as well as the regulation of dE2F1 expression by Tsc1.

In cells triple-mutant for *rbf1*, *tsc1*, and *de2f1*, I found that the observed elevation in cell death in *rbf1* and *tsc1*double-mutant cells was suppressed, which suggests that the cooperation between Rbf1 and Tsc1 is dE2F1-dependent. Moreover, by using a reporter construct for dE2F1 activity, PCNA-GFP, and performing *in situ* hybridization with anti-sense RNA probes of dE2F1 target genes, *rnrS*, *Cyclin E*, and *PCNA*, I showed that activities of *de2f1* downstream target genes were activated by *tsc1* mutations, suggesting that Tsc1 also regulates dE2F1 target gene expression. Through clonal analysis of loss-of-function mutant

alleles of the canonical Tsc pathway genes, I found that Tsc1 regulates dE2F1 via the Tsc pathway, specifically *tsc/rheb/Tor/s6k*.

Finally, my RTq-PCR result showed that the regulation of dE2F1 protein expression by Tsc1 is at post-transcriptional level. To address whether the regulation is at the level of translation, I cloned the 5' untranslated regions (UTRs) of *de2f1* mRNA variants, *de2f1-RA-α* and *de2f1-RA-β*, and incorporated them upstream of GFP proteins to generate translational reporters that monitor the effect of the 5' UTR. The results from these translational reporters in transgenic flies suggest that Tsc1 regulates dE2F1 expression at both the level of translation, through the 5'UTR of *de2f1-RA-β* mRNA, and protein stability.

## ABSTRAIT FRANÇAIS

Le suppresseur de tumeur du Rétinoblastome, Rb, est un régulateur du cycle cellulaire qui est actif dans la phase précoce G1, prévenant le passage en phase S. Pour ce faire Rb inhibe le facteur de transcription E2F, l'empêchant d'activer l'expression de gènes requis pour le passage de la phase G1 à la phase S et pour la synthèse d'ADN. Dans nos tests génétiques initiaux, cherchant des gènes interagissant avec la mutation *rbf1*, l'homologue de *rb* chez *Drosophila Melanogaster*, un des gènes identifié fut *tsc1*, qui est également un gène suppresseur de tumeur qui régule la traduction et la croissance cellulaire. Nous avons découvert que dans les cellules des disques imaginaux des yeux, les mutations *tsc1* et *rbf1* ont un effet synergique sur l'augmentation du taux de mort cellulaire et promeuvent l'entrée en phase ectopique S. Il fut également découvert que le taux de la protéine dE2f1 augmente dans les cellules mutantes du disque des yeux, ce qui implique que Tsc1 est un régulateur négatif de l'expression de dE2F1. Le but de ma thèse était de caractériser la régulation de l'expression de dE2F1 par Tsc1 et la relation de synergie entre Rbf1 et Tsc1.

Dans les cellules triples mutantes pour *rbf1*, *tsc1*, et *de2f1*, j'ai trouvé que l'augmentation du taux de mort cellulaire observé disparaissait dans les cellules doubles mutantes *rbf1* et *tsc1*, ce qui suggère que la coopération entre Rbf1 et Tsc1 est dE2F1-dependante. Egalement, en utilisant un gène rapporteur de l'activité de *de2f1*, PCNA-GFP, et en réalisant des hybridations *in situ* avec des sondes ARN anti sens, *rnrS*, *Cyclin E*, and *PCNA*, j'ai montré que l'activité de la région en aval des gènes cibles de *de2f1* était activé par la mutation *tsc1*,

suggérant que Tsc1 régule également l'expression des gènes cible de dE2F1. Par l'analyse de clones possédant des allèles mutants perte de fonction pour les gènes de la cascade canonique Tsc, j'ai trouvé que Tsc1 régule dE2F1 par le biais de la cascade Tsc, spécifiquement *tsc1/rheb/Tor/s6k*.

Finalement, le résultat de ma RTq-PCR a montré que la régulation de l'expression de la protéine dE2F1 par Tsc1 se fait au niveau post transcriptionnelle. Pour déterminer si la régulation se fait au niveau de la traduction, j'ai cloné les régions 5' non traduites (5'UTR) de variants de l'ARNm de *de2f1*, *de2f1-RA-α* et *de2f1-RA-β* et les ai incorporés en amont de la protéine GFP pour générer des gènes rapporteurs traductionnels nous permettant de visualiser l'effet du 5'UTR. Les résultats obtenus avec ces gènes rapporteurs traductionnels transgéniques suggèrent que Tsc1 régule l'expression de dE2F1 à la fois au niveau de la traduction, à travers le 5'UTR de la forme β de *de2f1-RA*, et au niveau de la stabilité de la protéine.

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# INTRODUCTION

## Thesis Overview

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Rb is an important regulator of the cell cycle whose best characterized function is to bind and inhibit E2F transcription factors [1, 2]. E2Fs activate the expression of genes that are necessary for cell cycle progression from G1 to S-phase and DNA replication [3]. Inactivation of Rb is crucial in development of cancers, which is believed to be caused by deregulated E2F activity (reviewed in [4]). Using the developing eyes of *Drosophila melanogaster*, the aim of our lab has been to find novel genetic interactions that associate with *rbf1*. We have found that many known inhibitors of major growth pathways showed genetic interactions with *rbf1* mutations. One of the genes identified was *tuberous sclerosis complex 1* (*tsc1*), an upstream inhibitor of Target of Rapamycin (Tor) [5], which is a central regulator of translation that inactivates 4E-BP but activates S6K [6, 7]. Like Rb, Tsc1 is also a tumour suppressor, and mutations of Tsc1 in humans cause the growth of benign tumours called hamartomas, which can be attributable to the function of Tsc1 in cell growth regulation. It should be noted that cell growth is a distinct process from cell proliferation as growth entails accumulation in cellular mass. However, the mechanisms connecting the two processes remain largely unclear (reviewed in [8]). In eye imaginal disc cells, we found that *rbf1* and *tsc1* double mutations lead to apoptosis at the morphogenetic

furrow (MF) and the anterior part of the eye disc. Moreover, we found that *rbf1* and *tsc1* double mutant cells undergo ectopic S-phase entry in the MF. The finding of the synergistic effects between *rbf1* and *tsc1* on cell survival and proliferation not only provides a novel link between two pathways that associate with tumorigenesis but also cell proliferation regulation and cell growth control.

During our investigation on the cooperative relation between Tsc1 and Rbf1, we found that Tsc1 negatively regulates the protein expression of dE2F1, the *Drosophila* homologue of activator E2Fs. This discovery, along with the discovered cooperative effects between *rbf1* and *tsc1*, provide evidence on dE2F1 being an important regulator of the cell cycle that translates growth-promoting signal downstream of the Tsc pathway.

In this thesis, I used a genetic approach to demonstrate that the cooperative interactions between Rbf1 and Tsc1 is dE2F1 activity-dependent. I showed that the loss of dE2F1 suppresses the increased apoptosis in *rbf1* and *tsc1* double mutant cells. By comparing the expression of dE2F1 target genes, I showed that in *rbf1* and *tsc1* double mutant cells, dE2F1 activity is further increased than *rbf1* single mutant cells. I also addressed some outstanding questions on the regulation of dE2F1 by Tsc1, such as what are the mediators downstream of Tsc1 and its mechanism of regulation on dE2F1. The work shown in this thesis would contribute to understand better the regulations on cell proliferation and growth involved during *Drosophila* eye development as well as expanding the genetic pathways that impinge on Rbf1 and dE2F1.

**Background**

**The *Drosophila* eye morphology**

An adult fly compound eye appears as a large aggregate of much smaller eyes called ommatidia [9]. The interior of a single ommatidium is precisely patterned, which can be described as a lattice, and are consisted of four cone cells, two primary, six secondary, three tertiary pigment cells, and three bristle cells [10, 11]. Further inside an ommatidium are eight photoreceptors, R1 to R8 [12], each of which possesses a light-gathering structure called rhabdomere [13]. The photoreceptors transmit signals to the central nervous system via their axons, with the axons of R1-R6 terminating at the outer layer of the optic lobe while the axons of R7 and R8 penetrate into the medulla [14, 15]. All these specialized neural cells that make up the ommatidia are differentiated from the epithelial cells of the larval eye primordia called the eye imaginal discs [9].

**The eye imaginal disc is composed of proliferating epithelial cells**

The eye imaginal disc is a monolayer of epithelial cells that is formed from an infolding of the ectoderm [16]. The eye imaginal disc is also called eye-antennal imaginal disc because it is attached to the antenna disc at the anterior end. At the posterior end, the eye disc is connected to the CNS by a nerve stalk.

Throughout embryonic development, until the second instar stage, the eye discs enlarge by cell proliferation, called the first mitotic wave [17]. In the late third instar stage, although proliferation continues, cells at the farthest posterior region begin to differentiate and organize into ommatidial precursors. This onset of neural differentiation is marked by a dorsal-ventral indentation at the apical surface called the morphogenetic furrow (MF) [12]. Neural patterning proceeds

until the early pupal stage, during which the eye disc eventually unfolds to expose the apical surface of the ommatidial cells and develops into the compound eye [9].

**Neural patterning is initiated in the morphogenetic furrow**

The MF is a physical indentation formed as a result of cellular apical constriction [12] that starts at the very posterior end of the eye disc during late third instar and travels toward the anterior until early pupal stage. As a consequence, cells in different parts of the eye disc relative to the furrow are in different phases of the cell cycle: cells anterior to the MF are undifferentiated and asynchronously dividing; cells arrest in G1 phase in the MF as well as the immediate region anterior to the furrow [18]; cells posterior to the furrow are post-mitotic and differentiating ommatidial precursor cells. Differentiation begins at the region immediately posterior to the furrow, where cells take on fate to become photoreceptor R8 precursors [19]. In a sequential manner, R8 precursors then recruit other photoreceptor precursors from the surrounding pool of undetermined cells to form developing ommatidia. The sequence of photoreceptor recruitment is as follow: R8, R2/5, R3/4, R1/6, and lastly R7. Developing photoreceptors are further differentiated the farther they are from the MF and undergo a series of conformational changes while recruitment takes place [20, 21]. Photoreceptors R1, R6, and R7 are recruited after the second mitotic wave, where undetermined cells undergo one extra round of synchronized mitosis posterior to the furrow.

**Photoreceptor Differentiation Requires the Egfr pathway**

Differentiation of the *Drosophila* eye photoreceptors is dictated by epidermal growth factor receptor (Egfr) signalling through the Ras-MAPK pathway [22, 23]. The sequential differentiation of the photoreceptors, which begins with R8, is achieved by paracrine signalling through an Egfr ligand, Spitz [24]. The transduction of Spitz is by the activation of the Ras-MAPK pathway [25]. Initially expressed in R8 and the first two recruited photoreceptors, R2 and R5, which are in the posterior region of the MF, Spitz in short ranges induces the neighbouring cells to differentiate into the subsequent photoreceptors. The induced differentiating photoreceptor precursors then start to express Spitz themselves and, accordingly, signal the surrounding undetermined cells to differentiate. Importantly, the state or time in which a cell receives the induction signal determines its fate [26]. Therefore, to prevent untimely induction of cells anterior to the MF, differentiating photoreceptors also express Argos [27], which is an inhibitor of the Egfr pathway. Argos acts in longer range than Spitz to inhibit activation of Efgr signalling in cells anterior to the furrow. However, in proximity to differentiating cells where Spitz activity is high, Argos is unable to inhibit Egfr signalling. Other conserved signalling pathways also play a role on photoreceptor development. Developing photoreceptors posterior to the MF express Hedgehog, which diffuses to the anterior to induce expression of Decapentaplegic (Dpp) and Atonal (Ato). Dpp acts on undifferentiated cells just anterior to the furrow causing them to enter a pre-proneural state [28], whereas Ato is required for the differentiation of R8 [29].

**The *Drosophila* eye imaginal discs as a tool for studying genetics**

The well characterized developing *Drosophila* eyes have been used as a tool to study numerous topics in biology [30]. Since pattern formation of the ommatidial precursors proceeds as a furrow that moves from posterior to anterior, all early patterning events are laid out in a single imaginal disc. Therefore, cells in different regions of the eye disc relative to the MF are in different phases of the cell cycle, which provides an advantage on examining genes that are implicated in cell cycle regulation. Since the eye disc is a monolayer of epithelial cells, immunostaining with antibodies is both facilitated and easy to analyze. For example, it has been shown that S-phase cells can be visualized by injecting or incubating imaginal discs with BrdU [19]. Furthermore, since cell fates in the eye are determined by positional cues, mosaic analysis can be used in the eye to determine a given cell's requirement for a gene of interest. Lastly, the *Drosophila* eyes are not essential organs for a fly's viability, and flies with severely deformed or missing eyes can still develop into adult stage. Therefore, genetic mutations that are otherwise lethal can be better studied using the *Drosophila* eyes.

**Retinoblastoma protein inhibits E2F transcription factors**

Retinoblastoma (Rb) family proteins are important regulators of cell cycle progression and survival (reviewed in [31, 32]). Orthologs of pRb exist in all metazoans where their functions are evolutionarily conserved (reviewed in [33]). Members of the retinoblastoma protein family in mammals, pRb, p107, and p130, contain a pocket domain needed for binding to other polypeptides. Their best-known molecular function is to physically interact with E2F family proteins and transcriptionally repress E2F-regulated target genes. In mammals there are E2F-1

to 8, which are classified into activators and repressors: E2F-1 to 3a are activators and E2F-3b to 8 are repressors. Rb family proteins form distinct complexes with E2Fs *in vivo*: pRb binds to E2F-1 to 4, p107 binds to E2F-4, and p130 binds to E2F-4 and 5 (reviewed in [34]). Genome-wide expression studies revealed that genes involved in various biological processes, such as cell cycle progression, survival, and development, are regulated by E2F family proteins [35-37]. E2F proteins are best characterized as transcription factors that activate the expression of S-phase genes, and intriguingly, pro-apoptotic genes. As a consequence, the loss of Rb family genes in mice results in developmental defects that are often associated with uncontrolled S-phase entry and ectopic cell death [38-40]; although the context or the presence of other growth factors determines which outcome the cells undertake [41]. Importantly, reducing E2F activity largely suppresses the Rb mutant phenotypes, indicating that deregulated E2F activity is responsible for the defects [42, 43]. Overall, E2F family proteins are the key molecular targets of Rb family proteins and responsible for the developmental consequence of Rb inactivation.

**Retinoblastoma inactivation in cancers**

The long-term consequence of Rb inactivation in mammals is tumorigenesis. In humans, the loss of Rb is believed to be a critical step for retinoblastoma development. Moreover, Rb is believed to be functionally inactivated in most, if not all, cancers (reviewed in [44]). In mice, *Rb* heterozygosity (*Rb*+/-) results in the formation of pituitary and thyroid tumors [38, 45-48]. The wild type copy of the *Rb* gene is lost in these tumors, illustrating the importance of *Rb* as a tumor

suppressor gene. Moreover, conditional knockout of *Rb* and an additional member of the *Rb* family gene, *p107* or *p130*, in mouse retina is sufficient to promote retinoblastoma development [49-52]. Similar to the developmental phenotype, deregulated E2F plays a major role during tumorigenesis in *Rb* mutant mice. In a pituitary tumor model, the loss of *E2f-1* or *E2f-3* reduces the frequency of tumor development [53, 54]. More recently, the importance of E2F family proteins in human cancer is further illustrated by the findings that E2F family proteins themselves are often deregulated in many types of cancers (reviewed in [4]). Clearly, E2F family proteins play a critical role during tumorigenesis and also contribute to the developmental defects observed in Rb mutant animals.

Although it is clear that studying the function of E2F is crucial to understand the biology of Rb mutant animals and cancers, it has been difficult to dissect the *in vivo* roles of E2F family genes in mammals. One of the difficulties is the fact that E2F family proteins can functionally compensate for each other, which is particularly true for the "activator E2Fs" (reviewed in [55]). This is best demonstrated by a recent study showing that a single "activator E2F", E2F-3a, is sufficient to support embryonic and post-natal development in mice, and the expression of E2F-3b or E2F-1 under the control of E2F-3a promoter can perform the role of E2F-3a [56]. This study suggests that the unique developmental functions of "activator E2Fs" are largely determined by their expression patterns and not by the differences of their protein sequences. Interestingly, *Drosophila melanogaster* has only a single "activator E2F" and "repressor E2F", dE2F1 and dE2F2 respectively. The function of dE2F1 is also evolutionarily conserved and

represents the three "activator E2Fs" in mammals. dE2F1 is required for cellular proliferation and controls DNA damage-induced cell death, activities that are shared by the three "activator E2Fs" in mammals (reviewed in [33]). Since dE2F1 is the sole member carrying out the function of three E2Fs in mammals, it is possible that the regulation of dE2F1 expression is more complex and tightly controlled in flies. However, the regulatory mechanism that controls dE2F1 expression in *Drosophila* is poorly understood.

