The PP2C phosphatase Alphabet is a general negative regulator of MAPK signaling in Drosophila

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Abstract

The Mitogen-Activated Protein Kinase (MAPK) pathways are evolutionarily conserved signaling units implicated in the transmission of extracellular cues to intracellular compartments. The ERK, JNK and p38 pathways are the best described MAPK cascades. Their function relies on a core module of three kinases, composed of a MAPK Kinase Kinase (MAPKKK), a MAPK Kinase (MAPKK) and a MAPK, which becomes sequentially phosphorylated upon mitogenic, proinflammatory or stress signals. These modules are used in various developmental contexts and are implicated in the maintenance of homeostasis in adult organisms. Although most of the core components of MAPK signaling pathways have been discovered, the molecular mechanisms that control signaling strength, duration, location and termination remain poorly characterized. Using a genetic screening approach in Drosophila, our research group has identified several new loci potentially implicated in the regulation of ERK/MAPK signaling. Therefore, my doctoral research has focused on the characterization of one of these loci, that we renamed *alphabet* (*alph*). The *alph* locus encodes a functional Ser/Thr phosphatase, which is highly homologous to mammalian PP2Cα/β. In a first set of experiments, I demonstrated that Alph phosphatase activity was required for the negative regulation of ERK/MAPK signaling at a step downstream of the small GTPase Ras and possibly upstream of ERK/MAPK during Drosophila development. In yeasts, plants and mammals, PP2C phosphatases are

implicated in the downregulation of JNK and p38 Stress-Activated Protein Kinase (SAPK) pathways. Accordingly, I showed that Alph activity was also implicated in the inhibition of SAPK signaling at a step upstream of the MAPKKs Hemipterous and Licorne and potentially downstream of the small GTPase Rac1. In addition, biochemical evidence suggests that MAPKKKs such as Slipper/MLK, dDLK and Tak1 are candidate substrates for Alph. Finally, transcriptional profiling in *alph* mutant flies predicts resistance to environmental stresses and bacterial challenge, two biological responses regulated by SAPK pathways. Taken together, these results demonstrate the general inhibitory role of Alph on MAPK signaling in Drosophila.

Résumé

Les voies de signalisation de type MAPK ont été hautement conservées au cours de l'évolution et sont principalement impliquées dans la transmission de signaux extracellulaires vers les compartiments intracellulaires. Les voies ERK, JNK et p38 sont les cascades de type MAPK les mieux décrites et reposent sur l'activation d'un module de trois kinases par phosphorylation séquentielle. En effet, lorsqu'un signal mitogénique, proinflammatoire ou de stress est perçu par la cellule, une MAPK Kinase Kinase (MAPKKK) phosphorylera une MAPK kinase (MAPKK) qui phosphorylera ensuite une MAPK. Ces modules sont utilisés dans une multitude de contextes développementaux ainsi que pour le maintien de l'homéostasie chez les organismes adultes. Bien que la majorité des constituants de base des modules MAPK soient connus, nous possédons peu d'information concernant les mécanismes moléculaires impliqués dans le contrôle de la force, la durée, la localisation et la terminaison du signal. Par le biais de cribles génétiques chez la Drosophile, notre équipe a identifié plusieurs nouveaux loci potentiellement impliqués dans la régulation de la voie de signalisation ERK/MAPK. Par conséquent, l'objectif principal de mes recherches doctorales a porté sur la caractérisation d'un de ces nouveaux loci, que nous avons renommé *alphabet* (*alph*). Le gene *alph* encode une Ser/Thr phosphatase ayant une forte homologie de séquence avec PP2Cα/β de mammifère. Dans un premier temps, j'ai démontré que l'activité

phosphatase d'Alph était requise pour l'inhibition de la voie ERK/MAPK en aval de Ras et possiblement en amont de ERK/MAPK. Chez les levures, les plantes et les mammifères, les phosphatases de type PP2C sont principalement impliquées dans l'inactivation des voies JNK et p38. De façon similaire, j'ai démontré dans un deuxième temps qu'Alph agit aussi comme régulateur négatif de ces deux voies chez la Drosophile, possiblement en aval de Rac1 et en amont des MAPKKs Hemipterous et Licorne. De plus, des évidences biochimiques suggèrent que l'activité négative d'Alph se situe au niveau des MAPKKKs Slipper/MLK, dDLK et Tak1. Finalement, le profil d'expression génique des mouches mutantes pour le gène *alph* prédit que ces dernières devraient être résistantes à des stress environnementaux et à l'infection bactérienne, deux réponses biologiques régulées par les voies JNK et p38. En conclusion, l'ensemble de ces résultats démontre la fonction inhibitrice générale de la phosphatase Alph au niveau des modules MAPK.

Acknowledgement

A few years ago, when I was looking for a lab to start a Ph.D degree, my only concern was to find a place where I could learn how to study signal transduction. I met a few researchers, but one looked particularly motivated, dedicated and concerned about the success of his students. However, he was working with flies…

I was not particularly interested in fly genetics but this researcher was very convincing… Today, I would like to express my gratitude to this researcher, Marc Therrien, for first convincing me to come in his lab. In addition, I thank him for providing me with an exceptional environment to study signal transduction. Finally, and most importantly, I am grateful to him for showing me how model organisms such as Drosophila can be used to quickly answer scientific questions. If I would be given the chance to go back in time and choose a lab to start a Ph.D, I would do the exact same choice.

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…Louis est finalement né le 14 décembre à 20h58, juste avant mon dépôt de thèse…

Publications arising from work of thesis

1) Alphabet, a Ser/Thr phosphatase of the protein phosphatase 2C family, negatively regulates Ras/MAPK signaling in Drosophila.
Baril C., Therrien M.
Dev Biol. 2006 Jun 1;294(1):232-45.

This publication reports the identification and characterization of a new negative regulator of the Ras/MAPK pathway in Drosophila, namely Alphabet. The work presented in this chapter is essentially my own. Marc Therrien did the mapping of the *alph* locus and the sequencing of *alph* predicted exons of the four alleles recovered in the screen. I wrote the first draft of this manuscript.

2) Alphabet is a negative regulator of stress signaling in Drosophila
Baril C., Sahmi M., Stronach B. and Therrien M.
Manuscript in preparation.

This manuscript reports the negative function of Alph in the SAPK pathways. The work presented in this chapter is essentially my own. Genetic analyses in Figures 1B and 2 were done by Dr Beth Stronach. Immunoblot analyses in Figures 4A and B were done by Dr Malha Sahmi with my assistance. I wrote this manuscript.

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List of abbreviations

Alph	Alphabet
AMP	Antimicrobial Peptide
ASK1	Apoptosis Signal-Regulating Kinase 1
Asp	Aspartate
ATP	Adenosine Triphosphate
BDGP	Berkeley Drosophila Genome Project
Ben	Bendless
BMP	Bone Morphogenetic Protein
Boss	Bride of Sevenless
BSA	Bovine Serum Albumin
Bsk	Basket
CA	Constitutively activated
CA1-5	Conserved Area 1-5
cAMP	Cyclic Adenosine Monophosphate
CBP	Calmodulin Binding Peptide
CC	Coiled-coil
CD domain	Common Docking domain
Cdc25	Cell Division Cycle 25
Cdc37	Cell Division Cycle 37
Cdc42	Cell Division Cycle 42
CDK2/6	Cyclin-Dependent Kinase 2/6
CDMC	Canadian Drosophila Microarray Center

cDNA	Complementary DNA
CKA	Connector of Kinase to AP-1
CNA	Calcineurin A
CNB	Calcineurin B
CNK	Connector Enhancer of KSR
CRIC	Conserved Region in CNK
CRKII	Chicken Retroviral kinase II
c-TAK1	Cdc25C-associated Kinase 1
Cys	Cysteine
Da	Dalton
DA	Dopaminergic
DD	Death Domain
DER	Drosophila EGF receptor
dFADD	FAS-associated Death Domain
Dif	Dorsal-related Imunity Factor
DLK	Dual Leucine Zipper Kinase
DNA	Deoxyribonucleic Acid
Dok	Downstream of kinase
DOS	Daughter of Sevenless
Dpp	Decapentaplegic
Dredd	Death related ced-3/Nedd2-like protein
DRK	Downstream of Receptor Kinases
DSHB	Developmental Studies Hybridoma Bank
DSP	Dual Specificity Phosphatase

EAD	Eye-Antennal imaginal Disc
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
ERBB2	Erythroblastic leukemia viral oncogene homolog 2
ERK	Extracellular signal-Regulated Kinase
ETS	E26-Specific
EYA	Eyes Absent
FAF1	Fas-associating Factor 1
FBS	Fetal Bovine Serum
FC	Fold change
FEM-2	Feminization of XX and XO animals
FLP	Flippase
FRT	FLP recognition target
GAB	Grb2-associated Binder
Gap1	GTPase-activating Protein 1
GDP	Guanosine Diphosphate
GEF	Guanine Exchange Factor
GFP	Green Fluorescent Protein
GMR	Glass Multimer Reporter
Grb2	Growth actor Receptor-Bound protein 2
GST	Glutathione-S-Transferase
GTP	Guanosine Triphosphate
H_2O_2	Hydrogen peroxide
Hep	Hemipterous

HePTP	hematopoietic PTP
HOG	High Osmolarity Glycerol
Hog1	High Osmolarity Glycerol 1
HSP	Heat shock protein
Hyp	Hyphen
IKK	IκB kinase
IL1/2	Interleukin 1/2
IMD	Immune deficiency
IMP	Impede Mitogenic Propagation
INDAC	International Drosophila Array Consortium
IQGAP1	Isoleucine/Glutamine GTPase activating ptotein 1
ird5	immune response deficient 5
JAK	Janus Kinase
JBD	JNK binding domain
JIP	JNK-Interacting Protein
JLP	JNK-associated Leucine zipper Protein
JNK	c-Jun N-terminal Kinase
Jsap1	JNK/SAPK-Associated Protein 1
KDN	KSR Dominant Negative
KO	Knock-out
KSR	Kinase Suppressor of Ras
LAR	Leucocyte common Antigen-Related
Lic	Licorne
LMW-PTP	Low Molecular Weight PTP

L-o-f	Loss of function
LPS	Lipopolysaccharide
LRRK2	Leucine-Rich Repeat Kinase 2
Lys (K)	Lysine
LZK	Leucine Zipper Kinase
MAPK	Mitogen-Activated Protein Kinase
MAPKK (MKK)	Mitogen-Activated Protein Kinase Kinase
MAPKKK	Mitogen-Activated Protein Kinase Kinase Kinase
Mbc	Myoblast City
MEF	Mouse Embryonic Fibroblast
MEK	MAP kinase/ ERK Kinase
MEKK	MEK Kinase
MF	Morphogenetic Furrow
MKP	MAPK Phosphatase
MLK	Mixed-Lineage Kinase
Mmp1	Matrix Metalloproteinase 1
MORG1	MAPK Organizer-1
MP1	MEK partner-1
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Mass Spectrometry
Msn	Misshapen
Mts	Microtubule Star
Nbp2	Nap1 Binding Protein
NFκB	Nuclear Factor κB

NIK	Nck-Interacting Kinase
ORF	Open Reading Frame
OSBP	Oxysterol-Binding Protein
OSM	Osmosensing scaffold for MEKK3
p(dp)MAPK	diphosphorylated form of ERK/MAPK
PAK	p21-activated Kinase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Parkinson disease
PDHc	Pyruvate dehydrogenase complex
PDK	Pyruvate Dehydrogenase Kinase
PDP	Pyruvate Dehydogenase Phosphatase
PDPc	Pyruvate Dehydogenase Phosphatase catalytic subunit
PDPr	Pyruvate Dehydogenase Phosphatase regulatory subunit
PDZ	PSD-95/DLG-1/ZO-1
PGRP	Peptidoglycan Recognition Protein
PH	Pleckstrin Homology
PHD	Plant Homeodomain
Phyl	Phyllopod
PI3K	Phosphoinositide-3 kinase
PINK	PTEN-induced kinase 1
pJNK	diphosphorylated form of JNK
PKA	Protein Kinase A

PKC	Protein Kinase C
Pnr-Gal4	pannier-GAL4
POPX1/2	Partner of PIX 1/2
POSH	Plenty of SH3s
PP2C	Protein Phosphatase 2C
pp38	diphosphorylated form of p38
PpD3	Protein Phosphatase D3
PPM	Phosphoprotein Phosphatase Mg^{2+}-Dependent
PPM1D	Protein Phosphatase Magnesium-dependent 1 Delta
PPP	Phosphoprotein Phosphatase
Ppt1	Protein Phosphatase T 1
PR	Photoreceptor
ProtA	Protein A
PSTP	Protein Serine/Threonine Phosphatase
Ptc1/2/3	Phosphatase type 2C 1/2/3
PTEN	Phosphatase and Tensin homolog deleted on chromosome Ten
PTP	Protein Tyrosine Phosphatase
PTP1B	Protein Tyrosine Phosphatase 1B
Ptp2/3	Protein Tyrosine Phosphatase 2/3
PTP-ER	PTP- Enhancer of Ras
PTP-SL	STEP-like PTP
pTyr	Phospho-Tyrosine
Puc	Puckered

Pyo	Polyoma
Raf	Rapidly growing Fibrosarcoma
Ras	Rat Sarcoma
RdgC	Retinal DeGeneration C
Rel	Relish
Rho	Ras Homolog
RIP1	Receptor Interacting Protein 1
RIR	Raf-Inhibitory Region
RNAi	Ribonucleic Acid Interference
ROS	Reactive Oxygen Species
RSK	p90 Ribosomal S6 Kinase
RTK	Receptor Tyrosine Kinase
S2	Schneider's Drosophila Line 2
SAM	Sterile α motif
SAPK	Stress-activated Protein Kinase
SAPKK	SAPK Kinase
SAPKKK	SAPK Kinase Kinase
SCF	Skp1/Cullin/F-box protein
Sef	Similar expression to FGF
SEM	Sevenmaker
SEM	Standard Error of the Mean
Ser	Serine
Sev	Sevenless receptor
SH2	Src-Homology 2

SH3	Src-Homology 3
SK3-1	Suppressor of KDN 3-1
SkpA	S-phase kinase-associated protein 1A
Slpr	Slipper
Smad	Homologs of both the drosophila protein, mothers against decapentaplegic (MAD) and the C. elegans protein SMA. The name is a combination of the two.
SOD	Superoxide Dismutase
Sos	Son Of Sevenless
SPAG9	Sperm-associated Antigen 9
Spry	Sprouty
Src	Rous Sarcoma virus
STAT	Signal Transducers and Activators of Transcription
STE	Homologs of yeast sterile 7, sterile 11 and sterile 20 kinases
STEP	Striatal-Enriched PTP
SUR-8	Suppressor of Let-60 ras
TAB1/2/3	Tak1 binding protein 2
TAE	Tris-acetate-EDTA
Tak1	TGFβ-activated Kinase 1
TAP	Tandem Affinity Purification
TC	Thorax Closure
TCA	Trichloroacetic Acid
TGFβ	Tumor Growth Factor β

Thr	Threonine
TKL	Tyrosine Kinase-Like
TNFα	Tumor Necrosis Factor α
TotA/C	Turandot A/C
Tpl-2	Tumour Progression Locus-2
TPR	Tetratricopeptide Repeat
TRAF	TNF Receptor-associated Factor
Tyr	Tyrosine
UAS	Upstream Activation Sequence
Ub	Ubiquitin
ubc13	Ubiquitin-Conjugating Enzyme 13
UCH-L1	Ubiquitin Carboxyl-terminal Hydrolase L1
UeV1a	Ubiquitin-conjugating Enzyme E2 Variant 1
UTR	Untranslated Region
UV	Ultraviolet
Wip1	Wild-type p53-Inducible Phosphatase 1
Wnd	Wallenda
WT	Wild-Type

Chapter 1: Literature review

1.1 Preface

In the early 70's, Dr. Martin Rodbell first introduced the principle of signal transduction. He believed that cells were cybernetic systems made up of three distinct molecular components: discriminators, transducers, and amplifiers. The discriminator, or cell receptor, receives information from outside the cell; a cell transducer processes this information across the cell membrane and the amplifier intensifies these signals to initiate reactions within the cell or to transmit information to other cells. In an elegant set of experiments, he showed that the binding of glucagon to membrane receptors induced the production of GTP, which stimulated the activity of a guanine nucleotide protein. In the language of signal transduction, the guanine nucleotide protein, activated by GTP, was the principal component of the transducer, which was the crucial link between the discriminator and the amplifier (Rodbell, 1980). Alfred G. Gilman would then define the biochemical nature of the guanine nucleotide protein, which is now best known as G-protein (Gilman, 1987). Their cooperative efforts "for their discovery of G-proteins and the role of these proteins in signal transduction in cells" was rewarded in 1994 when they both received the Nobel Prize in Physiology or Medecine (Marx, 1994). Their pioneering work ultimately led to the discovery of a range of other signal transduction pathways, which are all implicated in crucial developmental processes and maintenance of cellular homeostasis in adult organisms.

Although most of the core components of signaling pathways have been discovered, we are still dealing with an enormous problem: activation of a given signaling pathway generates multiple cellular outputs. Therefore, how is specificity achieved? Although researchers have started looking for mechanisms implicated in pathway specificity, much of the work remains to be done. While the discovery of signal transduction was the concern of the 20th century, the elucidation of mechanisms that dictate specificity within signaling pathways will probably be the issue of the 21st century.

The laboratory in which I spent the last years as a Ph.D student is studying molecular mechanisms regulating the Mitogen-Activated Protein Kinase (MAPK) signaling pathways using *Drosophila melanogaster* (Drosophila) as a model system. The main objective of my Ph.D was to characterize the function of a new negative regulator of MAPK signaling using biochemical and genetic tools in Drosophila. Therefore, in the first part of the literature review, I summarize the actual knowledge concerning the basic core module of MAPK pathways in mammals as well as their physiological importance. In addition, I describe emerging molecular mechanisms implicated in the regulation of MAPK pathways. In the second part of the literature review, I explain how Drosophila can be used as a model system to decipher molecular aspects of the MAPK pathways. Finally, in the last part, I describe the rationale and aims of my Ph.D project.

1.2 Mammalian MAPK signaling pathways

The Mitogen-Activated Protein Kinase (MAPK) signaling pathways are conserved in all eukaryotes. In mammals, at least four families of MAPK have been characterized: Extracellular signal-Regulated Kinases 1 and 2 (ERK1/2), c-Jun N-terminal Kinase (JNK), p38 MAPK and ERK5 (Qi and Elion, 2005). Their activity relies on a basic core of three kinases consisting of a MAPK, a MAPK activator (MAPKK) and a MAPKK activator (MAPKKK), which are involved in a phosphorelay (Chapter 1, Figure 1). Upon activation by extracellular stimuli, diverse mechanisms such as protein-protein interactions, post-translational modifications and subcellular relocalization increase MAPKKKs' catalytic activity. Once catalytically active, these Ser/Thr kinases will phosphorylate MAPKKs on two Ser residues in their activation segment, thereby generating an active MAPKK. The core module contains a closing component, MAPK, which requires phosphorylation of its TXY motif by MAPKKs in order to be converted into an active kinase (Schaeffer and Weber, 1999).

MAPKs are proline-directed Ser/Thr kinases, which phosphorylate a panel of cytosolic proteins as well as transcription factors. MAPKs interact with their activators, substrates and inactivators, via a conserved region called the common docking (CD) domain. This domain features a stretch

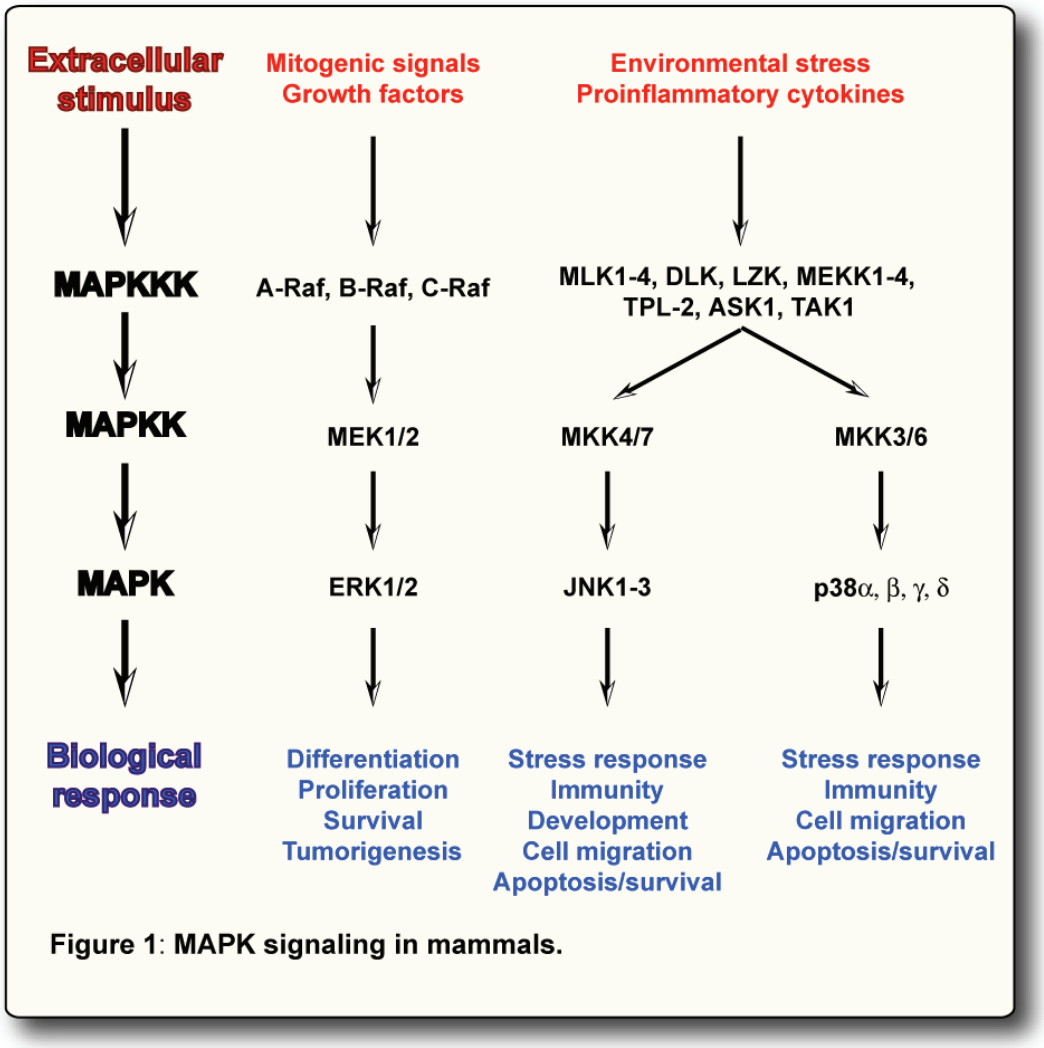

Figure 1: **MAPK signaling in mammals.**

of negatively charged amino acid opposing the active centre and is located in the C-terminal portion of the protein. However, the CD domain does not determine the specificity of docking interaction. The ED site, another motif near the CD domain constituted of negatively charged amino acids, appears to mediate the docking specificity to a specific MAPK (Tanoue and Nishida, 2003). Two docking sites for MAPKs on MAPK-interacting molecules have been described: the D-domain and the FXFP motif. The D-domain contains a cluster of positively charged amino acids surrounded

by hydrophobic amino acids and is located outside the catalytic domain of the MAPK-interacting molecule such as MAPKKs and MAPK Phosphatases (MKPs). The conserved FXFP motif is more commonly found on downstream transcriptional targets of MAPKs and is also required for efficient phosphorylation by MAPKs (Tanoue and Nishida, 2002).

MAPK modules, via activation of specific target proteins, are involved in cellular processes such as proliferation, stress response and cell death, and in multi-cellular processes, including differentiation, development, learning, memory and immune functions (Ashwell, 2006; Davis, 2000; Krens et al., 2006; Sweatt, 2004; Wada and Penninger, 2004). In addition, abnormal activity of various MAPKs is associated with inflammation, cancer and degenerative diseases (Kohno and Pouyssegur, 2006; Manning and Davis, 2003; Reddy et al., 2003).

1.2.1 The Extracellular signal-Regulated Kinase pathway

1.2.1.1 ERK module

The Extracellular signal-Regulated Kinase (ERK) pathway is mainly involved in intracellular transmission of growth and mitogenic stimuli integrated by Receptor Tyrosine Kinases (RTKs) (Chapter 1, Figure 1 and (Lewis et al., 1998)). The first step leading to full activation of the ERK

signaling cassette involves the MAPKKK Raf. Three Raf isoforms exist in mammals: A-Raf, B-Raf and C-Raf (Raf-1). The series of events leading to Raf activation are complex and still incompletely understood. They involve protein-protein interactions, phosphorylation/ dephosphorylation events, conformational changes and subcellular relocalization. Much of the work regarding Raf activation was done using the C-Raf isoform. A simplified scenario for C-Raf activation includes the following sequence of events. In quiescent cells, C-Raf adopts an autoinhibitory conformation whereby the N-terminal regulatory half binds to the catalytic domain and inhibits its activity. Following mitogenic signals, the small GTPase Ras becomes GTP loaded and binds the Ras Binding Domain in the N-terminal portion of C-Raf. Since Ras is a membrane bound protein, C-Raf relocalizes to the plasma membrane. Although membrane relocalization of C-Raf is essential, it is not sufficient for full activation of the kinase. Indeed, dephosphorylation/phosphorylation events occur on C-Raf at the plasma membrane, which result in conformational changes of the protein and generate a fully active kinase (for details on Raf activation, see (Rapp et al., 2006; Wellbrock et al., 2004)). Activated C-Raf then binds to and phosphorylates the dual specificity kinases MEK1 and 2, which in turn phosphorylate ERK1/2 within a conserved TEY motif in their activation loop (Rubinfeld and Seger, 2005). Activated ERK1/2 have numerous substrates (~160), which are located in all compartments of the cell (Yoon and Seger, 2006).

1.2.1.2 ERK physiological roles

Studies using various model organisms and cellular assays showed that the Raf/MEK/ERK signaling pathway is involved in fundamental cellular processes such as cell cycle control, differentiation, survival and cell migration (Pearson et al., 2001; Widmann et al., 1999). In addition, aberrant Raf/MEK/ERK signaling has been reported in several tumours. For instance, ~15% of cancers bear oncogenic mutations in Ras (Davies et al., 2002) and ERK is hyperactivated in nearly 30% of cancers (Hoshino et al., 1999). Recently, activating mutations in B-Raf were found in various human cancer cell lines, with predominance in melanoma tumours (Davies et al., 2002). These findings have prompted research on the discovery of new pharmacological inhibitors of Ras, Raf and MEK. However, none of the small molecule inhibitors synthesized until now has gone through phase II clinical trials with success (Kohno and Pouyssegur, 2006).

1.2.2 Stress-Activated Protein Kinases

1.2.2.1 SAPK modules

Stress-Activated Protein Kinase (SAPK) pathways comprise the JNK and p38 MAPK signaling modules. Environmental stresses (osmotic and heat shock, oxidative stress, UV irradiation, etc.) and inflammatory cytokines are extracellular stimuli responsible for activation of these pathways (Chapter 1, Figure 1 and (Kyriakis and Avruch, 1996)). JNK and

p38 signaling cascades also share a panel of MAPKKKs grouped in two families according to the classification of the human kinome (Manning et al., 2002): Tyrosine Kinase-Like (TKL) and Homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases (STE). The Mixed-Lineage Kinases group (MLK1 to 4), Dual-Leucine Zipper Kinases (DLK) and TGFβ-Activated Kinase 1 (Tak1) belong to the TKL family while Apoptosis Signal-Regulating Kinase 1 (ASK1), MEK kinases group (MEKK1 to 4), p21-Activated Kinases (PAK) and Tumour Progression Locus 2 (Tpl-2) are part of the STE group. In general, these Ser/Thr kinases are locked in an inactive conformation by homodimer formation, intramolecular interactions or inhibitory proteins. Generation of an active MLK, DLK, PAK or MEKK commonly requires the binding to a GTPase member of the Rho family such as Rac1 or Cdc42 as well as phosphorylation events (Fanger et al., 1997; Gallo and Johnson, 2002; Hagemann and Blank, 2001; Kumar et al., 2006). The proposed model for ASK1 activation involves its dissociation from the inhibitory protein thioredoxin and an autophosphorylation event on a Thr residue present in the activation loop (Hayakawa et al., 2006). For full activation, Tak1 requires the binding of two subunits TAB1 and TAB2 or TAB3, which by an unknown mechanism promote the phosphorylation of Tak1 (Chen et al., 2006b). In their active conformation, MAPKKKs of the SAPK pathways will phosphorylate the MAPKKs MKK3/6, which are upstream activators of p38 MAPK, or MKK4/7, which are specific for JNK. JNK and p38 become activated when

phosphorylated on the TPY or TGY motif in the activation loop respectively. They will then phosphorylate various cytoplasmic and nuclear proteins depending on the cellular context (Chen et al., 2001).

1.2.2.2 SAPK physiological roles

The p38 and JNK pathways have been implicated in numerous physiological processes: stress and immune response, cell migration, differentiation, development, apoptosis, survival, proliferation and tumorigenesis (Davis, 2000; Zarubin and Han, 2005). In this section, I briefly describe those functions that have been demonstrated in both cellular assays and whole organisms.

The SAPK pathways were first described as molecular sensors of environmental stresses in cell culture (Kyriakis and Avruch, 1996). These stressing agents include high osmolarity, radiation and changes in the cellular redox state. In some circumstances, stress-dependent activation of these pathways leads to cell death. However, in certain cases, activation promotes cell survival, probably due to transcriptional activation of detoxifying genes. It is possible that the cell type, the nature of the stressing agent, the duration of SAPK activation or the activity of parallel signaling pathways dictate the SAPK-dependent cellular outcome on apoptosis (Liu and Lin, 2005). Intriguingly, viable knock-out (KO) mice are available for the three JNK and four p38 genes, but no data exist yet on

their potential susceptibility to certain stresses (Kuida and Boucher, 2004). However, in other model organisms such as *D. melanogaster* (Craig et al., 2004; Wang et al., 2003a), *A. thaliana* (Mishra et al., 2006), yeasts (Gacto et al., 2003; Millar, 1999) and *C. elegans* (Oh et al., 2005), the SAPK pathways appear to be important for adaptation to environmental stresses.

