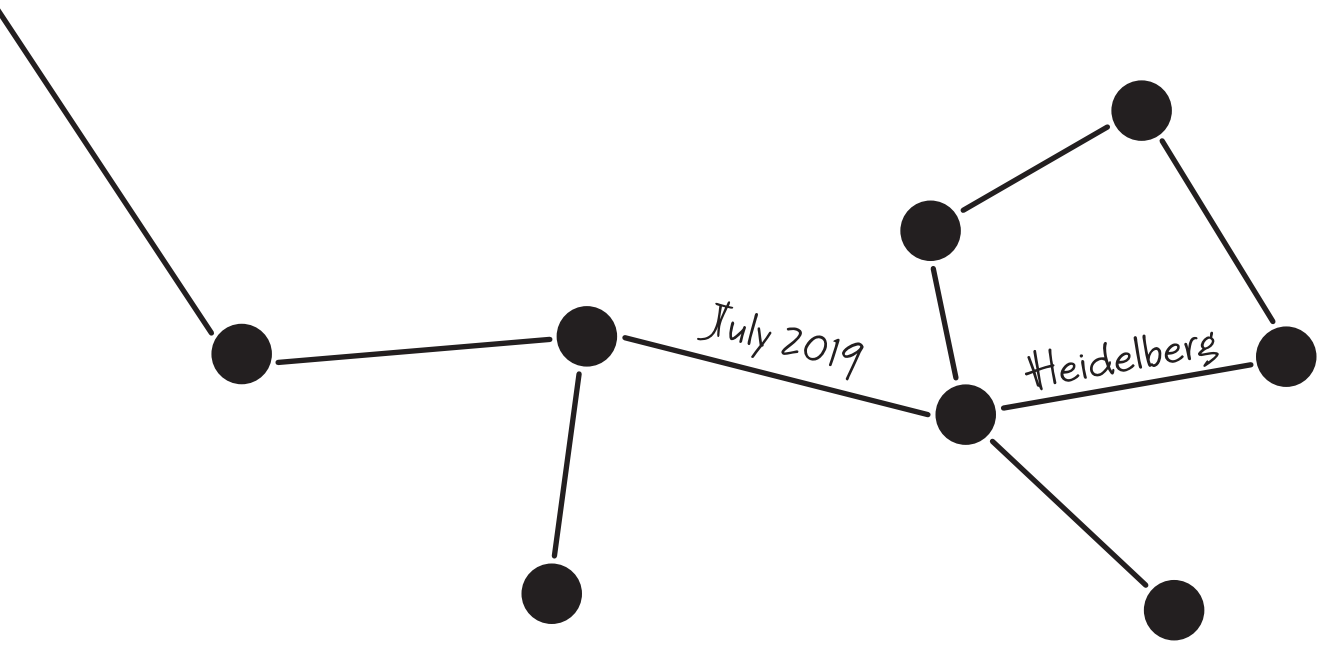
DOCTORAL THESIS

Sebastian I. Florescu



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Ras Mapk Growth Effectors in Drosophila

presented by
M.Sc. Sebastian I. Florescu
born in: Targoviste, Romania

Referees: Prof. Dr. Bruce A. Edgar

Prof. Dr. Ursula Klingmüller

Prof. Dr. Ingrid Lohmann

CONTENTS

Summary 1

Zusammenfassung 3

i. introduction

I.1 Growth Control and Growth Pathways 5

General Growth Control 5

Growth Pathways 5

I.2 Ras-Mapk Pathway 8

Mapk Pathways 8

Activation Mechanisms and Inhibitors 8

Substrates and Biological Functions 9

I.3 Mapk Activated Protein Kinases 14

The Mapk Interacting Kinase, Mnk 14

The p90 Ribosomal S6 Kinase, Rsk 15

Mitogen and Stress Activated Kinase, Msk 17

I.4 Ras-Mapk Promotes Growth in Drosophila 20

Ras Promotes Growth in Imaginal Discs 20

Active Ras Drives Growth through Myc and Pi3k 20

Mapk Phosphorylation is Required for Cell Growth 21

Insulin Activates Erk Mapk and Promotes Cell Growth 21

p38 Mapk Promotes Cell Growth via ToRC1 21

Pvr Activates Erk Mapk and Promotes Cell Growth 21

Ras-Mapk Drives Proliferation of Adult Midgut Progenitors 22
Ras-Mapk Drives Growth and Proliferation in the Adult Midgut 22
I.5 Ras-Mapk Promotes Cancerous Growth 24
Mapk Drives Growth and Alters Cell Identities 24
Oncogenic Cooperation 24
Inflammation and EMT 25
Jnk-Ras Duo Driving Tumorigenesis 25
Screening Platform for Anticancer Agents 26
Ras on the Brain 26
I.6 ToR Pathway 27
The ToR Pathway 27
Upstream Regulators of ToRC1 27
Cellular Processes Downstream of ToRC1 29
The ToRC2 Signaling Network 30
I.7 Mammalian and Drosophila Intestine 34
Intestinal Architecture and Cell Types 34
Intestinal Signaling Pathways 37
Mammalian Intestine 37
Notch Pathway 37
Wingless Pathway 38
Jak/Stat Pathway 38
Ras-Mapk Pathway 39
Jnk Pathway 40
Hippo Pathway 41
Redox Regulation 42
Additional Pathways 42

I.8 Aim of Study 44

ii. results

II.1 New Mapk Growth Effectors in Insect Cells 45

Mapk Activity is Required for Normal Insect Cell Growth 45

Mapkapks are Required for Normal and Insulin induced Insect Cell Growth . 46

Mapkapks are Required for Normal and Egfr induced Insect Cell Growth ... 47

II.2 Mapk Activates ToR Downstream of RTK in Drosophila Cells 61

Mapk Activates ToRC1 in Insect Cell Culture 61

Mapk Activity is Required for Basal and GF-induced ToRC1 Activity 62

Mapk Pathway Activation does not Require ToRC1 or Mapkapk 63

II.3 Mapk Growth Effectors in Drosophila Intestine 68

Mapk Driven Growth in the Adult Midgut is Partially ToR Dependent 68

Ras-Mapk Drives Growth in the Adult Intestine also through Ets21c 69

Ras-Mapk Promotes Nutrient Independent Growth in the Larval Intestine .. 70

II.4 Mapk Growth and Autophagy Phenotypes in Fat Body 80

Ras Activation Cannot Rescue Growth Deficiency of Fat Body Cells 80

Mapk Cannot Suppress Starvation Induced Autophagy in Fat Body Tissue ... 81

iii. discussion

III.1 Mapk Implications in Growth and Cancer 86

How Does Mapk Drive Growth? 86

Proposed Model 86

Mapk Signaling Mechanisms 91

Cancer Implications 92

III.2 New Growth Effectors in Insect Cells, the Mapkapks 93

Context 93

Aim 94

New Findings 94
Significance 95
III.3 Mapkapks in Translation and Growth 97
III.4 Mapk Activates ToR in Insect Cells 100
Context 100
Aim 101
New Findings 101
Significance 103
III.5 Mapk ToR Crosstalk and Convergence 105
Mapk ToR Pathway Crosstalk 105
Mapk ToR Pathway Convergence 106
III.6 Mapk Needs ToR to Fully Promote Midgut Cell Growth 108
Context 108
Aim 109
New Findings 109
Significance 111
III.7 Mapk Bypasses Nutrition and Promotes Larval Cell Growth 113
Context 113
Aim 113
New Findings 114
III.8 Future Perspectives 116

iv. methods

IV.1 Drosophila Life Cycle and Genetics 120
IV.2 Drosophila Gal4 Gene Expression System 121
IV.3 Flp Gal4 Lineage Tracing 122
IV.4 Genotypes and Gene Induction 123

Driver Lines 123
UAS Transgenes 123
IV.5 Immunohistochemistry 124
IV.6 Cell Treatment and Protein Extraction 125
Lysis Buffer Preparation 125
Cell Seeding and Treatments 125
Cell Lysis 126
IV.7 Immunoblotting 128
Reagents and Buffers 128
Electrophoresis 128
Blotting 130
Protein Detection and Visualisation 131
IV.8 Flow Cytometry 133
Cell Sample Preparation 133
FlowJo Analysis 134

v. references

List of Referenced Publications 135-158

SUMMARY

The evolutionary conserved Erk Mapk (mitogen activated protein kinase) pathway coordinates essential cellular functions including cell survival, division, growth, motility and differentiation [9,61]. To execute such intricate functions, Erk regulates transcription factors impinging on gene expression, and a vast assortment of cytosolic and nuclear substrates coordinating other aspects of cellular metabolism. As part of the polyvalent nature of this pathway's functionality, Erk Mapk has been firmly established as a growth promoter in different contexts [3,69,70,151-153]. However, despite a vast literature, the nature of the effectors and interactions underlying Mapk-driven growth remains poorly understood. Therefore, the question that sparked this study was- how does Mapk drive growth? Does it invoke a single growth mechanism or (like other metabolic decisions) it relies on the concerted action of multiple effectors acting at different stages and/or in different developmental contexts?

Our study brings forward a model according to which Erk Mapk may promote growth in insect cells via two mechanisms. A first Mapkapk-driven mechanism (Mapk activated protein kinase) that may directly promote translation or activate other effectors (like ToR) in order to do so. And a second ToRC1-dependent mechanism (target of rapamycin complex1) which promotes biosynthetic pathways and eventually growth. ToRC1 integrates five major inputs (growth factors, amino acids, energy, stress and oxygen) and accordingly regulates anabolic pathways (like protein and lipid synthesis) as well as catabolic pathways (like autophagy) [154]. Our study supports a ToR-dependent mechanism as we learned that in cultured insect cells, Ras-Mapk appears to be sufficient and required for ToRC1 activation (II-2, III-4/5), while in the animal's intestine Ras-Mapk depends on ToRC1 activity to fully promote growth (II-3, III-6). Furthermore, we found that Ras-Mapk activation in the developing intestine acts as a potent growth and proliferation promoter, even under conditions of protein starvation (II-4, III-6)—a phenotype previously attributed to ToRC1 [47]. Consistently, both Erk and one of its targets (Rsk) were found to positively regulate ToRC1 in mammalian cells [30-36]. As mentioned, Mapk pathways are firmly wired into the cell's metabolic framework by phosphorylating a varied assortment of target proteins in the nucleus as in the cytoplasm. Among these substrates are the Mapkapks [10,11]. Our study also supports a Mapkapk-dependent growth mechanism, as our in vitro assays reveal that three Mapkapks (Mnk, Rsk, Msk) are required for insect cell growth under normal and growth factor stimulated conditions (II-1, III-2/3). Furthermore, mammalian studies have attributed a significant extent of Mapk functionalities to the activation of downstream Mapkapks (III-3).

There is hardly any cellular stimulus that doesn't feed into Mapk and ToR pathways. It is easy to see how connecting them would be advantageous not only for tissue homeostasis and regeneration but also for keeping developmental and metabolic decisions in sync. Mapk and ToR pathways are often hijacked by different cancers to initiate and grow tumors, and eventually metastasize. Dysregulation of Mapkapks is also associated with multiple human diseases including cancer (I-3). The ability of Ras-Mapk to drive growth and initiate tumors has been exploited in designing fly-based screening platforms for potential anticancer agents (I-5). The significance of our study and others is therefore far reaching, not only for understanding of how cells integrate multiple inputs to grow and dynamically coordinate developmental with metabolic decisions, but also towards designing more effective therapies targeting tumor growth.

ZUSAMMENFASSUNG

Der evolutionär konservierte Erk Mapk-Weg koordiniert wesentliche zelluläre Funktionen, einschließlich Zellüberleben, Teilung, Wachstum, Motilität und Differenzierung [9,61]. Um solche komplizierten Funktionen auszuführen, regelt Erk Transkriptionsfaktoren, die auf die Genexpression auftreffen, und eine ausgedehnte Auswahl an zytosolischen und nuklearen Substraten, die andere Aspekte des Zellstoffwechsels koordinieren. Als Teil der polyvalenten Natur dieser Signalweg-Funktionalität ist Erk Mapk als Wachstumsförderer in unterschiedlichen Kontexten fest etabliert [3,69,70,151-153]. Trotz einer umfangreichen Literatur bleibt die Art der Effektoren und Interaktionen, die dem Mapk-getriebenen Wachstum zugrunde liegen, schlecht verstanden. Daher war die Frage, die diese Studie ausgelöst hat: Wie funktioniert Mapk Wachstum? Gibt es einen einzigen Wachstumsmechanismus an oder (wie andere metabolische Entscheidungen) beruht er auf der konzertierten Aktion mehrerer Effektoren, die auf verschiedenen Stufen und/oder in unterschiedlichen Entwicklungskontexten handeln?

Unsere Studie bringt ein Modell vor, nach dem Erk Mapk das Wachstum von Insektenzellen über zwei Mechanismen fördern kann. Ein erster Mapkapk-getriebener Mechanismus (Mapk-aktivierte Proteinkinase), der die Translation direkt fördern oder andere Effektoren (wie ToR) aktivieren kann, um dies zu tun. Und ein zweiter ToRC1-abhängiger Mechanismus (Target/Ziel von Rapamycin-Komplex1), der Biosynthesewege und schließlich Wachstum fördert. ToRC1 integriert fünf wichtige Inputs (Wachstumsfaktoren, Aminosäuren, Energie, Stress und Sauerstoff) und reguliert dementsprechend anabole Wege (wie Protein- und Lipidsynthese) sowie katabolische Wege (wie Autophagie) [154]. Unsere Studie unterstützt einen ToR-abhängigen Mechanismus, wie wir gelernt haben, dass in kultivierten Insektenzellen Ras-Mapk für die ToRC1-Aktivierung (II-2, III-4/5) ausreichend und erforderlich ist, während im Tierdarm Ras-Mapk davon abhängt Auf die Aktivität von ToRC1, um das Wachstum vollständig zu fördern (II-3, III-6). Darüber hinaus haben wir festgestellt, dass die Ras-Mapk-Aktivierung im Entwicklungsdarm auch unter den Bedingungen des Proteinverhungers (II-4, III-6) als potenter Wachstums- und Proliferations-Promotor wirkt - ein Phänotyp, der zuvor ToRC1 zugeschrieben wurde [47]. Konsequent wurden sowohl Erk als auch eine der Erk-Targets (Rsk) gefunden, um ToRC1 in Säugetierzellen positiv zu regulieren [30-36]. Wie bereits erwähnt, sind Mapk-Wege fest in das metabolische Rahmen der Zelle eingebunden, indem sie eine abwechslungsreiche Sortierung von Zielproteinen im Zellkern wie im Zytoplasma phosphorylieren. Unter diesen Substraten sind die Mapkapk [10,11]. Unsere Studie unterstützt auch einen Mapkapk-abhängigen Wachstumsmechanismus, da unsere in vitro Assays zeigen,

dass drei Mapkapks (Mnk, Rsk, Msk) für das Insektenzellwachstum unter normalen und Wachstumsfaktor stimulierten Bedingungen erforderlich sind (II-1, III-2/3)). Darüber hinaus haben Säugetierstudien ein signifikantes Ausmaß der Mapk Funktionalitäten zur Aktivierung von Downstream Mapkapks (III-3) zugeschrieben.

Es gibt kaum einen zellulären Stimulus, der sich nicht in Mapk- und ToR-Wege einbringt. Es ist leicht zu sehen, wie ihre Verbindung nicht nur für die Gewebshomöostase und die Regeneration vorteilhaft wäre, sondern auch für die Entwicklung von Entwicklungs- und Stoffwechselentscheidungen. Mapk- und ToR-Wege werden oft von verschiedenen Krebsarten entführt, um Tumore zu initiieren und zu wachsen und schließlich zu metastasieren. Dysregulation von Mapkapks ist auch mit mehreren menschlichen Krankheiten einschließlich Krebs (I-3) assoziiert. Die Fähigkeit von Ras-Mapk, das Wachstum zu fördern und Tumore zu initiieren, wurde bei der Gestaltung von Fliegen-basierten Screening-Plattformen für potentielle Antikrebsmittel (I-5) ausgenutzt. Die Bedeutung unserer Studie und anderer ist daher weitreichend, nicht nur für das Verständnis davon, wie die Zellen mehrere Inputs integrieren, um die Entwicklung mit metabolischen Entscheidungen zu wachsen und dynamisch zu koordinieren, sondern auch auf die Entwicklung effektiverer Therapien, die auf das Tumorwachstum abzielen.

I. INTRODUCTION

I-1 GROWTH CONTROL AND GROWTH PATHWAYS

General Growth Control

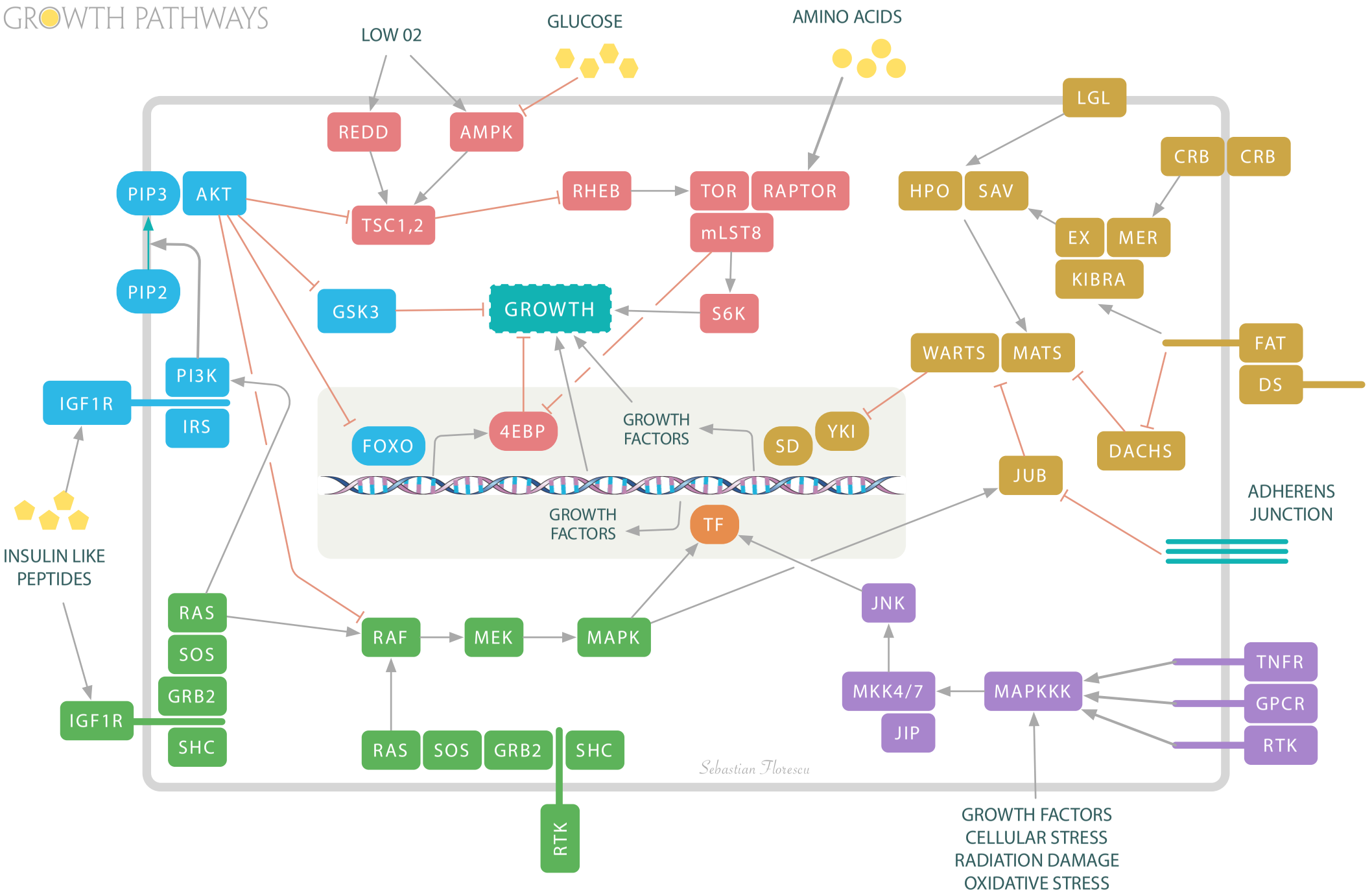
In unicellular organisms, cell growth and proliferation are mainly determined by the nutritional content of the surrounding medium. In multicellular organisms, however, growth, proliferation and survival are differentially regulated in different tissues. This is achieved by providing a relatively constant supply of nutrients through a circulatory system, and an assortment of signals instructive for cell growth, proliferation and survival. Cell growth [mass accumulation] and cell proliferation (cell division) are separable processes controlled through separate mechanisms. A cell can grow without dividing, like a postmitotic neurone, or divide without growing, like a fertilised egg undergoing cleavage divisions. Cell cycle progression tends to be a all-or-none process triggered by a certain threshold of mitogenic signaling. In contrast, most cells, whether in or out from the cycle, must maintain a constant balance between their anabolic [biosynthetic] and catabolic (degradative) pathways to maintain their biological function. A cell's size and growth rate is determined by a balance between buildup of macromolecules (through biosynthesis or uptake) and their loss (through degradation or secretion), which is dynamically controlled by changing levels of growth factor signaling.

Growth Pathways

One major regulator of cell growth and cell size is the IGF/Pi3k/Akt/ToRC1 pathway. IGF is a limiting growth factor acting both systemically (overexpression during development leads to larger animals) and locally (overexpression in the adult leads to cell hypertrophy). The key growth-regulating pathway activated downstream of IGF is Pi3k/Akt/ToRC1. ToRC1 is a major signaling hub receiving inputs from at least four major cues - amino acid levels, stress, energy and oxygen - and integrating these with signals from several pathways. Through this concerted action, ToRC1 activity promotes multiple biogenic processes, including nutrient uptake and protein and lipid synthesis, and also inhibits catabolic processes such as autophagy [154]. An-

other positive regulator of cell growth and cell size is the transcription factor Myc. Myc promotes biogenic pathways by increasing ribosomal RNA levels, nucleolar size, protein biogenesis, and the metabolic reprogramming required for cell growth [203,224,225]. Importantly, Myc and Pi3k differentially activate biogenic pathways, with Myc driving and increase in cell protein and ribosome content, while Pi3k strongly stimulates lipogenesis [224]. The Hippo pathway is another regulator of tissue/organ size, controlling cell proliferation and apoptosis, and thereby cell number [226]. The main downstream effector of this pathway is the transcriptional co-activator Yap, which functions to promote cell survival and proliferation. Hippo controls production of cells and sustains a certain level of mass likely through integration with biogenic pathways. Accordingly, Hippo has been found to crosstalk in drosophila and mammals with ToR signaling (with Yap activating ToR by decreasing Pten levels) and with Myc [226-228].

The 'Growth Pathways' figure (next page) illustrates the main pathways promoting growth in drosophila as in mammals. They function as part of an interconnected network being employed differently in different cell types and developmental stages. They are hardwired into the cell's metabolism which is constantly sustained by many millions of reactions occurring every second.


GROWTH PATHWAYS
LOW 02
GLUCOSE
AMINO ACIDS
REDD
AMPK
PIP3
AKT
TSC1,2
RHEB
TOR
RAPTOR
mLST8
PIP2
GSK3
GROWTH
S6K
PI3K
IRS
IGF1R
FOXO
4EBP
GROWTH FACTORS
SD
YKI
GROWTH FACTORS
TF
INSULIN LIKE PEPTIDES
RAS
SOS
GRB2
SHC
IGF1R
RAF
MEK
MAPK
RAS
SOS
GRB2
SHC
RTK
LGL
HPO
SAV
CRB
CRB
EX
MER
KIBRA
FAT
DS
WARTS
MATS
DACHS
JUB
ADHERENS JUNCTION
JNK
MKK4/7
JIP
MAPKKK
TNFR
GPCR
RTK
GROWTH FACTORS
CELLULAR STRESS
RADIATION DAMAGE
OXIDATIVE STRESS
Sebastian Floreseu

I-2 RAS-MAPK PATHWAY

Mapk Pathways

Mitogen activated protein kinase (Mapk) pathways are among the most ancient signaling pathways. All eukaryotic cells have multiple Mapk pathways that convert extracellular stimuli into a wide range of cellular responses, including survival, growth, proliferation, motility and differentiation. 14 Mapks have been characterised so far into 7 groups, though the most studied among these are the Erk, p38 and Jnk pathways. They are also conserved between mammals and drosophila. These conventional Mapk pathways comprise a cascade of sequentially acting kinases: a Mapk (Ser/Thr kinase), a Mapkk (a Thr/Tyr kinase) and a Mapkkk (a Ser/Thr kinase). Extracellular stimuli lead to Mapkkk activation as a result of its phosphorylation or interaction with a GTP-binding protein of the Ras/Rho family. The Mapkkk then phosphorylates and activates the Mapkk. The Mapkk in turn phosphorylates and activates the Mapk at a conserved Thr-X-Tyr motif located in the activation loop of the kinase domain [229,230].

Activation Mechanisms and Inhibitors

The Erk Mapk pathway responds to growth factors, including epidermal growth factor (EGF), platelet derived growth factor (PDGF) and nerve growth factor (NGF), and in response to insulin [232]. The Erk pathway also responds to ligands of the heterotrimeric G-protein coupled receptors (GPCRs), cytokines, osmotic stress and microtubule disorganisation [231]. In drosophila, the Egfr Mapk pathway responds to four different ligands: Gurken, Spitz, Keren and Vein. Gurken is used during embryogenesis, while the other three are employed at other developmental stages [61]. Activation of the Erk module occurs primarily through receptor tyrosine kinase receptors (RTKs). Ligand binding triggers receptor dimerisation and activation, and consequently autophosphorylation of Tyr residues in the intracellular domain [233]. These phosphorylations create new binding sites for proteins with SH2 (Src homology 2) or PTB (phosphotyrosine binding) domains, such as Grb2 (growth factor receptor bound protein 2). The primary Ras activation route occurs at the plasma membrane and is initiated by Sos [son of sevenless], a guanine nucleotide exchange factor (GEF). Once Sos is recruited to the plasma membrane via its interaction with Grb2, it stimulate Ras to exchange its bound GDP for GTP and become activated. Activated Ras can then directly interact with its targets, among which is the Mapkkk Raf. Activated Raf in turn phosphorylates and activates the Mapkk Mek,

that finally phosphorylates the Mapk Erk at Thr and Tyr residues in a conserved Thr-Glu-Tyr (TEY) motif in its activation loop.

Whereas Raf and Mek have a restricted substrate specificity, Erk targets a very broad assortment of cytoplasmic and nuclear targets to execute pathway tasks [234,235]. Multiple RTK receptors converge on this signaling cascade in drosophila as in mammals. Different RTKs do however use different adaptor proteins. For example, while Grb2 can directly bind to the Egf receptor, it requires co-adaptors for binding to other RTKs, like Dof for Fgf receptors and Shc for the insulin receptor. However, once Sos is recruited by Grb2, all pathways converge on the same signaling cascade [236].

Mapk inhibitors have been developed since the mid 1990's and extensively used to implicate Mek/Erk in diverse cellular processes. One class of such inhibitors is typified by the Mek inhibitor U0126 [237], which is not competitive with respect to ATP and appears to interact with the unphosphorylated kinase more strongly than with the phosphorylated species. This interaction prevents Mek phosphorylation and/or the conformational transition required for its activation [238]. Additional noncompetitive inhibitors were developed through the years and have entered clinical trials as potential anticancer agents [80].

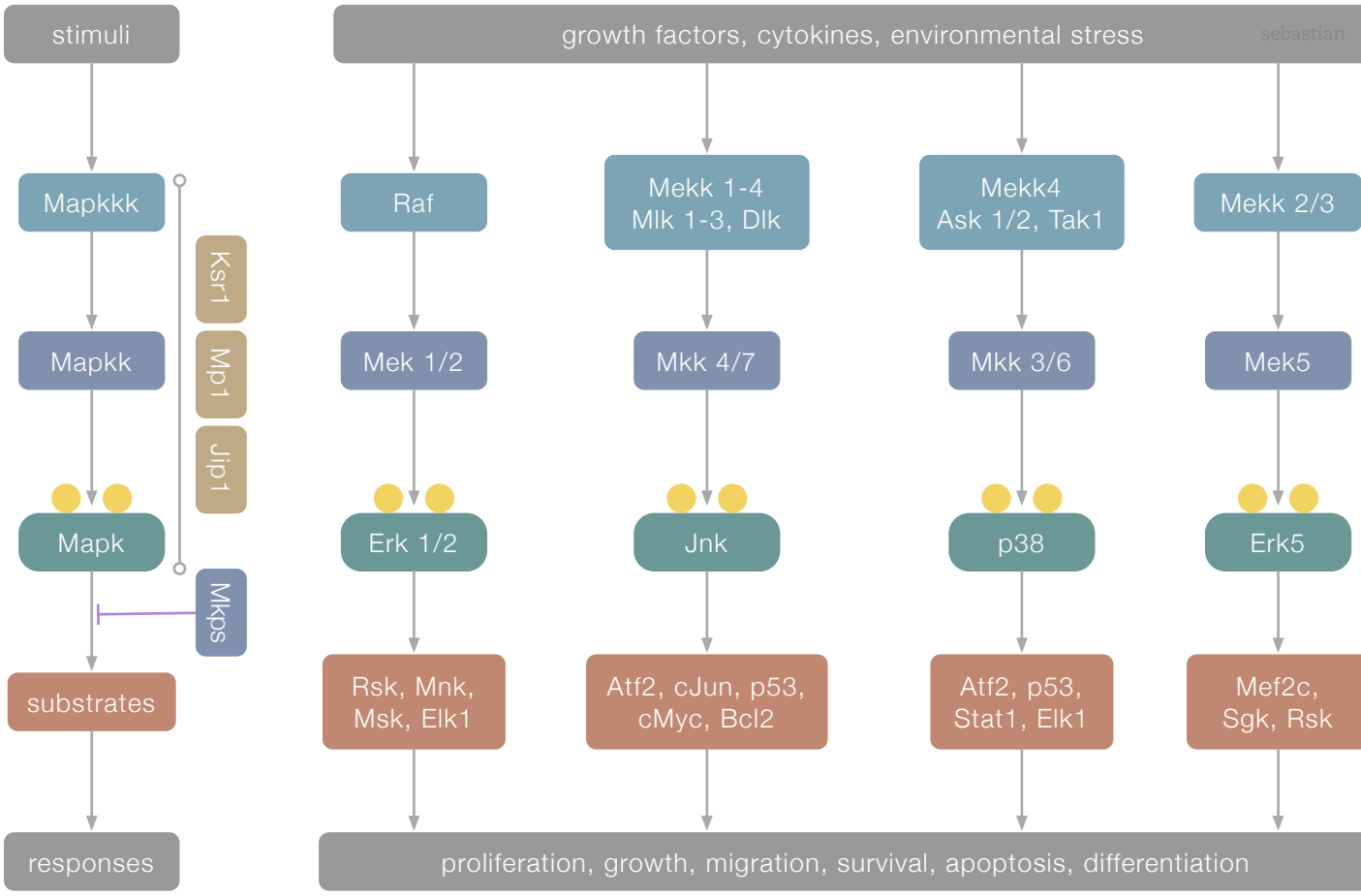
Substrates and Biological Functions

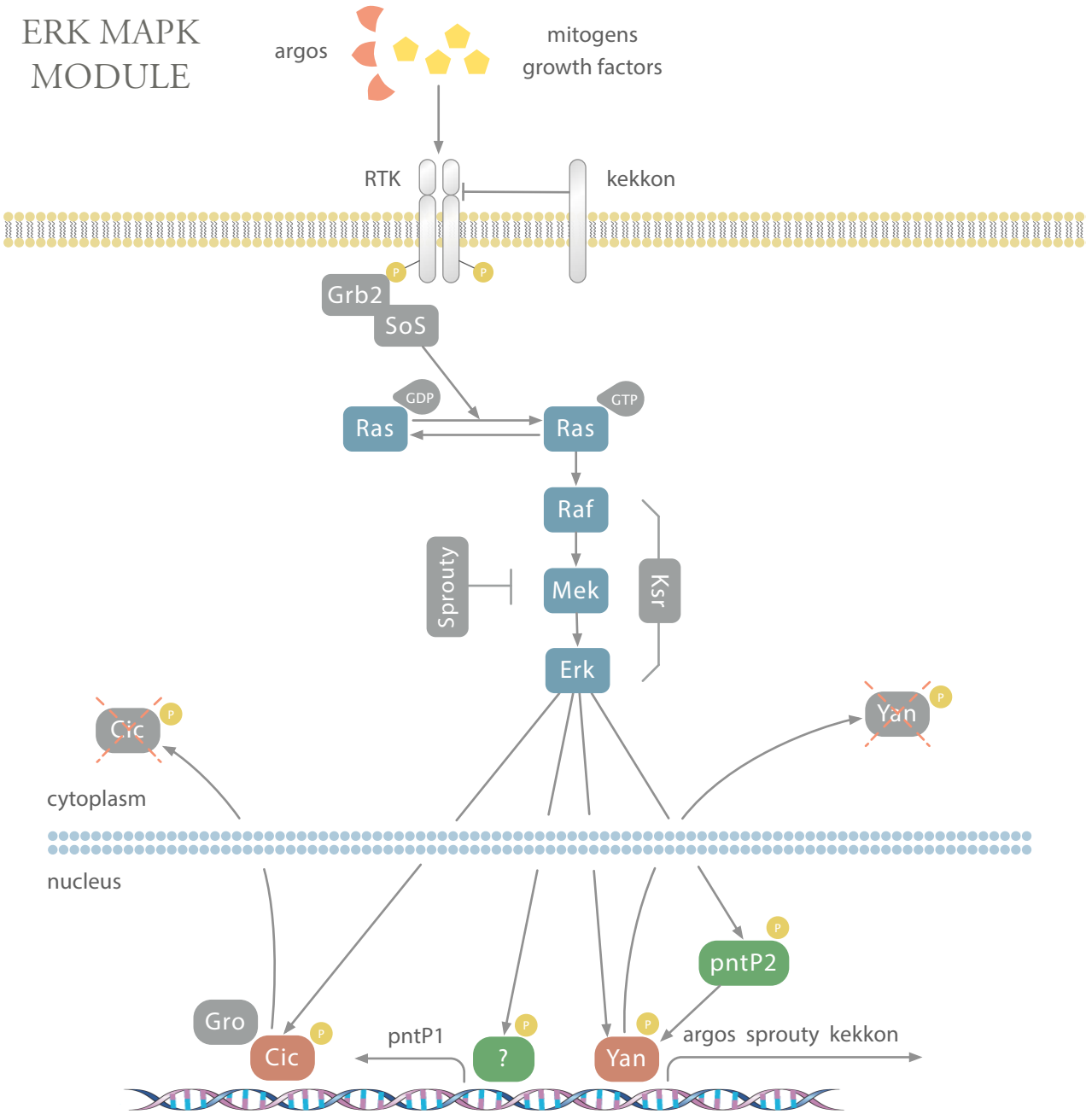
In quiescent cells, most if not all Erk Mapk pathway components are located in the cytoplasm. Upon activation, a significant proportion of Erk translocates into the nucleus [239,240]. Although not completely understood, studies have revealed multiple facets of this mechanism involving release from cytoplasmic anchors, phosphorylation and dimerisation, nuclear retention and a novel nuclear translocation sequence (NTS) located in the kinase domain [241,242]. Upon activation, Erk Mapk phosphorylates a large ensemble of targets in the cytoplasm as well as in the nucleus. Many of these targets have been well characterised in mammalian cells. Some of them are in the cytoplasm (death associated protein kinase DAPK, Tsc2, Rsk, Mnk), some are in the nucleus (Elk1, myocyte enhancer factor 2 Mef2, Stat3, Fos and Myc), whereas other are associated with membranes (CD120, Syk, calnexin) or the cytoskeleton (neurofilaments and paxillin)[243].

In drosophila, most Mapk driven phenotypes have been described through its function as a transcriptional regulator. Mapk impinges on transcription through two main mechanisms, by

phosphorylating and stimulating transcriptional activators and by phosphorylating and inhibiting transcriptional repressors. One transcriptional activator stimulated by Mapk is Pointed (Pnt). Pointed is produced in two alternative forms, PntP2 that requires Mapk phosphorylation for activation, and PntP1 who is constitutively active but requires Mapk for its transcriptional induction [244-246]. As mentioned, Mapk also targets transcriptional repressors. Two such repressors are Yan and Capicua. Phosphorylation of these repressors by Mapk leads to their inactivation and nuclear export, thus allowing expression of many target genes (Yan 247,248 Cic 249-252). Among these targets is the ETS domain transcription factor Ets21c, which is transcriptionally suppressed by capicua in the adult intestine and gains in expression following Egfr activation in this tissue [54]. The functional consequences of Mapk cytoplasmic targets in drosophila are just beginning to be understood. One example being the RNA-binding protein How whose phosphorylation by Mapk facilitates its dimerisation and binding of target RNAs [253].

The Erk Mapk pathway has been implicated in diverse cellular functions, including cell survival, growth, proliferation, motility and differentiation. In drosophila, Erk Mapk pathway is used repeatedly in various context such as dorsoventral patterning, wing vein determination and oogenesis [61]. One of the best characterised functions facilitated by the Erk Mapk module is cell proliferation. Erk is rapidly activated by mitogenic stimuli and is required for normal cells to progress from G1 to S phase of the cell cycle. Erk promotes cell cycle progression through several mechanisms [254]. For example, Erk directly interacts with the cyclin dependent kinase Cdc2 [255]. Furthermore, multiple cell cycle genes were found to be transcriptionally induced in drosophila upon capicua downregulation [a transcription factor normally inhibited upon Erk Mapk activation][54].


stimuli
growth factors, cytokines, environmental stress
sebastian
Mapkkk
Raf
Mekk 1-4
Mlk 1-3, Dlk
Mekk4
Ask 1/2, Tak1
Mekk 2/3
Ksr1
Mapkk
Mek 1/2
Mkk 4/7
Mkk 3/6
Mek5
Mp1
Jip1
Mapk
Erk 1/2
Jnk
p38
Erk5
Mkps
substrates
Rsk, Mnk,
Msk, Elk1
Atf2, cJun, p53,
cMyc, Bcl2
Atf2, p53,
Stat1, Elk1
Mef2c,
Sgk, Rsk
responses
proliferation, growth, migration, survival, apoptosis, differentiation


ERK MAPK MODULE
argos
mitogens
growth factors
RTK
kekkon
P
P
Grb2
SoS
GDP
Ras
GTP
Ras
Raf
Sprouty
Mek
ksr
Erk
Cic
P
Yan
P
cytoplasm
nucleus
pntP2
P
Gro
Cic
P
pntP1
?
P
Yan
P
argos sprouty kekkon

Mapk Figure 1. Mapk signalling cascades leading to activation of Mapkapks. Mitogens, cytokines and cellular stresses promote the activation of different Mapk pathways which in turn phosphorylates a multitude of cytoplasmic and nuclear substrates. All eukaryotic cells posses multiple Mapk pathways which coordinately regulate cell survival, division, growth, motility and differentiation. Each group of conventional Mapks consists of a sequentially acting kinase cascade: a Mapk, a Mapk kinase (Mapkk) and a Mapkk kinase (Mapkkk). Among the Mapk substrates are members of the Mapkapk (Mapk activated protein kinase) family, including Rsk, Mnk and Msk, which act as an additional amplification step of the Mapk modules and control a wide range of biological functions.

Mapk Figure 2. The Ras-Erk Mapk module in drosophila. Mitogens and growth factors bind to RTK (receptor tyrosine kinase) receptors triggering receptor dimerisation and activation. Egfr (epidermal growth factor receptor) is such an RTK receptor functioning upstream of the Ras-Erk module, being expressed at various stages during drosophila development and is an integral part of this study's work. As activating ligands for the Egfr receptor, function in drosophila Spitz, Keren, Gurken and Vein. Upon activation, the RTK receptors alter their conformation, leading to phosphorylation of tyrosine residues on their intracellular domains. This creates binding sites for adaptor proteins, such as Grb2 which in turn recruits the GEF (guanine nucleotide exchange factor) protein Sos. Sos promotes GDP for GTP exchange on Ras leading to its activation. Active Ras can now interact with its substrates among which is the Mapkkk (Mapk kinase kinase) Raf. Activated Raf phosphorylates and activates the Mapkk Mek, which in turn phosphorylates and activates the Mapk Erk. Ksr functions as a scaffold to increase the efficacy of signaling. Most Ras-Erk functionality in drosophila has been attributed to transcriptional regulation. The cardinal transcription factors acting downstream of Ras-Erk signaling are the activators PointedP1 and Pointed P2, and the repressors Capicua and Yan. PntP2 is activated by Mapk phosphorylation, while PntP1 is constitutively active but needs Mapk for its transcriptional induction. Yan and Cic are constitutive repressors which, upon Mapk phosphorylation, are inactivated and removed from the nucleus. Argos is part of a negative feedback loop initiated by the pathway and acts to sequester active ligands, thereby preventing receptor activation. Two additional feedback loops act to attenuate signaling, kekkon binds to and prevents receptor activation, whereas sprouty inhibits the intracellular module at different steps. Our proposed model adds additional effectors and functionality to the canonical Erk Mapk module.

I-3 MAPK ACTIVATED PROTEIN KINASES

As described in the previous section, Mapk pathways are firmly wired into the cell's metabolic mechanisms. To facilitate basic cell functions like survival, proliferation and growth, Erk and the other Mapk pathways phosphorylate a varied assortment of target proteins in the nucleus as in the cytoplasm. Among these substrates are the Mapk activated protein kinases (Mapkapks) [10,11]. Two of them were found to be involved in translation control, namely the Mnks (Mapk interacting kinases) [12] and the Rsks (p90 ribosomal S6 kinases) [13]. The drosophila Rsk ortholog was shown to be involved in modulation of circadian behaviour and memory formation [14-16]. Drosophila Mnk homologue is called Lk6, and was shown to be important for eIF4E phosphorylation, developmental rate and organism size [104,17-19]. Drosophila homologue of another Mapkapk (Msk) is called Jil1, is essential for viability, and it functions to maintain euchromatic domains while counteracting heterochromatinisation and gene silencing [20,21].

The Mapk Interacting Kinase, Mnk

The drosophila Mnk homologue is called Lk6 and it phosphorylates the eukaryotic translation initiation factor eIF4E in vivo. In contrast to its mammalian counterparts, Lk6 binds Erk but not p38 and is activated by the Erk pathway but not by stress-activated p38 pathway [219]. eIF4E controls a crucial step in cap-dependent translation initiation and is critical for cell growth. eIF4E phosphorylation is required in drosophila for normal growth and development [256]. Lk6 loss-of-function mutants have reduced eIF4E phosphorylation, reduced viability, slower development and reduced adult size, providing evidence that Lk6 is required for organism growth and development [220]. The effect of Lk6 on growth may be nutrient dependent. Accordingly, it was found that Lk6 function is dispensable on a high protein diet (analogous to mammals, where loss of Mnk didn't cause a growth phenotype), whereas Lk6 loss-of-function causes a significant growth reduction when the food amino acid content is reduced [221]. At the drosophila neuromuscular junction, Mnk has been implicated together with ToR in regulating the synthesis and localisation of synaptic glutamate receptors, a process essential to synaptic plasticity [222]. Mnk is suggested to act through its target eIF4E to regulate translation initiation and synaptic levels of glutamate receptor. Furthermore, Lk6 has been implicated in the development of Parkinson's disease [223]. Parkinson's disease is caused by loss of dopaminergic neurons and associated with alpha-synuclein phosphorylation and inclusion

body formation. Lk6 has been found to promote alpha-synuclein phosphorylation, to accelerate neurodegeneration and thereby to shorten the lifespan of drosophila [223].

Mammals have two Mnk genes, Mnk1 and Mnk2. Both Mnks are expressed in all adult tissues, with lower than average levels in the brain and higher levels in the skeletal muscle [105]. Both Mnk genes produce two isoforms, a long form (Mnk1A and Mnk2A) with a predominantly cytoplasmic localisation, and a short one (Mnk1B and Mnk2B) without a Mapk binding motif, equally distributed between the nucleus and the cytoplasm [106-108]. Although their involvement in the general translation control is unclear, several studies in mammalian cell culture have linked Mnks to the translation initiation complex. Whereas mToRC1 promotes eIF4E by inhibiting 4EBP, Mnk may promote cap-dependent translation initiation by directly phosphorylating eIF4E [109,110]. Stress and mitogenic stimuli which activate Erk and p38 promote eIF4E binding to eIF4G in the translation initiation complex, and subsequently Mnk dependent phosphorylation of eIF4E [109,111,112]. eIF4E phosphorylation appears to promote cancer cell proliferation in vitro [113] and enhance the oncogenic potential of eIF4E in vivo [114,115]. eIF4E mediated translational regulation of the antiapoptotic protein Mcl1 is believed to be important for tumorigenesis [114]. Furthermore, eIF4E phosphorylation was shown to be particularly important for the inflammatory response and tumor progression by regulating the translation of mRNAs encoding inflammatory molecules (Ccl2 and Ccl7) and matrix metalloproteases (Mmp3 and Mmp9), respectively [116].

The p90 Ribosomal S6 Kinase, Rsk

The Rsk family comprises a group of highly related Ser/Thr kinases including four vertebrate isoforms and a single orthologue in drosophila. Rsks have been involved in cellular functions mediated by the Ras-Mapk pathway, including cell growth, proliferation and survival. Rsks are known to be activated by stimuli such as growth factors, peptide hormones and neurotransmitters [257]. Among Rsk nuclear targets are histones and transcription factors like Creb, Atf4, Fos and Jun [258].

One context in which drosophila Rsk has been characterised is the circadian molecular oscillator. Organisms posses endogenous clocks (or circadian oscillators) that enable them to synchronise their metabolism with diurnal environmental cycles. The circadian molecular clocks usually rely on transcriptional and post-transcriptional autoregulatory feedback loops in which clock proteins are being modified, translocate to the nucleus and regulate expression

of clock genes [259-261]. In drosophila, the molecular clock consists of three feedback loops which involve rhythmic changes in the Clock (Clk) transcription factor activity and in the production of clock proteins such as Period (Per) and Timeless (Tim) [262,263]. Additionally, the molecular clocks in flies and mammals rely on post-transcriptional mechanisms to keep time. Prominent among these is the phosphorylation of clock proteins, which regulates their stability, nuclear entry and ability to regulate clock gene transcription [264]. Important enzymes regulating the drosophila clock feedback loops include casein kinase 1e (CK1e), casein kinase 2 (CK2), glycogen synthase 3b (Gsk3b), at least two protein phosphatases (PP2A and PP1) and an E3 ubiquitin ligase called Slimb [265,266]. In this context, drosophila Rsk was found to bind to casein kinase 2 and to be required in clock neurons for normal circadian periodicity [267]. Furthermore, Erk binding to the Rsk protein were shown to be required for normal Rsk phosphorylation and modulation of circadian behaviour [268].

Rsk is considered to be a one of the main effector kinases functioning downstream of the Erk Mapk module. Conversely, Rsk was found to negatively regulate Erk Mapk signaling in drosophila. Rsk null mutants are viable, but they exhibit enhanced Erk-dependent differentiation phenotypes, such ectopic photoreceptor and vein cell formation. Conversely, Rsk gain-of-function mutants strongly suppressed Erk dependent differentiation [14]. Rsk is thought to suppress Erk activity by acting as a cytoplasmic anchor and preventing Erk from entering the nucleus.