Like Rb, Rbf1 is the major regulator of dE2F1 in flies. Most of the *rbf1* mutant phenotypes are believed to be due to deregulated dE2F1 and can be rescued by a hypomorphic mutant allele of *de2f1* [57]. Because of its simplicity and conserved developmental function, the *Drosophila* Rb/E2F is considered as a simplified version of mammalian Rb/E2F. Although *rbf1* mutations are not sufficient to promote tumor phenotype in *Drosophila*, recent genetic studies revealed that Rbf1/dE2F1 plays a crucial role when proliferation and/or survival are compromised by various tumor-promoting mutations. For example, dE2F1 is required by *hippo* mutant cells to overcome the developmentally regulated cell cycle arrest in eye imaginal discs [58]. Moreover, dE2F1-dependent cell death limits the growth promoting effect of the *archipelago* mutations in the eye, and cooperates with low EGFR activity to promote cell death [41, 59]. Interestingly, although the *Drosophila p53* (*dp53*) does not genetically interact with *rbf1* during development, dE2F1 and p53 cooperate to promote DNA damage-induced cell death as they do in mammalian systems [60]. Overall, Rbf1/dE2F1 can either promote and/or limit the proliferation of cells that carry tumor-promoting

mutations in flies.

**Tuberous sclerosis complex 1 (Tsc1) tumour suppressor gene**

Tuberous Sclerosis Complex 1 (Tsc1) is a tumor suppressor gene whose *in vivo* function was first identified in *Drosophila melanogaster* as a regulator of cell size and proliferation (reviewed in [61]). In humans, mutations of *tsc1* cause tuberous sclerosis syndrome, a disorder characterized by the growth of benign tumors called hamartomas (reviewed in [62]). Hamartoma growth is found in multiple organ sites in tuberous sclerosis syndrome, and, in some cases, lesions of giant cells, such as giant cell astrocytomas, are found. *tsc1* encodes a protein product with two putative coiled-coil domains [63], which forms a heterodimer complex *in vivo* with Tsc2, a GTPase activating protein [63, 64]. Since Tsc1 and Tsc2 function as a complex [65], mutations of either *tsc1* or *tsc2* result in similar, if not identical, phenotypes. Previous reports have shown that *tsc1* mutant cells in numerous *Drosophila* tissues are larger than wild type cells. In addition, flow cytometry data showed that *tsc1* mutant cells spend less time in G1 phase but retain normal ploidy [66]. Although previous reports demonstrated that Tsc1 inactivation perturbs the cell cycle profile, the mechanism by which Tsc1 controls the cell cycle as well as cell size is not well understood. Other recently emerged roles for Tsc1 and Tsc2 include cell adhesion, endocytosis, transcription, and differentiation [67-70], highlighting the importance of the Tsc pathway.

**The Tsc pathway regulates translation through Tor**

The Tsc1/2 complex is a negative regulator of the Ras superfamily GTPase, Ras Homolog Enriched in Brain (Rheb) [71]. Rheb is inactivated by the

GTPase activity of Tsc2, which renders Rheb GDP-bound. In its active form, GTP-bound Rheb activates the serine/threonine kinase, Target of Rapamycin (Tor), by binding to its catalytic site and enabling Tor to stay in its active configuration [72]. First identified in yeast as the targets of the drug rapamycin [73], the Tor genes are evolutionarily conserved throughout the metazoans as a central regulator of cell growth, mRNA translation, and ribosome biogenesis (reviewed in [74]). Importantly, deregulated Tor activity is also found in many cancers (reviewed in [75]).

The Tor kinase functions in multi-protein complexes called TORC1 and TORC2 [76]. TORC1 regulates growth and overall translation by phosphorylating two key downstream effectors, eIF-4E binding protein (4E-BP) and ribosomal S6k. Under favourable conditions, such as nutrient abundance, TORC1 activates S6k through phosphorylation promoting translation. Moreover, active TORC1 phosphorylates 4E-BP causing 4E-BP to dissociate from eIF-4E to promote cap-dependent translation [77]. The less understood TORC2 is involved in actin organization and acts as an activator of the Akt kinase, an important kinase of the insulin receptor pathway that has an inhibitory role on Tsc2 [78]. Therefore, the Tsc/Rheb/Tor pathway plays a central role in translation and cell growth regulation by integrating signals from nutrients and growth factors.

**Project Rationale**

The primary goal of my thesis was to characterize the genetic interactions between *tsc1* and *rbf1* as well as the Tsc1 regulatory pathway on dE2F1 expression discovered by our lab. The important facets that I examined in regard

to these observations were: determination of whether the genetic interactions between *tsc1* and *rbf1* are dE2F1-dependent; investigation of the effects by Tsc1 on the expression of genes downstream of dE2F1; uncovering the downstream mediators of Tsc1 on dE2F1 regulation; and the mechanism by which Tsc1 regulates dE2F1. The rationale for studying each of these aspects is summarized below.

**Determining whether the interaction between *rbf1* and *tsc1* is dE2F1-dependent**

Previously, our lab has found that a crosstalk exists between the *Drosophila* Egfr pathway (DER) and Rbf1 on cell survival [41]. This evidence of a major growth pathway interacting with Rbf1 has led us to search for other growth pathways that genetically interact with *rbf1*. Cell death level and ectopic S-phase entry were used as the markers of genetic interaction as they are the hallmark phenotypes of deregulated E2F activities when Rb is inactivated, and were visualized by immunostaining with anti-cleaved Caspase 3 and anti-BrdU, respectively.

Our clonal analysis showed that cells double mutant for *rbf1* and *tsc1* in the eye imaginal disc are more susceptible to undergo cell death and unscheduled S-phase entry. Since it has been well documented that *rb* inactivation-induced cell death is E2F-dependent [79], I investigated whether the observed increase in cell death level in *rbf1 tsc1* double mutant cells was also dE2F1-dependent. I addressed this question by observing the cell death level with cleaved Caspase 3 antibody staining in *rbf1 tsc1 de2f1* triple mutant clones in the eye imaginal disc.

**Examining if Tsc1 has a regulatory effect on the expression of dE2F1 proteins and their transcriptional activities**

Since Rb is best known for inhibiting E2F activities, and because we observed that *rbf1* genetically interacts with *tsc1*, I sought to determine if Tsc1 has an effect on dE2F1 expression as dE2F1 is required for the elevated cell death level caused by *rbf1* and *tsc1* double mutations. This investigation would address, at least in part, the underlying basis of the interaction between *tsc1* and *rbf1*. Interestingly, I found that dE2F1 protein level increased in the absence of Tsc1. Since E2F transcription factors activate the expression of genes required for S-phase entry, it was important to examine if Tsc1 regulation extends to dE2F1 transcriptional activities. To address this question, I used a reporter construct of PCNA promoter activity, PCNA-GFP [80], whose GFP protein expression is under the control of the promoter of PCNA, a dE2F1 target gene [81], in *tsc1* mutant background. To examine other dE2F1 target genes and whether *rbf1 tsc1* double mutations have a cooperative effect on the transcription of these genes, I performed *in situ* hybridization with anti-sense RNA probes of *Cyclin E*, *rnrS*, and *PCNA* [81, 82].

**Determining the downstream mediators of Tsc1 regulation on dE2F1**

Finding the downstream mediators of Tsc1 regulation on dE2F1 expression was critical to better understand this regulatory pathway. To address this question, I selected genes of the canonical Tsc pathway, *rheb, Tor*, *4ebp*, and *s6k*, as the candidate mediators downstream of Tsc1. Since Tor promotes translation by inactivating 4E-BP and activating S6k, the Tsc pathway genes seemed likely to be involved in the regulation of dE2F1 protein expression by Tsc1. I used the loss-

of-function alleles of *rheb*, *Tor*, *4e-bp*, and *s6k* to conduct clonal analysis in the eye imaginal disc. The effects were determined by dE2F1 protein level as well as *rbf1* mutations-induced cell death.

**Mechanism of Tsc1 regulation on dE2F1 expression**

One of the most important aspects of characterizing the regulation of dE2F1 expression by Tsc1 was to address if it is at the level of transcription, translation, or protein stability. Since the canonical Tsc1 pathway is involved in translation regulation, I hypothesized that Tsc1 regulates dE2F1 at the translational level. Reports on translational repression of E2Fs are scarce, with cMyc regulated miRNA clusters translationally regulating E2F1 expression in human testis being one of the few [83]. Therefore, in this regard, establishing another example of dE2F1 regulation at the level of translation would be quite significant.

## RESULTS

**_tsc1_ and _rbf1_ mutations cooperate to promote S-phase entry and cell death**

Ectopic S-phase entry and cell death are well-established *Rb* loss-of-function phenotypes. To address the question whether growth-promoting mutations could alter the *Rb* mutant phenotypes, we sought to determine the effects of inactivating the *Drosophila* ortholog of Tuberous Sclerosis Complex 1 (Tsc1) in an *rbf1* mutant background. To test this, *tsc1* mutant clones were generated in wild type or *rbf1* mutant eye discs (Figure 1). Since homozygous *rbf1* null flies die at the first instar larval stage, we used an *rbf1* hypomorphic allele, $rbf1^{120a}$. Mitotic *tsc1* mutant clones were generated by expressing Flippase (FLP) with an eye-specific driver and marked by the absence of GFP. Thus, GFP

negative clones in wild type background have only *tsc1* mutations while GFP negative clones in the *rbf1*$^{120a}$ background have both *rbf1* and *tsc1* mutations. Third instar larval eye discs were dissected and immunostained with anti-BrdU antibodies. During normal eye development in *Drosophila*, S- phase cells, which can be labeled with BrdU, are found at the anterior portion of the eye imaginal disc where cells are asynchronously dividing, and immediately posterior to the MF where some cells undergo an extra S-phase called the Second Mitotic Wave (Figure 1A). At the MF, asynchronously dividing precursor cells arrest in G1 and begin differentiation process. Therefore, normally, there is no BrdU incorporating cells at the MF. Surprisingly, in clones that are double mutant for *rbf1* and *tsc1*, ectopic S-phase cells were readily observed at the MF (Figure 1C). Since we can occasionally detect *rbf1* mutant cells entering S-phase at the MF, we compared the number of ectopic BrdU positive cells at the MF between *rbf1* single and *rbf1 tsc1* double mutant clones. We normalized the number of ectopic BrdU positive cells by the clone size, which is measured by the number of the pixels in images taken at the same magnification. Clones that do not contain ectopic BrdU positive cells are excluded from the analysis. We determined that, on average, 3.7 ± 2.2 ectopic S-phase cells/1000 pixels are present in the *rbf1* clones while 12.4 ± 5.6 ectopic S-phase cells/1000 pixels cells are present in the *rbf1 tsc1* double mutant clones, showing more than 3-fold increase. This result indicates that Rbf1 and Tsc1 cooperatively regulate G1 to S- phase transition. Next, we stained for dying cells with anti-cleaved Caspase 3 antibodies (C3). *rbf1* mutant cells undergo apoptosis at the anterior region of the MF, and this is not observed in the wild

type eye disc (Figure 1B). It had been previously reported that this developmentally regulated cell death in *rbf1* mutant eye discs is dE2F1-dependent [41]. *tsc1* mutant cells also undergo apoptosis just anterior to the MF though the level of cell death is much lower than what is observed in $rbf1^{120a}$ eye discs. However, in clones that are double mutant for *rbf1* and *tsc1*, we observed a great increase in C3 staining at the MF and the anterior region of the eye disc (Figures 1B and 1C). Therefore, we concluded that Rbf1 and Tsc1 synergistically promote survival as well as G1 arrest during *Drosophila* eye development.

**Tsc1 regulates dE2F1 protein expression post-transcriptionally**

Rbf1 is best characterized as a regulator of dE2F1 transcription factors whose activity promotes both S-phase entry and apoptosis. Since we observed that *tsc1* mutations are able to enhance both ectopic S-phase entry and cell death phenotypes in *rbf1* mutant cells, we sought to determine if dE2F1 itself is deregulated by *tsc1* mutations. Eye discs containing *tsc1* mutant clones were generated as described previously and immunostained with an anti-dE2F1 antibody. We observed that the intensity of dE2F1 staining is clearly stronger in *tsc1* homozygous mutant clones throughout the eye disc, both in dividing and differentiating cells (Figure 2A and Figure 3A). Moreover I detected similar increase in antenna and wing discs, indicating that the effect on dE2F1 protein expression is not tissue-specific (Figures 3B and 3C). Importantly, the intensity of dE2F2 staining, the only other member of the E2F family in *Drosophila*, is unchanged in *tsc1* mutant cells (Figure 2A), indicating that the effect of *tsc1* mutations on dE2F1 expression is specific. To confirm the immunostaining result,

I performed immunoblot assays using protein extracts from eye imaginal discs comprised mostly of *tsc1* mutant cells (for full genotype see Materials and Methods). Consistent with the immunostaining experiments, dE2F1 protein level is higher in *tsc1* mutant eye discs than in control discs while no difference is detected in dE2F2 protein level (Figure 2B). To determine whether Tsc1 regulates the level of *de2f1* RNA, I performed real-time quantitative PCR (RTq-PCR). RNA was isolated from eye discs of the same genotypes used for immunoblot. I designed *de2f1* specific primers that span an intron and amplified portions of two exons (second and third exons or fifth and sixth exons) to distinguish the PCR products from cDNA and genomic DNA. *charybdis* (*chrb*), a previously reported Tsc1 regulated gene is used as a positive control [84]. Similar to the published result, I observed that the level of *chrb* RNA is increased by 11-fold in *tsc1* mutant eye discs (Figure 2C). However, I could not detect any significant changes in *de2f1* RNA level in *tsc1* mutant eye discs (Figure 2C). Therefore, I concluded that Tsc1 regulates dE2F1 expression at post-transcriptional level.

**Transcription of dE2F1 target genes is activated in *tsc1* mutant cells**

Next, I examined whether the transcription of a dE2F1 target gene is activated in *tsc1* mutant cells. To address this question, I used a reporter construct, *PCNA-GFP*, whose GFP expression is under the control of the *PCNA* promoter, a well-established dE2F1 target gene. As shown in Figure 4, GFP expression is increased in *tsc1* mutant cells in the posterior portion of the eye disc, suggesting that, at least in this region, the increase of dE2F1 protein is sufficient to activate the transcription of a target gene. Importantly, the abnormal BrdU positive cells

observed in the same region of *tsc1* mutant clones are scarcely present (Figure 1A), indicating that the increase in dE2F1-reporter activity is not an indirect consequence of ectopic S-phase cells. I also sought to determine if *tsc1* mutations could further activate dE2F1 target gene expression in *rbf1* mutant cells. My attempt to compare dE2F1 target gene expression between *rbf1* single and *rbf1 tsc1* double mutant eye discs by RTq-PCR did not yield any conclusive results (data not shown). This was somewhat expected since a substantial number of *rbf1 tsc1* double mutant cells, presumably cells with hyperactive dE2F1, undergo cell death (Figure 1B and Figure 5A). Therefore, I decided to perform an *in situ* hybridization experiment, hoping to detect specific changes in a subset of surviving *rbf1 tsc1* double mutant cells. Expression patterns of dE2F1 target genes *(rnrS, Cyclin E*, and *PCNA*) were determined using antisense RNA probes. In wild type eye discs, the expression pattern of these target genes resembles that of BrdU staining since their transcription is activated during the G1/S phase transition (Figure 4B left panel). In *rbf1* mutant eye discs, dE2F1 target genes are strongly expressed at the MF where dE2F1 protein expression is normally high (Figure 4B middle panel). It is probable that, in *rbf1* mutant eye discs, dE2F1 target gene expression is mainly controlled by dE2F1 protein level since cell cycle-dependent regulation by Rbf1 is absent. Interestingly, in *rbf1 tsc1* double mutant eye discs, dE2F1 target genes are strongly expressed both at the MF and in the anterior region of the eye disc (Figure 4B right panel). It can be reasoned that, since *rbf1* mutant cells at the MF already express a high level of dE2F1 protein (previously shown in [41]), there is only a small margin for dE2F1 target gene

expression to be further activated by *tsc1* mutations. However, in the anterior region of the eye disc where the dE2F1 protein expression is normally kept low [41], *tsc1* mutations can have a greater effect on dE2F1 activity and target gene expression. As a consequence, dE2F1 target genes are strongly expressed both at the MF and in the anterior region of *rbf1 tsc1* double mutant eye discs, reaching the threshold of expression before undergoing cell death. Supporting this idea, ectopic cell death in *rbf1 tsc1* double mutant eye discs is mainly observed at the MF and in the anterior region of the eye disc (Figure 5A). Interestingly, I could not detect much increase in dE2F1 target gene expression in the posterior region of *rbf1 tsc1* double mutant eye discs, somewhat contradicting the result obtained by the PCNA-GFP reporter construct (Figure 4A). One explanation is that the *in situ* hybridization experiment is not as sensitive and quantitative as the PCNA-GFP reporter construct. I also found that the residual Rbf1 proteins in the hypomorpic $rbf1^{120a}$ allele are mostly expressed in the posterior region of the MF, explaining why cells in this region do not show as much of an increase in dE2F1 target gene expression (Figure 5B). Nevertheless, these results indicate that *tsc1* mutations can activate dE2F1 target gene expression in the wild type and *rbf1* mutant backgrounds.