Several lines of evidence implicate the SAPK pathways in cell migration and cell shape changes, which are processes known to trigger cytoskeleton reorganization. First, SAPK activation by various stimuli in a multitude of cell lines correlates with increased cell motility. Conversely, inhibition of SAPK signaling using small molecule inhibitors or dominant-negative SAPK impairs cell migration. Second, SAPK substrates include several cytoskeleton-associated proteins and signaling molecules (Huang et al., 2004). Although cellular assays demonstrated that both SAPK pathways are involved in cell migration, only the JNK signaling cascade was studied concerning this aspect of cellular behaviour at the organismal level. In multicellular organisms, movement of epithelia, which includes active cytoskeleton reorganization, is critical for effective completion of embryogenesis. Closure of the mouse neural tube and later of the eyelid involves movement of epithelial cells. In both cases, inactivation of the JNK pathway leads to defective cytoskeleton reorganization, inhibition of cell motility and embryonic defects (Xia and Karin, 2004).

The SAPK pathways are also key players in the cellular response to microbial infection. They both promote the production of inflammatory cytokines such as IL-2 and TNFα by activated immune cells. The use of JNK KO mice also demonstrated the importance of this pathway in appropriate activation of T-lymphocytes, while p38 KO mice had no abnormalities relative to immune function. However, *mkk3-/-* or *mkk6-/-* mice, which are also viable, have defects in T cells maturation, display low levels of TNFα-induced cytokine production by fibroblasts or are defective for T cell activation. The involvement of SAPK pathways in the activation of the immune response prompted research on the discovery of pharmacological inhibitors. For instance, the administration of SB203580 and SP600125, which inhibits certain isoforms of p38 and JNK respectively, had beneficial effects on animal models of inflammatory diseases (Ashwell, 2006; Manning and Davis, 2003).

1.3 Regulation and specificity within the MAPK modules

MAPK modules are reiteratively used throughout development and adult life to produce a variety of biological responses. The generation of a specific response probably implies that intrinsic differences exist in the intracellular signaling network or that cells interpret MAPK activation based on their distinct developmental histories (or both). In any case, cellular mechanisms must regulate the strength, the duration, the localization and the termination of MAPK signaling, which most likely

dictate specificity. Some of these mechanisms have started to emerge. This part describes three important ways for the regulation of the MAPK pathways, namely dephosphorylation, the use of scaffolding molecules and ubiquitination.

1.3.1 Regulation through dephosphorylation

Reversible phosphorylation was identified as a critical regulatory mechanism of glycogenolysis around the 1950's (Fischer and Krebs, 1955) and was subsequently demonstrated to have critical implications in many other biological systems (Cohen, 2002a). Phosphorylation is a chemical process in which a phosphate group is added to an organic molecule, for instance proteins, lipids or sugars. Phosphorylation on proteins occurs predominantly on serine (Ser), threonine (Thr) and tyrosine (Tyr) residues at a ratio of about 1000/100/1 (Raggiaschi et al., 2005). Protein phosphorylation is a reversible reaction that involves two classes of enzymes that constitute about 2-3% of all eukaryotic genes (Ensembl v39 and (Arena et al., 2005)): kinases and phosphatases. Kinases catalyse the transfer of a phosphate group from ATP to an acceptor amino acid side chain of a protein while phosphatases catalyze the hydrolysis of the phosphoester bond.

Phosphorylation, which is a key modification used in signal transduction, is often viewed as a molecular switch. Indeed, the addition of

a negatively charged phosphate group at a specific place on a protein can engender a conformational change that would either activate or inactivate the enzyme, depending on the cellular context. In addition, phosphorylation can promote or disrupt protein-protein interactions. It can also stabilize or promote degradation of a protein as well as facilitate or inhibit the movement of proteins to different subcellular compartments. However, this switch is more likely to be provided with a timer and a dimmer since it appears that the duration and magnitude of phosphorylation is a key issue in the regulation of signal transduction.

Through phosphorylation, the cell controls many important biological processes such as proliferation, differentiation, apoptosis, migration and metabolism. The delicate balance between kinase and phosphatase activity is thus of high importance to maintain cellular homeostasis. Any gene alteration that would affect this equilibrium would generally result in illness such as cancer, diabetes and neurodegenerative diseases. Activation of MAPK pathways occurs through multiple phosphorylation events and phosphatases are key modulators of signaling, most of the time by inhibiting signal propagation and sometimes by having the opposite effect.

1.3.1.1 Phosphatases in general

Protein phosphatases are essential components of cellular signaling networks and are conserved both in prokaryotes and eukaryotes. Protein phosphatases are divided in two categories based on substrate specificity: Protein Tyrosine Phosphatases (PTPs) and Protein Serine/Threonine Phosphatase (PSTPs). The catalytic mechanism by which the phosphate group is removed is different for PTPs and PSTPs. Crystallographic studies indeed revealed that PSTPs use a metal-bound water molecule as a nucleophile to attack the phosphorus atom on Ser/Thr residues while most PTPs utilize an internal Cys or Asp residue as a nucleophilic compound (Barford, 1996; Barford et al., 1994). One interesting feature of PTPs is that they possess a deeper catalytic cleft than PSTP, which can accommodate the phenol side chain of a Tyr residue. On the other hand, Ser or Thr side chains are too short to reach the base of the catalytic cleft. In addition to the variation in the structure and catalytic mechanism, PTPs and PSTPs also differ at the level of the structure of the holoenzyme: while PTPs are single polypeptide chains, most PSTPs are holoenzymes composed of a catalytic subunit and one or more regulatory subunits (Barford, 1996; Zhang, 1998). Regulation of PTPs appears to be in part orchestrated by specific domains or motif within the protein itself. For example, 79 of 107 PTPs of the human genome have an additional module such as an SH2, SH3, PH or PDZ domain (Alonso et al., 2004).

1.3.1.2 Protein Tyrosine Phosphatases

1.3.1.2.1 Classification

PTPs constitute approximately 70% of the human phosphatome (Arena et al., 2005). Sequence alignment of the catalytic domain of PTPs revealed four groups: Classical PTPs, Low Molecular Weight PTP (LMW-PTP), Cdc25 and Asp-based PTPs (For details on classification, see (Alonso et al., 2004; Kennelly, 2001)). Classical PTPs constitute the largest group and contain all PTPs that are strictly tyrosine-specific. This group can further be subdivided into the transmembrane PTPs and intracellular PTPs, which include LAR and CD45 as well as PTP-1B and STEP, respectively. This class also comprises the Dual Specificity Phosphatases (DSPs) that can dephosphorylate Ser/Thr/Tyr residues. Famous DSPs include the MAP Kinase phosphatases and PTEN. LMW-PTPs are ~20 kDa enzymes that appear to be required for cell growth regulation (Raugei et al., 2002). Cdc25 dual-specificity phosphatases are cell cycle regulators. Each Cdc25 isoform targets adjacent Thr and Tyr on specific Cyclin-Dependent Kinases (Honda et al., 1993). Finally, the Asp-based PTPs constitute a novel class of eukaryotic PTPs and include the Eyes absent (EYA) protein. Asp-based PTPs are believed to employ a nucleophilic Asp (instead of a Cys) for the hydrolysis of phosphate groups (Rebay et al., 2005).

1.3.1.2.2 General features of PTPs

1.3.1.2.2.1 Substrate trapping

One challenging objective has been to identify potential substrate(s) for a given phosphatase. With the engineering of the substrate trapping strategy, many targets of PTPs were discovered. Indeed, mutations of certain critical residues within the catalytic pocket of PTPs generate catalytically dead enzymes that are locked onto their substrates. The enzyme-substrate complex is sufficiently stable to be purified by standard methods (for review, see (Blanchetot et al., 2005). For example, mutating the nucleophilic Cys to Ser in many PTPs results in a substrate-trapping version of the protein (Furukawa et al., 1994; Jia et al., 1995; Sun et al., 1993). In addition, changing the Asp in the WPD loop, another critical residue involved in catalysis, generates an even more potent trap (Flint et al., 1997)

1.3.1.2.2.2 Sensitivity to oxidation

For many decades, phosphatases were seen essentially as non-regulated, constantly active proteins. This paradigm was strongly challenged when researchers discovered that inactivation of PTPs occurred in an oxidizing environment (Meng et al., 2002). Indeed, due to its microenvironment, the catalytic Cys of PTPs is highly susceptible to oxidation. Oxidation of this Cys abrogates its nucleophilic property and blocks the capacity of these enzymes to dephosphorylate their substrates

(den Hertog et al., 2005; Tonks, 2005). This modification of PTPs is reversible and appears to serve as a regulatory mechanism in many circumstances. For example, it has been reported recently that oxidation of MKPs is an important regulatory mechanism of TNFα-induced JNK activation. Indeed, TNFα induces the production of reactive oxygen species (ROS), which then lead to inactivation of MKPs and sustained JNK activation in specific cell lines (Kamata et al., 2005).

1.3.1.3 Protein Serine/Threonine Phosphatases

1.3.1.3.1 Classification

PSTPs represent roughly 30% of the human phosphatome (Arena et al., 2005). Based on amino acid sequence identity and biochemical properties, these enzymes can be grouped in two families: the PPP and PPM families (for details on the classification, see (Barford, 1996; Cohen, 1997; Kennelly, 2001)). Although these two families have disparate polypeptide sequences, their tertiary structure revealed great similarities in their active site, suggesting a common catalytic mechanism (Das et al., 1996).

1.3.1.3.2 PPP phosphatases

Eukaryotic PPPs are the most abundant Ser/Thr Phosphatases and are subdivided in four categories termed PPP1, PPP2A, PPP2B and

PPP5. The PPP1 family comprises the PP1, PPQ, PPY, PPZ and the yeast Glc7 enzymes. The best characterized member of this family is PP1. PP1 enzymes act as a heterodimer, constituted of a catalytic (PP1c) and a regulatory (PP1r) subunit. PP1c can form complexes with over 50 PP1r in a mutually exclusive manner. Although there is only four PP1c isoforms in mammalian cells, the dimerization with PP1r generates various holoenzymes that have distinct substrate specificity and subcellular location (Cohen, 2002b). The PPP2A family is composed of PP2A, PP4, PP6 and PPH. PP2A is by far the most characterized enzyme. The native form of PP2A exists as a heterotrimer composed of a conserved catalytic C subunit, a conserved scaffolding A subunit and a variable B subunit. The B subunits are grouped in three families each with multiple isoforms, such that approximately 50 combinations are possible. While the B subunits appear to modulate the catalytic activity of the C subunit and to contribute to substrate specificity, the A subunit is involved in coordinating the assembly of the PP2A holoenzymes and in recruiting additional proteins to the complex (Sontag, 2001). The diversity in PP1 and PP2A holoenzymes most probably explains the involvement of these enzymes in a variety of biological processes. In addition, various small molecule inhibitors of PP1 and PP2A have been discovered, among which microcystin and okadaic acid are the most famous (Cohen, 2002b; Sontag, 2001).

The PPP2B family is essentially composed of calcineurin, a Ca^{2+} and calmodulin activated enzyme with limited substrates compared to PP1

and PP2A. Purified calcineurin is a heterodimer composed of a catalytic subunit, calcineurin A (CNA), and a regulatory subunit, calcineurin B (CNB). In the absence of Ca^{2+}, CNA is maintained in an autoinhibitory form. Upon Ca^{2+} binding to CNB, a structural change occurs on CNA that permits the binding of calmodulin and the complete activation of the holoenzymes. Numerous functions have been assigned to calcineurin such as its involvement in the immune response, cardiac hypertrophy and regulation of ion channels in the brain. Interestingly, cyclosporine, which is a powerful immunosuppressant used to prevent graft rejection, is a specific inhibitor of calcineurin (Rusnak and Mertz, 2000).

Finally, the PPP5 family comprises the Drosophila (PpD3) and mammalian PP5, the yeast Ppt1 and the more distantly related Drosophila RdgC phosphatase. The PP5 phosphatases contain a catalytic, regulatory and targeting subunit in a single polypeptide chain. The regulation and targeting of PP5 are determined by the N-terminal tetratricopeptide repeat (TPR) domain. PP5 phosphatases possess a very low basal activity due to blockage of the catalytic site by the TPR domain. However, binding of arachidonic acid and HSP90 (and possibly other unknown molecules) to the TPR relieves the inhibition and leads to activation of the phosphatase (Chinkers, 2001). This family of phosphatases appears to regulate the cellular response to stress (Morita et al., 2001; Zhou et al., 2004) and glucocorticoids (Chen et al., 1996; Silverstein et al., 1997) as well as DNA repair (Zhang et al., 2005).

1.3.1.3.3 PPM phosphatases

The PPM family contains the Pyruvate Dehydrogenase Phosphatases (PDPs) and PP2C phosphatases. Pyruvate dehydrogenase (PDHc) is a 9-million Dalton (Da) complex composed of three repeated subunits (E1, E2, E3) involved in the catabolism of pyruvate into Acetyl-CoA. Acetyl-CoA can then enter the Krebs cycle and participate into the production of ATP. Together with glycolysis and oxidative phosphorylation, PDHc is a vital complex involved in cellular respiration (Fernie et al., 2004). Reversible phosphorylation is one way by which PDHc is regulated and involves two types of enzymes: Pyruvate Dehydrogenase Kinases (PDKs) and Pyruvate Dehydogenase Phosphatases (PDPs). PDK-dependent phosphorylation of three critical Ser residues on the E1 subunit inactivates the enzyme while PDPs are involved into the reactivation of the complex via dephosphorylation of these Ser. Mammalian PDPs are heterodimeric proteins containing a catalytic subunit (PDPc) and a regulatory subunit (PDPr). Enzymatic activity of PDPs relies on sufficient amount of Ca^{2+} and Mg^{2+} (Maj et al., 2006). Apparently, the function of PDPs may not be restricted to PDHc reactivation since it has recently been shown that such phosphatases also dephosphorylate Smad proteins and inactivate the BMP/DPP signaling pathway in both Drosophila and mammalian cells (Chen et al., 2006a).

In contrast to other Ser/Thr phosphatases, PP2Cs are monomeric enzymes for which relatively little is known about what governs the activity, localization and substrate specificity. PP2Cs' catalytic domain is about 300 residues in length and contains 11 subdomains in which 8 absolutely conserved residues reside. The crystal structure of human PP2Cα revealed that the 8 invariant residues of the catalytic domain are located near the two Mg^{2+} ions present in the catalytic site (Das et al., 1996). This confirmed previous results demonstrating that PP2Cs required metal ions for catalytic activity. PP2C phosphatases have been involved in many biological processes such as progression through the cell cycle, p53 regulation and stress response (Schweighofer et al., 2004) and will be discussed in more detail in the next section.

1.3.1.4 Phosphatases of the MAPK pathways

Compared to the multiplicity of kinases involved in the regulation of the MAPK pathways, very few phosphatases were found to reverse these phosphorylation events. In addition, even though nearly every component of the MAPK module is phosphorylated, most of the phosphatases reported to date act on the MAPKs. In the next section, I describe phosphatases implicated in the dephosphorylation of MAPK module (ERK/JNK/p38) components. A description of these phosphatases is shown in Table I (Chapter 1).

1.3.1.4.1 Dual Specificity Phosphatases (DSPs)

In mammals, there are 10 catalytically active classical DSPs and all of them have been shown to dephosphorylate and inactivate at least one of the classical MAPKs. These DSPs, also known as MAP Kinase Phosphatases (MKPs), remove phosphate on the Thr and Tyr residues of the TXY motif present in the activation loop of MAPKs by a mechanism similar to PTPs. However, in contrast to PTPs, the catalytic cleft of DSPs is shallower permitting the dephosphorylation of the Thr residue. It was reported that the binding of ERK2 to MKP3 increases the catalytic activity of the phosphatase by 40-fold, a phenomenon found later to be common to many DSPs. Interestingly, the level of MKPs is very low in unstimulated cells, but upon activation of MAPK pathways, there is an increase in the transcription of MKP genes, thus involving these phosphatases in a negative feedback loop (For a review on MKPs, see (Farooq and Zhou, 2004; Theodosiou and Ashworth, 2002)).

1.3.1.4.2 PTPs and PSTPs: alone or in combination

STEP, PTP-SL and HePTP have all been shown to specifically dephosphorylate the Tyr residue in the activation loop of ERK1/2 in vitro and in vivo (Pulido et al., 1998; Saxena et al., 1999). In addition, PTP-ER, a close homologue in Drosophila, has been genetically shown to act as a negative regulator of the ERK pathway. Inactivating mutations in this gene increase Ras-mediated signaling, resulting in extra R7 neurons in the

Table I. Phosphatases of the MAPK pathways*

Phosphatases	Classification	Targets	Functions	References
Hematopoietic PTP (HePTP)	PTP	Inactivation of ERK (and p38?)	Dephosphorylation of ERK on the Tyr residue in the activation loop	(Gronda et al., 2001; Pettiford and Herbst, 2000; Saxena et al., 1999; Saxena et al., 1998; Wang et al., 2003; Wang et al., 2005)
Striatal-Enriched PTP (STEP)	PTP	Inactivation of ERK	Same as HePTP	(Pulido et al., 1998)
PTP STEP-like (PTP-SL)	PTP	Inactivation of ERK	Same as HePTP	(Pulido et al., 1998)
PP2A	PSTP (PPP; PP2A)	Inactivation of ERK	Dephosphorylation of ERK on the Thr residue in the activation loop	(Wang et al., 2003; Wang et al., 2005)
		activation of KSR	Dephosphorylation of 14-3-3 binding sites on KSR	(Ory et al., 2003)
		Activation of RAF	Dephosphorylation of 14-3-3 binding sites on RAF-1	(Ory et al., 2003)
		Activation of RAF	Reactivation of RAF-1	(Dougherty et al., 2005)
		Inactivation of p38	Dephosphorylation of p38	(Alvarado-Kristensson and Andersson, 2005; Lee et al., 2003)
PP5	PSTP (PPP; PP5)	Inactivation of RAF	Dephosphorylation of Ser338 on RAF-1	(von Kriegsheim et al., 2006)
Wip1	PSTP (PPM; PP2C)	Inactivation of p38	Dephosphorylation of p38 on the Thr residue in the activation loop	{Harrison, 2004 #676}
PP2Cα	PSTP (PPM; PP2C)	Inactivation of p38/JNK	Dephosphorylation of p38/JNK on the Thr residue in the activation loop	(Harrison et al., 2004)
PP2Cβ	PSTP (PPM; PP2C)	Inactivation of TAK1	Dephosphorylation of TAK1	(Hanada et al., 2001; Li et al., 2003)
PP2Cε	PSTP (PPM; PP2C)	Inactivation of TAK1	Dephosphorylation of TAK1	(Li et al., 2003)
Partner of PIX 1/2 (POPX1/POPX2)	PSTP (PPM; PP2C)	Inactivation of PAK	Dephosphorylate Ser residues on PAK	(Koh et al., 2002)

*DSPs are not included in this table. For a description of MAPK DSPs, refer to Farooq and Zhou, 2004.

Drosophila eye (Karim and Rubin, 1999a). Interestingly, in Hela cells, a dual specificity phosphatase complex (DSP complex), constituted of HePTP and PP2A, appears to be required for dephosphorylating the Tyr and Thr residue of ERK2 respectively in the presence of cholesterol (Wang et al., 2003b). The formation of the DSP complex relies on the scaffolding molecule Oxysterol-Binding Protein (OSBP). In the presence of cholesterol, OSBP assembles the complex and leads to ERK2 dephosphorylation (Wang et al., 2005). In addition to their negative role within the MAPK pathways, PP2A phosphatases are also essential for growth factor activation of the ERK pathway via dephosphorylation of specific Ser residues on Raf-1 and KSR1 (Ory et al., 2003). Moreover, PP2A is involved in the reactivation of Raf-1 following inhibitory phosphorylations by ERK (Dougherty et al., 2005). Taken together, these results are in agreement with previous findings in Drosophila, showing that the catalytic subunit of PP2A has both positive and negative activity in the Ras/MAPK signaling pathway (Wassarman et al., 1996).

In various organisms, PP2C phosphatases have been shown to downregulate the p38 and JNK pathways alone or in combination with PTPs. The activation of the High Osmolarity Glycerol (HOG) pathway, which is one of the five MAPK pathways present in S. cerevisiae, is essential for osmoregulation. Similar to JNK and p38 in mammals, Hog1 MAPK becomes phosphorylated on the TXY motif in the activation loop under hyperosmotic stress conditions (Saito and Tatebayashi, 2004). Two

groups of phosphatases have been shown to inactivate specifically Hog1. The Tyr residue in the activation loop of Hog1 is targeted by the Ptp2 and Ptp3 Tyr phosphatases, while the Thr residue is dephosphorylated by the PP2C phosphatases Ptc1, Ptc2 and Ptc3. It has been demonstrated that while Ptc1 maintains low basal activity of Hog1, Ptc2 and Ptc3 are responsible for setting the maximal limit to which stress can activate Hog1. In addition, although Ptc2 and Ptc3 directly contact Hog1, Ptc1 appears to be recruited to the MAPK via the adaptor protein Nbp2. A similar scenario occurs in the yeast S. pombe, where heat shock-dependent activation of the Hog1 homolog Spc1 is downregulated by both PTPs and PP2C phosphatases (Martin et al., 2005).

In mammals, PP2C phosphatases act at different levels within the SAPK module. PP2Cα can dephosphorylate and inactivate p38, JNK and MKK3/6/4/7 after activation by environmental stresses. In addition, PP2Cβ and ε have been found to dephosphorylate the SAPKKK TAK1 after cytokine treatment. A pair of PP2C phosphatases, POPX1 and POPX2 have also been shown to dephosphorylate PAK, which mediates signaling downstream of Rho GTPases (Schweighofer et al., 2004; Tamura et al., 2006). Recent findings showed that the PPM1D gene, which encodes the Wip1 PP2C phosphatase, is frequently amplified in human breast tumors. In addition, *wip1*$^{-/-}$ mice are resistant to mammary tumorigenesis induced by ERB2 and Ras. Wip1 is a p53-induced phosphatase that selectively

dephosphorylates a Thr residue in the activation loop of p38 and mediates a negative feedback loop of the p38/p53 signaling pathway. It is postulated that Wip1 operates through p38 since pharmacological inhibition of this SAPK restores ERBB2/Ras-induced mammary tumors formation in *wip1*$^{-/-}$ mice (Harrison et al., 2004). These observations not only demonstrate the physiological relevance of PP2C phosphatase as regulators of SAPK in multicellular organisms but also provide justification for a search for PP2C chemical inhibitors as potential anticancer drugs (Belova et al., 2005).

1.3.2 Regulation through scaffolding molecules

The coordination of signaling pathways is in part mediated by scaffolding molecules, which are multidomain-containing proteins that serve as meeting platforms for enzymes and their substrates. Scaffolds promote or attenuate signaling in different ways (for review, see (Dard and Peter, 2006)):

1) They can promote enzyme/substrate interaction by bringing them in close vicinity or by concentrating them at a specific cellular location;
2) They can have an impact on pathway activation by spatial and temporal restriction of the enzyme/substrate complex;
3) They can orient or allosterically modify the enzyme so that it is catalytically more active;
4) They can recruit additional protein to the enzyme/substrate complex in order to modify the kinetics of the enzymatic reaction.

Table II. Scaffolds of the MAPK pathways

Scaffolds	Pathway	Binding partners	Functions	References
Kinase Suppressor of Ras (KSR)	ERK	RAF/MEK/ERK	RTK- dependent activation of ERK	(Nguyen et al., 2002; Roy et al., 2002; Therrien et al., 1996)
Connector-Enhancer of KSR (CNK)	ERK	RAF	RTK- dependent activation of ERK	(Douziech et al., 2003; Therrien et al., 1998; Ziogas et al., 2005)
	JNK	RhoGEFS/MLK2/MKK7	Rho-dependent activation of JNK	(Jaffe et al., 2005)
MEK partner-1 (MP1)	ERK	MEK1/ERK1	Endosomal activation of ERK	(Schaeffer et al., 1998; Teis et al., 2002; Wunderlich et al., 2001)
MAPK Organizer-1 (MORG1)	ERK	RAF/MEK/ERK	Serum, LPS and phorbol ester activation (Endosomal?) of ERK	(Vomastek et al., 2004)
MEKK1	ERK	RAF-1/MEK1/ERK2	Activation, compartmentalization, proteasomal degradation	(Karandikar et al., 2000)
Paxillin	ERK	RAF-1/MEK/ERK	HGF-dependant activation of ERK/ lamellipodia formation	(Ishibe et al., 2003)
Suppressor of Let-60 ras (SUR-8)	ERK	RAS/RAF	EGF-dependant activation of ERK	(Li et al., 2000; Sieburth et al., 1998)
Similar expression to FGF (Sef)	ERK	MEK/ERK	Inhibition/prevents nuclear localization of ERK	(Torii et al., 2004)
Oxysterol-binding protein (OSBP)	ERK	HePTP/PP2A	Cholesterol-dependent inhibition of ERK	(Wang et al., 2005)
Isoleucine/Glutamine GTPase activating ptotein 1 (IQGAP1)	ERK	MEK/ERK	EGF activation of ERK	(Roy et al., 2004; Roy et al., 2005)
β-arrestin-1	ERK	RAF-1/MEK1/ERK2	GPCR activation of ERK and cytoplasmic retention	(DeFea et al., 2000)
β-arrestin-2	ERK	RAF-1/MEK1/ERK3	GPCR activation of ERK and cytoplasmic retention	(Luttrell et al., 2001)
	JNK	ASK1/MKK4/JNK3	GPCR activation of JNK3	(McDonald et al., 2000)
Chicken Retroviral kinase II (CRKII)	JNK	HPK1/MKK4/JNK	RAC1-dependent activation of JNK	(Girardin and Yaniv, 2001)
JNK interacting protein 1 (JIP1)	JNK	MLK2&3/DLK/MKK7/JNK1&2/MKP7	JNK activation/inhibition and kinesin cargo	(Dickens et al., 1997; Verhey et al., 2001; Whitmarsh et al., 1998; Willoughby et al., 2003; Yasuda et al., 1999)
JIP2	JNK	MLK2&3/DLK/MKK7/JNK1&3/MKP7	JNK activation/inhibition and kinesin cargo	(Verhey et al., 2001; Whitmarsh et al., 1998; Willoughby et al., 2003; Yasuda et al., 1999)
	p38	MKK3/p38	p38 activation and kinesin cargo	(Buchsbaum et al., 2002; Schoorlemmer and Goldfarb, 2002; Verhey et al., 2001)
JIP3	JNK	MEKK1/MLK3/MKK7/JNK	LPS-dependent activation of JNK and kinesin cargo	(Kelkar et al., 2000; Matsuguchi et al., 2003; Verhey et al., 2001)
JIP4	p38	ASK1/MKK3&6/p38	p38 activation and kinesin cargo	(Kelkar et al., 2005)
JNK-associated leucine zipper protein (JLP)	JNK	MEKK3/MKK4/JNK	JNK activation and kinesin cargo	(Lee et al., 2002)
Osmosensing scaffold for MEKK3 (OSM)	p38	RAC1/MEKK3/MKK3	Osmotic shock-dependent activation of p38	(Uhlik et al., 2003)
Plenty of SH3 (POSH)	JNK	RAC1/MLK/MKK4&7/JNK	RAC1-dependent activation of JNK	(Xu and Greene, 2006)

The ability of scaffolds to restrict signaling provides specificity and significantly influences signal output. At least 20 molecular scaffolds are implicated in the regulation of MAPK pathways in higher eukaryotes (see Chapter 1, Table II). The next section describes the function of three important MAPK scaffolds, namely KSR, CNK and JIP.

1.3.2.1 Kinase Suppressor of Ras

Kinase Suppressor of Ras (KSR) was originally identified as a positive regulator of the Ras/MAPK signaling pathway by genetic means in Drosophila and C. elegans (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995). Amino acid sequence comparison showed that KSR is conserved in mammals and has high sequence homology with Raf (Therrien et al., 1995). KSR proteins generally contain five conserved areas (CA1 to CA5) (Therrien et al., 1995). From N-terminal to C-terminal, KSR contains a stretch of 40 amino acids unique to KSR (CA1), a proline-rich region that may serve as an SH3 domain binding site (CA2), a cysteine-rich region (CA3) and an area rich in Ser and Thr, which also contains a MAPK consensus binding site (CA4). Finally, in its most C-terminal part, KSR contains a putative kinase domain (CA5), which led to initial speculation that KSR was a functional kinase. However, among the three signature motifs required for proper kinase activity (VAIK in subdomain III, HRD in subdomain VIb and DFG in subdomain VII; (Boudeau et al., 2006)), only the DFG motif is preserved in all KSR

orthologs (Therrien et al., 1995). In addition, functional assays trying to demonstrate the kinase activity of KSR were mostly unsuccessful (Denouel-Galy et al., 1998; Michaud et al., 1997; Sugimoto et al., 1998; Yu et al., 1998). This led to the hypothesis that the function of KSR within the Ras/MAPK pathway was independent of its catalytic function.

Intensive efforts concerning the elucidation of KSR functions have led to the identification of multiple interacting proteins, which are apparently required for intact ERK signaling. Indeed, KSR was found to be constitutively bound to MEK1/2 (Denouel-Galy et al., 1998; Muller et al., 2000; Yu et al., 1998), while Raf and ERK are recruited to KSR in a Ras-dependent manner (Cacace et al., 1999; Therrien et al., 1996; Xing et al., 1997). It is likely that these interactions are required for ERK signaling since Raf and ERK activation is impaired in *ksr1-/-* MEFs following EGF treatment (Kortum et al., 2006; Lozano et al., 2003). In Drosophila, KSR is constitutively bound to MEK and Raf and is required for phosphorylation of MEK by Raf (Roy et al., 2002). Other proteins such as 14-3-3, HSP70/90, cdc37, c-TAK1, PP2A and IMP have also been found in a complex with KSR and appear to regulate its function by having an effect on its stability, localization and/or phosphorylation (Kolch, 2005). Taken together, these results led to the proposal that KSR might coordinate the assembly of the ERK/MAPK module and facilitate signal propagation within the pathway.

In vivo studies using *ksr1-/-* mice suggest that the scaffolding property of KSR is required for Ras-dependent skin cancer development, for ERK-dependent induction of arthritis as well as for learning and memory. *ksr1* null mice are viable without major developmental defects. However, EGF-mediated Raf-1/ERK activation is impaired in *ksr1-/-* MEFs. In addition, Ras^{V12}-induced skin tumorigenesis is substantially diminished when *ksr1* is deleted (Lozano et al., 2003). *ksr1-/-* mice are also less susceptible to develop arthritis compared to wild type littermates when injected with arthritogenic antiserum. This phenotype correlates with decreased activation of ERK by proinflammatory cytokines in *ksr1* null MEFs (Fusello et al., 2006). Finally, ERK-dependent form of synaptic plasticity is impaired in *ksr1* null mice and this correlates with hippocampal memory impairments. Interestingly, the scaffolding function of KSR1 appears to be specific for PKC/Ras but not cAMP/PKA-dependent activation of ERK in hippocampal neurons (Shalin et al., 2006).