Rsks have been associated with memory formation in mammals and flies [269,270]. Two studies in drosophila have indicated that Rsk facilitates normal brain function by regulating synapse architecture, and that it does so by inhibiting the Erk Mapk pathway [271,272,274]. Both Ras and Erk are enriched at the synaptic boutons of the drosophila neuromuscular junction (NMJ), and Ras was found to support bouton formation [273]. Drosophila Rsk mutant larvae exhibit enhanced bouton numbers, whereas Rsk overexpression in motoneurons reduces bouton numbers [274], indicating that Rsk has a negative effect on bouton formation. Genetic epistasis studies have further revealed that this inhibitory function is mediated by suppressing the Erk Mapk pathway. A second study described Rsk as a regulator Erk activity, synaptic function and axonal transport in drosophila motoneurons [272]. Mouse and fly Rsk knockout models display various deficits in learning and memory [269,270]. Loss-of-function mutations in human Rsk2 cause the Coffin Lowry syndrome, characterised by facial and skeletal abnormalities and by mental retardation in affected males [275]. Drosophila Rsk loss-of-

function mutants display altered neuronal Erk Mapk activity and have defects in synaptic morphology and function [272]. An impaired anterograde axonal transport of mitochondria is also observed. Loss of Rsk function enhances Erk activity in the neuronal soma, while decreasing Erk activity in axons and the presynapse.

Rsks are expressed ubiquitously in every human tissue tested, predominantly in the kidney, pancreas, lungs, heart, skeletal muscle and brain [82]. Multiple studies in mammalian cells have linked Rsk function to translation control. Rsk was originally identified as an in vitro ribosomal protein S6 (rpS6) kinase [83,84]. Later studies have identified S6k as the main rpS6 kinase, but shown that Rsk also contributes to S6k phosphorylation in vivo, and it does so in a ToR independent manner [85]. Rsk mediated rpS6 phosphorylation was found to promote cap-dependent translation initiation. A second mechanism through which Rsk may promote translation and drive growth is by activating ToRC1. Rsk, and its activator Erk, were found to phosphorylate tuberous sclerosis complex component Tsc2, thereby negatively regulating its guanine activating protein (GAP) activity towards the small GTPase Rheb [30-33]. Activated Rheb, in turn, stimulates ToRC1 activity. Another way in which Rsk and Erk stimulate ToRC1 is by phosphorylating Raptor, an important interacting partner of ToRC1 [34-36]. A third mechanism for Rsk driven translation relies on phosphorylation and inhibition of Gsk3 [86], which prevents the suppression of translation initiation factor eIF2B [87]. By phosphorylating and inhibiting Gsk3, Rsk may also indirectly promote ToRC1, as Gsk3 and the Lkb1 activated kinase Ampk both phosphorylate and activate Tsc2 [88,89]. Finally, Rsk was shown to phosphorylate eIF4B and eEF2K, and thereby promote translation inhibition and elongation, respectively [38,90].

Mitogen and Stress Activated Kinase, Msk

The drosophila Msk homologue, Jil1, is essential for viability [96,276] and was found to phosphorylate histone 3 at Ser10 (H3S10ph) and thereby maintain gene expression by preventing the spread of heterochromatin and gene silencing. Thus, Jil1 localises to euchromatic regions of chromosomes and mediates H3S10 phosphorylation at interphase [20,96]. Multiple studies have led to a model where gene expression is regulated by a dynamic balance between euchromatic marks, such as H3S10ph, and heterochromatic marks, such as H3K9me2 (created by the methyltransferase Su(var)3-9) [277-281]. By creating H3S10ph marks, Jil1 maintains an active state of chromatic and prevents the spread of heterochromatic marks (especially H3K9me2) and gene silencing. H3S10ph itself is not required for transcription or gene activa-

tion, but rather to counteract the gene silencing effect of H3K9 dimethylation. A study on salivary glands of drosophila larvae indeed revealed that the H3S10ph mark is enriched at active genes whereas the H3K9me2 is largely associated with inactive genes [282]. Jil1 affects a vast number of genes, as in Jil1 null mutant larvae about 1500 genes changed their expression level by at least two fold. About half of these genes were downregulated (and had an increased density of H3K9me2 marks) whereas the other half were upregulated (and had a lower density of H3k9me2 marks).

Jil1 is also an important player in the regulation of telomeric chromatin in drosophila. Telomere elongation is needed in all eukaryotes with linear chromosomes as cellular polymerases cannot proceed in a 3' to 5' direction. Maintaining telomere length is important to avoid progressive loss of genetic material from the chromosome ends and for the assembly of the telomere capping complex (shelterin in telomerase telomeres or terminin in drosophila) [283,284]. Failure of capping complex assembly signals to the DNA damage machinery which, by trying to repair the damage, fuses chromosomal ends and thereby creates genomic instability [285]. Two mechanisms ensure the elongation of telomeres in eukaryotes, one relying on telomerase activity and one relying on transposons activity. Each of these mechanisms has at least two additional layers of control providing for their efficacy. A first level of control involves regulation of the expression and function of telomerase subunits [or the transposons], while a second level involves epigenetic control of telomeric chromatin [286,287]. The drosophila telomere elongation mechanism is based on the transposition of three retrotransposons, HetA, Tart and Tahre [288-290]. The transposition mechanism of these transposons involves an RNA intermediate which implies that each transposition will increase the copy number of the element and thereby telomere length. The telomeres of most eukaryotes are comprised of two modules, a terminal protective cap and the telomeric (distal) domain. The telomeric domain is flanked by a subtelomeric (proximal) domain composed of telomere associated sequences (TAS) and with different chromatin characteristics [291,292]. The telomeric domain is made up by either telomerase repeats (in telomerase organisms) or by retrotransposon repeats (HetA, Tart and Tahre, HTT in drosophila). The subtelomeric TAS sequences nucleate a compacted chromatin state (characterised by the presence of H3K27me3 and Polycomb) which is restrictive of gene expression at the telomeres [293,294]. In this context, Jil1 was found to localise to the HTT array and to positively regulate the expression of telomeric retrotransposons [295,296]. Jil1 supports retrotransposon expression by protecting the

telomeric domain from the spread of repressive chromatin from the adjacent subtelomeric domain [297].

In mammals, Msk1 and Msk2 are ubiquitously expressed, predominantly in brain, heart, placenta and skeletal muscle [91]. Msks contain a functional NLS conferring them a nuclear localisation in both serum starved and stimulated cells [91]. In cells, mitogens and stress stimuli lead to Erk and p38 Mapk pathways activation. Both Erk and p38 in turn were found to positively regulate Msk by phosphorylating the same sites [91-93]. Conversely, expression of Msk was found to regulate ectopically expressed p38 and Erk localisation, indicating that Msks may control the cellular localisation of their upstream activators. Msks were found to be important regulators of gene expression in mammals, by playing active roles in transcriptional regulation and chromatin remodelling in response to stress and mitogens [298,299]. Having a similar substrate specificity with Rsk, Msk might influence translation through similar targets. For example, both Rsk and Msk were found to phosphorylate the transcription factor Creb in mammalian cells [91,93]. Activated Creb in turn drives expression of immediate early (IE) genes, such as Fos, Jun and Egr1 [100]. Additional transcription factors targeted by Msk include NFkB and Stat3 [101,102]. Importantly, Msk was suggested to phosphorylate the translational inhibitor 4EBP1 [103], providing another convergence point between Mapk activated kinases and ToRC1 (see also III-5).

I-4 RAS-MAPK PROMOTES GROWTH IN DROSOPHILA

In developing organs, such as the eye or the wing, Ras Mapk signaling covers different functionalities. In dividing cells it promotes cell cycle progression and growth, while in postmitotic cells, it supports survival and differentiation.

Ras Promotes Growth in Imaginal Discs

The Ras GTPase acts as an essential signaling node, linking extracellular signals to intracellular mechanisms driving cell growth, proliferation, survival, and identity [70]. Ras was shown to promote growth in vivo in the drosophila wing and eye. Reducing Ras activity through null or dominant negative mutations, slows growth, decreases cell size, and increases cell death as a result of cell competition. While Ras mutant cells can still proliferate to some extent, they have poor viability and cannot properly differentiate. Conversely, activating Ras increases the growth rate and cell size. Similar to Myc and Pi3k growth promoters, Ras activation shortens the G1 cell cycle phase, likely as a consequence of increased growth, but cell division rates remain constant due to compensatory G2 elongation. Ras downregulation causes opposing effects [69,151,152].

Active Ras Drives Growth through Myc and Pi3k

Stimulation of the Egfr receptor in the wing by its ligand Vein, activates Ras signaling and is required for growth. Activated Ras drives growth and promotes G1/S progression, in part, by activating two growth promoting pathways, Raf/Mapk and Pi3k. Using pathway specific mutations, it was shown that Ras stimulation of either of the two pathways has a similar growth promoting effect. Raf/Mapk activation, in turn, further acts to increase Myc protein level, a potent growth promoter. Egfr/Mapk/Myc and Insulin/Pi3k signaling, however, do not crosstalk in this tissue. Raf/Mapk also determines, independently of Myc, cell identities in imaginal discs. Although mutationally activated Ras stimulates both Pi3k and Myc growth pathways, endogenous Ras does not increase Pi3k signaling (nor does Pi3k increase Raf/Mapk), and only modestly increases Myc protein levels, hinting towards additional growth promoters. Furthermore, the growth defect caused by loss of Ras activity in the wing can be rescued to a large extent by increasing Raf/Mapk, but not Pi3k singling [152]. Cell growth driven by Raf/Mapk, Myc and/or Pi3k, increases cyclin E protein levels, which is believed to stimulate G1/S progression [69,70].

Mapk Phosphorylation is Required for Cell Growth

Mapk phosphorylation status is also important for cell growth and division in the developing wing and eye tissues. Phosphorylated Mapk is able to promote cell growth and division in these tissues, while non-phosphorylated Mapk drives division, but fails to promote growth. This requirement for Mapk phosphorylation points towards downstream growth effectors, responsive to activated Mapk [153,300].

Insulin Activates Erk Mapk and Promotes Cell Growth

Insulin stimulation activates both Pi3k/Akt and Mek/Erk pathways in drosophila schneider cells. Pi3k/Akt seems to function upstream of Mek/Erk, as inhibition of Pi3k/Akt lowers both basal and insulin-induced Mek/Erk activities. Insulin activation promotes, through both Pi3k/Akt and Mek/Erk pathways, G1/S progression and proliferation of schneider cells, with Pi3k/Akt having an additional contribution of increasing cell size. These activities are also required for maintaining normal proliferation rates and cell size under basal conditions [7,8].

p38 Mapk Promotes Cell Growth via ToRC1

The stress activated p38 Mapk is a positive regulator of insect and mammalian cell growth, and a positive regulator of ToRC1 (ToR complex 1) and MK2 (Mapk activated kinase 2) activities. In fact, ToRC1 mediated cell growth requires p38 pathway activity in both insect and mammalian cells. p38 promotes growth through two distinct mechanisms, one relying of ToRC1-S6k activity, and the other on Mk2 activity. The mechanism through which p38 activates ToRC1 is not fully understood. p38 appears to function upstream of Rag GTPases (ToRC1 activators, in response to amino acids) [301], but downstream of, or in parallel to, Rheb GTPase (ToRC1 activator, in response to various stimuli) [41].

Pvr Activates Erk Mapk and Promotes Cell Growth

The drosophila homologue of mammalian Pdgf/Vegf receptor family, PVR, was found to promote cell growth in culture. Pvf2 and Pvf3 ligands act redundantly to activate Pvr and downstream Ras signaling. Activated Ras, in turn, drives growth by concomitantly activating Pi3k and Mapk, and subsequently, ToR pathway. Normal growth of S2R+ cells in culture does not require insulin receptor activation, but it rather depends on ToR and Mapk pathway activities. Pvr/Ras driven growth primarily relies on activation of Raf/Mapk pathway, but also on Pi3k/Akt pathway activation. In turn, both of these pathways are then likely able to stimulate ToR-

C1 activity and thereby trigger cell growth. An alternate ToR-independent growth-promoting mechanism, functioning downstream of Ras, could not be excluded so far [3].

Ras-Mapk Drives Proliferation of Adult Midgut Progenitors

In drosophila as in many other insects, adult appendages and internal organs form from larval progenitor cells during metamorphosis. The drosophila midgut [equivalent to the mammalian small intestine] develops from a stem cell population called adult midgut progenitors (AMPs). AMPs first appear in the embryo along with differentiating enterocytes (ECs), and continue to divide and differentiate during larval development, at the end of which the AMPs and their daughters fuse to form the adult midgut during metamorphosis. During larval development, the AMPs undergo extensive proliferation in two phases, first to expand throughout the tissue, and then to generate AMP cell clusters which eventually fuse at the end of the larval stage to bring about the adult midgut during metamorphosis. Two critical pathways regulating the proliferation and differentiation of AMPs during development are Notch and Egfr-Mapk. Notch functions as a differentiation factor throughout midgut development. In the larval intestine, low Notch activity allows formation of enteroendocrine (EE) cells and AMPs, while high Notch activity promotes differentiation of enterocytes (ECs) and peripheral cells (PCs) of the AMP clusters. Similarly, in the adult intestine, low (or lack of) Notch activity allows ISC proliferation and EE cell formation, while high Notch directs EC differentiation [117,118]. The second master controller of midgut progenitors is the Egfr-Ras-Mapk pathway, which is necessary and limiting for AMP proliferation. Expression of the Egfr ligand Vein by the visceral muscles activates Ras-Mapk and stimulates AMP proliferation in the early larva, while expression of the stronger Egfr ligands Spitz and Keren by the AMPs themselves assures their proliferation during late larval stages [50,51].

Ras-Mapk Drives Growth and Proliferation in the Adult Midgut

Similar to the mammalian small intestine and colon, the adult drosophila midgut undergoes dynamic self-renewal [117,118]. This is accomplished by resident ISCs which divide to generate new ISCs and committed progenitors called enteroblasts (EBs). Unlike their mammalian counterparts, the transit amplifying cells, the EBs don't usually divide but rather differentiate into two functional cell types, the absorptive enterocytes (ECs) and secretory enteroendocrine cells (EEs). ECs grow very large and endoreplicate their genomes up to ploidy levels of 32c, and therefore constitute the bulk of the intestinal epithelium. A recent study further suggested that EEs are not formed from Su(h)+ EBs, but rather from a different pre-EE progenitor, and

that the transcription factor Prospero is essential for ISC commitment towards the EE fate [302]. The gut turn-over rate is approximated at 1-2 weeks [118]. However, in response to midgut damage (mechanical, chemical, bacterial), ISC proliferation and EB differentiation are enhanced up to 100 fold to ensure tissue regeneration [119-125]. Egfr pathway acts as a major growth and proliferation factor during midgut homeostasis and regeneration. Egfr signaling is stimulated by three ligands, Vein produced by muscle cells surrounding the midgut, Spitz and Keren produced by the midgut epithelial cells, and shows high levels of activity in the progenitor cells, ISCs and EBs. Furthermore, loss of Egfr signaling blocks ISC growth and division, whereas constitutively activated Ras accelerates the growth of ISCs and post-mitotic enteroblasts [51-53,126]. Damage or stress to the midgut epithelium increases the expression of Egfr ligands and rhomboids (intramembrane proteases that activate some Egfr ligands), and consequently Ras-Mapk activity (especially in ISCs). This increase in Ras-Mapk activity is essential for midgut regeneration, especially upon bacterial infection, as it coordinates ISC growth and division, proper morphogenesis of new enterocytes, and delamination of damaged enterocytes [52,122]. Together with the Jak/Stat pathway, which functions as a major mitogenic and differentiation factor in the midgut [119,127-131], Egfr pathway is highly activated by midgut damage and essential for tissue regeneration [51-53,126]. Two other damage/stress sensing pathways, Hippo and Jnk, were found to promote Egfr and Jak/Stat activation and implicitly tissue regeneration [119,123,126,131-134].

I-5 RAS-MAPK PROMOTES CANCEROUS GROWTH

Mapk Drives Growth and Alters Cell Identities

A hallmark of tumor development within a tissue is the escape from the stringent homeostatic regulation resulting from the continuous exchange of signals between the cells, the extracellular matrix and the local environment. Such an evasion endows tumor cells with the ability to grow and proliferate autonomously, and eventually alter their adhesion and migrate away from their site of origin. Clones of cells with elevated Raf-Mapk signaling in developing drosophila epithelia are able to mimic such tumor-characteristic transformations, such that they exhibit enhanced growth and altered cell adhesion and identities, thereby minimising contact with neighbouring wild type cells. In contrast, other growth signals, such as the Pi3k pathway and Myc, do not regulate cell adhesion and identities [70]. The ability of Ras/Mapk to exert such effects, together with its capacity to stimulate both mentioned growth promoting signals, highlight its biological dominancy in regulating cell fate, growth and proliferation, and may underly the strong synergy between Ras and other growth promoting oncogenes in vivo.

Oncogenic Cooperation

Human tumors display a high degree of heterogeneity in cell types and genetic makeup. Cooperation among these tumor cell communities, between the tumor and its microenvironment, and between different oncogenic genetic lesions plays a defining role in tumorigenesis and cancer progression [303,304]. An epitome of such cooperation aiding tumorigenesis is the development of epithelial cancer in the drosophila larval eye epithelium, driven by expression of activated alleles of Ras or Notch within clones of tissue mutant for the cell polarity regulator, Scribbled (Scrib). Such drosophila born tumors bare remarkable parallels to human cancers, including overgrowth, failure to differentiate, invasion and metastasis [305-307]. Clones expressing activated Ras moderately overgrow [151]. Clones mutant for scribbled lose apicobasal polarity and die [305,308]. In contrast, scribbled clones also expression activated Ras become malignant lethal tumors [305,309]. The ability of activated Ras to convert Scrib mutant clones into cancerous tumors is mediated by the downstream Raf-Mek-Erk cascade, and not by cardinal side-pathways, such as Pi3k, Ral, Rho or Rac [305]. Although the oncogenic effect of Ras-Raf in these tumors is not completely understood, it involves more than just blocking apoptosis and enhancing cell cycle progression [305].

Inflammation and EMT

Overgrowth and invasion of these tumors is aided by an inflammation response. Hemocytes (blood cells) are recruited to the tumors and secrete an inflammatory cytokine, TNF, which activates Jnk in the tumor cells. Jnk activation triggered cell death in Scrib mutant cells [eliminating them], but it promotes overgrowth and invasion in Scrib mutant cells harbouring a second activating mutation in Ras or Notch [309-311]. Jnk endows tumors with invasive potential by promoting a process called epithelial-to-mesenchymal transition (EMT). EMT is a developmental process essential for morphogenesis, organogenesis and wound healing, that is frequently coopted by cancer cells to gain stem cell-like properties and metastatic potential [312]. Localised inflammation triggered by interactions between tumour cells and associated stromal cells plays a defining role in EMT induction. Whereas the Hippo pathway is required for tumor overgrowth, Jak-Stat pathway (activated by inflammatory cytokines like Il6 and TNF), together with Jnk and TGFb pathways are the main regulators of proliferation and the EMT programme [313-316]. These pathways target transcription factors, including the Zeb, Snail, Twist and NFkB, that activate EMT associated processes. Such processes include repression of E-cadherin expression and induction of invasion promoting genes such as matrix metelloproteinase 1 (Mmp1), Paxillin and Filamin [317].

Jnk-Ras Duo Driving Tumorigenesis

Jnk is believed to play a more central part in tumor overgrowth and invasion by activating expression of the invasion genes just mentioned and activating several additional pathways. Thus, Jnk promotes expression of Il6-like cytokines, the drosophila unpaireds Upd1-3 (activating Jak-Stat signaling), expression of Egf-like growth factors (activating Ras-Mapk signaling), expression of morphogens like Dpp (activating Tgfb signaling) and Wingless (activating Wnt/Wg signaling), and finally Jnk stimulates Yorkie activity (transcriptional co-activator in Hippo signaling) [318-321]. Among Jnk mobilised targets, two zinc-finger transcription factors, chinmo (chronologically inappropriate morphogenesis) and fruitless, are able to prime cells for oncogenic transformation in the eye as in the intestine. Both chinmo and fruitless are induced by Jnk in the tumors and can cooperate with activated Ras or Notch in promoting eye tumorigenesis [322]. Together with activated Ras, chinmo can also induce intestinal neoplasia in drosophila.

Screening Platform for Anticancer Agents

Using the Ras-driven tumor model, a drosophila-based screening platform was developed for rapid and economic identification of potential anti-cancer agents. In vivo screening for such compounds using animal tumor models would be much more efficient than the conventional pharmaceutical in vitro approach. Arguments pertaining to cost, time constrain, and conservation of signaling pathways make drosophila an ideal system for large-scale screening. One such tumor model successfully converted to a screening platform is the Ras-Scrib model mentioned above [323]. This model is based on the induction of GFP+ tumors in drosophila larvae upon expression of activated Ras in Scrib mutant clones. These tumors engage in uncontrolled growth and invade surrounding tissues in late larval stages. Using standard microscopy techniques, thousands of chemicals can be tested for tumor suppressing functions. The extent of the GFP signal (produced by the growing tumors) acts as a reproducible parameter to estimate the efficacy of these chemicals at blocking tumor growth.

Ras on the Brain

Activated Ras drives oncogenic growth also in the mammalian and drosophila brain, where it cooperates with activated Pi3k-Akt signaling to generate malignant gliomas. The mammalian brain is comprised of neurons and supportive glial cells. Many glial cell types retain their proliferative potential, including differentiated astrocytes, glial progenitors, and multipotent neural stem cells. Ras-Mapk and Pi3k-Akt pathways are essential for the proliferation and self-renewal of these cell types and for overall brain development and function [324]. These pathways are, however, also frequently activated in malignant gliomas. Gliomas are the most common tumors in the central nervous system and can arise from oncogenic genetic lesions in differentiated glial cells, glial progenitors or stem cells [325,326]. These tumors rapidly grow and infiltrate the brain, are resistant to standard chemotherapies and are largely incurable. Activation of Ras-Mapk or Pi3k-Akt alone cannot transform glial cells. However, co-activation of both pathways in drosophila glia and glial precursors gives rise to neoplastic, invasive glial cells that create tumor-like growths. These malignant cells can be transplanted between animals and represent a cell type specific cancer model mimicking human gliomas. Ras-Mapk and Pi3k-Akt initiate malignant neoplastic transformation by coercing additional pathways, commonly mutated in human gliomas, including ToR, Myc, G1 Cyclin-Cdks, and Rb-E2F [327]. This combinatorial genetic network drives abnormal cell division, growth and migration, eventually leading to malignant transformation. Genes within this network represent therefore potential therapeutic targets.

I-6 TOR PATHWAY

The ToR Pathway

All organisms have evolved signaling mechanisms that dynamically regulate anabolic and catabolic pathways according to nutrient availability. Perhaps the most important of these mechanisms is the one anchored by the protein kinase ToR. The ToR pathway responds to diverse environmental cues and strongly impacts most cell behaviours including survival, growth and proliferation [154]. Moreover, ToR was implicated in determining lifespan of multiple organisms, including worms, flies and mice [162]. Due to its outsized role in cell metabolism, many other pathways converge on ToR to exert their own cellular programs. Abnormal ToR pathway functionality is associated with many human diseases, including cancer, obesity, diabetes and neurodegeneration. Consequently, significant efforts are being made to understand ToR functionality in cell and organism metabolism and aging, as well as to develop pharmacological inhibitors as potential anti-cancer agents.

ToR is the target of rapamycin or sirolimus, a macrolide produced by Streptomyces Hygroscopicus bacteria. The ToR kinase pairs with several protein partners and forms two distinct complexes, ToRC1 and ToRC2, which exhibit different sensitivities to rapamycin, as well as different upstream inputs and downstream outputs. ToRC1 consists of ToR, Lst8 (lethal with Sec13 protein 8), Raptor (regulatory associated protein of ToR) and Pras40 (Proline rich Akt substrate of 40kDa). ToRC2 consists of ToR, Lst8, Rictor (rapamycin insensitive companion of ToR) and Sin1 (stress activated Mapk interacting protein 1) [163,164]. The mammalian/mechanistic ToR complexes (mToRC1/2) share two additional components: Deptor (Dep domain containing ToR interacting protein) and the Tti1/Tel2 complex [165,166], with mToRC2 also containing Protor (protein observed with rictor)[167].

Upstream Regulators of ToRC1

ToRC1 is remarkable in its ability to integrate five major inputs (growth factors, amino acids, energy, stress and oxygen) and appropriately coordinate anabolic pathways, including nutrient intake, protein and lipid synthesis, as well as catabolic pathways such as autophagy [154]. ToRC1 pathway was found to function in drosophila downstream of insulin signaling, and to control cell growth in culture [1-3] and in vivo [4,44,45].

Most upstream pathways regulate ToRC1 through the tuberous sclerosis (Tsc) complex, consisting of Tsc1 and Tsc2. The Tsc complex functions as a GTPase activating protein (GAP) for the small GTPase Rheb (Ras homologue enriched in brain). GTP-bound Rheb is a strong activator or ToRC1, whereas Tsc by converting Rheb to its GDP-bound state acts as an inhibitor of ToRC1 [47,168-171].

Growth factors such as insulin and insulin-like growth factors activate ToRC1 through the InR-Pi3k-Akt pathway [172,173]. Additional growth and inflammatory pathways were found to activate mToRC1 in mammals. The Erk Mapk and its effector Rsk activate mToRC1 by targeting the upstream Tsc2 and the mToRC1 scaffold Raptor [30-36]. Pro-inflammatory cytokines such as Tnfa (tumor necrosis factor alpha) activate mToRC1 via IKKb (IkB kinase beta)-dependent phosphorylation and inhibition of Tsc1 [174]. The Wnt pathway, a major regulator of cell growth, proliferation, polarity and differentiation, activates mToRC1 by inhibiting Gsk3b which normally phosphorylates and stimulates Tsc2 [175].

Amino acids also stimulate ToRC1 and must be present, in order for any upstream signal, including growth factors, to activate ToRC1 [176-178]. Their presence stimulates ToRC1 interaction with Rag GTPases which then promotes ToRC1 translocation from a poorly defined cytosolic location to the surface of lysosomes. There, the Rags GTPases dock onto a multiprotein complex called the Ragulator and bring ToRC1 into close proximity to the Rheb GTPase, which is found throughout the endomembrane system [177,178]. Finally, GTP-loaded Rheb will activate ToRC1. Thus, Rags and Rheb constitute a molecular AND gate which ensures that upstream pathways acting through Tsc/Rheb activate ToRC1 only if amino acids are also present. ToRC1 recruitment to the lysosomal surface and downstream signaling also depends on a v-ATPase dependent mechanism that senses the presence of amino acids in the lysosomal lumen [179]. As you can see, amino acid signaling to ToRC1 is a complex process and it implicates additional effectors, including Map4k3 (mitogen activated protein kinase kinase kinase kinase, 180), Vps34 (vacuolar protein sorting 34, 181) and Ipmk (inositol polyphosphate monokinase, 182), which we are only now beginning to understand.

Oxygen and energy levels also have a strong impact on ToRC1 activity, as evidenced by multiple studies in mammalian cells. Hypoxia or a low energy state activate the Ampk kinase [AMP activated protein kinase] and the transcription factor Redd1, which in turn inhibit mToRC1 activity [183,184]. Ampk acts by phosphorylating Tsc2 and increasing its GAP activity towards

Rheb, and by phosphorylating Raptor and allosterically inhibiting mToRC1. Redd1 stimulates Tsc2 through a poorly defined mechanism [185-187]. DNA damage also inhibits mToRC1 via several mechanisms, all of which depend on p53-mediated transcription. Notably, induced expression of Tsc2 and Pten downregulates the entire Pi3k-mToRC1 pathway, whereas the sestrin-dependent induction of Ampk inhibits mToRC1 by acting on Tsc2 and Raptor [188,189].

Finally, phosphatidic acid (PA) was also identified as an activator of mToRC1 acting, at least partially, by stabilising the ToR complexes [190,191]. Although its role as a ToRC1 regulator is unclear, providing exogenous PA or expressing PA-producing enzymes, like phospholipase D1 (Pld1) and Pld2, activates ToRC1 [192].

Cellular Processes Downstream of ToRC1

Protein synthesis is the best characterised process downstream of ToRC1. Two ToRC1 targets implicated in translation control are 4EBP (eIF4E binding protein) and S6k (rpS6 kinase). 4EBP competes with eIF4G for a shared binding site on eIF4E and thereby prevents assembly of the eIF4F translation initiation complex (eIF4A-eIF4G-eIF4E), hindering cap-dependent translation initiation [193]. ToRC1 phosphorylates and inhibits 4EBP and thereby promotes eIF4F assembly and translation initiation. Although 4EBP null flies grow and develop normally, expression of an activated form of 4EBP reduces cell size and its co-expression with known growth promoters (e.g. Pi3k/Akt) reduces their growth effects [194]. Activation of S6k by ToRC1 mediated phosphorylation leads through multiple effectors to an increase in transcription, translation initiation and translation elongation [195]. Most flies null for S6k die during development, and the ones that survive are smaller then wild-type flies [196]. ToRC1 also promotes protein synthesis by stimulating RNA polymerases. ToRC1 phosphorylates and activates Tif1A, promoting its interaction with PolI and expression of ribosomal rRNA [197]. ToRC1 phosphorylates and inhibits Maf1, a PolIII repressor, and thereby promotes expression of 5S rRNA and transfer tRNA [198,199].

ToRC1 also promotes lipid synthesis, which is required for proliferating cells to generate membranes. SREBP transcription factor is a master regulator of lipogenic genes involved in fatty acid and cholesterol synthesis. ToRC1 promotes lipogenesis by stimulating SREBP expression, processing and activity [200-203]. Additionally, ToRC1 supports adipogenesis by promoting the expression and activity of PPARg (peroxisome proliferator activated receptor gamma) [182,204].

All these biosynthetic pathways require a significant energy input. The main energy currency used in biosynthetic reactions is ATP. ToRC1 promotes ATP production by stimulating glycolysis, which generates the activated carrier molecules ATP and NADH from sugars. ToRC1 achieves this by increasing the transcription and translation of HIF1a (hypoxia inducible factor 1 alpha), a positive regulator of glycolytic genes [200,205-207]. Glycolysis generates pyruvate which is then imported in mitochondria for further oxidation to CO2 and H2O. ToRC1 also promotes mitochondrial biogenesis and oxidative function by mediating the nuclear association between PGC1a (PPARg co-activator 1 alpha) and the transcription factor YY1 (ying yang 1)[208].

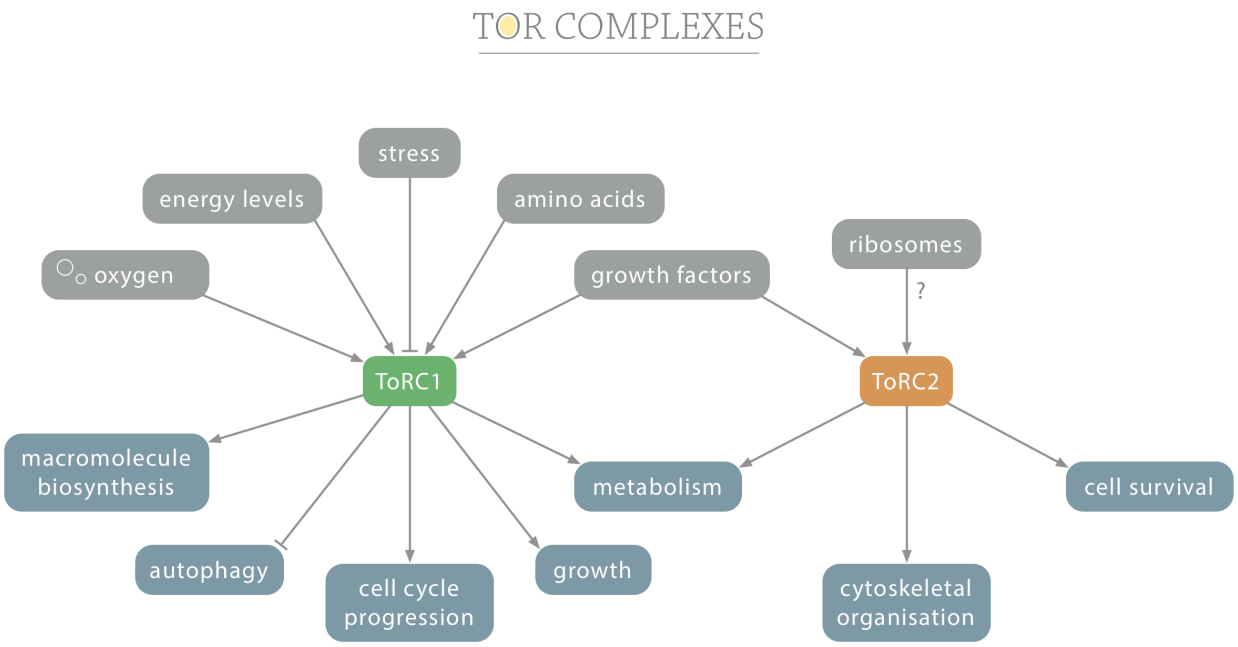
ToRC1 also regulates catabolic pathways. Autophagy is the main degradative process in cells. It supports normal cellular function by recycling damaged proteins and organelles, and it promotes survival when nutrient levels are low, by generating new building blocks from degraded cellular components. Upon starvation or ToRC1 inhibition, autophagosomes form and engulf cellular proteins and organelles. They subsequently fuse with lysosomes and break down the captured cellular components into new usable building blocks. In mammals, ToRC1 phosphorylates and suppresses Ulk1/Atg13/Fip200, a kinase complex required for autophagy [209-211]. Additionally, ToRC1 regulates Dap1 (death associated protein 1), a suppressor of autophagy [212]. ToR signaling was also shown to be necessary and sufficient to suppress starvation induced autophagy in the drosophila fat body (a nutrient storage organ analogous to the vertebrate liver)[48].

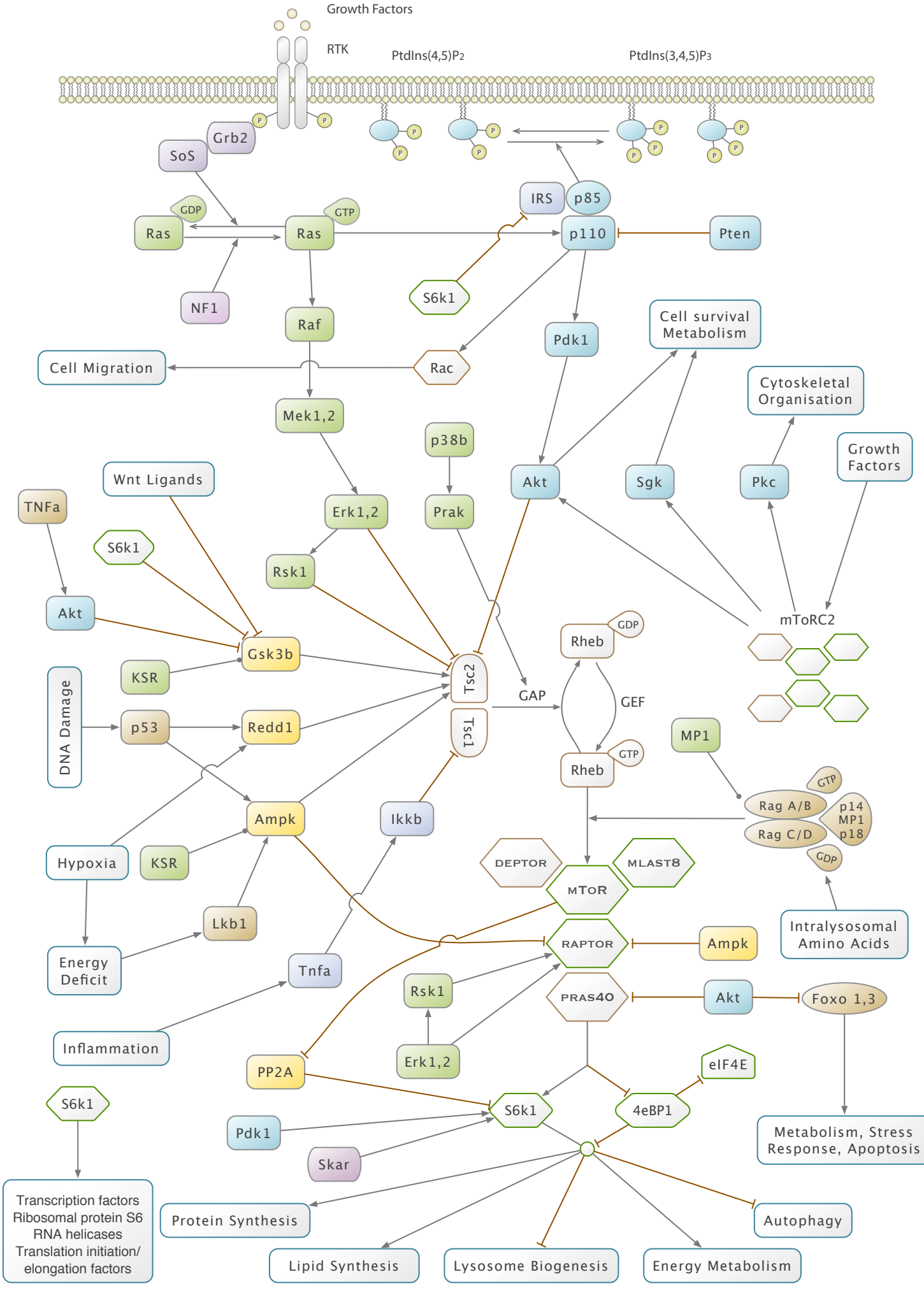
ToRC1 further impacts catabolic pathways by negatively regulating lysosome formation. Lysosomes are multifunctional organelles capable of degrading most cellular components. ToRC1 phosphorylates the transcription factor TFEB and prevents it from entering the nucleus and activating lysosomal genes [213].

The ToRC2 Signaling Network

The ToRC2 complex is much less studied than ToRC1. ToRC2 is insensitive to acute treatment with rapamycin and to nutrients, but does respond to growth factors such as insulin. Upon growth factor stimulation, ToRC2 binds ribosomes in a Pi3k-dependent manner and becomes activated [214]. ToRC2 regulates multiple cell functions, such as survival, growth, proliferation and motility. To achieve this, ToRC2 controls several AGC kinase family members, including

Akt, Sgk (serum and glucocorticoid induced kinase) and Pkc (protein kinase c) [176]. Akt regulates cell survival, growth and proliferation through multiple targets. For its full activation, Akt needs to be phosphorylated by Pdk1 and by ToRC2 [215]. ToRC2 also directly targets Sgk in mammalian cells, a regulator of ion transport and growth [216]. In contrast to Akt, Sgk activity is completely blocked by ToRC2 loss. Finally, ToRC2 activates Pkc which, together with effectors such as paxillin and Rho GTPases, control the actin cytoskeleton and thereby cell shape [167,217]. In drosophila, a central component of both ToRC1 and ToRC2 complexes is Lst8. Although it is the only ToR binding partner conserved in both complexes, Lst8 is required for ToRC2 but not for ToRC1 function [218]. Furthermore, ToRC2 was found to promote cell-autonomous growth in drosophila through a mechanism independent of Akt phosphorylation [218].




Growth Factors
RTK
PtdIns(4,5)P2
PtdIns(3,4,5)P3
Grb2
SoS
Ras
GDP
Ras
GTP
NF1
IRS
p85
p110
Pten
S6k1
Raf
Pdk1
Cell survival
Metabolism
Cell Migration
Rac
Cytoskeletal
Organisation
Mek1,2
p38b
Growth
Factors
Wnt Ligands
Akt
Sgk
Pkc
TNFa
Erk1,2
Prak
S6k1
Rsk1
mToRC2
Akt
Gsk3b
Rheb
GDP
KSR
Tsc2
GAP
GEF
DNA Damage
p53
Redd1
Tsc1
MP1
Rheb
GTP
Rag A/B
GTP
p14
MP1
p18
Rag C/D
GDP
Ampk
Ikkb
Hypoxia
KSR
DEPTOR
MLAST8
mTOR
Lkb1
Intralysosomal
Amino Acids
RAPTOR
Ampk
Energy
Deficit
Tnfa
Rsk1
PRAS40
Akt
Foxo 1,3
Inflammation
Erk1,2
eIF4E
PP2A
S6k1
4eBP1
S6k1
Pdk1
Metabolism, Stress
Response, Apoptosis
Skar
Transcription factors
Ribosomal protein S6
RNA helicases
Translation initiation/
elongation factors
Protein Synthesis
Autophagy
Lipid Synthesis
Lysosome Biogenesis
Energy Metabolism

ToR Figure 1. ToRC1 and ToRC2 complexes. The ToR kinase nucleates two distinct protein complexes named ToRC1 and ToRC2. ToRC1 responds to growth factors, amino acids, energy level, oxygen and stress. It promotes growth by inducing anabolic pathways, such as protein and lipid synthesis as well as ATP production, and by repressing catabolic pathways, such as autophagy. It also promotes cell cycle progression. ToRC2 responds to growth factors and regulates cell survival, metabolism and the cytoskeleton.

ToR Figure 2. The key signaling nodes that regulate ToRC1 and ToRC2. ToRC1 is remarkable in its ability to integrate five major inputs (growth factors, amino acids, energy, stress and oxygen) and appropriately coordinate anabolic pathways, including nutrient intake, protein and lipid synthesis, as well as catabolic pathways such as autophagy. Protein synthesis is the best characterised process downstream of ToRC1. ToRC1 also promotes lipid synthesis, which is required for proliferating cells to generate membranes. All these biosynthetic pathways require a significant energy input. ToRC1 promotes ATP production by stimulating glycolysis, which generates the activated carrier molecules ATP and NADH from sugars. ToRC1 also regulates catabolic pathways. Autophagy is the main degradative process in cells. It supports normal cellular function by recycling damaged proteins and organelles, and it promotes survival when nutrient levels are low, by generating new building blocks from degraded cellular components. ToRC2 is insensitive to acute treatment with rapamycin and to nutrients, but does respond to growth factors such as insulin. ToRC2 regulates multiple cell functions, such as survival, growth, proliferation and motility. To achieve this, ToRC2 controls several AGC kinase family members, including Akt, Sgk (serum and glucocorticoid induced kinase) and Pkc (protein kinase c).

I-7 MAMMALIAN AND DROSOPHILA INTESTINE

Intestinal Architecture and Cell Types

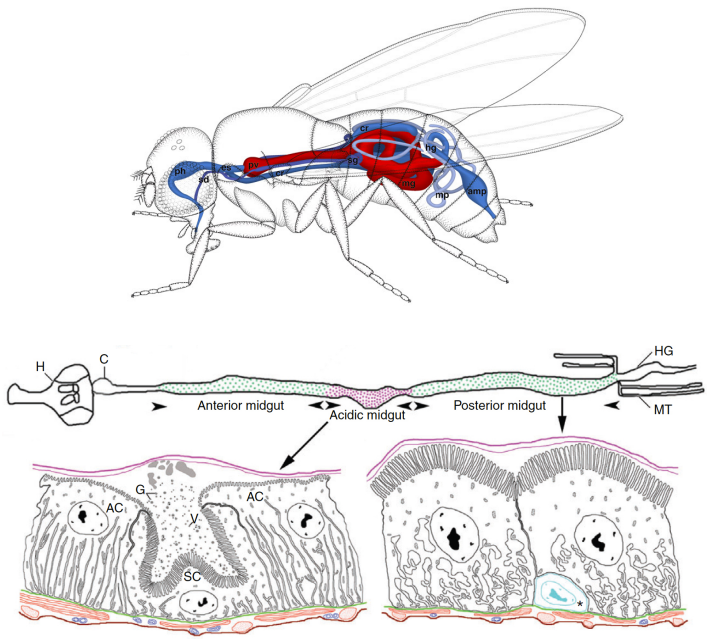
The development, growth, regeneration and reproduction of every animal relies on constant structural and functional maintenance of its tissues and organs. Homeostasis of most adult organs depends on small populations of tissue specific stem cells that can self-renew and at the same time generate differentiated cells to replace the aged or damaged ones. Mammalian cellular repertoire comprises more than 200 different terminally differentiated cell types, who exhibit great lifespan variability. Examples include the intestinal epithelium (about 5days), erythrocytes (about 120days), heart muscle cells, lens cells and most neurons (life long). One of the most rapidly renewing tissues is the lining of the small intestine, outpacing all other tissues in the vertebrate body. The high turnover rate, the structural and functional conservation across species, and the availability of corresponding genetic tools make the intestinal epithelium a very attractive and tractable system for the study of cell proliferation and differentiation, and cell-niche interactions.

A specialised simple epithelium lines the intestinal lumen and performs the primary function of digestion and nutrient absorption. The mammalian intestine is composed of proliferative crypts (harbouring the stem cells) and villi (which contain specialised differentiated cell types). The crypts contain stem cells and their progeny, the transit amplifying (TA) cells. TA cells spend approximately two days in the crypt, during which they divide 4/5 times, before differentiating into other specialised cell types. The progeny of these dividing cells migrate upwards from the crypt base towards the surface of the villi (finger-like protrusions into the gut lumen), where all the cells seem to be fully differentiated [Wright 1984]. Three types of differentiated epithelial cells cover these villi: absorptive enterocytes, mucus-secreting goblet cells, and hormone-secreting enteroendocrine cells.

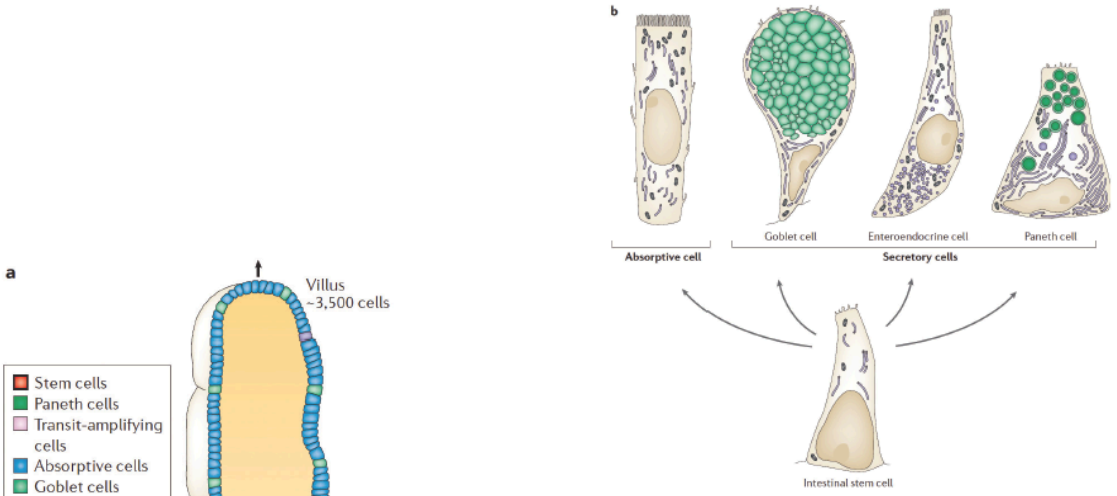
The drosophila intestinal (midgut) epithelium bares close resemblance to the mammalian intestine, in structural organisation as in signaling mechanisms responsible for tissue homeostasis and regeneration. The availability of powerful genetic tools combined with a relatively simple tissue architecture have allowed the identification of several stem cell population retained during adulthood, as well as transient stem cell population functioning during drosophila development. The drosophila midgut is maintained throughout adulthood by about 1000 intestinal stem cells (ISCs) [117,118]. The epithelium has an apico-basal polarity and is

composed of a pseudostratified layer of cells projecting into the gut lumen. The ISCs reside on the basal surface in direct contact with the basement membrane, which separate the gut epithelium from the surrounding visceral muscles. The ISCs are the only midgut epithelial cells that undergo mitosis under normal homeostatic conditions. ISCs divide asymmetrically to generate new stem cells and transient progenitors called enteroblasts (EBs). The immature EBs commit towards differentiation and produce absorptive enterocytes (ECs) and hormone-secreting enteroendocrine (EE) cells. As they mature, ECs grow and endoreplicate their genomes several times, reaching ploidy levels of 32c or more, and take up the bulk of the intestinal epithelium. A recent study suggested that EEs are not formed from Su(h)+ EBs, but rather from a different pre-EE progenitor, and that the transcription factor Prospero is essential for ISC commitment toward the EE fate [302].