**dE2F1 is required for the ectopic cell death induced by *rbf1* and *tsc1* mutations**

To determine if the cooperative effect on cell death by *rbf1* and *tsc1* mutations is dE2F1-dependent, I used an allele with an FRT chromosome carrying both *tsc1* and *de2f1* mutations. For this allele, the $tsc1^{f01910}$ allele that

contains a piggyBac transposable element inserted in the intron 6 of *tsc1* locus was employed. Generating $tsc1^{f01910}$ clones in $rbf1^{120a}$ eye discs produces a similar increase in the level of ectopic cell death observed in Figure 1 (Figure 6A). When $tsc1^{f01910}$ and $de2f1^{729}$ double mutant clones are generated in $rbf1^{120a}$ eye discs, I noticed that the sizes of *tsc1 de2f1* double mutant clones are much smaller than that of *tsc1* single mutant clones (compare Figure 6A and 6B). The sizes of *tsc1 de2f1* double mutant clones in the wild type background are also small (data not shown), indicating that the loss of *de2f1* severely compromises proliferation of *tsc1* mutant cells. Occasionally, I was able to obtain $rbf1^{120a}$ mutant eye discs with substantial sizes of the *tsc1 de2f1* double mutant clones. I performed C3 staining to measure the level of cell death in *rbf1*, *tsc1*, and *de2f1* triple mutant cells in these eye discs. Interestingly, the prevailing cell death phenotype observed in *rbf1 tsc1* double mutant cells at the MF is no longer present in *rbf1 de2f tsc1* triple mutant cells (Figure 6B). This result demonstrates that the increased level of ectopic cell death observed in *rbf1 tsc1* double mutant cells is dE2F1-dependent.

**Rheb regulates dE2F1 expression and dE2F1-dependent cell death**

Next, I asked if the known downstream regulators of TSC1 could regulate dE2F1 expression. I first determined the effect of *rheb* loss-of-function mutations on dE2F1 expression by generating mitotic mutant clones of *rheb* in the eye disc. Rheb is a Ras superfamily GTPase whose activity is negatively regulated by Tsc1. As shown in Figure 7A, dE2F1 protein level is reduced, though not absent, in *rheb* mutant cells. This is best observed at the MF where dE2F1 expression is normally high [85]. I then asked if Rheb is required for the increased dE2F1

expression in *tsc1* mutant cells. dE2F1 protein level is also reduced in *tsc1 rheb* double mutant cells (Figure 7A), indicating that Rheb is an important downstream regulator of TSC1 controlling dE2F1 expression. I concluded that, although not essential, Rheb regulates dE2F1 expression during eye development, and is clearly required for dE2F1 upregulation in *tsc1* mutant cells. Since Rheb controls dE2F1 expression, I next tested if Rheb is also required for dE2F1-dependent cell death. To test this, I generated *rheb* mutant clones in the $rbf1^{120a}$ mutant eye disc where deregulated dE2F1 produces a stripe of apoptotic cells at the anterior region of the MF (Figure 1A and [41, 86]). As shown in Figure 7B, this stripe of cell death is interrupted by *rheb* mutant clones. Moreover, the ectopic cell death observed in *rbf1 tsc1* double mutant cells is completely suppressed by *rheb* mutations. These results indicate that Rheb is an important regulator of dE2F1-dependent cell death as well as dE2F1 expression.

**Tor, but neither S6k nor 4E-BP, is required for dE2F1 expression during *Drosophila* eye development**

Rheb activates the Tor serine/threonine kinase, which through phosphorylation, can either inhibit 4EBP or activate S6k. I examined whether these proteins downstream of Rheb also participate in dE2F1 regulation. To address this question, *Tor*, *s6k*, and *4ebp* mutant clones were generated in the eye disc. Similar to what is observed in *rheb* mutant clones, dE2F1 expression is reduced, but not absent, in *Tor* mutant clones, indicating that Tor participates in regulating dE2F1 expression during eye development (Figure 8A). Importantly, dE2F2 expression is unchanged in *Tor* mutant clones (data not shown). Based on

this observation, I had hypothesized that dE2F1 expression levels would decrease in *s6k* mutant clones and/or increase in *4ebp* mutant clones. Surprisingly, dE2F1 expression is unchanged in either *4ebp* or *s6k* mutant clones (Figure 8B). These results suggest that Tor is required for proper dE2F1 expression during eye development while 4EBP and S6k are dispensable.

**S6k is required for the effect of Tsc inactivation on dE2F1 expression and dE2F1- dependent cell death**

The fact that the loss of neither *4ebp* nor *s6k* has an effect on dE2F1 expression might indicate a functional redundancy between the two genes. Alternatively, an unknown factor downstream of Tor might regulate dE2F1 expression during development. Nevertheless, I assessed whether S6k is required for the increase of dE2F1 expression observed when Tsc1 is inactivated. I aimed to generate mitotic clones that are double mutants for *tsc1* and *s6k*. However, because *tsc1* and *s6k* are on the opposite arms of the third chromosome, I used a mutant allele of *tsc2* (or *gig* in *Drosophila*), which is on the same chromosomal arm as *s6k*. Tsc1 and Tsc2 function together as a heterodimer, and mutations of *tsc1* or *tsc2* yield very similar phenotypes [64-66]. As expected, dE2F1 expression is elevated in *gig* mutant clones (Figure 9A). Furthermore, similar to what was observed in *tsc1* mutant clones in the $rbf1^{120a}$ mutant background, the level of ectopic cell death was increased in *gig* mutant clones generated in $rbf1^{120a}$ mutant eye discs (Figure 9B). Surprisingly, the effects of *gig* mutations on dE2F1 expression and ectopic cell death are completely suppressed by *s6k* loss-of-function mutations. I observed that the level of dE2F1 expression in *s6k gig*

double mutant clones is unchanged compared to the control (Figure 9A), and the ectopic cell death observed in *rbf1 gig* double mutant cells is completely absent in *rbf1 gig s6k* triple mutant cells (Figure 9B). Moreover, I observed that the basal level of dE2F1-dependent cell death normally present in the $rbf1^{120a}$ mutant eye disc (the stripe of cell death, Figure 1B) is also suppressed (Figure 9B). These results indicate that *s6k* is required for both the elevation of dE2F1 expression upon Tsc inactivation and the increased level of cell death in *rbf1 gig* double mutant cells. In summary, my genetic studies led us to conclude that Tsc1 and Tsc2 regulate dE2F1 expression and dE2F1-dependent cell death via the canonical Rheb/Tor/S6k pathway during *Drosophila* eye development.

**Tsc1 regulates dE2F1 expression at the translational level via the 5'UTR of *de2f1-RA-β* and likely also on protein stability**

Through immunostaining and immunoblot, I showed that dE2F1 protein expression increases in *tsc1* mutant cells (Figure 2A, Figure 2B, and Figure 3A). My RTq-PCR result suggests that Tsc1 post-transcriptionally regulates dE2F1 expression (Figure 3C). I then asked if Tsc1 regulation for dE2F1 expression is at the level of translation. I designed reporter constructs of *de2f1* 5' UTR to address this question –more specifically whether this regulation is through the 5' UTR of *de2f1*. However, there are three variants of *de2f1* mRNA: *de2f1-RA*, *de2f1-RB*, and *de2f1-RC*, each differs in the 5' UTR but all encode the same protein product. In both wild type and *tsc1* mutant eye imaginal discs, *de2f1-RA* transcript is the most abundant form based on my RTq-PCR result (data not shown). Therefore, I first tested if *de2f1-RA* is the determining transcript for the expression outcome

assuming that translational regulation is the mechanism. Furthermore, an original research paper by Sawado T. *et al* reported that there are two alternative transcription start sites in *de2f1-RA* [87], thus further yielding two variants of *de2f1-RA* that differ in the 5' UTR. This is different from the Flybase annotated sequence which denotes only one transcription start site. Indeed, my PCR results on cDNA extracted from the eye discs are in accordance with the work by Sawado T. *et al* (data not shown). For this thesis, I called the two *de2f1-RA* variants, *de2f1-RA-α* and *de2f1-RA-β*. Not only the 5' UTRs of *de2f1-RA-α* and *de2f1-RA-β* differ in length, with the 5' UTR of *de2f1-RA-β* being 400 nucleotides longer than that of *de2f1-RA-α*, but *de2f1-RA-β* 5' UTR also contains more ATG and Stop codons (see Appendix for the nucleotide sequences of *de2f1-RA-*α and *de2f1-RA-β* 5' UTRs).

I generated *de2f1-RA* 5'UTR reporter constructs, UAS-dE2F1-RA-α-GFP and UAS-dE2F1-RA-β-GFP, whose GFPs contain the 5' UTR of *de2f1-RA-α* or *de2f1-RA-β* respectively (refer to Materials and Methods for further details). The 5' UTR constructs were injected into *y w* embryos to generate transgenic flies. The 5' UTR reporters were expressed by Gal4 driver, and I compared the GFP expression levels between wild type and *tsc1* mutant clones in the eye imaginal discs (for full genotypes see Materials and Methods). *UAS-GFP-de2f1* [88] was used as control, whose GFP proteins are fused with the full-length coding sequence of wild type *de2f1*. Interestingly, the GFP expression level of UAS-dE2F1-RA-α-GFP, which is the reporter for *de2f1-RA-α* 5' UTR, does not change in *tsc1* mutant cells (Figure 10A). Note that larger cell size should be

distinguished from higher GFP intensity as *tsc1* homozygous mutant cells are bigger. In contrast, UAS-dE2F1-RA-β-GFP expression, in which the GFPs were under the control of *de2f1-RA-β* 5' UTR, increases in *tsc1* mutant cells (Figure 10B). Moreover, the GFP expression of the control, UAS-GFP-dE2F1, also increases in *tsc1* mutant cells (Figure 10C). These surprising results imply that Tsc1 regulates dE2F1 expression, at least in part, through the 5' UTR of *de2f1-RA-β* and likely also on protein stability. More experiments with an appropriate control are needed to validate whether Tsc1 regulates dE2F1 protein stability (see Future Experiments).

As an alternative approach to test whether Tsc1 regulation on dE2f1 expression is at the level of translation, we turned to *Drosophila* S2 cells for potential biochemical assays, such as the polysome assay. S2 cells were treated with *tsc1* RNAi or rapamycin for *tsc1* knock-down and *Tor* inactivation, respectively, and extracted for proteins to perform immunoblotting. Interestingly, there was no increase in dE2F1 expression level in *tsc1* RNAi treated S2 cells (Figure 10D). The observed increase in phosphorylated S6k level implies that *tsc1* knock-down was successful. In addition, dE2F1 expression did not decrease in rapamycin treated S2 cells (Figure 10E, compare to Figure 8A). Therefore, S2 tissue culture cells could not be used for biochemical assays to test whether dE2F1 regulation by Tsc1 is at the level of translation (see Summary and Discussions).

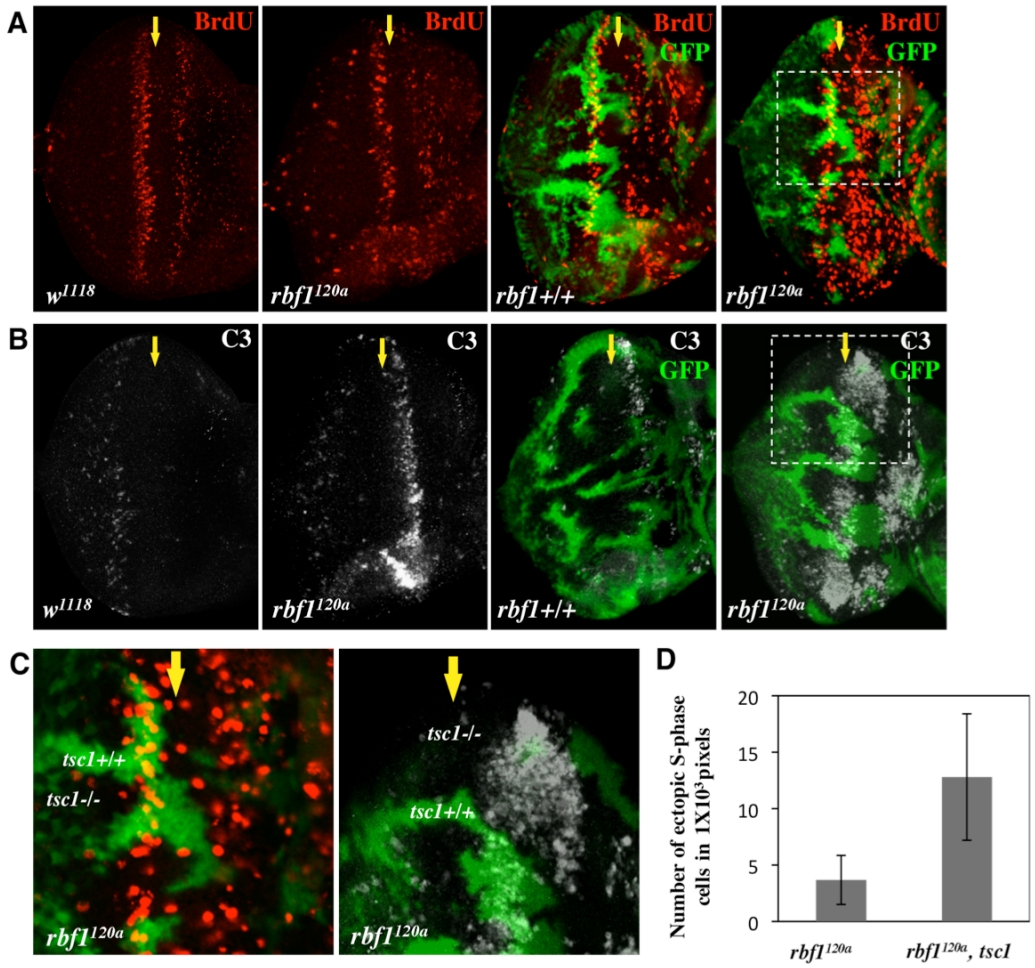



**Figure 1. *tsc1* and *rbf1* mutations cooperate to promote S-phase entry and cell death during eye development.** *tsc1*$^{R453X}$ mutant clones are generated in wild type and *rbf1*$^{120a}$ mutant eye discs by FLP-induced mitotic recombination. Wild type clones are marked with GFP (green) and the absence of GFP indicates *tsc1*$^{R453X}$ mutant clones. Control ($w^{1118}$) and *rbf1*$^{120a}$ eye discs without *tsc1*$^{R453X}$ mutant clones are also presented. The position of the Morphogenetic Furrow (MF) is indicated by a yellow arrow. (A) Third instar eye discs of indicated genotypes are treated with BrdU, and S-phase cells are visualized by anti-BrdU antibody (red). (B) To visualize apoptotic cells in the eye discs of the same genotypes, antibodies that recognize the cleaved form of Caspase 3 (C3) are used (white). (C) Images of higher magnification of the eye discs containing *tsc1* mutant clones in *rbf1* mutant background are shown. Note that the cells with both *tsc1* and *rbf1* mutations ectopically enter S-phase at the MF, and the C3 staining is stronger in the double mutant clones. (D) Numbers of ectopic BrdU positive cells within the MF are counted and normalized by the sizes of clones. The clone sizes are determined by counting the numbers of pixels that encompass the region between the first and second mitotic waves. Total of 12 *rbf1* single and 20 *rbf1 tsc1* double mutant clones are analyzed. The error bars indicate standard deviation.