1.3.2.2 Connector eNhancer of KSR

Connector eNhancer of KSR (CNK) was first identified as a positive regulator of Ras/MAPK signaling in a KSR-dependent screen in Drosophila (Therrien et al., 1998). CNK is evolutionary conserved and contains multiple protein-protein interaction domains. From N-terminal to C-terminal, CNK is constituted of five conserved regions: a Sterile α motif (SAM), a Conserved Region In CNK (CRIC), a PSD95/DlgA/Zo-1

homology domain (PDZ), a Pleckstrin Homology (PH) and two Proline-rich regions (Therrien et al., 1998). Genetic epistatic analysis in Drosophila showed that the activity of CNK is required downstream of Ras and upstream of, or parallel to Raf. In support of these data, Rocheleau et al. recently showed that *C. elegans* CNK promotes LIN-45/Raf activation at a step between the dephosphorylation of inhibitory sites in the regulatory domain and activating phosphorylation in the kinase domain (Rocheleau et al., 2005). Biochemical studies in Drosophila and mammals reported that CNK is a Raf binding protein (Lanigan et al., 2003; Therrien et al., 1998; Ziogas et al., 2005) and that RNAi depletion of CNK inhibits ERK activation by RTKs (Anselmo et al., 2002; Bumeister et al., 2004; Douziech et al., 2003). These results suggested that CNK is required for Raf activation.

Further analyses in Drosophila revealed that CNK has a dual function with respect to Raf activation. Indeed, the N-terminal region of CNK contains two domains (SAM and CRIC), which are essential for Raf activation, while the C-terminal part of CNK mediates an inhibitory effect. The negative effect of the C-terminal part of CNK was mapped to a 40 amino acid region, hereafter called the Raf-Inhibitory Region (RIR). The RIR was shown to prevent signaling leakage from Raf to MEK in the absence of upstream signaling (Douziech et al., 2003). RTK triggering has at least two consequences on CNK-mediated Raf activation: the inhibition of Raf via the RIR is relieved by a Src kinase-dependent mechanism

(Laberge et al., 2005) and the N-terminal part of CNK integrates positive cues that are essential for Raf activation (Douziech et al., 2003). Recently, the N-terminal part of CNK has been shown to mediate its positive effect on Raf via the recruitment of KSR and a new SAM domain-containing protein called Hyphen (Hyp). It was demonstrated that KSR promotes Ras-dependent Raf activation by a mechanism that is dependent on its kinase domain, but independent of its scaffolding property or catalytic activity (Douziech et al., 2006). Hyp, which was also identified as a positive regulator of RTK signaling by genetic screening in Drosophila (Roignant et al., 2006), was found to mediate the interaction between CNK and KSR (Douziech et al., 2006). In summary, CNK appears to be required for Raf catalytic function by placing the kinase in close vicinity with proteins required for its activation. In mammals, the antagonistic property of CNK on the Ras/ERK pathway is likely to be conserved. Indeed, overexpression of human CNK (hCNK) has been shown to inhibit Ras/ERK signaling (Lanigan et al., 2003). In addition, hCNK apparently serves as a molecular platform for Raf-1 and Src kinase and potentially mediates Src-dependent activation of Raf (Ziogas et al., 2005). Further experiments are required to fully establish the molecular mechanism by which mammalian CNK regulates Raf.

1.3.2.3 The JIP proteins

The JNK interacting proteins (JIPs) are scaffolding molecules implicated in the assembly of the core module of the JNK and p38 pathways. In mammals, four genes encode JIP proteins: *jip1*, *jip2*, *jip3/jsap1* and *jip4*. Interestingly, the *jip4* gene encodes at least three proteins (JIP4, JLP, and SPAG9) that differ in utilization of 5′ exons but share common 3′ exons. The JIP1 and JIP2 proteins share extensive sequence homology, having an SH3 and PTP domain in C-terminal and a JNK binding domain (JBD) in the N-terminal part. Although highly divergent from JIP1/2, JIP3/JSAP and JIP4 gene products are related in sequence. Both proteins share an extended coiled-coil domain and a leucine zipper in the N-terminal region together with a JBD, a JNK phosphorylation domain, and a putative transmembrane domain (for review, see (Whitmarsh, 2006)).

Cell transfection experiments demonstrated that the JIP1/2 scaffolding molecules mediate JNK activation by simultaneously binding JNK1/2, MKK7 as well as members of the MLK group of MAPKKK (Whitmarsh et al., 1998). In addition, JIP2 also promotes p38 signaling by binding MLK3/p38δ (Schoorlemmer and Goldfarb, 2002) or MLK3/MKK3/p38α (Buchsbaum et al., 2002). Both JIP1 and JIP2 can bind the phosphatase MKP7, which leads to reduced activation of the JNK pathway (Willoughby et al., 2003). However, the generation of *jip1* KO

mice fully established the physiological relevance of the scaffold. Indeed, JIP1 is essential for obesity-induced JNK activation in fat and muscle (Jaeschke et al., 2004) as well as for the neuronal activation of JNK upon excitotoxic stress (Whitmarsh et al., 2001). Depending on the cellular context, JIP3/JSAP1 was found to increase JNK activation by interacting with MLK3/MKK7/JNK (Kelkar et al., 2000), MEKK1/MKK4/JNK (Ito et al., 1999) or ASK1/MKK4/7/JNK (Kelkar et al., 2005) proteins in cotransfection experiments. In addition, JIP3 is required for JNK activation downstream of Toll-like receptor and fibronectin stimulation (Matsuguchi et al., 2003) as well as for the morphogenesis of the telencephalon in mice (Kelkar et al., 2003). Finally, the JIP4 protein was reported to bind to ASK1/MKK3/MKK6/p38αβ (Kelkar et al., 2005), while JLP interacted with MEKK3/MKK4/JNK1 or p38α (Lee et al., 2002). One interesting fact about JIP proteins is their ability to interact with the light chain of microtubule motor protein Kinesin-1 and to be transported as cargo along the microtubule network within cells. This suggests that JIP proteins may not only serve as an anchoring platform for members of the JNK/p38 core module but may also be required for spatial regulation of the SAPK pathways (Whitmarsh, 2006).

1.3.3 Regulation through ubiquitination

Ubiquitination is the process by which ubiquitin (Ub), a highly conserved 76-amino acid peptide universally found in eukaryotic cells, is added to a target protein. Ubiquitination occurs through sequential steps

catalyzed by activating (E1), conjugating (E2) and ligase (E3) enzymes. The first step implicates the ATP-dependent formation of thiol ester bond from the C-terminus of Ub to the active Cys of Ub-activating enzyme E1. Then, Ub is transferred to the active Cys in the Ub-conjugating enzyme E2. Finally, Ub is attached to a Lys residue on the target protein in an E3-dependent manner. The best understood function of ubiquitination is proteolysis, whereby Lys48-linked polyubiquitin chains allow recognition by the 26S proteasome. However, proteins can also be monoubiquitinated or polyubiquitinated through alternative (e.g., K6, K11, K29 and K63) linkages, and such modifications are thought to control protein activity or localization (Pickart and Fushman, 2004). The regulation of the MAPK pathways is partly achieved by Ub modifications (for review, see (Laine and Ronai, 2005)), which either target protein for degradation or influence the catalytic activity of the targeted protein. Some elegant examples are described below for the Ras/ERK pathway.

Ras is a small membrane bound GTPase implicated in the activation of the ERK pathway. Mammalian cells contain three ubiquitously expressed Ras isoforms, H-, K-, and N-Ras. Numerous evidences point toward a compartmentalized activation of the ERK pathway via differential subcellular localization of Ras (Mor and Philips, 2006). It was recently demonstrated that ubiquitination might be important for compartmentalized signalization via the Ras/ERK pathway. Indeed, in mammalian cells, H-Ras and N-Ras but not K-Ras are constitutively di-ubiquitinated via the

non-canonical Lys63 on ubiquitin. Ubiquitination promotes endosomal association of Ras and also inhibits its capacity to activate the ERK pathway probably by altering the capacity of Raf to bind Ras (Jura et al., 2006). Upon activation, Ras also mediates an Ub-dependent positive feedback mechanism on the ERK pathway via the Impede Mitogenic Propagation (IMP) protein. IMP is an E3 ligase, which maintains KSR in a triton-insoluble fraction and impedes signal transmission from Raf to MEK. Ras activation promotes auto-ubiquitination of IMP and facilitates KSR-dependent engagement of MEK by activated Raf (Matheny et al., 2004).

Mekk1 is a MAPKKK mainly implicated in the activation of the SAPK pathways. However, it also has the capacity to regulate the ERK pathway in certain cellular context. In addition to its kinase domain, Mekk1 also possesses a Plant Homeodomain (PHD) in its N-terminal part, which is highly related to the RING domain required for the ligase activity of E3 RING ligases. It was shown that the PHD domain of Mekk1 displays E3 ubiquitin ligase activity toward ERK1 and ERK2 under hyperosmotic conditions. Apparently, an intact kinase and PHD domain are required for in vivo ubiquitination of ERKs and subsequent degradation (Lu et al., 2002). In addition, auto-ubiquitination of Mekk1 inhibits the Mekk1-dependent activation of ERK and JNK. In this particular situation, ubiquitination of Mekk1 does not target the protein for degradation. It is likely that auto-ubiquitination affects the kinase activity of Mekk1 or its association with downstream substrates (Witowsky and Johnson, 2003).

1.4. Drosophila: a model system to study MAPK signaling pathways

1.4.1. Drosophila and its advantages

1.4.1.1 Gene conservation

For over a century, the fruit fly *Drosophila melanogaster* has been used as a model for exploring the mysteries of eukaryotic biology. Drosophila is a suitable organism for research because of its short generation time (~10 days), the possibility of mass culture at low cost and the availability of powerful genetic tools. In 2000, the complete sequence of the Drosophila genome was released and revealed very interesting features (Adams et al., 2000). First, a comparative analysis of the predicted proteins encoded by these genes suggested that half of the fly proteins show similarity to mammalian proteins (BLAST cutoff: $E<10^{-10}$). This is in accordance with previous studies showing conservation for most of the signaling pathways such as the Notch, Wingless, Hedgehog, MAPK and TGFβ pathway. In addition, in many cases, a single Drosophila gene serves the function of multiple related family members in mammals. Drosophila geneticists are thus less likely to encounter the problem of redundancy found in vertebrates. Finally, approximately 75% of the genes associated with human diseases have related sequence in Drosophila (Rubin et al., 2000) and gene disruption in Drosophila phenocopies various human pathologies (Bier, 2005).

1.4.1.2 Rapid forward and reverse genetics

Many signaling pathways that guide basic developmental processes in vertebrates are conserved in invertebrates such as Drosophila. The ease with which Drosophila can be genetically manipulated has led to the molecular characterization of these signaling pathways and the results, in most of the cases, can be directly applied to vertebrate systems. Indeed, flies benefit from a wide range of methods for carrying out molecular genetic research. These include P-element mediated transgenesis (Rubin and Spradling, 1982), which permits the generation of fly transgenics in less than two months, a gene-overexpression system that is based on the yeast Gal4/UAS system (Brand and Perrimon, 1993), which allows a restricted gene expression in time and space and the FLP/FRT site-specific mitotic recombination system (Golic and Golic, 1996; Xu and Rubin, 1993), which among other things enables the characterization of recessive lethal alleles. These methods are useful for forward and reverse genetic strategies.

Forward genetics or genetic screening has proven to be extremely powerful to decipher signal transduction pathways. For example, one can generate a particular phenotype in fly by modulating a known signaling pathway and then screen for mutant loci, which either enhances or suppresses this phenotype. The output of such a screen is a list of loci each containing a set of alleles that modify the original mutant phenotype.

This type of strategy was particularly successful for the identification of new regulators of the Notch, Hedgehog, Wingless, TGFβ and MAPK signaling pathways, which are conserved in mammals (St Johnston, 2002).

A particular gene can also be mutated, and the phenotype of such gene alteration analyzed for instructive phenotypes (change in morphology, behavior, cellular identity, gene expression, etc.). This strategy, also called reverse genetics, is particularly appealing since roughly 55% of all Drosophila genes are disrupted by transposable elements (Thibault et al., 2004). In addition, a way to perform gene targeting by homologous recombination in Drosophila was recently described (Rong and Golic, 2000). Finally, the combination of the RNA interference (RNAi) technology and GAL4/UAS system now permits gene silencing in restricted tissues and in a defined time frame (Kennerdell and Carthew, 2000; Lee and Carthew, 2003). This is particularly useful for studying genes required for late developmental events. In this system, depletion of the protein can be achieved after the development of most tissues and evaluation of a true loss-of-function phenotype can be performed in a fully developed organism.

Thus, Drosophila was and is still a versatile model system to molecularly define signaling pathways. In the next sections, I describe the

cellular and/or organismal contexts for which the Drosophila RTK/Ras/MAPK, JNK and p38 pathways are crucial. For many decades, these biological contexts served for the characterization of MAPK pathways using forward and reverse genetics.

1.4.2 ERK/MAPK signaling pathway during Drosophila eye development

Many developmental processes in Drosophila require signaling via the Ras/MAPK pathway (Shilo, 2003). However, one of the most powerful systems to study this signaling cascade is the developing fly eye. This system is appealing for a few reasons. First, the cellular sequence of events leading to a WT adult eye is fairly well understood and implicates a variety of biological processes such as differentiation, proliferation, cell shape changes, cell survival and apoptosis. Thus, the same tissue is useful to study various aspects of the Ras/MAPK signaling pathway. In addition, the outer and inner architecture of the WT compound eye is simple and well characterized. The external morphology of the eye resembles a honeycomb-like structure composed of ~800 identical facets, each comprising a defined complement of cells (Chapter 1, Figure 2A). Modulating the Ras/MAPK signaling intensity (or other signaling pathways) usually disturbs this stereotyped architecture and leads to the so-called “rough eye phenotype”. This phenotype is easily observable under a stereomicroscope and can be studied at the cellular level in adult or

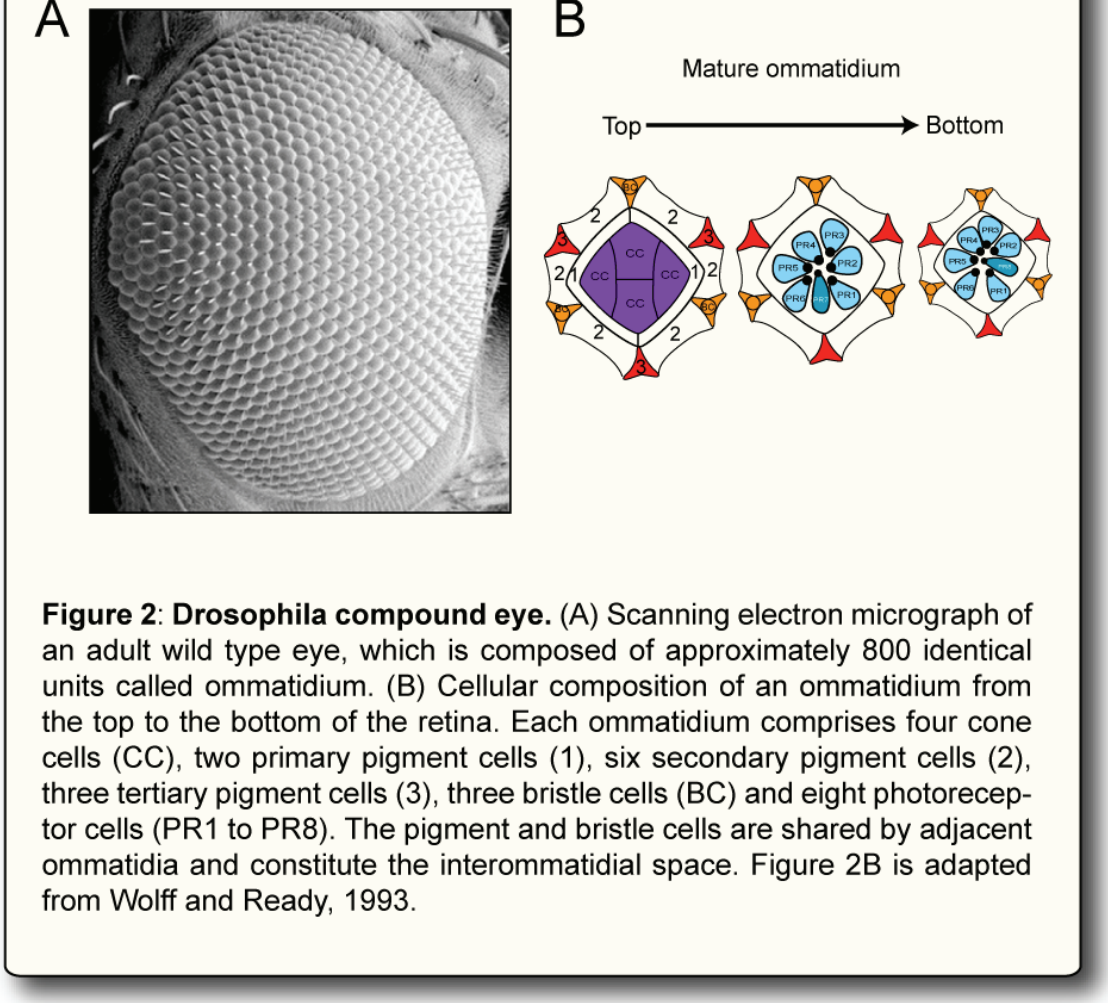

Figure 2: **Drosophila compound eye.** (A) Scanning electron micrograph of an adult wild type eye, which is composed of approximately 800 identical units called ommatidium. (B) Cellular composition of an ommatidium from the top to the bottom of the retina. Each ommatidium comprises four cone cells (CC), two primary pigment cells (1), six secondary pigment cells (2), three tertiary pigment cells (3), three bristle cells (BC) and eight photoreceptor cells (PR1 to PR8). The pigment and bristle cells are shared by adjacent ommatidia and constitute the interommatidial space. Figure 2B is adapted from Wolff and Ready, 1993.

developing flies. The Drosophila eye is also dispensable for viability and a number of tools are available for its manipulation. Finally, virtually all of the genes required for proper Drosophila eye development are conserved, and many perform similar functions in a wide range of species (Firth et al., 2005; Wolff and Ready, 1993).

1.4.2.1 Drosophila eye development

The Drosophila compound eye is composed of ~800 identical units called ommatidium. Each ommatidium comprises a definite number of cells arranged in an invariant pattern. Eight photoreceptor cells (PR1-PR8) send axons in the Drosophila brain and are implicated in the detection of visual cues. An ommatidium also contains four lens-secreting cone cells, two primary pigment cells and is surrounded by an hexagonal lattice of 12 cells constituting the interommatidial space (which includes sensory bristle, secondary and tertiary pigment cells). The specific arrangement and number of cells within each ommatidium is important to preserve the wild type architecture of the eye (see Chapter 1, Figure 2B) (Wolff and Ready, 1993).

The Drosophila life cycle consists of a number of stages: embryogenesis, three larval stages (1^{st} instar, 2^{nd} instar and 3^{rd} instar larvae), a pupal stage, and finally the adult stage. During larval stages, a set of presumptive tissues called imaginal discs will undergo pattern formation and ultimately give rise to various adult body parts. The presumptive eye and antennal tissues are formed late during embryogenesis and are referred to as the eye-antennal imaginal disc (EAD). The EAD is a sac-like structure consisting of a monolayer of epithelial cells enclosed within the peripodial membrane. Little information is known about the development of the EAD before the third instar larval

stage except that cellular growth and proliferation are asynchronous and continuous. In the middle of the third instar larval stage, an apical constriction of the disc called the morphogenetic furrow (MF) starts moving from the posterior edge of the eye disc and sweeps across the entire eye field (Chapter 1, Figure 3A). Unpatterned cells that are ahead of the furrow are still proliferating while cells inside the furrow undergo cell cycle G1 arrest. Cells within the MF also undergo shape changes and epithelial reorganisation, forming distinct ‘rosettes’ and ‘arcs’. The initial rosette contains 15–20 cells, but this reduces to approximately 9 cells that form arcs, which then close up to give clusters of 6–7 cells: the future PR8 and PR2–PR5 cells, plus one or two cells that will be ejected from the maturing ommatidium (the so-called mystery cells). PR8 is the first cell to enter the differentiation program. Each newly specified PR8 cell acts as a founder cell for one ommatidium and initiates the sequential recruitment of other cells. After PR8 is committed, PR2 and PR5, PR3 and PR4, will sequentially integrate the ommatidium (Chapter 1, Figure 3B). At this point, all uncommitted cells posterior to the furrow go through a last round of mitosis called the “second mitotic wave”. Then, PR1 and PR6 followed by PR7 are recruited to the ommatidium. Finally, the non-neuronal cone cells are incorporated into the ommatidium. By the end of larval life, the most mature clusters, which are at the edge of the posterior part, have gathered the adult complement of eight photoreceptors and four cone cells. During the first three days of pupal stage, pigment and bristle cells will join the ommatidium and start building the interommatidial lattice. At

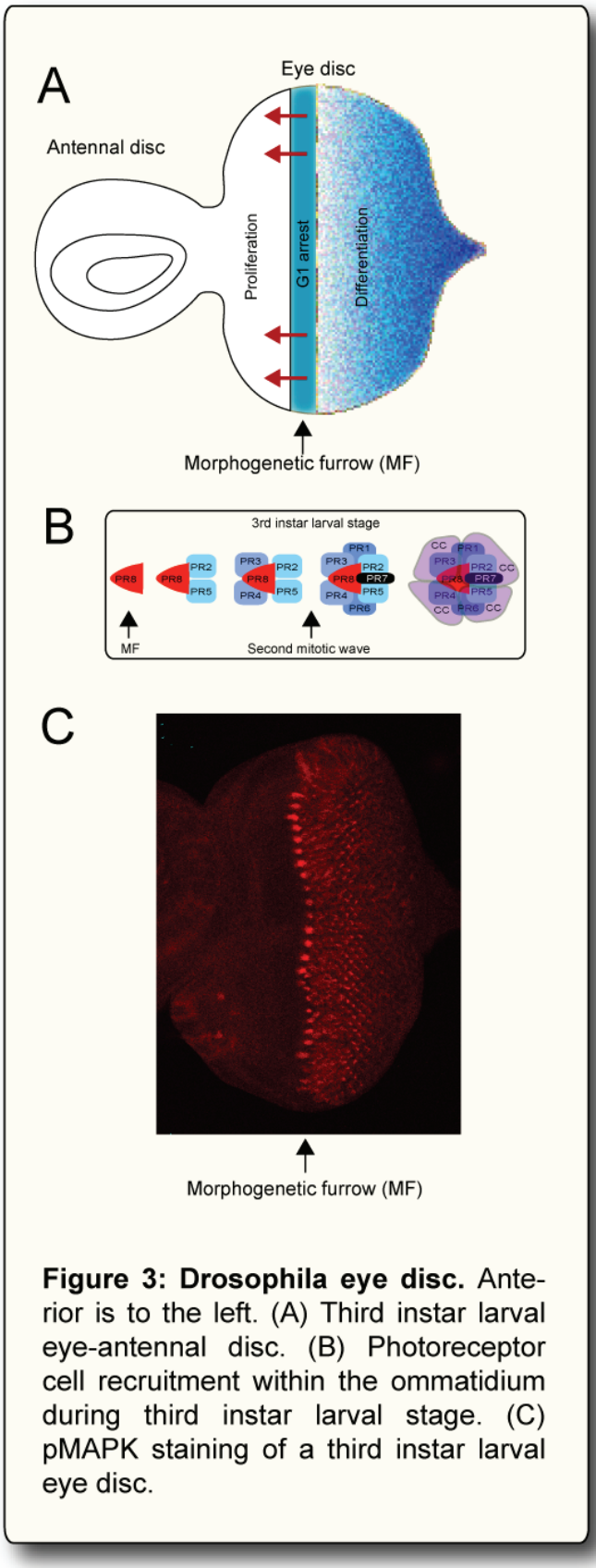

Figure 3: Drosophila eye disc. Anterior is to the left. (A) Third instar larval eye-antennal disc. (B) Photoreceptor cell recruitment within the ommatidium during third instar larval stage. (C) pMAPK staining of a third instar larval eye disc.

the beginning of pupariation, the retina has more cells than it will ultimately need. Hence, cells that are uncommitted at the end of retinal pattern formation will be eliminated through programmed cell death. Cell death appears to eliminate cells that have failed to establish proper contacts with their neighbouring counterparts. Finally, in the last three days of pupal development, retinal cells will elaborate their specialized cellular products and

structures (Wolff and Ready, 1993).

1.4.2.2 ERK/MAPK signaling is crucial for photoreceptor differentiation

Mosaic analysis early during eye development revealed the absence of a clonal relationship between ommatidial cells (Ready et al., 1976). Instead, the recruitment of cells occurs in a stereotyped manner by inductive cellular interactions involving a complex network of signaling pathways. Signaling via the Ras/Raf/MEK/MAPK (Ras/MAPK) module is crucial for many aspects of eye development. In approximate order of appearance, the Ras/MAPK signaling cascade functions in the proliferation of undifferentiated cells anterior to the furrow (and also in the second mitotic wave), the initiation (but not the progression) of the MF, epithelial cell adhesion in the MF, the differentiation of multiple cell types and cell survival posterior to the MF. (Frankfort and Mardon, 2002; Voas and Rebay, 2004). However, the best described and understood function of the Ras/MAPK pathway is during retinal differentiation.

1.4.2.2.1 DER/Sev and photoreceptor differentiation

The progression of the MF is responsible for the initiation of photoreceptor differentiation, which starts with the PR8. Genetic analyses revealed that Drosophila EGF receptor (DER)-dependent Ras/MAPK

signaling is essential for the differentiation of all photoreceptors (Freeman, 1996; Xu and Rubin, 1993), except the PR8. Indeed, the selection of a PR8 among a pool of uncommitted cells, the adequate spacing of PR8 as well as the differentiation into a functional neuron are possible into *DER -/-* clones (Frankfort and Mardon, 2002; Rodrigues et al., 2005).

DER is a RTK present at the surface of all precursor cells posterior to the MF, which activates the Ras/MAPK cascade. During ommatidial differentiation, successive cycles of photoreceptor recruitment through the DER pathway take place. The PR8 is the coordinator of all photoreceptors recruitment by serving as an initial source of DER ligand. Among the four ligands of DER (Spitz, Keren, Vein, Gurken), Spitz is the most widely expressed and is implicated in cell fate specification in the eye. Activated Spitz is secreted in the immediate extracellular environment of the PR8 and will bind DER receptors at the surface of nearby cells, resulting in the differentiation of all other photoreceptors (Chapter 1, Figure 4). Spitz availability to PR8-surrounding cells is tightly regulated and will be discussed in section 1.4.2.2.2.1 (Kurada and White, 1999; Shilo, 2005). As DER-dependent photoreceptor differentiation occurs posterior to the MF, cells start expressing a dynamic pattern of phosphorylated ERK/MAPK as shown using an antibody that recognizes the diphosphorylated form of ERK/MAPK (pMAPK; Chapter 1, Figure 3C). Indeed, the most prominent pMAPK staining appears in the row of photoreceptor clusters immediately

posterior to the MF while more posterior ommatidial rows show less staining (Gabay et al., 1997).

Activation of DER by Spitz is required but not sufficient for full PR7 differentiation. Signaling via the Ras/MAPK pathway must also be stimulated via a second RTK, the Sevenless (Sev) receptor (Chapter 1, Figure 4). Although required only in the PR7 precursor, the Sev RTK is located on the apical surface of a number of ommatidial precursor cells: the precursors of the R3, R4, R7 and the cone cells. The close vicinity of presumptive PR7 with PR8, which expresses the Sev ligand Bride of Sevenless (Boss) at its cell surface, will lead to PR7 differentiation. Since other Sev-expressing cells do not contact the ligand-presenting PR8 cell, the receptor is not activated in these cells. The necessity of this combinatorial Ras/MAPK activating mechanism is not fully understood. One hypothesis is that the PR7 requires higher level or sustained activation of the Ras/MAPK pathway for cell fate commitment. Indeed, overexpression of activated Ras/MAPK pathway component in cone cells resulted in the recruitment of extra PR7 cells per ommatidium. On the other hand, due to this combinatorial mechanism, PR7 differentiation is highly dependent on fluctuation of Ras/MAPK signaling and is the first photoreceptor to disappear when the pathway is inhibited (Nagaraj and Banerjee, 2004; Raabe, 2000).

1.4.2.2.2 Additional players for photoreceptor differentiation

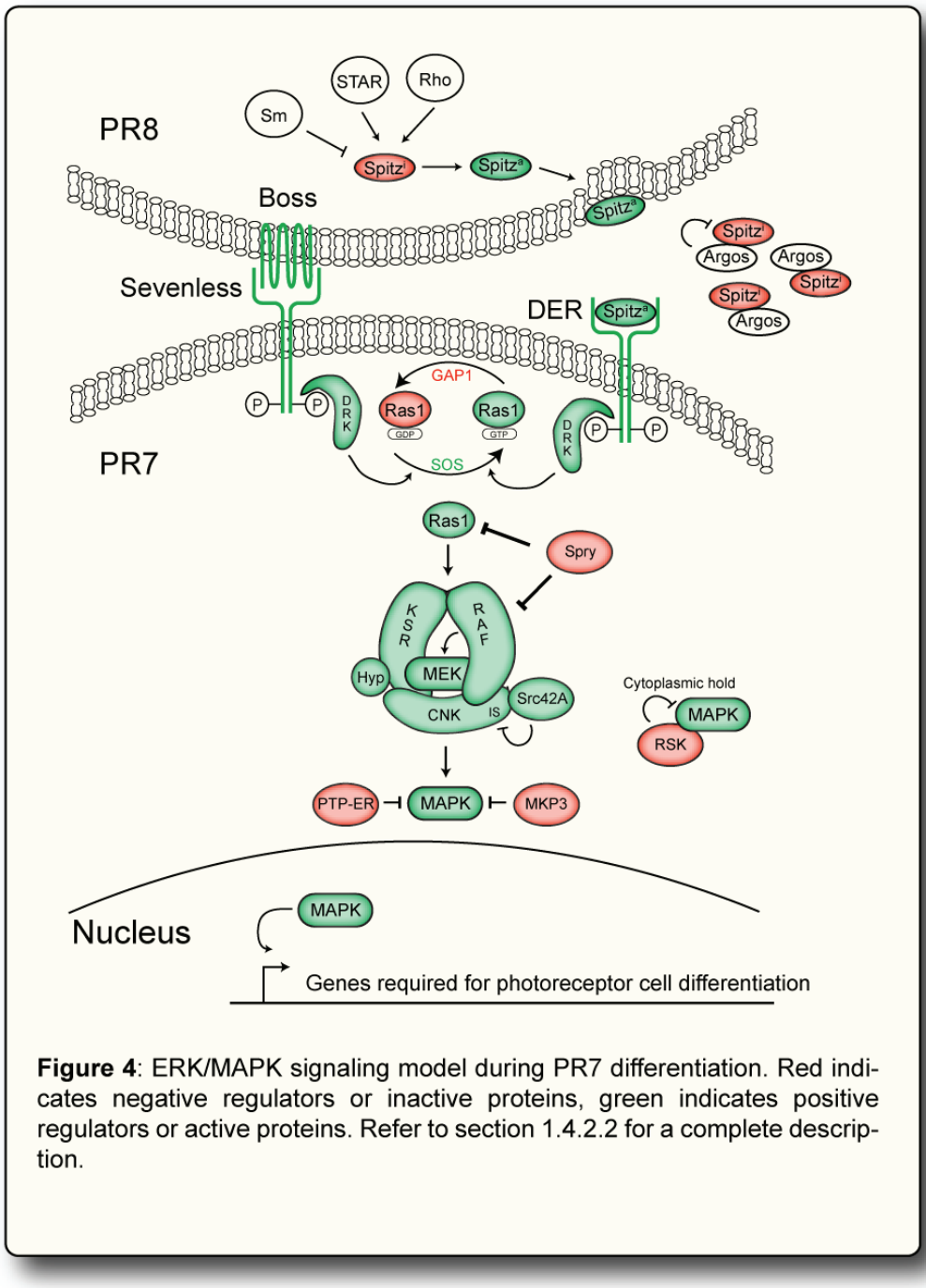

Figure 4: ERK/MAPK signaling model during PR7 differentiation. Red indicates negative regulators or inactive proteins, green indicates positive regulators or active proteins. Refer to section 1.4.2.2 for a complete description.