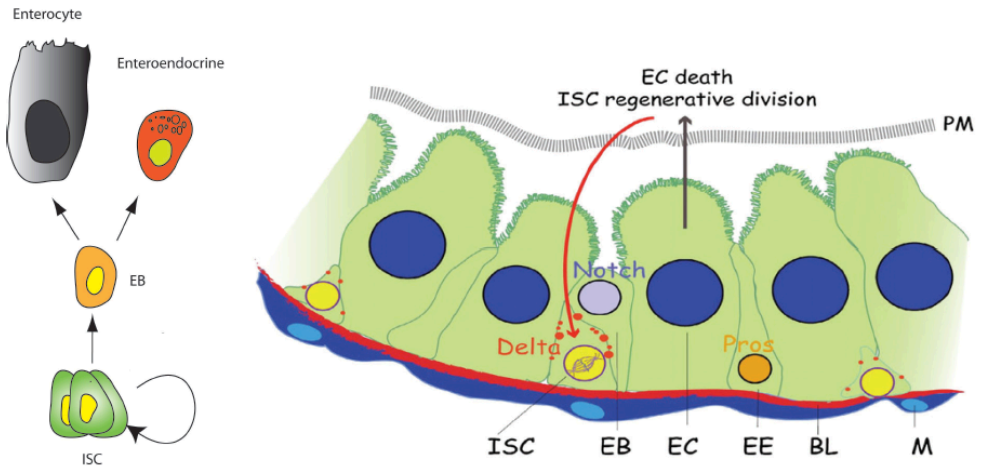
The different cell types in the drosophila midgut can be identified based on morphology and expression of specific marker genes. ISCs have a small nucleus, are diploid, and express the Notch ligand Delta. EBs are diploid, have a small nucleus, and express Su(h) activated Notch target genes, such as the transcriptional reporter Su(h)GBE-LacZ. ECs are polyploid, have a large nucleus and express the transcription factor Pdm1 and the non-muscle myosin 1A. EEs are diploid, have a small nucleus, and express the transcription factor Prospero. The midgut epithelium is enveloped on the outside by two layers of mesodermally-derived visceral muscle formed by orthogonally oriented actin-myosin fibres, whereas the inside of the midgut is coated by a chitinous peritrophic membrane, constituting a barrier against ingested food and microorganisms [328].



Top, digestive system of the adult Drosophila, dorso-lateral aspect. Bottom, line diagram of the adult gut showing the intraluminal pH in various midgut regions; and the general organisation of the adjacent epithelial cells showing the arrangement of the peritrophic membrane {in magenta}, epithelial cells {in black}, basement membrane {in green}, muscle fibres {in red}, stem cells {in light blue} and serosal barrier {in brown}. ph~pharynx, pv~proventriculus, sg~salivary glands, mg~midgut, hg~hindgut, mp~malpighian tubules, es~oesophagus, cr~crop, gc~gastric caeca, ac~absorptive cell, g~granules, v~vesicles, sc~secretory cell, h~head, c~cardia. Top. Hartenstein, Atlas of Drosophila development, 1993. Bottom: Shanbhag and Tripathi, J. Exp. Biol. 2009



Distribution of epithelial cell types in the mammalian small intestine. A villus with one of the crypts that contribute to self-renewal of its epithelium. Stem cells lie at the crypt base mixed with the Paneth cells. Above the stem cells are transit amplifying cells (dividing progenitors, some of them already partially differentiated); and above these, in the neck of the crypt and on the villus, lie post-mitotic differentiated cells (absorptive cells, goblet cells and enteroendocrine cells). There are four classes of terminally differentiated cells. Absorptive cells have a brush border (a dense array of microvilli) on their apical surface. The other three classes are all secretory: goblet cells secrete mucus, enteroendocrine cells are smaller and secrete various gut hormones, and paneth cells secrete antibacterial proteins. Crosnier et al. Nat. Genetics 2006



Schematic of the adult drosophila midgut. ISCs in the adult drosophila proliferate throughout the life of the animal, renewing themselves and also generating transient cells called enteroblasts {EB}, which can differentiate directly into enterocytes {EC} or enteroendocrine cells {EE} without division. ECs are large, polyploid absorptive cells, that comprise the bulk of the intestinal epithelium. The Pdm1 TF and non-muscle Myosin 1A are specific markers for differentiated ECs. EEs are small secretory cells that express neuroendocrine markers such as prospero {pros}, and hormones {allatostatin, tachykinin} which drive peristalsis by controlling the contraction of a sheath of visceral muscle {VM} that surrounds the intestine. The drosophila midgut maintains about 2000 ISCs and EBs that can be identified by their small size and basal location, as well as by expression of escargot {esg} a TF specific to diploid progenitor cells. H. Jiang and B. A. Edgar Exp. Cell Research 2011

Intestinal Signaling Pathways

The intestinal epithelium is constantly being exposed to damaging agents including mechanical and chemical insults from the ingested food, as well as biological attacks from opportunistic microorganisms. Therefore, aged or damaged cells are constantly being replaced to ensure the structural and functional integrity of the intestinal epithelium. More than 300 million cells are produced in the mouse small intestine every day. The following paragraph briefly describes the mammalian intestine, while all following paragraphs deal with the signaling pathways underlying homeostasis and regeneration in the drosophila intestine (midgut).

Mammalian Intestine

In the mammalian small intestine, Wnt and Notch pathways jointly control the proliferation and cell fate commitment of intestinal stem cells. Components of both pathways are chiefly being expressed in the neighbourhood of the crypt base, in the stem cell region. Wnt ligands, including Wnt 3,6,9 and correspond- ing receptors frizzled 5,6,7 and LRP 5,6 are expressed by the crypt epithelial cells [329]. The same expression pattern also applies to Notch, Delta and Hes proteins [330,331]. Mammalian intestinal stem cells depend on both Wnt and Notch signals in combination to maintain their proliferative state. Furthermore, Wnt signaling seems to be able to switch on Notch activity, whereas the converse does not seem to apply [332,333]. Possible explanation for the prevalence of intestinal cancers caused by Wnt pathway mutations, as compared to Notch mutations. With regard to cell fate commitment, Wnt has been reported to impart progenitor cells with the potential (but not the obligation) to fallow a secretory fate [334,335]. Notch signaling, on the other hand, promotes secretory fate commitment [332,333]. Thus, within the Wnt activated population, some cells express Dl and escape Notch activation (Wnt+Notch-) and become committed to a secretory fate, while others fail to express Dl and have Notch activation imposed on them (Wnt+Notch+) and continue to divide without differentiating, whereas Wnt-Notch+ cells are converted to absorptive enterocytes [336].

Notch Pathway

The effect of Notch signalling in mediating asymmetric stem cell divisions and favouring absorptive fate commitment is conserved in the vertebrate and fly intestine. However, Notch influence on ISC self-renewal and proliferation is opposite in the two systems. Notch activation in the drosophila midgut inhibits ISC proliferation and promotes EC differentiation, whereas loss of Notch activity in progenitors blocks EC production and leads to an expansion

of ISC-like and EE cell populations [117,118]. Midgut progenitors and early differentiated cells all express the Notch receptor, but the Delta ligand is expressed only in the ISCs [118,337]. Midgut ISCs delivering high levels of Notch signalling to their EB daughters drive them into the EC cell fate, while low (or absent) Notch activation supports ISC self-renewal or EE fate specification [337].

Wingless Pathway

Similar to the mammalian system, but less dramatic, drosophila Wnt/Wg pathway activation promotes ISC proliferation in the midgut, while its disruption results in ISCs that divide more slowly and turn-over faster compared to wild-type [338,339]. However, these effects are much milder compared to the phenotypes evoked by other pathways with a mitogenic function in the midgut, like Jak/Stat or Egfr. Wg ligand expressing foci include the foregut/midgut and the midgut/hindgut boundaries, as well as the visceral muscle surrounding the midgut [338,340].

Jak/Stat Pathway

One other especially important signaling pathway in the midgut is Jak/Stat (Janus Kinase/ Signal Transducer and Activator of Transcription). It regulates stem cell growth, proliferation and differentiation. Major components of the Jak/Stat pathway in drosophila include three leptin-like cytokines (unpaireds Upd, Upd2, Upd3) which bind to a transmembrane receptor (domeless) and thereby stimulate drosophila Jak (hopscotch) to phosphorylate both the receptor and other Jak molecules. The resulting phosphorylated receptor-Jak complexes form binding sites for drosophila Stat (Stat92e) which also becomes activated by phosphorylation and translocates into the nucleus as dimers to regulate target gene expression [122,341]. Jak/Stat acts as a differentiation factor stimulating both EC and EE cell fate commitment. In fact, midgut stem cell clones mutant for Jak/Stat pathway fail to undergo normal differentiation [119,342]. Another important functional role of Jak/Stat regards its involvement in midgut regeneration upon damage. Various types of tissue damage or bacterial infection trigger cytokine expression in the midgut which leads to Jak/Stat activation in the progenitor population stimulating both ISC proliferation and EB differentiation [50,51,119,121]. At the same time, damaged ECs induce Jak/Stat activation in other neighbouring ECs, triggering production of antimicrobial peptides like Drosomycin 3. Upd, Upd2 and Upd3 are secreted by ECs in the affected epithelium, with Upd being produced also by progenitors and the visceral muscle, leading to Jak/Stat activation in the progenitor compartment, stimulating their proliferation

and differentiation in order to repopulate the damaged epithelium [119,121,122,131]. In contrast to the requirement for Jak/Stat pathway activity during midgut regeneration, its function is not essential for stem cell proliferation in healthy animals. However, Jak/Stat pathway activity is a constant presence in the progenitor population, which together with its essential role in cell fate commitment highlight its importance for normal homeostasis and hint towards additional functions it might perform in the midgut.

Ras-Mapk Pathway

A highly conserved mitogenic pathway operating in the midgut is the Egfr-Ras-Mapk pathway. Egfr signaling is one of the central pathways coordinating cell remodelling during development in a broad range of multicellular organisms, with diverse implications in regulation of cell proliferation, differentiation and survival in several Drosophila tissues [61]. Ras-Mapk was found to be a key regulator for the proliferation of adult midgut progenitors (precursors of midgut epithelial cells, including ISCs) during larval development [50,51]. In the adult midgut, Ras-Mapk signaling is essential for ISC proliferation and maintenance [51-53,129]. Midguts stained with antibodies against di-phospho-ERK, the activated Mapk, revealed Egfr/Mapk activity specifically in the ISCs and EBs. Furthermore, various types of epithelial damage or infection trigger the expression of several Egfr ligands in the Drosophila midgut. Thus, Spitz and Keren are expressed in ECs, as well as proteases called Rhomboids which cleave and activate these Egfr ligands. Another Egfr ligand, called Vein, is expressed in the visceral muscle and acts redundantly with the other ligands to activate Egfr signaling in ISCs and regulate their proliferation, as well as in ECs where it is required to coordinate delamination and anoikis upon infection. The Jak/Stat and Egfr pathway synergize in ISCs to promote their proliferation. In fact, these pathways are capable of activating each other's ligands. Upd2 and Upd3 expressed by damaged ECs lead to Jak/Stat activation in the muscle and positively stimulate growth factor Vein production, indirectly regulating ISC proliferation [52,119,121]. Similar to Jak/Stat signaling, the Egfr pathway is required for ISC maintenance and division; but unlike Jak/Stat, Egfr signaling is not directly involved in cell fate specification or differentiation toward ECs or EEs. Epistasis analyses reveal that Egfr signaling is required for positive stimulation of ISC proliferation upon Jak/Stat activation, Jnk signaling, as well as for the expansion of the progenitor pool observed after Notch inhibition [51-53]. Egfr's function during development and homeostasis seems also to be conserved in the mouse intestine [343]. Furthermore, the Egfr pathway is frequently activated in cancer, making it a prime target for cancer

therapy. Various combinatorial approaches employing Egfr and Pi3k inhibitors in conjunction with traditional chemotherapy are currently being tested [344,345].

Jnk Pathway

Two additional pathways essential for a proper regenerative response to various types of damage or infection of the midgut epithelium are Jnk (Jun N-terminal Kinase) and Hippo pathways. Although the initial damage sensing mechanisms are not completely understood, Jnk pathway activation and Hippo pathway inactivation both stimulate cytokine and growth factor expression, leading to Jak/Stat and Egfr/Mapk activation and thereby initiating a regenerative response. A variety of environmental challenges, including oxidative stress, damage the intestinal epithelium and activate Jnk signalling which is involved in compensatory cell proliferation, aiding regeneration and increasing stress tolerance and longevity in flies and worms. Furthermore, Jnk also promotes a proliferative response fallowing injury in mammals, and it was demonstrated to be required in ISCs to maintain gut homeostasis in aging flies [123,346]. Jnk pathway activation in the midgut is part of a complex response tailored to the type of sustained damage. Thus, direct damage to the midgut epithelial cells by bacteria such as Pseudomonas entomophila, activates Jnk mostly in ECs, whereas oxidative stress triggered by chemicals like paraquat or bleomycin, or by bacteria like Ecc15 lead to Jnk activation in both the ECs and the progenitor population [119,121,122,131]. EC-specific activation likely facilitates their removal from the epithelium through a caspase-independent mechanism stimulating ISC division (prolonged Jnk activation leads to significant cell death), whereas stem cell-specific activation upregulates stress-response genes to deal with the oxidative damage [347]. Among Jnk downstream effectors are AP1 transcription factors including Jun and Fos which promote its mitogenic effect [53]. A Fos homolog in Drosophila, Kayak, is required by both Egfr and Jnk to stimulate ISC proliferation, suggesting that these two pathways may converge on the AP1 transcription factors to mediate their effects on stem cell proliferation [123]. Intrinsic and environmental challenges contribute to the loss of tissue homeostasis in aging animals, mainly due to a decline in the regenerative capacity of the resident stem cells [348]. As an integral part of this process, increased Jnk activity in the progenitor population contributes to the loss of homeostasis in the aging drosophila intestine, by promoting excessive proliferation and defective differentiation, due to ectopic Notch activation caused by a failure to suppress Dl expression in stem cell daughters [53,123]. A tight control of this pathway is essential for maintaining a proper balance between stem cell proliferation and tumor suppressor mechanisms carrying an anticancer function. Such a control is partially ensured in healthy

animals through expression of a Jnk feedback inhibitor called puckered, preventing excessive ISC proliferation [123]. However, in aged or stressed animals, this balance is gradually lost, increasing the likelihood of neoplastic transformation.

Hippo Pathway

Another evolutionarily conserved pathway regulating cell growth, proliferation and survival, is the Hippo pathway. It is implicated in organ size control, tissue regeneration, stem cell self-renewal, and its deregulation is associated with several types of cancer [349,350]. Hippo pathway acts through a kinase cascade, whereby the sterile-20-like kinase Hippo forms a complex with the ww domain adaptor protein Salvador. Hippo phosphorylates and activates the DBF family kinase Warts, which forms a complex with the adaptor protein Mats. Warts phosphorylates the transcriptional co-activator Yorkie, which is then bound by 14-3-3 proteins and excluded from the nucleus. When in the nucleus, Yki collaborates with transcription factors like Scalloped, Homothorax and Teashirt, activating transcription of growth-promoting and apoptosis-inhibiting genes, like bantam, CycE, Diap1, Expanded and Four jointed. Upstream of these kinases is the cadherin Fat and the Ferm domain proteins Expanded and Merlin, and a ww domain protein called Kibra that promotes Expanded-Merlin interactions, enhancing Hpo signaling [351,352]. Hippo's extensive functional repertoire also includes a central role in regulating intestinal stem cells in drosophila. While under normal homeostatic conditions, Hippo restricts ISC proliferation in the adult midgut, its inactivation upon tissue damage triggers, in a similar manner to Jnk pathway, both cytokine and growth factor expression leading to Jak/Stat and Egfr/Mapk pathways activation in ISCs, respectively, where they function synergistically to promote proliferation [349,353]. Hippo pathway inactivation (or Yki overexpression) in the ISCs or the differentiated ECs stimulates stem cell proliferation (more strongly in case of ECs), accompanied by a correspondingly increased differentiation rate. Intestinal stress upregulates Yki activity both in the progenitor and enterocyte populations as well as Yki target gene expression [353,354]. Furthermore, Yki activity is essential in the progenitors in order to mount a proper proliferative response to bacterial infection. Hippo pathway inactivation in turn, triggers expression of cytokines and growth factors further propagating the signaling response to damage (mentioned above). Increased Yki activity upon damage, together with its ability to promote non-autonomous cell proliferation when expressed in ECs, suggest that Hpo functions as a stress detection system in the midgut, sensing perturbations in cell adhesion or structural integrity and setting in motion a regenerative response accordingly [353,354]. The Hpo pathway seems to be structurally and functionally conserved also in the

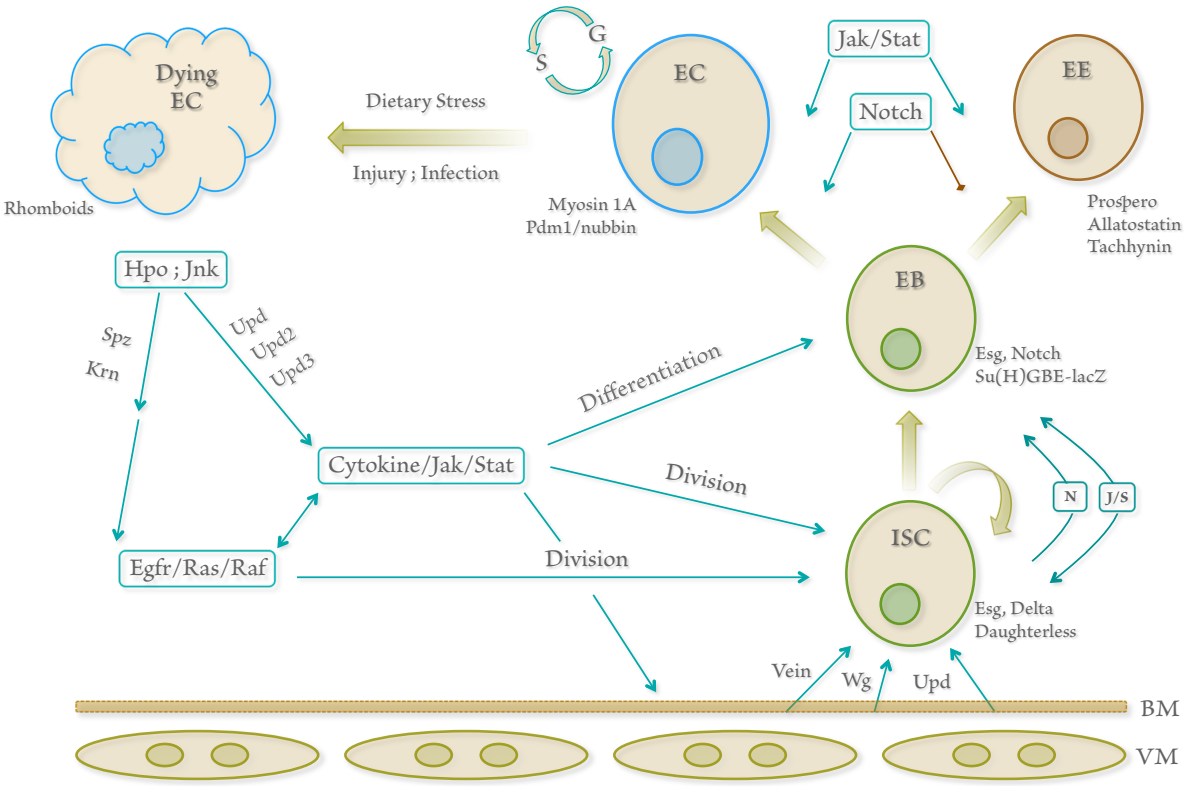
mammals, where Yap (Yki orthologue) has been shown to positively stimulate ISC proliferation and crypt formation, as well as being required for proper regeneration fallowing chemical-induced damage [355].

Redox Regulation

One important factor regulating ISC proliferation in the midgut is the dynamically maintained redox state. Two key players mediating such regulation are Nrf2 and its negative regulator Keap1, together ensuring low ROS (reactive oxygen species) levels in ISCs under basal homeostasis and a corresponding increase in ROS upon damage. High ROS levels over an extended period, however, contribute to ectopic proliferation and misdifferentiation of progenitors, culminating with the loss of tissue homeostasis [356].

Additional Pathways

More layers of regulation are achieved through additional pathways, including Bmp/Tgfb, Hedgehog, InR/Tor and PDGF/VEGF receptor (PVR) signaling. For example, one PVR ligand (Pvf2) stimulates ISC proliferation in aged midguts, whereas InR/Pi3k signalling is a known activator of cell growth and proliferation in healthy guts, as in regenerating guts following damage [55,121,123,357]. Befitting as a major growth promoting pathway, ToRC1 signalling also proved to be essential for ISC maintenance and differentiation in the drosophila midgut. ToRC1 activity is believed to be low in ISCs and high in EBs, due to Notch-mediated suppression of Tsc2. ToRC1 activity in ISCs is kept low to prevent their overgrowth and precocious differentiation, while higher ToRC1 activity in EBs supports their growth and differentiation into ECs. EE cell formation requires low ToRC1 activity [55-57].



Feedback mechanism regulating adult midgut homeostasis and regeneration in drosophila. As in humans, differentiated ECs and EEs are short lived and turn-over roughly weekly, though this rate varies greatly according to diet, enteric microbiota, and age. Mature midgut cell loss triggers activation of the Jnk pathway and inactivation of the Hippo pathway in the ECs, both of which are able to induce the expression of mitogenic cytokines (Upds) and growth factors (Egfs) in the damaged midgut. The cytokines and Egfs, in turn, activate the Jak/Stat and Egfr/Ras/Mapk pathways respectively in the progenitor cells, where these stimuli function synergistically to promote ISC division. In addition, the Jak/Stat signaling also promotes EB differentiation. Thus, in response to damage, the ISCs rapidly generate new midgut cells to maintain epithelial homeostasis.

I-8 AIM OF STUDY

The evolutionary conserved Ras-Mapk pathway has been associated with cell growth and proliferation in multiple cell types and organisms [3,9,69,70,151-153]. As a central regulator of these processes, Mapk is often hijacked by different cancers in mammals and flies to initiate and grow tumors, and eventually metastasise (see I-5). However, even with such wide reaching implications in development and cancer, a complete mechanism underlying Ras-Mapk growth function remains undefined. Therefore, the objectives we wanted to clarify through this study were: [1] Test if Ras-Mapk indeed functions as a growth promoter in different drosophila cells and tissues. [2] Determine if Ras-Mapk is able to activate the known metabolic driver ToRC1 (as potential intersection points have been identified in mammalian cells), and if Ras-Mapk driven growth depends on ToRC1 activity. [3] Identify any additional mechanisms through which Ras-Mapk may drive growth. As potential candidates, the Mapkapks (Mapk activated protein kinases) attracted my interest as they are widely used in varied systems as secondary effectors of Mapk modules.

In this study, I employed genetic and molecular tests to reveal that Mapk can indeed promote growth in insect cells, in the adult intestine and in the developing (larval) intestine (even under nutrient starvation). Cell culture assays confirmed that Ras-Mapk can stimulate ToRC1 activity, whereas intestinal assays uncovered a partial dependency of Ras-Mapk driven growth on ToRC1 activity. Consistently, both Erk and one of its targets (Rsk) were found to positively regulate ToRC1 in mammalian cells [30-36]. Importantly, I also identified three Mapk activated kinases that function as new growth effectors for the Ras-Mapk module in insect cells. These Mapkapks appear to be required for cell growth under normal and growth factor stimulated conditions.

II. RESULTS

II-1 NEW MAPK GROWTH EFFECTORS IN INSECT CELLS

II-1A Mapk Pathway Activity is Required for Normal Insect Cell Growth in Culture

context The canonical ToR pathway has been previously shown to control cell growth in drosophila S2 cells [1,2], S2R+ cells [3], and in vivo [4,44,45]. ToR was found to function downstream of insulin initiated receptor tyrosine kinase signaling to control drosophila cell growth in vivo [4,44,45]. Insulin drives drosophila cell growth in vivo [5], as well as in culture [6-8]. However, whereas insulin stimulates drosophila S2R+ cell growth in culture, RNAi mediated knockdown of insulin pathway components (InR, Irs, p60 or p110) does not affect the cell size [3]. This indicates that the insulin pathway is not rate-limiting for size control in this cell line. On the other hand, Pvr-Ras signaling pathway was found to affect the size of drosophila cells in culture. As part of a screen, dsRNAs targeting Pvr (Pdgf/Vegf receptor), Sos, Grb2, Ras, Ksr were found to reduce S2R+ cell size in culture [3]. Moreover, insulin stimulation of schneider cells has been observed to activate not only Akt, but also the Erk pathway [7,8]. AIM In light of these findings, we wanted to closer investigate the role Ras-Mapk pathway plays in regulating drosophila cell growth.

approach For this purpose, cultured S2R+ schneider cells were treated with growth factors and/or inhibitors for different Ras-Mapk pathway components. Modulators of ToR pathway activity were used in parallel as positive controls. Changes in proliferation and cell size (mean diameters) were subsequently measured for all treatment conditions over time. Parameters were acquired using the Nexcelom cellometer system, which employs image based cytometry for cell analysis. The schneider cells were cultured in medium with or without serum, and pretreated with kinase inhibitors for 40min prior to addition of various growth factors.

results Cell culture treatments with kinase inhibitors for Mek (U0126, 100 uM) or Erk (GDC0994, 50 uM), in absence of serum, significantly lowered the proliferation and average cell sizes compared to untreated control samples (figure 1A,B). The same proliferation and cell size impairments were observed upon treatment with these Mapk(k) inhibitors in the presence of

1% serum (figure 2A-C). Cell culture treatments with ToRC1 inhibitor rapamycin (10 uM) or with ToRC1,2 inhibitor torin1 (10 uM), as positive controls, in the presence or absence of serum, similarly reduced proliferation and average cell sizes compared to untreated controls (figures 1A-B, 2A-C). Treatment of the S2R+ cell cultures with Pdgf (platelet derived growth factor, 1ug/ml) or with Vegf (vascular endothelial growth factor, 1ug/ml) in the presence or absence of serum did not significantly affect growth rates relative to untreated controls (figures 1C-D, 2D-F). Consistent with previous data, pre-treatment of cell cultures, in the presence or absence of serum, with Mapk pathway inhibitors for Mek (U0126, 100 uM) or Erk (GDC0994, 50 uM), prior to addition of Pdgf/Vegf growth factors (1ug/ml), visibly lowered the proliferation rates and average cell sizes compared to untreated or growth factor-only treated controls (1C-D, 2D-F). Similarly, pre-treatment with ToR pathway inhibitors rapamycin or torin, also reduced the growth rates of S2R+ cells in the presence or absence of Pdgf/Vegf growth factors (1C-D, 2D-F). All together, these data indicate that Mapk pathway activity is required for normal growth of S2R+ cells in culture.

Il-1B Mnk, Rsk, Msk are Required for Normal and Insulin induced Insect Cell Growth

context Mapks are evolutionary conserved Ser/Thr kinases that control essential cellular functions, including gene expression, mitosis, metabolism, mobility, survival, apoptosis and differentiation [9]. These functions are performed through phosphorylation of many substrates, among which are members of a Ser/Thr kinase family termed Mapk activated protein kinases (Mapkapk) [10,11]. Two of them were found to be involved in the control of translation, namely the Mnks (Mapk interacting kinases) [12] and the Rsks (p90 ribosomal S6 kinases) [13]. The drosophila Rsk ortholog was shown to be involved in modulation of circadian behaviour and memory formation [14-16]. Drosophila homologue for Mnk1,2 is called Lk6, and was shown to be important for eIF4E phosphorylation, developmental rate and organismal size [17-19]. Drosophila homologue for another Mapkapk (Msk) is called Jil1, it is essential for viability, and it functions to maintain euchromatic domains while counteracting heterochromatinisation and gene silencing [20,21].

aim Investigate a potential role for Mapk activated protein kinases in regulating insect cell growth in culture. Furthermore, are these kinases involved in driving cell growth downstream of established growth promoters, such as the insulin pathway?

approach S2R+ cells were cultured in standard insect medium supplemented with 1% serum. Cultures were then either left untreated (controls) or pre-treated with various kinase inhibitors and further incubated for 40min prior to addition of insulin or Pdgf growth factors. Modulators of ToR pathway activity were used in parallel as positive controls. Changes in proliferation and cell size (mean diameters) were subsequently measured for all treatment conditions over time.

results Addition of Mapkapk kinase inhibitors to growing S2R+ cells in culture strongly reduced their proliferation and average cell sizes compared to untreated controls. Such cell growth impairment phenotypes were observed upon treatment with the Mnk inhibitor CGP57380 (500 uM), Rsk inhibitor BID1870 (500 uM) and with the Msk inhibitor Ro318220 (500 uM). The Msk inhibitor proved to be the most effective at reducing cell sizes. Treatment with the ToRC1 inhibitor rapamycin (10 uM), as a positive control, also consistently reduced cell proliferation and sizes (figure 3A-C). Insulin stimulation (10 ug/ml) of the cultured schneider cells triggered an increase in proliferation and cell size compared to untreated control, as previously shown (Lizcano et al 2003). Surprisingly, pre-treatment with Mnk/Rsk/Msk inhibitors invoked similar cell proliferation and size reduction effects even in the presence of insulin treatment, compared to untreated or insulin-only treated controls. As before, pre-treatment with the ToRC1 inhibitor rapamycin, as a positive control, consistently impaired cell growth (figure 3D-F). Stimulation of S2R+ cell cultures with Pdgf (platelet derived growth factor) had no significant effect on cell proliferation and average sizes. Consistent with previous data (3A-F), pre-treatment of the cell cultures with Mnk/Rsk/Msk inhibitors markedly lowered cell proliferation and average cell sizes, regardless of whether Pdgf growth factor was added or not (figure 3G-I). Taken together, these data highlight an important role for the Mnk/Rsk/Msk Mapk activated kinases in the control of homeostatic and insulin-induced insect cell growth in culture.

II-1C Mnk, Rsk and Msk are Required for Normal and Egfr induced Insect Cell Growth

context/aim Previous experiments have revealed a determining role for three Mapkapk kinases in the control of drosophila cell growth in culture (see II-1B). Furthermore, the growth promoting function of the established insulin pathway also appears to depend on the activity of these kinases. To further investigate the role of Mapk pathway and of Mapk activated protein kinases in growth control, cultured cells were stimulated for native Egfr signalling and concomitantly treated with various Mapkapk kinase inhibitors. The scope of these assays be-

ing to address if native activation of drosophila Egfr pathway promotes cell growth in culture, and to establish the requirement of Mapk activated protein kinases for Egfr-driven growth.

approach Schneider S2 cells stably expressing endogenous drosophila Egf receptor [22] were cultured in standard medium supplemented with 1% serum. Cultures were then either left untreated (controls) or pre-treated with various kinase inhibitors and further incubated for 40min prior to addition of Spitz conditioned medium. Modulators of ToR pathway activity were used in parallel as positive controls. Changes in proliferation and cell size (mean diameters) were subsequently measured for all treatment conditions over time.

results As observed in previous experiments (see figure3), treatment of cultured schneider cells with kinase inhibitors (500uM) for Mnk (CGP57380), Rsk (BID1870), or Msk (Ro318220) reduced the average cell sizes compared to untreated control (figure 4A,B). ToRC1 inhibition using rapamycin (10 uM) likewise reduced average cell sizes relative to untreated control. Stimulation of the cultured S2 cells with native drosophila Egfr ligand Spitz increased the average cell size compared to untreated control. Pre-treatment with kinase inhibitors for Mnk/Rsk/Msk, however, consistently reduced the average cell sizes in both untreated and Spitz-stimulated cell cultures (figure 4C,D). Taken together, these results further suggest an important role for Mnk/Rsk/Msk kinases in the control of cell size downstream of the Egfr pathway in drosophila schneider cells.

In a different experiment, schneider S2 cells were pretreated with rapamycin (10 uM) for 30min, before insulin or Egf growth factor (10 ug/ml) were added to the preexisting cultures. Following overnight incubation, the cells were stained with Hoechst 33342 and analysed by flow cytometry. Cell size measurements (based on forward scatter FCS values) show that insulin-stimulated cells are slightly bigger than control untreated cells. Conversely, rapamycin treated cells show a negative shift in the acquired histograms, towards smaller cell sizes (figure 5A). Egf treated S2 cells show a similar mild increase in cell sizes compared to untreated controls, whereas rapamycin treatment reproducibly induced a negative shift in the acquired histograms, towards smaller cell sizes (figure 5B). A comparison of the mean FSC values for S2R+ cell samples treated in the same manner revealed similar effects (figure 5C). Cell cycle phase distribution analysis of schneider cells stimulated as described above revealed that insulin treatment increased the proportion of cells in S and G2, while rapamycin treatment slightly increased the G1 cell population in control and Egf-treated samples (figure 5D). Compared to

native Egfr stimulation with Spitz ligand, treatments with Egf or Pdgf/Vegf growth factors proved less effective at eliciting cell size changes in culture.

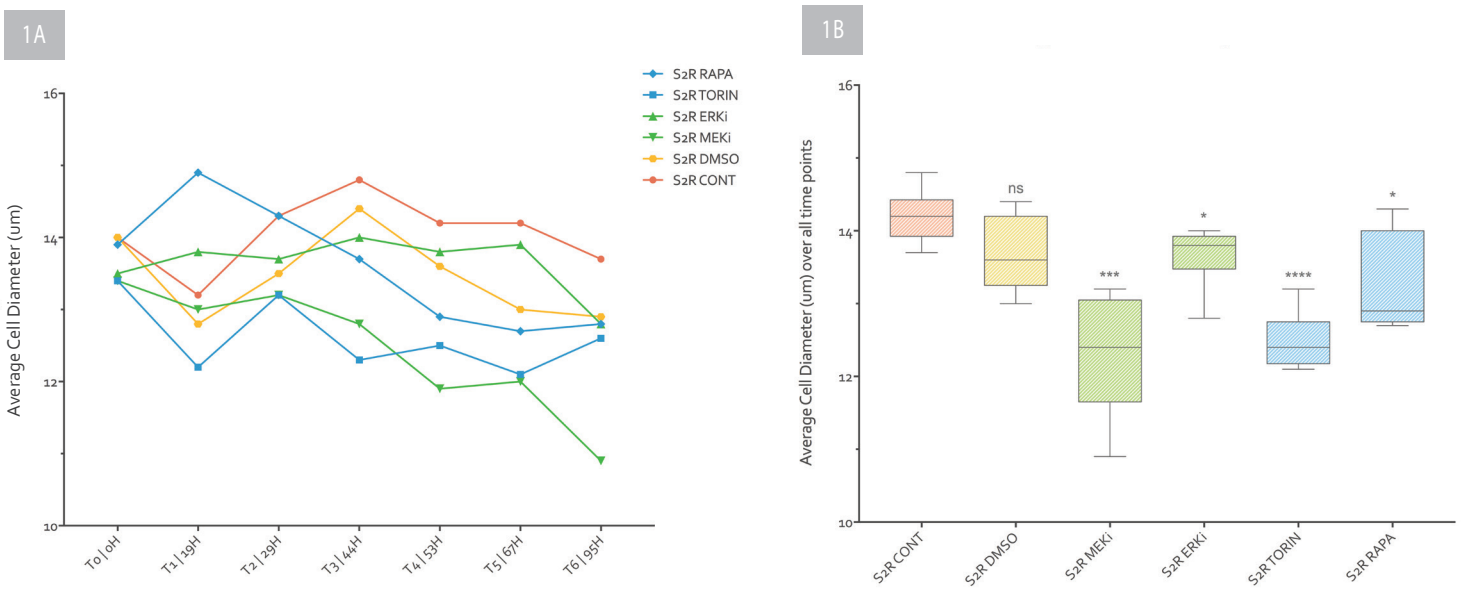
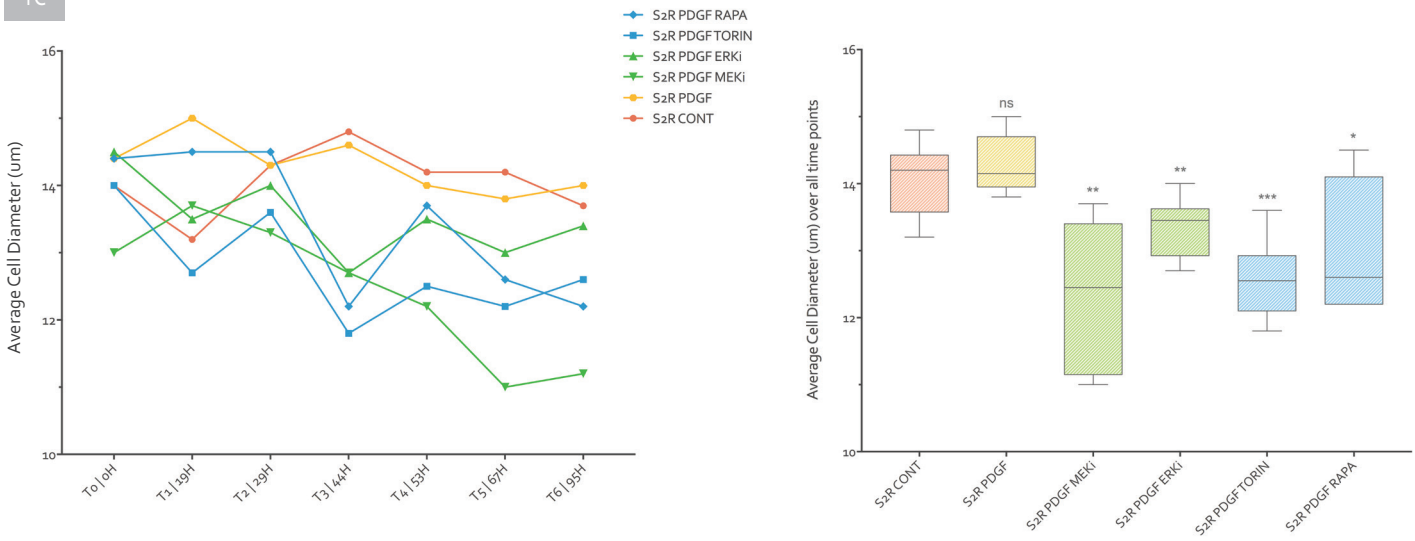


FIGURE 1 Effects of Ras/Mapk and ToR pathway inhibitors on the cell size of cultured schneider cells. Mapk pathway controls cell size in a hemocyte-like cell line. S2R+ cell cultures for all indicated conditions were established in medium without serum, and pre-treated with inhibitors for 40 min, prior to addition of various growth factors. Mean cell diameters were subsequently measured for all samples over time. Cell density and diameter measurements were made for a sample population of 1200-1600 cells for each treatment condition and timepoint.

FIGURE 1A-B Cell size (mean diameter) measurements of inhibitor treated S2R+ cells over time. 1A shows the mean cell diameters for every sample over time, and 1B averages the mean diameters for each sample over all time points. Average size of control untreated cells (S2R Cont) over time is marked in red. Average size of DMSO treated cell samples across the same timepoints is shown in yellow. The cell sizes for S2R+ cells treated with Meki (U0126 Mek inhibitor 100 uM) or Erki (GDC0994 Erk inhibitor 50 uM) are marked in green. Rapamycin (ToRC1 inhibitor 10 uM) and torin (ToRC1,2 inhibitor 10 uM) treated samples are illustrated in blue. Error bars indicate the standard error of the mean. Statistical significance relative to untreated control was determined by student's t test (*p<0.05,**p<0.01,***p<0.001,****p<0.0001).

Treatment of S2R+ cell cultures with Map kinase inhibitors for Mek or Erk significantly lowered the average cell sizes compared to control samples. Treatment with ToR pathway inhibitors rapamycin or torin (positive controls, color coded in blue) similarly reduced the average cell sizes, as shown before (Stocker et al 2003, Patel et al 2003, Sims et al 2009).

1C



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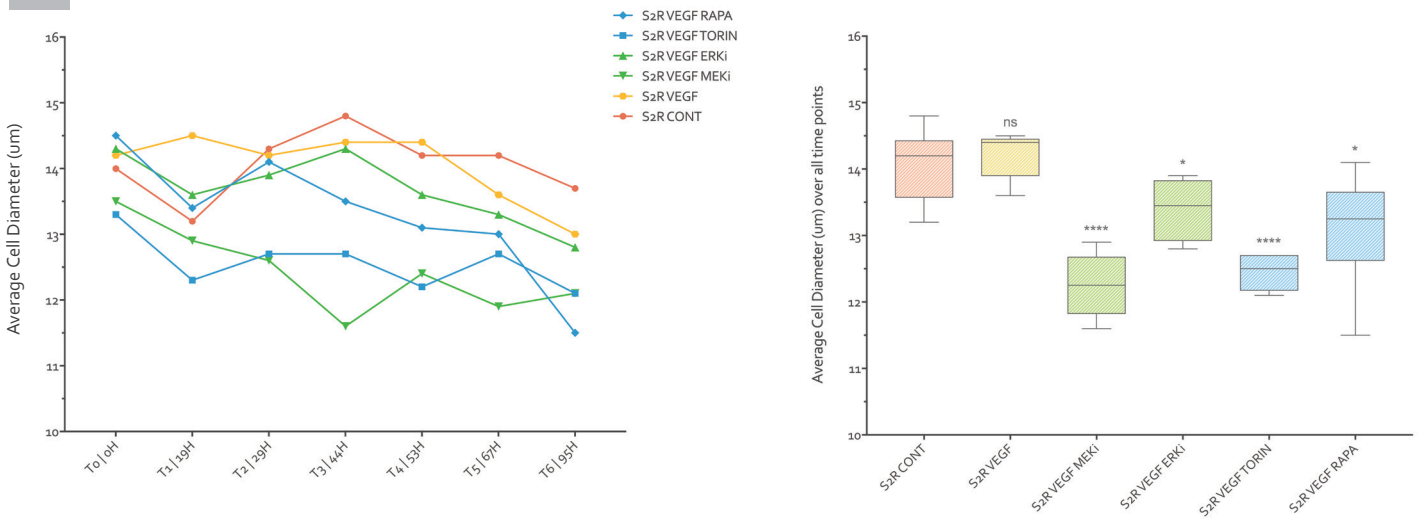


FIGURE 1C-D Effects of Ras/Mapk and ToR pathway inhibitors on the cell size of Pdgf/Vegf growth factor treated schneider cells. Cell size (mean diameter) measurements of inhibitor and Pdgf/Vegf treated S2R+ cells over time (Pdgf/Vegf = platelet derived/vascular endothelial growth factor 1ug/ml). 1C illustrates average cell sizes for Pdgf and inhibitor treated samples over time. 1D shows average cell sizes of Vegf and inhibitor treated samples over time. Average size of control untreated cells (S2R Cont) over time is marked in red. Average size of Pdgf/Vegf treated cell samples across the same timepoints is shown in yellow. The cell sizes for S2R+ cells treated with Pdgf/Vegf and Meki (U0126 Mek inhibitor 100 uM) or with Pdgf/Vegf and Erki (GDC0994 Erk inhibitor 50 uM) are marked in green. Samples treated with Pdgf/Vegf and rapamycin (ToRC1 inhibitor 10 uM) or with Pdgf/Vegf and torin (ToRC1,2 inhibitor 10 uM) are illustrated in blue. Error bars indicate the standard error of the mean. Statistical significance relative to controls was determined by student's t test (*p<0.05,**p<0.01, ***p<0.001,****p<0.0001). Treatment with the Mek or Erk inhibitors (green curves and bars) triggered as before a reduction in the average cell sizes compared to controls (marked in red and yellow). Treatment with ToR inhibitors (marked in blue) revealed similar effects.

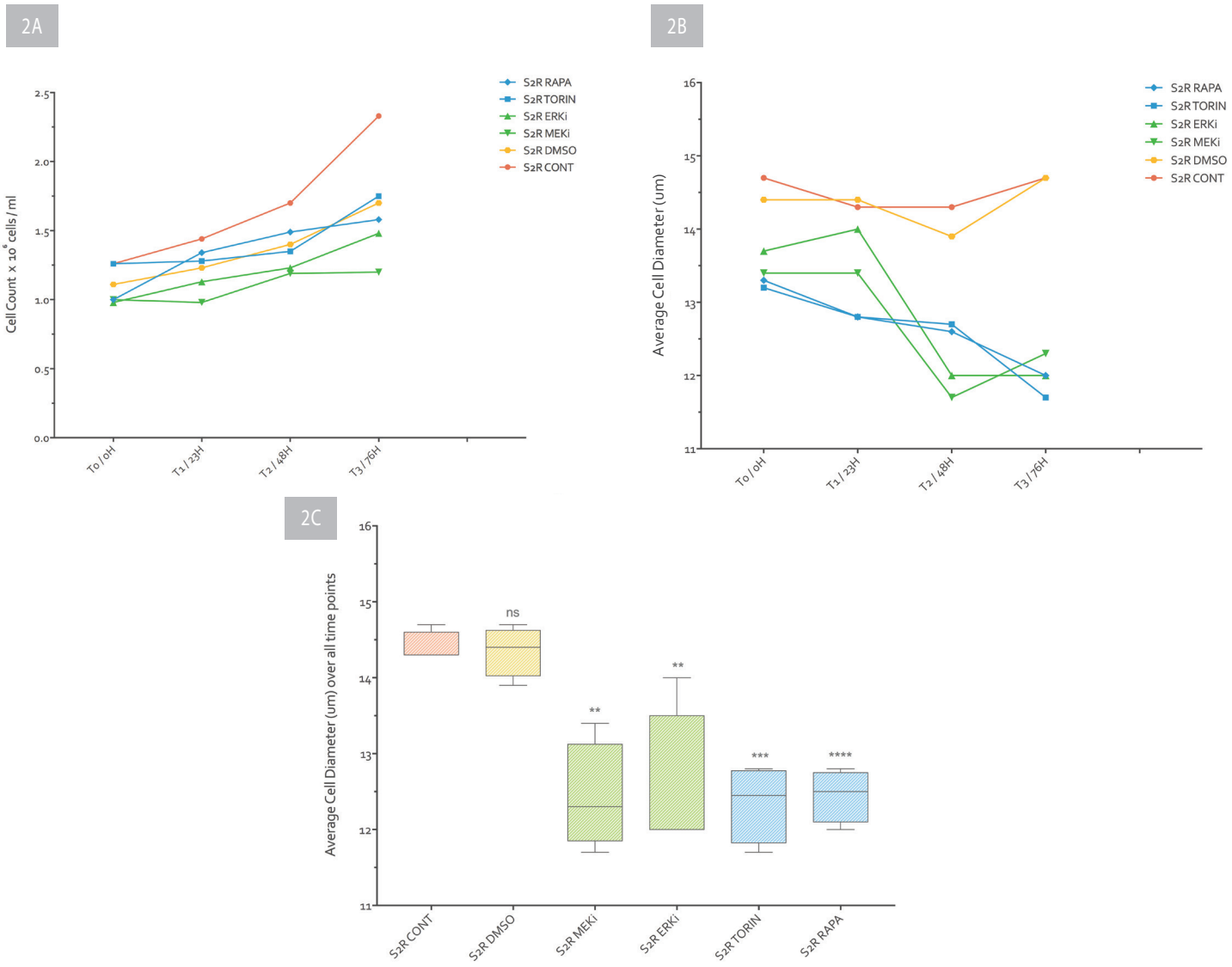


FIGURE 2 Effects of Ras/Mapk and ToR pathway inhibitors on the cell size of serum and growth factor fed schneider cells. Mapk pathway activity is required for growth of cultured insect cells. S2R+ cell cultures for all indicated conditions were established in medium with 1% serum. Cell cultures were pre-treated with the indicated inhibitors for 40 min, prior to addition of Pdgf (platelet derived growth factor). Cell proliferation and mean diameters were subsequently measured for all samples over time. Cell density and diameter measurements were made for a sample population of 1200-1600 cells for each treatment condition and timepoint.