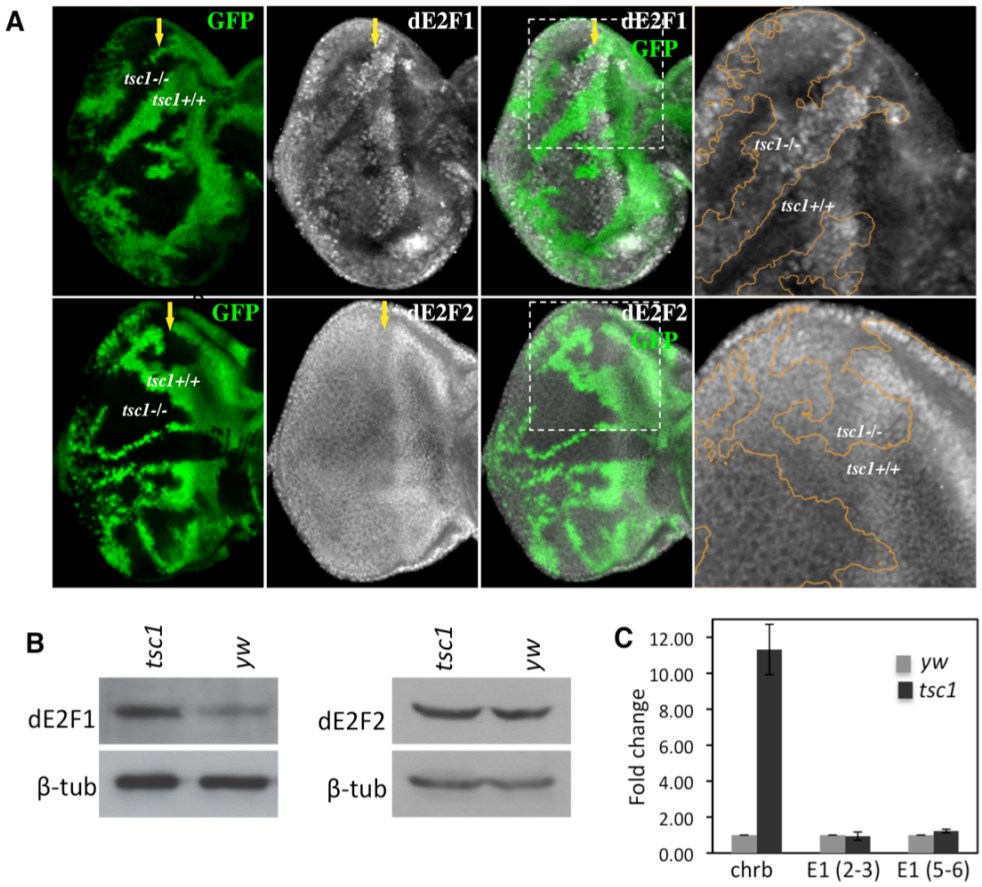



**Figure 2. Tsc1 regulates dE2F1 protein expression post-transcriptionally.** (A) *tsc1*$^{R453X}$ mutant clones are generated in the eye disc as described previously and immunostained with anti-dE2F1 or anti-dE2F2 antibodies. Images of higher magnification for the indicated areas (dotted line) are presented in the rightmost panel. The orange line indicates the clonal boundary. Position of the MF is indicated by a yellow arrow. Note that the intensity of dE2F1 staining is stronger in *tsc1* mutant clones, whereas dE2F2 staining is unaltered. (B) The protein level of dE2F1 and dE2F2 in *tsc1* mutant eye discs is determined by immunoblot. Eye-antenna imaginal discs that are mostly comprised of *tsc1* mutant cells are used. *β*-tubulin is used as a loading control. (C) Quantitative real-time PCR is used to compare the level of *de2f1* RNA in the control (*yw*) and *tsc1* mutant eye discs. The average fold difference of three independent triplicated experiments is presented. Primers for *de2f1* were designed to span an intron, covering either the second and third exons, E1 (2- 3) or the fifth and the sixth exons E1 (5-6). *charybdis* (*chrb*), whose expression is known to be upregulated by *tsc1* mutations, is used as a positive control. The error bars indicate standard deviation of the three independent experiments, ± 1.40 for Chrb, ± 0.23 for E1 (2- 3), and ± 0.10 for E1 (5-6).

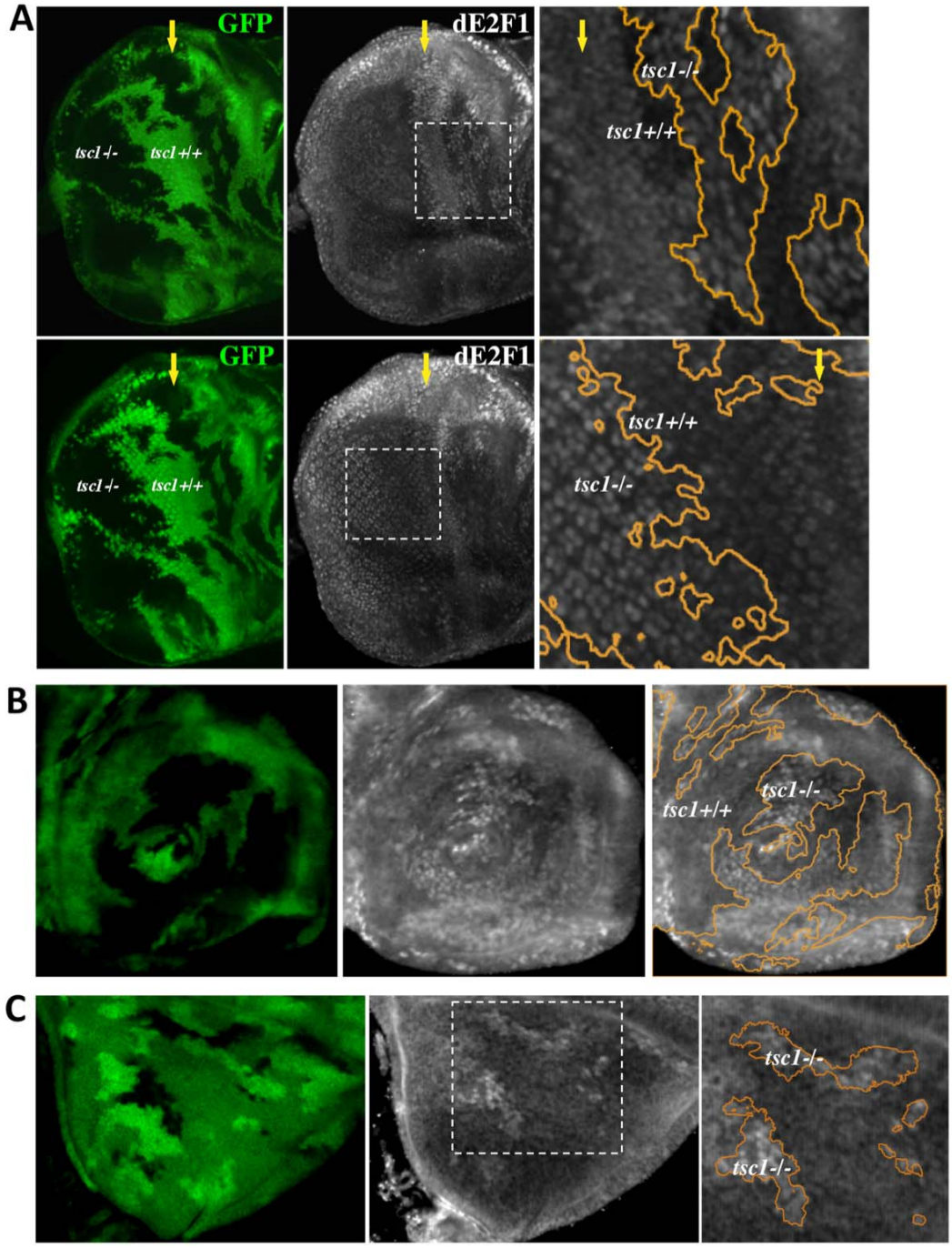



**Figure 3 Tsc1 regulates dE2F1 protein level both in proliferating and differentiating cells in imaginal discs** (A) *tsc1*$^{R453X}$ mutant clones are generated in the eye disc as described previously and immunostained with anti-dE2F1 antibodies. Images of higher magnification are presented in the rightmost panel for the dotted areas. The orange line indicates the clonal boundary. Yellow arrow indicates where the MF is located. Images of a single eye disc were taken at different focal planes to contrast cells at the anterior or the posterior part. Note that dE2F1 staining is higher in *tsc1*mutant cells that are asynchronously dividing

(region anterior to the MF, upper panel) and post-mitotic (region posterior to the MF, bottom panel). (B) Mutant clones of *tsc1*$^{R453X}$ are generated in the antenna disc in the same manner as previously described. Note that dE2F1 staining is higher in the mutant clones. (C) *tsc1*$^{R453X}$ mutant clones are generated in the wing disc by heat shock driven FLP. Wing-notum region is shown. GFP marks wild type cells. Magnified view of the area enclosed in dotted line is shown in the rightmost panel. The orange line indicates the clonal boundary. Note that dE2F1 staining is stronger in *tsc1* mutant clones. Cells with highest GFP expression are the twin spots.

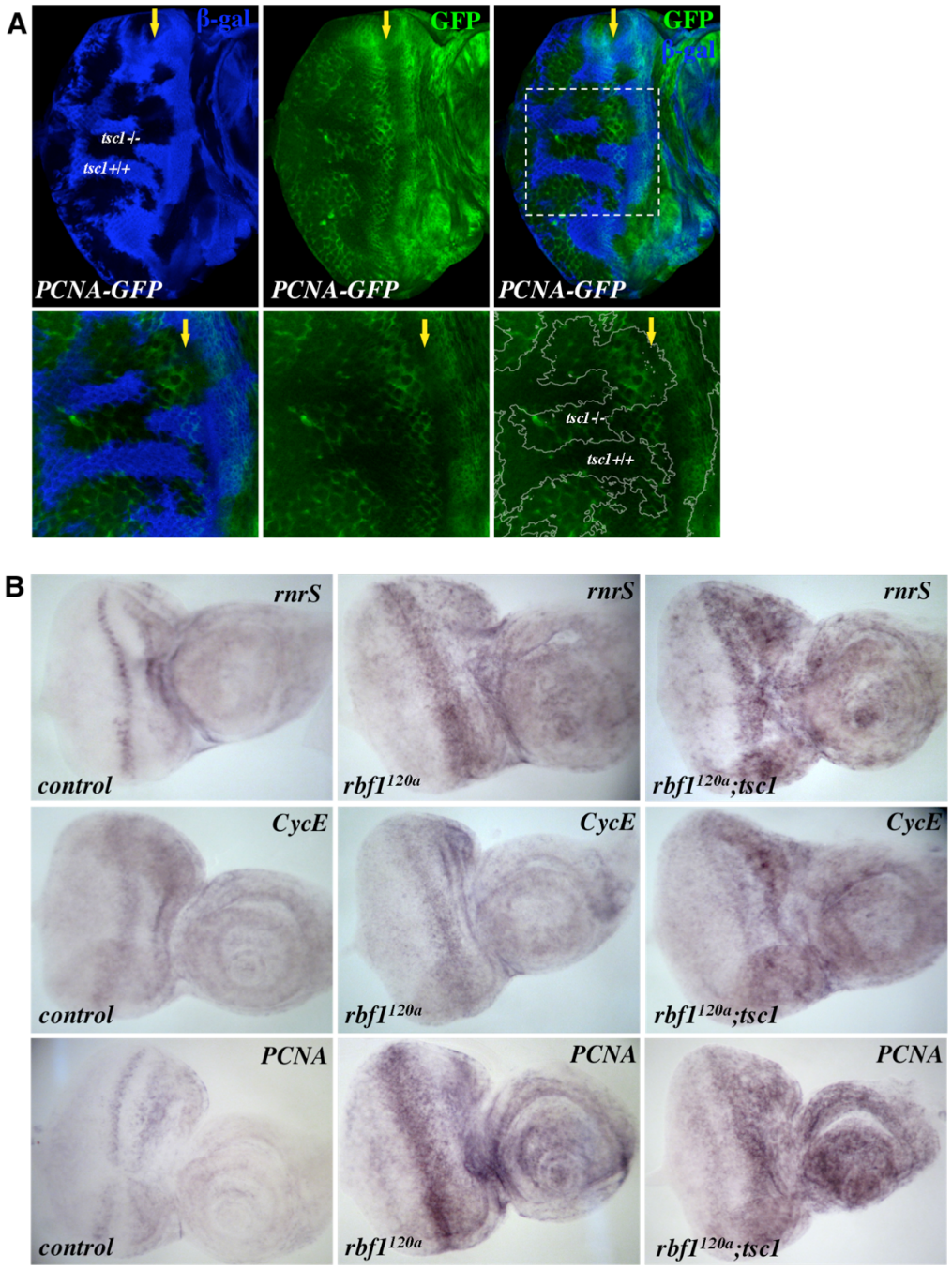



**Figure 4. Transcription of dE2F1 target genes is activated in *tsc1* mutant cells.** (A) Mitotic clones of $tsc1^{R453X}$ are generated in the eye disc of *PCNA-GFP* transgenic flies. *PCNA- GFP* is a reporter construct where GFP (green) is expressed under the control of the *PCNA* promoter, a known dE2F1 target. Wild type clones are marked by the presence of β- galactosidase for this experiment (blue). Note that GFP expression is increased in *tsc1* homozygous mutant clones at the posterior of the MF. Images of higher magnification of the mitotic clones are also shown (lower panel). (B) *In situ* hybridization assay is used to compare

expression patterns of three dE2F1 target genes, *rnrS, CycE*, and *PCNA*. *rbf1 tsc1* double mutant eye discs are generated as described previously. Since *rbf1 tsc1* double mutant eye discs are generated by mitotic recombination using a recessive cell lethal mutation, the control and *rbf1* eye discs are generated by inducing mitotic recombination between the wild type FRT chromosome against the same recessive cell lethal mutation (see Materials and Methods for the full genotypes). Note the expression of *rnrS, CycE,* and *PCNA* in $rbf1^{120a}$ eye discs is highest at the MF. In contrast, strong expression of *rnrS*, *CycE,* and *PCNA* is observed both at the MF and in the anterior region of the *rbf1 tsc1* double mutant eye disc.

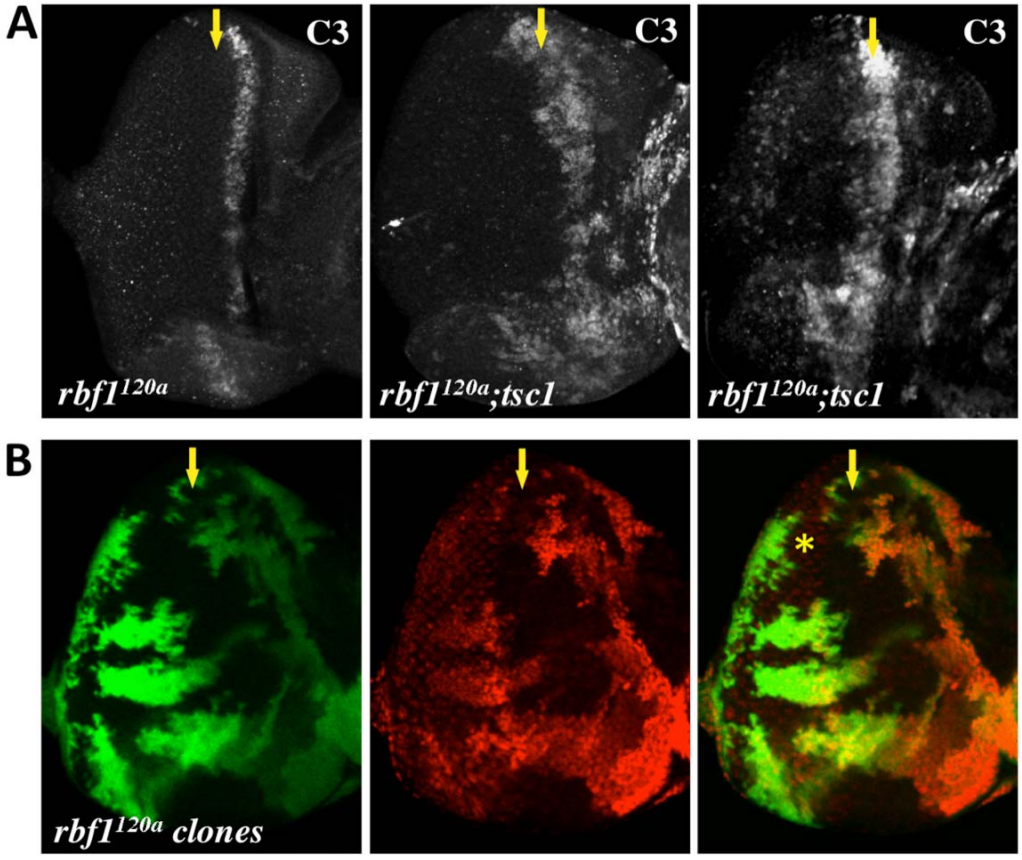



**Figure 5. The pattern of ectopic cell death in eye imaginal discs that are composed mostly of *rbf1$^{120a}$ tsc1* double mutant cells.** (A) *rbf1* single or *rbf1 tsc1* double mutant eye discs are generated by mitotic recombination using a recessive cell lethal mutation as described in *in situ* experiment. Apoptotic cells are visualized by anti-Caspase 3 (white). *rbf1* mutant cells undergo apoptosis at the anterior part of the MF (left panel) whereas *rbf1 tsc1* double mutant cells undergo apoptosis at the MF and the anterior region of the eye disc (two different eye discs of the same genotype are shown in the middle and the right panels). (B) *rbf1* mutant clones are generated as described previously using FLP recombinase. GFP marks wild type cells. The eye disc was immunostained with anti-Rbf1 (red). Note the weak but visible Rbf1 staining in *rbf1$^{120a}$* mutant clones in the region posterior to the MF (yellow asterisk).