The Drosophila eye has been used to study the morphogenetic processes occurring during development. However, geneticists have also used this biological system to seek new regulators of a given signaling pathway, to molecularly define a new signaling pathway or to identify

crosstalk between signaling pathways. Many conserved components of the Ras/MAPK pathways have been isolated using genetic approaches in Drosophila eye. The next section highlights most of the proteins that branch on the RTK/Ras/Raf/MEK/MAPK cascades during eye development (Chapter 1, Figure 4).

1.4.2.2.2.1 At the level of RTKs

DER activation is regulated by the availability of the Spitz ligand in the extracellular environment of the PR8. The Star/Rhomboid/Small Wing set of proteins has been shown to control the amount of active Spitz ligand secreted by the PR8. Spitz is first produced as an inactive ligand in the endoplasmic reticulum (ER). Spitz association with Star facilitates its translocation from the ER to the Golgi apparatus. Once in the Golgi, Spitz is cleaved by an intramembrane protease of the Rhomboid family to yield the active form of the ligand. Another order of regulation is brought by the Small Wing phospholipase Cγ. This protein has been shown to inhibit Spitz secretion by retaining the ligand in the ER compartment. The extracellular availability of Spitz is also modulated by Argos, which encodes a secreted protein with an atypical EGF-like motif at its carboxy terminus. Its expression is induced by DER activation, constituting a negative feedback mechanism. Although previous studies found that Argos was a competitive inhibitor of Spitz for binding to DER, it was recently shown that Argos acts by directly sequestering the Spitz ligand

(Shilo, 2005). Up until now, no such mechanism exists for the regulation of Boss, the Sev ligand.

Various multi-domain containing adaptor molecules have been implicated in bridging RTK activities to Ras during Drosophila eye development (DOS/GAB, DShc, DRK/Grb2). However, the role of Downstream of Receptor Kinases (DRK), the homolog of mammalian Grb2, is best characterized during retinal differentiation. DRK is a SH2/SH3 domains-containing protein that is constitutively bound to the guanine exchange factor Son of Sevenless (Sos). Upon RTK activation, the DRK/Sos complex is recruited to the plasma membrane and will favor the exchange of GDP for GTP on the small membrane bound GTPase Ras (Bonfini et al., 1992; Olivier et al., 1993; Simon et al., 1993). GTP loaded and activated Ras then participates in the activation of Raf. Another protein, called GTPase Activating Protein 1 (GAP1), was also demonstrated to be required for proper eye development. GAP1 is a negative regulator of the Ras/MAPK pathway and promotes the intrinsic GTPase activity of Ras (Gaul et al., 1992).

1.4.2.2.2.2 At the level of Raf/MEK

Kinase Suppressor of Ras (KSR) and Connector eNhancer of KSR (CNK) are among proteins found to positively regulate the Ras/MAPK pathway downstream of Ras ((Therrien et al., 1995; Therrien et al., 1998);

see also sections 1.3.2.1 and 1.3.2.2). KSR is a Raf-like kinase implicated in RTK-mediated signaling during Drosophila eye development. However, KSR appears to be devoid of kinase activity. By its ability to interact with both Raf and MEK and facilitate the phosphorylation of MEK by Raf, KSR serves as a molecular platform in the Ras/MAPK pathway. The scaffolding property of KSR with respect to Raf and MEK appears to depend on CNK and a novel SAM domain protein called Hyp (Douziech et al., 2006; Roignant et al., 2006). In the absence of RTK signaling, CNK serves as a platform for the KSR/MEK pair as well as for Raf, which is maintained in an inhibitory state by an inhibitory sequence present in the C-terminal portion of CNK. RTK activation leads to CNK phosphorylation on a Tyr near the inhibitory sequence and subsequent recruitment of a Src-like molecule called Src42A. The binding of Src42A to the C-terminal part of CNK releases the inhibition on Raf, while the N-terminal part of CNK promotes signaling via KSR and Hyp (Douziech et al., 2003; Laberge et al., 2005). The PP2A Ser/Thr phosphatase (Ory et al., 2003; Wassarman et al., 1996), 14-3-3 proteins (Chang and Rubin, 1997; Kockel et al., 1997; Wilker and Yaffe, 2004) and HSP90/Cdc37 protein (Cutforth and Rubin, 1994; Grammatikakis et al., 1999) are also positively required for RTK-mediated retinal differentiation at the level of Raf activation.

Sprouty (Spry) is a general inhibitor of RTK-mediated Ras signaling. However, the point at which Spry blocks MAPK activation remains controversial and the evidence to date suggests the existence of multiple

mechanisms that depend on the cellular context and/or the identity of the RTK. For instance, epistasis analyses during eye development showed that the activity of Spry is required downstream of DER and upstream of Ras. However, in other systems, Spry appears to act at the level of Raf. One possible model for Spry-mediated inhibition of DER is the following. Upon activation of the pathway, gene expression of Spry is upregulated, and the protein translocates to the cytoplasmic membrane where it is phosphorylated by a Src-like kinase. Then, Spry terminates the signal by inhibiting Ras or Raf activation by a mechanism that remains to be characterized (Mason et al., 2006).

1.4.2.2.2.3 At the level of ERK/MAPK

PTP-ER was identified as a negative regulator of the Ras/MAPK pathway during retinal differentiation. It encodes a Tyr phosphatase similar to PTP-SL, He-PTP and STEP and was shown to bind to and specifically dephosphorylate ERK/MAPK (Karim and Rubin, 1999a). Another negative regulator of the pathway is encoded by DMKP3, a dual specificity phosphatase also required for RTK-mediated photoreceptor differentiation. Given that homologs in mammal specifically dephosphorylate ERK, it is likely that DMKP3 acts by a similar mechanism in flies (Kim et al., 2004; Kim et al., 2002). Finally, the p90 Ribosomal S6 kinase (RSK) also acts as a negative regulator of the Ras/MAPK pathway by binding to MAPK and

inhibiting its nuclear translocation in the nucleus in a kinase-independent manner (Kim et al., 2006a).

1.4.3 The Drosophila SAPK pathways

1.4.3.1 JNK pathway

In Drosophila, the JNK pathway controls various biological processes such as tissue morphogenesis, wound healing, immune and stress response, longevity, planar cell polarity, apoptosis and synaptic plasticity. This collection of JNK-dependent processes in Drosophila highlights the fundamental nature of this pathway for basic cellular functions in multicellular oraganisms. The diverse biological processes enumerated above all require the basic core module of the JNK pathway. This module is composed of JNK itself or Basket (Bsk), one of the two upstream Ser/Thr kinases Hemipterous (Hep) or MKK4 as well as a member of a panel of SAPKKKs, namely Slipper (Slpr; Drosophila MLK), Tak1, Tak12, Wallenda/dDLK, Pk92b/dASK1 or Mekk1. JNK pathway conservation in Drosophila permitted a faster characterization of complex biological processes such as tissue morphogenesis and immune functions. In addition, genetic screening for genes implicated in JNK-dependent biological processes also identified new regulators of the pathway (Stronach, 2005).

1.4.3.1.1 JNK pathway involvement in tissue morphogenesis

Embryonic dorsal closure in Drosophila is a morphogenetic episode that involves the migration of lateral epithelium towards the dorsal midline of the embryo (Chapter 1, Figure 5A-D). Before closure, the dorsal part of the embryo is covered by the amnioserosa, a tissue that disintegrates during later stages of development. At the onset of dorsal closure, the epidermis on both sides of the embryo will migrate towards the midline of the embryo and eventually attach to seal the embryo in a protective layer of epidermal cells. The JNK pathway plays a pivotal role during embryonic dorsal closure by modulating the actin cytoskeleton and the transcription of key genes. Loss-of-function mutations in genes required for adequate JNK signaling generally produce a large, dorsal anterior hole in the secreted larval cuticle (hereafter called “dorsal open phenotype”) and result in embryonic lethality (Harden, 2002; Jacinto et al., 2002; Stronach, 2005). However, hypomorphic mutations in components of the JNK pathway usually permit completion of embryogenesis, but result in thorax closure (TC) defects during later stages of development. Flies with TC failure feature a cleft in the dorsal midline of the thorax. In addition, the area of the notum and scutellum is often reduced and misses bristles (Chapter 1, compare Figure 5E and F and (Zeitlinger and Bohmann, 1999)). Interestingly, the process of wound healing in Drosophila and mammals also depends on JNK signaling and implicates morphogenetic

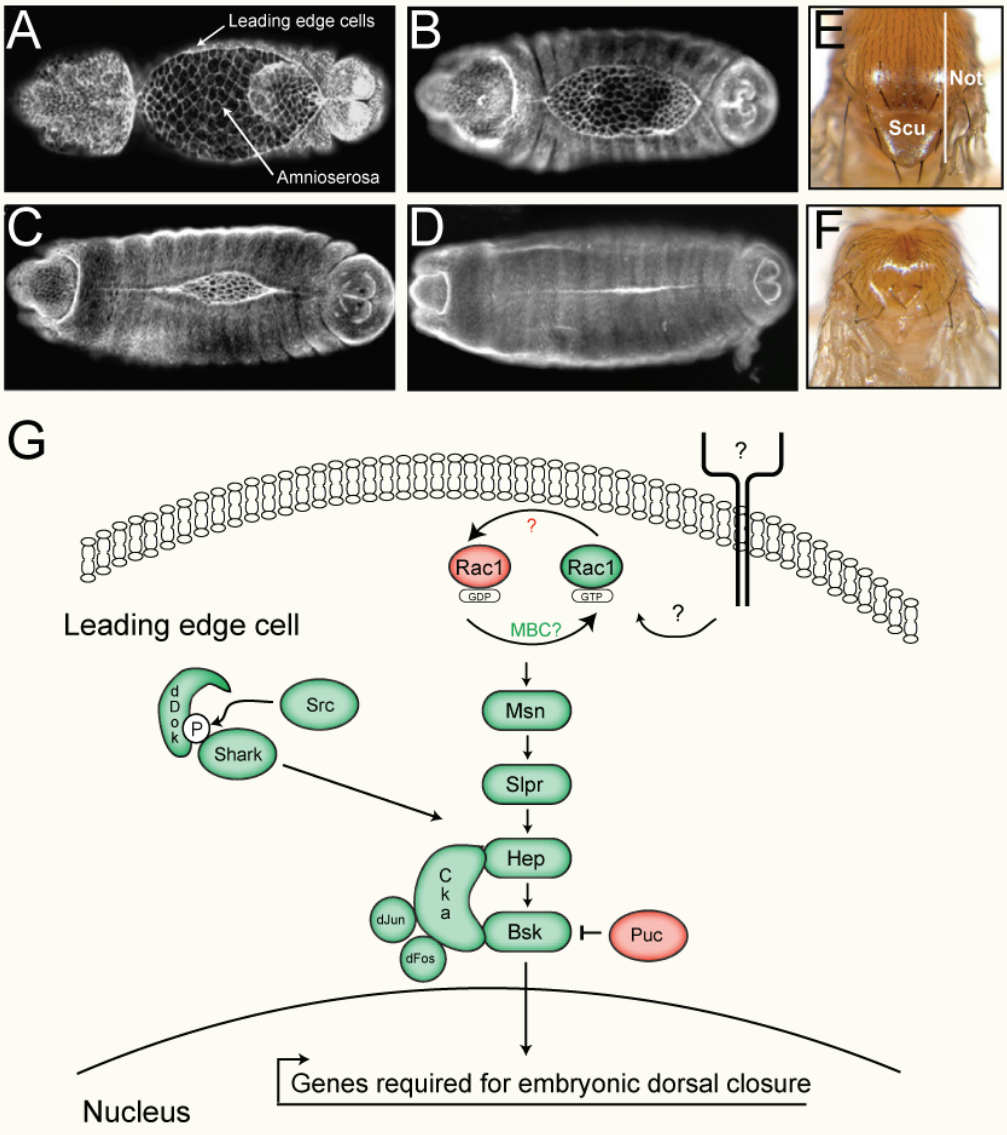

Figure 5: JNK-dependent morphogenetic processes in Drosophila. (A-D) Dorsal closure of the embryo. Dorsal views of progressively older embryos stained with anti-phosphotyrosine antibodies to show closure of the epidermis over the large, flat cells of the amnioserosa. These pictures are taken from Harden N., 2002 with the permission of Blackwell Publishing LTD. (E,F) Thorax closure. E shows a wild type notum (Not) while F shows thorax closure defects. Note the smaller notum and the absence of scutellum (Scu) as well as the loss of bristles in F. (G) JNK signaling model in the leading edge cells during embryonic dorsal closure. See section 1.4.3.1.1 for description. Red indicates negative regulators or inactive proteins and green indicates positive regulators or active proteins.

movements similar to embryonic dorsal closure (Martin and Parkhurst, 2004).

Embryos with inactivating mutations in *hep* or *bsk* are unable to complete closure and have a lethal dorsal open phenotype (Glise et al., 1995; Riesgo-Escovar et al., 1996). Based on this particular phenotype, a variety of additional molecules have been linked to the JNK pathway and are now integral components of this signaling cascade (see Figure 5G and (Harden, 2002; Jacinto et al., 2002; Stronach, 2005)). Although many members of the Rho family of small GTPase appear to regulate the process of dorsal closure, only Rac1 is required for JNK pathway activation upstream of Slpr (Genova et al., 2000; Ricos et al., 1999). Genetic analyses in Drosophila also identified Myoblast city (Mbc) as a putative activator of Rac1 during dorsal closure (Nolan et al., 1998). Mbc possesses a guanine exchange factor (GEF) domain called the DOCKER/CZH2/DHR2 domain, which probably promotes the exchange of GDP for GTP on Rac1 (Meller et al., 2005). Genetic and biochemical evidence suggested that the NIK (Nck-Interacting Kinase)-related kinase Misshapen (Msn) is acting upstream of Hep and possibly downstream of Rac1 during dorsal closure (Su et al., 1998). In addition, Slpr is apparently the sole SAPKKK required for embryonic dorsal closure in Drosophila and acts downstream of Rac1 and upstream of Bsk (Stronach and Perrimon, 2002). Indeed, loss-of-function mutations in the *slpr* locus generate a

lethal dorsal open phenotype while *tak1*, *mekk1* or *ddlk* alleles are viable with no apparent phenotype (Collins et al., 2006; Inoue et al., 2001; Vidal et al., 2001). The position of Slpr was further refined as the upstream activating kinase for Hep using a biochemical approach in S2 cells (Sathyanarayana et al., 2003). Biochemical analyses also suggested that the multi-domain containing protein Connector of Kinase to AP-1 (Cka) physically interacts with Hep and Bsk as well as two downstream targets of Bsk, namely dJun and dFos transcription factors. Cka may perform scaffolding functions since complex formation with Hep, Bsk, DJun and DFos result in BSK activation, which in turn phosphorylates and activates DJun and DFos (Chen et al., 2002). The *puckered* (*puc*) gene, which encodes a VH1-like dual-specificity phosphatase, is the sole direct negative regulator of JNK signaling found to date in Drosophila. This phosphatase specifically dephosphorylates Bsk on the TXY motif present in the activation segment and required for activity. Puc is a downstream transcriptional target of the JNK pathway and therefore participates in a negative feedback loop (Martin-Blanco et al., 1998). Finally, members of the non-receptor tyrosine kinase family, namely Shark, Src42A and Tec29, were shown to be important in the morphogenetic process of dorsal closure at a step upstream of Bsk (Fernandez et al., 2000; Tateno et al., 2000). The importance of Tyr phosphorylation is highlighted by the presence of an enrichment of pTyr staining at the leading edge of the moving epidermis. However, Shark, Src42A and Tec29 target substrate(s) have yet to be discovered. Recently, the adaptor molecule dDok was

shown to be required for dorsal closure at a step upstream of Shark. In addition, biochemical studies in Drosophila S2 cells showed that dDok physically interacts with Shark and that this interaction depends on Src-dependent Tyr phosphorylation of dDok (Biswas et al., 2006). Although much is known about the identity of JNK pathway core components implicated in dorsal closure, it is still unclear which signals initiate and terminate this morphogenetic movement.

1.4.3.1.2 JNK pathway involvement in immune function

Drosophila immune defense apparently relies solely on innate immunity response, which is of two types: cellular and humoral. The major site of immune response is the hemolymph, which is an open circulatory system where the movement of fluid is ensured by the dorsal vessel (a structure reminiscent of the mammalian heart). The hemolymph contains a few thousands hemocytes required for the cellular response to invading microorganisms. During larval stages, three lineages of hemocytes (plasmatocytes, lamellocytes and crystal cells) assure the appropriate cellular response. Plasmatocytes constitute 90% of the hemocyte population and are macrophage-like cells dedicated to phagocytosis. Lammelocytes and crystal cells constitute each 5% of the hemocyte population and are involved in encapsulation and melanization of microorganisms, respectively. In adult Drosophila however, only plasmatocytes are present in the hemolymph. The hallmark of the humoral

response is the secretion of antimicrobial peptides (AMPs) in the hemolymph by hemocytes and fat bodies (similar to the mammalian liver). AMPs are small cationic molecules with antibiotic properties on a large spectrum of microorganisms. AMP production depends on the activation of Nuclear Factor κB (NFκB)-related molecules called Dorsal-related Immunity Factor (Dif), Dorsal and Relish (Rel). While infection with Gram+ bacteria and fungus typically lead to the activation of the Toll pathway and subsequent activation of Dif and Dorsal, Gram- bacteria activate Rel, via the Immune Deficiency (IMD) pathway. However, an IMD-dependent efficient immune response also depends on the activation of the JNK pathway (Pinheiro and Ellar, 2006; Tzou et al., 2002; Wang and Ligoxygakis, 2006).

The IMD pathway broadly resembles the mammalian TNF pathway (Chapter 1, Figure 6). Following microbial recognition by specific peptidoglycan recognition proteins (PGRPs), the signal is transduced to Imd, a Death Domain (DD)-containing protein similar to mammalian Receptor Interacting Protein 1 (RIP) (Georgel et al., 2001; Lemaitre et al., 1995). Imd has the capacity to associate with another DD-containing protein called DFadd (Naitza et al., 2002). DFadd is complexed to the caspase Dredd and promotes the proteolytic processing of Dredd in an active protease (Hu and Yang, 2000). By a yet

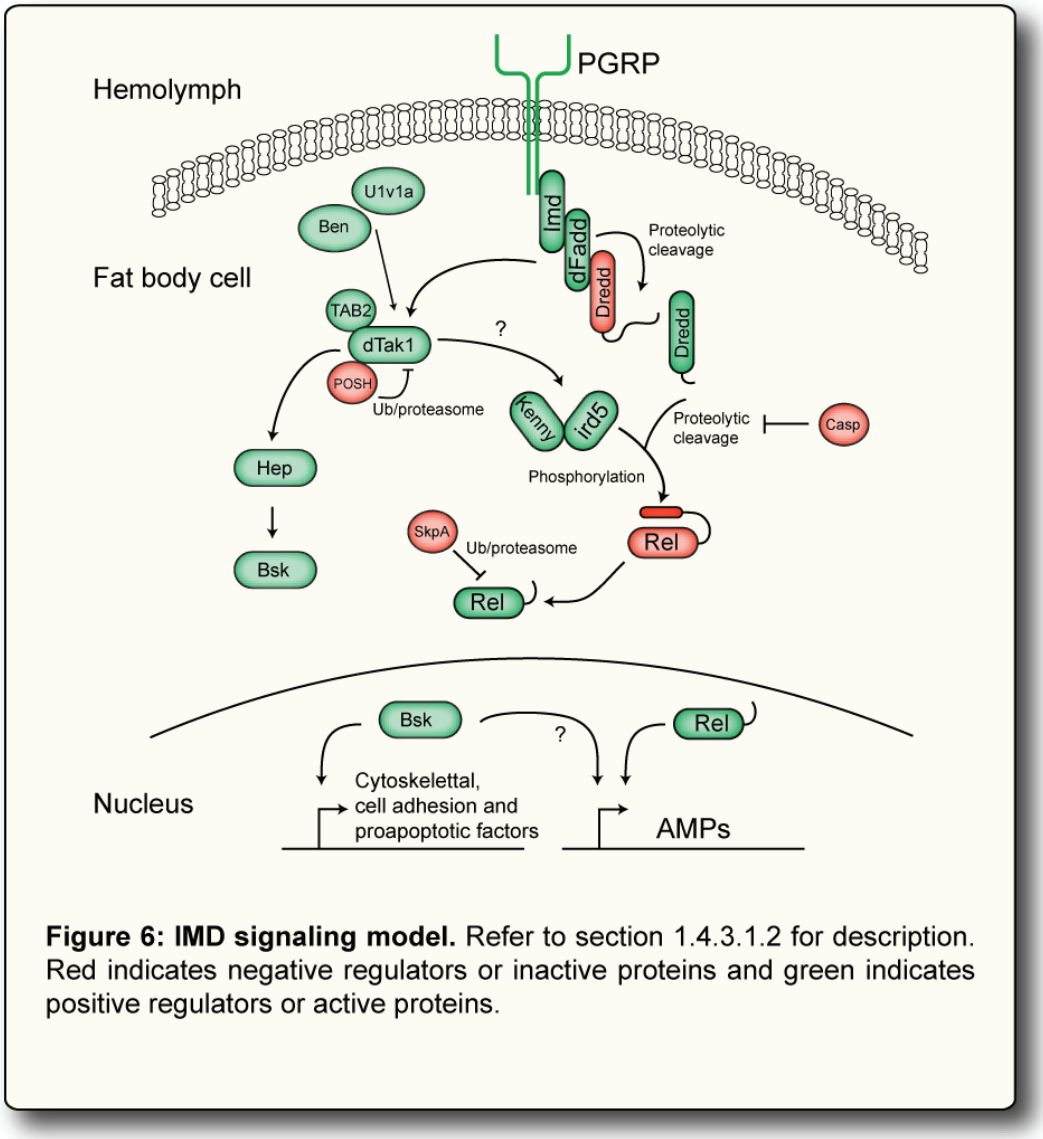

Figure 6: IMD signaling model. Refer to section 1.4.3.1.2 for description. Red indicates negative regulators or inactive proteins and green indicates positive regulators or active proteins.

undefined mechanism, the Imd/dFadd/Dredd complex activates the SAPKKK Tak1 (Vidal et al., 2001). Signaling between Imd and Tak1 also requires the ubiquitin-conjugating enzymes Bendless (Ben; mammalian homolog of ubc13) and Uev1A (Zhou et al., 2005). In mammals, these E2 enzymes are implicated in the synthesis of polyubiquitin chains linked through Lys63 of ubiquitin on TNF receptor-associated factor (TRAF) proteins, which are downstream effectors of the TNF, IL1 and Toll

receptors. This type of ubiquitination does not lead to degradation, but to activation of Tak1. This activation of Tak1 requires an associated protein, Tak1 Binding Protein 2 (TAB2), which specifically binds to Lys63 polyubiquitin chains through a conserved zinc finger domain (Chen et al., 2006b). Although TRAF proteins are conserved in Drosophila, none of them have been implicated in innate immunity. However, formation of a Tak1/TAB2 complex is required for the activation of the IMD pathway (Zhuang et al., 2006a). In Drosophila, Tak1 is also the target of Plenty of SH3s (POSH), a scaffolding molecule that possesses E3 ligase activity. It was reported that POSH is required for properly timed activation and termination of the IMD cascade by inducing the proteasomal degradation of Tak1 (Tsuda et al., 2005). Genetic analyses placed the IκB kinase (IKK) complex downstream of Tak1 during immune challenge. The IKK complex comprises the Ser/Thr kinase IKKβ (or ird5) as well as an associated protein IKKγ (Kenny) and mediates the phosphorylation of NFκB-related protein Rel and its subsequent endoproteolytic cleavage by active caspase Dredd (Lu et al., 2001; Rutschmann et al., 2000; Silverman et al., 2000; Stoven et al., 2000). Cleaved Rel is no longer retained in the cytoplasm and translocates to the nucleus where it activates the transcription of AMPs. Modulation of Rel activity occurs via two mechanisms. First, it is suggested that the level of Rel is downregulated by SkpA, an homolog of the mammalian and yeast Skp1 proteins that are components of Skp1/Cullin/F-box protein (SCF)-E3 ubiquitin ligase (Khush et al., 2002). Second, it was recently demonstrated that Caspar, a protein

homologous to mammalian Fas-associating factor 1 (FAF1) suppresses IMD-mediated immune response by preventing Dredd-dependent cleavage and nuclear translocation of Rel (Kim et al., 2006b).

As mentioned earlier, the IMD pathway also promotes immune resistance via activation of the JNK pathway. However, the molecular mechanism by which JNK pathway induces the Gram- bacterial immune response remains a subject of debate. Earlier signaling models implicated Tak1 in the activation of IKK/Rel and Hep/Bsk. The IKK/Rel branch induced the transcription of AMPs while the Hep/Bsk cascade induced genes coding for cytoskeletal, cell adhesion and proapoptotic factors. However, it was recently demonstrated that although Tak1 mutant flies are sensitive to bacterial challenge and fail to properly induce AMPs, the activation of Rel was normal in those flies (Delaney et al., 2006). These results strongly challenged the placement of Tak1 upstream of IKK complex and the hypothesis that activation of the JNK pathway is unable to trigger AMP production. It was also demonstrated that expression of an activated form of Hep rescues AMPs production in infected *tak1* mutant flies and that AMP gene expression upon infection is blocked in JNK mutant clones generated in fat bodies. These discrepancies may be of two sources. First, the original positioning of Tak1 upstream of IKK/Rel was based on the fact that Tak1 mutations blocked the constitutive activation of AMPs by IMD overexpression while IKK mutations blocked Tak1 induced AMPs production. However, if both the JNK and IKK/Rel were required for

induction of AMPs, disruption of either pathway would suppress any upstream activation. The second source of divergence may come from the fact that most studies implicating the JNK cascade in IMD pathway were done in S2 cells. Further studies in whole organisms are required to clarify the mode of action of the JNK pathway upon Gram- bacterial infection.

1.4.3.2 The p38 pathway

Although Drosophila was efficient at deciphering the ERK and JNK cascades, very little information was collected regarding the p38 pathway (Martin-Blanco, 2000; Stronach and Perrimon, 1999). Homology-based screening and functional complementation in yeast isolated Licorne (Lic) as being the Drosophila homolog of MKK3/6. One study reported the isolation of an atypical *lic* allele. The *hep* and *lic* loci are directly adjacent on the X chromosome and therefore a P-element insertion in the *hep* gene was used to generate an excision-induced deletion of both genes. By providing Hep function from a transgene, the specific impact of *lic* deletion was studied. *lic* gene was shown to be required during oogenesis for egg asymmetric development (Suzanne et al., 1999).

Using similar approaches as for *lic*, two Drosophila p38 genes were isolated, namely *p38a* and *p38b*. Cell-based assays in Drosophila S2 cells revealed that, as their mammalian counterparts, Drosophila *p38a/b* are activated by various environmental stresses including heat and

osmotic shock, UV and bacterial peptidoglycan (Han et al., 1998; Zhuang et al., 2006b). Characterization of the sole mutant allele of *p38a* revealed that it is required for resistance to dry starvation, heat shock and H_2O_2 treatments (Craig et al., 2004). In addition, expression of Mekk1 in flies is apparently required for p38-mediated heat and osmotic shock resistance (Inoue et al., 2001). Finally, reverse genetic experiments revealed that reduction in p38b activity disrupts Drosophila TGFβ signaling (called decapentaplegic or dpp). Indeed, overexpression of a dominant negative version of p38b generated wing defects similar to dpp loss-of function. In addition, phenotypes associated with constitutive activation of Dpp signaling were suppressed by p38b antisense RNA expression or the p38 pharmacological inhibitor SB203580 (Adachi-Yamada et al., 1999b). Given that mammalian TGFβ partly signals via Tak1/MKK3/p38, it is thus possible that similar functions are attributable to Dpp signaling during wing development (Hanafusa et al., 1999; Yamaguchi et al., 1995).

1.5. Rationale for experimentation

The MAPK pathways are evolutionarily conserved signaling modules implicated in various developmental processes and in the maintenance of homeostasis in adult organisms. Genetic alterations that lead to improper MAPK-dependent signaling generally lead to pathological conditions such as neuronal degeneration, inflammation disorders or cancer. Although most of the core components of this signal transduction cascade have been discovered, relatively little is known concerning their mode of regulation. For example, one specific extracellular stimulus is transduced by a MAPK module, but yet elicits different biological outputs depending on the cellular context. What are the molecular mechanisms that define the cellular context?

The use of genetically amenable organisms such as *D. melanogaster* is very useful for the rapid identification and characterization of new regulators of the MAPK pathways. In addition, half of the fly proteins have mammalian counterparts and it is therefore likely that a new MAPK regulator found in Drosophila would be conserved in mammals. Using a forward genetic approach in flies, my thesis director has isolated new putative regulators of the Ras/MAPK pathway in Drosophila. Two of them, namely KSR and CNK were found to be key positively acting components within the Ras/MAPK pathways in various organisms. In addition, he also identified a new locus that genetically behaved as a

Ras/MAPK negative regulator. The aim of my Ph.D was to characterize genetically and molecularly the function of this new gene.