FIGURE 2A-C Cell proliferation and size (mean diameter) measurements of inhibitor treated S2R+ cells over time. 2A displays cell counts for each treatment over time, 2B shows the mean cell diameters for every sample over time, and 2C averages the mean diameters for each sample over all time points. Proliferation/average size of control untreated cells (S2R Cont) over time is marked in red. Proliferation/average size of DMSO treated cell samples across the same timepoints is shown in yellow. The cell counts/sizes for S2R+ cells treated with Meki (U0126 Mek inhibitor 100 uM) or Erki (GDC0994 Erk inhibitor 50 uM) are marked in green. Rapamycin (ToRC1 inhibitor 10 uM) and torin (ToRC1,2 inhibitor 10 uM) treated samples are illustrated in blue. Error bars indicate the standard error of the mean. Statistical significance relative to untreated controls was determined by student's t test (*p<0.05,**p<0.01,***p<0.001,****p<0.0001).

Treatment of S2R+ cell cultures with Map kinase inhibitors for Mek or Erk significantly lowered the cell counts and the cell sizes compared to control samples. Treatment with ToR pathway inhibitors rapamycin or torin (positive controls, color coded in blue) also reduced proliferation rates and average cell sizes.

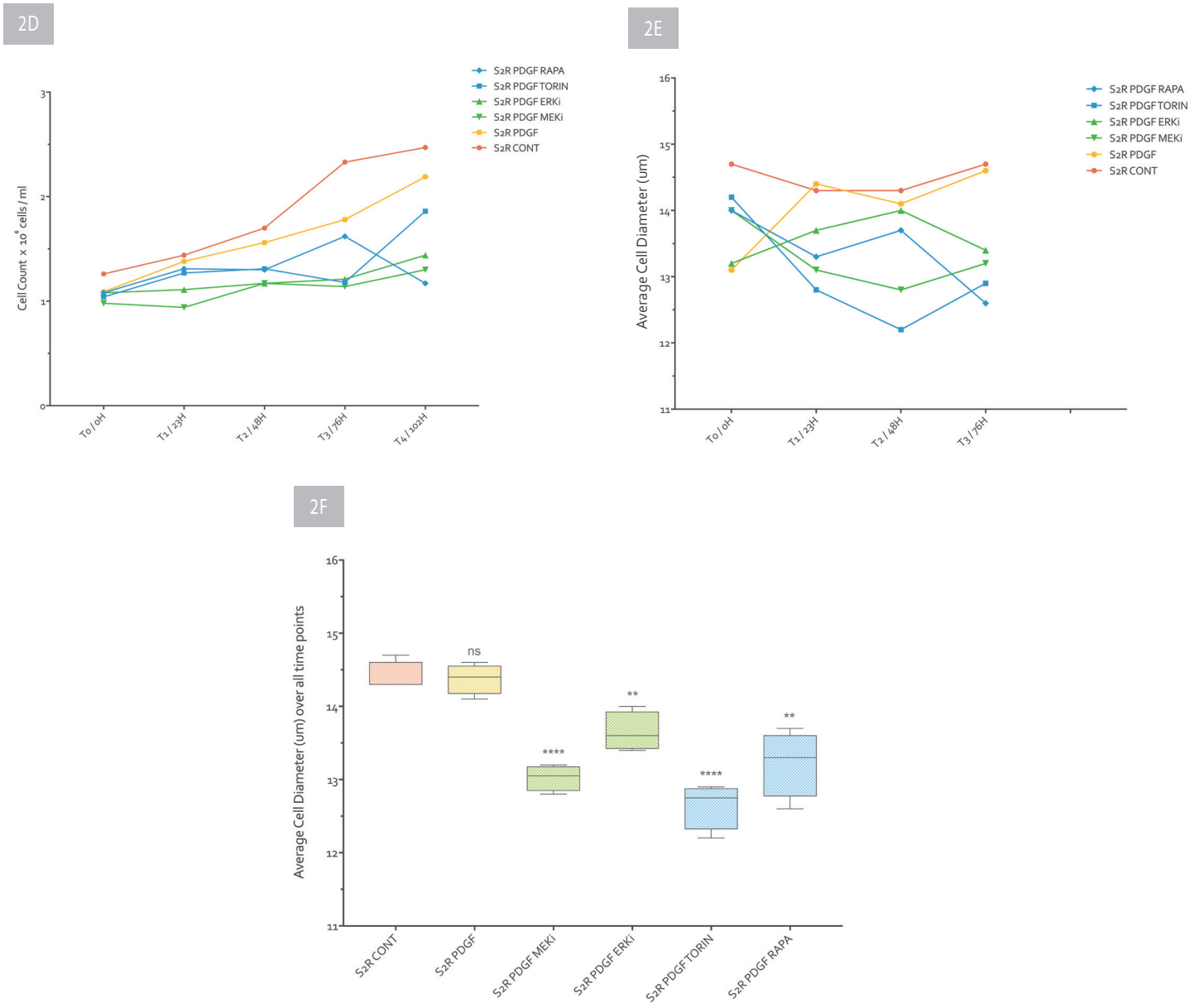


FIGURE 2D-F Cell proliferation and size (mean diameter) measurements of inhibitor and Pdgf (platelet derived growth factor) treated S2R+ cells over time. 2D displays cell counts for each treatment over time, 2E shows the mean cell diameters for every sample over time, and 2F averages the mean diameters for each sample over all time points. Proliferation/average size of control untreated cells (S2R Cont) over time is marked in red. Proliferation/average size of Pdgf treated cell samples (1ug/ml) across the same timepoints is shown in yellow. The cell counts/sizes for S2R+ cells treated with Pdgf and Meki (U0126 Mek inhibitor 100 uM) or with Pdgf and Erki (GDC0994 Erk inhibitor 50 uM) are marked in green. Samples stimulated with Pdgf and rapamycin (ToRC1 inhibitor 10 uM) or with Pdgf and torin (ToRC1,2 inhibitor 10 uM) are illustrated in blue. Error bars indicate the standard error of the mean. Statistical significance relative to controls was determined by student's t test (*p<0.05,**p<0.01,***p<0.001,****p<0.0001).

Treatment with Mapk pathway inhibitors consistently reduced cell proliferation and sizes compared to untreated control (S2R Cont, colored red) or to Pdgf treated control (S2R Pdgf, marked in yellow). Treatment with ToR pathway inhibitors (S2R Rapa and S2R Torin, positive controls marked in blue) also significantly reduced growth rates compared to controls.

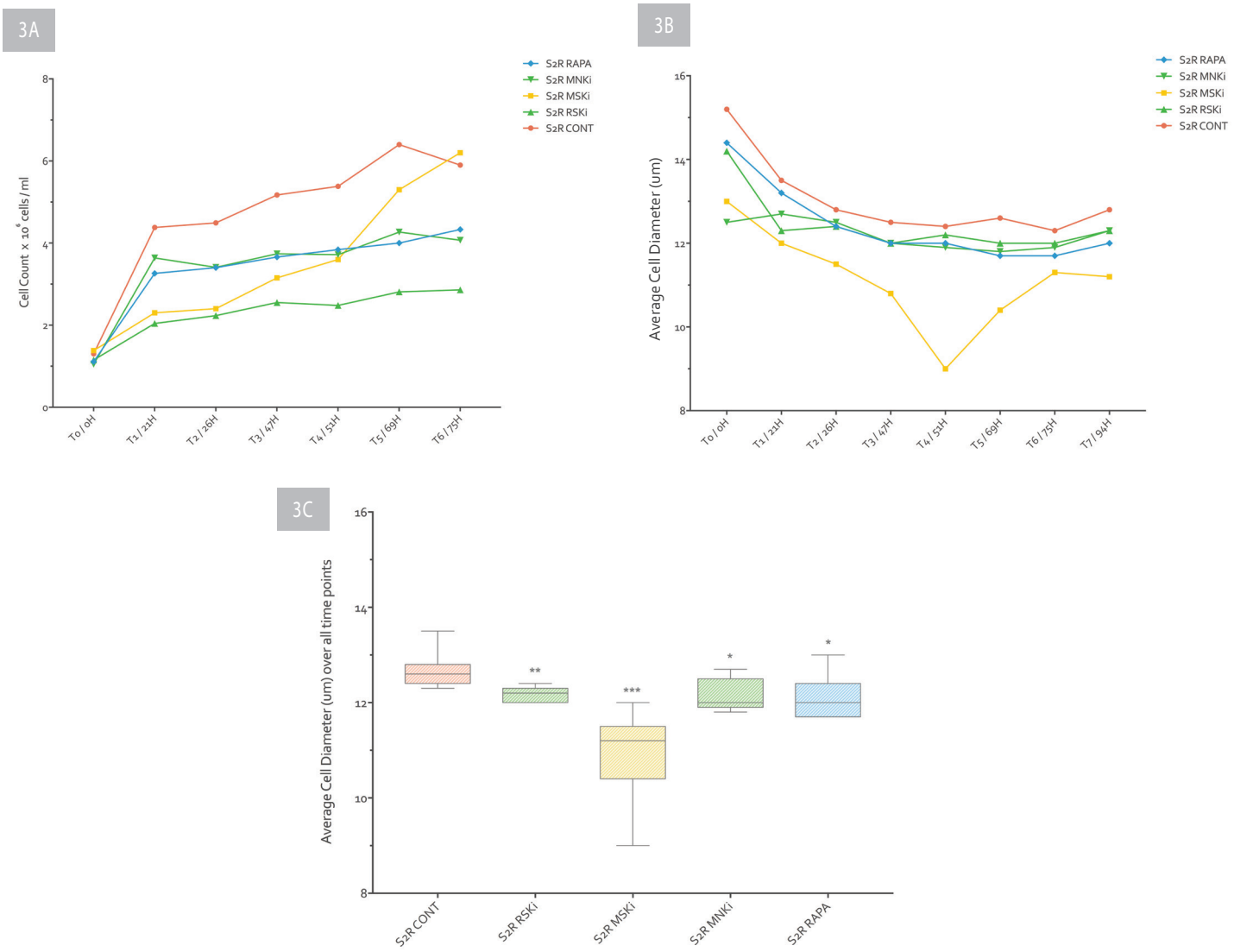


FIGURE 3 Effects on Mapkapk inhibitors on the proliferation and size of cultured schneider cells. Mapk activated protein kinases (Mapkapk) Mnk, Rsk and Msk control homeostatic and insulin driven growth in insect cells. S2R+ cell cultures for all indicated conditions were established in medium with 1% serum. Cell cultures were pre-treated with the indicated inhibitors for 40 min, prior to addition of various growth factors. Cell proliferation and mean diameters were subsequently measured for all samples over time. Cell density and diameter measurements were made for a sample population of 1200-1600 cells for each treatment condition and timepoint.

FIGURE 3A-C Cell proliferation and size (mean diameter) measurements of inhibitor treated S2R+ cells over time. 3A displays cell counts for each treatment over time, 3B shows the mean cell diameters for every sample over time, and 3C averages the mean diameters for each sample over all time points. Proliferation/average size of control untreated cells (S2R Cont) over time is marked in red. Proliferation/average size of Mski (R0318220 Msk inhibitor 500 uM) treated cell samples across the same timepoints is shown in yellow. The cell counts/sizes for S2R+ cells treated with Rski (Rsk inhibitor BID1870 500 uM) or Mnki (Mnk inhibitor CGP57380 500 uM) are marked in green. Rapamycin (ToRC1 inhibitor 10 uM) treated samples are illustrated in blue. Error bars indicate the standard error of the mean. Statistical significance was determined by student's t test relative to untreated control sample (*p<0.05,**p<0.01,***p<0.001).

Treatment of the S2R+ cell cultures with all three Mapkapk inhibitors (colored green and yellow) significantly reduced cell proliferation and sizes compared to control untreated cells (marked in red). Treatment with the Msk inhibitor (S2R Mski, shown in yellow) had the strongest effect. Treatment with the ToRC1 inhibitor rapamycin (positive control, shown in blue) also consistently reduced cell proliferation and sizes.

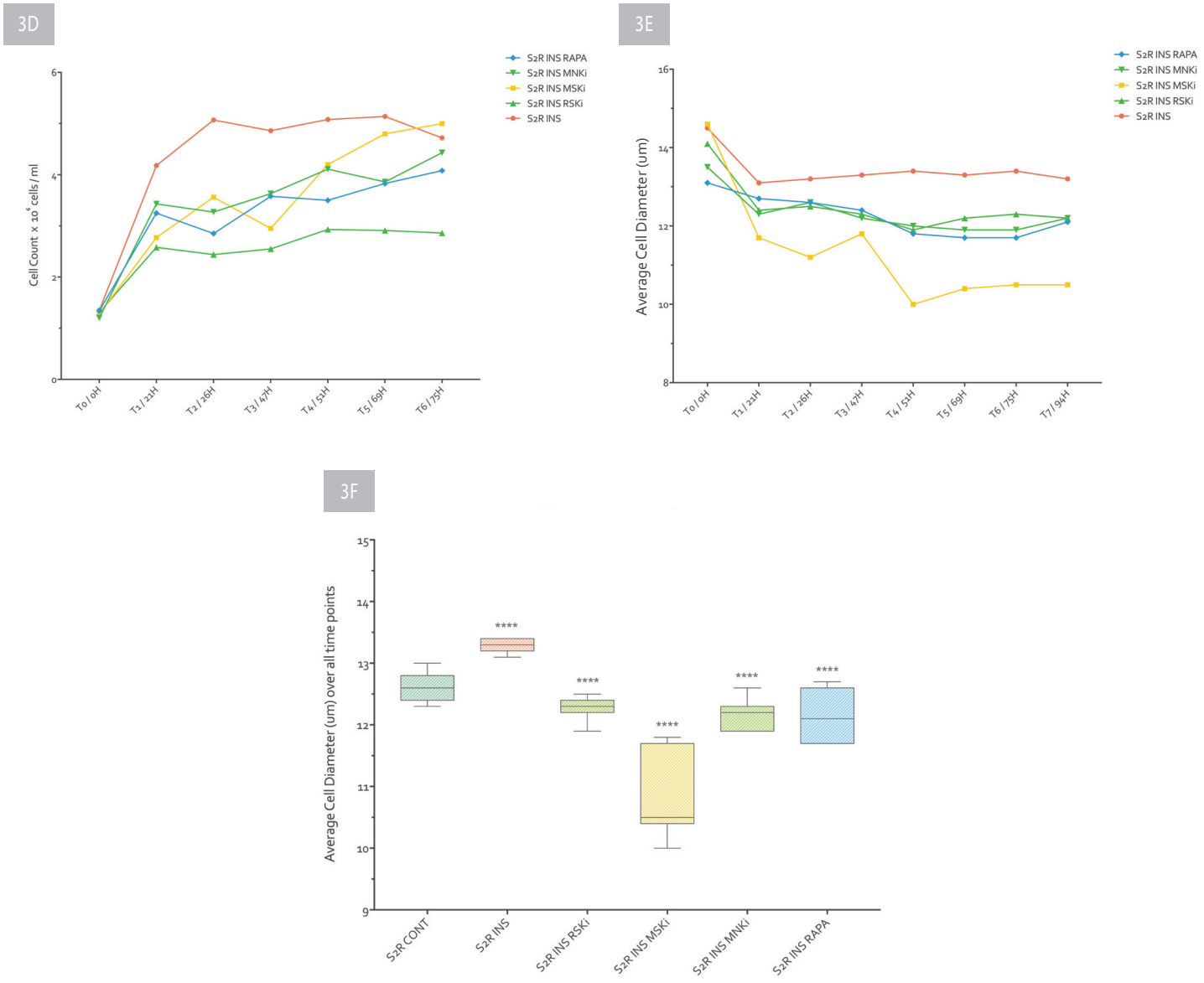


FIGURE 3D-F Cell proliferation and size (mean diameter) measurements of inhibitor and insulin treated S2R+ cells over time. 3D displays cell counts for each treatment over time, 3E shows the mean cell diameters for every sample over time, and 3F averages the mean diameters for each sample over all time points. Proliferation/average size of insulin only treated cells (S2R Ins, 10 ug/ml) over time is marked in red. Proliferation/average size of insulin and Mski (Ro318220 Msk inhibitor 500 uM) treated cell samples across the same timepoints is shown in yellow. The cell counts/sizes for S2R+ cells treated with insulin and Rski (Rsk inhibitor BID1870 500 uM) or with insulin and Mnki (Mnk inhibitor CGP57380 500 uM) are marked in green. Insulin + rapamycin (ToRC1 inhibitor 10 uM) treated samples are illustrated in blue. Error bars indicate the standard error of the mean. Statistical significance was determined by student's t test (*p<0.05,**p<0.01,***p<0.001,****p<0.0001).

Treatment with all three Mapkapk inhibitors (Mnki and Rski in green, and Mski in yellow) significantly reduced cell proliferation and sizes of untreated (3A-C) and insulin treated (3D-F) samples, compared to controls. As before, treatment with the Msk inhibitor (S2R Mski, yellow) had the strongest effect. Treatment with the ToRC1 inhibitor rapamycin (positive control, shown in blue) also consistently reduced cell growth.

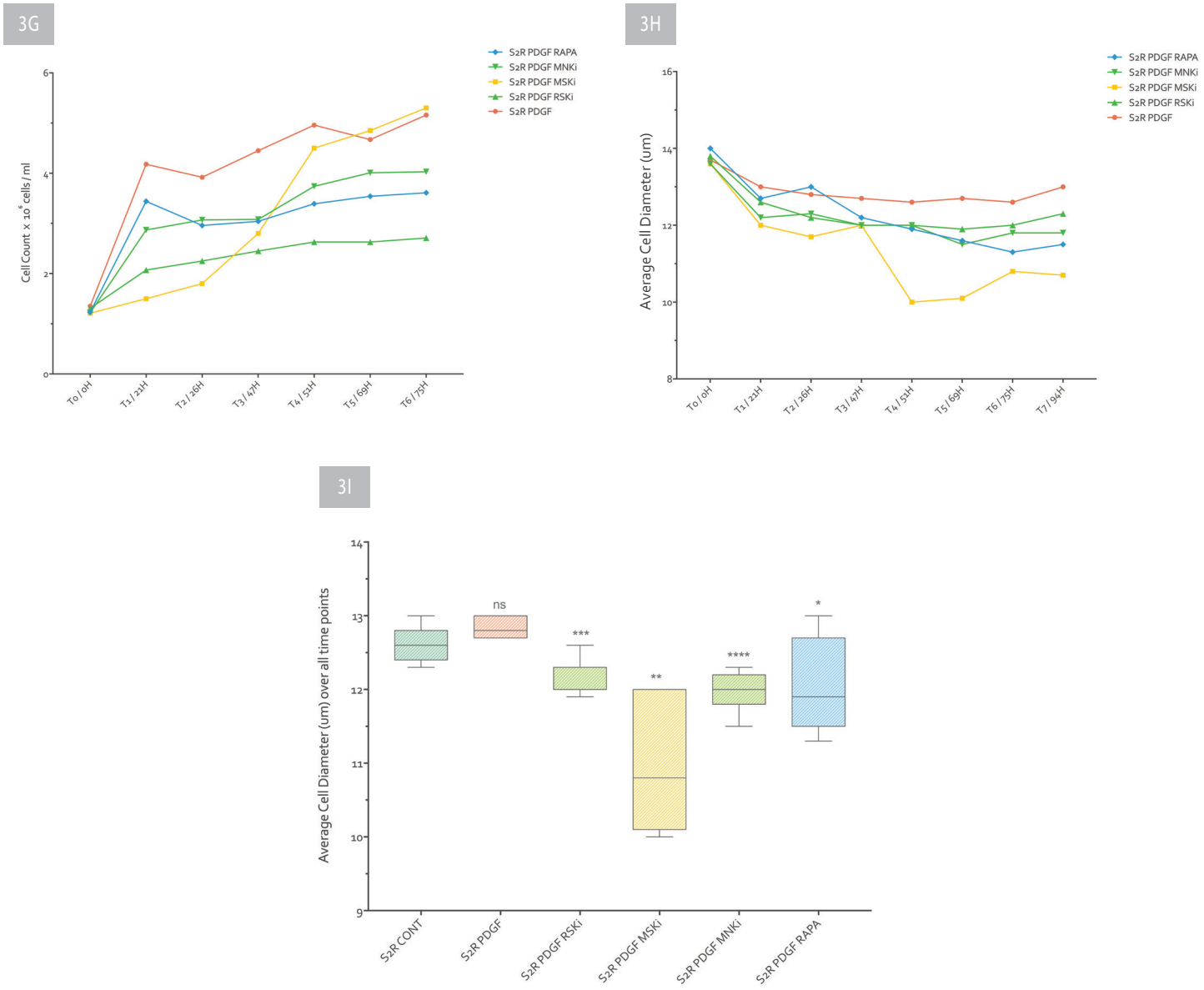
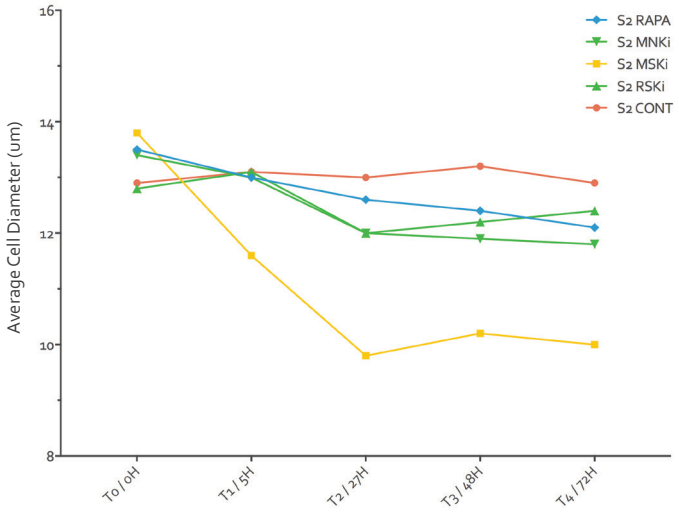


FIGURE 3G-I Cell proliferation and size (mean diameter) measurements of inhibitor and Pdgf (platelet derived growth factor) treated S2R+ cells over time. 3G displays cell counts for each treatment over time, 3H shows the mean cell diameters for every sample over time, and 3I averages the mean diameters for each sample over all time points. Proliferation/average size of Pdgf only treated cells (S2R Pdgf, 1ug/ml) over time is marked in red. Proliferation/average size of Pdgf and Mski (Ro318220 Msk inhibitor 500 uM) treated cell samples across the same time points is shown in yellow. The cell counts/sizes for S2R+ cells treated with Pdgf and Rski (Rsk inhibitor BID1870 500 uM) or with Pdgf and Mnki (Mnk inhibitor CGP57380 500 uM) are marked in green. Pdgf + rapamycin (ToRC1 inhibitor 10 uM) treated samples are illustrated in blue. Untreated sample (S2R Cont) is colored in cyan (3I). Error bars indicate the standard error of the mean. Statistical significance was determined by student's t test (*p<0.05,**p<0.01,***p<0.001,****p<0.0001).

Consistent with previous data, treatment with all three Mapkapk inhibitors (Mnki and Rski in green, Mski in yellow) significantly reduced cell proliferation and sizes of untreated (3A-C) or Pdgf treated (3G-I) samples compared to control samples (shown in red). Treatment with the ToRC1 inhibitor rapamycin (positive control, shown in blue) also consistently lowered the values for these parameters.

4A



4B

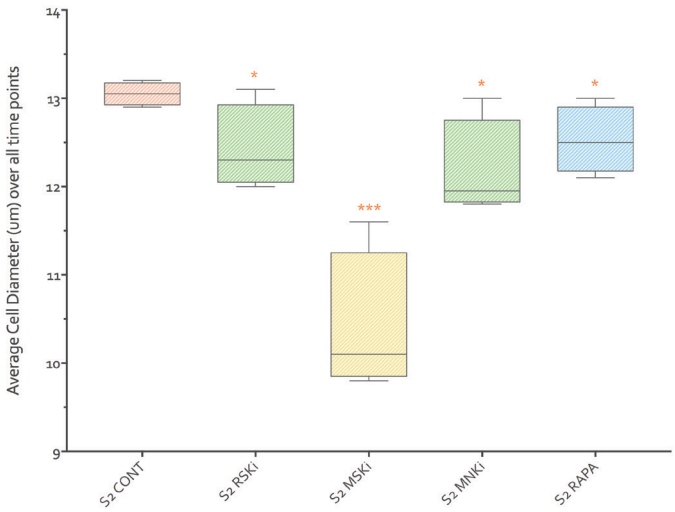
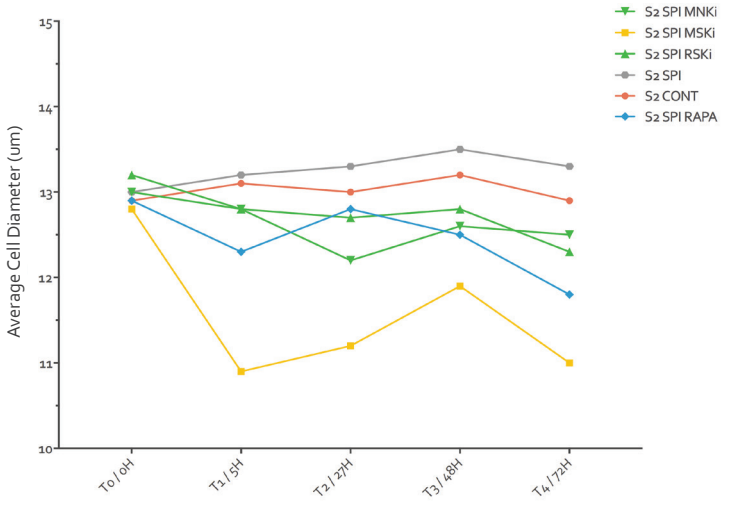


FIGURE 4 Effects of Mapkapk inhibitors on the cell size of untreated and Spitz treated schneider cells. Mapk activated protein kinases (Mapkapk) Mnk, Rsk and Msk control homeostatic and Egfr driven growth in insect cells. S2 cell cultures for all indicated conditions were established in medium with 1% serum. Cell cultures were pre-treated with the indicated inhibitors for 40 min, prior to addition of the Egfr ligand Spitz (Spi). Cell proliferation and mean diameters were subsequently measured for all samples over time. Cell density and diameter measurements were made for a sample population of 1200-1600 cells for each treatment condition and timepoint.

FIGURE 4A-B Cell size measurements (based on mean cell diameters) of inhibitor treated S2 cells over time. 4A shows the mean cell diameters for every sample over time, while 4B averages the mean diameters over all time points for each sample. Each measured point in 4A represents the average cell size (diameter) of the respective cell population with the indicated treatment at the mentioned time point. The average cell size of control untreated cells (S2 Cont) over time is shown in red. The cell sizes for S2 cells treated with Rski (Rsk inhibitor BID1870 500 uM) or Mnki (Mnk inhibitor CGP57380 500 uM) inhibitors are marked in green. Average sizes of S2 cells treated with Mski (Msk inhibitor Ro318220 500 uM) are colored yellow. Rapamycin (ToRC1 inhibitor 10 uM) treated samples are illustrated in blue. Error bars indicate the standard error of the mean. Statistical significance was determined by student's t test relative to untreated control sample (*p<0.05,**p<0.01,***p<0.001,****p<0.0001).

Treatment of the cultured schneider cells with the indicated inhibitors significantly reduced the average cell sizes compared to control untreated cells. Msk inhibitor proved most effective at reducing the average cell sizes.

4C



4D

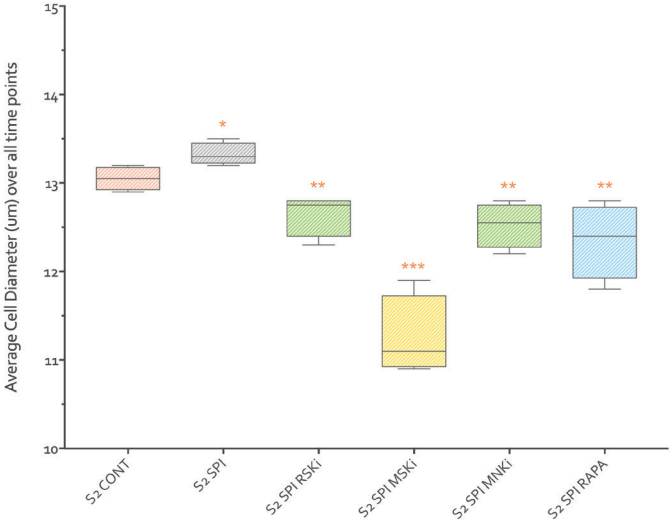
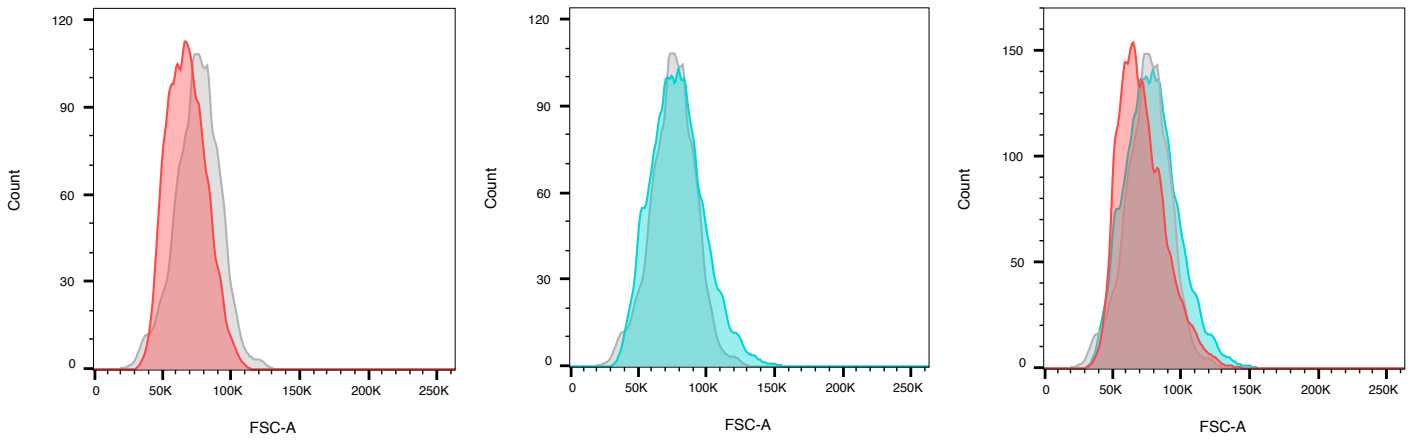


FIGURE 4C-D Cell size measurements (based on mean cell diameters) of inhibitor and Spitz treated S2/S2R+ cells over time. 4C shows the mean cell diameters for every sample over time, while 4B averages the mean diameters over all time points for every sample. Each measured point in 4C represents the average cell size (diameter) of the respective cell population with the indicated treatment at the mentioned time point. Average sizes of control untreated cells (S2 Cont) are shown in red. Spitz (only) treated cell samples are marked in grey. Average cell sizes for samples treated with Spitz and Rski (Rsk inhibitor BID1870 500 uM) or with Spitz and Mnki (Mnk inhibitor CGP57380 500 uM) are colored in green. Spitz and Mski (Msk inhibitor R0318220 500 uM) treated samples are shown in yellow, and Spitz + rapamycin (ToRC1 inhibitor 10 uM) treated samples in blue. Error bars indicate the standard error of the mean. Statistical significance was determined by student's t test relative to control (*p<0.05,**p<0.01,***p<0.001,****p<0.0001).

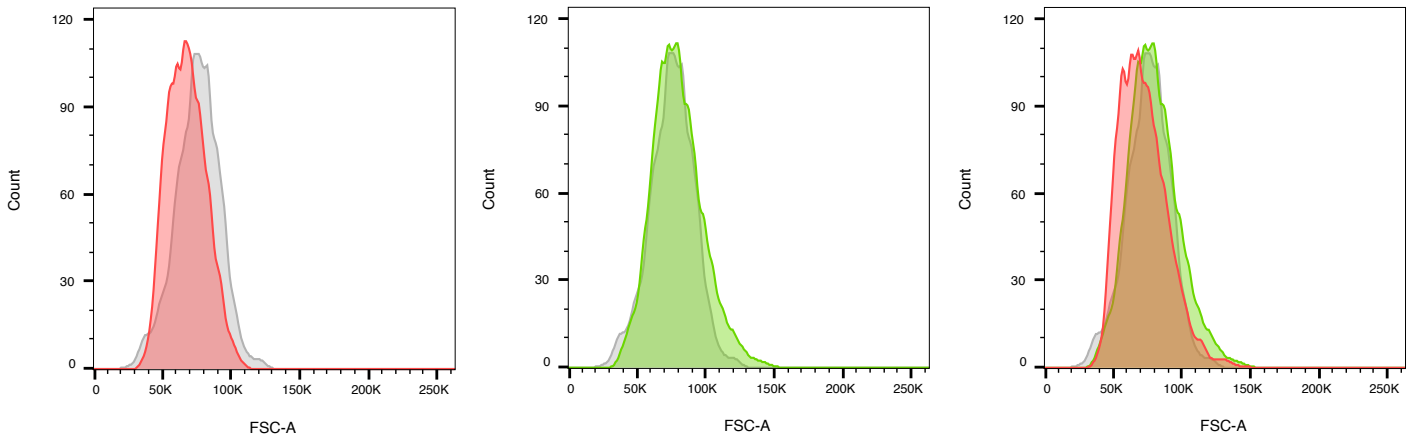
Addition of the Spitz Egfr ligand to the cell cultures significantly increased the average cell size compared to control untreated cells. Pre-treatment with the mentioned inhibitors, however, significantly reduced the average cell sizes (diameters) of untreated (4A-B) and Spitz treated (4C-D) cell samples compared to untreated or spitz-only treated controls.

5A



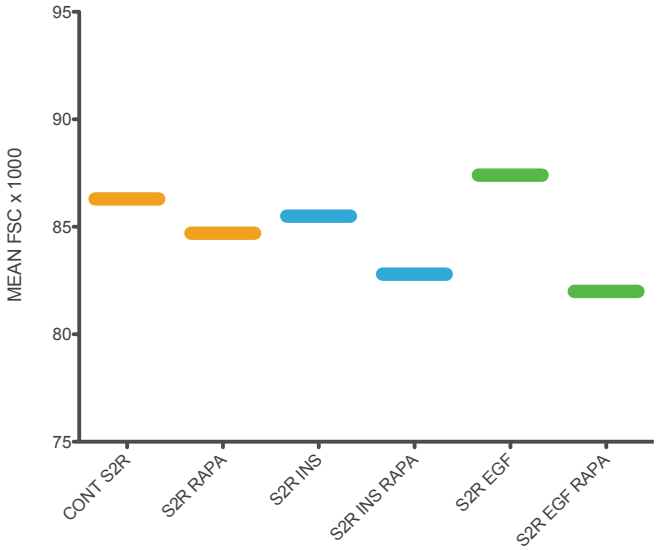
Alive Singlet CONT vs RAPA | Alive Singlet CONT vs INS | Alive Singlet CONT vs INS vs INS+RAPA

5B



Alive Singlet CONT vs RAPA | Alive Singlet CONT vs EGF | Alive Singlet CONT vs EGF vs EGF+RAPA

5C



5D

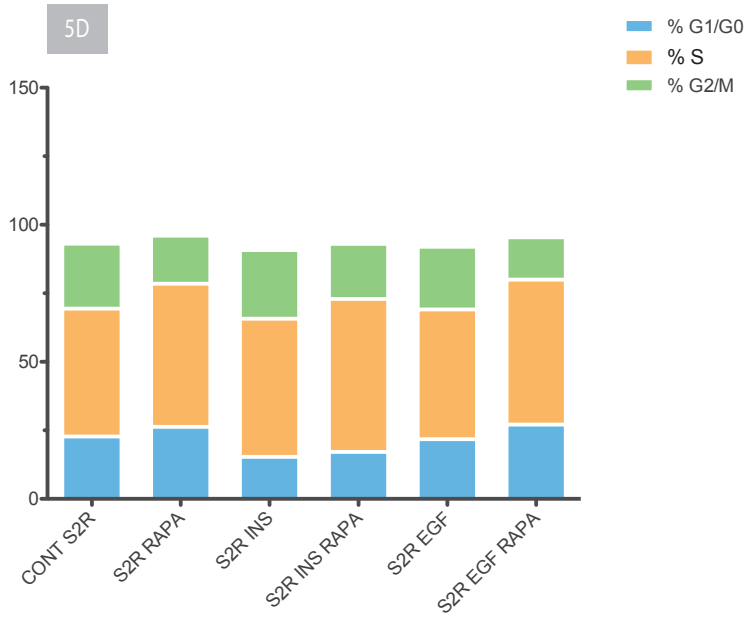


FIGURE 5 Flow cytometric analysis of growth factor-treated schneider cells. (A-D) Schneider S2 cells were pretreated with rapamycin (10 uM) for 30min, before insulin or Egf growth factors (10 ug/ml) were added to the preexisting cultures. Following overnight incubation, the cells were stained with Hoechst 33342 and analysed by flow cytometry. (A) S2 cell size measured by forward scatter (FCS) shows that insulin-stimulated cells (cyan) are slightly bigger than control untreated cells (grey). Conversely, rapamycin treated cells (red) show a negative shift in the acquired histograms, towards smaller cell sizes. (B) Egf treated S2 cells (green) show a similar mild increase in cell sizes compared to untreated controls (grey), whereas rapamycin treatment (red) reproducibly induced a negative shift in the acquired histograms, towards smaller cell sizes. (C) The mean FSC values for S2R+ cell samples treated in the same manner are shown. Control samples ± rapamycin are shown in orange, insulin-treated samples ± rapamycin in blue, and Egf-treated samples ± rapamycin in green. Egf treatment increased the mean FSC value of the cell population, whereas rapamycin addition lowered the mean FSC, indicative of reduced cell sizes. (D) Cell cycle phase distribution of S2R+ cells is shown. Insulin treatment (S2R Ins) increased the proportion of cells in S and G2, while rapamycin treatment slightly increased the G1 cell population in control (S2R Rapa) and Egf-treated samples (S2R Egf Rapa). Graphs were generated using the FlowJo software. For determination of cell cycle distribution, FlowJo uses the Watson pragmatic model to create gaussian distributions for the Go/G1 and G2/M peaks, and an S phase polynomial.

II-2 MAPK ACTIVATES TOR DOWNSTREAM OF RTK IN DROSOPHILA CELLS

II-2A Mapk Activates ToRC1 in Insect Cell Culture

context As mentioned, insulin signaling drives cell growth in drosophila in vivo [5, 23-27], as well as in cell culture [6-8, 28]. Upon insulin treatment of drosophila schneider cells, S6k, Pi3k and Akt as well as Mek and Erk kinases become activated [6-8, 29]. Furthermore, Erk has been involved in insulin stimulated schneider cell proliferation [7]. The canonical ToR pathway has been previously shown to function downstream of insulin signaling and to control drosophila cell growth in vivo [4] and in cell culture [1-3]. Strong activation of Ras-Erk pathway leads to mToRC1 activation in mammalian cells through Erk and Rsk signaling to Tsc and Raptor components. Egf, phorbol esters and constitutively active Ras mutants promote Erk and Rsk mediated phosphorylation of Tsc2, inhibiting its GAP function and thereby stimulating mToRC1 activity and tumorigenesis [30-33]. Similar stimuli promote Erk and Rsk phosphorylation of Raptor, likewise increasing mToRC1 activity [34-36]. AIM Based on these findings, we wanted to investigate if these pathways show a similar crosstalk in drosophila cultured cells.

approach S2R+ cells were cultured in standard insect medium. Cultures were then either left untreated (controls) or pre-treated with rapamycin (ToRC1 inhibitor, 10 uM) and further incubated for 40min prior to addition of insulin, Egf or Pma stimulating factors (10 ug/ml). Cells were subsequently lysed and the proteins separated by electrophoresis. Akt and S6k kinase phosphorylation was detected through immunoblotting assays with anti-phospho-Akt and anti-phospho-S6k antibodies.

results Insulin treatment of the cultured S2R+ cells triggers a strong induction of Akt phosphorylation compared to untreated control. Pre-treatment with rapamycin did not prevent insulin-stimulated Akt phosphorylation in these cells. In contrast to insulin treatment, stimulation of cell cultures with Egf epidermal growth factor or with Pma (phorbol-12-myristate-13-acetate, Mek agonist) had no obvious effect on Akt phosphorylation (figure 6A,C). Treatment of cell cultures with insulin or with Egf/Pma Ras-Mapk pathway agonists increased S6k phosphorylation compared to untreated control. Pre-treatment with rapamycin prevented the increase in phospho-S6k signal observed upon insulin, Egf and Pma stimulation (figure 6B,D). These data indicate that Ras-Mapk pathway agonists stimulate ToRC1 activity towards its target S6k, without increasing Akt activity.

II-2B Mapk Activity is Required for Basal and Growth Factor induced ToRC1 Activity

context/aim Previous experiments (see II-2A) revealed that Ras-Mapk pathway agonists stimulate ToRC1 activity toward S6k, likely independently of Akt activation. One of the next logical questions was if Ras-Mapk signaling is also required for ToRC1 activation under basal or stimulated conditions.

approach To tackle this hypothesis, schneider cell cultures were treated in a similar manner as described before. S2R+ cells were cultured in standard insect medium without serum. Cultures were then either left untreated (controls) or pre-treated with various Ras-Mapk or ToR pathway inhibitors for 40min. Following this incubation period, different growth factors were individually added to the pre-established cultures. All cell samples were subsequently lysed and the proteins separated by electrophoresis. Erk and S6k kinase phosphorylations were then detected through immunoblotting assays with anti-phospho-Erk and anti-phospho-S6k antibodies.

results Treatment of S2R+ cells with Mek inhibitor (U0126), or with Erk inhibitor (GDC0994) strongly reduced Erk phosphorylation levels compared to untreated control. Mek and Erk inhibitors triggered a similar reduction in Erk phosphorylation also in growth factor stimulated cell cultures, including Pdgf, insulin and Vegf treated cultures (figures 7A, 8A). This confirms the specificity and efficacy of these kinase inhibitors. Inhibition of Mek kinase triggered a stronger effect than inhibition of Erk kinase in this respect. Inhibition of ToR pathway activity with either ToRC1 inhibitor (rapamycin) or with ToRC1,2 inhibitor torin had no obvious influence on Erk phosphorylation level in untreated as well as growth factor-stimulated cultures (figures 7A, 8A).

Stimulation of cultured S2R+ cells with the well known Pi3k-ToR agonist insulin, increased S6k phosphorylation relative to untreated control, indicative of ToRC1 activation. Interestingly, stimulation with the well known Ras-Erk pathway agonist Pdgf, also led to a mild increase in S6k phosphorylation (figures 7B, 8B). Significantly, S6k phosphorylation induced by both insulin and Pdgf growth factors, was strongly reduced by inhibition of Mek kinase activity (and to a smaller extent by Erk inhibition). Treatment with well known ToR pathway inhibitors (as positive controls) such as rapamycin and torin, likewise abrogated S6k phosphorylation upon growth factor treatment (figures 7B, 8B). Taken together, these results indicate that Pdgf-Ras signaling stimulated ToRC1 activity in drosophila schneider cells, as evidenced

by an increase in its target S6k phosphorylation. Importantly, ToRC1 pathway activation by insulin and Pdgf growth factors depends to some extent on Mek-Erk pathway activation, as inhibition of this pathway reduced growth factor stimulated ToR target phosphorylation.

II-2C Mapk Pathway Activation does not Require ToRC1 or Mapkapk Activities

context It has previously been shown that insulin stimulation of schneider cells increases Erk phosphorylation [7,8]. Likewise, stimulation of schneider cells with the native drosophila Egfr ligand Spitz also increases Erk phosphorylation [22]. AIM Based on these findings and our previous results (II-2B), we wanted to know if ToRC1 or any of the Mapk activated protein kinases previously tested might also play a role in Erk activation.

approach S2 cell cultures expressing the native drosophila Egfr receptor were established in medium without serum for all indicated treatments. Cell cultures were pre-treated with kinase inhibitors for 40 min, prior to addition of growth factors. The cells were subsequently lysed and the proteins separated by electrophoresis. Erk phosphorylation was detected through western blotting with anti-phospho-Erk antibody. Kinase inhibitors: Rski (Rsk inhibitor BID1870), Mnki (Mnk inhibitor CGP57380), Mski (Ro318220 Msk inhibitor), Rapa (ToRC1 inhibitor).

results Treatment of schneider cell cultures with kinase inhibitors for Mnk, Rsk, Msk or ToRC1, caused no noticeable change in Erk phosphorylation level compared to untreated control. Similarly, no reduction in Erk phosphorylation was detected upon inhibitor treatment of the insulin- or spitz-stimulated cell cultures. This would indicate that Mapk pathway activation under normal or growth factor stimulated conditions does not require ToRC1 or the Mapk activated protein kinases Mnk, Rsk, Msk.