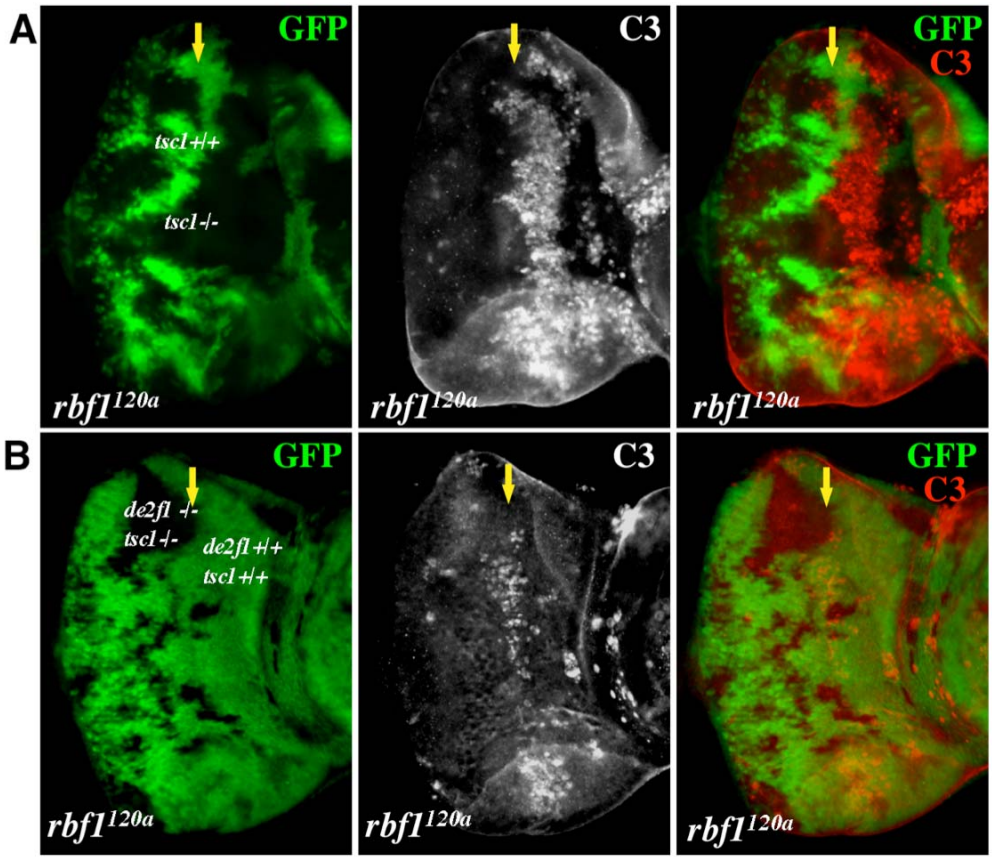
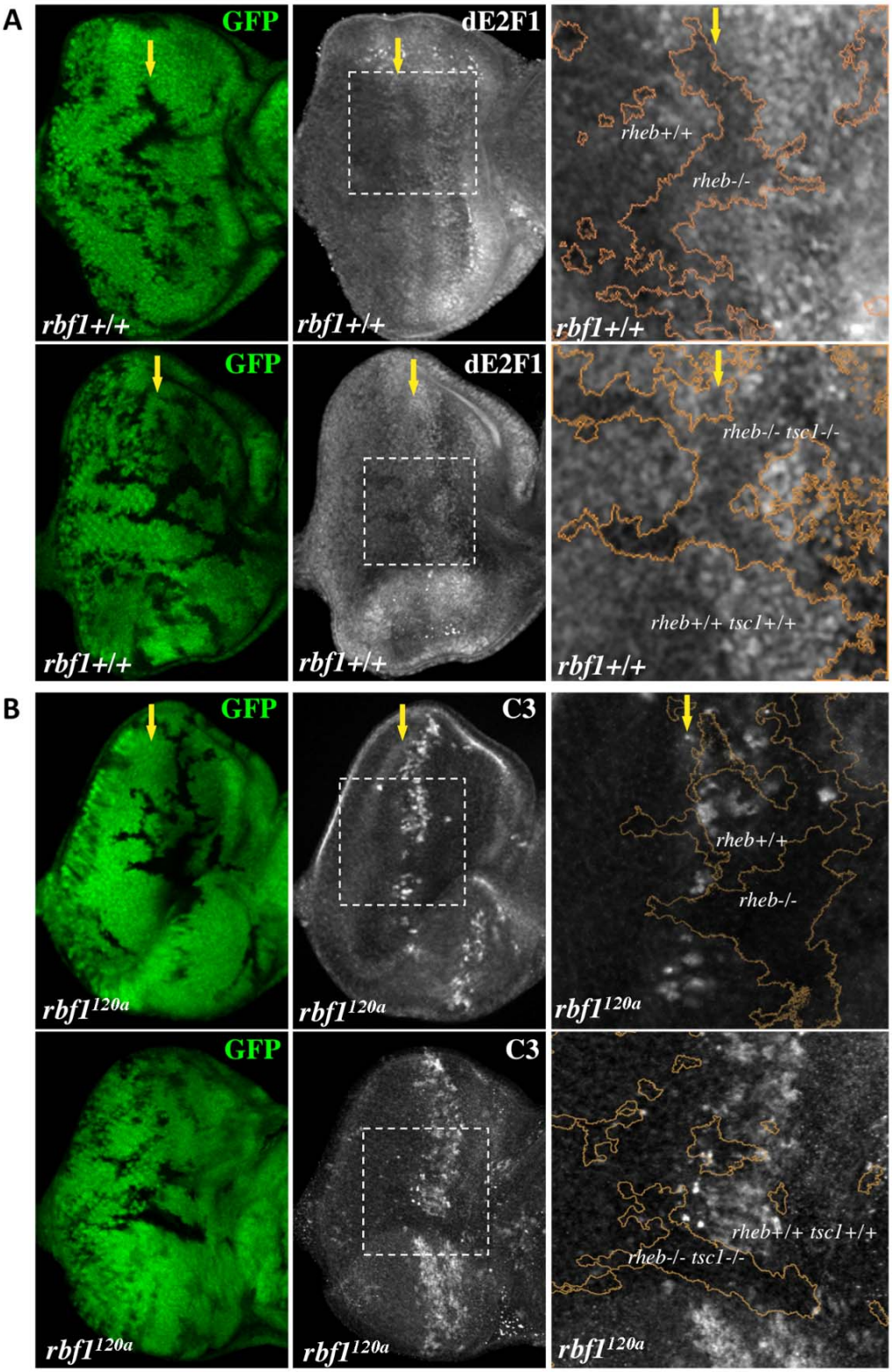



**Figure 6. dE2F1 is required for the ectopic cell death induced by *tsc1* mutations in the *rbf1*$^{120a}$ eye discs.** Mitotic clones of *tsc1*$^{f01910}$ single (A) or *tsc1*$^{f01910}$ and *de2f1*$^{729}$ double mutants (B) were generated in the *rbf1*$^{120a}$ mutant background. Wild type cells were marked with GFP (green). Apoptotic cells are visualized by immunostaining with C3. Note that the ectopic cell death induced by *tsc1* mutations in *rbf1* mutant eye discs is completely suppressed by *de2f1* mutations.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rheb+/+ tsc1+/+
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C3
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**Figure 7. Rheb promotes dE2F1 expression during eye development and dE2F1- dependent cell death in *rbf1* mutant eye discs.** (A) Mitotic clones of *rheb*$^{2D1}$ or double- mutant clones of *rheb*$^{2D1}$ and *tsc1*$^{R453X}$ are generated in the eye discs and immunostained with an anti-dE2F1 antibody (white). Images of higher magnification with outlined clonal boundaries (orange) is also shown. Note the reduced dE2F1 staining in *rheb* mutant clones. (B) *rheb*$^{2D1}$ or double-mutant clones of *rheb*$^{2D1}$ and *tsc1*$^{R453X}$ are generated in *rbf1*$^{120a}$ mutant eye discs and immunostained with C3 to visualize apoptotic cells (white). Note the discontinued stripe of cell death in *rheb* mutant clones.

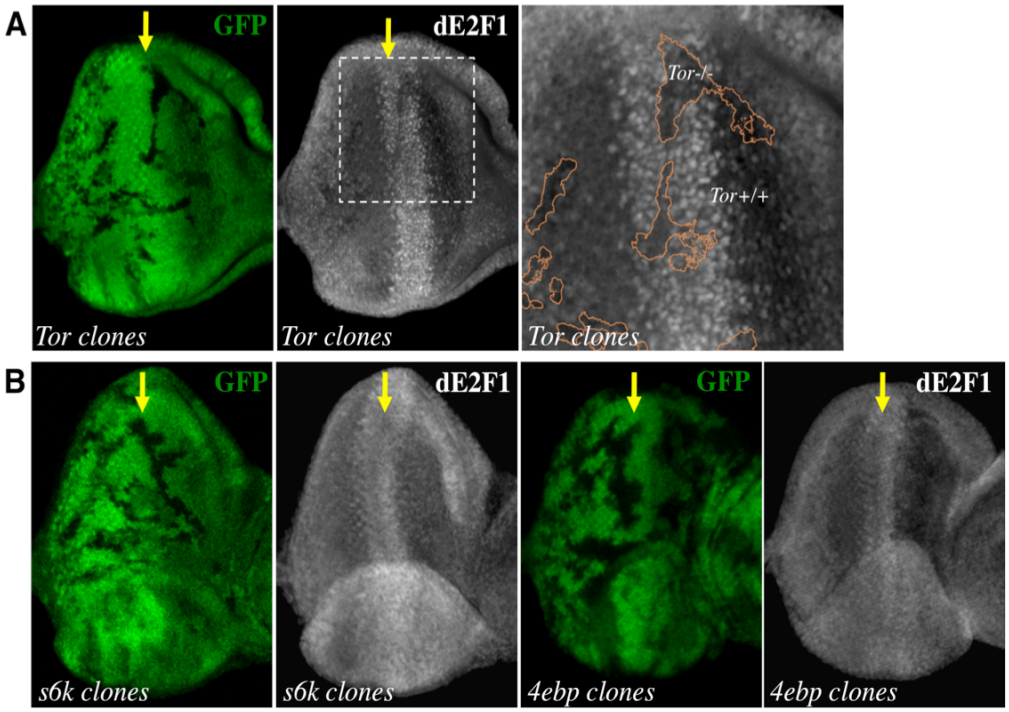



**Figure 8. Tor is required for dE2F1 expression during eye development but neither *s6k* or *4ebp* mutations affect dE2F1 expression.** (A) Mitotic clones of $Tor^{2L19}$ are generated in the eye discs and immunostained with an anti-dE2F1 antibody (white). Images of higher magnification with outlined clonal boundaries (orange) are also shown. dE2F1 staining is clearly reduced in the *tor* mutant clones. (B) $s6k^{l-1}$ or $4ebp^{null}$ mutant clones are generated as described previously. In contrast to *Tor* mutant clones, dE2F1 expression is unchanged in the mutant clones of either genotype.

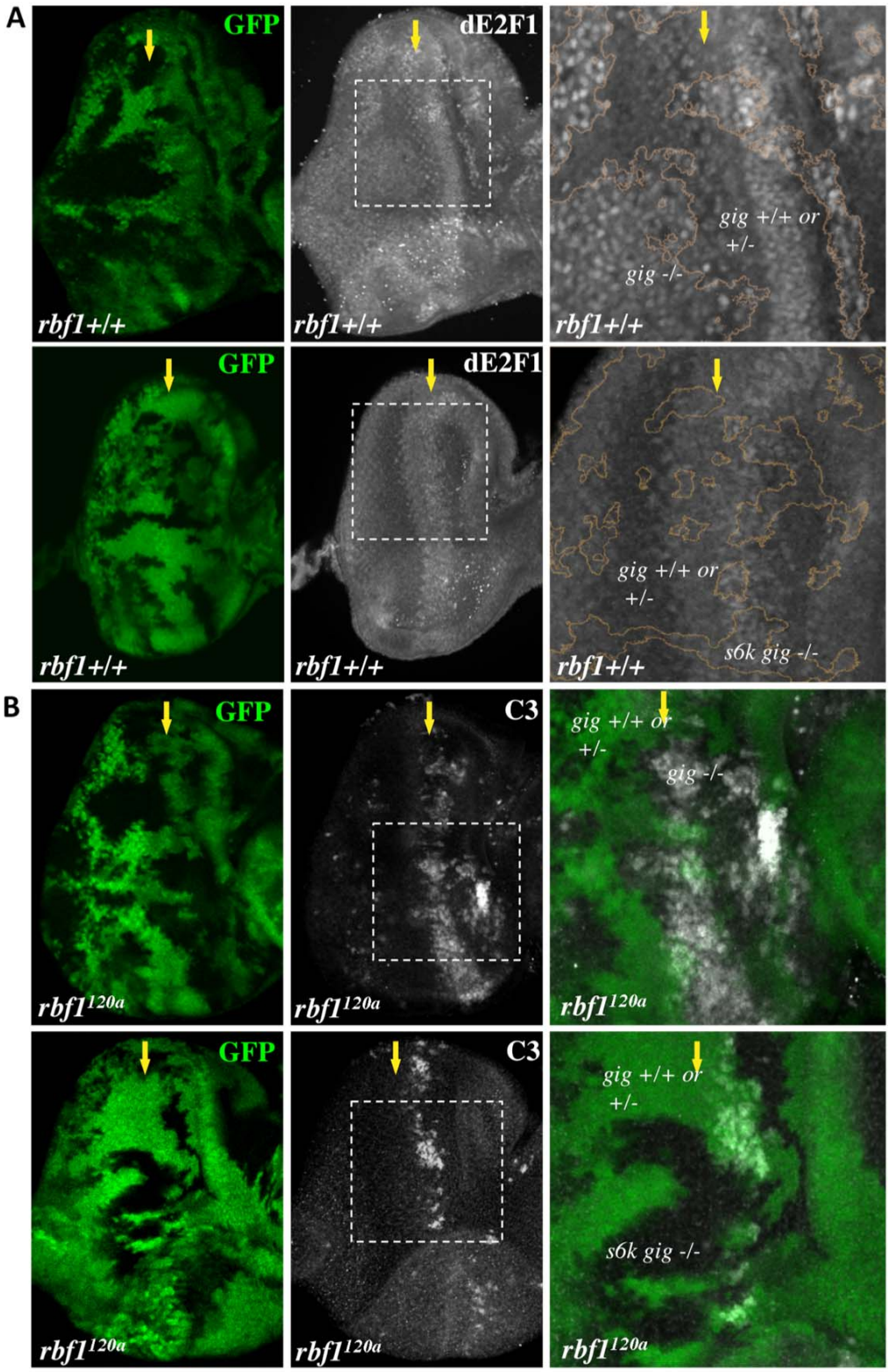
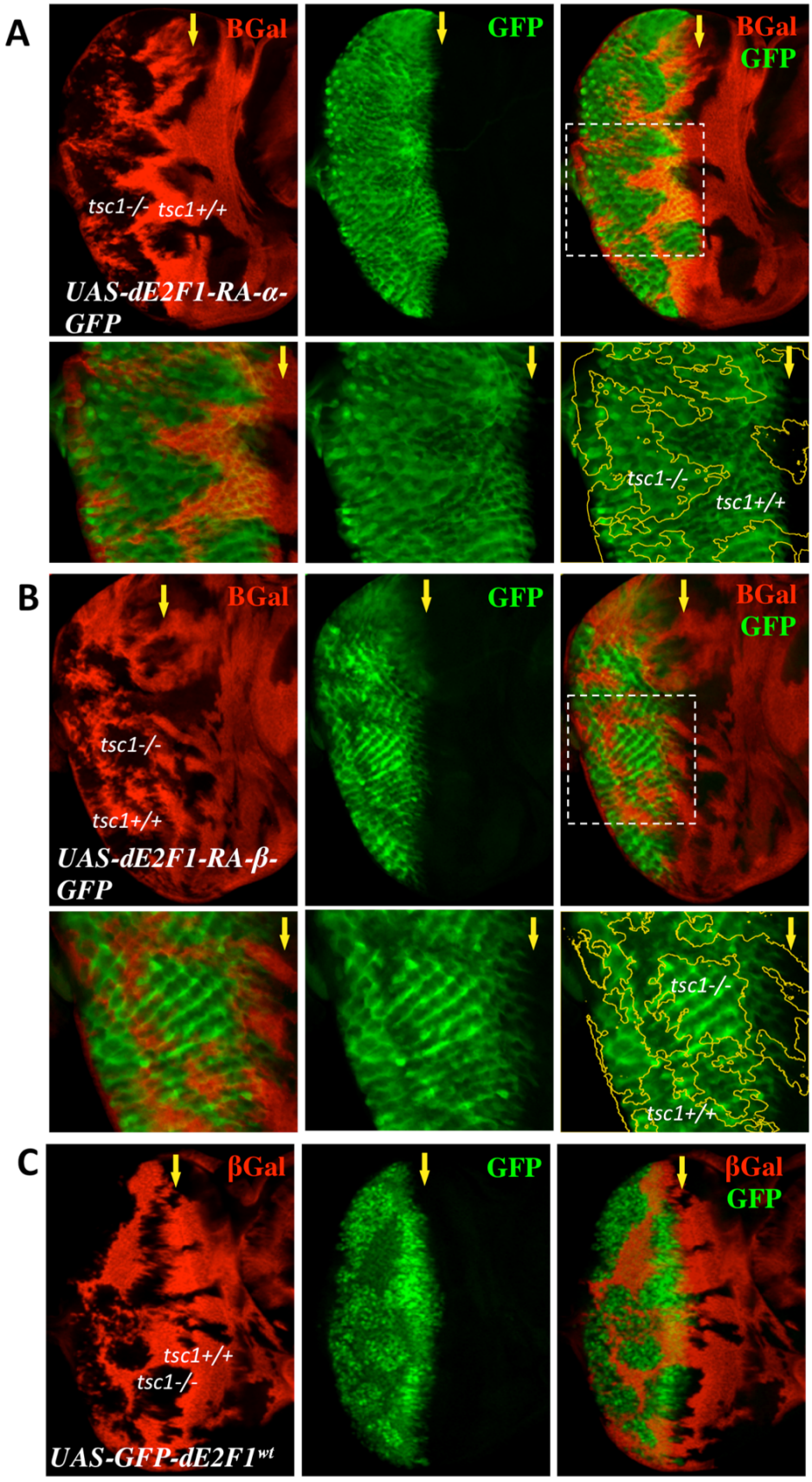