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Alphabet, a Ser/Thr phosphatase of the Protein Phosphatase 2C family, negatively regulates Ras/MAPK signaling in Drosophila.

Alphabet, a Ser/Thr phosphatase of the Protein Phosphatase 2C family, negatively regulates Ras/MAPK signaling in Drosophila

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2.1 Abstract

Signal transduction through the Ras/Mitogen-Activated Protein Kinase (MAPK) pathway depends on a diverse collection of proteins regulating positively and negatively signaling flow. We previously conducted a genetic screen in Drosophila to identify novel components of this signaling pathway. Here, we present the identification and characterization of a new gene, *alphabet* (*alph*), whose activity negatively regulates Ras/MAPK-dependent developmental processes in Drosophila and this, at a step downstream or in parallel to Ras. *alph* encodes a protein phosphatase 2C (PP2C) family member closely related to the mammalian PP2C alpha and beta isoforms. Interestingly, although *alph* gene product does not appear to be essential for viability, its elimination leads to weak, but significant developmental defects reminiscent of an overactivated Ras/MAPK pathway. Consistent with this interpretation, strong genetic interactions are observed between *alph* alleles and mutations in bona fide components of the pathway. Together, this work identifies a PP2C of the alpha/beta subfamily as a novel negative regulator of the Ras/MAPK pathway and suggests that these evolutionarily conserved enzymes play a similar role in other metazoans. Finally, despite the relatively large size of the *PP2C* gene family in metazoans, this study represents only the second genetic characterization of a PP2C in these organisms.

Keywords: signal transduction, Ras, MAPK, PP2C, phosphatase, Drosophila development

2.2 Introduction

The Raf/MEK/MAPK pathway is a pivotal route through which the small GTPase Ras transmits proliferation, differentiation or survival signals elicited by membrane receptors (Campbell et al., 1998; Chang and Karin, 2001; Katz and McCormick, 1997)). Upon activation, GTP-loaded Ras physically associates with Raf thereby initiating a complex series of events leading to Raf activation. This step triggers the well-documented activating phosphorylation cascade from Raf to MEK and from MEK to MAPK. Activated MAPK then moves to the nucleus or to other cytoplasmic locations and modulate by phosphorylation the activity of a large set of substrates that in turn will orchestrate a cell-specific response (Kolch, 2000)).

In addition to the three basic kinases, a surprisingly large number of regulatory components have been found to modulate the pathway and their characterization, although not complete, is unveiling complex mechanisms shaping signaling flow (Chong et al., 2003; Morrison and Davis, 2004; Kolch, 2005). Various strategies have been devised to identify new components of the Ras/MAPK pathway. For instance, one of these approaches exploited Drosophila eye development as an assay to isolate mutations in genes mediating or opposing Ras/MAPK signaling (St Johnston, 2002). During eye development, cell fate specification is governed by the reiterated use of EGFR-stimulated Ras/MAPK signaling

(Wolff and Ready, 1993). Therefore, up- or down-regulation of the pathway during eye development, both by natural or artificial means, alter cell differentiation and as a consequence, often leads to the visible roughening of the adult external eye surface. Based on this property, components of the pathway with gain-of-function or dominant-negative activity have been expressed in the eye and the resulting eye phenotypes were used in dominant modifier screens ((Dickson et al., 1996; Karim et al., 1996; Rebay et al., 2000; Therrien et al., 2000). Although these screens identified several new positively acting genes, significantly fewer negative regulators were uncovered by this approach. Nonetheless, as expected for a pathway that relies on phosphorylation events, two of the genetically-defined negative loci encoded phosphatases. One of these, microtubule star (Mts), corresponds to the catalytic subunit of protein phosphatase 2A (PP2A) and appears to negatively regulate an event between Ras and Raf (Wassarman et al., 1996). The second one, named Protein Tyrosine Phosphatase-Enhancer of Ras (PTP-ER), was found to specifically inactivate MAPK by dephosphorylating the tyrosine residue of the activation loop TEY motif (Karim and Rubin, 1999b). More recently, genetic studies revealed that the Drosophila dual-specificity phosphatase MKP-3 also negatively regulates the Ras/MAPK pathway during development (Kim et al., 2004).

We previously conducted a modifier screen based on a Kinase Suppressor of Ras (KSR)-dependent rough eye phenotype to identify

novel components of the Ras/MAPK pathway (Therrien et al., 2000). KSR is a Raf-related protein that is thought to act as a scaffold of the MAPK module by bridging Raf, MEK and MAPK together (Morrison and Davis, 2004). Expression of the isolated kinase domain of KSR during Drosophila eye development inhibits photoreceptor cell differentiation and produces a rough eye as it interferes with Ras-mediated MAPK activation owing to its presumed ability to sequester MEK (Therrien et al., 1996; Roy et al., 2002). We predicted that loss-of-function mutations in positively-acting components should worsen the KSR dominant negative (KDN) phenotype and thus should be recovered as *Enhancers of KDN*, whereas loss-of-function mutations in genes encoding negative regulators should fall within the *Suppressors of KDN* category. Fifteen groups of enhancers were isolated and the corresponding genes have been identified for several of these, including known positively-acting loci such as *egfr*, *Ras*, and *mapk* (Therrien et al., 2000). In contrast, only four groups of suppressors were isolated. Here, we report the identification and characterization of one of these groups called *Suppressor of KDN 3-1* (*SK3-1*). The *SK3-1* locus, renamed *alphabet* (*alph*), encodes a protein phosphatase 2C (PP2C) highly related to the mammalian PP2C alpha and beta isoforms. Alph activity appears to be dispensable for viability as mutations that eliminate or severely reduce catalytic function are homozygous viable. Nonetheless, homozygous mutant flies have phenotypic defects reminiscent of a hyperactivated Ras/MAPK pathway. In agreement with this interpretation, *alph* alleles dominantly enhanced phenotypes caused by activated

components of the Ras/MAPK and genetically interacted with mutations in bona fide components of the pathway, whereas ectopic expression of the wild type form had the opposite effect. Finally, our genetic interaction data suggest that Alph activity is required downstream or in parallel to Ras. Together, these findings identify a phosphatase of the PP2C family as a novel negative regulator of Ras/MAPK signaling in Drosophila.

2.3 Materials and methods

Genetics and fly stocks

Fly maintenance, genetic interaction studies and P[w^+] mapping were conducted according to standard procedures. The *PBac{PB}CG1906*^{c04528} and *EP* lines were obtained from the Bloomington and Szeged stock centers, respectively. The *sE-alph* and *UAS-alph* lines were generated by P-element-mediated germline transformation as described (Rubin and Spradling, 1982).

Homozygous mutant clones of *alph*$^{S-331}$ were generated by the FRT technique (Xu and Rubin, 1993) using the homologous *FRT*^{82B}, *Ubi-GFP* chromosome. Eye-specific clones were induced using the *eyFlp1.2* transgene (Therrien et al., 1998). *UAS-Ras*^{V12} flies were described previously (Karim and Rubin, 1998). Ras^{V12}- and Ras^{V12}/Alph-expressing clones were induced 72 hr after egg deposition (AED) by a 10 min heat shock at 37^{o}C. The *y,w,hs-flp*^{122} ; *Act>CD2>Gal4* ; *UAS-GFP* line was kindly provided by M. Miron and was previously described in Neufeld et al. (1998).

Molecular analysis of alph alleles

Genomic DNA from each *alph* homozygous viable alleles (*XS-88, S-331 and S-355*) was used to amplify by PCR the predicted exons of the *alph* locus (Chapter 2, Figure 2A). The exons were then directly

sequenced from the PCR products. Mutations were confirmed by sequencing a second PCR product from a distinct DNA sample.

Plasmids

alph cDNAs corresponding to the open reading frame (ORF) of transcripts RA/C/D and RB (Chapter 2, Figure 2) were amplified by PCR from the LD cDNA library (BDGP), cloned into the Not1 site of pBlueScript (BS) II (Stratagene) and entirely sequenced. The third variant (RE) was generated by PCR using a 3'-oligonucleotide encoding the C-terminal Alph-PE extremity (Chapter 2, Figure 2C). The three *alph* cDNAs were then moved in either *psE* (Dickson et al., 1992) or *pUAST* (Brand and Perrimon, 1994) P-element vectors.

The *pBS-alph* construct (transcript RA/C/D) was used as a template for mutagenesis (QuickChange^{TM}; Stratagene) to generate the three mutations identified in the screen (Chapter 2, Figure 2B) as well as a phosphatase-inactivating R189A mutation. Wild-type and mutant versions were then moved into *pGEX-4T3* (Amersham) to produce GST fusion proteins in BL21 cells (Novagen).

Alph antibodies and fly extracts

Rabbit polyclonal antibodies were raised (Sigma Genosys) against full-length Alph fused to GST as described above. Crude antiserum was

directly used in Western blots (1/2000) to detect endogenous Alph from either wild type or mutant fly protein extracts. The lysates were prepared by homogenizing tissues or whole animals of the indicated stage in NP-40 lysis buffer (Therrien et al. 1996). Anti-actin clone 4 (1/1000; Boehringer-Mannheim) was used to monitor protein loadings.

Phosphatase assay

Phosphatase assays were conducted in 50 µl of solution A (50 mM Tris-HCl pH 7.0, 0.1% β-mercaptoethanol) supplemented with 60 mM MgOAc, 1 mg/ml BSA and 1 µg ^{32}P-casein. Reactions were initiated by adding GST-Alph proteins (2.5 µg) and incubated at 30°C for 30 and 90 min. Reactions were stopped by adding 20% trichloroacetic acid (TCA). Non-precipitable [^{32}P] was determined by scintillation counting. ^{32}P-labeled phospho-casein was prepared according to McGowan and Cohen (1988).

Histology

Scanning electron microscopy and sectioning of adult fly eyes were performed as described by Wassarman et al. (2000) and (Tomlinson and Ready, 1987), respectively. Adult wings were mounted in Canada balsam (Sigma) on a glass slide.

Eye imaginal discs from third instar larvae or pupae (45h after pupal formation) were dissected in plain Schneider medium (Invitrogen), fixed in

PBS 1X + 4% paraformaldehyde for 15 min at room temperature and washed three times with PBT (PBS 1X + 0.2% Triton X-100). Primary antibodies were incubated in PBT + 2.5% fat-free dry milk overnight at 4ºC with gentle rocking. Primary antibodies and dilutions were: mouse anti-Cut (1/1000; DSHB), rabbit anti-Dlg (1/2000; Woods and Bryant, 1991), rabbit anti-BarH1 (1/200; Higashijima et al., 1992), rat anti-Elav (1/1000; DSHB), mouse anti-dpMAPK (1/1000; Sigma). Samples were then washed with PBT and incubated with appropriate species-specific Cy3-, Cy5-, or FITC-conjugated secondary antibodies (1/1000; Jackson Immuno-Research Laboratories) for 2 hr at room temperature in PBT + 2% BSA. Samples were again washed with PBT + 2% BSA, then mounted in Vectashield (Vector Laboratories) and analyzed by confocal microscopy.

In situ hybridization

Alph sense and antisense RNA probe (~700 bp) preparation, embryo fixation, post-fixation treatment and hybridization were performed as described in (Parthasarathy, 2005)(*in press*; also available at http://www.utoronto.ca/krause/). After hybridization, embryos were "electrowashed" at 100V for 30 min in wells of a 2% agarose gel (TAE 1X). They were then washed for 10 min in PBT (1X PBS, 0.1% tween-20) and blocked with 20% heat-inactivated FBS in PBT for 1 hr. Embryos were then incubated with anti-digoxigenin (Roche; 1/750) in 20% heat-inactivated FBS for 1hr and subsequently washed for 1hr in PBT.

Detection was done using the NBT/BCIP solution from Roche as described by the manufacturer.

2.4 Results

SK3-1 alleles enhance Ras/MAPK signaling during eye development

The *SK3-1* complementation group comprises four alleles (Therrien et al., 2000). The ability of an allele (*S-355*) to dominantly suppress the KDN rough eye phenotype is shown in Figure 1 (compare B and C). To confirm that the suppression reflected an augmented number of photoreceptor cells, we sectioned adult fly retinas and counted the number of ommatidia missing the R7 photoreceptor cell. In a *KDN* / + genetic background, 70% of the ommatidia were missing the R7 cell, whereas this number was reduced to 20% in a *KDN* / *SK3-1*$^{S-355}$ background (Chapter 2, Table 1). Similar results were found with a second allele (*S-331*, Chapter 2, Table 1). This effect was not specific to the R7 cells as the alleles also suppressed the loss of outer photoreceptors caused by KDN (data not shown). Together, these results suggested that the *SK3-1* locus encodes a protein that negatively regulates photoreceptor cell differentiation.

Given that photoreceptor cell differentiation depends on Ras/MAPK signaling, it is possible that the *SK3-1* alleles specifically increased signaling through this pathway. Consistent with this possibility, the alleles enhanced the rough eye phenotype caused by expression of a gain-of-function of Ras (Ras^{V12}; Chapter 2, Figure 1, compare D and E and (Therrien et al., 2000). The enhancement was not restricted to Ras^{V12} as

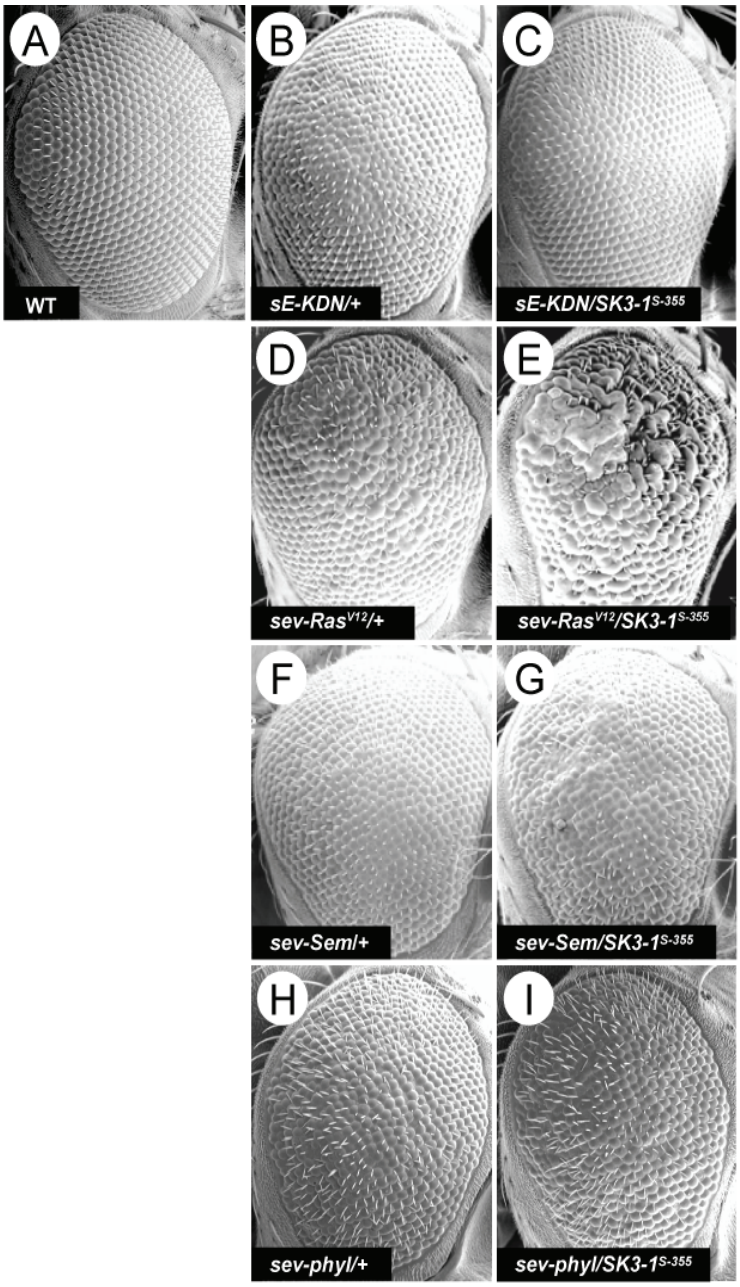


Figure 1. *SK3-1* modulates Ras/MAPK rough eye phenotypes. Scanning electron micrographs of adult eyes of the following genotypes: (A) *WT*. (B) *P[sE-KDN]/+*. (C) *P[sE-KDN]/+ ; SK3-1^{S-355}/+*. (D) *P[sev-RAS^{V12}]/+*. (E) *P[sev-RAS^{V12}]/+ ; SK3-1^{S-355}/+*. (F) *sev-Sem/+*. (G) *sev-Sem/SK3-1^{S-355}*. (H) *sev-phyl/+*. (I) *sev-phyl/SK3-1^{S-355}*. Anterior is to the right.

similar rough eye phenotypes produced by either an activated Sevenless receptor (Sev^{S11}; Basler et al., 1991), activated Raf (Tor^{4021}Raf; (Dickson et al., 1992) or activated MAPK (Sevenmaker [Sem]; (Brunner et al., 1994) were also augmented by the alleles (Chapter 2, Figure 1, compare F and G; and data not shown). Furthermore, tangential adult eye sections confirmed the ability of the *SK3-1* alleles to increase the number of extra R7 cells produced by the $MAPK^{SEM}$ transgene (Table 1 and data not shown).

Given that the effect of Ras^{V12} expressed in the eye does not appear to be modifiable by mutations in upstream components (Karim et al. 1996; and unpublished observation), it suggests that *SK3-1* activity is acting downstream or in parallel to Ras. However, similar conclusions could not be made with Tor^{4021}Raf or $MAPK^{SEM}$ as these activated variants still appear to be influenced by upstream events (Oellers and Hafen, 1996; Wassarman et al., 1996). We also tested the position of Alph activity with respect to a transcriptional target of the Ras/MAPK pathway known as *phyllopod* (*phyl*; (Chang et al., 1995; Dickson et al., 1995). Like activated signaling components of the Ras/MAPK pathway, overexpression of Phyl during eye development is sufficient to drive the R7 cell differentiation program in non-neuronal cells (Chang et al., 1995). Interestingly, unlike the other activated components, the *SK3-1* alleles did not enhance the rough eye or the number of additional R7 cells produced by Phyl overexpression (Chapter 2, Figure 1, compare H and I; and Table 1).

Table 1. *SK3-1* genetic interaction studies

Genotype	Mean number of R7 cells / ommatidium
WT	*1*
sE-KDN / +	0.30 ± 0.015 (n = 892)
sE-KDN / + ; SK3-1^{S-331} / +	0.76 ± 0.015 (n = 774)
sE-KDN / + ; SK3-1^{S-355} / +	0.80 ± 0.016 (n = 640)
sev-Sem / +	2.55 ± 0.034 (n = 811)
sev-Sem / + ; SK3-1^{S-331} / +	2.75 ± 0.037 (n = 538)
sev-Sem / + ; SK3-1^{S-355} / +	3.39 ± 0.049 (n = 412)
sev-phyl / +	1.71 ± 0.038 (n = 550)
sev-phyl / + ; SK3-1^{S-331} / +	1.72 ± 0.047 (n = 504)
sev-phyl / + ; SK3-1^{S-355} / +	1.70 ± 0.038 (n = 612)
SK3-1^{S-331}	1.04 ± 0.011 (n = 406)
PBac{PB}CG1906^{c04528}	1.03 ± 0.012 (n = 344)
yan^{P}	1.37 ± 0.029 (n = 411)
yan^{P}; SK3-1^{S-331} / +	1.86 ± 0.030 (n = 804)
yan^{P} / + ; SK3-1^{S-331}	1.14 ± 0.021 (n = 477)
yan^{P}; SK3-1^{S-331}	2.88 ± 0.069 (n = 288)
yan^{P}; PBac{PB}CG1906^{c04528}	2.70 ± 0.055 (n = 349)
PTP-ER$^{XE-3022/XE-2776}$	1.05 ± 0.009 (n = 733)
PTP-ER$^{XE-3022}$ / + ; SK3-1^{S-331}	1.08 ± 0.014 (n = 401)
PTP-ER$^{XE-3022/XE-2776}$; SK3-1^{S-331} / +	1.43 ± 0.029 (n = 538)
PTP-ER$^{XE-3022/XE-2776}$; SK3-1^{S-331}	1.91 ± 0.043 (n = 414)

Adult eyes of the indicated genotypes were sectioned and the mean number of R7 photoreceptor cells ±S.E.M. was determined from at least three eyes of independent flies per genotype. The number (n) of ommatidia analyzed is also indicated.

Taken together, these findings suggest that *SK3-1* activity is required at a step downstream or in parallel to Ras, but upstream of Phyl.

The SK3-1 locus encodes a homologue of the mammalian Protein Phosphatase 2C α and β isoforms

The four *SK3-1* alleles had been previously mapped meiotically to 3-100 on the third chromosome (Therrien et al. 2000). Two of the alleles

(*SK3-1*$^{XS-88}$, *SK3-1*$^{S-331}$) are homozygous viable, whereas the other two (*SK3-1*$^{S-292}$, *SK3-1*$^{S-355}$) were originally identified as recessive lethal alleles. However, outcrosses of the *SK3-1*$^{S-355}$ allele separated the *SK* locus from a secondary, but non-relevant recessive lethal mutation shared with the *SK3-1*$^{S-292}$ allele (data not shown). Like *SK3-1*$^{XS-88}$ and *SK3-1*$^{S-331}$, the outcrossed *SK3-1*$^{S-355}$ allele is homozygous viable. Similarly, the *SK* locus presents in the *SK3-1*$^{S-292}$ line is probably also homozygous viable as this line is viable when tested in complementation assays with the other three viable alleles.

To precisely position the *SK3-1* locus, we further mapped the ability of *SK3-1*$^{S-355}$ to suppress the KDN rough eye phenotype with respect to P[*w*$^{+}$]-element lines of the EP collection inserted in the area (Rorth, 1996). This high resolution mapping technique allowed us to place the *SK3-1* locus exactly at equal distance between the insertion sites of *EP(3)3522* and *EP(3)1005* located at cytological position 99A$_7$ and 99C$_4$, respectively (data not shown). Using the FlyBase Genome Browser tool (http://flybase.bio.indiana.edu), the P-elements were found to be separated by ~ 550 kb of genomic DNA. In the middle of this interval, five candidate genes (*CG1906*, *CG7567*, *CG7568*, *CG11470* and *CG31041*) spanning a 50kb stretch are predicted to lie (data not shown). One of these, *CG1906*, encodes a serine/threonine (Ser/Thr) phosphatase of the PP2C family. As the Ras/MAPK pathway depends on phosphorylation events, we suspected that it might be the relevant gene. To test this

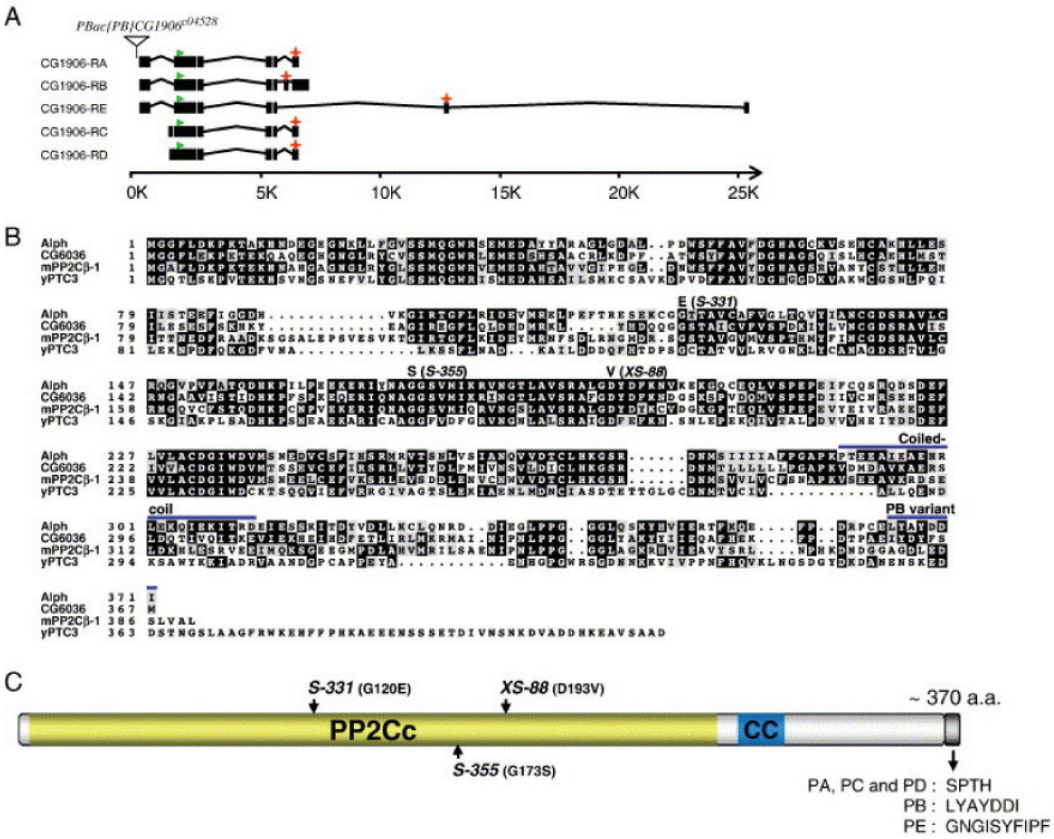


Figure 2. Molecular analysis of the *alphabet* gene. (A) The genomic organization of five predicted alternative transcripts (RA to RE; FlyBase) transcribed from the *CG1906/alph* locus is shown with green arrowheads and red stars representing the position of Start and Stop codons, respectively. (B) Amino acid sequence comparison of Alph and its closest Drosophila homologue, the CG6036 gene product, to mouse PP2Cβ-1 (NCBI accession #AAF56905) and S. cerevisiae Ptc3 (NCBI accession #P34221). Amino acid changes of *alph* mutant alleles are indicated on top of the sequence and a putative coiled-coil motif found in Alph (a.a 291-311) is highlighted in blue. (C) Schematic representation of the predicted Alphabet protein variants (~370 a.a. in length depending on their C-terminal tail [PA/C/D, PB and PE; FlyBase]) depicting the relative position of the catalytic domain (PP2Cc; a.a 13-282), the mutations found in *alph* alleles and the putative coiled-coil (CC) motif.

possibility, we isolated genomic DNA for each of the three viable alleles and sequenced the predicted exons of *CG1906* (Chapter 2, Figure 2A). In agreement with our hypothesis, all three alleles were found to have a single point mutation affecting a highly conserved residue within the catalytic domain (Chapter 2, Figures 2B and 2C), thereby providing

compelling evidence that the *SK3-1* locus corresponds to *CG1906*. In support for this conclusion, eye-specific expression of a *CG1906* cDNA fully rescued the ability of *SK3-1* alleles to dominantly modify the KDN or Ras^{V12} rough eye phenotypes (data not shown).

Five splicing variants are predicted to originate from the *CG1906* locus (Chapter 2, Figure 2A). These transcripts should give rise to three protein variants distinguishable by their unique C-terminal extremity (Chapter 2, Figure 2C). Amino acid sequence comparison to PP2C family members from other species identified mammalian PP2C alpha and beta isoforms as the closest homologues (~53% of amino acid identity, Chapter 2, Figure 2B, Suppl. Figure S1). Based on this similarity, the locus was renamed *alphabet* (*alph*). *Alph* transcripts appear to be widely distributed throughout embryogenesis (Chapter 2, Figure 3A). Furthermore, Alph proteins could be detected at various stages of fly development as well as in eye-antenal and wing imaginal discs (Chapter 2, Figure 3B). Although specific splicing variant expression remains to be determined, these results suggest that Alph activity is ubiquitously present in Drosophila.

During the course of this work, a PiggyBac transposon element (*PBac{PB}CG1906^{c04528}*) inserted ~100bp upstream of the first exon of the *CG1906-RA*, *-RB* and *-RE* transcripts (Chapter 2, Figure 2A) has been made available by Exelixis™ (Thibault et al., 2004). In contrast to the *SK3-1* mutations, this insertion eliminates Alph expression (Chapter 2, Figures

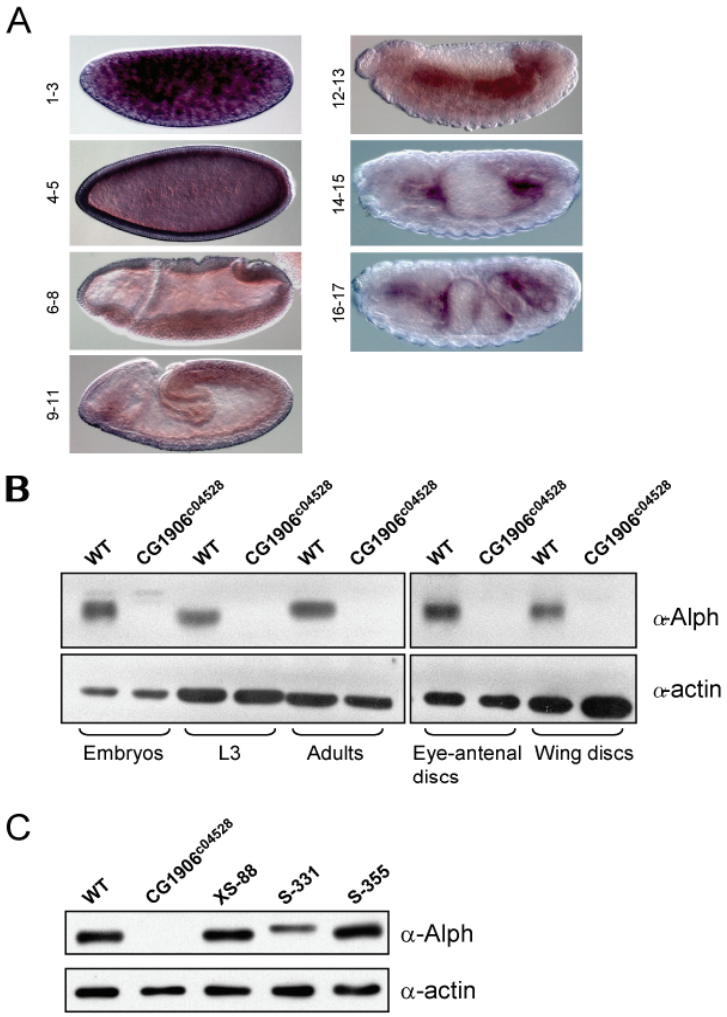


Figure 3. **Alph expression during Drosophila development.** (A) *In situ* hybridization of digoxigenin-labeled alph antisense RNA probe to wild-type embryos. Anterior is to the left and dorsal is up. Alph transcripts are detected at all stages (1-17) of embryogenesis and are widely distributed. The expression patterns are consistent with the high expression levels detected for CG1906 transcripts throughout embryogenesis by array profiling (Tomancak et al., 2002). Hybridization with a sense probe gave no specific signal (not shown). (B) Proteins lysates from the indicated sources (10 µg) and derived from wild-type (WT) or mutant (PBac{PB}CG1906c04528) genotypes were analyzed by Western blot using α-Alph polyclonal antibodies. Actin levels were monitored to control for equal protein loading. (C) Total adult fly extracts (30 µg) prepared from wild-type (WT) or the other indicated homozygous genotypes were analyzed as in B. The reduced level and mobility shift observed for the Alph^{S-331} protein is reproducible, but its significance is unknown.