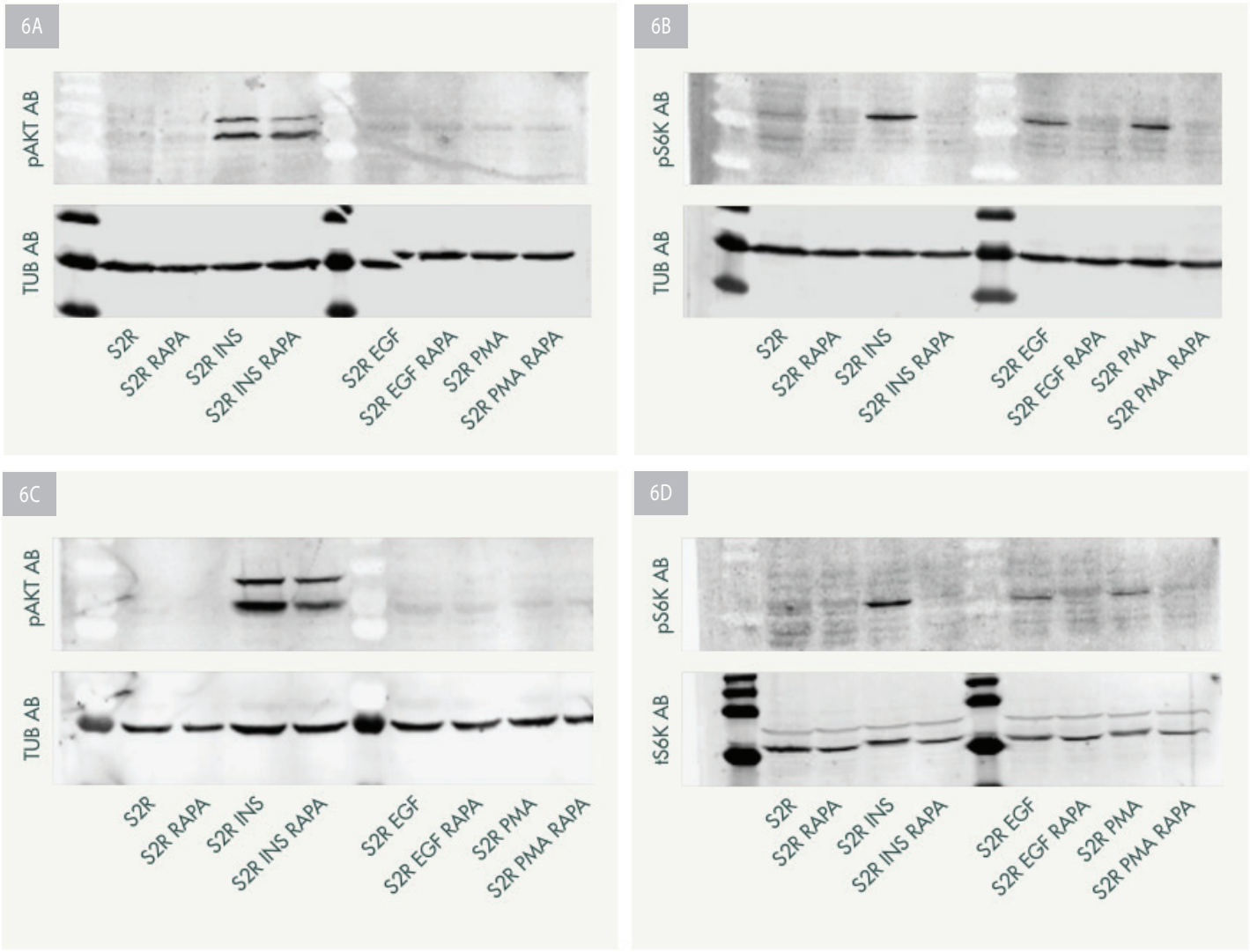


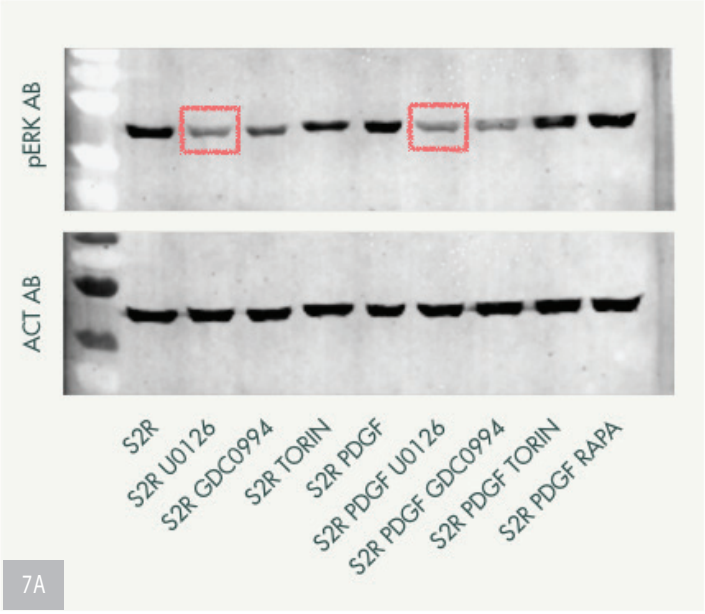
FIGURE 6 The effects of growth factor treatment on Akt and S6k phosphorylation, and their sensitivity to rapamycin treatment. Ras-Mapk pathway activates the ToRC1 complex in schneider cells. S2R+ cell cultures for all indicated conditions were established in medium without serum (as not to confound growth factor-triggered effects). Cell cultures were pre-treated with rapamycin (ToRC1 inhibitor) for 40 min, prior to addition of growth factors. Cells were subsequently lysed and the proteins separated by electrophoresis. Akt and S6k kinase phosphorylation was detected through immunoblotting assays with anti-phospho-Akt (pAKT AB) and anti-phospho-S6k (pS6k AB) antibodies. Loading controls: tubulin (Tub) and total S6k (tS6k).

FIGURE 6A Insulin treatment (10 ug/ml) of cultured S2R+ cells triggers a strong induction of Akt phosphorylation (S2R Ins) compared to untreated control (S2R). Pre-treatment with rapamycin (S2R Ins Rapa, 10 uM) did not prevent insulin-stimulated Akt phosphorylation in these cells. Treatment of cell cultures with Egf epidermal growth factor (10 ug/ml) or with Pma (phorbol-12-myristate-13-acetate, Mek agonist)(8-10 ug/ml), however, had no obvious effect on Akt phosphorylation (S2R Egf and S2R Pma). Tubulin levels were also probed for all samples as a loading control.

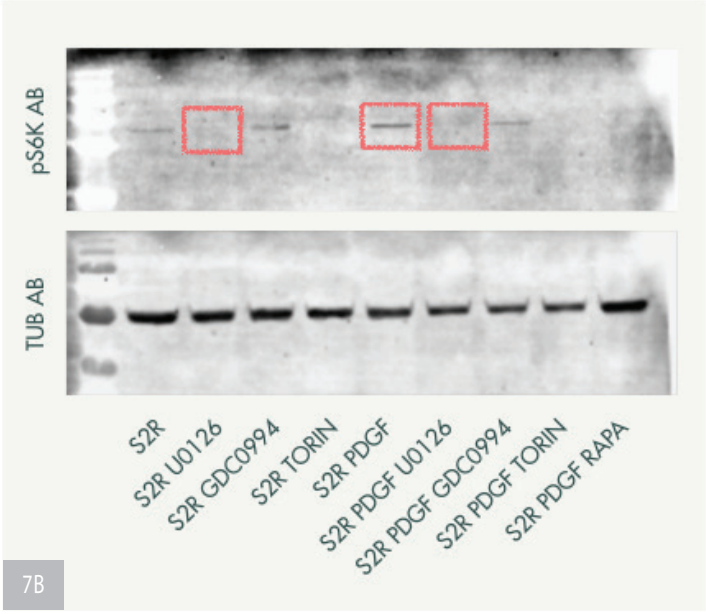
FIGURE 6B Treatment of S2R+ cells with insulin or with Ras-Mapk pathway agonists increases S6k phosphorylation compared to untreated control. Insulin treatment (10 ug/ml) triggered a strong increase in S6k phosphorylation (S2R Ins) compared to untreated control (S2R). Pre-treatment with rapamycin (10 uM) prevented the increase in phospho-S6k signal observed upon insulin treatment (S2R Ins Rapa). Similar to insulin, treatment of schneider cells with Ras-Mapk pathway agonists Egf (10 ug/ml) or Pma (10 ug/ml) visibly increased S6k phosphorylation relative to untreated control (S2R). Pre-treatment with rapamycin (10 uM) was again able to prevent the increase in S6k phosphorylation triggered by Egf or Pma (S2R Egf Rapa, S2R Pma Rapa). Tubulin levels were also determined for all samples as a loading control.

FIGURE 6C Similar treatment as described in 6A, S2R+ cells were treated with rapamycin and/or growth factors, and probed for Akt phosphorylation through immunoblotting. Insulin treatment, but not Egf or Pma treatments, significantly increased Akt phosphorylation compared to untreated control. Tubulin (Tub) and actin (Act) antibodies were used as loading controls.

FIGURE 6D Similar treatment as described in 6B, S2R+ cells were treated with rapamycin and/or growth factors, and probed for S6k phosphorylation through immunoblotting. Treatment of cell cultures with all three mitogenic factors insulin, Egf and Pma, visibly increased S6k phosphorylation compared to untreated control. Rapamycin pre-treatment efficiently abrogated the increase in S6k phosphorylation triggered by insulin, Egf or Pma. Total S6k levels (tS6k) were also determined for all samples as loading controls.



7A



7B

FIGURE 7 Assessment of Erk and S6k phosphorylation in schneider cells upon treatment with Pdgf growth factor and with Ras-Mapk/ToR pathway inhibitors. Mapk activity is required for basal and growth factor induced ToRC1 activity. S2R+ cell cultures for all indicated conditions were established in medium without serum. Cell cultures were pre-treated with kinase inhibitors for 40 min, prior to addition of Pdgf platelet derived growth factor. Cells were subsequently lysed and the proteins separated by electrophoresis. Erk and S6k kinase phosphorylation was detected through immunoblotting assays with anti-phospho-Erk (pErk) and anti-phospho-S6k (pS6k) antibodies. Kinase inhibitors: U0126 Mek inhibitor, GDC0994 Erk inhibitor, Rapamycin ToRC1 inhibitor, Torin ToRC1,2 inhibitor. Pdgf is platelet derived growth factor.

FIGURE 7A Treatment of S2R+ cells with U0126 Mek inhibitor (100 uM), or with GDC0994 Erk inhibitor (50 uM) strongly reduced Erk phosphorylation levels compared to untreated control or to Pdgf treated sample (1 ug/ml), confirming the specificity and efficacy of these kinase inhibitors. The Mek inhibitor U0126 triggered a stronger effect. Treatment with the ToR pathway inhibitor torin (10 uM) did not influence Erk phosphorylation in this system. Actin protein levels were also determined as a loading control (Act AB).

FIGURE 7B Treatment of unstimulated and Pdgf-stimulated (1 ug/ml) S2R+ cells with the Mek inhibitor U0126 (100 uM) abrogated S6k phosphorylation. Treatment with the ToR pathway inhibitors rapamycin or torin (10 uM) also reduced S6k phosphorylation relative to untreated or Pdgf-treated controls. Tubulin -Tub- protein levels were determined as loading control.

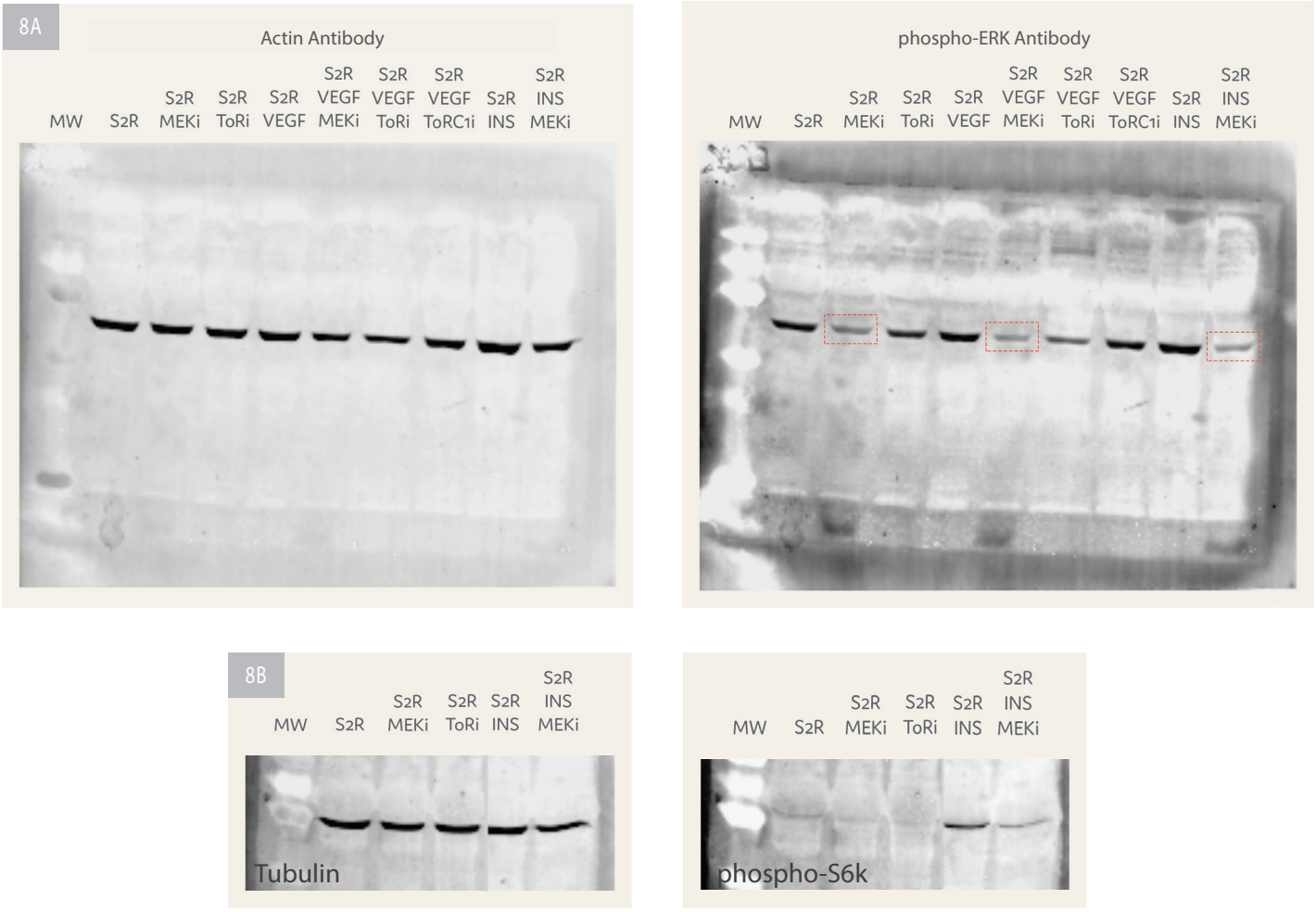
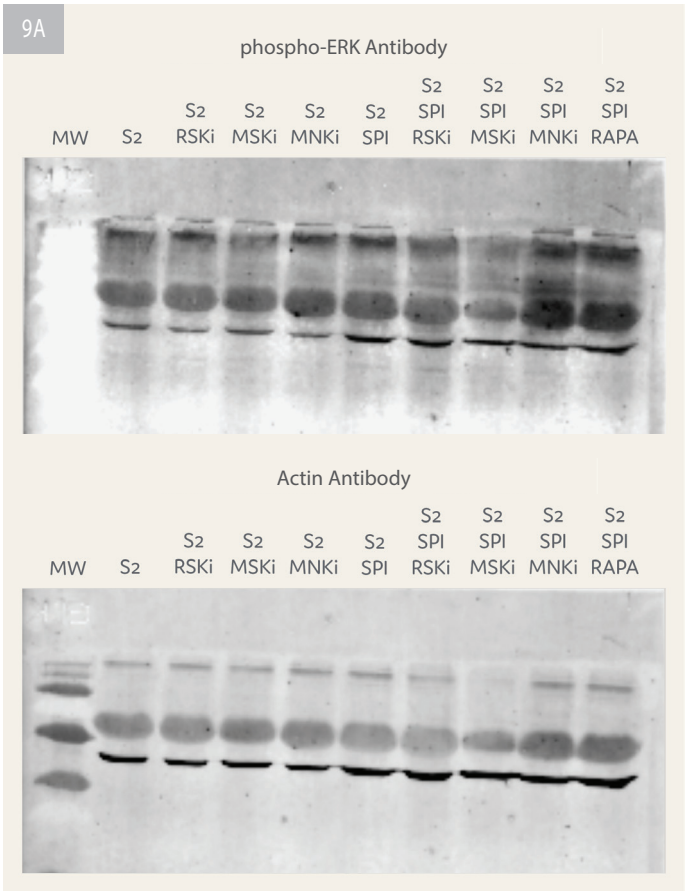


FIGURE 8 The effects of insulin and Vegf growth factor treatment on Erk and S6k phosphorylation in schneider cells, and their sensitivity to Ras-Mapk/ToR pathway inhibitors. Erk and S6k phosphorylation under basal or growth factor treatment conditions depends on Mek kinase activity. S2R+ cell cultures for all indicated conditions were established in medium without serum. Cell cultures were pre-treated with kinase inhibitors for 40 min, prior to addition of growth factors. Cells were subsequently lysed and the proteins separated by electrophoresis. Erk and S6k kinase phosphorylation was detected through immunoblotting assays with anti-phospho-Erk and anti-phospho-S6k antibodies. Kinase inhibitors: Meki is U0126 Mek inhibitor, ToRC1i is rapamycin ToRC1 inhibitor, ToRC1,2i is torin ToRC1,2 inhibitor. Vegf is vascular endothelial growth factor, Ins is insulin.

FIG 8A Treatment of S2R+ cells with Mek inhibitor (U0126 100 uM) strongly reduced Erk phosphorylation levels in unstimulated and insulin (10 ug/ml) or Vegf (1 ug/ml) growth factor stimulated cell cultures. ToR inhibition did not trigger such a reduction. Actin levels were used as loading controls. FIG 8B Insulin treatment (10 ug/ml) of cultured S2R+ cells increased as before S6k phosphorylation relative to untreated control. Pre-treatment with U0126 Mek inhibitor (100 uM), however, partially abrogated the effect of insulin on S6k phosphorylation, and reduced the phospho-S6k level compared to insulin-only treated sample. Tubulin protein levels were used as loading controls.



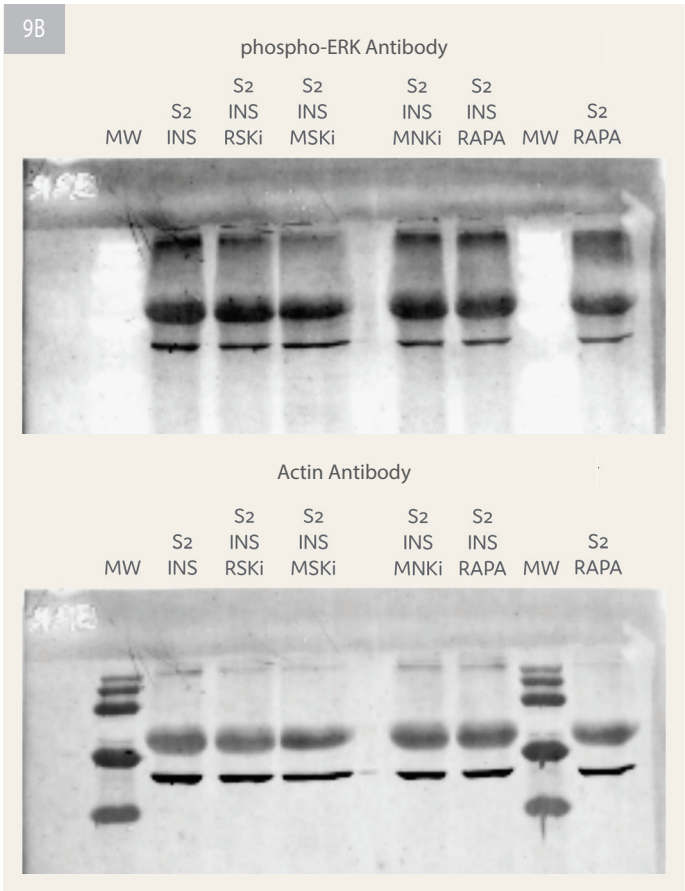


FIGURE 9 Erk phosphorylation levels in schneider cells upon treatment with insulin or native drosophila Spitz ligand, and the effects of Mapkapk (Mapk activated protein kinases) inhibitors. Basal as well as insulin and Egfr induced Erk phosphorylation does not require Mnk, Rsk, Msk or ToR kinase activities. S2 cell cultures expressing the native drosophila Egfr receptor were established in medium without serum for all indicated treatments. Cell cultures were pre-treated with kinase inhibitors for 40 min, prior to addition of growth factors. Cells were subsequently lysed and the proteins separated by electrophoresis. Erk phosphorylation was detected through western blotting with anti-phospho-Erk antibody. Kinase inhibitors: Rski (Rsk inhibitor BID1870), Mnki (Mnk inhibitor CGP57380), Mski (R0318220 Msk inhibitor), Rapa (ToRC1 inhibitor). Spi is the native Egfr ligand spitz, and Ins is insulin.

FIG 9A Treatment of the cultured schneider cells with Spitz ligand conditioned medium increased Erk phosphorylation level compared to untreated control. Pre-treatment with Mnk, Rsk or Msk (500 uM) inhibitors or with ToRC1 inhibitor rapamycin (10 uM) did not affect Erk phosphorylation levels in unstimulated or Spitz-stimulated cell cultures. Actin protein levels were detected as loading controls. FIG 9B Treatment with Mnk, Rsk, Msk or ToRC1 inhibitors did not reduce Erk phosphorylation levels in insulin-stimulated (10 ug/ml) schneider cells. Actin was detected as loading control.

II-3 MAPK GROWTH EFFECTORS IN DROSOPHILA INTESTINE

II-3A Mapk Driven Growth in the Adult Drosophila Midgut is Partially ToR Dependent

As described in the introductory part, the conserved Egfr pathway is a key determinant of cell growth and proliferation in the midgut, being essential for tissue homeostasis and regeneration [50-54]. A second conserved pathway important for growth and differentiation of midgut epithelial cells is ToR, Target of Rapamycin [55-57]. To better delineate the crosstalk between these central pathways in vivo, a series of experiments were performed to address ToR dependency for Egfr-driven growth and division in the adult midgut tissue.

To investigate Egfr-driven growth and division phenotypes and their dependency on ToR, in the ISC lineage, the esg-Gal4 UAS-GFP tub-Gal80^{TS} system (here-forth referred to as esg^{TS}) was used to express activated Egfr, Ras or Raf alleles in ISCs and their undifferentiated daughters, the EBs. Ras^{V12} (constitutively active Ras) expression in the progenitor compartment led to a pronounced increase in ISC mitotic activity accompanied by an expansion of the GFP-positive cell population and overall cell density (figure 10A). Area quantifications further revealed an overstimulated enterocyte (EC) growth and endoreplication. Additional treatment with the ToRC1 inhibitor rapamycin didn't have a significant effect on mitotic activity or Gfp+ (expressing cell) numbers. A noticeable change, however, was a decreased occurrence of large polyploid Gfp+ cells, although some large nuclei were still observed.

Expression of the activated receptor Egfr^{Top}, also with the esg^{TS} driver, had a relatively weak phenotype reflected in a mild increase in the ISC mitotic index, Gfp+ cells and EC growth (data not shown). This might be due to weaker expression or reduced potency of the transgenic construct, or the fact that its functional output relies on endogenous Ras rather than the transformed activated version. Raf gain-of-function genotypes yielded similar results as in the case of Ras mutant guts, although it produced fewer overgrown Gfp+ cells (figure 10B). Rapamycin treatment mildly reduced the Gfp+ cell density and average cell size.

To better characterise the growth promoting activity of Egfr signaling and distinguish it from its mitogenic effects, we overactivated Ras-Mapk signaling in the committed enteroblast progenitors. EBs are generally believed to have exited mitotic cycle and follow a differentiation pathway to either secretory EEs or commence endocycle and become absorptive ECs. Stimulation of the Mek-Erk pathway in EBs by expressing activated Ras^{V12S35} using the Su(h)^{TS} driver

(Su(h)-Gal4, UAS-CD8-GFP; tub-Gal80^{TS}) led to significant increase in the size of the expressing cells (appreciated based on nuclear area quantifications), compared to their control GFP-expressing siblings. The growth phenotype triggered by increased Mapk signaling appeared to be partially dependent on ToRC1 activity, as treatment with the inhibitor rapamycin significantly decreased the growth effect (figure 11).

Although rapamycin treatment was performed according to previous publications [55,57], additional controls were also tested in this study. Rheb (ToRC1 promoter) overexpression using the esg^{TS} progenitor-specific driver markedly increased the phosphorylation of the ToRC1 targets S6k and 4EBP, as compared to the GFP-expressing control guts. As previously described [Ip 2012], the p4EBP signal seems to highlight ToR activity in ECs, as well as in small nuclei-containing cells. Large polyploid GFP+ cells were also observed in Rheb-expressing guts. Concomitant rapamycin treatment of the Rheb-expressing animals partially masked the overgrowth phenotype and led to a reduction in p4EBP staining (figure 12A). Although the increase in phospho-specific signal was quantitatively higher in Rheb-expressing animals and lower in rapamycin-treated animals, as compared with controls, the staining pattern was not specific enough to warrant further investigation. To assess if there is an activation of ToR signaling downstream of Ras-Erk in this context, immunofluorescence and immunoblotting assays were performed. Although largely unsuccessful, some revealed an increase in phospho-S6k upon expression of activated Raf in the progenitors (figure 12B)

II-3B Ras-Mapk Drives Growth in the Adult Drosophila Intestine also through Ets21c

In drosophila as in other species, the ETS transcription factors are considered to be the predominant RTK-Ras-Mapk nuclear effectors [58]. One of these factors, Ets21c, was found to be transcriptionally induced the adult drosophila intestine upon downstream activation of Egfr signaling [54]. Additionally, preliminary experiments overexpressing Ets21c in the progenitor population revealed an increase in the rate of proliferation (data not shown). Based on this knowledge, we addressed Ets21c function in the midgut epithelium, with a focus on its growth effects.

To delineate the role of Ets21c in regulating enteroblast growth, the Su(h)^{TS} driver was used to overexpress or knockdown Ets21c (RNAi) in the adult intestine. Additionally, a possible Mapk dependency for Ets21c-driven phenotype was tested by co-expressing Mek-RNAi with Ets21c. Ets21c gain of function in the committed EB progenitors led to a significant size increase of

these cells, in comparison to their wild type siblings expressing GFP alone (figure 13). Sizes of the GFP+ expressing cells were measured based on accurate quantifications of individual cell areas from confocal z-stack projections. Although the cell size (area) of Ets21c-expressing cells is significantly increased compared to control cells, the effect is milder compared to the size increase triggered by activated Ras expression under the same conditions (see 10A).
Interestingly, not only Ets21c expression turned out to be sufficient to promote progenitor cell growth, but also required by these cells in order to achieve their normal size under homeostatic conditions. This is evidenced by a significant reduction in cell size prompted by Ets21c knockdown. Epistasis experiments revealed that this growth promoting effect triggered by Ets21c expression does not depend on Mapk activity, as co-expression of Mek-RNAi did not suppress the observed size increase (figure 13).

II-3C Ras-Mapk Promotes Nutrient Independent Growth in the Larval Intestine

The precursors for intestinal stem cells, called adult midgut progenitors (AMPs), first appear in the embryonic midgut epithelium amongst other epithelial cells that will differentiate into functional enterocytes (ECs) or enteroendocrine cells (EEs). AMP proliferation during larval development is highly sensitive to Egfr signaling and to changing nutritional conditions [50,46]. As the AMPs do not differentiate at these early stages, they constitute a good system to study Ras-Mapk mediated effects on growth and proliferation.

Therefore, we tested the resistance of AMPs to starvation conditions and if Ras-Mapk is able to provide an additional growth input dominant to nutrient availability that would allow AMPs to expand under starvation. Following 4h egg collection, larvae were allowed to grow until 72h AED (after egg deposition, larval stage L2), at which point they were starved of nutrients. The animals were then dissected, whether kept on normal diet or starved, at 120h AED (L3). Ras-Mapk pathway upregulation was achieved by overexpressing Ras^{V12S35} using the esg^{TS} driver, whereas Pi3k and ToR pathways were modulate by p110 or Rheb expression, respectively. Cell growth and division were quantified from z-stack projections, based on cell counts as well as cell cluster and area measurements.

In control animals, at 72h AED (after egg deposition) most AMPs were found in the midgut as dividing doublets. A subsequent surge in proliferation then ensured their dispersal through the epithelium and formation of characteristic clusters, known as AMP islands, at 120h AED. Upon starvation, the wild type controls showed a block in proliferation, with AMPs still pre-

senting as doublets throughout the tissue at 120h AED. In normally fed animals, providing a growth promoting signal in the form of activated Ras, led to a strong proliferative response with progenitor cell and cluster sizes (areas) increasing significantly compared to the fed controls (figure 13). In several animals, the AMPs appeared no longer restrained to clusters but rather dispersed through the epithelium.

Interestingly, Ras^{V12S35} expression with the esg^{TS} driver allowed the progenitors to expand even under starvation conditions, although the cluster sizes were not quite as big as the ones showcased by the fed Ras^{V12S35} expressing animals. Nevertheless, the starved Ras expressing guts closely resembled the fed wild type controls. The cell and cluster area quantifications confirm these observations (figure 13). Thus, activation of Ras-Mapk signaling in midgut progenitors in the developing intestine is able to provide an additional growth input which is partially dominant to nutrient availability.

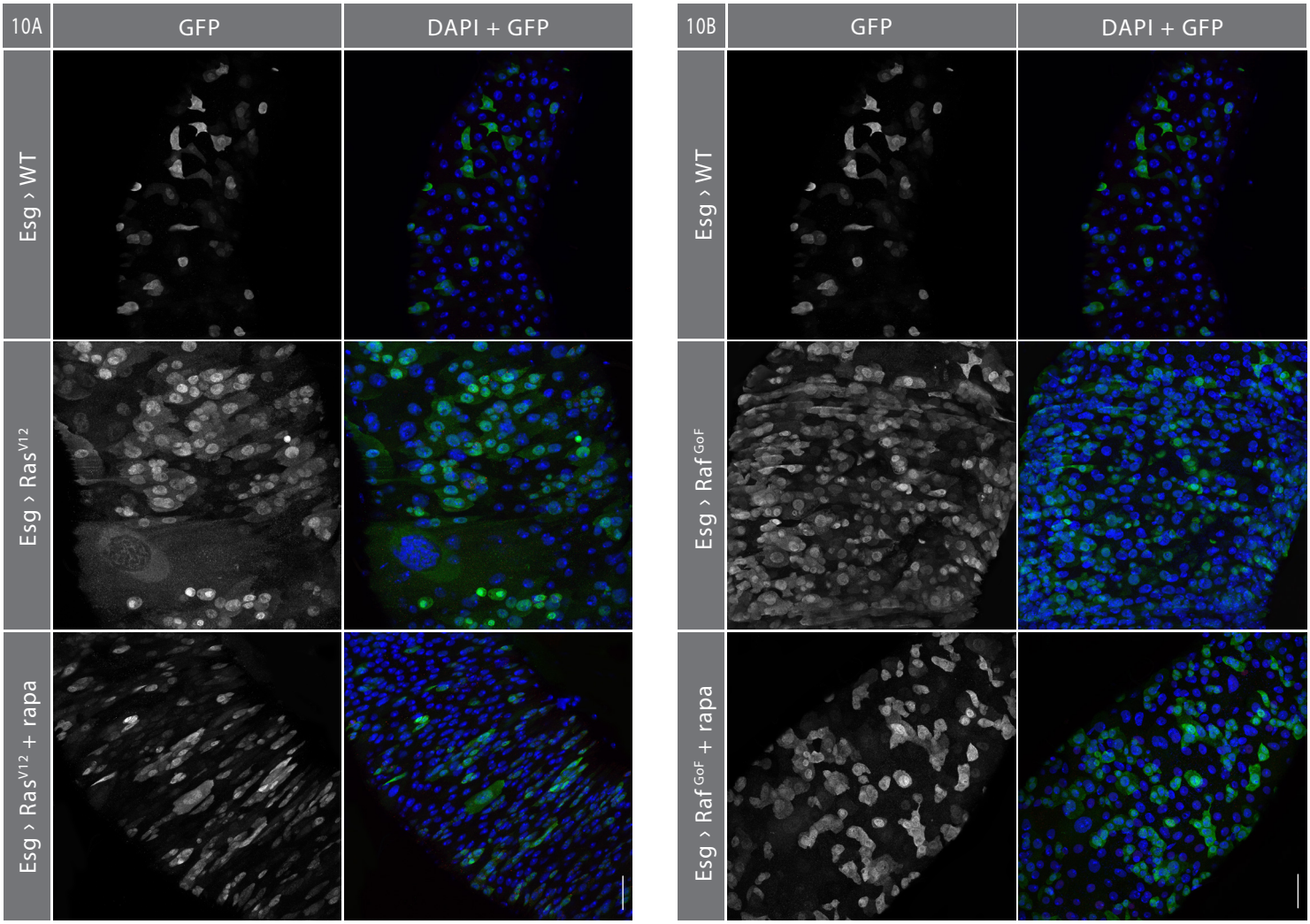
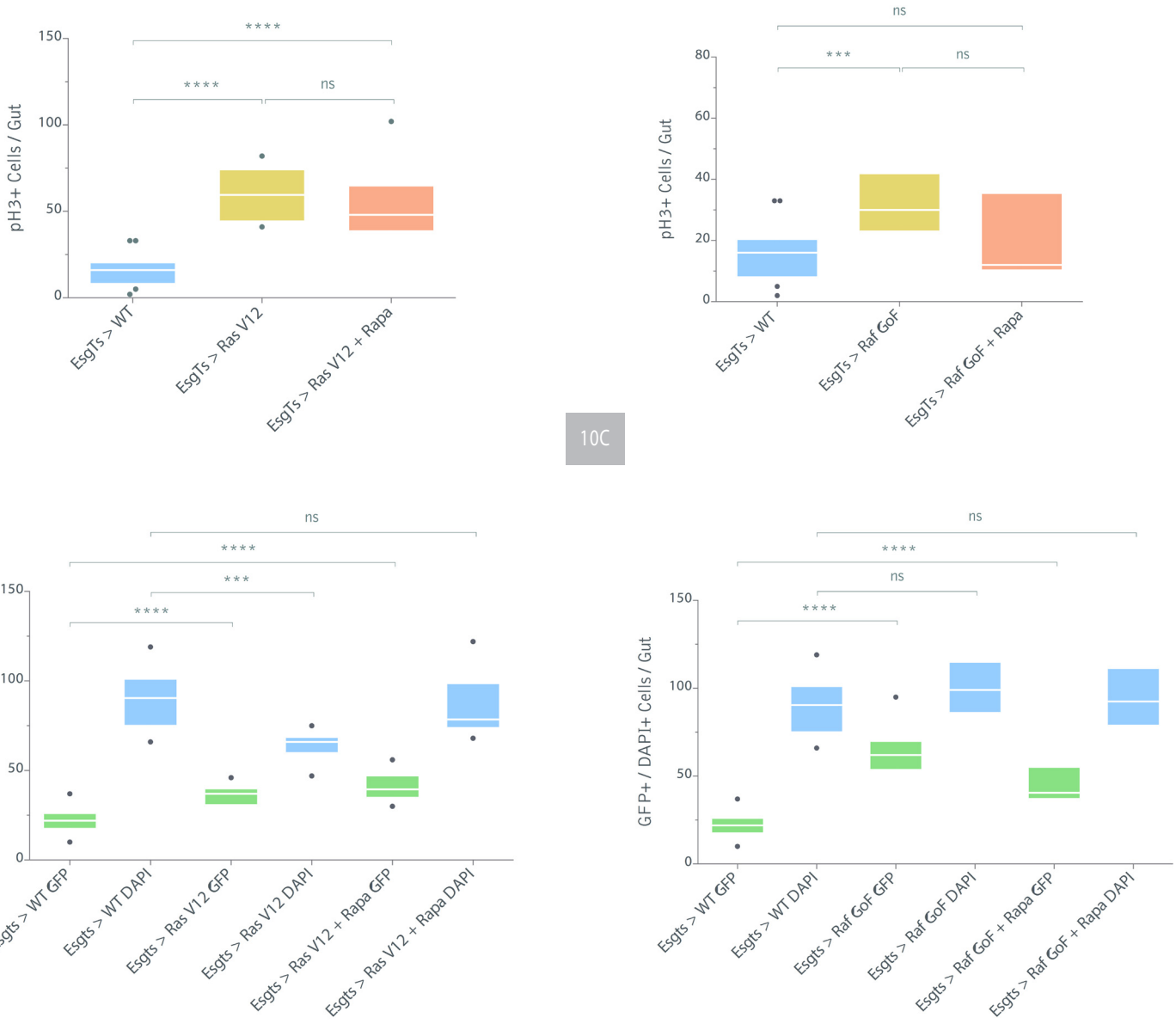
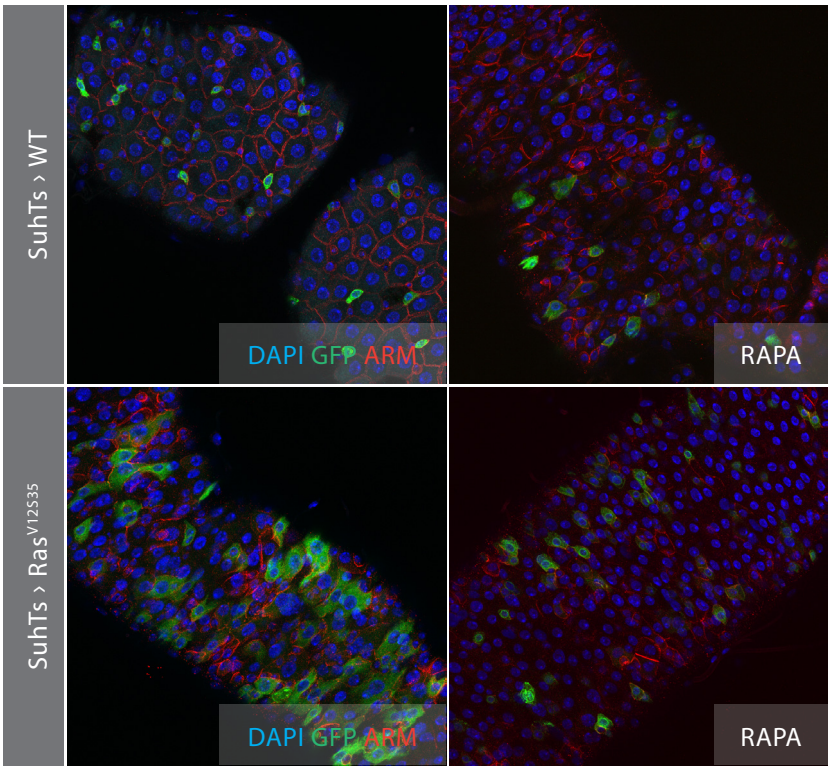


FIGURE 10A,B Egfr-Ras-Mapk activation promotes ISC proliferation independently of ToR. (10A) Ras^{V12} expression in ISCs and EBs using the esg^{TS} system. Esg+ progenitor cells are marked in green (grey) and nuclei stained with DAPI in blue. Esg-WT control guts expressing GFP alone, Esg-Ras^{V12} guts expressing activated Ras during a 5 day induction period at 29°C, rapa: animals treated with rapamycin during the induction period. Active Ras triggered a significant increase in the midgut ISC mitotic index (see 10C) and an expansion of the GFP+ cell population. Rapamycin treatment did not have a significant effect on Ras-induced proliferative activity. Fewer overgrown GFP+ cells were, however, observed following rapamycin treatment. (10B) Raf^{GoF} expression in ISCs and EBs using the esg^{TS} system. Same color convention as in 10A. Similar as with Ras expressing guts, expression of activated Raf in the progenitor compartment significantly enhanced ISC proliferation in the midgut, with or without concomitant rapamycin treatment. Scale bars represent 30 µm.



10C

FIGURE 10C Quantification of pH3+ (dividing), GFP+ (expressing), and DAPI+ cells per adult midgut of the indicated genotype. Midguts expressing activated Ras or Raf had significantly more mitotic cells and GFP+ cells then controls. Ras/Raf driven proliferation was not impaired by concomitant rapamycin treatment. Statistical significance was determined by student's t test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Error bars in each graph represent standard deviation.



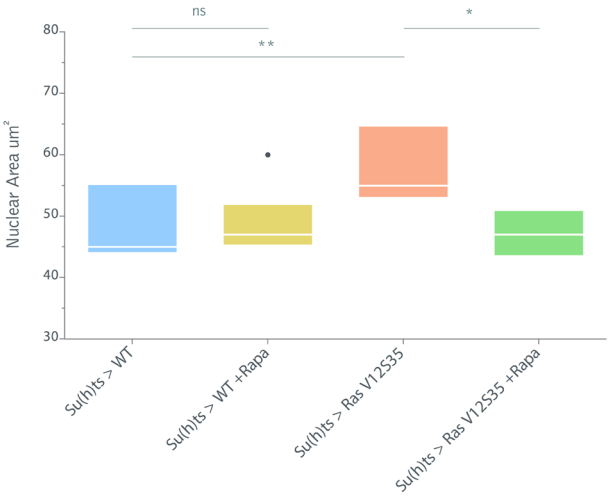


FIGURE 11 Ras driven growth of committed midgut progenitors is partially ToR dependent. The Su(h)^{TS} system was used for EB-specific expression of activated Ras (Suh^{TS}-Ras^{V12S35}) or GFP alone (Suh^{TS}-WT) during a 5 day induction period at 29°C. Rapa: rapamycin treatment during the induction period. Nuclei are marked by DAPI in blue, progenitor EBs are marked in green. Samples were additionally stained for armadillo (Arm) to better reveal cell boundaries. Cell sizes were measured based on accurate quantifications of individual cell areas from confocal z-stack projections. Expression of activated Ras in committed progenitors significantly stimulated their growth compared to controls. Rapamycin treatment impaired the growth of cells expressing Ras^{V12S35}. Statistical significance was determined by student's t test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Error bars in each graph represent standard deviation.

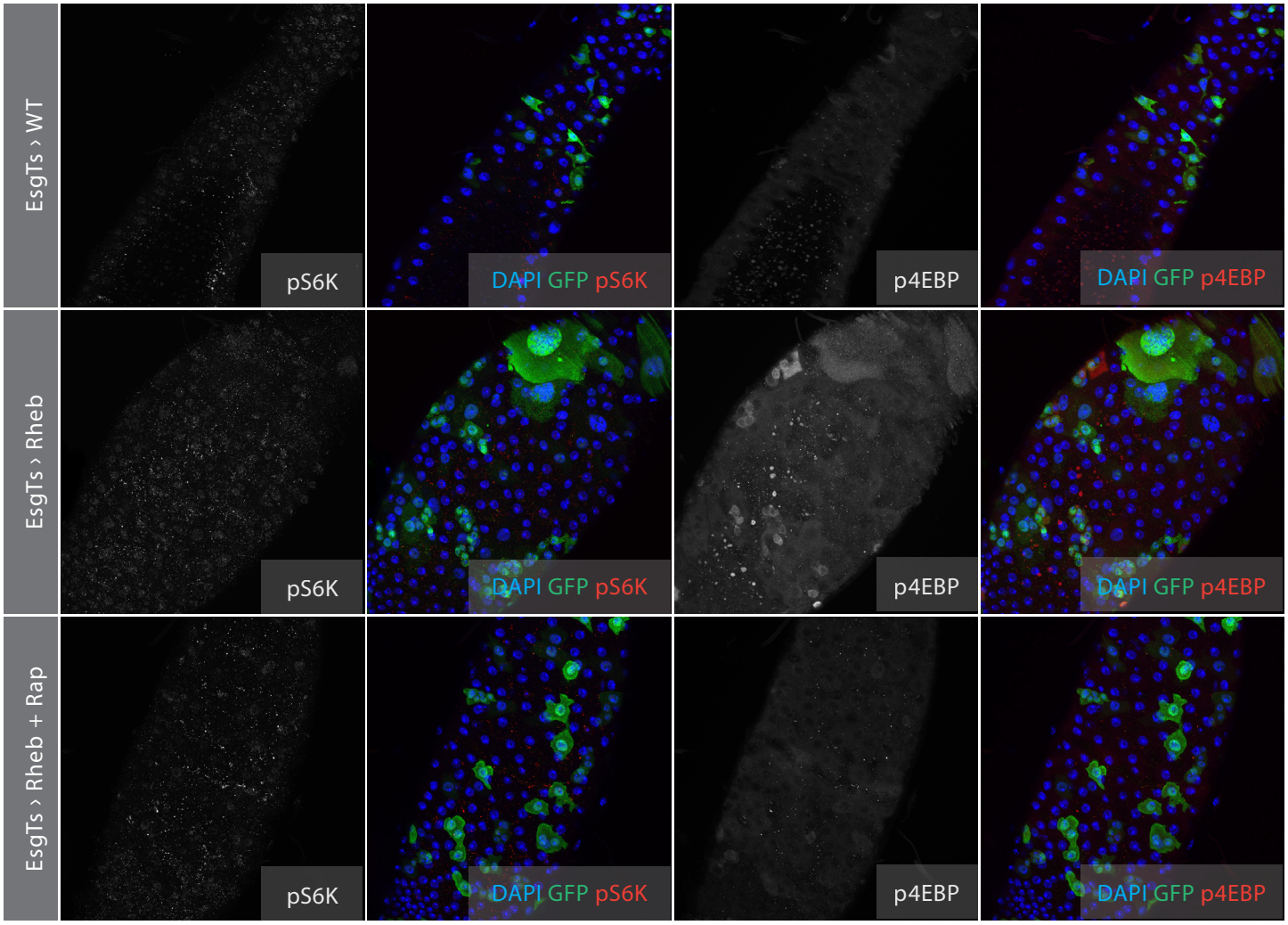


FIGURE 12A phospho-S6k and phospho-4EBP staining patterns in the midgut upon ToRC1 pathway modulation. EsgTs system was used to express Rheb or GFP alone in midgut progenitor cells (5 day induction at 29°C). Rap indicates rapamycin treatment during the induction period. pS6k/p4EBP stainings shown in separate grey images. Merges: DNA in blue, GFP+ expressing cells in green, and phospho-S6k/4EBP in red. Rheb expression in the progenitors (ISC/EBs) noticeably enhanced the overall phospho-4EBP signal and triggered the appearance of large overgrown GFP+ cells in the midgut, while rapamycin treatment reduces the phospho-4EBP staining and the occurrence of overgrown GFP+ polyploid cells.