**Figure 9. S6K is required for the effect of TSC inactivation on dE2F1**

**expression and dE2F1-dependent cell death in *rbf1*$^{120a}$ eye discs.** (A) Mitotic clones of *gig*$^{192}$ single or *s6k*$^{l-1}$ and *gig*$^{192}$ double mutant clones are generated in the eye discs. The intensity of the GFP indicates that GFP expressing clones are composed of two genotypes, wild type and heterozygous mutations (eg. *gig*+/+ or *gig*+/-). An anti-dE2F1 antibody (white) is used to determine the expression pattern of dE2F1. Images of higher magnification with outlined clonal boundaries (orange) are also presented. Note that dE2F1 expression is unchanged in *s6k gig* double mutant clones contrary to *gig* single mutant clones where dE2F1 level is clearly elevated. (B) *gig*$^{192}$ single or *s6k*$^{l-1}$ and *gig*$^{192}$ double mutant clones are generated in *rbf1*$^{120a}$ mutant eye discs and stained with C3 to visualize apoptotic cells. The increased level of apoptosis by *gig* mutations is suppressed by *s6k* mutations.


A
BGal
GFP
BGal
GFP
tsc1-/-
tsc1+/+
UAS-dE2F1-RA-α-GFP
tsc1-/-
tsc1+/+
B
BGal
GFP
BGal
GFP
tsc1-/-
tsc1+/+
UAS-dE2F1-RA-β-GFP
tsc1-/-
tsc1+/+
C
βGal
GFP
βGal
GFP
tsc1+/+
tsc1-/-
UAS-GFP-dE2F1wt

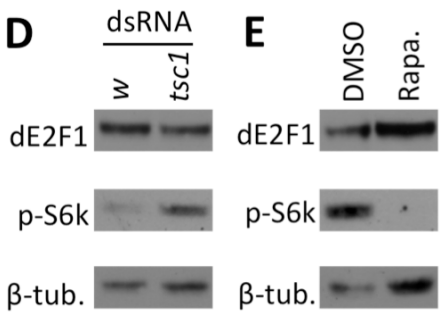



**Figure 10. Tsc1 regulates dE2F1 expression through the 5' UTR of beta-form *de2f1-RA* and neither knock-down of *tsc1* nor *tor* affects dE2F1 protein level in S2 *Drosophila* tissue culture cells.** (A) Mitotic clones of $tsc1^{R453X}$ are generated with the same approach in the eye disc of *GMR-Gal4,UAS-de2f1-RA-α-GFP* transgenic fly. UAS-dE2F1-RA-α-GFP is a reporter construct whose GFPs (green) contain the *de2f1-RA-α* 5' UTR. Note that GMR expression domain is posterior to the MF in the eye disc. Wild type clones are marked by the presence of β- galactosidase (red). Note that there is no difference in GFP expression between wild type and *tsc1* homozygous mutant cells. Images with higher magnification of the mitotic clones are also shown (lower panel). (B) *UAS-de2f1-RA-β-GFP* is expressed in the eye disc as described above. Note the higher GFP intensity in *tsc1* mutant cells compared to wild type cells. Higher magnification images are shown in bottom panels (C) The transgenic construct *UAS-GFP-de2f1,* whose GFPs are fused with the full length *de2f1* coding sequence, is also expressed with *GMR-Gal4* as described above. A different 5' UTR from *de2f1-RA* is embedded in the *UAS-GFP-de2f1* control construct. Note that GFP expression level is higher in *tsc1* mutant cells than wild type cells. (D) Immunoblot analysis of S2 cells treated with either *white* or *tsc1* dsRNA. Anti-phosphorylated S6k serves as the positive control and anti-B-tubulin as the loading control. Note that, in contrast to imaginal discs, dE2F1 protein expression is not increased in *tsc1* dsRNA treated cells when compared to *white* dsRNA treated cells. (E) Immunoblot analysis of S2 cells treated with DMSO or DMSO containing rapamycin. Note that dE2F1 expression is unchanged in rapamycin treated cells.

# DISCUSSION

## Summary and Discussions

The loss of Rb leads to hyperactivation of E2F family proteins, which is a crucial event during tumorigenesis. Here, I demonstrate that the *Drosophila* ortholog of Tsc1 tumor suppressor cooperates with Rbf1 to regulate dE2F1 activity during development. Tsc1 regulates dE2F1 expression at the level of translation and likely also on protein stability. The loss of *tsc1* cooperates with *rbf1* mutations to promote unscheduled S-phase entry and cell death. This effect of *tsc1* mutations on dE2F1 expression requires the components of canonical Tsc/Rheb/Tor pathway which are major regulators of cellular growth. My study provides evidence to suggest that dE2F1 is an important protein that couples growth signals to cell cycle progression.

Recent studies have identified that pro-proliferative and pro-apoptotic activities of dE2F1 are engaged by various *Drosophila* tumor suppressor genes, such as *hippo* and *archipelago* [58, 59]. The findings of my thesis work add *tsc1/2* tumor suppressor genes to this list. Previously, dE2F1 or Cyclin E overexpression is shown to bypass starvation induced G1 arrest at least in endoreduplicating tissues [89]. Moreover, similar to dE2F1, expression of Cyclin E is elevated in *tsc1* mutant cells in eye imaginal discs [64-66]. Perhaps, restricting the expression of cell cycle regulators, such as dE2F1 and Cyclin E, is a part of the molecular mechanisms by which nutrient deprivation induces G1 arrest. Interestingly, overexpression of dE2F1 or Cyclin E does not overcome starvation-induced G1 arrest in larval neuroblasts, indicating that, in mitotic cells, neither dE2F1 nor

Cyclin E is the limiting factor [89]. Consistent with this observation, we could not observe any appreciable increase in the size of *rheb* or *Tor* mutant clones in *rbf1* mutant background, suggesting that multiple factors contribute to the proliferative defect observed in *rheb* or *Tor* mutant cells in imaginal discs.

Interestingly, despite the elevated level of dE2F1 and Cyclin E, *tsc1* mutant clones have relatively normal patterns of BrdU staining at the MF and a limited amount of ectopic cell death. It is likely that the activity of dE2F1 in *tsc1* mutant cells is normally restricted by the presence of Rbf1. The fact that the increase in ectopic S-phase entry and apoptosis by *tsc1* mutations can be only observed in the *rbf1* mutant background supports this idea. Moreover, the Tsc/Rheb/Tor pathway may be modulating the amount of dE2F1 needed for cellular division in proportion to the cell size during development. Supporting this idea, previous studies have demonstrated that *tsc1* or *tsc2* mutant cells spend less time in G1, a phenotype commonly observed in cells with elevated dE2F1 activity [64-66, 90]. It is conceivable that the elevated level of dE2F1 proteins in *tsc1* or *tsc2* mutant cells allows them to go through G1 to S-phase transition faster where Rbf1 is normally inactivated by Cyclin Dependent Kinases (CDKs).

Despite being the only "activator E2F" in *Drosophila*, it is still unclear how dE2F1 expression is regulated during development. A recent study reported that Cul4(Cdt2) E3 ubiquitin ligase mediates destruction of dE2F1 in S-phase, a mechanism that regulates dE2F1 expression in a cell cycle dependent manner [91]. My findings here suggest that the expression of dE2F1 is also regulated by a growth-controlling network. Furthermore, my preliminary data suggest that the

Tsc/Rheb/Tor pathway regulates dE2F1 at the level of translation, specifically through the 5' UTR of *de2f1-RA* β-variant, and protein stability, which, however, requires a control to validate (see Future Experiments). The finding that S6k is involved in this process supports the idea of translational control since S6k directly phosphorylates and regulates proteins involved in translation, such as RpS6, eIF4B, and eEF2K to list a few (reviewed in [92]). Further, my evidence showed that the loss of Tsc causes dE2F1 protein to become more stable, which would suggest that the Tsc pathway promotes dE2F1 protein turnover. Interestingly, Rb also influences the stability of E2F-1 by binding to the motif of E2F-1that mediates ubiquitin-mediated proteasome degradation, hence inhibiting E2F-1 degradation [93]. In S2 cells, neither *tsc1* RNAi nor Rapamycin (*Tor* inhibitor) treatment had the same effect on dE2F1 expression observed in imaginal discs (Figure 10D and 10E). It is probable that S2 cells lack factors necessary for dE2F1 regulation that are present *in vivo*. Nevertheless, it is important to note that this effect on dE2F1 expression is specific since dE2F2 expression is unchanged in *tsc1*, *rheb* or *Tor* mutant cells (Figure 2A and data not shown). Curiously, the requirement of S6k to regulate dE2F1 is limited to the context in which Tsc is inactivated. The loss of *s6k* in the wild type background has no effect on dE2F1 expression while *rheb* or *Tor* mutations reduce the level of dE2F1 proteins in the eye disc (Figures 7A and 8A). In mammals, it has been demonstrated that the translation of specific mRNAs can be mTor-dependent but not S6k- dependent [94]. The molecular mechanism in which S6k promotes dE2F1 expression only when Tsc is inactivated is presently unclear and warrants

further investigation.

Another interesting finding in my study is that *s6k* mutations suppress the dE2F1-dependent cell death normally present in *rbf1* mutant eye discs (Figure 9B). *s6k* mutations alone did not alter the dE2F1 expression level at least in the wild type background. Although it is not formally tested, this raises a possibility that the Tsc/Tor/S6k pathway controls dE2F1-dependent cell death without altering dE2F1 expression. Interestingly, the crosstalk between the InR/Tor and the EGFR signaling pathways during *Drosophila* eye development has been recently established [95]. InR/Tor signalling regulates the timing of neuronal differentiation in the eye disc by modulating EGFR activity. Since the EGFR pathway is an important determinant of dE2F1-dependent cell death [41], S6k might promote dE2F1-dependent cell death by modulating the EGFR pathway. Thus, it is conceivable that the cooperative effect between *tsc1* and *rbf1* mutations may be the consequence of multiple changes that include the increase in dE2F1 expression.

In cancer cells, it is generally thought that the loss of Rb function is the most common mechanism of deregulating E2F activity. However, in some types of cancers, amplification of E2F genes or overexpression of E2F family proteins have been observed (reviewed in [4]). Moreover, in a subtype of human retinoblastoma where Rb is already deficient, E2f-3 proteins are also overexpressed [96]. These observations suggest that E2F family genes themselves can be directly targeted and deregulated during tumorigenesis. It will be

interesting to investigate if Tsc1/2 or other tumor suppressors and oncogenes regulate the expression of E2F family proteins to promote tumorigenesis.

**Future Experiments**

The mechanism of Tsc regulation on dE2F1 requires further investigation as the GFP expression of UAS-GFP-dE2F1 transgenic construct, which was originally tested as the control for the 5'UTR reporters of *de2f1-R-α* and *-β*, surprisingly had increased in *tsc1* mutant cells. Moreover, a control for the UAS-GFP-dE2F1 construct was not taken into consideration. Since Tsc1 regulation on endogenous protein has shown to be specific to dE2F1, a transgenic construct employing dE2F2 can be used as the appropriate control for UAS-GFP-dE2F1 to validate Tsc regulation on dE2F1 protein stability. I have generated a construct expressing GFP-dE2F2, whose *de2f2* full-length coding sequence is fused to GFP and under the control of *UAS* sequence. UAS-GFP-dE2F2 will be expressed with GMR-Gal4, the same driver used to express UAS-GFP-dE2F1, and GFP intensities will be compared between $tsc1^{R453X}$ and wild type cells in the eye imaginal discs. If GFP expression does not change in *tsc1* mutant cells of *UAS-GFP-de2f2* transgenic flies, it would support the idea that Tsc1 regulates dE2F1 protein stability. In addition, a report by Shibutani ST. *et al* showed that during S-phase, the PCNA-interacting-protein (PIP) motif in dE2F1 mediates dE2F1 protein degradation by the ubiquitin-proteasome system [88]. The reported transgenic flies expressing PIP motif mutant dE2F1, either with conserved amino residues change or deletion of the entire motif, UAS-GFP-$dE2F1^{PIP3A}$ or UAS-GFP-$dE2F1^{PIP\text{-}7\ del}$ respectively, can be employed to test whether the PIP motif is

responsible for Tsc regulated dE2F1 turnover. If the increase of GFP expression in *tsc1* mutant cells, as seen in *UAS-GFP-de2f1* transgenic flies, is suppressed in either *UAS-GFP-de2f1*$^{PIP3A}$ or *UAS-GFP-de2f1*$^{PIP\text{-}7\ del}$ transgenic flies, it would suggest that the dE2F1 PIP motif is required for Tsc-mediated degradation of dE2F1 protein.

A biochemical approach to investigate Tsc translational regulation on dE2F1 is to perform polysome assay on lysates from *Drosophila* Kc cells transfected with *de2f1-RA-α* or *-β* 5' UTR construct. Since *tsc1* or *tor* knock-down in S2 cells showed no effect on dE2F1 expression, Kc cells from *Drosophila* embryos will be used instead. However, prior to performing polysome assay, whether *tsc1* RNAi or rapamycin has an effect on endogenous dE2F1 expression in Kc cells will be tested. Kc cells would be suitable for this experiment because it has been shown that *de2f1-RA* is expressed in Kc cells [87, 97]. Furthermore, in a reported DNA microarray experiment, *de2f1* is identified as one of the most differentially expressed genes between S2 and Kc cells [98], raising a possibility that the expression of dE2F1 is differentially regulated between the two cell types. *de2f1-RA* is the most abundant form of *de2f1* transcript in the eye discs and, through the 5' UTR of its β-form, I showed that Tsc regulates dE2F1 protein expression; therefore, it is likely that *tsc1* or *tor* knock-down would cause endogenous dE2F1 protein level to increase or decrease, respectively, in Kc cells.

Since we saw an increase in GFP expression in *tsc1* mutant cells of *UAS-de2f1-RA-β-GFP* transgenic fly eye imaginal discs, a polysome assay on the lysates of

*tsc1* knock-down Kc cells is expected to show *de2f1-RA-β* 5' UTR shifting towards heavier fractions, which is the result of increasing number of ribonucleoproteins binding to the mRNA molecules. The polysome assay is an even more convincing technique to demonstrate that Tsc repression on dE2F1 is at the level of translation.