3B and 3C) and thus possibly corresponds to a null mutation. Furthermore, this line is homozygous viable and is also viable over our four alleles or over a chromosomal deletion of the region (data not shown). Therefore, these data strongly suggest that the *alph* gene is not essential for viability. However, as slight eye and wing developmental defects are observed in homozygous *alph* alleles (see below), it indicates that Alph activity is important for the development of at least these two tissues.

Alph is a catalytically active phosphatase

Before characterizing further the role of Alph with respect to the Ras/MAPK pathway, we verified whether it is catalytically active and if so, examined the effect that the mutations recovered in the screen have on catalytic activity. To accomplish this, we separately fused to GST the wild type and the three mutant versions of Alph encoded by the *XS-88*, *S-331* and *S-355* alleles and evaluated their respective phosphatase activity. We also generated a phosphatase-dead control (R189A) based on a similar mutation previously shown to inactivate catalytic function of other PP2Cs (Chin-Sang and Spence, 1996; Takekawa et al., 1998). The GST fusion proteins were purified from bacterial lysates (Chapter 2, Figure 4A) and assayed using ^{32}P-labelled phospho-casein as a substrate. As shown in Figure 4B, the wild type protein displayed phosphatase activity while, as expected, the R189A variant was inert. Interestingly, all three mutations recovered in the screen had an impaired catalytic function and among which, the G120E mutant derived from the *S-331* allele was found to be

devoid of activity (Chapter 2, Figure 4B). Together, these results demonstrated that Alph is an active phosphatase and that the alleles recovered in the KDN screen are loss-of-function mutations.

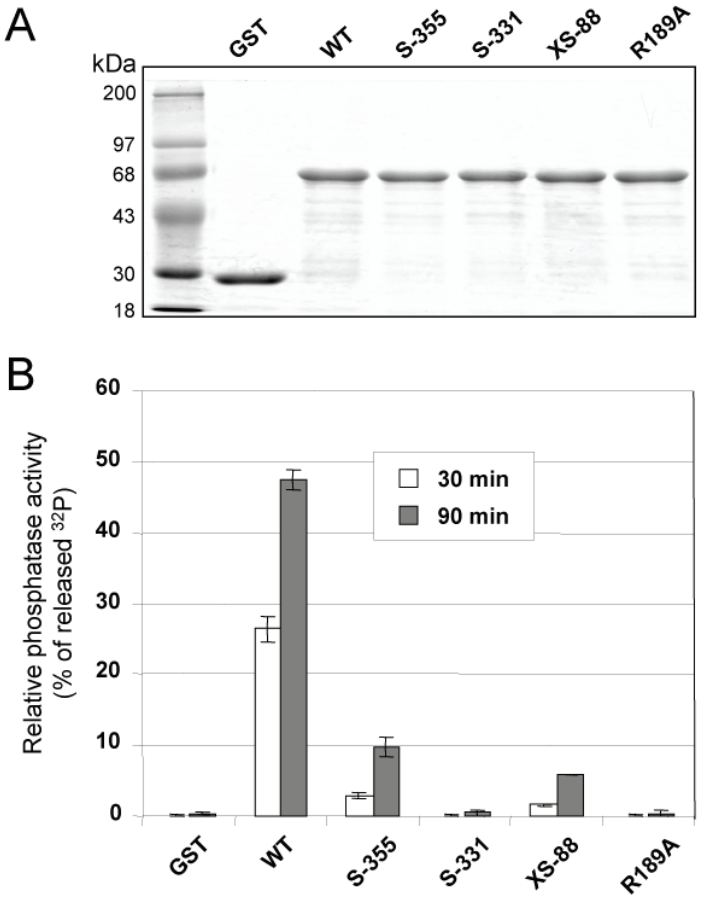


Figure 4. *alph* encodes a catalytically active phosphatase. (A) GST-Alph fusion protein variants were expressed and purified from E. coli lysates. Integrity and quantity of the GST protein preparations were assessed by loading 5 µg of proteins per sample on a 10% SDS-PAGE. Proteins were revealed by Coomassie staining. (B) Relative phosphatase activity is determined as the ratio of ^{32}P released over the total input of ^{32}P-casein in the presence of 2.5 µg of the indicated GST fusion proteins. Measurements were made after 30 and 90 min.

Alph negatively regulates cell fate specification during eye development

We were intrigued by the fact that the alleles recovered in the screen dominantly modified the KDN or Ras^{V12} rough eye phenotypes and yet the homozygotes displayed no externally visible phenotype in their eyes (not shown). One explanation for this observation could be that Alph activity is redundant with other proteins and that Ras-mediated events are only mildly altered in its absence in a wild type background, whereas in dosage-sensitive conditions, such as in KDN- or Ras^{V12}-expressing developing eyes, slight variations in signaling flow become perceptible. Nonetheless, we verified whether mild internal defects reminiscent of an up-regulated Ras/MAPK pathway could be detected in the homozygous lines. We sectioned adult eyes of $alph^{S-331}$ homozygotes and looked for the presence of additional R7-like photoreceptor cells, which is a classical hallmark of an over-activated Ras/MAPK pathway (Zipursky and Rubin, 1994). As reported in Table 1, the $alph^{S-331}$ allele indeed exhibited a low (4%), but significant number of extra R7 cells (for example, see Chapter 2, Figure 5D). Similar findings were made with the other alleles, including the *PBac{PB}CG1906*^{c04528} transposon line (Chapter 2, Table 1 and data not shown). Moreover, defects were not restricted to photoreceptor cells as staining of pupal eye discs with a cone cell marker (α-Cut) showed additional or missing cone cells in approximately 5% of the $alph^{S-331}$ ommatidia (Chapter 2, Figure 5, compare B and E). Mispositioned primary pigment cells (α-Bar, Chapter 2; Figure 5, compare C and F) as well as

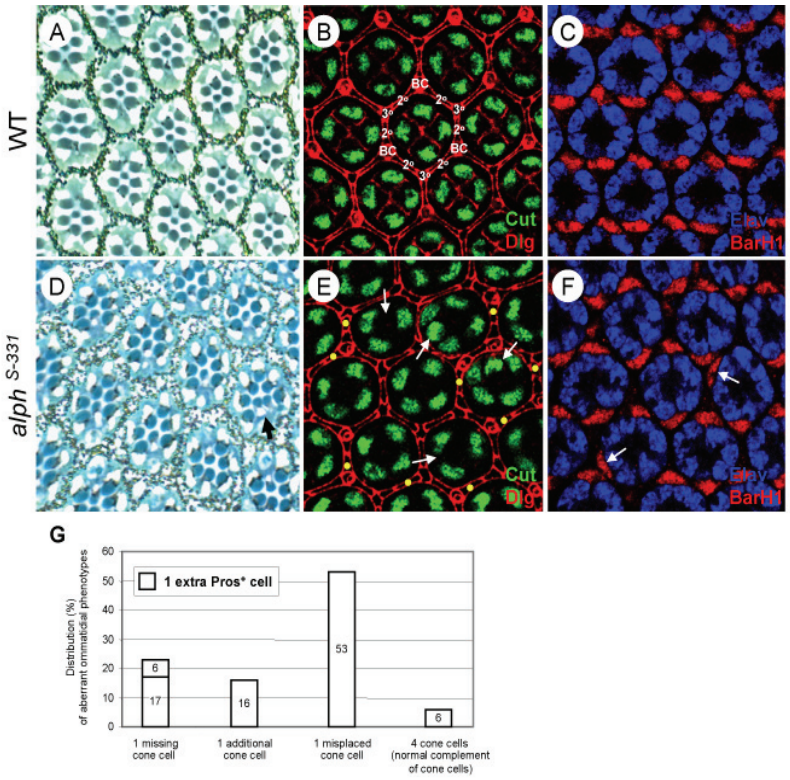


Figure 5. *alph* homozygous mutant ommatidia have abnormal numbers of differentiated cells. Upper panels (A, B, C) correspond to WT, while lower panels (D, E, F) correspond to *alph*$^{S-331}$ homozygous eye tissues. (A, D) Tangential sections of adult eyes at the R7 photoreceptor cell level. The arrow in D points to an ommatidium containing an additional R7 cell (the smaller size and the central location of the rhabdomere is characteristic of this class of photoreceptors). (B, E) immunofluorescence stainings of 45h pupal eye discs using α -Cut (reveals cone cell nuclei) and α-Dlg (reveals the outlines of secondary [2°] and tertiary [3°] pigment cells as well as bristle cells [BC]). A normal ommatidium contains four Cut-positive cells surrounded by twelve additional accessory cells (six 2° and three 3° pigment cells and three bristle cells) distributed in a stereotypical manner (Wolff and Ready, 1993). *alph*$^{S-331}$ mutant eyes have ommatidia with missing or additional cone cells (arrows). They also display additional pigment cells (yellow dots) or mispositioned 3° pigment and bristle cells. (C, F) Immunofluorescence stainings of 45h pupal eye discs using α-BarH1 (primary pigment cell marker) and α-Elav (neuronal marker) to highlight mispositioned (arrows) primary pigment cells in *alph*$^{S-331}$ mutant eyes. (G) Histogram showing the distribution of defects associated with 231 abnormally-constructed ommatidia with respect to cone cell / R7 cell differentiation. The data have been obtained by staining 45h pupal eye discs with α-Dlg (at a focal plane revealing cone cell outlines) and α-Pros (to reveal cells engaged towards the R7 cell fate).

additional secondary and tertiary pigment cells (α-Dlg, Chapter 2, Figure 5 compare B and E) were also observed. Therefore, the differentiation of extra eye-specific cells in the *alph* mutant eyes is consistent with an up-regulation of the Ras/MAPK pathway. Finally, given that ectopic activation of the pathway in cone cell precursors has been shown to lead to their transformation into supernumerary R7-like cells (Fortini et al., 1992), we investigated whether such an event might be occurring in the *alph* mutant eyes. Using antibodies to concomitantly reveal cone cell outlines (α -Dlg) and R7 cell-like nuclei (α-prospero; Kauffmann et al., 1996), we analyzed 231 abnormally constructed ommatidia (additional, missing or mispositioned cone cells) from pupal eye discs of the $alph^{S-331}$ genotype. Strikingly, approximately three out of four ommatidia with a missing cone cell (54 occurrences) also had an extra R7-like cell (Chapter 2, Figure 5G), thereby strongly suggesting that cone cell to R7 cell transformation is indeed taking place in Alph mutant eyes.

alph genetically interacts with other components of the Ras/MAPK pathway

Although mild, the defects observed in Alph mutant eyes provided compelling evidence that the encoded phosphatase plays a negative role on the Ras/MAPK pathway. To verify this hypothesis further, we tested if *alph* could genetically interact with bona fide negative regulators of the pathway such as *yan*, which encodes an ETS domain-containing transcriptional repressor (Lai and Rubin, 1992) and *PTP-ER* (Karim and

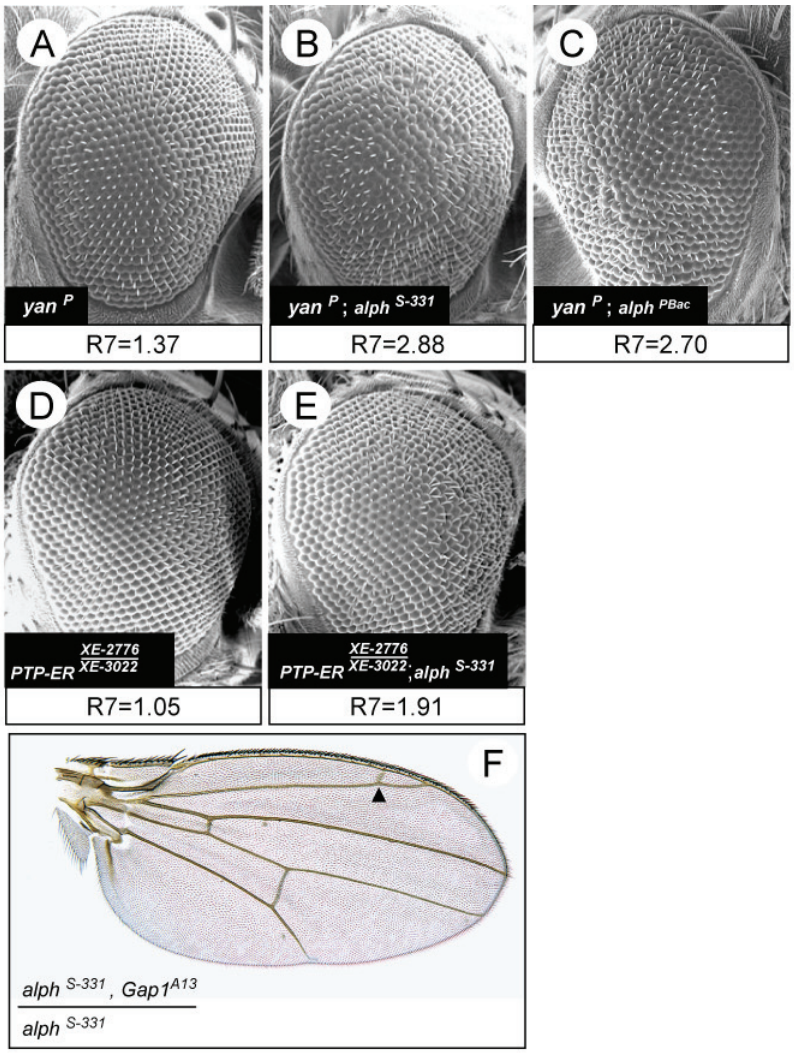


Figure 6. *alph* alleles genetically interact with *bona fide* negative regulators of the RAS/MAPK pathway. Scanning electron micrographs of adult eyes of the following genotype: (A) yan^{P} homozygote (B) yan^{P} ; $alph^{S\text{-}331}$ double homozygotes (C) yan^{P}; $alph^{PBac\{PB\}CG1906c04528}$ double homozygotes. (D) $PTP\text{-}ER^{XE\text{-}3022}/PTP\text{-}ER^{XE\text{-}2776}$. (E) $PTP\text{-}ER^{XE\text{-}3022}/PTP\text{-}ER^{XE\text{-}2776}$; $alph^{S\text{-}331}$ double homozygotes. The mean number of R7 cells per ommatidium is shown at the bottom of each micrograph. (F) Micrograph of an adult wing homozygous for $alph^{S\text{-}331}$ and heterozygous for $Gap1^{A13}$. The arrowhead points to a typical location (distal end of the L2 vein) where additional wing vein material is occurring in this genotype. In contrast to $Gap1^{A13}$ heterozygotes which do not have any wing vein defects, $alph^{S\text{-}331}$ homozygotes show a low penetrance of wings with extra vein material (~1%) appearing at the indicated location. Strikingly, this number rose to ~50% when a $Gap1^{A13}$ heterozygous allele is introduced in an $alph^{S\text{-}331}$ homozygous background.

Rubin, 1999). As predicted, we found that one copy of the *alph*$^{S-331}$ allele enhanced from 37 % to 86 % the number of extra R7 cells observed in a homozygous viable hypomorphic allele of *yan* (*yan*^{P}, Chaper 2, Table 1). Strikingly, when tested as a double homozygote, 100 % of the ommatidia had extra R7 cells with a mean number of close to three R7 cells per ommatidium (Chaper 2, Table 1), which visibly increased eye roughness (Chapter 2, Figure 6, compare A and B). Similar results were obtained using the *PBac{PB}CG1906*^{c04528} allele (Chapter 2, Figure 6C).

Analogous analyses were conducted with *PTP-ER*. Like *alph*, homozygous or trans-heterozygous, *PTP-ER* alleles have a mild effect on cell fate specification in the eye (Karim and Rubin, 1999; Table 1). Remarkably, when tested as double homozygotes, *PTP-ER*$^{XE-3022}$ displayed a complete synthetic lethality with *alph*$^{S-331}$ (data not shown). Moreover, using a *PTP-ER* trans-heterozygous configuration (*XE-3022*/*XE-2776*) that did not show synthetic lethality when placed in an *alph*$^{S-331}$ homozygous background, a significant increase of extra R7 cells was nonetheless observed compared to the respective single homozygotes (Chapter 2, Table 1 and Figures 6D and 6E).

Genetic interactions were also observed with general components of the pathway and these interactions were not restricted to the eye. For example, a heterozygous *Gap1* allele (*Gap1*^{A13} / +) introduced in the *alph*$^{S-331}$ background not only increased the number of extra R7 cell (data not

shown), but also induced the formation of additional wing vein material (Chapter 2, Figure 6F). As wing vein development in Drosophila is governed by EGFR-elicited Ras/MAPK signaling (Schweitzer and Shilo, 1997), it suggests that Alph negatively regulates this process as well. Finally, another significant example was the finding that *alph* alleles suppressed the lethality associated with a hypomorphic allele of *Raf* (Therrien et al., 2000).

Table 2. *sE-alph* genetic interaction studies

Genotype	Mean number of R7 cells / ommatidium
sE-KDN / +	0.30 ± 0.015 (n = 892)
sE-KDN / + ; sE-alph / +	0.00 ± 0.000 (n = 482)
sev-Sem / +	2.55 ± 0.034 (n = 811)
sev-Sem / sE-alph	1.48 ± 0.031 (n = 546)
sev-phyl / +	1.71 ± 0.038 (n = 550)
sev-phyl / sE-alph	1.70 ± 0.034 (n = 622)
yan^{P}	1.37 ± 0.029 (n = 411)
yan^{P} *; sE-alph / +*	1.05 ± 0.012 (n = 283)
yan^{P} *; sE-alph*	1.00 ± 0.003 (n = 359)
sE-alph	1.00 ± 0.000 (n = 348)

Adult eyes of the indicated genotypes were sectioned and the mean number of R7 photoreceptor cells ±S.E.M. was determined from at least three eyes of independent flies per genotype. The data were obtained using a sE-alph line (#3; 3rd chromosome insertion) expressing the alph-RB transcript. Identical results were observed with multiple independent lines expressing the same transcript as well as with lines expressing either of the two other variants (RA/C/D and RE).

Forced expression of Alph during eye development antagonizes Ras/MAPK signaling

To complement the genetic interactions presented above, we verified whether Ras/MAPK-dependent events could be specifically altered by overexpressing Alph during eye development. To this end, we separately introduced the three spliced variants of *alph* downstream of the *sE* expression cassette in a P-element vector to drive transgene expression in a subset of differentiating cells in the eye, including cone cell and R7 cell precursors (Basler et al., 1991). Surprisingly, although several transgenic lines were obtained, none of these displayed external or internal eye defects (data not shown and Chapter 2, Table 2). This apparent lack of activity might be due to the fact that endogenous levels of Alph are not limiting. Therefore, forced expression of Alph in a wild type background might either not affect or negligibly reduce Ras/MAPK signaling. Given that expression of activated or dominant-negative components of the Ras/MAPK pathway sensitizes the pathway to the point that slight variations in signaling efficiency become detectable, we tested whether the expression of *alph* transgenes in such contexts would reveal their activity. Consistent with this hypothesis, overexpression of any of the three *alph* splicing variants enhanced eye roughness as well as the loss of R7 cells observed in the *sE-KDN* flies (Chapter 2, Figure 7, compare A and B; Table 2; and data not shown). Conversely, Alph expression suppressed Sev^{S11} (data not shown), Ras^{V12} (Chapter 2, Figure 7, compare C and D), Tor^{4021}Rafc (data not shown) or $MAPK^{SEM}$ (Chapter 2,

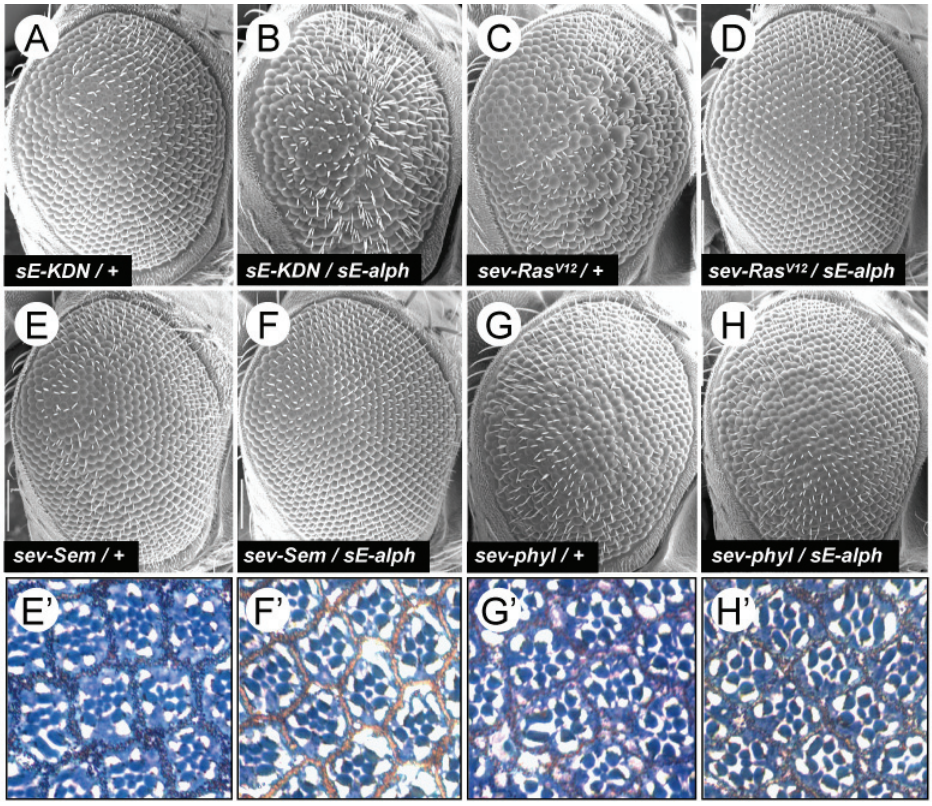


Figure 7. Overexpression of wild-type Alph modulates Ras/MAPK-dependent eye phenotypes. Scanning electron micrographs of adult eyes of the following genotypes: (A) *P[sE-KDN]/+*. (B) *P[sE-KDN]/+ ; P[sE-alph]/+*. (C) *P[sev-RAS^{V12}]/+*. (D) *P[sev-RAS^{V12}]/+ ; P[sE-alph]/+*. (E) *P[sev-Sem]/+*. (F) *P[sev-Sem]/P[sE-alph]*. (G) *P[sev-phyl]/+*. (H) *P[sev-phyl]/P[sE-alph]*. Anterior is to the right. (E' to H') Apical adult eye sections. Genotypes are as in E to H, respectively.

Figure 7, compare E and F) rough eye phenotypes as well as it reduced the number of additional R7 cells produced by MAPK^{SEM} (Chapter 2, Table 2 and compare Figures 7E' and 7F'). In agreement with its role upstream of *phyl*, Alph expression did not suppress the rough eye produced by *phyl* expression (Chapter 2, Figure 7, compare G and H) nor did it decrease the mean number of extra R7 cells present in those eyes (Chapter 2, Table 2 and compare Figures 7G' and 7H'). Moreover, the effect of Alph

expression was not restricted to phenotypes produced by overexpression as it also efficiently suppressed the rough eye phenotype and the extra R7 cells generated in a *yan*^{P} homozygous mutant background (Chapter 2, Table 2 and data not shown).

Alph overexpression blocks Ras-induced MAPK activation

The genetic tools that have been used to functionally link Alph to the Ras/MAPK pathway could not unambiguously determine whether it is acting upstream or downstream of MAPK. To address this point, we probed wild type and *alph*$^{S-331}$ third instar eye-antennal discs with an anti-phospho-MAPK antibody specific for the di-phosphorylated form of MAPK (dpMAPK; Gabay et al., 1997) and compared the respective levels of endogenously activated MAPK. If Alph was genuinely acting upstream of MAPK, elimination of its activity should specifically augment endogenous dpMAPK levels. We conducted multiple staining experiments, but failed to detect any significant difference between wild type and *alph*$^{S-331}$ genotypes (data not shown). We repeated those experiments in eye discs containing *alph*$^{S-331}$ homozygous mutant clones adjacent to wild type tissue to see if slight elevation of dpMAPK levels could then be detected, but again no significant variation was observed (Chapter 2, Figure 8A). Although these negative results would imply that Alph is acting downstream or in parallel to MAPK, they are probably inconclusive given the weak eye phenotype associated with the homozygous mutant alleles (Chapter 2, Figure 5). Indeed, it is possible that a slight elevation of dpMAPK levels undetectable

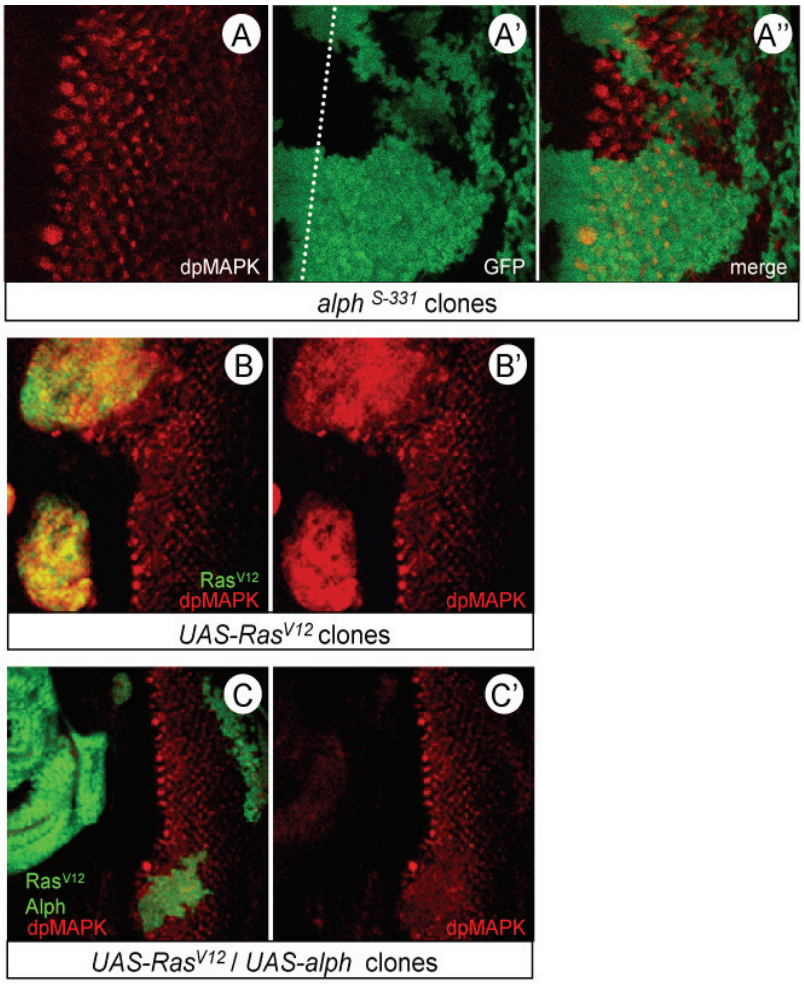


Figure 8. Alph overexpression blocks Ras^{V12}-induced MAPK activation. (A – C) Third instar larval eye discs were stained with α-dpMAPK to detect the di-phosphorylated (dp) actived form of MAPK. Anterior is to the left. (A) *alph*$^{S-331}$ homozygous mutant clones are marked by the absence of GFP staining. According to the intensity of the fluorescence signal, dpMAPK levels does not appear to be significantly elevated in homozygous mutant patches (GFP-negative. compared to adjacent heterozygous or homozygous wild-type areas. The dotted line represents the position of the morphogenetic furrow (Wolff and Ready, 1993). (B) As previously reported (Prober and Edgar, 2002), clonal expression of Ras^{V12} (clones are marked by GFP) leads to rounded clones appearing as cell masses which intensely stain for active MAPK. The yellow signal is caused by merging GFP and dpMAPK (Cy3) signals. (C) Clonal expression of Alph along with Ras^{V12} not only strongly attenuates Ras^{V12} dpMAPK levels, but also prevents the clones from adopting a rounded shape (this latter phenomenon also appears to depend on MAPK signaling (Prober and Edgar, 2002).

by immunofluorescence is responsible for the observed phenotype. Alternatively, it is possible that Alph is acting on MAPK itself and thus as the anti-dpMAPK antibody is specifically recognizing the dually phosphorylated form of MAPK on the TEY motif, a specific increase of the threonine's phosphorylated state would be undetectable.

We addressed the question differently and ask whether the ability of Alph ectopic expression to suppress the Ras^{V12} rough eye phenotype could be correlated with a reduction in Ras-induced dpMAPK levels. To accomplish this, we generated random clones of cells expressing Ras^{V12} during eye development using the Gal4-dependent "flip-out" technique (Struhl and Basler, 1993) and evaluated the ability of Alph co-expression to suppress dpMAPK production. As shown in Figure 8, high levels of dpMAPK associated with Ras^{V12}–expressing clones (panels B and B') were dramatically reduced upon Alph co-expression (panels C and C'). Similar findings were made using an activated Raf construct (data not shown). Although these results do not prove that Alph is acting upstream of MAPK, they support this view.

2.5 Discussion

In this paper, we identified and characterized a new gene encoding a Ser/Thr phosphatase of the PP2C family, named *alph*, which appears to oppose RTK-driven Ras/MAPK signaling during Drosophila development at a step downstream or in parallel to Ras. Its ability to antagonize EGFR and Sevenless activity suggests that its inhibitory effect is not restricted to a single RTK. As its substrate(s) is/are currently unknown, it remains unclear whether Alph activity is required after signal transmission to down-regulate specific components of the pathway or whether it is involved in maintaining background signaling levels prior to activation. Alternatively, it might be acting in a parallel pathway that in turn influences Ras/MAPK signaling.