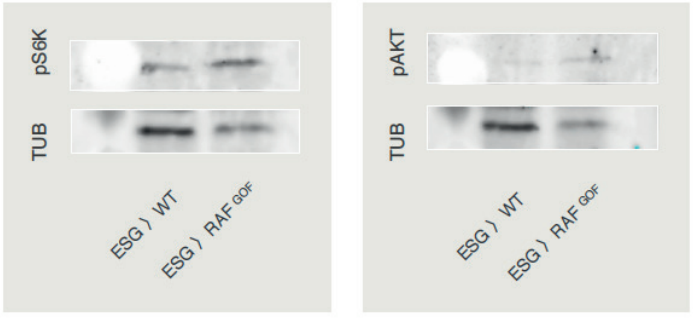


FIGURE 12B Expression of activated Raf in the midgut progenitors induces S6k phosphorylation. EsgTs system was used to express Raf^{GoF} (activated Raf allele) in midgut ISC/EBs, over a 3 day induction period at 29°C. Following transgene expression, the midguts were dissected and homogenised. Proteins were then extracted and separated by size through electrophoresis. Following blotting, the indicated antibodies were used to detect protein phosphorylation. Raf gain-of-function in the progenitors promoted S6k phosphorylation (pS6k), indicative of increased ToRC1 activity. Additional blots and controls should confirm this result. Phosphorylation levels were quantified relative to tubulin loading control.

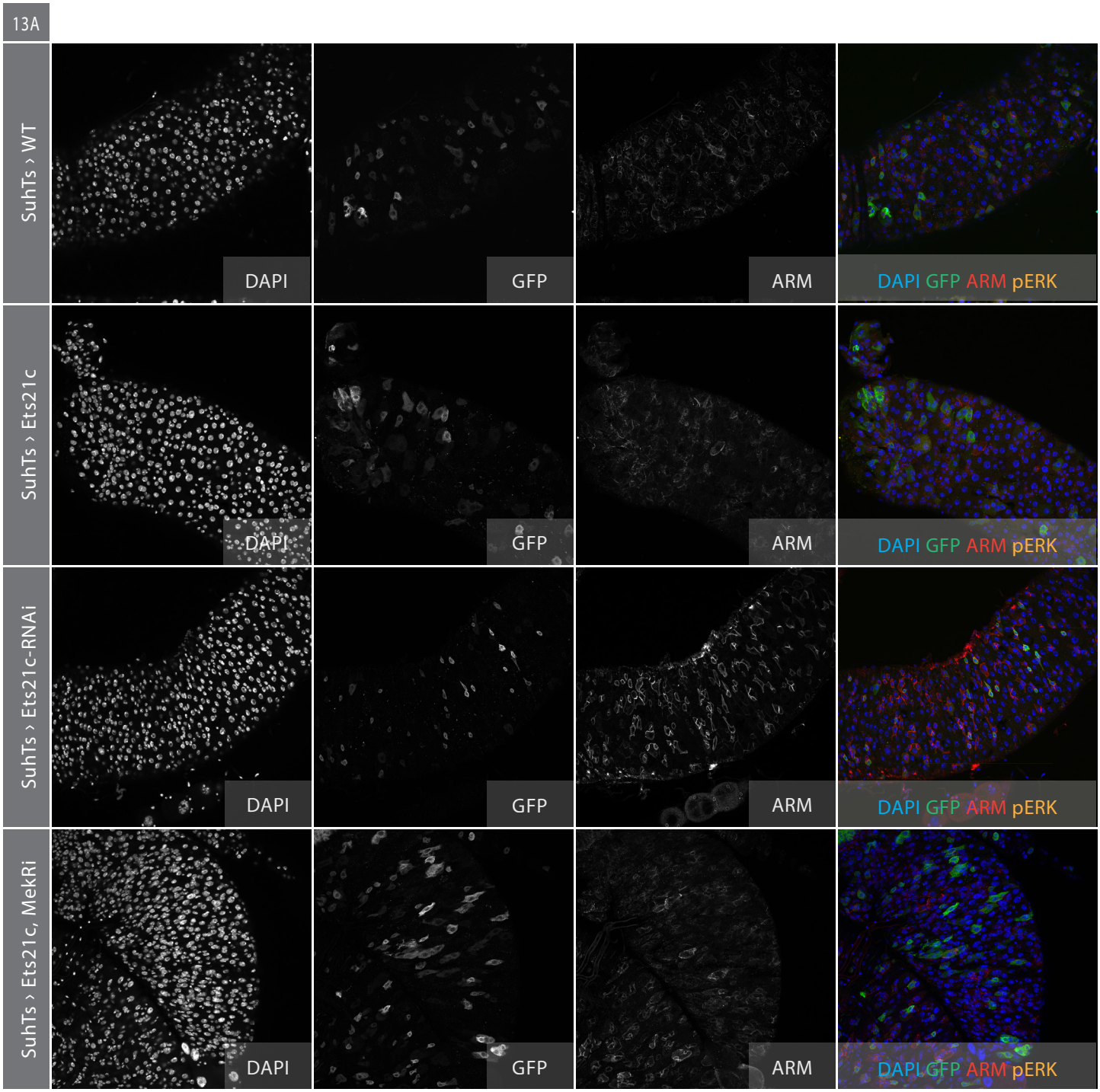


FIGURE 13 See legend on next page.

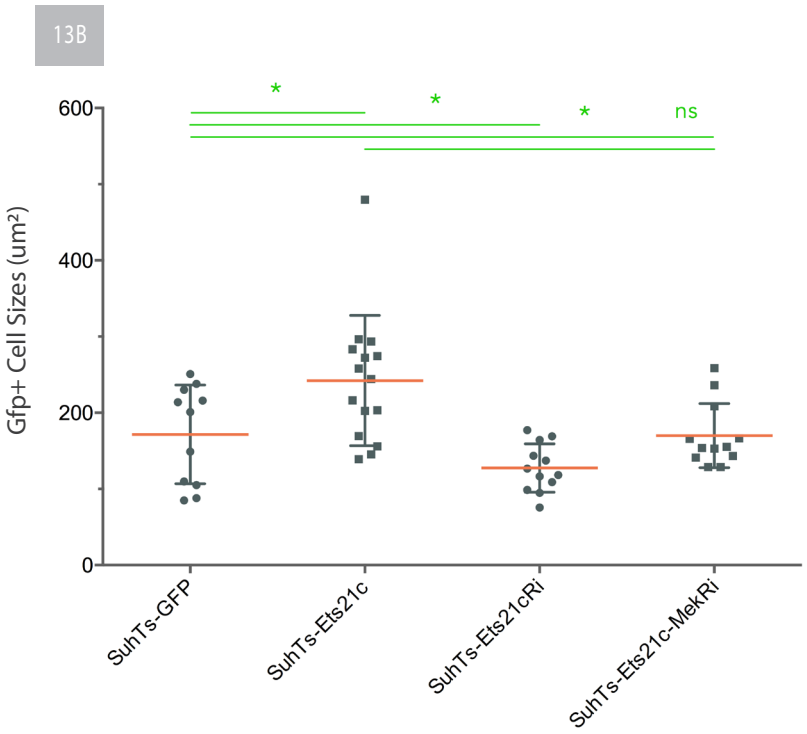
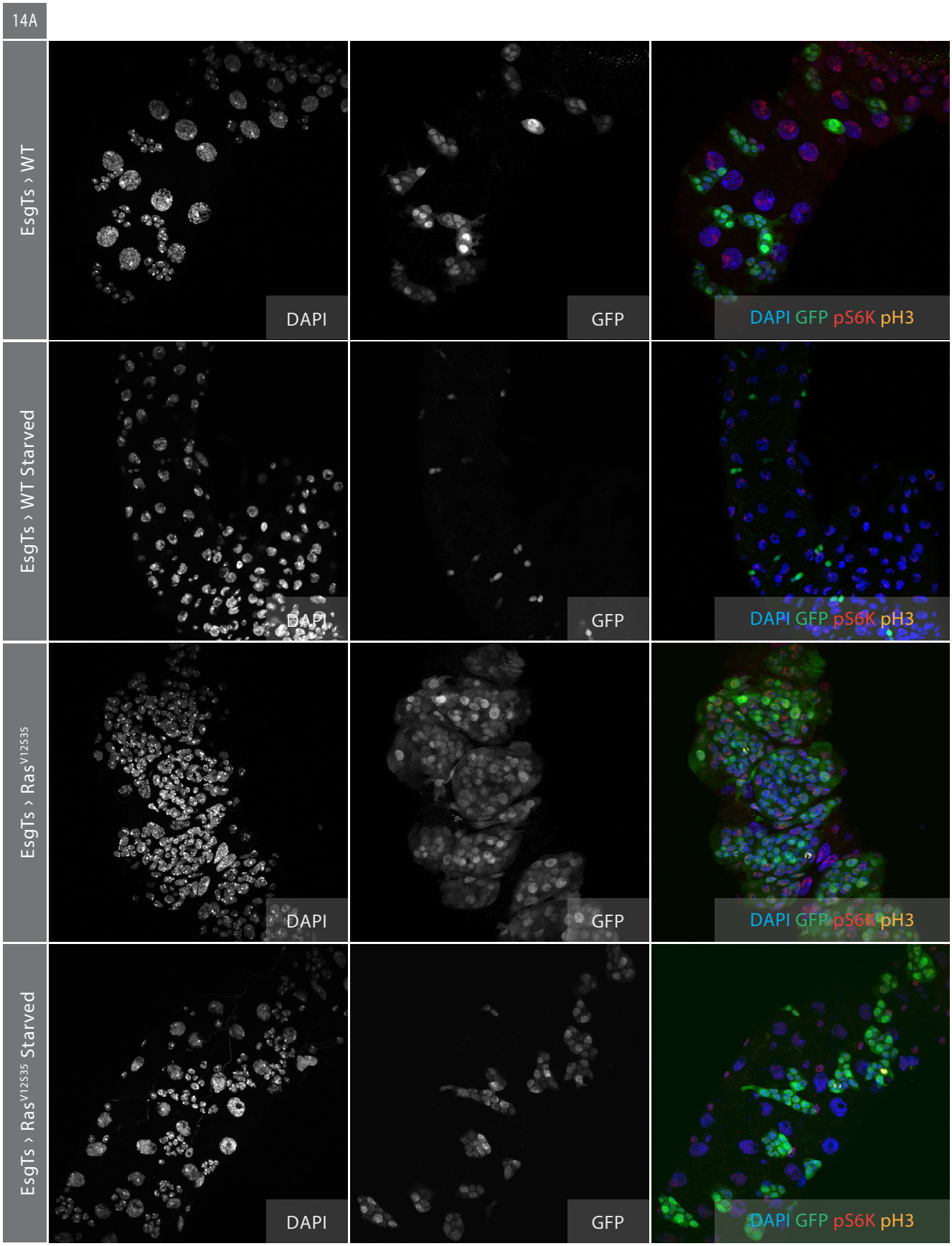


FIGURE 13 Ets21c is required and sufficient to drive EB progenitor growth in the adult intestine. The Su(h)Ts system was used drive transgene expression specifically in EBs during a 3 day induction period at 29C. 13A DAPI marks nuclear DNA in blue, EBs are colored in green, armadillo (Arm) is in red, and phospho-Erk in yellow. Row1 (first up) control expressing GFP alone, row2 midguts expressing Ets21c in the committed EB progenitors, row3 Ets21c RNAi mediated knockdown in EBs, row4 co-expression of Ets21c and Mek-RNAi in EBs. 13B Cell sizes were measured based on accurate quantifications of individual cell areas from confocal z-stack projections. Ets21c expression in the committed progenitors led to a significant size increase in comparison to their wild type siblings expression GFP alone. Furthermore, Ets21c appears to be required by these cells in order to achieve their normal size under homeostatic conditions, as Ets21c knockdown triggered a significant reduction in cell size. The epistasis test (row4) revealed that the growth promoting effect prompted by Ets21c expression does not critically depend on Mek kinase activity, as Mek-RNAi did not significantly impair the Ets21c-driven size increase. Statistical significance was determined by student's t test (*p<0.05,**p<0.01,***p<0.001,****p<0.0001). Error bars in each graph represent standard deviation.


14A
EsgTs › WT
DAPI
GFP
DAPI GFP pS6K pH3
EsgTs › WT Starved
DAPI
GFP
DAPI GFP pS6K pH3
EsgTs › RasV12S35
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14B

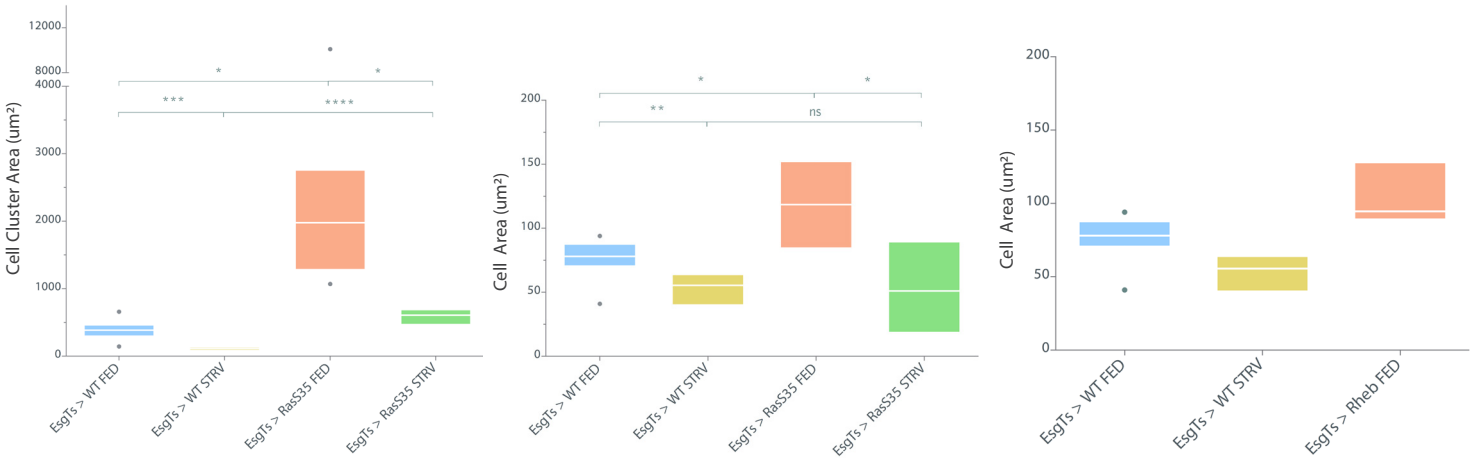


FIGURE 14 Ras-Mapk signaling promotes midgut progenitor cell growth in absence of nutrients in the larval gut. Sample preparation: embryos were collected for 4 hours at 25C on grape juice agar plates and transferred to 18C; L1 larvae were transferred after hatching at 48h after egg deposition (AED) (24h at 25C) into new vials on standard laboratory food in a density controlled manner and maintained at 18C; few hours before 6d AED (132h 5.5d) (72h at 25C), late L2 larvae were transferred into 20% sucrose solution for the starved condition and maintained at 29C for transgene induction; animals were dissected few hours before 10d AED (9.5d) (120h at 25C) for immunofluorescent staining. The esgTs system was used to achieve conditional transgene expression in the adult midgut progenitors (AMPs) in the larval gut. (14A) DAPI stained nuclei are in blue, GFP+ progenitors are depicted in green, phospho-S6k in red, and pH3+ (dividing) cells in yellow. (14B) Cell and cluster area quantifications were performed on confocal z-stack projections for at least 15 guts per sample. Upon starvation, the wild type controls showed a block in proliferation, with AMPs failing to expand and remaining as doublets throughout the tissue (14A: esgTs-WT starved, 14B: yellow bars). In animals on normal diet, expressing activated Ras in AMP progenitors led to a strong proliferative response with progenitor cell and cluster sizes increasing significantly compared to the fed GFP-expressing controls (14A: esgTs-Ras^{V12S35}, 14B: red bars). Ras expression allowed the progenitors to proliferate to a certain extent and increase cluster sizes, even in animals deprived of nutrients (14A: esgTs-Ras^{V12S35} starved, 14B: green bars). Rheb (positive ToRC1 regulator) expression in the progenitors also significantly increased progenitor cell sizes (14B red bar). Statistical significance was determined by student's t test (*p<0.05,**p<0.01,***p<0.001,****p<0.0001). Error bars in each graph represent standard deviation.

II-4 MAPK GROWTH AND AUTOPHAGY PHENOTYPES IN FAT BODY

II-4A Ras Activation Cannot Rescue Growth Deficiency of Fat Body Cells

context Reduction of InR/Pi3k activity in differentiated tissues of drosophila larvae leads to cell autonomous reduction in cell growth and DNA endoreplication [46]. Reducing ToR activity triggers similar inhibitory effects [44,45]. Conversely, activation of InR/Pi3k or of Rheb/ToR signaling increases cell size and nuclear DNA content in many larval tissues, including the gut and the fat body. Moreover, InR/Pi3k as well as Rheb are capable of bypassing the dietary requirement for cell growth, and can function as growth promoters even in animals starved for protein [46,47].

aim To better understand Ras-Mapk involvement in growth related processes and its possible connection to ToR signaling, we tested its ability to promote cell growth in fat body tissue under fed and nutrient/ToR-deficient conditions.

approach To determine if Ras-Mapk is able to rescue the growth of cells deficient for ToRC1 activity, the Adh-Gal4 driver was used to express activated Ras in all fat body tissue concomitant with ToRC1 inhibition via rapamycin treatment. An additional experiment addressed the clonal ability of Ras-Mapk activation to induce a growth phenotype in fat body tissue under fed or nutrient starved conditions. In this latter experiment, clones hyperactive for Ras-Mapk or ToRC1 signaling were generated in fat body tissue using the Flp/Gal4 technique (Basler 1993, Zipursky 1997, Neufeld 1998). In both experiments, cell sizes of transgene expressing and control non-expressing cells were estimated based on individual cell area quantifications from confocal projections.

results A visible change in fat body cells deprived of protein was an aggregation of lipid vesicles, effect also observed upon treatment with rapamycin. Treatment with the known ToRC1 inhibitor rapamycin led to a decrease in the average cell size in fat body tissue. This size defect was not rescued by concomitant ectopic expression of activated Ras using the Adh-Gal4 driver, indicating that either Ras alone is not sufficient to promote growth in this tissue, or that it requires ToR activity in order to do so (figure 15). The second experiment revealed that clonal Rheb overexpression significantly increased the size (cell area) of these endoreplicating cells under both fed and starved conditions (figure 16). Fat body cells overexpressing Rheb encompassed approximately 1.5x the area of neighbouring control cells. In contrast to the cell size in

crease triggered by Rheb, RasV12 expressing cells did not show a significant growth change compared to control cells in neither the fed nor the starved conditions (figure 16).

II-4B Mapk Cannot Suppress Starvation Induced Autophagy in Fat Body Tissue

context Autophagy is the main degradative process in eukaryotic cells, allowing cells to maintain homeostasis by recycling damaged proteins and organelles, and to adapt to nutrient starvation by breaking down and recycling cellular components. Starvation induces a rapid autophagic response in the larval fat body, a nutrient storage organ analogous to the vertebrate liver. Furthermore, activation of Pi3k-Rheb-ToR signaling is necessary and sufficient to suppress starvation-induced autophagy in the fat body [48,49].

aim Based on the rapid response of fat body tissue to starvation and the effect of ToR pathway activity in this context, we wanted to assay whether clonal Ras-Mapk activation can rescue the starvation-induced autophagic response in this tissue. Ras mediated autophagy phenotype would give an indication of its influence on ToR activity in this regard.

approach Clones hyperactive for Ras-Mapk or ToRC1 signaling were generated in fat body tissue using the Flp/Gal4 technique (as in II-4A), under both fed and starved conditions. Autophagy induction was detected using Lysotracker Red staining [48]. The lysotracker probes are fluorescent acidotropic probes commonly used for labelling and tracking acidic organelles in live cells.

results Upon starvation, larval fat body cell growth is arrested, lipid droplets aggregate and autophagy is rapidly induced. Fat body from starved larvae displayed an intense granulated lysotracker staining, whereas fed animals showed only a faint diffuse staining pattern (figure 17). Clonal expression of Rheb suppressed autophagy in starved animals in a cell autonomous manner, indicating that ToR signaling is sufficient to prevent starvation-induced autophagy (as shown before, 48). Unlike Rheb, however, we found that clonal expression of constitutively active Ras did not suppress starvation-induced autophagy, indicating that Ras signaling is not capable to circumvent the autophagic response induced by starvation in fat body tissue (figure 17).

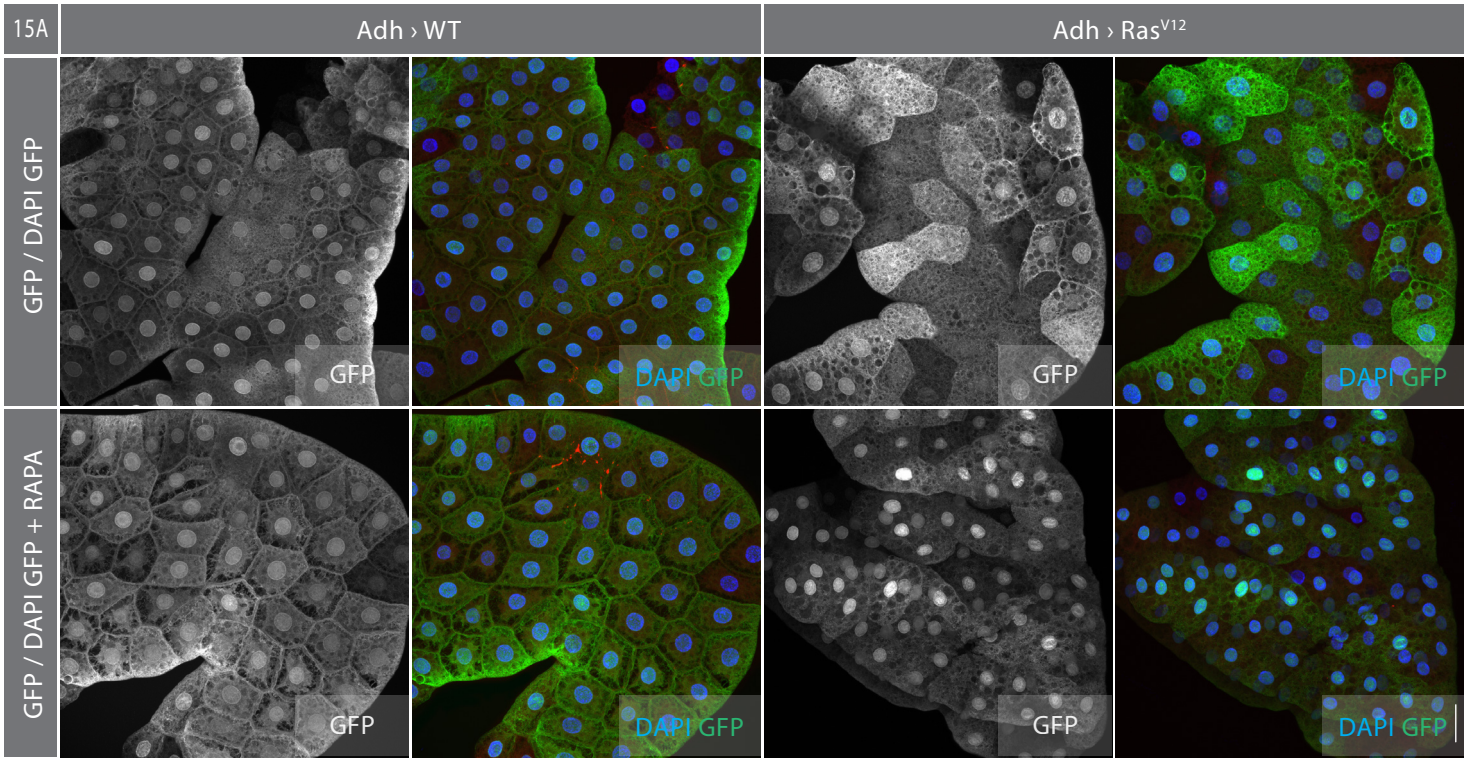
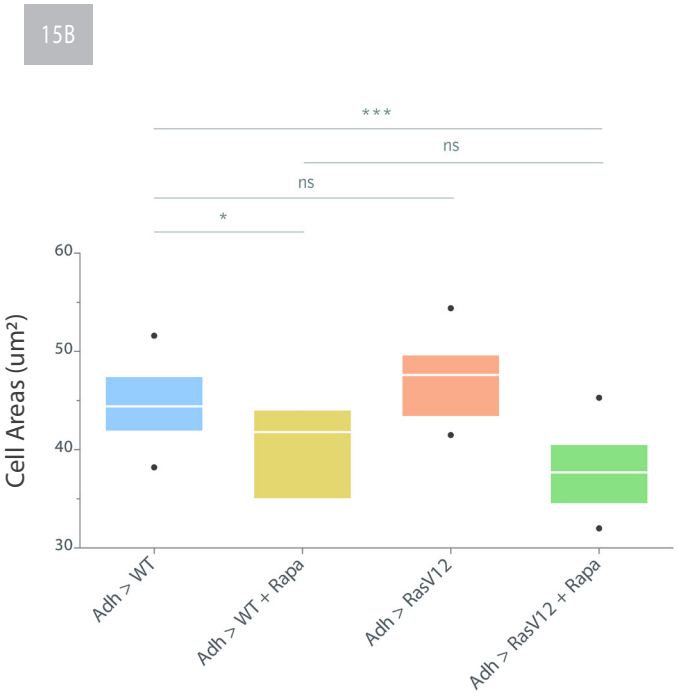
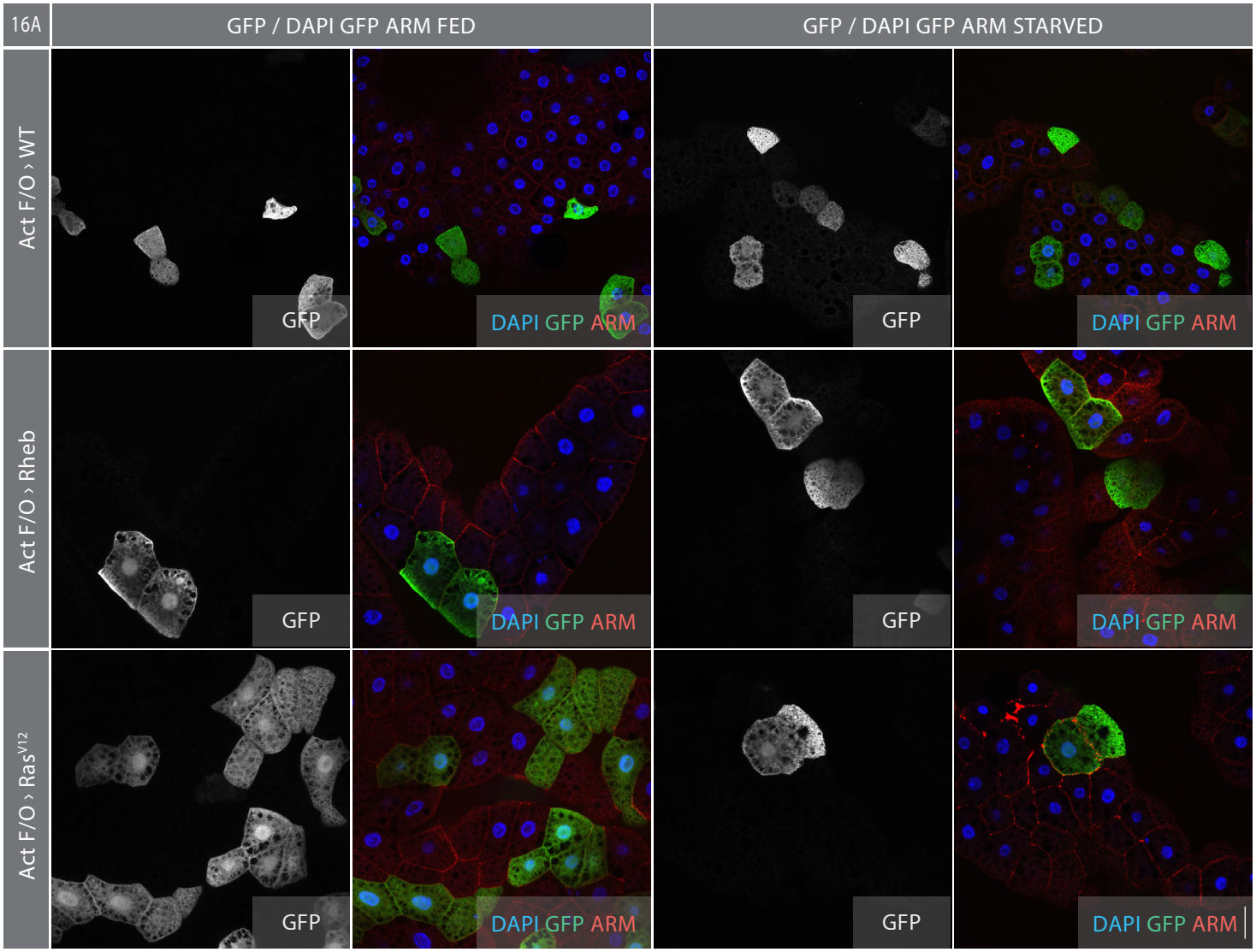


FIGURE 15 Ras expression in fat body tissue of rapamycin treated larvae. The Adh-Gal4 driver was used to achieve transgene expression in all fat body cells. Following a 3 hour egg collection, L2 larvae were transferred at 72 hours AED (after egg deposition) into vials with fresh fly food with or without rapamycin. L3 larvae were then collected at 90 hours AED for analysis. DAPI stained nuclei are depicted in blue, and GFP+ expressing cells in green. Cell sizes were measured based on accurate quantifications of individual cell areas from confocal z-stack projections. Scale bar represents 50 um. Rapamycin treatment led to a significant decrease in the average cell size in fat body tissue (15A Adh-WT + Rapa, 15B yellow bar) compared to untreated controls (16A Adh-WT, 16B blue bar). This growth defect was not rescued by concomitant expression of activated Ras (15A Adh-RasV12 + Rapa, 15B green bar). Statistical significance was determined by student's t test (*p<0.05,**p<0.01, ***p<0.001,****p<0.0001). Error bars in each graph represent standard deviation.





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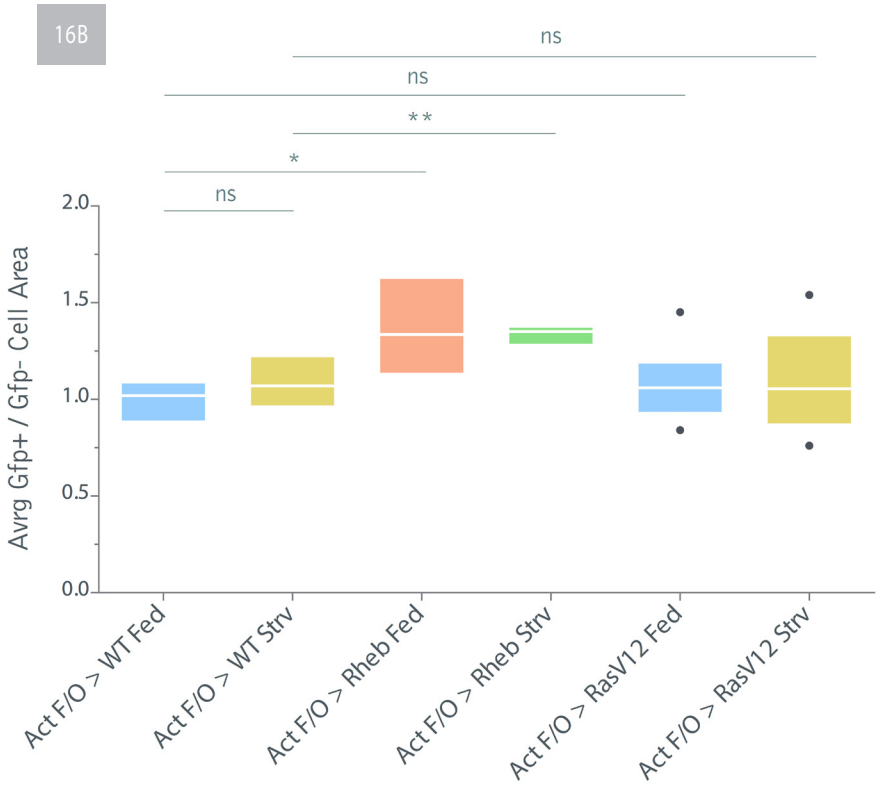


FIGURE 16 Activated Ras expression cannot rescue growth defect of protein-deprived fat body cells. Cell clones expressing Rheb or RasV12 were induced in fat body tissue using the Flp-Gal4 system. DAPI-stained nuclei are shown in blue, GFP+ expressing cell clones are visible in green, and armadillo (bCatenin) in red to better delineate cell boundaries. Animals were starved of dietary protein from L2 stage at 80 hours AED until L3 at 94 hours AED. Clonal Rheb overexpression significantly increased the size of these endoreplicating cells under both fed and starved conditions (16A ActFO-Rheb middle row, 16B red and green bars). Fat body cells overexpressing Rheb encompassed approximately 1.5 times the area of neighbouring control cells. In contrast to the cell size increase triggered by Rheb, Ras expressing cells did not show any significant growth change compared to neighbouring control cells in neither the fed or the starved conditions (ActFO-RasV12). Cell sizes were measured based on accurate quantifications of individual cell areas from confocal z-stack projections. Quantifications are expressed as ratios of average GFP+ (expressing Ras/Rheb) cell areas versus the average GFP- non-expressing cell areas. Scale bar represents 50 um. Statistical significance was determined by student's t test (*p<0.05,**p<0.01,***p<0.001, ****p<0.0001).

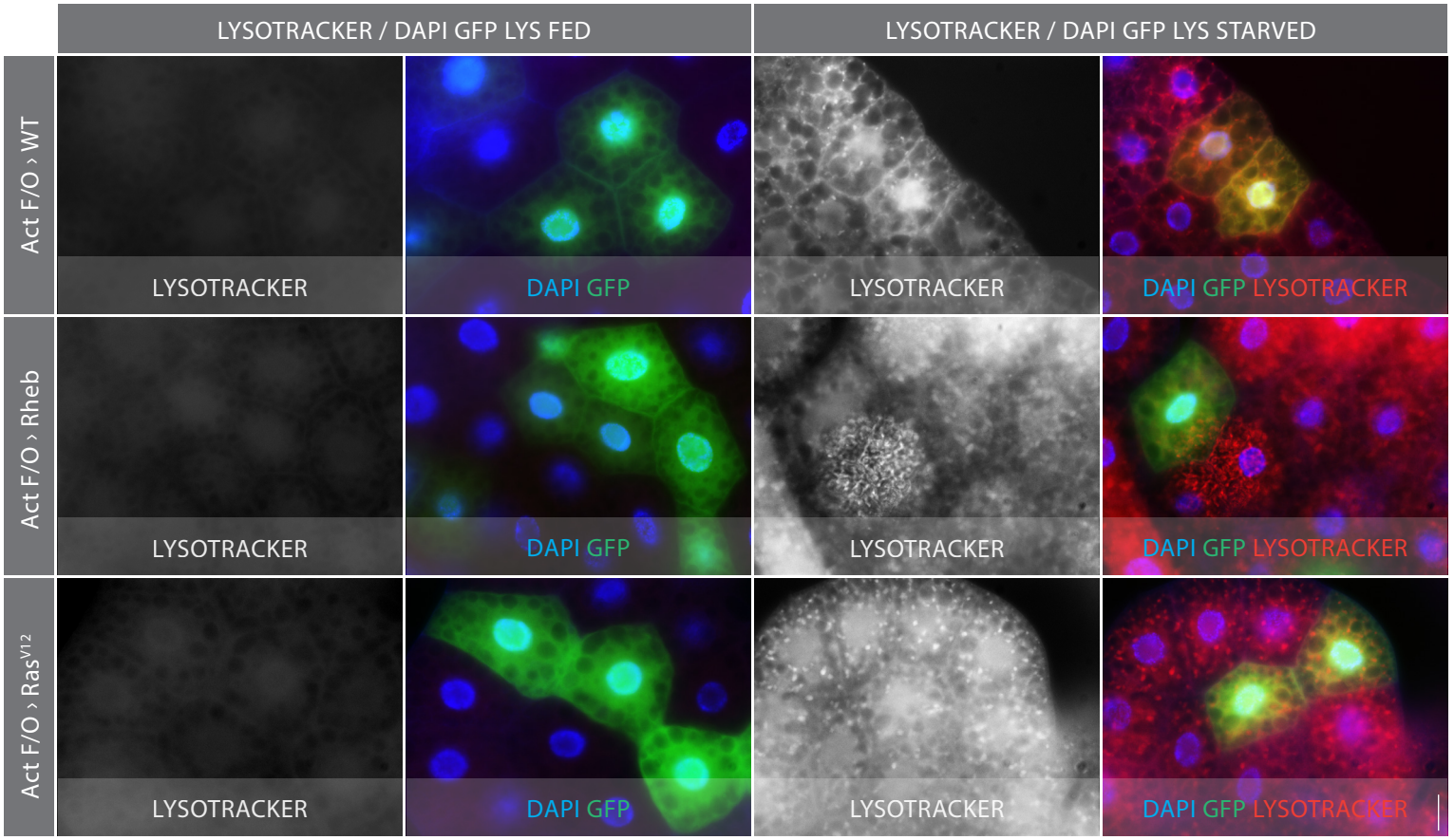


FIGURE 17 Ras expression cannot rescue starvation induced autophagy in larval fat body tissue. Heat shock independent spontaneous Flp induced expression of Gal4 transcription factor was used to clonally activate UAS-controlled transgenes. L3 larvae were starved of dietary protein in 20% sucrose at 86h AED for 4h prior to dissection and staining with lysotracker. DAPI-stained DNA is shown in blue, GFP+ expressing cells are in green, and lysotracker staining in red as an indicator of autophagy. The lysotracker probe consists of a fluorophore linked to a weak base that selectively accumulates in cellular compartments with a low internal pH, and can be used as an indicator of autophagy. Upon starvation, larval fat body cell growth is arrested and autophagy is rapidly induced (ActFO-WT, upper row). Fat body tissue from starved animals displayed an intense granulated lysotracker staining, whereas fed animals show only a faint diffuse staining pattern (upper row, ActFO-WT fed vs starved). Clonal expression of Rheb suppressed autophagy (lysotracker staining) in starved animals in a cell autonomous manner (ActFO-Rheb, middle row), as shown before by Scott and Neufeld 2004. Unlike Rheb, however, clonal expression of activated Ras didn't suppressed the starvation induced autophagic response. Scale bar represents 50um.

III. DISCUSSION

III-1 MAPK IMPLICATIONS IN GROWTH AND CANCER

How Does Mapk Drive Growth?

The evolutionary conserved Mapk pathways coordinate essential cellular functions, including cell survival, proliferation, growth, motility and differentiation [9]. To execute such intricate functions, Mapks regulate transcription factors impinging on gene expression, as well as a variety of cytosolic substrates coordinating other aspects of cellular metabolism. As part of the polyvalent nature of this pathway's functionality, Ras-Mapk has been firmly established as a growth promoter in different contexts [3,69,70,151-153]. However, despite a vast literature on Mapk pathways, the nature of the effectors and interactions underlying Mapk-driven growth remains a matter of inference from an assortment of disparate systems. Studies based mostly on mammalian systems suggest multiple interactions that may play a role in Mapk driven growth. In our study we focused on two types of effectors, the ToRC1 complex (target of rapamycin complex 1) and the Mapk activated protein kinases Mapkapks.

ToRC1 is remarkable in its ability to integrate five major inputs (growth factors, amino acids, energy, stress and oxygen) and appropriately coordinate anabolic pathways, including nutrient intake, protein and lipid synthesis, as well as catabolic pathways such as autophagy [154]. Importantly, both Erk Mapk and one of its targets (Rsk) were found to positively regulate ToRC1 in mammalian cells through direct and indirect mechanisms [30-36]. Furthermore, mammalian studies have attributed a significant extent of Mapk functionalities to the activation of downstream Mapkapks. They are presented as an additional amplification step in the Mapk catalytic cascades, increasing the range of actions regulated by Mapk modules (III-2). Two of the Mapkapk were found to be involved in translation control, namely the Mnks (Mapk interacting kinases])[12] and the Rsks (p90 ribosomal S6 kinases) [13].

Proposed Model

This study suggests a model that describes Mapk-ToR crosstalk in drosophila and brings forth new potential Mapk growth effectors, the Mapkapks. According to this model, Erk Mapk may

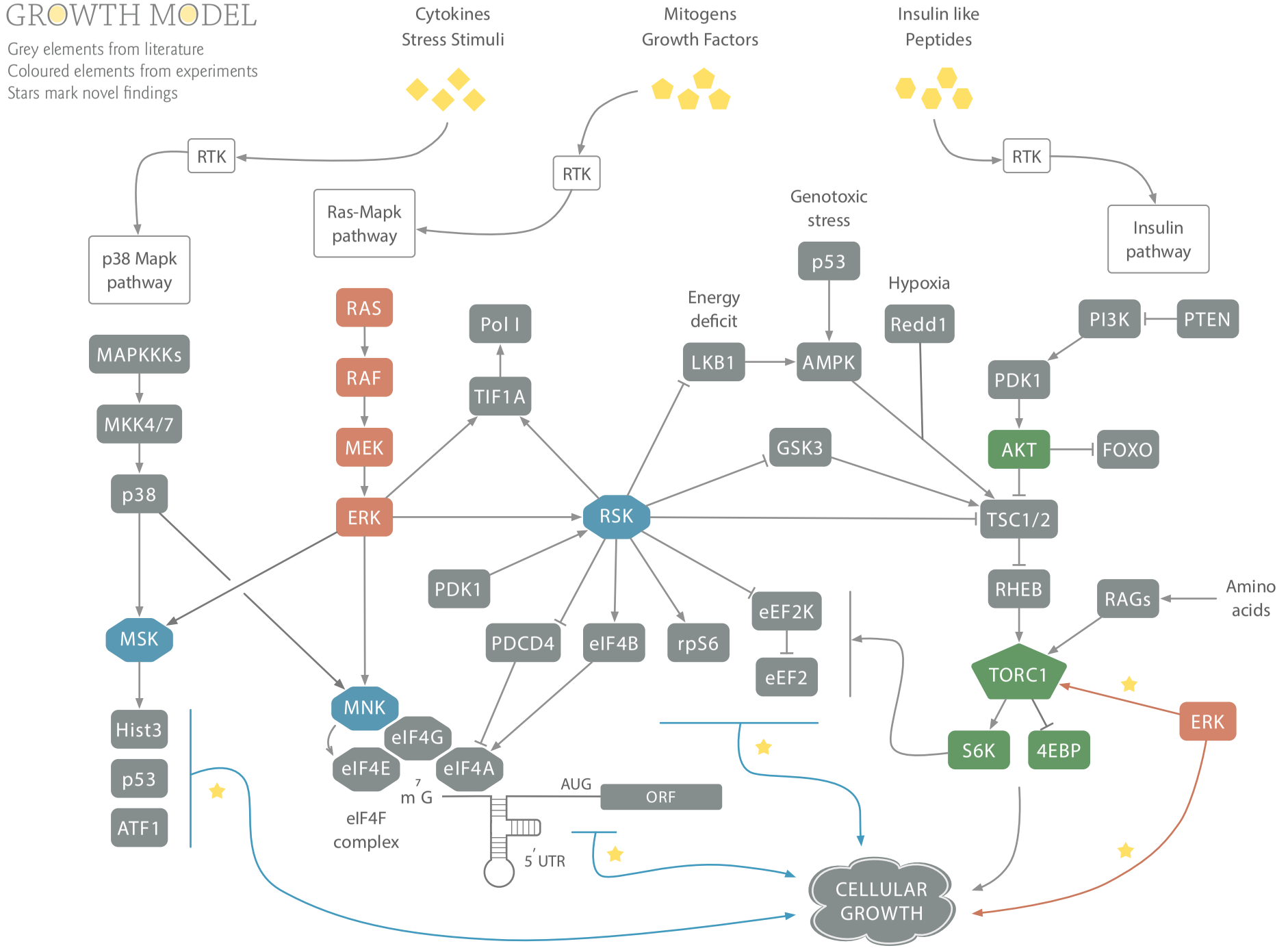
promote growth in insect cells via two mechanisms. A first mechanism relies on Mapkapk activation that might directly promote translation or activate another pathway, such as ToR, in order to do so. The connection to Mapkapks would integrate Ras-Mapk pathway activity with nuclear signaling and chromatin remodelling processes (see III-2). A second mechanism may rely on ToRC1 activation, which in turn promotes biosynthetic pathways and eventually growth. The connection to ToRC1 would integrate Ras-Mapk pathway activity with the cellular and systemic metabolic state, and initiate Mapk-driven cell functions (requiring translation) only if the nutritional and energetic context is favourable.

Experimental evidence in support of the first mechanism comes from our in vitro assays, which show that three Mapkapks (Mnk, Rsk, Msk) are required for insect cell growth downstream of RTK signaling (II-1, III-1, III-2). Although mammalian studies identify these Mapkapks as Erk Mapk effectors [Rsk 82-85, Msk 91-97, Mnk 105-111], it remains to be experimentally tested if they are indeed directly targeted by Erk Mapk in insect cells. Two of them, Rsk and Mnk, were already identified as Erk interaction partners in insect cells [14-16, 17-19, 104].

Experimental evidence in support of the second mechanism relying on ToRC1 comes from our in vitro and in vivo assays. In cultured insect cells, Ras-Mapk appears to be sufficient and required for ToRC1 activation (II-2, III-3, III-4), while in the animal's intestine Ras-Mapk depends on ToRC1 activity to fully promote growth (II-3, III-5). Furthermore, Ras-Mapk activation in the developing intestine acts as a potent growth and proliferation promoter, even under conditions of protein starvation (II-4, III-6). Ras-Mapk is thus able to replicate a phenotype previously attributed to ToRC1 functionality [47]. If this Ras-Mapk growth phenotype is indeed dependent on ToRC1 remains to be seen.

Both Mapk and ToR pathways are evolutionary conserved and key players in the regulation of multiple cellular functions. There is hardly any cellular stimulus that doesn't feed into these pathways. It is easy to see how connecting these pathways would be advantageous not only for tissue homeostasis and regeneration but also for keeping developmental and metabolic decisions in sync. Multiple convergence points between these pathways (III-4) seem to argue for the validity of this hypothesis.