The 5' UTR of *de2f1-RA-β* can be further examined to identify the sequences within the 5' UTR required for Tsc regulation, such as by deletion series analysis of the *de2f1-RA-β* 5' UTR. Truncated 5' UTR sequences can be made into reporter constructs, such as by fusing with GFPs or luciferases; the reporter activities can eventually be measured and compared between *tsc* mutant and wild type backgrounds. This experiment would uncover the elements in the *de2f1-RA-β* 5' UTR that are required for regulation by Tsc, and potentially, the effector downstream of S6k that directly interacts with the 5' UTR. Lastly, a *de2f1* 3' UTR reporter construct can be generated to examine whether Tsc also acts through the 3' UTR to regulate translation of *de2f1* mRNA. All three *de2f1* mRNA variants share the same 3' UTR, which is 1265 nucleotide in length. Similar to the 5' UTR reporter constructs, the 3' UTR of *de2f1* can be cloned, set under the control of UAS-Gal4, and its activity can be visualized with a GFP reporter.

## MATERIALS AND METHODS

### Fly Stocks

All crosses have been performed at 25°C. The *rbf1* mutant allele, $rbf1^{120a}$, and *de2f1* allele, $de2f1^{729}$, are described previously [47, 48]. The *tsc1* alleles used in

this study are *tsc1*$^{R453X}$, a gift from Dr. Hariharan [84], and *tsc1*$^{f01910}$ (Exelixis collection, Harvard Medical School). The mutant alleles of the TSC/Rheb/Tor pathway used in this study are as follows: *Tor*$^{2L19}$ *FRT40A* and *4ebp*$^{null}$ are gifts from P. Lasko [85, 86]. *s6k*$^{l-1}$ *FRT80B* is a gift from D.J. Pan [64]. The *gig*$^{56}$ *FRT80B*, *FRT82B rheb*$^{2D1}$, and *s6k*$^{l-1}$ *gig*$^{192}$*FRT80B* alleles were kindly provided by J.M. Bateman [65]. The *4ebp*$^{null}$ *FRT40A*, *FRT82B de2f1*$^{729}$ *tsc1*$^{f01910}$, and *FRT82B rheb*$^{2D1}$ *tsc1*$^{R453X}$ alleles were generated by meiotic recombination. For the double mutant alleles, presence of both mutations is verified by genetic complementation tests using multiple mutant alleles. For example, presence of both *s6k* and *gig* mutations in *s6k*$^{l-1}$*gig*$^{192}$ *FRT80B* alleles were verified by crossing the alleles to *gig*$^{52}$, *gig*$^{192}$, *s6k*$^{l-1}$ and *s6k*$^{p\{PZ\}07084}$ alleles individually. Translational reporters of *de2f1* 5' UTR, *UAS-de2f1-RAα-GFP* and *UAS-de2f1-RAβ-GFP*, were generated using TOPO® Cloning approach described below. *UAS-GFP-de2f1*$^{w.t.}$, the control for the translational reporter experiment, was a gift from R.J. Duronio [91].

**Clonal Analysis**

Flippase (FLP) was expressed from the *eyeless* promoter to generate mitotic clones in the eye. To examine clones in *rbf1* mutant animals, the X chromosome carrying *rbf1*$^{120a}$ and an *ey-FLP* transgene was used. Following are the full genotypes of larvae analysed:

**Mutant clones in the wild type background**

*y w eyFlp/ + or Y; FRT82B GFP*$^{ubi}$*/ FRT82B tsc1*$^{R453X}$

*y w eyFlp/ + or Y; FRT82B GFP*$^{ubi}$*/ FRT82B rheb*$^{2D1}$

*y w eyFlp/ + or Y; FRT82B GFP$^{ubi}$/ FRT82B rheb$^{2D1}$ tsc1$^{R453X}$*

*y w eyFlp/ + or Y; GFP$^{ubi}$ FRT40A/ Tor$^{2L19}$ FRT40A*

*y w eyFlp/ + or Y; GFP$^{ubi}$ FRT80B/ s6k$^{l-1}$ FRT80B*

*y w eyFlp/ + or Y; GFP$^{ubi}$ FRT40A/ 4ebp$^{null}$ FRT40A*

*y w eyFlp/ + or Y; GFP$^{ubi}$ FRT80B/ gig$^{56}$ FRT80B*

*y w eyFlp/ + or Y; GFP$^{ubi}$ FRT80B/ s6k$^{l-1}$ gig$^{192}$ FRT80B*

*rbf1$^{120a}$ FRT19A/ GFP FRT19A; eyFLP/+*

**Mutant clones in the *rbf1$^{120a}$* background**

*w rbf1$^{120a}$ eyFlp/ Y; FRT82B GFP$^{ubi}$/ FRT82B tsc1$^{R453X}$*

*w rbf1$^{120a}$ eyFlp/ Y; FRT82B GFP$^{ubi}$/ FRT82B tsc1$^{f01910}$*

*w rbf1$^{120a}$ eyFlp/ Y; FRT82B GFP$^{ubi}$/ FRT82B de2f1$^{729}$ tsc1$^{f01910}$*

*w rbf1$^{120a}$ eyFlp/ Y; FRT82B GFP$^{ubi}$/ FRT82B rheb$^{2D1}$*

*w rbf1$^{120a}$ eyFlp/ Y; FRT82B GFP$^{ubi}$/ FRT82B rheb$^{2D1}$ tsc1$^{R453X}$*

*w rbf1$^{120a}$ eyFlp/ Y; GFP$^{ubi}$ FRT80B/ gig$^{56}$ FRT80B*

*w rbf1$^{120a}$ eyFlp/ Y; GFP$^{ubi}$ FRT80B/ s6k$^{l-1}$ gig$^{192}$ FRT80B*

**Immunoblot, real-time quantitative PCR and *in situ* hybridization**

*y w eyFlp/ Y; FRT82B [W+] l(3)cl-R3/ FRT82B* (controls)

*y w eyFlp/ Y; FRT82B [W+] l(3)cl-R3/ FRT82B tsc1$^{R453X}$*

*w rbf1$^{120a}$ eyFlp/ Y; [W+] l(3)cl-R3/ FRT82B*

*w rbf1$^{120a}$ eyFlp/ Y; [W+] l(3)cl-R3/ FRT82B tsc1$^{R453X}$*

**PCNA-GFP in *tsc1* mutant clones**

*y w eyFlp/ PCNA-GFP; FRT82B LacZ$^{arm}$/ FRT82B tsc1$^{R453X}$*

**5' UTR translational reporters in *tsc1* mutant clones**

*GMR-Gal4 UAS-de2f1-RA-α-GFP; FRT82B LacZ$^{arm}$/ FRT82B tsc1$^{R453X}$*

*GMR-Gal4 UAS-de2f1-RA-β-GFP; FRT82B LacZ$^{arm}$/ FRT82B tsc1$^{R453X}$*

*GMR-Gal4 UAS-GFP-de2f1$^{w.t.}$; FRT82B LacZ$^{arm}$/ FRT82B tsc1$^{R453X}$*

**Immunostaining and Microscopy**

The antibodies used in this study are: anti-dE2F1 (1/1000) [41], anti-dE2F2 (1/1000) [66], anti-RBF1 (1/100) from Dyson Lab, anti-C3 (1/200, Cell Signaling), anti-GFP-FITC (1/200, abcam), anti-β-galactosidase (Developmental Studies Hybridoma Banks [DSHB]), and anti- ELAV (DSHB). For immunostaining, third-instar eye discs were fixed in 4% formaldehyde for 20 minutes at room temperature (eye discs immunostained for anti-dE2F1 were fixed at 4°C for 30 minutes) and washed twice with 0.3% PBST (0.3% Triton X-100 in PBS) and once with 0.1% PBST (0.1% Triton X-100 in PBS). Fixed eye discs were incubated in primary antibody with 0.1% PBST and 5% normal goat serum (NGS) at room temperature for 3 hours. After four washes with 0.1% PBST, eye discs were incubated in secondary antibody with 0.3% PBST and 5% NGS at room temperature for 2 hours. Immunostained eye discs were then washed five times with 0.1% PBST at room temperature and mounted for confocal microscopy imaging (Zeiss LSM).

**BrdU Labelling**

Third instar larval eye discs were dissected in room temperature Schneider's medium and subsequently incubated in 0.2 mg/ml BrdU (in Schneider's medium) for 1 hour at room temperature. Eye discs were washed once for 5 minutes in Schneider's and twice on ice in PBS. Eye discs were fixed in paraformaldehyde

(4%) on ice for 30 minutes. After fixation, eye discs were washed twice in 0.3% PBST (0.3% Triton X-100 in PBS) for 5 minutes each at room temperature then proceeded to dehydration in 100% methanol for 20 minutes on ice. Eye discs were rehydrated by sequential treatments with 7:3, 1:1, and 3:7 of methanol to 0.3% PBST at room temperature for 5 minutes each. Rehydrated eye discs were washed twice in 0.3% PBST for 5 minutes each then incubated in 2N HCl (diluted in 0.1% Triton X-100 in PBS) for 30 minutes at room temperature. After two 10-minute washes in 0.1% PBST, eye discs were incubated in primary anti-BrdU (1/50) for 3 hours at room temperature. Secondary immunostaining and microscopy procedures were performed according to the steps described in Immunostaining and Microscpy.

***In situ* hybridization**

For *in situ* hybridization experiments, eye-antennal discs were prepared as described previously [57]. Anti-sense RNA probes were generated using cDNA clones LD41588, LD17578, and LD45889 for *rnrS, CycE,* and *PCNA* respectively. After hybridization, Alkaline Phosphatase conjugated anti-DIG antibodies were used to detect DIG labeled anti- sense RNA probes. For each target genes, more than 20 eye antennal discs were analyzed and the representative images were chosen to be presented.

**Immunoblotting**

40 eye discs of *tsc1* mutant and control animals were dissected and used for Western blot as previously described [41]. For proteins extracted from the S2 tissue culture cells, the antibody against the phospho-specific form of S6k (Cell

Signaling, Cat#. 9206) was used to monitor the effect of *tsc1* depletion and Rapamycin treatment.

**Real-Time Reverse Transcriptase PCR**

The average of three independent experiments of triplicate-PCR reaction is presented. Total RNA was isolated from 40 eye-antenna eye discs with RNeasy Mini kit (QIAGEN) according to manufacturer's protocol, and reverse transcribed using DyNAmo cDNA Synthesis Kit (Finnzymes) according to manufacturer's instructions. Quantitative PCR reactions were performed with DyNAmo Flash SYBR Green qPCR Kit (Finnzymes). Quantification was determined by comparative threshold cycle method (CT) on Bio-Rad CFX Manager software. Both *rp49* and *β-tubulin* were used as normalization controls in a single experiment. All primers were designed with Primer3 (Whitehead Institute for Biomedical Research primer3 shareware [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi]). Primer pairs used are:

chrb1F (AACTGCAGGCTCAGCTACG)

chrb1R (CGCTCTCGAACTCAATGAAG)

de2f12-3F (CAGCACCACCACCAAAATC)

de2f12-3R (ACTGCTAGCCGTATGCTTCTG)

de2f15-6F (TACAGCCATGACCGCAAC)

de2f15-6R (GTTCAGCGCATACGGATAGTC)

tubulin-F (ACATCCCGCCCCGTGGTC)

tubulin-R (AGAAAGCCTTGCGCCTGAACATAG)

Rp49-F (TACAGGCCCAAGATCGTGAAG)

Rp49-R (GACGCACTCTGTTGTCGATACC)

**Cloning 5'UTR and Making GFP Reporter Constructs**

5' UTRs of *de2f1-RA-α* and *-β* were amplified from clone LD36172 and *y w* cDNA that was prepared from third larval eye discs respectively using Phusion® Hi-Fidelity PCR kit (Finnzymes). 6 μl TOPO® Cloning reaction containing 5 ng of PCR product was set up according manufacturer's protocol. The reaction was allowed to incubate at room temperature for 15 minutes and transferred onto ice. 2 μl of TOPO® Cloning reaction mix (Invitrogen) was transformed with One Shot® TOP10 chemically competent *E. coli* (Invitrogen). After verification for the presence of *de2f1-RA-α* or *-β* 5'UTR insertion by sequencing and restriction digestions, LR reaction was performed using LR Clonase™ II enzyme mix kit (Invitrogen) per manufacturer's instructions. The destination vector used was pTWG (a Gateway vector by T. Murphy, The Carnegie Institution of Washington, Baltimore, MD). 5-α Competent *E. coli* cells (NEB) were used for transformation. DNAs were extracted with QIAGEN Plasmid Midi Kit (Qiagen) following manufacturer's protocol and used for injecting embryos.

**UAS-dE2F1-RA-α-GFP and UAS-dE2F1-β-GFP 5' UTR Reporter Constructs**

Cloned *de2f1-RA-α* or *de2f1-RA-β* 5' UTR sequences were placed upstream of GFP reporters, thus the GFP transcripts contained *de2f1-RA-α* or *-β* 5' UTR. Transcription of *de2f1-RA-α-GFP* and *de2f1-RA-β-GFP* was controlled by the *UAS* sequence. UAS-dE2F1-RA-α-GFP and UAS-dE2F1-RA-β-GFP constructs were expressed with GMR-driven Gal4.

**RNAi and Rapamycin Treatment of S2 Cells**

One set of S2 cells were treated with either *white* or *tsc1* double strand RNA for 4 days. Another set of S2 cells were treated with DMSO or DMSO containing rapamycin (20nM) for 16 hours. Proteins were extracted for immunoblot.

## AUTHOR CONTRIBUTIONS

I contributed the majority of the work presented in this thesis. This study was initiated and designed by Dr. Nam-Sung Moon who first found the synergistic effects between *rbf1* and *tsc1* mutations on increased level of apoptosis and ectopic S-phase entry in the *Drosophila* eye disc cells. Works shown in Figures 2-5 and Figures 7-10 were done by me. Dr. Moon contributed Figure 1, Figure 6, and parts D and E of Figure 10. The fly line *FRT82B de2f1*$^{729}$ *tsc1*$^{f01910}$ was generated and provided by Brandon N. Nicolay and Maxim V. Frolov of University of Illinois Chicago that made Figure 6 possible. The French version of my thesis abstract was translated by Jeremy Magescas and edited by Jeff Boisclair-Lachance.

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REFERENCES

1. Mihara K, Cao XR, Yen A, Chandler S, Driscoll B, et al. (1989) Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. Science 246: 1300-1303.

2. Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR (1991) The E2F transcription factor is a cellular target for the RB protein. Cell 65: 1053-1061.

3. Mudryj M, Devoto SH, Hiebert SW, Hunter T, Pines J, Nevins JR (1991) Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. Cell 65: 1243-1253.

4. Chen HZ, Tsai SY, Leone G (2009) Emerging roles of E2Fs in cancer: an exit from cell cycle control. Nat Rev Cancer 9: 785-797.

5. Gao X, Zhang Y, Arrazola P, Hino O, Kobayashi T, et al. (2002) Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. Nat Cell Biol 4: 699-704.

6. Miron M, Lasko P, Sonenberg N (2003) Signaling from Akt to FRAP/TOR target both 4E-BP and S6K in *Drosophila melanogaster*. Mol Cell Biol 23: 9117-9126.

7. Zhang H, Stallock JP, Ng JC, Reinhard C, Neufeld TP (2000) Regulation of cellular growth by the *Drosophila* target of rapamycin dTOR. Genes Dev. 14: 2712-2724.

8. Neufeld TP, Edgar BA (1998) Connections between growth and the cell cycle. Curr Opin Cell Biol 10: 784-790.

9. Haynie JL, Bryant PJ (1986) Development of the eye-antenna imaginal disc and morphogenesis of the adult head in *Drosophila melanogaster*. J Exp Zool 237: 293-308.

10. Cagan RL, Ready DF (1989) The emergence of the order in the *Drosophila* pupal retina. Dev Biol 136: 346-362.

11. Harris WA, Stark WS, Walker JA (1976) Genetic dissection of the photoreceptor system in the compound eye of *Drosophila melanogaster*. J Physiol 256: 415-439.

12. Ready DF, Hanson TE, Benzer S (1976) Development of the *Drosophila* retina, a neurocrystalline lattice. Dev Biol 53: 217-240.

13. Wolken JJ, Capenos J, Turano A (1957) Photoreceptor Structure. III. *Drosophila melanogaster*. J Biophys Biochem Cytol 3: 441-448.

14. Koch C, Laurent G (1999) Complexity and the nervous system. Science 284: 96-98.

15. Gong Q, Rangarajan R, Seeger M, Gaul U (1999) The Netrin receptor Frazzled is required in the target for establishment of retinal projections in the *Drosophila* visual system. Development 126: 1451-1456.