Given that the signaling mechanism of the Ras/MAPK pathway depends on multiple Ser/Thr phosphorylation events, it is surprising that only two genes (*mts* and *alph*) encoding Ser/Thr phosphatases have thus far been identified through modifier screens in Drosophila (Wassarman et al., 1996; this study). Although it is possible that very few phosphatases are involved in reversing activating phosphorylation events, this situation might in fact be due to functional redundancy among particular Ser/Thr phosphatases. This could also explain why Alph activity is dispensable for viability and that homozygous mutant flies exhibit relatively weak phenotypes. Functional redundancy has indeed been observed among

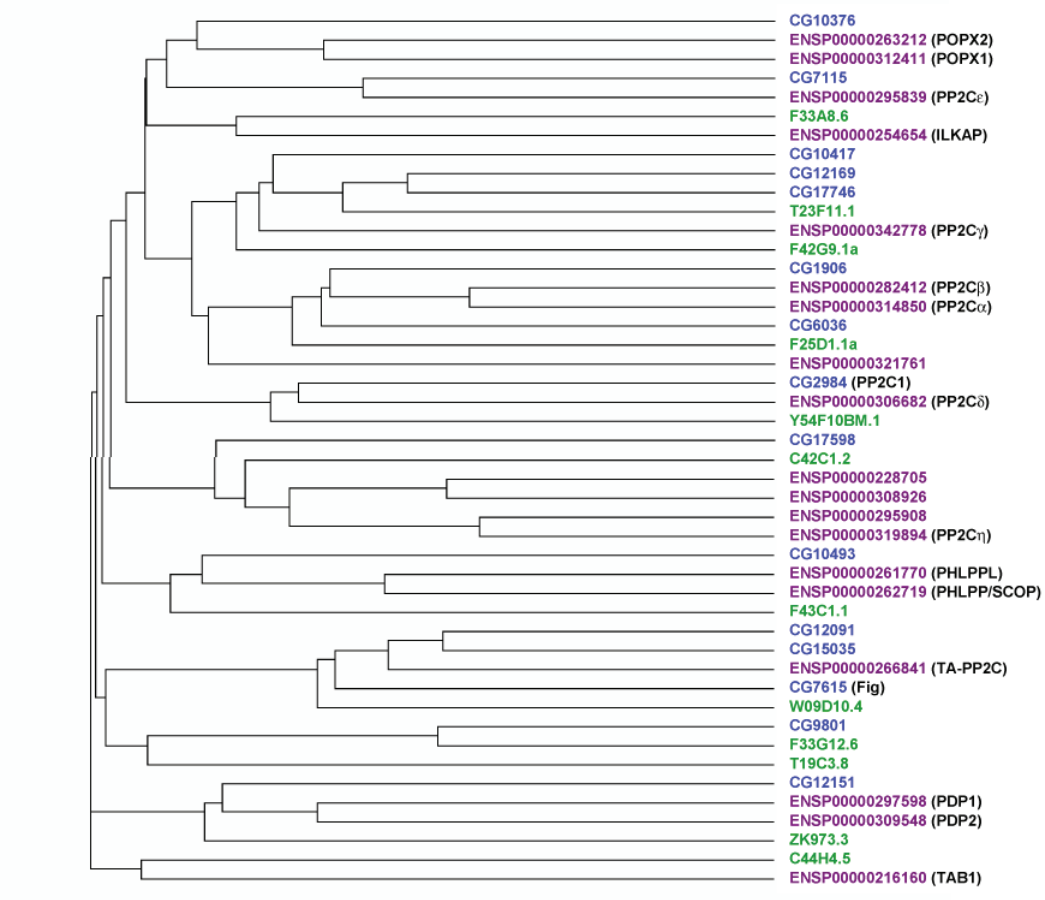
homologous PP2C phosphatases in yeasts (Young et al. 2002; Leroy et al., 2003). Although not mutually exclusive, high gene dosage might also explain the failure to identify phosphatases through modifier screens as most heterozygous mutations recovered in such screens (null and hypomorphic alleles) might not be strong enough to alter rough eye phenotypes. In this regard, it is interesting to note that the *PBac{PB}CG1906*^{c04528} line, which probably corresponds to a null mutation (Chapter 2, Figure 3), modified KDN and Ras^{V12} accordingly when tested as a heterozygote, but its effect was significantly weaker than the alleles recovered in the screen (data not shown). Nonetheless, when tested as a homozygote, it completely suppressed KDN and very strongly enhanced Ras^{V12} (data not shown). These observations suggest that the alleles isolated in the screen have some dominant negative activity.

Based on amino acid sequences and crystallographic studies, Ser/Thr phosphatases have been categorized into two families: the PPP family, which includes the PP1, PP2A and PP2B subfamilies; and the PPM family, which comprises the PP2C and the pyruvate dehydrogenase phosphatase (PDPs) subfamilies (Das et al., 1996). Functional studies conducted in yeasts, plants and mammalian cell lines have linked PP2Cs to a wide range of intracellular functions, including signal transduction, cell cycle progression, DNA damage checkpoint, and RNA splicing (Schweighofer et al., 2004). Their involvement is probably even wider as most functional studies are still limited to a small proportion of the PP2C

repertoire. Given its homology to mammalian PP2C α/β isoforms, it is likely that Alph also plays some of the roles that have been attributed to these enzymes. For instance, growing evidence indicates that mammalian PP2C α/β down-regulate stress-activated protein kinase (SAPK) pathways by dephosphorylating specific core kinases of the JNK and p38 pathways (Tamura et al., 2002). This latter connection to SAPK pathways is highly probable as genetic analysis of related PP2Cs in S. cerevisiae and in plants indicated that this is one of their major functions (Saito and Tatebayashi, 2004; Schweighofer et al., 2004). In light of this putative function for Alph, it will be interesting to determine whether its ability to affect Ras/MAPK signaling is related to a role in SAPK signaling.

Database searches coupled to the Simple Modular Architecture Research Tool (SMART; Letunic et al., 2004) identified fifteen genes encoding phosphatases of the PPM family in Drosophila (Chapter 2, Suppl. Table 1). Fourteen of these correspond to PP2Cs and all except one have mammalian counterparts. Surprisingly, even though metazoans have several *PP2C*-encoding genes (Chapter 2, Suppl. Figure S1), very little has been learned genetically about their respective roles. Indeed, only the C. elegans *fem-2* gene, which encodes a PP2C regulating sex determination, has thus far been characterized genetically (Chin-Sang and Spencer, 1996). Our work therefore represents only the second genetic study of a *PP2C* gene in a metazoan. The availability of additional transposon-disrupted *PP2C* loci (Chapter 2, Suppl. Table 2) as well as

RNAi-based approaches should now accelerate the characterization of this class of phosphatases in metazoans.



Supplemental Fig. S1. Dendrogram of PPM enzymes found in *D. melanogaster* **(15 entries [blue]),** *C. elegans* **(12 entries [green]), and** *H. sapiens* **(19 entries [purple]).** Protein identifiers correspond to the nomenclature used at the Ensembl Project (www.ensembl.org). Proteins containing a PP2C catalytic domain were first identified using the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de). The dendrogram was then generated by the ClustalW software (Chenna et al., 2003. Nucl Acids Res 31, 3497-500) using default parameters. Except for Alph, names in parentheses correspond to the most commonly used protein name found in the literature and retrievable at NCBI. Among all PPM-encoding gene products identified thus far in metazoans, only two of these, Alph and Fem-2 (highlighted in red), have been characterized genetically.

CG #	Closest mammalian homologues (E value)	Cytomap position	Mutant Alleles[c]	Length	Protein domain architecture	PP2Cc TM LRR CC
CG1906 (*alph*)	PP2Cβ (e^{-88})[a]	99B4-5	Yes	372		
CG6036	PP2Cβ (e^{-83})[a]	97A9	No	367		
CG12169	PP2Cγ (e^{-59})[b]	61C5	No	352		
CG17746	PP2Cγ (e^{-55})[b]	63C1	Yes	371		
CG10417	PP2Cγ (e^{-66})	41E5	No	663		
CG10376	POPX1 (e^{-41})	37A1	Yes	428		
CG2984 (*PP2C1*)	PP2Cδ (e^{-71})	4C12-13	Yes	1428		
CG7115	PP2Cε (e^{-54})	28D1	Yes	524		
CG17598	PP2Cη (e^{-43})	20C1	No	651		
CG12151	PDP1 (e^{-81})	7B7	Yes	475		
CG10493	PHLPP/SCOP (e^{-12})	37B12-13	No	955		
CG12091	TA-PP2C (e^{-71})	62A3	No	321		
CG15035	TA-PP2C (e^{-59})	7B2	No	374		
CG7615 (*fig*)	TA-PP2C (e^{-46})	99C1	No	314		
CG9801	*n/a*	85A9-10	No	709		

Supplemental Table S1. The PPM gene family in Drosophila. The 15 Drosophila PPM-encoding genes and their respective cytological position (FlyBase: http://flybase.bio.indiana.edu) are presented. Related genes are highlighted by green shading. Human PP2C catalytic domains were used to define the closest mammalian homologues. Protein architectures (shown on scale relatively to each other) were determined using the SMART architecture query tool.

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Chapter 3: Manuscript #2

Alphabet is a negative regulator of stress signaling in Drosophila

Alphabet is a negative regulator of stress signaling in Drosophila

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3.1 Abstract

The Jun N-terminal kinase (JNK) and p38 pathways, also known as Stress-Activated Protein Kinase (SAPK) pathways, are signaling units reiteratively used throughout development and adult life of metazoan. While they serve a host of functions, they have been mostly characterized for their ability to control apoptosis, immune function and environmental stress responses. We recently identified a Drosophila Ser/Thr phosphatase of the PP2C family, named Alphabet (Alph), which acts as a negative regulator of the classical Ras/MAPK pathway. Here, we show that Alph also plays an inhibitory role with respect to Drosophila SAPK signaling. Our results suggest that Alph negative influence resides upstream of, or in parallel to the MAPKKs *hep* and *lic*. Moreover, biochemical evidence suggests that MAPKKKs such as Tak1, Slpr and dDLK are potential Alph substrates. Finally, we found that *alph* alleles generate a pattern of gene expression that would predict immune and/or stress resistance, two biological responses regulated by SAPK. Together, our work suggests that Alph may serve as a general attenuator of MAPK signaling pathways in flies.

3.2 Introduction

The c-Jun N-terminal Kinase (JNK) and p38 pathways are evolutionarily conserved Mitogen-Activated Protein Kinase (MAPK) signaling cascades involved in many developmental processes as well as in homeostasis maintenance in adult organisms. Also called Stress-Activated Protein Kinase (SAPK) pathways, their misregulation often leads to neurodegenerative diseases, immune dysfunction and cancer (Manning and Davis, 2003; Zarubin and Han, 2005).

SAPK pathways are constituted of a three-kinase core module (MAPKKK→ MAPKK→ MAPK/SAPK) that becomes sequentially phosphorylated upon activation by cellular stress or inflammatory cytokines (Kyriakis and Avruch, 1996). Several kinases occupy the MAPKKK position. They include Mixed-Lineage Kinases (MLK1-4), Dual-Leucine Zipper Kinase (DLK), Leucine Zipper Kinase (LZK), MEK Kinases (MEKK1-4), Apoptosis Signal Regulating Kinase-1 (ASK1) and TGFβ-Activated Kinase-1 (TAK1). Upon activation, these kinases phosphorylate MAPKKs (or MKKs) specific for p38 or JNK pathways. For instance, MAPKKK-mediated phosphorylation of MKK3/6 leads to p38 activation while phosphorylation of MKK4/7 promotes JNK-dependent signaling. Activated MAPKKs phosphorylate a threonine and tyrosine residue in the activation loop of JNK or p38, thereby triggering their catalytic activation (Davis, 2000; Zarubin and Han, 2005). Given the variety of extracellular

signals and MAPKKKs involved in SAPK activation, cells must possess mechanisms to elicit specific cellular responses. These mechanisms are currently being unraveled and appear to depend on specific scaffolds (Dard and Peter, 2006) and other types of proteins such as phosphatases (Theodosiou and Ashworth, 2002) and components of the ubiquitin/proteasome complex (Laine and Ronai, 2005) that shape signaling paths and modulate internal signaling flow. Nonetheless, the way these proteins work together to produce specific outputs remains largely unknown.

SAPK pathways and their constituents have been well conserved during the evolution of metazoans, thereby making simpler and genetically amenable organisms useful models to identify and characterize the function of novel components. For instance, a number of developmental processes in Drosophila such as embryonic dorsal closure, pupal thorax closure and the establishment of ommatidial polarity in the developing retina have proven to be powerful systems for deciphering the molecular events linked to JNK-dependent signaling (Jacinto et al., 2002; Noselli and Agnes, 1999; Zeitlinger and Bohmann, 1999). More recently, SAPK pathways were shown to be critical for stress and immune resistance in flies (Craig et al., 2004; Delaney et al., 2006; Stronach and Perrimon, 1999; Wang et al., 2003a). As in mammals, SAPK pathways in Drosophila can be subdivided into two branches: the Basket (Bsk; JNK homolog) pathway that uses Hemipterous (Hep; MKK7 homolog) or dMKK4 as

MAPKKs, and the p38 pathway that comprises two p38 isoforms (p38a and p38b) and one MAPKK named Licorne (Lic; MKK3/6 homolog). Several MAPKKKs have also been identified in flies. They correspond to Slipper (Slpr; Drosophila MLK), Wallenda (Wnd; Drosophila DLK/LZK), Tak1, Tak12, Mekk1 and dASK1 (Stronach, 2005). Mutant alleles have been isolated for most of these and genetic evidence not only showed their role in SAPK pathways, but also linked some of them to specific developmental events or stress responses. For example, the Drosophila MLK homolog Slpr is an essential regulator of JNK-dependent epithelial cell migration such as those observed during embryonic dorsal closure or pupal thorax closure (Polaski et al., 2006; Stronach and Perrimon, 2002). On the other hand, Tak1 is critical for SAPK-dependent innate immune response (Vidal et al., 2001), while Mekk1 showed a clear ability to regulate p38-mediated environmental stress responses such as resistance to heat or oxidative stress (Inoue et al., 2001). Recently, loss-of-function mutations recovered in the *wnd* gene linked the encoded DLK/LZK homolog to JNK-dependent synaptic growth (Collins et al., 2006). Although specific roles have been attributed to MAPKKKs, redundancy has also been observed (Polaski et al., 2006).

We previously isolated mutations in a gene encoding a Ser/Thr phosphatase of the PP2C family closely related to mammalian PP2Cα/β isoforms (Baril and Therrien, 2006). Genetic analysis of the locus,

renamed *alphabet* (*alph*), demonstrated its role as a negative regulator of the classical Ras/MAPK pathway during Drosophila eye and wing development. While its target substrates had not been identified, Alph was found to be catalytically active and all recovered mutant alleles impeded if not abrogated phosphatase activity *in vitro*. Interestingly, functional characterization of PP2Cs in mammals, yeast and plants, have identified several of their family members, including the PP2Cα/β related proteins, as potent negative regulators of both JNK and p38 pathways (Saito and Tatebayashi, 2004; Schweighofer et al., 2004). Here, we show that Alph is also negatively regulating SAPK-dependent signaling in Drosophila. Our epistatic analysis suggests that Alph functions upstream of the MAPKKs Hep and Lic, but possibly at the level of various MAPKKKs, which would be consistent with Alph ability to regulate distinct SAPK-dependent events.

3.3 Results

alph inactivation increases SAPK-dependent signaling during Drosophila development

Loss-of-function alleles of *hep* or *lic* cause lethality due to early developmental defects of the egg or the embryo, respectively (Glise et al., 1995; Suzanne et al., 1999). To determine if Alph activity negatively influences SAPK signaling, we tested whether *alph* loss-of-function alleles could rescue the lethality associated with *hep* or *lic* hypomorphic alleles. hep^{G0107} and lic^{G0252} alleles have a P-element transposon inserted in the 5'UTR of their respective gene, which presumably results in reduced protein levels. The *hep* and *lic* loci are located on the X chromosome and therefore the viability of mutant hemizygous males is strongly compromised. Indeed, hep^{G0107}/ *Y* males die at the pupal stage, while only 3% of lic^{G0252}*/Y* males reach adulthood (http://flybase.bio.indiana.edu; and Chapter 3, Figure 1A). We previously showed that *alph* loss-of-function mutants, which include three alleles ($alph^{XS-88}$, $alph^{S-331}$ and $alph^{S-355}$) with single amino acid change that disrupted catalytic activity and a piggyBac insertion ($alph^{PBac}$) that completely abolished expression, are homozygous viable with no discernable external phenotypes (Baril and Therrien, 2006). Interestingly, introduction of a homozygous $alph^{S-331}$ allele into either a hep^{G0107} or lic^{G0252} mutant background strongly suppressed hemizygous male lethality (Chapter 3, Figure 1A). Although to a weaker extent, similar

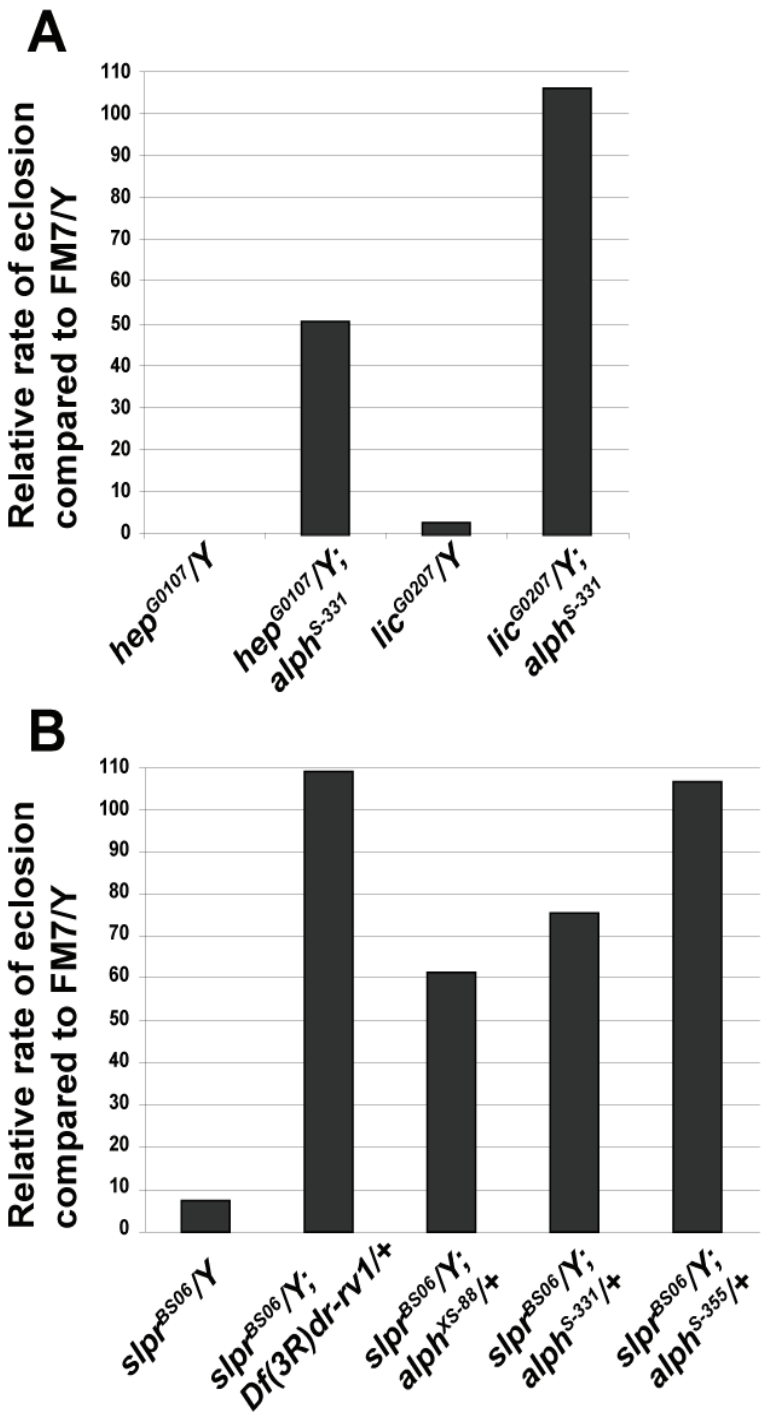

Figure 1: *alph* mutations rescue the viability of *hep, lic* and *slpr* alleles. Lethality rescue experiments. The number of hep^{G0107}/Y, lic^{G0252}/Y or $slpr^{BS06}/Y$ flies with or without *alph* alleles were scored and compared to the number of males carrying the FM7 balancer chromosome (*FM7/Y*) in the same cross. The results are represented as percentage.

results were obtained when any of the four *alph* alleles mentioned above were tested as heterozygotes (data not shown).

In Drosophila, the *slipper* (*slpr*) locus encodes the closest homologue of mammalian MLKs and activates both the JNK and p38 pathways *in vitro*. In addition, Slpr appears to activate the JNK pathway via Hep, although it is not clear if it induces p38 signaling through Lic (Sathyanarayana et al., 2003; Stronach and Perrimon, 2002). The $slpr^{BS06}$ allele contains a premature stop codon at amino acid 47, which presumably generates a null mutation (Polaski et al., 2006). $slpr^{BS06}$ hemizygous males are semi-viable with a proportion of 8% that emerge as adults. During the course of this work, we uncovered a chromosomal deletion on the right arm of the 3rd chromosome (*Df(3R)Dr-rv1*) that dominantly restored viability to $slpr^{BS06}$ males (Chapter 3, Figure 1B). The *Df(3R)Dr-rv1* deficiency deletes approximately 40 genes at cytological position 99A7-99B11, including the *alph* locus. Hence, we tested whether mutations in the *alph* gene would have a similar effect. As expected, we found that heterozygous *alph* alleles rescued the viability of $slpr^{BS06}$ hemizygous males (Chapter 3, Figure 1B). The ability of *alph* mutants to reverse the lethality associated with *hep*, *lic* and *slpr* alleles is thus consistent with elevated SAPK signaling during Drosophila development. Therefore, these results suggest that *alph* acts as a negative regulator of stress signaling pathways.

The morphogenetic processes of embryonic dorsal closure and pupal thorax closure require intact JNK signaling. Loss-of-function mutations in genes required for adequate JNK signaling generally produce a large, dorsal anterior hole in the secreted larval cuticle and result in embryonic lethality (Jacinto et al., 2002). However, hypomorphic mutations in components of the JNK pathway usually permit completion of embryogenesis, but result in thorax closure (TC) defects during later stages of development. Flies with TC failure display a cleft in the dorsal midline of the thorax. In addition, the area of the notum and scutellum is often reduced and has reduced bristle numbers (Zeitlinger and Bohmann, 1999). It has been previously reported that overexpression of $Slpr^{WT}$ in dorsal developing tissues using the *pannier-GAL4* (*pnr-GAL4*) driver resulted in a mild TC defect ((Polaski et al., 2006) and Chapter 3, Figure 2, compare A and B). This phenotype depends on the strength of JNK signaling as inactivation of one copy of the negative regulator *puckered* (*puc*), which encodes a Bsk-specific dual-specificity phosphatase (Martin-Blanco et al., 1998), strongly enhanced TC defects (Chapter 3, Figure 2C). Interestingly, we found that heterozygous alleles of *alph* also enhanced the $Slpr^{WT}$ overexpression TC phenotype (Chapter 3, Figure 2 compare D and E to B; note the reduction of scutellar area). To quantify the severity of the TC phenotype, we counted the number of scutellar bristles for each genotype. While the expression of $Slpr^{WT}$ generally results in the loss of one or two bristles, removing one copy of the *puc* gene strongly reduced the area of the scutellum and no bristle were recovered in this region

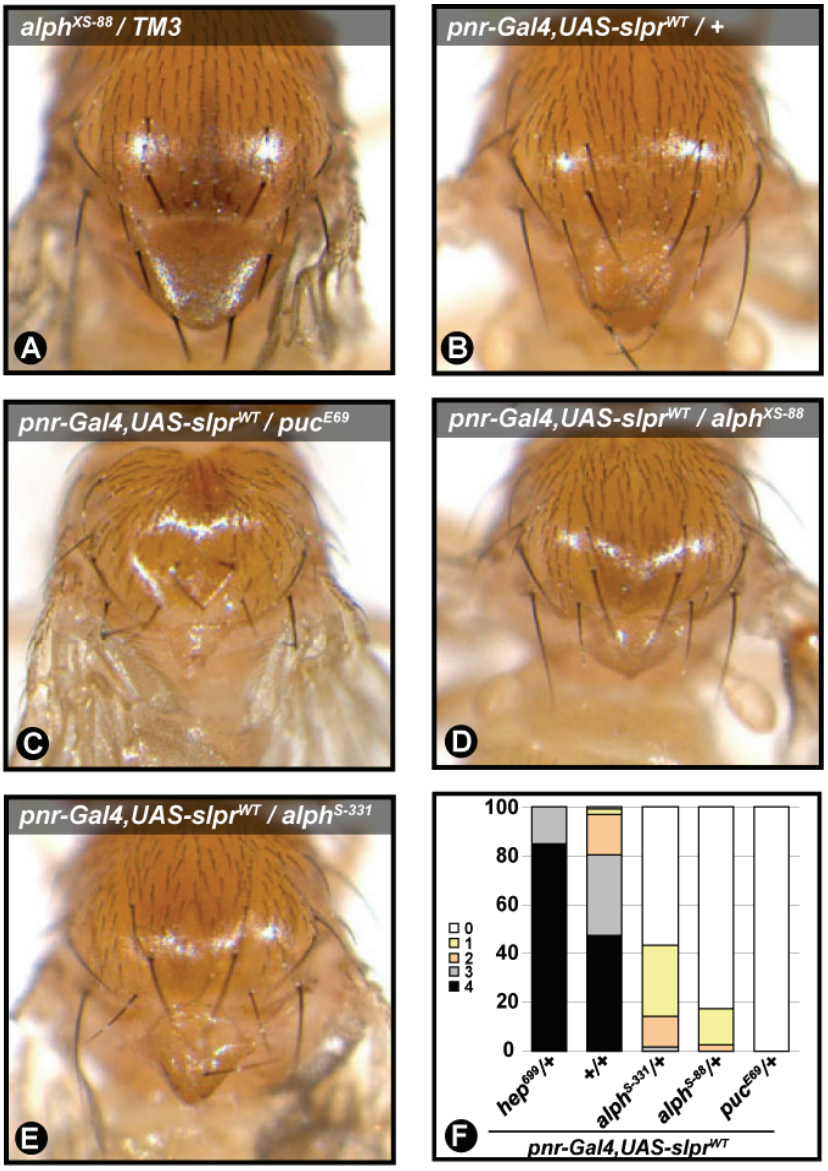

Figure 2: *alph* alleles enhance thorax closure defects generated by Slpr expression. The *pannier-Gal4* driver was used to direct the expression of wild type Slpr ($Slpr^{WT}$) in dorsal tissues during development. Modulation of thorax closure defects was evaluated for each heterozygous allele. (A-E) A representative image is shown for each genotype. Image shown in A represents a wild type thorax. (F) Quantification of thorax closure defects by monitoring the number of post-vertical scutellar bristles. Wild type flies have four scutellar bristles while mutants with thorax closure defects generally loose a few or entirely miss these bristles. In an otherwise wild type (+/+) background, Slpr overexpression generates a thorax closure defect that is accompanied with a loss of one to two bristles to a penetrance of about 50%.

(Chapter 3, Figure 2F). *alph* alleles also reduced the number of scutellar bristles to one or none. As an additional control for these genetic interactions, a *hep* loss-of-function allele (*hep*^{699}) suppressed the loss of scutellar bristles induced by Slpr^{WT} expression (Chapter 3, Figure 2F). Together, these results indicate that *alph* alleles increase SAPK-mediated signaling during embryogenesis and suggest that it may normally regulate thorax closure during Drosophila development.

alph acts upstream of hep and lic

To determine the position where Alph activity is required in SAPK signaling, we used the developing Drosophila eye as an assay system. GMR-driven eye-specific expression of Rac1^{WT}, which encodes a small GTPase of the Rho family that acts upstream of *slpr* in the JNK pathway, provokes a JNK pathway-dependent rough eye phenotype mainly in the posterior part of the eye ((Nolan et al., 1998; Stronach and Perrimon, 2002) and Chapter 3, Figure 3A). As shown previously, the phenotype is enhanced by mutations in *puc* and suppressed by mutations in *bsk* or *slpr* (Chapter 3, Figure 3, compare A to B, C and D; (Stronach and Perrimon, 2002)). Interestingly, the rough eye phenotype of *GMR-Rac1* flies is not fully penetrant and a proportion of these flies exhibit a relatively normal-looking eye. However, introduction of *alph* alleles into the *GMR-Rac1* background generates a fully penetrant and enhanced rough eye phenotype as shown in Figure 3E. In agreement with this result, overexpression of WT *alph* strongly suppressed *GMR-Rac1*, while the

mutant version was devoid of suppressing activity and was even slightly enhancing the phenotype (Chapter 3, Figure 3, compare A to F and G). Given that a wild type version of Rac1 was used in this assay, we could not determine whether the effects of *alph* mutations or overexpression were taking place upstream or downstream of *Rac1*. However, these results provide additional evidence that *alph* negatively regulates SAPK signaling.