Model for Mapk driven growth in insect cells. Ras-Mapk pathway activation downstream of RTK receptors (including InR and Egfr) stimulates ToRC1, a strong promoter of cell growth. Additionally, Mapk activated protein kinases (Mnk, Rsk, Msk), previously identified as downstream effectors of Mapks*, also function as growth promoters in insect cells. Therefore, depending on the developmental timing, tissue type and regenerative needs, Ras-Mapk may rely on multiple effectors to drive growth. *14-16,83-85,91-93,96-99, 109-112


GROWTH MODEL
Grey elements from literature
Coloured elements from experiments
Stars mark novel findings
Cytokines
Stress Stimuli
Mitogens
Growth Factors
Insulin like
Peptides
RTK
RTK
RTK
p38 Mapk
pathway
Ras-Mapk
pathway
Insulin
pathway
Genotoxic
stress
p53
Hypoxia
Redd1
Energy
deficit
LKB1
AMPK
PI3K
PTEN
PDK1
AKT
FOXO
GSK3
RAS
RAF
MEK
ERK
Pol I
TIF1A
RSK
TSC1/2
MAPKKKs
MKK4/7
p38
MSK
PDK1
PDCD4
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eEF2K
eEF2
RHEB
RAGs
Amino
acids
TORC1
ERK
S6K
4EBP
MNK
eIF4G
eIF4E
eIF4A
eIF4F
complex
m7G
AUG
ORF
5′UTR
Hist3
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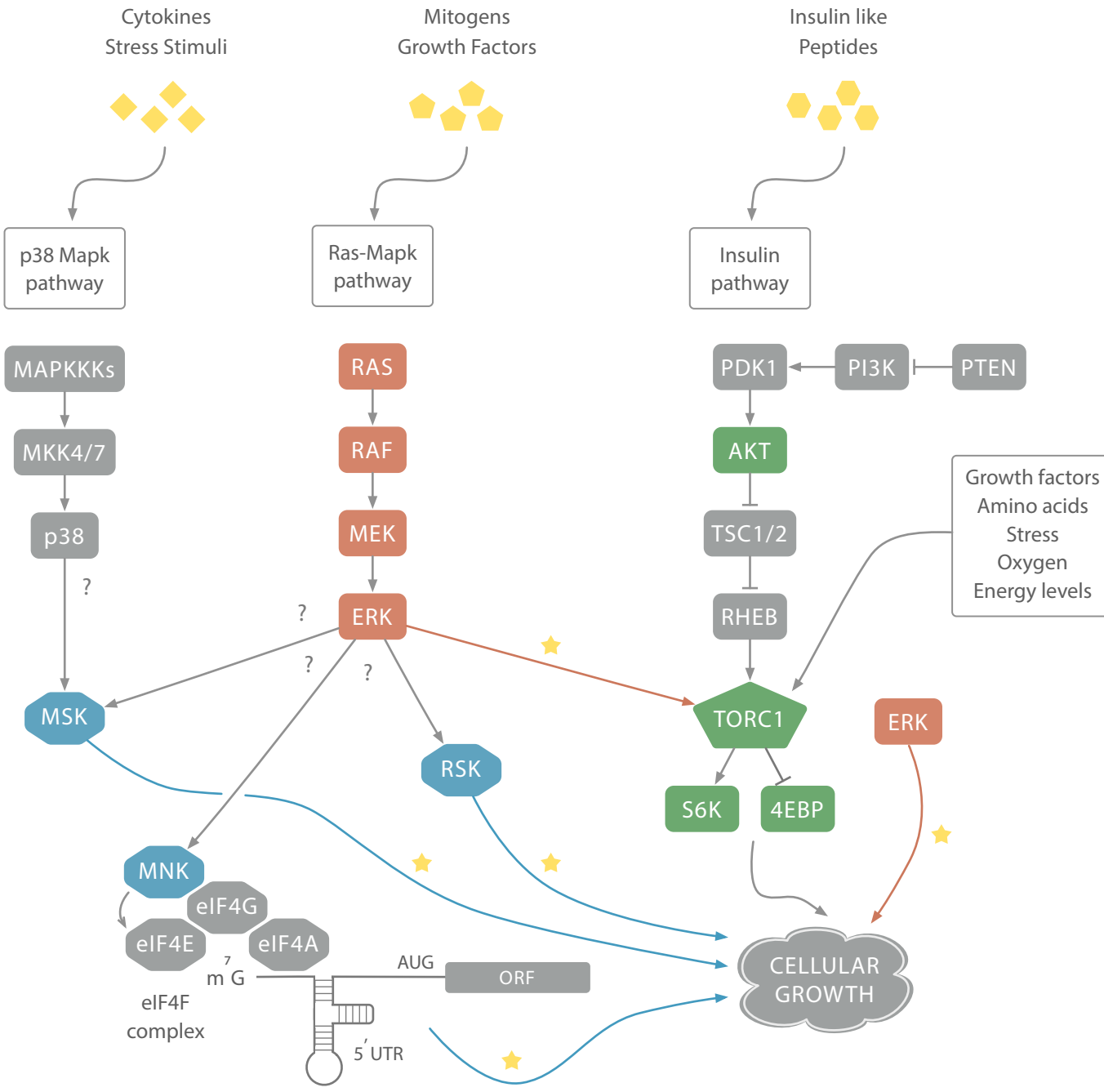
CELLULAR
GROWTH

GROWTH MODEL

Simplified to emphasise the novel elements in insect cells
Grey elements from literature
Coloured elements from experiments
Stars mark novel findings



Mapk Signaling Mechanisms

Ras-Mapk activation generates two categories of cellular responses: transcriptional and non-transcriptional. Both types of responses unravel in different stages, involve lateral signaling and activate feedback loops which eventually restrict signaling activity. The transcriptional response relies on both activators [like Pointed] and inhibitors (like Capicua, Yan and Groucho) of gene expression, and supports functions such as proliferation, survival and differentiation. The sequential nature of the transcriptional response is nicely illustrated during drosophila eye development. During photoreceptor recruitment, low and transient Mapk activation triggers PointedP2 (PntP2) which promotes (directly or indirectly) transcription of the constitutively active PntP1 [155]. The system therefore switches from a regime that depends on Mapk phosphorylation to one that relies on PntP1 protein stability. Crosstalk at the transcriptional level occurs via convergence of different transcription factors at the promoter/enhancer region of target genes [156-158], via transcriptional induction of components of other pathways (e.g. InR regulation by Cic, 54), and via regulation of transcription co-factors implicated in other pathways (e.g. Groucho regulation by Cic, 159).

The non-transcriptional Mapk response mainly relies on phosphorylation of an assortment of nuclear and cytoplasmic targets, and supports functions such as survival, chromatin remodelling, cell cycle progression, translation and cytoskeletal organisation. The sequential nature of the non-transcriptional responses is nicely illustrated by events triggered in Rat2 cells upon Egf stimulation [160]. There, multiple phosphorylation waves of the scaffold protein Shc1 trigger first the recruitment of the adaptor Grb2 and activation of mitogenic pathways. A second phosphorylation wave activates a negative feedback loop via Akt-mediated recruitment of a phosphatase. A third Shc phosphorylation wave terminates the signal and leads to cytoskeletal reorganisation. Crosstalk at the non-transcriptional level mainly occurs via Mapk mediated phosphorylation of components of other pathways. An epitome of such crosstalk is Ras-Mapk activation of ToRC1 and Mapkapk effector kinases (III-1to4). The more elaborate circuitry may facilitate a more versatile and adaptable signalling outcome. Mapk growth function is likely executed through non-transcriptional mechanisms that may activate effectors including ToRC1 and Mapkapks in a context dependent manner, demanded by the tissue type and developmental timing.

Cancer Implications

As key mechanisms for regulating cell growth and proliferation, Mapk and ToR pathways are often hijacked by different cancers to initiate and grow tumors, and eventually metastasise. Extensive crosstalk between these pathways is advantageous not only in a developmental context but also for cancerous growth. In cases which I describe as collaborative transformation, where oncogenesis appears to be driven by both pathways, pathway crosstalk and convergence limits the use of single target drugs. For example, cancer cell lines harbouring Pi3k/Pten mutations show a reduced cytostatic response to Akt/ToR inhibitors if they additionally acquire Ras/Raf mutations [39,77]. Furthermore, in cases known as oncogene addiction, where transformation appears to be driven by a single pathway, the effectiveness of single agents if limited by cross-inhibition mechanisms between Mapk and ToR pathways. For example, treatment of breast cancer cell lines harbouring high levels of activated Erk and Egfr with Mek inhibitors increases Akt activity and is cytostatic rather then cytotoxic [78,79].

As introduced by our model, the Mapkapks may also function as Mapk growth effectors in insect cells. Dysregulation of these kinases is associated with multiple human diseases including cancer. For example, Rsk2 mutations are the underlying cause for CLS (Coffin Lowry Syndrome), an X-linked mental retardation syndrome characterised by psychomotor retardation and facial, hand and skeletal malformations [81]. Furthermore, Mnk directly phosphorylates the translation initiation factor eIF4E. eIF4E phosphorylation in turn was found to be important for eIF4E oncogenic potential [113]. eIF4E is believed to exert its tumorigenic activity by suppressing apoptosis (e.g. via Mcl1, 114) and by promoting translation of inflammatory molecules (Ccl2 and Ccl7) and matrix metalloproteases (Mmp3 and Mmp9), which play roles in inflammation and tumor progression, respectively [116].

The significance of studies such as the one described here is therefore far reaching, not only for our understanding of how cells integrate multiple RTK-derived signals to grow and dynamically coordinate developmental with metabolic decisions, but also towards designing more effective therapies targeting tumor growth.

III-2 NEW GROWTH EFFECTORS IN INSECT CELLS, THE MAPKAPKS

This section discusses the results presented in II-1.

Context

The dogma. Mapk (mitogen activated protein kinase) and ToR (target of rapamycin) pathways are key mechanisms through which cells control their survival, proliferation, growth and motility. Both pathways are frequently mutated in a variety of human cancers, and extensive signaling crosstalk and convergence between these pathways limits the efficacy of single agent therapeutic approaches. The ToR kinase pairs with several protein partners and forms two distinct complexes, ToRC1 and ToRC2, which exhibit different sensitivities to the macrolide rapamycin, as well as different upstream inputs and downstream outputs. ToRC1 is remarkable in its ability to integrate five major inputs [growth factors, amino acids, energy, stress and oxygen] and appropriately coordinate anabolic pathways, including nutrient intake, protein and lipid synthesis, as well as catabolic pathways such as autophagy [154]. The canonical ToR pathway was found in drosophila to function downstream of insulin initiated receptor tyrosine kinase RTK signaling, and control cell growth in culture [1-3] as well as in vivo [4,44,45]. For a better understanding of general growth control and growth promoting pathways, please refer to the introductory chapter.

It's not just insulin. As described in the introduction, insulin is not the only growth promoting pathway, and knockdown of insulin pathway components (InR, IRS, p60, p110) in insect schneider cells does not significantly affect their size [3]. On the other hand, Pvr-Ras signaling pathway was found to affect the size of drosophila cells in culture (Pvr, Pdgf/Vegf receptor signaling). As part of a screen, knockdown of Ras-Mapk pathway components, including Pvr, Sos, Grb2, Ras, Ksr was found to reduce insect cell size in culture [3]. Moreover, even insulin stimulation of schneider cells has been observed to activate not only Akt, but also the Erk pathway [7,8].

Conspiring to promote growth. The evolutionary conserved Mapk (mitogen activated protein kinase) pathways coordinate essential cellular functions, including cell survival, proliferation, growth, motility and differentiation [9]. Although 14 Mapks have been characterised, the most extensively studied are Erk, Jnk and p38 isoforms. To execute such intricate functions, Mapks regulate transcription factors impinging on gene expression, as well as a variety of cytosolic substrates coordinating other aspects of cellular metabolism. Among these substrates

are the Mapk activated protein kinases (Mapkapks) [10,11]. Two of them were found to be involved in translation control, namely the Mnks (Mapk interacting kinases) [12] and the Rsks (p90 ribosomal S6 kinases) [13]. The drosophila Rsk ortholog was shown to be involved in modulation of circadian behaviour and memory formation [14-16]. Drosophila Mnk homologue is called Lk6, and was shown to be important for eIF4E phosphorylation, developmental rate and organism size [17-19]. Drosophila homologue of another Mapkapk (Msk) is called Jil1, is essential for viability, and it functions to maintain euchromatic domains while counteracting heterochromatinisation and gene silencing [20, 21].

Aim

Emergent scope of study. Although Ras-Mapk pathway was found to promote growth in different tissues in drosophila (see introduction chapter), it doesn't significantly affect the transcription of growth genes [54]. Furthermore, extensive crosstalk between Mapk and other growth promoting pathways has been described in mammalian cells. Prominently, Erk Mapk activates ToRC1 pathway both directly and indirectly through its effector Rsk [30-36]. In addition, as stated above, several Mapk activated protein kinases have been associated with translational control. Together, these lines of evidence point toward non-transcriptional effectors stimulated by Mapk to promote cell growth. Therefore, we tested in our study several kinases as potential Mapk downstream growth effectors. We also asked if these kinases are involved in driving cell growth downstream of different RTK pathways, such as insulin and Egfr.

New Findings

Mek/Erk activity is important for growth. Kinase inhibitors for Mek and Erk have been used extensively to implicate Erk Mapk in various biological functions. Since their initial introduction in the mid 1990's, several Mek inhibitors have been developed and entered clinical trials as potential anticancer agents [80]. We found that treatment of schneider cells with kinase inhibitors for Mek or Erk, in presence or absence of serum, lowered the proliferation and average cell sizes [mean diameters] compared to untreated controls (figures 1A,B 2A-C). The same effects were observed in the presence of added Pdgf/Vegf growth factors (figures 1C,D 2D-F). A similar inhibition of cell proliferation and size was obtained by inhibiting ToR signaling with rapamycin of torin, as positive controls. These results indicate that Mek/Erk kinase activity is relevant for these cells to maintain proper growth in culture.

Mapkapk (Mapk activated protein kinases) are important for normal and insulin stimulated growth. In order to investigate some of the connections described above, cell cultures were treated under the same conditions with various Mapkapk kinase inhibitors. We discovered that some of these, targeting Mnk, Rsk or Msk proved to have similar inhibitory effects on cell proliferation and size, as did Mek or ToR inhibition (figure 3A-C). Insulin stimulation of the cultured schneider cells triggered an increase in proliferation and cell size compared to untreated control, as previously shown [6]. Surprisingly, pre-treatment with Mnk/Rsk/Msk inhibitors invoked similar cell proliferation and size reduction effects even in the presence of insulin, compared to untreated or insulin-only treated controls. The same effects were observed in the presence of added Pdgf growth factor (figure 3G-I). As before, pre-treatment with the ToRC1 inhibitor rapamycin, as a positive control, consistently impaired cell growth (figure 3D-F). Taken together, these data highlight an important role for these Mapk activated kinases in the control of homeostatic and insulin-induced insect cell growth in culture.

Mapkapk kinases are important for normal and Egfr stimulated growth. Having observed that Mapkapks may act as growth effectors downstream of insulin singling, we also wanted to test other RTKs for such a dependency. Thus, we natively activated Egfr-Mapk signaling in cultured schneider cells (expressing the receptor) by stimulation with the drosophila Egfr ligand Spitz. Previous work showed that stimulation of such Egfr-expressing cells with Spitz does indeed lead to Erk Mapk activation [22]. Native activation of drosophila Egfr-Mapk signaling slightly increased the cell size (mean diameters measured over several time points) compared to cells not treated with Spitz ligand. Inhibition of Mapkapks, however, again reduced cell sizes in Spitz treated and non-treated cells (figure 4A-D). This result places these kinases as potential growth effectors functioning downstream of drosophila Egfr signaling.

Significance

Our findings suggest a considerable involvement of three Mapkapk kinases towards growth promoting processes, downstream of insulin and Egfr RTK signaling. Although described as Mapk activated kinases in mammals, it remains to be seen if these Mapkapks indeed function downstream of Mek/Erk in insect cells. Two of them, Rsk and Mnk, have already been described as Erk interacting partners in drosophila [14-19]. The requirement for these kinases towards growth in insect cells also reveals a certain degree of conservation between insects and mammals with regard to Mapkapk functionalities.

Our hypothesis that Mapkapks may be important for insect cell growth fits with previous studies, introduced at the beginning of this section and detailed in the following section. These studies bring forward three main points: 1. Mapk activity drives growth in vivo and it's activated by Egfr and insulin in vitro 2. Mapkapks function downstream of Map kinases and 3. Mapkapks have been implicated in promoting transcription (Msk) and translation (Mnk, Rsk).

Our results, therefore, extend such findings to insect cells and propose a new mechanism of Mapk driven growth through activation of Mapkapk kinases. These, in turn, might directly promote translation or activate other pathways, such as ToR, in order to do so. The mechanistic framework that may support these processes is described in the following section. The significance of these and other findings is far reaching not only for our understanding of how cells integrate multiple RTK-derived signals to grow, but also towards designing more effective therapies targeting tumor growth. Indeed, several Mapkapks were found to be mutated in different human diseases. For example, Rsk2 mutations are the underlying cause for CLS (Coffin Lowry Syndrome), an X-linked mental retardation syndrome characterised by psychomotor retardation and facial, hand and skeletal malformations [81].

III-3 MAPKAPKS IN TRANSLATION AND GROWTH

The results discussed in the previous section bring forward new potential growth promoters in insect cells, the Mapk activated protein kinases (Mapkapks). Mammalian studies have attributed a significant extent of Mapk functionalities to the activation of downstream Mapkapks. They are presented as an additional amplification step in the Mapk catalytic cascades, increasing the range of actions regulated by Mapk modules. The activation mechanisms, substrates and biological functions of these kinases are described in the introduction chapter. Here, I emphasise on their ability to promote translation and drive growth, as potential mechanisms supporting the results discussed in the previous section.

One Mapkapk extensively studied in mammals is Rsk (p90 ribosomal S6 kinase). The Rsk family includes four vertebrate isoforms and a single drosophila ortholog. Rsks are expressed ubiquitously in every human tissue tested, predominantly in the kidney, pancreas, lungs, heart, skeletal muscle and brain [82]. Numerous studies link Rsks to basic cellular functions, including cell cycle progression and cell proliferation, protein synthesis and cell growth, and cell survival. The drosophila Rsk ortholog was shown to be involved in modulation of circadian behaviour and memory formation [14-16]. Rsk was originally identified as an in vitro ribosomal protein S6 (rpS6) kinase [83, 84]. Later studies have identified S6k as the main rpS6 kinase, but shown that Rsk also contributes to S6k phosphorylation in vivo, and it does so in a ToR independent manner [85]. Rsk mediated rpS6 phosphorylation was found to promote cap-dependent translation initiation. A second mechanism through which Rsk may promote translation and drive growth is by activating ToRC1. Rsk, and its activator Erk, were found to phosphorylate tuberous sclerosis complex component Tsc2, thereby negatively regulating its guanine activating protein (GAP) activity towards the small GTPase Rheb [30-33]. Activated Rheb, in turn, stimulates ToRC1 activity. Another way in which Rsk and Erk stimulate ToRC1 is by phosphorylating Raptor, an important interacting partner of ToRC1 [34-36]. A third mechanism for Rsk driven translation relies on phosphorylation and inhibition of Gsk3 [86], which prevents the suppression of translation initiation factor eIF2B [87]. By phosphorylating and inhibiting Gsk3, Rsk may also indirectly promote ToRC1, as Gsk3 and the Lkb1 activated kinase Ampk both phosphorylate and activate Tsc2 [88, 89]. Finally, Rsk was shown to phosphorylate eIF4B and eEF2K, and thereby promote translation inhibition and elongation, respectively [38,90].

A second Mapkapk found to be conserved in mammals and drosophila is Msk (mitogen and stress activated kinase). Msk family includes two vertebrate genes (Msk1 and Msk2) and a single ortholog in drosophila (Jil1). Similar to Rsk, it is expressed ubiquitously in human tissues, with a higher preponderance in the heart, brain, skeletal muscle and placenta [91]. Msks have a predominant nuclear localisation and are potently activated by mitogens and stress stimuli that promote Erk and p38 activation [91-93]. Although mice deficient for Msk1,2 do not display obvious phenotypes [94,95], deletion of the Msk ortholog Jil1 in drosophila is lethal, perhaps due to lower genetic redundancy [96,97]. In drosophila, Jil1 phosphorylates histone H3 at Ser10 and is a key regulator of chromatin structure, by facilitating the nucleosomal response and thereby gene relaxation and activation [98,99]. Having a similar substrate specificity with Rsk, Msk might influence translation through similar targets. For example, both Rsk and Msk were found to phosphorylate the transcription factor Creb in mammalian cells [91,93]. Activated Creb in turn drives expression of immediate early (IE) genes, such as Fos, Jun and Egr1 [100]. Additional transcription factors targeted by Msk include NFkB and Stat3 [101,102]. Importantly, Msk was suggested to phosphorylate the translational inhibitor 4EBP1 [103], providing another convergence point between Mapk activated kinases and ToRC1 (see also III-4).

A third Mapkapk documented in our study and tied to translational control is Mnk (Mapk interacting kinase). Mnk family includes two vertebrate genes and a single ortholog in drosophila (Lk6)[104]. Both Mnks are expressed in all adult tissues, with lower than average levels in the brain and higher levels in the skeletal muscle [105]. Both Mnk genes produce two isoforms, a long form (Mnk1A and Mnk2A) with a predominantly cytoplasmic localisation, and a short one (Mnk1B and Mnk2B) without a Mapk binding motif, equally distributed between the nucleus and the cytoplasm [106-108]. Whereas ToRC1 promotes eIF4E by inhibiting 4EBP, Mnk may promote cap-dependent translation initiation by directly phosphorylating eIF4E [109,110]. Stress and mitogenic stimuli which activate Erk and p38 promote eIF4E binding to eIF4G in the translation initiation complex, and subsequently Mnk dependent phosphorylation of eIF4E [109,111,112]. eIF4E phosphorylation appears to promote cancer cell proliferation in vitro [113] and enhance the oncogenic potential of eIF4E in vivo [114,115]. eIF4E mediated translational regulation of the antiapoptotic protein Mcl1 is believed to be important for tumorigenesis [114]. Furthermore, eIF4E phosphorylation was shown to be particularly important for the inflammatory response and tumor progression by regulating the translation

of mRNAs encoding inflammatory molecules (Ccl2 and Ccl7) and matrix metalloproteases (Mmp3 and Mmp9), respectively [116].

III-4 MAPK ACTIVATES TOR IN INSECT CELLS

This section discusses the results presented in II-2.

Context

Insulin signaling activates Erk and promotes cell growth. The insulin pathway constitutes the main nutrient sensing mechanism in drosophila, and coordinates cellular metabolism, proliferation and growth [60]. Mutation of insulin pathway components phenocopies the effects of nutrient starvation, whereas pathway overactivation can drive growth and proliferation even upon lack of nutrients [44,46,59]. Insulin promotes growth in drosophila in vivo [5,23-27] as well as in cell culture [6-8,28]. Furthermore, insulin stimulation of cultured schneider cells activates several effector kinases, including Akt, S6k, Mek and Erk [6-8,29]. Activation of the Mapk Erk contributes to insulin driven proliferation in these cells [7], confirming Erk as one of the kinases functioning downstream of this growth promoting pathway.

Pvr signalling also activates Erk and promotes cell growth. The drosophila homologue of mammalian Pdgf/Vegf receptor (PVR) was found to promote schneider cell growth in culture. Pvf2 and Pvf3 ligands act redundantly to activate Pvr and downstream Ras signaling. Activated Ras, in turn, drives growth by concomitantly activating Pi3k and Mapk, and subsequently, ToR pathway [3]. Normal growth of S2R+ cells in culture does not require insulin receptor activation, but it rather depends on ToR and Mapk pathway activities.

ToR functions downstream of insulin and Pvr to promote growth. The best characterised effector pathway activated by insulin and promoting growth is the canonical ToR pathway (introduced in III-1). Thus, ToR promotes cell growth in drosophila in vivo [4] and in cell culture [1-3]. Pvr/Ras driven cell growth relies on activation of Raf/Mapk and Pi3k/Akt pathways, and is dependent on ToR pathway activity [3].

Mapk positively regulates ToR. Strong activation of Ras-Erk pathway leads to mToRC1 activation in mammalian cells through Erk and Rsk signaling to Tsc and Raptor components. Egf, phorbol esters and constitutively active Ras mutants promote Erk and Rsk mediated phosphorylation of Tsc2, inhibiting its GAP function and thereby stimulating mToRC1 activity and tumorigenesis [30-33]. Similar stimuli promote Erk and Rsk phosphorylation of Raptor, likewise increasing mToRC1 activity [34-36]. Furthermore, the stress activated p38 Mapk is a positive regulator of insect and mammalian cell growth, and a positive regulator of ToRC1 (ToR

complex 1). In fact, ToRC1 mediated cell growth requires p38 pathway activity in both insect and mammalian cells. The mechanism through which p38 activates ToRC1, however, is not fully understood [41]. Further details on Mapk-ToR signaling in the following section 'Mapk ToR Crosstalk and Convergence'.

Aim

The findings described above inevitably lead to the consideration that Ras-Mapk may function downstream of insulin and Egfr signaling to promote ToRC1 activity and drive growth. Hence, we investigated this possibility by stimulating Ras-Mapk signaling in drosophila cell culture and quantifying ToRC1 target phosphorylation as a readout of its activity. Additionally, we tested if ToRC1 or any of the Mapk activated kinases might play a role in activating the canonical Erk pathway.

New Findings

Ras-Mapk signaling is a potential ToRC1 activator in insect cells. Stimulation of cultured schneider cells with insulin, a known Pi3k/Akt and ToR pathway agonist, led to a marked increase in activating phosphorylations of the Akt kinase and of the ToRC1 target S6k (figures 6, 7B), as shown before [6]. Surprisingly, when we stimulated the cells with Egf growth factor or with the known Mek agonist Pma, we also observed an increased S6k phosphorylation, with no obvious effects on Akt phosphorylation (figure 6). Furthermore, pre-treatment with the ToRC1 inhibitor rapamycin prevented the increase in S6k phosphorylation upon stimulation with insulin, Egf or Pma. This indicates that Egfr signaling may be able to stimulate ToRC1 activity toward its target kinase S6k in cultured schneider S2/S2R+ cells. Furthermore, stimulating S2R+ cells with the known Ras-Mapk agonist Pdgf also led to a mild increase in S6k phosphorylation (figures 7B, 8B). Thus, it is possible that signalling initiated by several RTK receptors, including InR, Egfr and Pvr, may be used in insect cells to stimulate ToRC1 at different times and to different extents. This would make sense if we consider the specificity of RTK pathways during development, reflected in temporally and spatially restricted activation patterns. Temporally, some RKTs like Torso are specifically expressed at a certain developmental stage, whereas others like Egfr are more dynamically expressed. Spatially, some RKTs like Torso and Egfr are broadly expressed, while others like Heartless and Breathless Fgf receptors are restricted to particular tissues [61]. Most developmental programs controlled by these RTKs rely on cellular growth, and therefore the ability to coordinate developmental decisions with growth promoting pathways, like ToR, could prove especially useful. The level of pathway acti-

vation may depend on factors such as ligand expression, ligand processing and presentation, feedback loops, and co-option of a secondary transcriptional response.

Ras-Mapk is important for ToRC1 activation by growth factors in insect cells. Having seen that Egfr signaling may stimulate ToRC1 activity in cultured schneider cells, likely independently of Akt, we asked if it might also be required for ToRC1 activation under basal or growth factor stimulated conditions. For this purpose, we used Mek and Erk chemical inhibitors to down-regulate signaling through the Raf/Mek/Erk Mapk pathway. As expected, cell treatment with these inhibitors efficiently reduced Erk phosphorylation under normal or growth factor (insulin, Pdgf, Vegf) stimulated conditions (figures 7A, 8A). ToRC1 inhibition with rapamycin or ToRC1,2 inhibition with torin had no obvious effect on Erk phosphorylation under these conditions. Significantly, insulin and Pdgf stimulated phosphorylation of the ToRC1 target S6k was strongly reduced by the inhibition of Mek (and to a smaller extent by Erk inhibition). Treatment with well known ToR pathway inhibitors [as positive controls] such as rapamycin and torin, likewise abrogated S6k activation by these growth factors (figures 7B, 8B). Taken together, these results indicate that RTK driven Ras-Mapk signaling stimulates ToRC1 activity in drosophila schneider cells, as evidenced by an increase in its target S6k phosphorylation. Importantly, ToRC1 pathway activation by insulin and Pdgf growth factors depends to some extent on Mek-Erk activity, as their inhibition reduced growth factor stimulated ToRC1 target phosphorylation. This finding further supports the hypothesis described in the previous paragraph suggesting an integration of RTK and ToRC1 signaling.

Mapk pathway activation does not require ToRC1 or Mapkapk activities. It has previously been shown that insulin stimulation of schneider cells increases Erk phosphorylation [7,8]. Likewise, stimulation of schneider cells with the native drosophila Egfr ligand Spitz also increases Erk phosphorylation [22]. Based on these findings and our previous results, we wanted to know if ToRC1 or any of the Mapk activated kinases (Mapkapk) previously tested might also play a role in Erk activation. Treatment of schneider cells with kinase inhibitors for Mnk, Rsk, Msk or ToRC1, did not reduce Erk phosphorylation levels in basal or in growth factor (insulin, spitz) stimulated cell cultures (figure 9). This would indicate that Mapk pathway activation under normal or growth factor stimulated conditions does not require ToRC1 or the Mapk activated protein kinases Mnk, Rsk, Msk.

Significance

Our findings described in the previous sections bring forward several potential mechanisms through which Erk Mapk might promote cell growth, namely through stimulation of three Mapkapks, previously identified as Erk targets. This section proposes yet another mechanism (or an extension of the previous) for Mapk driven growth, namely by stimulation of ToRC1 activity. Erk Mapk might positively regulate ToRC1 directly, through mechanisms described in the following section, and/or indirectly by activating Mapkapks which in turn can activate ToRC1, through mechanisms described in the previous section.

As introduced at the beginning of this section, connection points between Mapk and ToR have been previously described in mammalian cells [30-36]. Moreover, the the stress activated p38 Mapk was also found to function as a positive regulator of ToRC1 in mammalian and insect cells [41]. Our findings, therefore, bring yet another piece of the puzzle by showing that Ras-Mapk signaling is sufficient and required for ToRC1 activation in schneider cells. It appears that some level of connectivity between these two pathways that are deeply anchored into the cell's inner workings, has been conserved throughout evolution. Such connectivity may be important not only for tissue homeostasis and regeneration but also for keeping developmental and metabolic decisions in sync. Multiple convergence points between these pathways seem to argue for the validity of such thinking.

Both Mapk and ToR pathways are evolutionary conserved and key players in the regulation of multiple cellular functions. There's hardly any cellular stimulus that doesn't feed into these pathways. Understanding their individual and collaborative contribution to cellular homeostasis is imperative for our scientific advancement and the development of future therapeutic approaches.

Indeed, both Mapk and ToR pathways are frequently mutated in human cancers. Due to cross-activation and convergence between these pathways [detailed in the following section], tumors often develop resistance to drugs targeting only one pathway. For example, cancer cell lines harbouring Pi3k/Pten mutations show a reduced cytostatic response to Akt/ToR inhibitors if they additionally acquire Ras/Raf mutations [39,77]. Even in cases of oncogene addiction, where transformation appears to be driven by a single pathway, the effectiveness of single agents if limited by cross-inhibition mechanisms between Mapk and ToR pathways. For exam-

ple, treatment of breast cancer cell lines harbouring high levels of activated Erk and Egfr with Mek inhibitors increases Akt activity and is cytostatic rather then cytotoxic [78,79].

Last but not least, primary and immortalised cell lines provide a more versatile and tractable system for investigating not only basic cellular functions but also the signaling pathways coordinating these processes. An epitome for their advantages being the use of high throughput methods to manipulate and monitor signaling pathways in culture, which led to the identification of hundreds of new signaling pathways components, and of new small molecule inhibitors for future therapeutic approaches.

How Mapk ToR crosstalk may occur mechanistically in insect cells, is an important question. The intricate ways in which Ras-Mapk may feed into the ToR pathway are briefly described in the following section.

III-5 MAPK TOR CROSSTALK AND CONVERGENCE

Ras-Mapk and Pi3k-Akt-ToR pathways have been shown to crosstalk extensively in different cell types and organisms. A clear and complete definition of the underlying mechanism warrants, however, further research. A comprehensive survey of the published literature reveals five potential intersection points.

[i1,i2] Strong activation of Ras-Erk pathway leads to mToRC1 activation in mammalian cells through Erk and Rsk signaling to Tsc and Raptor components. Egf, phorbol esters and constitutively active Ras mutants promote Erk and Rsk mediated phosphorylation of Tsc2, inhibiting its GAP function and thereby stimulating mToRC1 activity and tumorigenesis [30-33]. Similar stimuli promote Erk and Rsk phosphorylation of Raptor, likewise increasing mToRC1 activity [34-36]. Our bioinformatic analysis indeed revealed conserved Erk targeted phosphorylation sites in drosophila Tsc2 and Raptor. Furthermore, another study pointing in such a direction regards Pi3k-Akt input towards ToRC1 activity. It shows that mutating the Akt targeted sites in insect Tsc2 has no impact on Tsc2 function in vivo [67]. This suggests that Pi3k may stimulate ToRC1 differently (through Pras40 for example) and/or that additional upstream activators feed into the pathway. One could also think that Erk might directly phosphorylate S6k and thereby promote transcription as well as translation initiation and elongation. However, our experiments show that Mapk driven S6k phosphorylation seems to be sensitive to rapamycin, which implies that the effect on S6k depends on ToRC1 activation. This doesn't exclude the possibility that Erk or one of its target kinases phosphorylate other sites in S6k which are not detected by this antibody.

[i3] Another appealing avenue involves phosphorylation and inactivation of another ToRC1 target, 4EBP (eIF4E binding protein). In support of this hypothesis, we know that Erk activation in mammalian cells, either by the phorbol ester TPA or by constitutively active Mek, leads to phosphorylation of 4EBP1 and its dissociation from eIF4E, which in turn promotes translation initiation [37]. 4EBP inactivation in this manner is blocked by inhibitors of Mek or ToRC1, implying that Mek/Erk may act through ToRC1 to inactivate 4EBP, or that 4EBP inactivation requires sequential phosphorylation by both kinases.

[i4] Additional interactions that can bridge Mapk and ToR pathways are mediated by two scaffold proteins. MP1 protein scaffolds Mek and Erk at late endosomes and is required for sus-

tained Erk activity, but it also scaffolds Rag GTPases to the lysosome, and thereby permits ToRC1 activation [62-64]. In response to growth factors, the Mapk scaffold Ksr translocates from the cytoplasm to the cell membrane and mediates the co-localisation of Raf, Mek and Erk, needed for Erk activation [62]. Well, it turns out that Ksr also interacts with ToR, Raptor, Rictor and the Tsc2 activating kinases Ampk and Gsk3 [65,66].

[i5] A final mechanism through which Ras-Mapk may feed into Pi3k-Akt-ToR pathway involves positive regulation of the lipid kinase Pi3k by the GTPase Ras. Ras binding to drosophila Pi3k is dispensable for viability, but it is required for maximal Pi3k activation towards growth processes. One function highly dependent on growth is egg production, and it is dramatically lowered in Pi3k mutant flies. Flies harbouring such Pi3k mutations that prevent Ras binding are also underdeveloped compared to their wild type siblings [68]. Two other studies in drosophila inspect Ras, Myc and Pi3k crosstalk towards growth. Stimulation of the Egfr receptor in the drosophila wing by its ligand Vein, activates Ras signaling and is required for growth. Activated Ras drives growth and promotes G1/S progression, in part, by activating two growth promoting pathways, Raf/Mapk and Pi3k. Although mutationally activated Ras stimulates both Pi3k and Myc growth pathways, endogenous Ras does not increase Pi3k signaling (nor does Pi3k increase Raf/Mapk), and only modestly increases Myc protein levels, hinting towards additional growth promoters [69,70].

In addition to the pathway crosstalk described above, studies in mammals have revealed multiple convergence points between Erk Mapk and ToR pathways. In fact, Erk, Rsk and S6k often target the same substrates to promote survival, proliferation, metabolism and motility. The following five convergence points stand out:

[c1] Foxo (forkhead box O) proteins regulate the expression of apoptotic proteins and cell cycle regulators that suppress cell survival and proliferation. Erk phosphorylates Foxo and promotes its interaction with the E3 ubiquitin ligase MDM2, thereby directing Foxo's proteasome mediated degradation [71]. The ToRC2 targets Akt and Sgk [serum and glucocorticoid regulated kinase] likewise phosphorylate Foxo promoting its interaction with 14-3-3 proteins. Binding by 14-3-3 sequesters Foxo in the cytosol, precluding it from entering the nucleus and initiate quiescence and apoptotic gene expression programs [72].

[c2] Myc transcription factor was shown to be activated by oncogenic Ras in the drosophila wing [70] and to be an essential effector for ToRC1 driven growth. Myc functions as an obligate heterodimer with Max to drive survival and growth gene expression programs. The related transcription factor Mad1 competes with Max for Myc binding, precluding expression of survival and growth genes. Erk mediated phosphorylation stabilises Myc [73]. Rsk and S6k phosphorylate Mad promoting its ubiquitination and degradation, and thereby induction of pro-survival and growth genes [74].

[c3] Bad is a pro-apoptotic Bcl2 family protein. Hypophosphorylated Bad neutralises Bcl2 pro-survival proteins, and thereby releases Bax and Bak to induce apoptosis at the mitochondria. PKA, PKB (aka Akt), PKC as well as Rsk and S6k promote cell survival by phosphorylating multiple sites on Bad and stimulating 14-3-3 binding and sequestration in the cytosol, away from the mitochondria and Bcl2 family pro-survival proteins [75].

[c4] Inactivation of Gsk3 by Wnt, insulin and other growth factors, releases its inhibition of pro-survival, proliferation and motility proteins. Gsk3 also inhibits ToRC1 by phosphorylating and activating Tsc2 [63]. Erk and Rsk sequentially phosphorylate and inactivate Gsk3 [76]. In different cell types, Pka, Pkb, Pkc and S6k also phosphorylate this inhibitory site.

[c5] Tif1A transcription initiation factor stimulates PolI activity and thereby rRNA synthesis. Interestingly, Tif1A is positively regulated by Erk, Rsk and ToRC1 [135,136], which suggests that Ras/Mapk may collaborate with ToRC1 to promote rRNA synthesis in response to growth factors.

III-6 MAPK NEEDS TOR TO FULLY PROMOTE MIDGUT CELL GROWTH

Context

The discovery of intestinal stem cells (ISCs) in the adult drosophila midgut has established the drosophila intestine as a model system for studying stem cell mediated tissue homeostasis and regeneration. Similar to the mammalian small intestine and colon, the adult drosophila midgut undergoes dynamic self-renewal [117,118]. This is accomplished by resident ISCs which divide to generate new ISCs and committed progenitors called enteroblasts (EBs). Unlike their mammalian counterparts, the transit amplifying cells, the EBs don't usually divide but rather differentiate into two functional cell types, the absorptive enterocytes (ECs) and secretory enteroendocrine cells (EEs). ECs grow very large and endoreplicate their genomes up to ploidy levels of 32c, and therefore constitute the bulk of the intestinal epithelium. The gut turn-over rate is approximated at 1-2 weeks [118]. However, in response to midgut damage (mechanical, chemical, bacterial), ISC proliferation and EB differentiation are enhanced up to 100 fold to ensure tissue regeneration [119-125].

Egfr pathway acts as a major growth and proliferation factor during midgut homeostasis and regeneration. Egfr signaling is stimulated by three ligands, Vein produced by muscle cells surrounding the midgut, Spitz and Keren produced by the midgut epithelial cells, and shows high levels of activity in the progenitor cells, ISCs and EBs. Furthermore, loss of Egfr signaling blocks ISC growth and division, whereas constitutively activated Ras accelerates the growth of ISCs and post-mitotic enteroblasts [51-53,126]. Together with the Jak/Stat pathway, which functions as a major mitogenic and differentiation factor in the midgut [119,127-131], Egfr pathway is highly activated by midgut damage and essential for tissue regeneration [51-53,126]. Two other damage/stress sensing pathways, Hippo and Jnk, were found to promote Egfr and Jak/Stat activation and implicitly tissue regeneration [126, 132-134, 119, 123, 131].

Befitting as a major growth promoting pathway, ToRC1 signalling also proved to be essential for ISC maintenance and differentiation in the drosophila midgut. ToRC1 activity is believed to be low in ISCs and high in EBs, due to Notch-mediated suppression of Tsc2. ToRC1 activity in ISCs is kept low to prevent their overgrowth and precocious differentiation, while higher ToRC1 activity in EBs supports their growth and differentiation into ECs. EE cell formation requires low ToRC1 activity [55-57].

Aim

Having seen that Egfr pathway functions as a potent growth and proliferation stimulus in the midgut epithelium, and that a link between Egfr/Mapk and ToRC1 has already been established in vitro (see III-3/4), we asked if part of its growth effect might be mediated by ToRC1 activity. Therefore, we replicated some of the phenotypes described in the above references, involving Egfr pathway activation, but with concomitant downregulation of ToRC1 signaling.

New Findings

In accordance with previous findings [51], over-activating Egfr-Ras-Mapk pathway in the midgut had a strong proliferative and growth effect. When we expressed constitutively active Ras or Raf in the progenitor cell population (ISCs and EBs), we observed a significant increase in ISC mitotic activity and overall cell density. Area quantifications also revealed an enhanced growth of polyploid cells, believed to be enterocytes. Treatment of the midguts expressing activated Ras/Raf with the ToRC1 inhibitor rapamycin didn't have a significant effect on the increased mitotic activity, but it visibly reduced the occurrence of overgrown transgene-expressing cells (figure 10).

Having noticed a possible effect of rapamycin treatment on Ras-Mapk driven growth, we tested a similar dependency in the committed progenitor cells (EBs) only. As EBs don't usually divide, a change in their growth potential will be easier to observe and quantify. And indeed, expressing a Mapk-specific activated Ras isoform in the EBs led to a significant increase in the size of the expressing cells, compared to their control Gfp-expressing siblings. Importantly, this Ras-Mapk driven growth appeared to partially dependent on ToRC1 activity, as treatment with rapamycin reduced the growth phenotype (figure 11).

These findings indicate that Ras-Mapk activation can drive cell cycle progression and cell division to some extent independently of ToR. However, for exerting its full growth promoting function, Ras-Mapk may need ToRC1 activity. The observed proliferation observed upon Ras-mapk activation with ToRC1 inhibition may be explained by two mechanisms. A first mechanism relies on additional growth promoting effectors functioning downstream of Ras-Mapk. The Mapk activated protein kinases discussed in the sections III-1 and III-2 would make for likely candidates. A second mechanism may arise from synthetic interactions that the activated oncogenic Ras, but not the native endogenous Ras, may allow. Evidence in support of the second mechanism come from studies in the developing wing, where it was found that activat-

ed Ras can indeed coarse both Pi3k and Myc to drive growth, whereas endogenous Ras does not increase Pi3k signaling and only modestly increases Myc protein levels [70]. However, in our experiments we used a Ras allele specific for the Raf-Mapk pathway, minimising lateral signaling. Thus, the first mechanism relying on secondary growth effectors, such as the Mapkapks, seems more likely. Nevertheless, the growth reduction observed upon ToR inhibition clearly points towards a partial ToR dependency (downstream or in parallel) of Ras-Mapk driven growth in the midgut epithelium.

Much of Egfr-Mapk pathway functionality in drosophila has been associated with transcriptional regulation, and the ETS transcription factors are considered to be the predominant RTK-Ras-Mapk nuclear effectors [58]. Ets transcription factors function downstream of RTK pathways to control the expression of a varied assortment of genes with wide reaching effects on cellular behaviour. One such factor, Ets21c, is transcriptionally suppressed by capicua in the adult intestine and gains in expression following Egfr activation and capicua inhibition [54]. Additionally, preliminary experiments overexpressing Ets21c in the progenitor population revealed an increase in the rate of proliferation (data not shown). Based on this knowledge, we addressed Ets21c function in the midgut epithelium, with a focus on its growth effects. Overexpression of Ets21c in the committed progenitors (EBs) led to a small but significant size increase of these cells relative to their wild type siblings expressing Gfp alone. Conversely, RNAi mediated knockdown of Ets21c in EBs slightly reduced their size (figure 13). Thus, as evidenced so far, Ets21c has a mitogenic effect on the intestinal stem cell population and appears to be sufficient and required to promote growth of committed non-dividing progenitors. However, the growth phenotypes observed upon Ets21c knockdown or overexpression were far less potent than the ones caused by Ras or Raf modulation (e.g. Ras overexpression in figure 10A, knockdown data not shown). Furthermore, loss of capicua-mediated target gene repression upon Egfr-Mapk activation is considered to be the predominant mechanism for Mapk dependent transcriptional regulation in the midgut [54]. Among capicua targets, however, are mostly cell cycle regulators such as Cdc25 and cyclin E. Moreover, growth genes such as those coding for Myc, insulin or ToR pathway components were not found to be upregulated in capicua depleted progenitor cells (except for InR, 54). These findings clearly point towards non-transcriptional mechanisms for Mapk driven growth, and further support our results bringing forward ToRC1 and Mapkapks as potential Mapk growth effectors.

Significance

Mapk pathways are conserved in all eukaryotic cells and are among the core signaling pathways fundamental to cellular and organismal functionality. Most adult tissues have resident somatic stem cells which are crucial for tissue homeostasis and regeneration upon damage. This is particularly evident in high-turnover tissues such as the mammalian intestine or drosophila midgut. In these tissues, proliferation of the resident stem cells (ISCs) is dynamically controlled by an assortment of signaling pathways responding to various growth and stress stimuli (see also beginning of this section). Egfr signaling is required to maintain the normal proliferative and growth capacity of drosophila ISCs, and essential for midgut regeneration upon damage [51-53,126]. However, we don't completely understand how these pathways are integrated to maintain tissue functionality and promptly respond to challenges. Our findings reveal one aspect of such integration, by exposing a possible dependency of Ras-Mapk driven growth on ToRC1 activity in the midgut epithelium. As ToRC1 inhibition didn't completely abrogate Ras-Mapk driven growth under normal conditions (figures 10,11) or after pathogenic infection (ToR null cell clones activated for Ras, 161), additional growth effectors could also be considered. The Mapkapks discussed in sections III-1 and III-2 being potential candidates.

The Egfr pathway is also critical for the development, homeostasis and cancerous transformation of the mammalian intestine and colon [137-139]. Given its conserved role in regulating mammalian and fly ISC proliferation, understanding how Egfr executes its functions in the fly midgut will greatly facilitate the development of targeted therapies against colorectal cancer (CRC) and other afflictions. One such therapy targeting Egfr with monoclonal antibodies, such as cetuximab and panitumumab, is already used for patients with CRC [140,141]. Bellow I will discuss the advantages and challenges of targeting Ras-Mapk and Pi3k-ToR pathways as potential anti-cancer therapies.

One advantage for using pathway inhibitors stems from the fact that Ras-Erk and Pi3k-ToR pathways are frequently abnormally regulated in human cancer, and their activation is typically implicated in unrestricted proliferation and decreased sensitivity to apoptosis-inducing agents. Both pathways also crosstalk with Wnt and p53, which are also critically involved in cell growth, aging, oncogenesis and metastasis [142]. A second advantage is that inhibitors targeting components of Ras-Erk and Pi3k-ToR pathways have been developed and extensively tested as potential anti-cancer agents but also for use against other proliferative diseases in-

cluding premature aging. Oftentimes, cancer cells acquire resistance to single agents due to crosstalk and convergence between these pathways, in which case a combined approach simultaneously targeting both pathways would be needed. Studies such as ours aimed at clarifying the extent and context dependence of such pathway crosstalk are imperative for overcoming this challenge. A third advantage that warrants the use of pathway inhibitors comes from their use in combating the resistance cancers often acquire to conventional therapies such as chemotherapy and radiotherapy, perhaps due to emergence of cancer initiating cells (CICs) [143-146].

One challenge of using pathway inhibitors comes from the high number of target genes (easily in the 1000's) regulated by these pathways. Their inhibition, therefore, may be detrimental to many cell types, unless one could target the inhibitors specifically to the transformed cells. A second challenge is the extensive crosstalk between Ras-Mapk and Pi3k-ToR pathways, and between these and other critical pathways including Wnt, p53, Jak/Stat, NFkB and TgfB which can be directly and indirectly regulated by Erk and Akt phosphorylation [147]. Thus, inhibition of Ras-Mapk and/or Pi3k-ToR may lead to deregulation of these secondary pathways. A third challenge is that most inhibitors act as cytostatic and not cytotoxic agents, which avoids massive toxicity problems but also limited their use as anti-cancer agents. To circumvent this issue, inhibitors could be used together with cytotoxic chemotherapeutic drugs or radiation therapy that affect rapidly growing cancer cells.