16. Chan LN, Gehring W (1971) Determination of blastoderm of cells in *Drosophila melanogaster*. Proc Nat Acad Sci USA 68: 2217-2221.

17. Becker HJ (1957) Über roentgenmosaikflecken und defektmuationen am auge von *Drosophila* und die entwicklungsphysiologie des auges. Z indukt abstamm vererbrung 88: 333-373.

18. Horsfield J, Penton A, Secombe J, Hoffman FM, Richardson H (1998) *decapentaplegic* is required for arrest in G1phase during *Drosophila* eye development. Development 125: 5069-5078.

19. Wolff T, Ready DF (1991) The beginning of pattern formation in the *Drosophila* compound eye: the morphogenetic furrow and the second mitotic wave. Development 113: 841-850.

20. Tomlinson A (1985) The cellular dynamics of pattern formation in the eye of *Drosophila*. J Embryol Exp Morph 89: 313-331.

21. Tomlinson A, Struhl G (1999) Decoding vectorial information from a gradient: sequential roles of the receptors Frizzled and Notch in establishing planar polarity in the *Drosophila* eye. Development 126: 5725-5738.

22. Dominguez M, Wasserman JD, Freeman M (1998) Multiple functions of the EGF receptor in *Drosophila* eye development. Curr Biol 8: 1039-1048.

23. Begemann G, Michon A-M, van der Voorn L, Wepf R, Mlodzik M (1995) The *Drosophila* orphan nuclear receptor Seven-up requires the Ras pathway for its function in photoreceptor determination. Development 121: 225-235.

24. Freeman M (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. Cell 87**:** 651–660.

25. Greenwood S, Struhl G (1999) Progression of the morphogenetic furrow in the *Drosophila* eye: the roles of *hedgehog*, *decapentaplegic* and the *raf* pathway. Development 126: 5795-5808.

26. Freeman M (1997) Cell determination strategies in the *Drosophila* eye. Development 124: 261-270.

27. Freeman M, Klambt C, Goodman CS, Rubin GM (1992) The *argos* gene encodes a diffusible factor that regulates cell fate decisions in the *Drosophila* eye. Cell 69: 963-975.

28. Blackman RK, Sanicola M, Raftery LA, Gillevet T, Gelbart WM (1991) An extensive 3' cis-regulatory region directs the imaginal disk expression of Decapentaplegic, a member of the TGF-beta family in *Drosophila*. Development 111: 657-666.

29. Jarman AP, Grell EH, Ackerman L, Jan LY, Jan YN (1994) *atonal* is the proneural gene for *Drosophila* photoreceptors. Nature 369: 398-400.

30. Tanya Wolff (2007) Dissection techniques for larval and pupal *Drosophila* eyes. Cold Spring Harb Protoc doi: 10.1101/pdb.prot4715

31. Dyson N (1998) The regulation of E2F by pRB-family proteins. Genes Dev 12: 2245-2262.

32. Polager S, Ginsberg D (2008) E2F - at the crossroads of life and death. Trends Cell Biol 18: 528-535.

33. van den Heuvel S, Dyson NJ (2008) Conserved functions of the pRB and E2F families. Nat Rev Mol Cell Biol 9: 713-724.

34. Sutcliffe JE, Korenjak M, Brehm A (2003) Tumour suppressors – a fly's perspective. Eur J Cancer 39: 1355-1362.

35. Muller H, Bracken AP, Vernell R, Moroni MC, Christians F, et al. (2001) E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev 15: 267-285.

36. Dimova DK, Stevaux O, Frolov MV, Dyson NJ (2003) Cell cycle-dependent and cell cycle-independent control of transcription by the Drosophila E2F/RB pathway. Genes Dev 17: 2308-2320.

37. Ishida S, Huang E, Zuzan H, Spang R, Leone G, et al. (2001) Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. Mol Cell Biol 21: 4684-4699.

38. Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, et al. (1992) Effects of an Rb mutation in the mouse. Nature 359: 295-300.

39. Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van der Valk M, et al. (1992) Requirement for a functional Rb-1 gene in murine development. Nature 359: 328-330.

40. Lee EY, Chang CY, Hu N, Wang YC, Lai CC, et al. (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature 359: 288-294.

41. Moon NS, Di Stefano L, Dyson N (2006) A gradient of epidermal growth factor receptor signaling determines the sensitivity of rbf1 mutant cells to E2F-dependent apoptosis. Mol Cell Biol 26: 7601-7615.

42. Tsai KY, Hu Y, Macleod KF, Crowley D, Yamasaki L, et al. (1998) Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb- deficient mouse embryos. Mol Cell 2: 293-304.

43. Ziebold U, Reza T, Caron A, Lees JA (2001) E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos. Genes Dev 15: 386- 391.

44. Sherr CJ (1996) Cancer cell cycles. Science 274: 1672-1677.

45. Harrison DJ, Hooper ML, Armstrong JF, Clarke AR (1995) Effects of heterozygosity for the Rb-1t19neo allele in the mouse. Oncogene 10: 1615-1620.

46. Hu N, Gutsmann A, Herbert DC, Bradley A, Lee WH, et al. (1994) Heterozygous Rb-1 delta 20/+mice are predisposed to tumors of the pituitary gland with a nearly complete penetrance. Oncogene 9: 1021-1027.

47. Du W, Dyson N (1999) The role of RBF in the introduction of G1 regulation during Drosophila embryogenesis. EMBO J 18: 916-925.

48. Duronio RJ, O'Farrell PH, Xie JE, Brook A, Dyson N (1995) The transcription factor E2F is required for S phase during Drosophila embryogenesis. Genes Dev 9: 1445-1455.

49. MacPherson D, Sage J, Kim T, Ho D, McLaughlin ME, et al. (2004) Cell type-specific effects of Rb deletion in the murine retina. Genes Dev 18: 1681-1694.

50. Robanus-Maandag E, Dekker M, van der Valk M, Carrozza ML, Jeanny JC, et al. (1998) p107 is a suppressor of retinoblastoma development in pRb-deficient mice. Genes Dev 12: 1599-1609.

51. Chen D, Livne-bar I, Vanderluit JL, Slack RS, Agochiya M, et al. (2004) Cell-specific effects of RB or RB/p107 loss on retinal development implicate an intrinsically death- resistant cell-of-origin in retinoblastoma. Cancer Cell 5: 539-551.

52. Zhang J, Schweers B, Dyer MA (2004) The first knockout mouse model of retinoblastoma. Cell Cycle 3: 952-959.

53. Yamasaki L, Bronson R, Williams BO, Dyson NJ, Harlow E, et al. (1998) Loss of E2F-1 reduces tumorigenesis and extends the lifespan of Rb1(+/-)mice. Nat Genet 18: 360- 364.

54. Ziebold U, Lee EY, Bronson RT, Lees JA (2003) E2F3 loss has opposing effects on different pRB-deficient tumors, resulting in suppression of pituitary tumors but metastasis of medullary thyroid carcinomas. Mol Cell Biol 23: 6542-6552.

55. Stevaux O, Dyson NJ (2002) A revised picture of the E2F transcriptional network and RB function. Curr Opin Cell Biol 14: 684-691.

56. Tsai SY, Opavsky R, Sharma N, Wu L, Naidu S, et al. (2008) Mouse development with a single E2F activator. Nature 454: 1137-1141.

57. Du W (2000) Suppression of the *rbf* null mutants by a *de2f1* allele that lacks transactivation domain. Development 127: 367-379.

58. Nicolay BN, Frolov MV (2008) Context-dependent requirement for dE2F during oncogenic proliferation. PLoS Genet 4: e1000205.

59. Nicholson SC, Gilbert MM, Nicolay BN, Frolov MV, Moberg KH (2009) The archipelago tumor suppressor gene limits rb/e2f-regulated apoptosis in developing Drosophila tissues. Curr Biol 19: 1503-1510.

60. Moon NS, Di Stefano L, Morris EJ, Patel R, White K, et al. (2008) E2F and p53 induce apoptosis independently during Drosophila development but intersect in the context of DNA damage. PLoS Genet 4: e1000153.

61. Pan D, Dong J, Zhang Y, Gao X (2004) Tuberous sclerosis complex: from Drosophila to human disease. Trends Cell Biol 14: 78-85.

62. Inoki K, Corradetti MN, Guan KL (2005) Dysregulation of the TSC-mTOR pathway in human disease. Nat Genet 37: 19-24.

63. Ito N, Rubin GM (1999) *gigas*, a *Drosophila* homolog of tuberous sclerosis gene product-2, regulates the cell cycle. Cell 96: 529-539.

64. Gao X, Pan D (2001) *TSC1* and *TSC2* tumor suppressors antagonize insulin signaling in cell growth. Genes Dev 15: 1383-1392.

65. Potter CJ, Huang H, Xu T (2001) *Drosophila Tsc1* functions with *Tsc2* to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. Cell 105: 357-368.

66. Tapon N, Ito N, Dickson BJ, Treisman JE, Hariharan IK (2001) The *Drosophila* tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. Cell 105: 345-355.

67. Soucek T, Pusch O, Weinecke R, DeClue JE, Hengstschlager M (1997) Role of the tuberous sclerosis gene-2 product in cell cycle control. Loss of the tuberous sclerosis gene-2 induces quiescent cells to enter S phase. J Biol Chem 272: 29301-29308.

68. Xiao GH, Shoarinejad F, Jin F, Golemis EA, Yeung RS (1997) The tuberous sclerosis 2 gene product, tuberin, functions as a Rab5 GTPase activating protein (GAP) in modulating endocytosis. J Biol Chem 272: 6097-6100.

69. Lamb RF, Roy C, Diefenbach TJ, Vinters HV, Johnson MW, et al. (2000) The TSC1 tumour suppressor harmartin regulates cell adhesion through ERM proteins and the GTPase Rho. Nat Cell Biol 2: E76-78.

70. Henry KW, Yuan X, Koszewski NJ, Onda H, Kwiatkowski DJ, et al. (1998) Tuberous sclerosis gene 2 product modulates transcription mediated by steroid hormone receptor family members. J Biol Chem 273: 20535-20539.

71. Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, et al. (2003) Rheb is a direct target of the tuberous sclerosis tumours uppressor proteins. Nat Cell Biol 5: 578-581.

72. Long X, Lin Y, Ortiz-Vega S, Yonezawa K, Avruch J (2005) Rheb binds and regulates the mTOR kinase. Curr Biol 15: 702-713.

73. Heitman J, Movva NR, Hall MN (1991) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science 253: 905–909.

74. Jacinto E, Hall MN (2003) TOR signalling in bugs, brain and brawn. Nat Rev Mol Cell Biol 4: 117-126.

75. Inoki K, Corradetti MN, Guan K-L (2005) Dysregulation of the TSC-mTOR pathway in human disease. Nat Genet 37: 19-24.

76. Loewith R, Jacinto E, Wullschleger S, Sorberg A, Crespo J, et al. (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell 10: 457-468.

77. Sonenberg N, Hinnebusch AG (2009) Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell 136: 731-745.

78. Wullschleger S, Loewith R, Hall MN (2006) TOR signalling in growth and metabolism. Cell 124: 471-484.

79. Denchi EL, Helin K (2005) E2F1 is crucial for E2F-dependent apoptosis. EMBO 6: 661-668.

80. Thacker SA, Bonnette PC, Duronio RJ (2003) The contribution of E2F-regulated transcription to *Drosophila PCNA* Gene function. Curr Biol 13: 53-58.

81. DeGregori J, Kowalik T, Nevins JR (1995) Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1-S-regulatory genes. Mol Cell Biol 15: 4215-4224.

82. Royzman I, Whittaker AJ, Orr-Weaver TL (1997) Mutations in *Drosophila* DP and E2F distinguish G1-S progression from an associated transcriptional program. Genes Dev 11: 1999-2011.

83. Novotny GW, Sonne SB, Nielsen JE, Jonstrup SP, Hansen MA, et al. (2007) Translational repression of E2F1 mRNA in carcinoma *in situ* and normal testis correlates with expression of the miR-17-92 cluster. Cell Death Differ 14: 879-882.

84. Harvey KF, Mattila J, Sofer A, Bennett FC, Ramsey MR, et al. (2008) FOXO-regulated transcription restricts overgrowth of Tsc mutant organs. J Cell Biol 180: 691-696.

85. Frolov MV, Moon NS, Dyson NJ (2005) dDP is needed for normal cell proliferation. Mol Cell Biol 25: 3027-3039.

86. Tanaka-Matakatsu M, Xu J, Cheng L, Du W (2009) Regulation of apoptosis of *rbf* mutant cells during Drosophila development. Dev Biol 326: 347-356.

87. Sawado T, Hirose F, Takahashi Y, Sasaki T, Shinomiya T, et al. (1998) The DNA replication-related element (DRE)/DRE-binding factor system is a transcriptional regulator of the *Drosophila E2F* gene. Jour Biol Chem 273: 26042-26051.

88. Shibutani ST, de la Cruz AFA, Tran V, Turbyfill III WJ, Reis T, et al. (2008) Intrinsic negative cell cycle regulation provided by PIP box-and cul4$^{cdt2}$-mediated

destruction of E2f1 during S phase. Dev Cell 15: 890-900.

89. Britton JS, Edgar BA (1998) Environmental control of the cell cycle in Drosophila: nutrition activates mitotic and endoreplicative cells by distinct mechanisms. Development 125: 2149-2158.

90. Neufeld TP, de la Cruz AF, Johnston LA, Edgar BA (1998) Coordination of growth and cell division in the Drosophila wing. Cell 93: 1183-1193.

91. Shibutani ST, de la Cruz AF, Tran V, Turbyfill WJ, 3rd, Reis T, et al. (2008) Intrinsic negative cell cycle regulation provided by PIP box- and Cul4Cdt2-mediated destruction of E2f1 during S phase. Dev Cell 15: 890-900.

92. Ruvinsky I, Meyuhas O (2006) Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. Trends Biochem Sci 31: 342-348.

93. Campanero MR, Flemington EK (1997) Regulation of E2F through ubiquitin-proteasome-depdendent degradation: stabilization by the pRB tumour suppressor protein. Proc Natl Acad Sci USA 94: 2221-2226.

94. Pende M, Um SH, Mieulet V, Sticker M, Goss VL, et al. (2004) S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. Mol Cell Biol 24: 3112-3124.

95. McNeill H, Craig GM, Bateman JM (2008) Regulation of neurogenesis and epidermal growth factor receptor signaling by the insulin receptor/target of rapamycin pathway in *Drosophila*. Genetics 179: 843-853.

96. Orlic M, Spencer CE, Wang L, Gallie BL (2006) Expression analysis of 6p22 genomic gain in retinoblastoma. Genes Chromosomes Cancer 45: 72-82.

97. Sasaki T, Sawado T, Yamaguchi M, Shinomiya T (1999) Specification of regions of DNA replication initiation during embryogenesis in the 65-kilobase DNApolalpha-dE2F locus of *Drosophila melanogaster*. Mol Cell Biol 19: 547-555.

98. Neal SJ, Gibson ML, So AK, Westwood JT (2003) Construction of a cDNA-based microarray for *Drosophila melanogaster*: a comparison of gene transcription profiles from SL2 and Kc167 cells. Genome 46: 879-892.

# APPENDIX

*de2f1-RA-α* Sequence (5'-3')

CACACACATGTGCGAGCAGAAAACACGTTACGAG
GGGAAAATCGACGGAAAAATCCGAAAAAAAATAG
AAAAGCTAACTGAAAAGAGTACAAGACTGAG

*de2f1-RA-β* Sequence (5'-3')

CAAAAAGCCGCAAAAGACTGCCTCTGCAAGTAAA
AGATTCAAGTCGCTGAGTAGCAATAGCAAATGCTC
TAAAAAATCAAGAATAAAATAAAATAAAAGTGCG
GGCGTGTAACAATTATGTGATATCTACAACAAATG
CTCGAACTGCTGACGTCACGCACAGAATCGTAGAA
TATATAGAGAATAAAGAAGAGAAACGAAAACGTC
GCGTAGTAAAGTTAAAAGTTCCCACACCACCGACG
TACTCACACTCGCACACACACAGACTGAGTCAAAC
GAGTAAGAAGCAGCAGCACATTATACGTGGAATA
CAAAAAGTAACCGACAGAAAAGGCAAAACGCCCG
CAAAAAAATGAAAAATTTGCAAATATGAAACGTGT
GTGATATTCAAAAAGAGCAAGAAGAAGCAGGCG
GCGGTGGAAAAAAAAGCAGAGCAATTTGTTGTGG
TTGCGTGTGATATATAGAAAGCAAGTAAAACCAAC
AAAAATTCCATTTTTAG