III-7 MAPK BYPASSES NUTRITION AND PROMOTES LARVAL CELL GROWTH

Context

The drosophila midgut is formed during embryogenesis and retained through the larval development. During larval development, the midgut epithelium consists of two distinct cell populations: differentiated larval enterocytes ECs which grow in both size and ploidy through endocycles, and undifferentiated adult midgut progenitors AMPs which remain diploid, divide extensively and appear as scattered islets of cells. Towards the end of this stage, the fly enters metamorphosis as the larval structures undergo a remodelling process in preparation for pupariation and formation of the adult animal. At this time, most midgut cells delaminate from the basement membrane and visceral muscle as they are being shed into the gut lumen. The AMPs and their daughters, however, fuse to form the future adult midgut epithelium through a series of well coordinated steps which define five phases of AMP activity. During larval development, Egfr-Ras-Mapk signaling was shown to be necessary and limiting for AMP proliferation. Early AMP proliferation is stimulated by visceral muscle derived Vein expression (weak Egfr ligand), whereas the stronger Spitz and Keren Egfr ligands are produced by the AMPs themselves and provide a mitogenic stimulus during late larval stages [46,50].

Reduction of InR-Pi3k activity in differentiated tissues of drosophila larvae leads to cell autonomous reduction in cell growth and DNA endoreplication [46]. Reducing ToR activity triggers similar inhibitory effects [44,45]. Conversely, activation of InR-Pi3k or of Rheb-ToR signaling increases cell size and nuclear DNA content in many larval tissues, including the gut and the fat body. Moreover, InR-Pi3k as well as Rheb are capable of bypassing the dietary requirement for cell growth, and can function as growth promoters even in animals starved for protein [46,47].

Aim

AMP proliferation during larval development is highly sensitive to Egfr signaling and to changing nutritional conditions [46,50]. As the AMPs do not differentiate at these early stages, they constitute a good system to study Ras Mapk mediated effects on growth and proliferation. Thus, we tested if Ras-Mapk pathway activation could also replicate this starvation-independent growth observed upon Rheb overexpression.

New Findings

In control animals, at 72h AED (after egg deposition) most AMPs were found in the midgut as dividing doublets. A subsequent surge in proliferation then ensured their dispersal through the epithelium and formation of characteristic clusters, known as AMP islands, at 120h AED. Upon starvation, the wild type controls showed a block in proliferation, with AMPs still presenting as doublets throughout the tissue at 120h AED. In normally fed animals, providing a growth promoting signal in the form of activated Ras, led to a strong proliferative response with progenitor cell and cluster areas increasing significantly compared to the fed controls (figure 13). In several animals, the AMPs appeared no longer restrained to clusters but rather dispersed through the epithelium. A novel and surprising finding was that expression of activated Ras allowed the progenitors to expand to some extent even under starvation. The starved Ras expressing guts closely resembled the fed wild type controls.

Thus, activated Ras acts as a potent growth and proliferation promoter in the larval intestine under fed conditions. Particularly promising was our novel result showing that Ras expression in the developing intestine is able to provide a growth input which is partially dominant to nutrient requirement. Considering that we expressed a Ras allele which specifically activates the Raf-Mek-Erk kinase cascade, it is highly likely that the observed growth and proliferation phenotypes are a consequence of Erk activation. Based on our previous results (III-1,3,5), we could hypothesise that Mapkapk or ToRC1 kinases might act downstream of activated Erk to drive the observed growth. Moreover, ToRC1 seems a particularly appealing candidate, as expression of its activator Rheb in this tissue was previously found to drive a similar phenotype [47]. Another important growth promoter and a known convergence point between Mapk and ToR signaling is Myc (see III-4). However, it is unlikely that Myc is the main growth driver in this context, as its expression in larval tissues fails to promote growth under starvation conditions [46]. Other known pathways including Notch, Hedgehog, Wingless, Dpp and Jak/Stat were also tested for driving growth and cell cycle progression in larval endoreplicating tissues (which constitute most of the drosophila larva), and found not to be required [148,149].

I think that Ras-Mapk exerts its growth promoting function mainly through non-transcriptional mechanisms by activating different effectors, such as ToRC1 and Mapkapk kinases, in a context dependent manner demanded by the tissue type and developmental timing. Similarly, Mapk nuclear signaling relies on several mechanisms including chromatin remodelling and regulation of several transcriptional factors. We know that Ras-Mapk activation and function-

ality during development is strongly context dependent (see introductory chapter). Therefore, it would not be surprising to find that its growth functionality relies on more than one effector, whose expression might depend on the developmental timing and tissue type. For example, if ToRC1 activation by Erk is mediated by Rsk dependent Tsc2/Raptor phosphorylation, then Erk activation will not replicate ToRC1 phenotypes in tissues where Rsk expression is low. This could be the case for fat body tissue (a nutrient storage organ analogous to the vertebrate liver). There, we expressed activated Ras but did not observed a significant growth phenotype, as we did upon Rheb expression (see II-4). Perhaps Ras-Mapk ability to tap into ToRC1 activity for promoting growth is restricted to progenitor-like cells which haven't yet exited mitotic cell cycles and differentiated. Thus, a future challenge is to identify the primary context-specific growth effectors activated by Ras-Mapk, especially in tissues with relevance to human health, such as the intestine. Future experiments could employ different ways to activate Ras-Mapk signaling, with concomitant ToRC1 or Mapkapk modulation, and subject the animals to starvation conditions. In a similar context, Pi3k activation cannot drive growth in ToR mutant larvae [Lande and Neufeld, personal communication].

III-8 FUTURE PERSPECTIVES

Based on the results provided by our study and available literature, the following five avenues stand out and are worth further investigation.

[1] Mapkapks as novel growth promoters. Our results presented in section II-1 and discussed in sections III-1,2 bring forward new potential growth effectors in insect cells, the so-called Mapk activated protein kinases (Mapkapks). Three of these kinases (Mnk, Rsk, Msk) had robust growth phenotypes in cultured drosophila schneider cells. Using chemical inhibitors and measuring cell numbers and diameters at several time points, we found that these Mapkapks are important for normal and insulin stimulated proliferation of schneider cells. A first extension of these results would be to confirm the specificity of these inhibitors with RNAi-mediated knockdown of the respective kinases. Also, cells derived from knockout animals (when these are not lethal) could prove informative for validating substrates and biological functions. A second important aspect to address is whether these Mapkapks are indeed activated by Mapks. Two of them, Rsk and Mnk, have already been described as Erk interacting partners in drosophila [14-19]. Monitoring phosphorylation of the activation loop of Mapkapk kinase domains upon Ras-Mapk modulation should confirm or exclude their identity as Mapk downstream effectors. Quantification of Mapkapk target phosphorylation (such as eIF4E for Mnk) could also informative in this regard. A third question to ask is how are these Mapkapks promoting growth? Are they activating ToRC1 or do they promote anabolic pathways through parallel mechanisms? Chemical or genetic manipulation of Mapkapk activities combined with detection of ToRC1 target phosphorylation (such as phospho-S6k and phospho-4EBP) would provide such clues. Concomitant modulation of Mapkapk and ToRC1 activities coupled with cell division and size measurements would reveal possible dependencies, and if their combined phenotypes are synergistic or additive.

[2] Mapk ToR crosstalk mechanism. The results described in sections II-2/3 and discussed in sections III-3/4/5 reveal a connection between Ras-Mapk and ToRC1 pathways that may be conserved in cultured insect cells as well as the living animal, and with relevance to Mapk-driven growth. An integration between Ras-Mapk as a main controller of cell-cycle progression, survival, growth and differentiation, and ToRC1 as a main nutrient sensing mechanism employed by all eukaryotic cells, would be highly advantageous for the dynamic adaptation of these cellular function according to the cell's metabolic state. Mapk-ToR crosstalk probably

predates the advent of insulin signaling in the course of evolution, as multicellular organisms needed a way to coordinate metabolism in communities of cells. Such an integration is also supported by our results in insect cells and similar findings in mammalian systems. In order to understand and successfully manipulate the cellular functions controlled by these pathways, we need a detailed view of their mechanistic interactions. I described the potential intersection points between Ras-Mapk and ToR pathways in section III-4. Experimental approaches could address the following questions: Does Ras-Mapk modulation disrupt the subcellular localisation of Tsc1 and Tsc2? Tagged Tsc1 and Tsc2 constructs (available in public stock collections) could be co-expressed and their subcellular localisation followed by standard microscopy. Does Ras-Mapk activation lead to Erk-dependent phosphorylation of Tsc2 or Raptor? To address this, Tsc2 and Raptor phosphorylation on Ser/Thr residues could be measured by immunoblotting from cells untreated or treated with Ras-Mapk agonists like PMA or Spitz. Additionally, cells could be pretreated with Mek/Erk inhibitors (like U0126) or dsRNAs to assess the dependency of the phosphorylation events on Mapk activity. In vitro kinase assays using purified proteins could also confirm the observed interactions. If such interactions are identified, the next question is which are the targeted sites in Tsc2/Raptor? This can be addressed by mass spectrometry analysis of immunoprecipitated Tsc2/Raptor from unstimulated and Ras-Mapk-stimulated cells. One interesting question would then be what are the functional effects of these phosphorylation events? Substitution mutants could then be used to assess the effects on ToRC1 target phosphorylation (immunoblotting), ToRC1 complex formation (co-immunoprecipitation) and overall cell behaviour like growth and proliferation. The same series of questions could be asked with regard to Rsk instead of Erk as a potential effector kinase.

[3] Measuring translation upon Mapk or Mapkapk modulation. The discordance between steady state mRNA levels and protein levels indicates that translational control plays a major role in the control of gene expression. This is particularly relevant for cell growth and proliferation genes. One way to characterise Mapk dependent translational control and its dependency on ToRC1 or Mapkapks is to monitor nascent protein production upon modulating these pathways. This can be achieved with a simple and robust chemical method for imaging nascent polypeptide chains in cultured cells or tissue explants. The method relies on a puromycin analog, o-propargyl-puromycin (OP-puro), which forms conjugates with nascent polypeptide chains [150]. These conjugates can be visualised or captured via a copper-catalysed azide-alkyne cycloaddition (CuAAC) reaction with fluorescent- or biotin-labelled azides, respectively.

One could test, for example, to what extent Ras-Mapk or Mapkapk modulation affects the rate of protein synthesis, and how this is influenced by simultaneous ToRC1 inhibition.

[4] Growth in the adult intestine. Our findings introduced in section II-3 and discussed in section III-5 reveal a possible dependency of Ras-Mapk driven growth in the adult midgut on ToRC1 activity. Indeed, Ras-Mapk activation in the committed progenitors led to a significant increase in cell size which was dependent to some extent on ToRC1 activity, as rapamycin treatment decreased the occurrence of overgrown cells typically associated with expressing activated Ras. An extension of these results would be to accurately measure ToRC1 pathway activity upon Ras-Mapk modulation in this tissue. I performed several experiments addressing this issue (see II-3), and although phospho-4EBP levels appeared to increase (indicative of higher ToRC1 activity) upon Ras or Rheb expression, the signal was not specific enough to draw reliable conclusions. Refinement of the immunofluorescence protocol may improve detection quality and allow a more accurate assessment of ToRC1 activity. Alternatively, one could also measure changes in ToRC1 activity upon Ras-Mapk modulation by using an immunoblotting assay. This would give a clear quantitative measure of ToRC1 activity (by detecting phospho-S6k or phospho-4EBP), but provide less information about the identity of the cells in which ToRC1 is being activated or inhibited (unless is coupled with cell sorting). Our results discussed in sections III-1,2 introduce Mapkapk kinases as potential growth promoters in insect cells, probably acting downstream of Mapk pathways. If their functions are conserved in the living animal, they may play a role for Ras-Mapk driven growth in the adult intestine. Therefore, these kinases could be tested for growth phenotypes in the midgut using constitutively active or null alleles together with cell type specific or clonal expression systems. Chemical inhibitors could also be used, though the results may be difficult to interpret.

[5] Nutrient independent growth. Our results presented in section II-4 and discussed in section III-6 bring forward a new growth function for Ras-Mapk in the larval intestine. Activation of Ras-Mapk triggered progenitor cell growth and proliferation even in animals starved of dietary protein. This suggests that Ras-Mapk is able to provide a growth input which is partially dominant to nutrient requirement. Although we expressed a Ras allele specifically activating the Raf-Mek-Erk pathway, the phenotype should also be confirmed using activated downstream components such as Raf. Monitoring Erk activation based on its dual phosphorylation status under these conditions could also be informative. The next step would be to identify the primary effector responsible for the growth phenotype. As discussed in previous sections, this

effector(s) acts likely at a translational rather then a transcriptional level, as part of the initial response following pathway activation. Based on our results, one could focus on ToRC1 and Mapkapks as potential candidates. Such an approach would employ initiating starvation followed by simultaneous activation of Ras-Mapk and inhibition of ToRC1 or Mapkapks in the larval intestine (using mutant alleles or chemical inhibitors). Testing if activated Mapkapks are capable of driving nutrient independent growth in the larval intestine by themselves would also be a novel result and complement the previous assay. Measurements of cell proliferation and cell areas would reveal any growth effects exerted by these candidates.

IV. METHODS

IV-1 DROSOPHILA LIFE CYCLE AND GENETICS

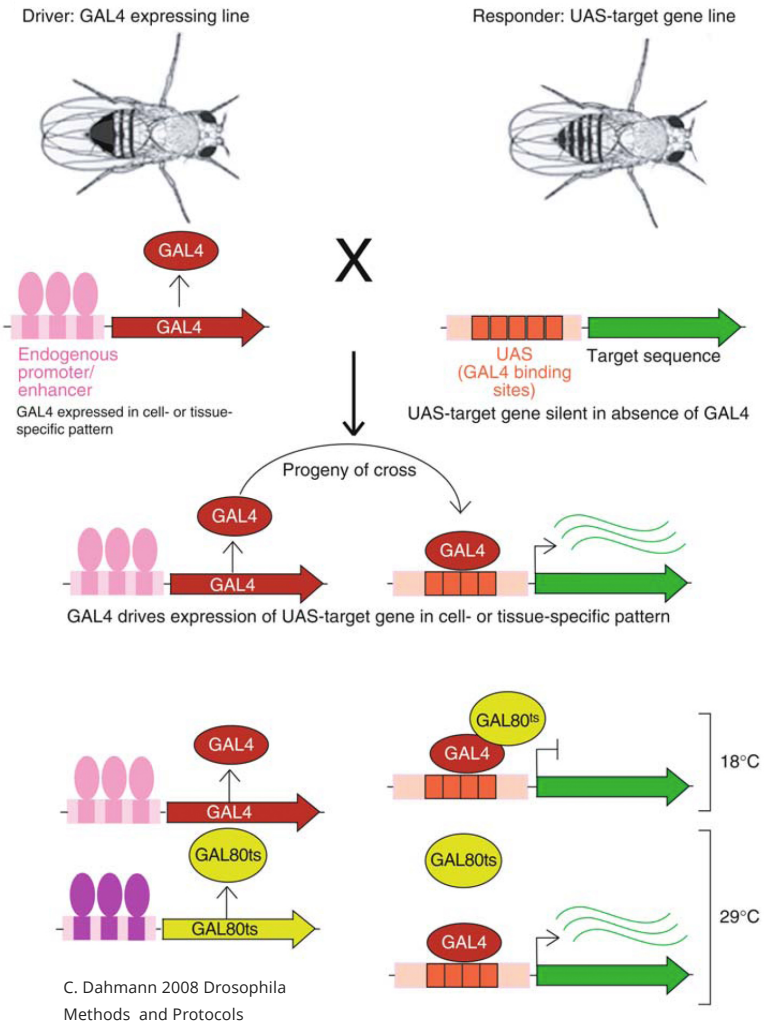
One advantage of drosophila melanogaster as a model organism is its rapid development. At 25C, its whole life cycle takes about 10 days. Flies deposit their eggs in the food, and after about 1 day of embryogenesis, the first instar larvae hatches. During the next 4 days, the larvae feeds avidly increasing about 200 times in mass. Following this extreme growth program, the third instar larvae stops feeding and searches for a dry place suitable for pupariation. Metamorphosis takes place in the pupal case during the next 4/5 days. Thus, after about 10 days from egg deposition, the adult fly ecloses measuring on average 3mm in length.

The fly's genome is distributed among 8 chromosomes: one pair of sex chromosomes (XX in females and XY in males) and three pairs of autosomes (denominated 2nd, 3rd and 4th). The Y chromosome consists almost entirely of heterochromatin and carries just a few genes required for male fertility, but not viability. Each gene is named after the mutant phenotype its disruption causes, with additional identifiers as superscripts or in brackets used to distinguish different alleles.

Versions of drosophila chromosomes have been developed (so-called balancers) that contain multiple inversions so as to suppress meiotic recombination with un-rearranged chromosomes. These balancers also harbour dominant mutations with an easily visible phenotype and recessive lethal or sterile mutations. Given their usefulness, balancers have been developed for each chromosome: the FM6/7 series for the X chromosome (where F stands for first chromosome and M for multiple inversions), CyO and SM5/6 for the 2nd chromosome, TM2/3/6 for the 3rd. There is no need for a balancer chromosome for the 4th chromosome as it does not undergo meiotic recombination - and there is no meiotic recombination in males.

IV-2 DROSOPHILA GAL4 GENE EXPRESSION SYSTEM

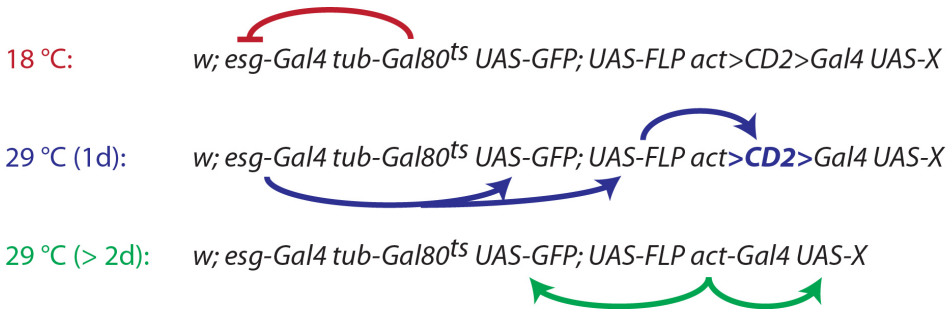
The Gal4 system is widely used in drosophila research for expressing any DNA sequence in a spatial and/or temporal controlled fashion [358]. The Gal4 system is build upon two components: (1) Gal4, a transcriptional activator from yeast that can be expressed in a tissue-specific manner and (2) a gene regulatory sequence that can be bound by Gal4 (UAS, upstream activation sequence). The two components are typically brought together by crossing one transgenic line carrying the Gal4 activator (the driver) and a second line carrying the UAS-linked transgene (the responder). The progeny of the cross that inherited both the Gal4 and UAS-transgene will express the transgene only in the tissues where Gal4 is expressed. A common way to control when Gal4 is being expressed is by ubiquitously co-expressing Gal80 protein, which binds and inhibits Gal4. Gal80 in turn can be made temperature sensitive so as to become inactive upon raising the temperature from 18C to 29C (commonly used in fly assays). The expression of any transgene can thus be confined to certain tissues (due to the tissue-specific expression of Gal4) and to certain times (due to a temperature shift that inactivates Gal80).



C. Dahmann 2008 Drosophila Methods and Protocols

IV-3 FLP GAL4 LINEAGE TRACING

The Esg^{TS} Flip Out (Esg^{TS} F/O) system is an adaptation of the Gal4 expression system just described. Esg^{TS} FO is a lineage tracing system in which the expression of the UAS-fused transgene is induced in a cell and its expression is then inherited in all the progeny derived from this initial cells [359]. The genotype supporting this type of expression control is detailed below. Briefly, Esg-Gal4 was expressed in ISCs and EBs under the temperature-sensitise control of Tub-Gal80^{TS}. Once activated by a temperature shift (from 18C to 29C), Gal4 drives expression of Flp recombinase which cuts out the CD2 cassette and thereby allows Act-driven Gal4 expression in all progenitors of that cell. Thus, any UAS-linked sequences will be transcribed in that cell lineage. As UAS-GFP is frequently common in these genomes, the system allows for an easy way to ascertain tissue renewal rates by simply scoring the fraction of GFP+ (newborn) cells. Identification of additional cell type specific markers can also be informative as to what types of differentiated cells are generated from the initially labelled cells, and in what proportions.



Flp Gal4 lineage tracing system. See text for details.

IV-4 GENOTYPES AND GENE INDUCTION

Flies were grown in vials with standard fly food [0.72% (w/v) agar, 7.2% (w/v) maize, 2.4% molasses, 7.2%(w/v) malt, 0.88%(w/v) soya, 1.464% (w/v) yeast and acid mix (1% propionic acid + 0.064% orthophosphoric acid)] media on 12-hour day-night cycle. For each cross, around 15 female and 5-6 male flies were used. All the fly stocks were maintained at 18°C. The expression of transgenes was achieved by using a temperature sensitive inducible UAS-Gal4 system. Crosses were set up and maintained at 18°C, the permissive temperature. 3-7 days old flies were shifted to 29°C for different times (usually 5d, unless otherwise specified).

Driver Lines

Esg^{TS} > esg-Gal4/CyO; tubGal80ts UAS-GFP/TM6B (Micchelli and Perrimon 2006)

Esg^{TS}F/O > esg-Gal4 tubGal80ts UAS-GFP/CyO; UAS-Flp>CD2>Gal4/TM6B (Jiang et al. 2009)

Tub^{TS} > tub-Gal4; tubGal80ts/TM3, ser (Romani et al 2009)(from Valeria Cavaliere lab)

$Su(H)^{TS}$ > Su(H)GBE- Gal4,UAS-CD8-GFP/CyO; tubGal80ts/TM6B,Tb

Adh > w- ; Adh-Gal4 / CyO; UAS-GFP / TM6B (B.A.Edgar lab)

UAS Transgenes

UAS-RAS^{v12s35} (Karim & Rubin, 1998)

UAS-Ets21c > IF / CyO ; Ets21c / MKRS (B.A.Edgar lab)

UAS-Ets21c-RNAi (VDRC KK103211)

UAS-Ets21c-RNAi > UAS-Ets21c-RNAi / CyO; TM6B / MKRS (B.A.Edgar lab)

UAS-Ets21c, Mek-RNAi > UAS-Mek-RNAi / CyO ; UAS-Ets21c / TM6B (Y. Jin)

UAS-Ras RNAi (Jiang et al. 2011)

$UAS\text{-}Ras^{V12}$ > y w, hsFlp122 ; UAS-RAS-V12 / CyO act-GFP

$UAS\text{-}Ras^{V12G37}$ > y, w, hsFlp122 ; UAS-Ras-V12G37 ; + (Ulrike Gaul)

$UAS\text{-}Ras^{V12S35}$ > y, w, hsFlp122 ; UAS-Ras-V12S35 ; + (Ulrike Gaul)

UAS-Raf^{GoF} > w ; Pin / CyO ; TM6b / Raf-GoF (B.A.Edgar lab)

UAS-Rheb > w-; UAS-Rheb #3; + (A. Teleman Lab)

UAS-Rheb-RNAi > UAS-Rheb-RNAi ; UAS-Dcr2 / CyO (Aida de la Cruz)

UAS-Rheb-RNAi > w ; UAS-Dcr2 / CyO ; UAS-Rheb-RNAi / TM6b (Aida de la Cruz)

IV-5 IMMUNOHISTOCHEMISTRY

(1) Dissection » 10-15 guts for each genotype in 1xPBS. During the dissections, the guts can be gathered already in eppendorf tubes containing 4% PFA and on ice

(2) Fixation » fresh 4% PFA/PBS (less than a few days old, kept at 40 or better freshly thawed), 30 minutes, at room temperature, on nutator

(3) Rinsing and washing » 1xPBS, 2 x 10 minutes

(4) Permeabilisation » freshly made 0.15% Triton in PBS (75ul Tx100 for 50ml), 30 min

(5) Rinsing and washing » 2 x 5 minutes with washing solution (0.1% Tween20 in 1xPBS)

(6) Blocking » 30 minutes in blocking solution (2.5% BSA, 0.1% Tween20, 10% normal goat serum, in 1xPBS). Prepare blocking pre-solution: 50ml washing solution + 2.5% BSA (1.2g for 50ml). Calculate how many samples you have: e.g. 10 x 500ul = 5ml needed, then add serum: e.g. 4500ul blocking pre-solution + 500ul normal gout serum

(7) Primary ABs » incubate o/n, 4^0C, antibodies diluted in blocking solution with normal goat serum; rotate tubes. Alternatively, incubate at RT for 3h rotating

(8) Next day, rinsed washed » 1xPBS, 0.2% BSA, 0.1% Tween (washing solution), 3 x 20 min

(9) Secondary antibodies » incubate 3 hours, room temperature (in the dark, covered with aluminium foil); secondary antibodies conjugated with Alexa fluorophores and diluted 1:1000 in blocking solution

(10) Rinsed then washed » 1xPBS, 0.1% Tween (washing solution), 1 x 20 minutes (in dark!)

(11) Washed in 1xPBS, 0.1% Tween, DAPI (diluted 1/100 from stock) for 10 minutes

(12) Washed out DAPI in 1xPBS, 0.1% Tween for 10 minutes

(13) Embedded in Vectashield (can use Vectashield with DAPI)

a. place 4 small drops of lab fat on edges of cover glass

b. bring samples on objective slide with a cut 1ml pipette tip

c. distribute the samples with a forceps

d. remove excess liquid (drain and use tissue)

e. put on cover glass with very little pressure

f. add Vectashield with the 200ul pipette tip and let it fill by capillary force

g. seal with nail polish and store until analysis at 40C in the dark

(14) Image processing: fluorescence micrographs were acquired with either of two confocal systems: Leica® TCS SP5 II and Zeiss® LSM 780 confocal inverted microscopes. Images were then processed using ImageJ® Fiji, Adobe Photoshop® and Adobe Illustrator®. Antibodies are listed under section IV-7, together with immunoblotting antibodies.

IV-6 CELL TREATMENT AND PROTEIN EXTRACTION

Lysis Buffer Preparation

10ml LB were prepared and stored as 2ml aliquots at -20C. 100ul LB was added to each sample (2x50ul aliquots).

(S1) 2.5ml (Laemmli+DTT; 1/4 of total vol) + (S2) 7.5ml H2O (with inhibitors) = 10ml (LB)

(S1) mix 900ul Laemmli (4x) with 100ul DTT; boil 5min at 95C; perform similar mix in 3x1ml

(S2) dissolve in 7.5ml H2O: 6 tablets phosphatase inhibitor cocktail + 6 tablets protease inhibitor cocktail + Na3VO4 (sodium orthovanadate)(MW 184, 20mM = 36.8mg) + NaF (MW 42, 50mM = 21mg) + b-glycerophosphate (optional, MW 216, 0.011 g/ml = 0.11g)

Cell Seeding

(1) Initial cultures were set up 2d prior to experiment startup. Cells were seeded in T25 flasks in medium without serum at about 70% confluence. 700uM CuSo4 were added to S2:sSpi cells to induce ligand production.

(2) After 2xON, the growing cells were spinned down from initial culture by centrifugation (10min 800 rpm). S2:Egfr (S2/S2R+) cells were then seeded in 6well plates, 2ml/well, in medium without serum, 1.5-2x10^6 cells/ml or semiconfluence. Cells were recovered by incubation for 20min in 25C incubator.

(3) After pelleting the S2:sSpi cells from initial culture, the supernatant was removed and stored (Spi conditioned medium), and the S2:sSpi cells passaged for further experiments. Store sSpi medium in fridge for about a week (consider NaN3 for long term).

Inhibitor Pretreatment

(4) After 20min recovery, inhibitors were added in individual wells, and the cells incubated for further 40min in 25C incubator.

Growth Factor Treatment

(5) After 40min inhibitor pretreatment, cells were resuspended from each well by pipetting and transfer to 15ml falcons.

(6) Growth factors and Spi-conditioned medium (3ml on top of original culture) were added to corresponding tubes, and further incubated for 10min in 25C incubator.

(7) After 12min GF treatment, falcon tubes were transferred on ice for 1min, before cell lysis.

Cell Lysis

(8) Cells were pelleted quickly by centrifugation at 1'500 rpm for 5min (set centrifuge at 4C, if possible), and placed on ice.

(9) Supernatant was discarded and 100ul lysis buffer added to each sample (for 2x 50ul aliquots, each for two gels), mix 10x, transfer to 2ml eppendorf tubes, and place tubes on ice.

(10) All sample tubes were boiled for 5min at 95C and quickly placed on ice.

(11) Each sample was aliquoted in 2x 50ul aliquots in two 2ml tubes, snap frozen in liquid nitrogen and stored at -80C.

GROWTH FACTOR	EFFECTIVE CONC.	COMPANY	CATALOG NR.
Epidermal growth factor, murine, natural	10 ug/ml	Invitrogen	53003018
Recombinant mouse EGF	10 ug/ml	Life Tech.	PMG8044
Insulin from bovine pancreas	10 ug/ml	Sigma Aldrich	IO516
Recombinant mouse PDGF	1 ug/ml	Life Tech.	PMG0044
Recombinant mouse VEGF	1 ug/ml	Life Tech.	PMG0114
Phorbol 12-myristate 13-acetate, PMA	10 ug/ml	Abcam	ab120297

INHIBITOR	IC50	POTENTIAL 2° TARGETS	EFF. CONC.	COMPANY	CATALOG
Mek inhibitor U0126	0.07uM	–	100 uM	Cell Signaling	9903
Mek inhibitor PD98059	4/50uM	–	100 uM	Cell Signaling	9900
Erk inhibitor GDC0994	1 nM	phosphoRsk	50 uM	Selleckchem	S7554
cOmplete protease inh.	–	–	1 tab/1.5ml	Roche	4693124001
PhosStop phosphatase inh.	–	–	1 tab/1.5ml	Roche	4906837001
Mnk inhibitor CGP57380	2.2 uM	–	500 uM	Selleckchem	S7421
Rsk inhibitor BID1870	25 nM	–	500 uM	Selleckchem	S2843
Msk+ inhibitor Ro318220	27 nM	Pkc Gsk3 S6k	500 uM	Selleckchem	S7207
ToR inhibitor, Rapamycin	0.1 nM	–	10 uM	Sigma Aldrich	R8781
ToR inhibitor, Torin1	4 nM	–	10 uM	Tocris	4247

CELL LINE	REFERENCE
[Schneider S2] The S2 cell line was derived from the primary culture of late stage (20-24h) drosophila embryos. S2 cells are round and display features that indicate an origin in a macrophage-like lineage. S2 cells can be grown at room temperature without CO_2 as a loose semi-adherent monolayer in tissue culture flasks and in suspension in shake flasks.	Schneider I. 1972 Cell lines derived from late embryonic stages of Drosophila melanogaster. J. Embryol. Exp. Morphol. 27: 353-365
[Schneider S2R+] Schneider receptor plus cells are derived from an S2 cell line. They originate, therefore, also from dissociated embryos near hatching. Like the parental S2 cells, they also display a hemocyte-like gene expression and are phagocytic. In contrast to the S2 cells which are round and semi-adherent, however, the S2R+ cells are flat and adherent cells. Moreover, S2R+ cells express the Frizzled receptor and respond to extracellular Wingless by upregulating b-catenin and e-cadherin levels.	Yanagawa S.I. et al. 1998 Identification and characterisation of a novel line of Drosophila Schneider S2 cells that respond to Wg signaling. J Biol. Chem. 273: 32353-32359
[S2:sSpi] S2 cells transfected with a construct encoding secreted Spitz protein, in which a termination codon was used to replace the dibasic putative cleavage signal. Expression is controlled by the inducible metallothionein promoter. Upon induction, significant amounts of the mature Spi protein are secreted in the medium. Cell line was a kind gift from Prof. Ben-Zion Shilo, Weizmann Institute of science, Israel.	Schweitzer R. et al. 1995 Secreted Spitz triggers the DER signaling pathway and is a limiting component in embryonic ectoderm determination. Genes & Dev. 9(12), 1518–1529
[S2:Egfr] S2 cells transfected with a construct encoding the type II drosophila Egf receptor (Der) under the control of the metallothionein promoter. Upon treatment with secreted Spi protein, the Egf receptor displays a dramatic increase in the level or tyrosine phosphorylation. Cell line was a kind gift from Prof. Ben-Zion Shilo, Weizmann Institute of science, Israel.	Schweitzer R. et al. 1995 Secreted Spitz triggers the DER signaling pathway and is a limiting component in embryonic ectoderm determination. Genes & Dev. 9(12), 1518–1529

10x RUNNING BFF	1x RUNNING BFF	10x TRANSFER BFF	1x TRANSFER BFF	1x PBST (0,1%)	5% BLOCKING MILK
30,2 g Tris	100 ml 10x RB	30,3 g Tris	100 ml 10x TB	100ml 10xPBS	10 g Milk Powder
188 g Glycine	900 ml dH_2O	144 g Glycine	200 ml Methanol	10ml 10% Twn	200 ml 1x PBST
100 ml 10% SDS		H_2O to 1L	700 ml H_2O	H_2O to 1L	
H_2O to 1L	Store all buffers at 40C		(or 10% MetOH)		

IV-7 IMMUNOBLOTTING

Reagents and Buffers

Cells were plated 2 days before treatments, at 70% confluence, in medium without serum to enhance GF response.

WB washing buffer: 1x PBST (0.1% Triton in 1x PBS).

WB separation buffers: running buffer (for SDS-PAGE, in house 25C), transfer buffer (for WB, house 25C).

WB blocking buffers: 5% Milk in PBST (membrane and secondary AB block), and 5% BSA in PBST (primary AB block). Store buffers at 4C and use for up to three days.

Gel solutions: APS(4C), Temed (commercial 4C), 30% Acrylamide (commercial 4C), Separating gel buffer, stacking gel buffer (in house 25C).

Inhibitors and growth factors for cell culture treatments.

Gel Preparation

Most proteins were separated and detected using 12,5% gels.

Commonly in lab stock: running buffer, separating gel buffer, stacking gel buffer, 30% acrylamide and Temed.

APS prep: solve 0,1g in 1ml water in a 1,5ml eppendorf tube. Store at 4C for 1-2 months.

The preparations of 10 ml separating gel and 8 ml stacking gel (table bellow) suffice for 2 gels.

Separating and stacking gel solutions were prepared acc. to indications in the summary table.

After APS addition, the separating gel was quickly mixed and poured, and isopropanol added to ensure an even surface.

After the separating gel solidifies (20min), the isopropanol was removed using tissues, APS was then added to the stacking gel solution and poured on top of the separating gel. Don't forget to add the plastic comb (with 15 well teeth) after pouring the stacking gel.

Separate Proteins via Electrophoresis

12.5% polyacrylamide gels were prepared according to specifications in the gel tables.

Chamber was filled with 1x running buffer (dilute from 10x stock with dH2O).

Sample mixing with hamilton (or 20ul pipet) very well.

20 ul sample (in LB) were loaded, 15 ul marker (PageRuler Plus protein ladder) per lane.

Electrophoresis run at 20 mA/gel, 200V(optional setting) for 1h.

Running buffer for SDS-PAGE (10X, 10L)

300g Tris base (0.25M)
1440g Glycine (1.9M)
1L 10% SDS

Separating gel buffer (4X, 1L)

181.7g Tris base (1.5M)
4ml 10% SDS
adjust pH to 8.8 with HCl

Separating gels for SDS-PAGE

	8.5%	10%	12.5%	15%
H_2O (ml)	4.7	4.2	3.4	2.4
30% Acrylamide (ml)	2.7	3.2	4	5
4X Separating gel buffer (ml)	2.6	2.6	2.6	2.6
APS (µl)	150	150	150	150
TEMED (µl)	7	7	7	7

10ml

the amount is sufficient for 2 mini gels.

add 1st
add 3rd
add 2nd
100µl add 5th
add 4th

Stacking gel buffer (4X, 500ml)

30.3g Tris base (0,5M)
20ml 10% SDS
adjust pH to 6.8 with HCl

Stacking gels for SDS-PAGE (4%)

5.2%

	50ml	100ml
H_2O (ml)	28.65	57.3
30% Acrylamide (ml)	8.6	17.2
4X Stacking gel buffer (ml)	12.75	25.5

for 2 mini gels take 10 ml of the prepared stacking gel mix and add 200 µl APS and 10 µl TEMED.
100

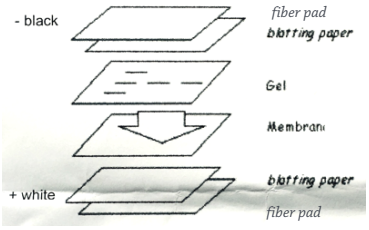
8mL
4.7 mL H_2O
1.2 mL Acrylamide
2 Stk gel 5ff
8 TEMED
80 APS

Transfer buffer for western-blotting (1L)

5.82g Tris base (48mM)
2.93g Glycine (39mM)
3.75 ml 10% SDS
200ml Methanol

1x LAEMMLI	1x LAEMMLI, PHOSPHO-PROTEINS
78 ul H2O	2 ul Protease inhibitor
2 ul Protease inhibitor mix	2 ul Vanadate
20 ul 5x Laemmli	5 ul NaF
Final Vol 100 ul	5 ul Phosphatase inhibitor
	10 ul phospho-b-glycerol
	20 ul 5x Laemmli
	56 ul H2O
	Final Vol 100 ul

Transfer Proteins to Membrane via Blotting



PVDF membrane was cut at same size as the gel and marked with a pencil one of the corners to recognise the loading order and the side with proteins (8.5 x 6.5 cm).

PVDF membranes were then activated by incubating in MetOH for 20s.

Membranes and gels were then incubated in 1x cold transfer buffer (TB) for 10m on a nutator.

Transfer sandwich was prepared as pictured, in a dish with TB; avoiding air bubbles between gel and membrane.

The transfer chamber was filled with TB and gel sandwich added (don't forget the ice box) for achieving better transfer, a magnetic bar was added and the transfer chamber was placed on a magnetic stirrer in the cold room.

Western blots were run at 30V overnight at 4C (or 100V 270mA for 1h at 4C).

Visualise Proteins with Ponceau Red

Proteins were visualised by incubation for 1min in ponceau red solution.

Washed 2-3x 10s with desalted water (membranes could be stored in-between filter papers at 4C for days).

Optional, to detect the proteins in the gel, place in Coomasie stain with EtOH 5% for few min.

Membranes were incubated in MetOH for 30s prior to blocking (when using PVDF membrane)

Block Membranes

Membranes were then transferred to 50ml eppendorf tubes for blocking and AB incubations.

Membranes were then incubated for 50min with 15ml membrane blocking buffer MB-BLK (5% Milk in 1x PBST = 5g Milk in 100ml PBST) at 25C on a roller.

Wash Membranes

Membranes were washed 3x 10min with 1x PBST at 25C on a roller (important to remove the milk, as the milk casein interferes with phospho-antibody detection).

Primary Antibody Incubation

Prepare 1AB blocking buffer 1AB-BLK: 5% BSA in 1x PBST (2.5g BSA in 50ml PBST).

Primary antibodies were diluted 1:1'000 in 1AB-BLK (usually two mixes, each in 10ml BLK bff)

1AB mixes were then added to corresponding falcon tubes (with membranes) and incubate d overnight at 4C on a roller (longer incubation of 2-3 days also possible). Check compatibility with IRDye-labelled 2ABs.

Wash Membranes

Membranes were washed 3x 10min with 1x PBST at 25C on a roller. Washing removes background.

Secondary Antibody Incubation

2AB blocking buffer 2AB-BLK was prepared: 5% Milk in 1x PBST (same as MB-BLK).
Dilute IRDye-labelled secondary antibodies 1:10'000 in 2AB-BLK (usually two mixes, each in 10ml BLK).
2AB mixes were then added to corresponding falcon tubes (with membranes) and incubated for 2h at 25C on a roller.
Generally, the control protein (tubulin, actin or total protein) is detected with IRDye700-labelled 2AB, and the (phospho)protein of interest with IRDye800-labelled 2AB (less background).

Wash Membranes

Membranes were again washed 3x 10min with 1x PBST at 25C on a roller.

Image Proteins

Imaging was done with the Odyssey Infrared Analyser.
Membrane were placed between two transparent plastic foils and with the marked protein side facing down on the Odyssey reader, avoiding air bubbles.
Own workspace was created and used throughout all assays in the Odyssey software.
Scanning area selected according to the positioning of the membrane on the scanner.
Scanning quality selected, generally for both 700 and 800 channels simultaneously, max reading options.
Initiated scan and best signal to noise ratio for each channel from offered scans.
Adjusted each channel individually, generally by taking the black and white and adjust brightness and contrast to achieve best signal, and save each channel in BW by selecting file > export image > save current image view > browse for file location and name.

PRIMARY ANTIBODY TARGET	HOST	DILUTION	COMPANY	CATALOG
Akt antibody	Rabbit	1:1000	Cell Signaling	9272
Phospho-drosophila Akt (Ser505)	Rabbit	1:1000	Cell Signaling	4054
Phospho-4EBP1 mAb	Rabbit	1:1000	Cell Signaling	2855
Phospho-drosophila p70 S6k Ab	Rabbit	1:1000	Cell Signaling	9209
Drosophila S6k antibody	Guinea pig	1:2000	A. Teleman Lab	—
Phospho-Erk mAb	Mouse	1:1000	Sigma Aldrich	M9692
p44/42 Erk mAb	Rabbit	1:1000	Cell Signaling	4695
aTubulin mAb	Mouse	1:800	Sigma Aldrich	T9026
Actin Ab	Rabbit	1:1000	Sigma Aldrich	A2066
Phospho-Mek (Ser 217/221)	Rabbit	1:1000	Cell Signaling	9154
GFP (only for IF)	Chicken	1:1000	Life Tech.	A10262
Phospho-H3 (Ser 10) (IF)	Mouse	1:200	Cell Signaling	9706
Phospho-H3 (Ser 10) (IF)	Rabbit	1:500	Abcam	5176
Armadillo (IF)	Mouse	1:100	DSHB	N27A1

SECONDARY ANTIBODY TARGET	HOST	DILUTION	COMPANY	CATALOG
Anti-mouse-DyLight680	Donkey	1:10'000	Rockland	610-744-002
Anti-mouse-DyLight800	Donkey	1:10'000	Rockland	610-745-002
Anti-rabbit-DyLight680	Goat	1:10'000	Rockland	611-144-002
Anti-rabbit-DyLight800	Donkey	1:10'000	Rockland	611-745-127
Anti-guineapig-DyLight680	Goat	1:10'000	Rockland	606-144-129
Anti-goat-IRDye700	Rabbit	1:10'000	Rockland	605-430-002
Anti-chicken IgG Alexa 488 (IF)	Goat	1:1000	Invitrogen	A11039
Anti-mouse IgG Alexa 568 (IF)	Goat	1:1000	Invitrogen	A11031
Anti-rabbit IgG Alexa 633 (IF)	Goat	1:1000	Invitrogen	A21070
Anti-guineea pig IgG Alexa 568 (IF)	Goat	1:1000	Mol. Probes	A11075

IV-8 FLOW CYTOMETRY

Schneider S2/S2R+ cells were pretreated with rapamycin (10 uM) for 30min, before insulin or Egf growth factor (10 ug/ml) were added to the preexisting cultures. Following overnight incubation, the cells were stained with Hoechst 33342 and analysed by flow cytometry. Cell size measurements were based on forward scatter FCS values. FACS analysis was performed with BD FACS Canto II. The DAPI channel was chosen. 10000 events were acquired per sample at a slow acquisition speed. SSC-A/FSC-A, DAPI-W/DAPI-A and DAPI-A/FSC-A displays were set to gate single cell population sequentially. The histogram of FSC-A was used to show the cell size population. The histogram of DAPI-A provided a cell cycle profile.

Preparation of Cell Samples

(1) Cell seeding and inhibitor/growth factor treatment as described in section IV-6.
(2) Overnight incubation.
(3) Resuspended cells from each well by pipetting, transferred to falcons and placed on ice.
(4) Pelleted the cells by centrifugation at 800 rpm for 10min at 4C
(5) Discarded supernatant and resuspended cells in 1 ml medium without serum
(6) Added 10 µl Hoechst 33342 (0,5 mg/ml)
(7) Incubated 20min in the dark
(8) Pelleted cells by centrifugation at 800 rpm for 10min at 4C
(9) Resuspended cells in 1ml medium without serum and transferred to FACS tubes
(10) Analysed samples with the BD FACSCanto II flow cytometer

(3*) When working with adherent cells, proper detachment requires trypsinization
For a well grown T25 flask/dish of cells, add 2 ml trypsin solution
Incubate 3-5 min at RT
Inactivate trypsin by adding 5 ml of complete medium
Use a 5 ml pipette for washing the remaining cells from the walls of the flask
Transfer to a 15 ml falcon tube

FlowJo Analysis

> Basic gating of populations of interest and associated histograms can be generated in the DIVA software. However, for more comprehensive processing of the FACS data, experiments

were exported as fcs3 files and collated into groups, such that gates and statistics can be defined interactively and shared among members of each group.

> The common workflow for FlowJo data analysis includes: 1 » Load sample data files into a workspace. 2 » Organise sample data into experimental groups. 3 » View one prototype sample to define gates and statistics. 4 » Copy the gates and statistics to a group. 5 » Verify the analyses on all samples in s group. 6 » Layout multiple plots for all samples in a group. 7 » Generate a graphical report in the layout editor and table of statistics in the table editor.

> Using curve-fitting algorithms, FlowJo fits the DNA histogram into mathematical distributions, representing the populations of cells in each of the phases. Fitting parameters can be constrained and the percentage of cells in G1/G0, S, G2/M, E1, E2 are automatically calculated by applying the Cell Cycle Analysis platform to the gated populations.

> FlowJo fits the cell cycle data using one of two mathematical models: the Watson Pragmatic model or the Dean-Jett-Fox model. Both of them were used to define individual cell cycle phases. Both models fit the G1 and G2 with Gaussian curves. For the S-phase distribution, the Watson model subtracts the G0/G1 and G2/M portions of the data and builds a function that fits what remains, whereas the DJF model fits the S-phase with a second-degree polynomial.

> For cell cycle analysis, the common procedure includes: Launch the Cell Cycle platform. 1 » Choose a model to fit the data. 2 » Adjust the model; the fit of the model can be assessed by comparing the model to the DNA histogram and by a low Root Mean Squared (RMS) score. If the model does not fit, you can either change the model or constrain one of the model parameters to assist fitting. 3 » Apply the cell cycle node to other samples. 4 » Generate a graphical report in the Layout Editor and a statistics report in the Table Editor.

> For each experiment, control samples were first analysed, and constraining ranges for the peaks were initially set based on those samples. Also, the CV values (coefficients of variance) for the G1 and G2 peaks were generally set to equal values to minimise cytometer's measurement errors.

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sebastian-florescu.myportfolio.com