To assess the relative position of *alph* with respect to specific components of SAPK pathways, we monitored the impact of *alph* alleles on the rough eye phenotype obtained by overexpressing constitutively active (CA) *hep* or *lic* transgenes. To generate gain-of-function alleles of *hep* and *lic*, we changed activation loop Thr/Ser residues normally phosphorylated by SAPKKKs to phospho-mimetic residues. These mutations generate constitutively active enzymes, thereby making these kinases insensitive to upstream regulation. Expression of hep^{CA} in the developing eye produces a phenotype that is sensitive to the dose of downstream components, such as *bsk* and *puc* (Chapter 3, Figure 3; compare H to I and J), but is not affected by mutations in upstream components such as *slpr* (Chapter 3, Figure 3K). In contrast to the *Rac1* rough eye phenotype, *alph* loss-of-function alleles did not modify the hep^{CA} rough eye phenotype (Chapter 3, Figure 3L). Consistent with these results, *alph* overexpression also did not modify this phenotype (Chapter 3, Figure 3M). These findings therefore suggest that *alph* activity is

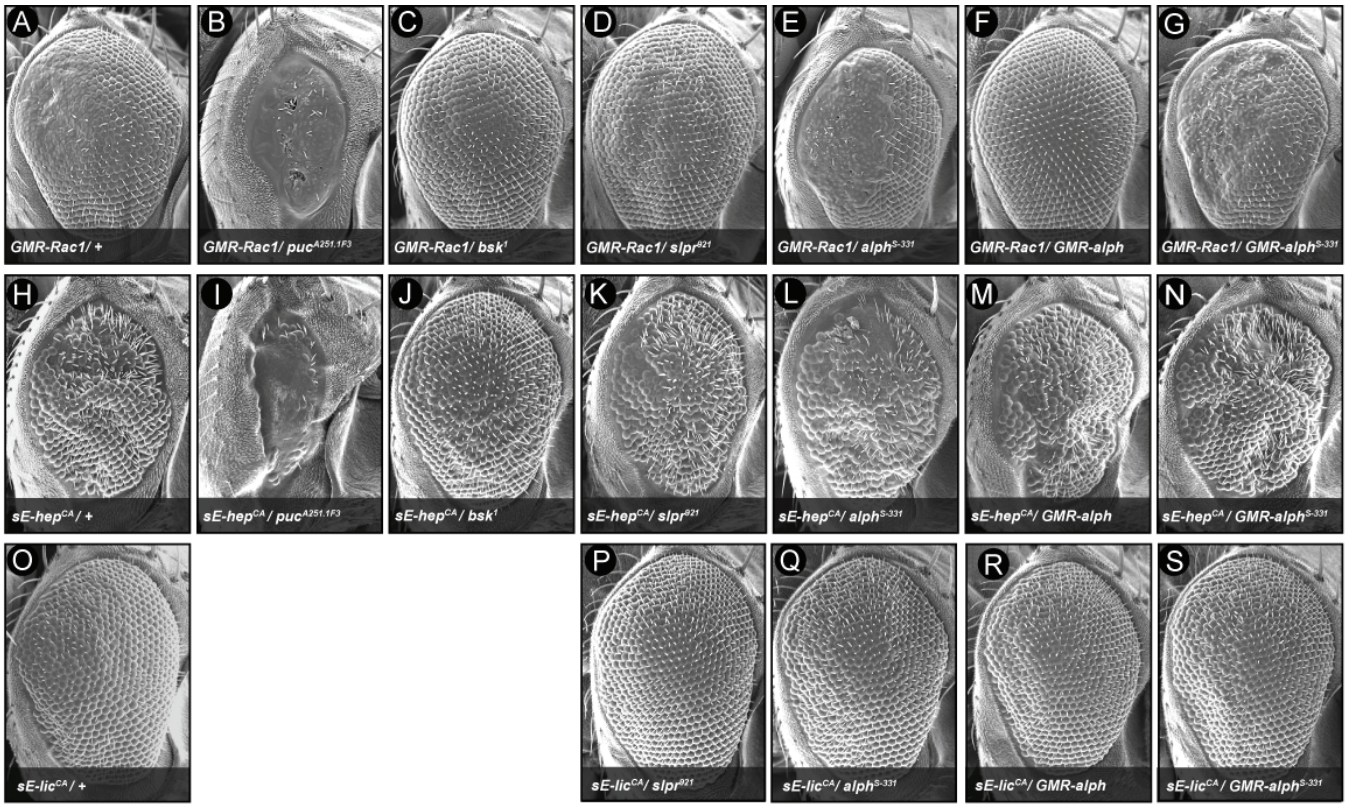

Figure 3: *alph* negative activity resides upstream of *hep* and *lic*. Scanning electron micrographs of adult eyes of the following genotypes: (A) *GMR-Rac1/+* (B) *GMR-Rac1/+*; $puc^{A251.1F3}$/+ (C) *GMR-Rac1/*bsk^{1} (D) $slpr^{921}$/+; *GMR-Rac1/+* (E) *GMR-Rac1/+*; $alph^{S-331}$/+ (F) *GMR-Rac1/+ ; GMR-alph /+* (G) *GMR-Rac1/+ ; GMR-*$alph^{S-331}$/+ (H) *sE-*hep^{CA} /+ (I) *sE-*hep^{CA} /+ ; $puc^{A251.1F3}$/+ (J) *sE-*hep^{CA} /bsk^{1} (K) $slpr^{921}$/+; *sE-*hep^{CA} /+ (L) *sE-*hep^{CA} /+ ; $alph^{S-331}$/+ (M) *sE-*hep^{CA} /+ ; *GMR-alph /+* (N) *sE-*hep^{CA} /+; *GMR-*$alph^{S-331}$/+ (O) *sE-*lic^{CA} /+ (P) $slpr^{921}$/+; *sE-*lic^{CA} /+ (Q) *sE-*lic^{CA} / $alph^{S-331}$ (R) *sE-*lic^{CA} */ GMR-alph* (S) *sE-*lic^{CA} */ GMR-*$alph^{S-331}$. Both *sE-*hep^{CA} and *sE-*lic^{CA} rough eye phenotypes were not modified by overexpression of wild type Alph using the sE promoter (data not shown). Anterior is to the right.

required at a step upstream of *hep*. Given the possibility that hep^{CA} overexpression may override the need for a parallel pathway, it is also possible that Alph functions in parallel to Hep, but not downstream of it. We also obtained a rough eye phenotype by overexpressing lic^{CA} during eye development (Chapter 3, Figure 3O). As for hep^{CA}, neither *alph* mutations nor *alph* overexpression altered this rough eye phenotype (Chapter 3, Figures 3Q and 3R). These data therefore suggest that *alph* activity is required at a step upstream of, or in parallel to *lic*.

Alph antagonizes SAPKKK-induced activation of Bsk in S2 cells

To further delineate the position of Alph in the SAPK pathways and identify putative substrates, we used a biochemical assay where the activation state of Bsk was monitored by immunoblots with a phospho-specific JNK antibody (pJNK). Expression of Hep^{CA} in S2 cells led to the phosphorylation of a Bsk reporter protein on its activating residues (Chapter 3, Figure 4A, lane 2). Consistent with the genetic interaction studies presented above, overexpression of Alph (or the mutant S-331 version) with Hep^{CA} did not alter pJNK levels (Chapter 3, Figure 4A, lanes 3 and 4). Therefore, this result biochemically places Alph at a step upstream or in parallel to Hep. Overexpression of Slpr in S2 cells is sufficient to induce its kinase activity thereby leading to Bsk-dependent signaling (Fig. 4B, lane 2). Given that *alph* genetically interacts with *slpr*, we tested whether Alph overexpression can modulate Slpr-induced Bsk activation. As shown in Figure 4B, this seems to be the case as co-

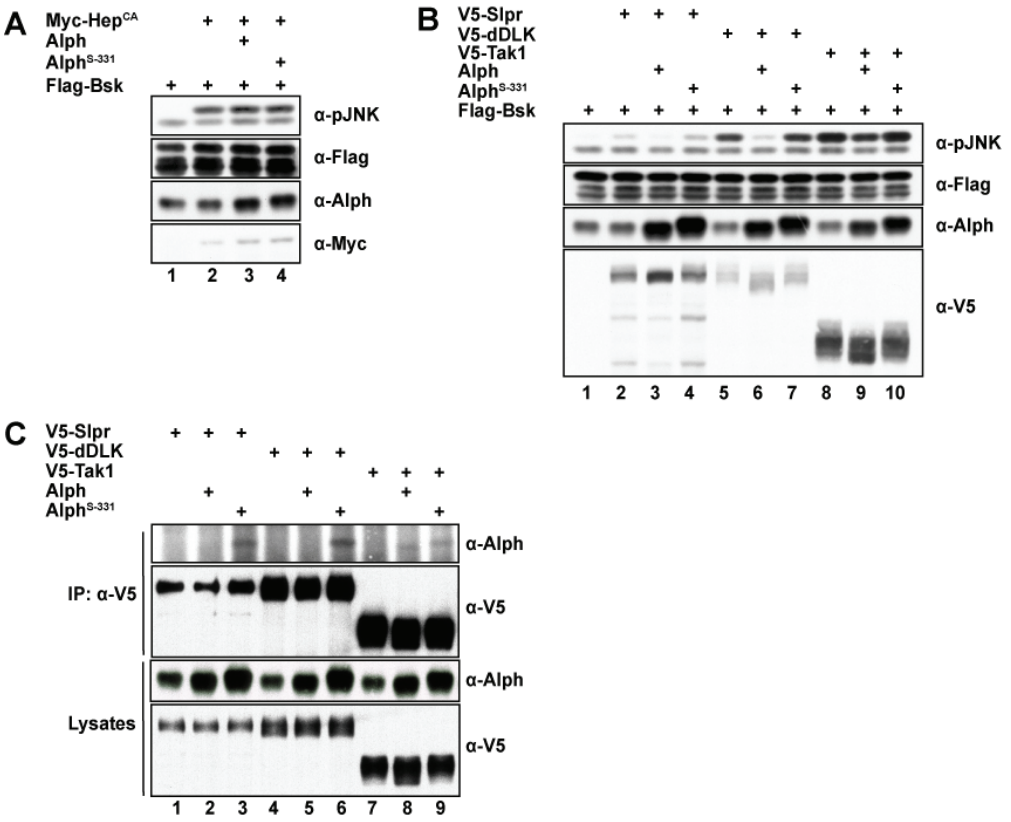

Figure 4: Alph negatively regulates the JNK pathway in S2 cells. S2 cells were transfected with the indicated plasmid combinations. (A, B) Activation of the JNK pathway was monitored by immunoblot analysis of cell lysates using an α-diphospho-JNK antibody that recognizes the dually phosphorylated form of Bsk in its activation segment. As the Alph constructs expressed in S2 cells do not contain an epitope tag, we monitored their expression level using an α-Alph antibody that also recognizes the endogenous protein. Therefore, lanes that do not overexpress the Alph constructs display the endogenous Alph contribution, whereas other lanes show an identical increased amount of Alph proteins. As previously reported (Baril and Therrien, 2006) and shown in this figure, the Alph^{S-331} proteins migrate slightly slower than the wild type variant. (C) Cell lysates were immunoprecipitated with an a-V5 antibody to determine whether SAKKKs can co-immunoprecipitate Alph. Lysates were also directly probed to monitor protein levels.

expression of Alph along with Slpr prevented Bsk phosphorylation, whereas the S-331 mutant version was inactive (Chapter 3, Figure 4B, compare lanes 2, 3 and 4). In mammalian cells, PP2C phosphatases related to Alph were previously shown to specifically dephosphorylate

Tak1 and inactivate IL-1 induced JNK pathways (Hanada et al., 2001; Li et al., 2003). We thus decided to verify whether Alph activity could also antagonize the ability of Drosophila Tak1 or DLK, which are relatively close structural homologs that are part of the tyrosine kinase-like family (Niedner et al., 2006), to induce Bsk phosphorylation. As shown in Figure 4B, Alph turned out to be very efficient at preventing dDLK-mediated Bsk activation (lanes 5 and 6) and also inhibited Tak1 activity, although to a lesser degree (lanes 8 and 9). Taken together, these results support the genetic results that position *alph* at a step upstream of Hep, by possibly dephosphorylating critical residues on SAPKKKs. However, as Hep was activated by introducing phospho-mimetic residues in its activation loop, we cannot rule out the possibility that Alph is acting directly on Hep.

Using co-immunoprecipitation assays in S2 cells, we next wanted to determine whether Alph could physically associate with the SAPKKKs. Interestingly, we found that Slpr and dDLK formed a stable complex only with the S-331 mutant version of Alph, whereas both the wild type and mutant versions could be detected forming a complex with Tak1 (Chapter 3, Figure 4C). While these results are consistent with a direct action of Alph on these SAPKKKs, it appears that the mutant version that had been originally isolated in a Ras/MAPK-dependent genetic screen (Baril and Therrien, 2006) may not be a simple loss-of-function but have some dominant effect on certain SAPKKKs.

Inactivation of alph produces a gene expression pattern that predicts immune and/or stress resistance

Using an oligonucleotide microarray system capable of assaying nearly every Drosophila gene, we monitored transcript expression profiles in *alph*$^{S-331}$ and *alph*^{PBAC} mutant flies compared to wild type flies. We used two criteria to catalog genes differentially expressed. First, we picked candidate genes only if their expression profile was similarly modified for both mutant strains compared to wild type. Second, we selected genes that were modulated by at least 1.3 fold with a maximum p-value of 0.001. These selection criteria generated a list of 311 genes, which is provided as supplementary data (Chapter 3, Figure S1).

Among the 311 hits selected, ~20% are related to immune and/or stress response (Chapter 3, Table 1). Moreover, while 60% of genes listed in Table I are modulated in a way that would suggest immune and/or stress resistance in *alph* mutants (Table 1; highlighted in blue), only 10% would predict the contrary (Table 1; highlighted in red). The remaining 30% are related to immune and/or stress response, but due to the lack of information we cannot conclude on their positive or negative requirement in those precise responses.

The TotA and TotC genes, which belong to the Turandot family members, are listed among the most upregulated genes (2.89 and 2.44

Table I. Immune and stress-related genes regulated by Alph

Pathogen recognition and Phagocytosis

Name		FC	p.value	Function	Family	References
CG4099	Sr-CI	1,66	5,5E-05	Defense	Sr-CI	(Pearson et al., 1995)
CG32912	PGRP-LD	1,40	0,00097	Defense	PGRP	(Steiner, 2004)

Melanization and Coagulation

CG5730	Annexin IX	1,41	0,00073	Defense	Annexin	(Boutros et al., 2002; De Gregorio et al., 2001; De Gregorio et al., 2002)
CG7890		0,59	0,00079	Defense	heparin-glucosamine 3-O-sulfotransferase	
CG16858	viking	1,48	0,00046	Defense	Collagen	(Rodriguez et al., 1996)
CG31832		0,70	0,00055	Defense	Fibrinogen	
CG32496		0,38	7,4E-05	Defense	Fibrinogen	

Proteolysis

CG3066	SP7	1,56	2,8E-05	Defense	Ser protease	(De Gregorio et al., 2001; De Gregorio et al., 2002; Karlsson et al., 2004)
CG5390		1,45	0,00012	Defense	Ser protease	(Karlsson et al., 2004; Vierstraete et al., 2004)
CG7532		1,82	0,0006	Defense	Ser protease	(De Gregorio et al., 2001; De Gregorio et al., 2002)
CG9672		1,68	0,00047	Defense	Ser protease	(De Gregorio et al., 2001; De Gregorio et al., 2002)
CG11841		1,60	0,00011	Defense	Ser protease	(De Gregorio et al., 2001; Irving et al., 2001)
CG15002	masquerade	0,66	0,00092	Stress	Ser protease	(Leemans et al., 2000)
CG16705	SP4	1,67	3,4E-05	Defense	Ser protease	(Boutros et al., 2002; De Gregorio et al., 2001; De Gregorio et al., 2002; Irving
CG17278		1,39	0,00083	Defense	Ser protease inhibitor	(De Gregorio et al., 2001; De Gregorio et al., 2002)
CG18030	Jon99Fi	0,42	0,00021	Defense	Ser protease	(Boutros et al., 2002; De Gregorio et al., 2001; De Gregorio et al., 2002)
CG31313		2,05	3,8E-05	Defense	Cys protease inhibitor	(Vierstraete et al., 2003)
CG33103	Papilin	1,78	2,1E-05	Defense/RAS	Ser protease inhibitor	(Asha et al., 2003)

Peptides

CG4250		2,06	2,1E-06	Defense	Unknown	(De Gregorio et al., 2001; De Gregorio et al., 2002)
CG6429		1,59	0,00016	Defense	Unknown	(De Gregorio et al., 2001; De Gregorio et al., 2002)
CG11314		2,52	8,7E-06	Defense/RAS	Unknown	(Asha et al., 2003; Karlsson et al., 2004)
CG11315		1,78	0,00035	Defense	Unknown	(Karlsson et al., 2004)
CG13323		1,78	2E-05	Defense	Unknown	(De Gregorio et al., 2001; De Gregorio et al., 2002)
CG13324		2,21	2,2E-05	Defense	Unknown	(De Gregorio et al., 2001; De Gregorio et al., 2002)
CG16844	IM3	1,64	0,00026	Defense	Unknown	(Boutros et al., 2002; Uttenweiler-Joseph et al.,
CG17107		1,83	0,00027	Defense	Unknown	(De Gregorio et al., 2001)
CG18106	IM2	1,60	9,3E-05	Defense	Unknown	(Boutros et al., 2002; De Gregorio et al., 2001; De Gregorio et al., 2002; Uttenweiler-Joseph et al.,

Table I. Immune and stress-related genes regulated by Alph (cont.)

Detoxification/Response to stress/Aging

Name		FC	p.value	Function	Family	References
CG1873	Ef1α100E	1,62	2,6E-05	Aging	Elongation factor	(Shepherd et al., 1989)
CG3972	Cyp4g1	0,72	0,00052	Detox	Cytochrome P450	
CG4105	Cyp4e3	2,84	0,00011	Detox/stress	Cytochrome P450	(Landis et al., 2004)
CG4181	GstD2	1,78	1,7E-05	Defense/Stress	Glutathione S-transferase	(Landis et al., 2004)
CG5873		1,46	0,00049	Defense/Stress	Peroxidase	
CG6214	dMRP	0,62	0,00017	Defense/Stress	ABC transporter	
CG6530	mthl3	1,63	0,00026	Stress/Aging	GPCR	
CG6649	ugt35b	0,65	6,2E-05	Stress/Aging	UDP-glycosyltransferase	(Zou et al., 2000)
CG6730	Cyp4d21	1,40	0,00092	Detox	Cytochrome P450	
CG6955	Lcp65Ad	0,73	0,0003	Stress	Cuticle	(Leemans et al., 2000)
CG7291	NPC2	1,38	0,00046	Aging	Niemann Pick C2	(Kang et al., 2002; Zou et al., 2000)
CG8505		1,81	9,1E-06	Aging	Cuticle	(Leemans et al., 2000)
CG8677		0,66	0,00068	Aging	Transcriptional repressor	(Seong et al., 2001)
CG10178		1,57	0,00014	Defense/Stress	UDP-glycosyltransferase	
CG10246	Cyp6a9	1,47	0,00027	Detox/RAS	Cytochrome P450	(Asha et al., 2003; Daborn et al., 2001; Landis et al., 2004)
CG12505		1,75	0,00013	Aging	Unknown	{Zou, 2000 #10}
CG31509	TotA	2,89	2,1E-07	Defense/Stress	Turandot	(Agaisse et al., 2003; Brun et al., 2006; Ekengren and Hultmark, 2001; Ekengren et
CG31508	TotC	2,44	2E-05	Defense/Stress	Turandot	(Brun et al., 2006; Ekengren and Hultmark, 2001)

Signal transduction

CG1225	RhoGEF3	0,55	2,5E-05	Defense/RAS	GEF	(Boutros et al., 2002)
CG1803	Regucalcin	1,72	7,7E-05	Defense	SMP-30	(Karlsson et al., 2004; Vierstraete et al., 2003; Vierstraete et al., 2004)
CG4950		2,24	4,6E-06	Defense	Receptor	
CG8561	Tartan	1,46	0,00013	Defense	Receptor	
CG16827	αPS4	0,68	0,00082	Defense/RAS	Receptor and RAS	(Asha et al., 2003; Crozatier et al., 2004)
CG31421	Takl1	1,37	0,00088	JNK	MAPKKK	
CG32602		0,63	0,00099	Defense	Receptor	

Others

CG3132	Ect3	1,56	0,00034	Defense	Beta-galactosidase	(De Gregorio et al., 2001; De Gregorio et al., 2002)
CG5080		1,41	0,00061	JNK	Unknown	(Jasper et al., 2001; Vierstraete et al., 2003)
CG5770		1,67	0,00062	Defense	Unknown	(Silverman et al., 2003)
CG6579		1,40	0,00028	RAS	Unknown	(Asha et al., 2003; Vierstraete et al., 2003)
CG9119		1,48	0,00046	Defense	Ester hydrolase	
CG14610		0,62	0,0003	RAS	Unknown	(Asha et al., 2003)

For gene selection criteria, refer to materials and methods. Genes colored in blue are modulated in a way that would predict immune and/or stress resistance (60%), while the ones colored in red would predict immune and/or stress sensitivity (10%). Genes colored in green are related to immune and/or stress response, but we do not know if their expression promotes or antagonizes these responses. Fold change (FC).

respectively). The TotA gene encodes a humoral factor, which is secreted from the fat bodies and accumulates in the body fluids. TotA is strongly induced under stressful conditions such as heat shock, UV irradiation, bacterial challenge or paraquat treatment. In addition, overexpression of the TotA protein generates heat resistant flies (Ekengren et al., 2001). Database search revealed that the Drosophila genome comprises eight Tot genes, which are all upregulated by stressful conditions (Ekengren and Hultmark, 2001). The TotA and TotM genes have been recently demonstrated to be upregulated following septic injury and stress conditions in a Mekk1-dependent manner (Brun et al., 2006). Moreover, activation of the JAK/STAT and Immune Deficiency pathway (TNF-like) following septic injury results in the upregulation of the humoral factor TotA in the fat bodies (Agaisse et al., 2003). We also noticed an increase in the transcription of genes predicted to encode small polypeptides ranging from 40 to 134 amino acid residues (Chapter 3, Table 1; Peptides). Other studies have reported an increase in the transcription of those genes following bacterial or fungal challenge (Boutros et al., 2002; De Gregorio et al., 2001; De Gregorio et al., 2002; Karlsson et al., 2004; Uttenweiler-Joseph et al., 1998). However, their function relative to fly immunity is not known, but they may represent a new class of antimicrobial peptides, which have antibiotic properties. Alternatively, they may participate in signaling as cytokines. In Drosophila, the JNK pathway is implicated in the cellular adaptation to stress, most likely by increasing genes related to detoxification, radical scavenging and protein folding (Jasper et al., 2001).

In addition, the JNK pathway is also critical to promote an adequate immune response via Tak1 MAPKKK (Delaney et al., 2006). Therefore, the modulation of genes required for stress and immune resistance in *alph* mutant flies may be due to increased JNK pathway signaling.

3.4 Discussion

SAPK pathways are signaling modules reiteratively used by various extracellular stimuli to communicate molecular information inside the cell. Once activated, SAPKs phosphorylate a panel of substrates that will modulate numerous cellular events. How a given stimulus generates a specific biological response? Some regulatory mechanisms have emerged as powerful systems to modulate the strength and the duration of SAPK signaling, which probably dictate stimulus specific responses. However, much remains to be unraveled concerning what confers signal specificity. In this article, we describe a new putative negative regulator of SAPK pathways in Drosophila. We found that inactivation of the *alph* locus rescued the viability of *hep*, *lic* and *slpr* alleles. In addition, we demonstrated genetically and biochemically that *alph* acts upstream of, or in parallel to *hep* and *lic*. Wild type Alph protein appears to physically associate with the SAPKKK Tak1, thereby suggesting that the negative activity of the phosphatase resides in part at this level. Finally, inactivation of *alph* in adult flies results in a transcript expression profile that would predict immune and/or stress resistance, two biological processes previously associated with increased SAPK signaling.

Alph may control crosstalk between ERK/MAPK and SAPK pathways

The *alph* locus was originally identified as a negative regulator of the Ras/MAPK pathway acting at a step downstream of the small GTPase

Ras and possibly upstream of ERK/MAPK (Baril and Therrien, 2006). However, these results have not ruled out the possibility that the impact of Alph on Ras/MAPK signaling is mediated via a parallel pathway. Numerous studies have reported crosstalk between ERK, JNK or p38 pathways in cell culture systems. In addition, interactions between ERK and JNK pathways were recently reported in an intact living tissue, the Drosophila developing eye. A first study showed that the clonal overgrowth of cells overexpressing oncogenic Raf was suppressed by JNK activation during Drosophila eye development. However, these Raf and JNK-expressing clones mediated hyperproliferation of adjacent WT tissues in a non-cell autonomous manner (Uhlirova et al., 2005). In a second study, loss of polarity genes (such as *scribble*, *dlg* and *lgl*) in Ras^{V12}-expressing clones drove overgrowth and metastatic behavior in a JNK-dependent manner (Igaki et al., 2006). In a previous report, we showed that mutation in *alph* increased Ras^{V12}-mediated rough eye phenotypes. It is thus possible that this enhancement is due to increased JNK signaling by a yet undefined mechanism.

Another hypothesis is that SAPK and ERK pathways share a common upstream activator that is a substrate for Alph. SAPKKKs are good candidates as they require phosphorylation events for full catalytic activation and have been implicated in crosstalk between SAPK and ERK. For instance, MLK3 is required for Ras-induced proliferation and mitogen activation of ERK (Chadee and Kyriakis, 2004b). The effect of MLK3 on

the ERK pathway is independent of MLK3 kinase activity since kinase-dead mutants are still capable of ERK activation. Instead, it was shown that MLK3 is implicated in the maintenance of a heterotrimeric complex containing B-Raf and C-Raf (Chadee et al., 2006). It was also demonstrated that activation of ERK by serum factors is strongly reduced in *mekk1-/-* mouse embryonic fibroblast (Yujiri et al., 1998) and that Mekk1 serves as a platform for Raf, MEK and ERK proteins (Karandikar et al., 2000). These studies together with our results demonstrating the physical interaction between Tak1 and Alph support the idea that Alph inhibits a common activator of the ERK and SAPK pathways, possibly the SAPKKKs.

Are alph mutants acting as TAB1-like proteins?

Tak1 was originally identified as a TGF-β-activated SAPKKK and was subsequently shown to play an important role in intracellular responses to pro-inflammatory cytokines, such as interleukin-1 and tumor necrosis factor (Chen et al., 2006b). In mammalian cells, Tak1 is interacting with two other proteins, namely Tak1-Binding Protein 1 (TAB1) and either TAB2 or the structurally related TAB3 (Cheung et al., 2004). TAB1 is apparently required for Tak1 activation (Shibuya et al., 1996), while TAB2/TAB3 mediate interactions with upstream signaling components (Cheung et al., 2004; Takaesu et al., 2000). Interestingly, sequence alignment and crystal structure analyses revealed that TAB1 N-terminal portion has great structural similarity with PP2Cα. However, this

PP2C domain is not functional due to the non-conservation of critical amino acid residues required for catalysis. It has thus been hypothesized that TAB1 acts as a pseudo-phosphatase regulating the availability of phosphorylated residues on downstream targets of Tak1 or on Tak1 itself (Conner et al., 2006). So far, no TAB1-like protein was identified in Drosophila. However, Drosophila TAB2 was recently reported to bind Tak1 and have a role in innate immunity induced by the JNK and NF-κB pathway (Zhuang et al., 2006a). Interestingly, the closest Drosophila homolog of human TAB1 protein is Alph. The three *alph* alleles recovered in the genetic screen possessed point mutations in highly conserved residues within the catalytic domain, which impaired phosphatase activity *in vitro* (Baril and Therrien, 2006). It is thus possible that *alph* alleles behave like a TAB1 molecule. In a normal cellular context, Alph would dephosphorylate and inactivate Tak1. In the case where Alph is mutated (XS-88, S-331, S-355), it would somehow protect Tak1 from being dephosphorylated by WT Alph or another redundant phosphatase. Another hypothesis is that these specific mutations in Alph render the phosphatase capable of binding and protecting other SAPKKKs such as dDLK or Slpr. These two latter scenarios would lead to increased SAPK signaling. Finally, in the case where Alph is depleted (PBac), more Tak1 molecules (and/or other SAPKKKs) would be phosphorylated or would be maintained phosphorylated for a longer period of time, thereby resulting in increased SAPK signaling. However, SAPK signaling may not be as high

as in the case of mutant Alph since redundant phosphatases may compensate.

Is alph implicated in stress and/or immune response?

In Drosophila, the SAPK pathways regulate the cellular response to environmental stress (Stronach and Perrimon, 1999). Indeed, overexpression of hep^{CA} in embryos enhanced the transcription of genes required for stress response (Jasper et al., 2001). In addition, elevation of JNK signaling in *puc* mutant flies increased their resistance to oxidative stress generated by paraquat, while an inactivating allele of p38a has the opposite effect (Craig et al., 2004; Wang et al., 2003a).

Environmental stress also includes bacterial, fungal and viral challenges and the JNK pathway has been reported to be crucial for immune response to Gram-negative bacteria via the TNF-like Immune Deficiency (IMD) pathway. Upon infection by Gram-negative (Gram-) bacteria, the death domain containing IMD protein initiates the sequential activation of Tak1 and the NFκB-like molecule Relish (Rel). Rel then enters into the nucleus and acts as a transcriptional activator of antimicrobial peptide genes (Pinheiro and Ellar, 2006). Although Tak1 is clearly implicated in the response to Gram- bacteria, conflicting results exist concerning its mode of action in the IMD pathway. Indeed, microarray experiments in S2 cells and genetic analyses showed that Tak1 regulates antimicrobial peptide production via the direct

phosphorylation and activation of Rel (Boutros et al., 2002; Vidal et al., 2001) and/or the direct activation of the JNK pathway (Delaney et al., 2006; Kallio et al., 2005). In the light of these studies, it is thus possible that Tak1 mediates part of its effects on immune response via direct activation of the JNK pathway.

Since *alph* inactivation increased SAPK signaling and modulated gene transcription in a way that would predict immune and/or stress resistance, we are interested in testing the effect of various stressful conditions on *alph* mutant flies. Our prediction is that *alph* alleles will be resistant to oxidative and/or heat shock treatment for example. As wild type flies recover very rapidly from Gram- bacterial infection and since no lethality is associated with such a challenge, we cannot evaluate whether *alph* alleles confer resistance to bacterial infection simply by looking at the associated lethality phenotype. However, our second prediction is that overexpression of $Alph^{WT}$ in adult Drosophila should increase their sensitivity to Gram- bacteria. Finally, since *tak1-/-* and *imd-/-* flies die 2-3 days post-infection with Gram- bacteria (Lemaitre et al., 1995; Vidal et al., 2001), it would be interesting to test whether *alph* mutants rescue this lethality phenotype.

Supplementary Table S1: Candidate genes

Name	p.value	FC	synonym
CG31509	2,1E-07	2,9	Turandot A
CG4105	1,1E-04	2,8	cyp4e3
CG17820	1,3E-06	2,8	female-specific independent of transformer
CG7298	6,9E-05	2,8	
CG7542	4,7E-07	2,5	SP71
CG11314	8,7E-06	2,5	
CG15353	7,5E-05	2,5	
CG31508	2,0E-05	2,4	Turandot C
CG12998	4,2E-04	2,4	
CG32950	1,9E-05	2,4	CG14245
CG10910	5,7E-05	2,3	
CG4950	4,6E-06	2,2	CT15890
CG13324	2,2E-05	2,2	
CG7171	8,5E-07	2,2	Urate oxidase
CG10297	2,9E-05	2,1	DCP5
CG13636	1,3E-05	2,1	
CG4250	2,1E-06	2,1	
CG31313	3,8E-05	2,0	
CG7216	1,2E-05	2,0	Adult cuticle protein 1
CG15043	8,9E-07	2,0	
CG8299	6,6E-05	2,0	SP65
CG12045	2,4E-04	1,9	
CG2958	8,2E-05	1,8	lectin-24Db
CG4087	7,6E-04	1,8	rpP2
CG17107	2,7E-04	1,8	
CG7532	6,0E-04	1,8	
CG30042	6,8E-05	1,8	CG8496
CG17005	5,8E-06	1,8	NA
CG16836	1,2E-05	1,8	NA
CG8505	9,1E-06	1,8	NA
CG15155	6,2E-05	1,8	
CG13116	3,8E-04	1,8	NA
CG13057	3,6E-06	1,8	retinin
CG13323	2,0E-05	1,8	
CG4181	1,7E-05	1,8	Glutathione S transferase D2
CG11315	3,5E-04	1,8	
CG33103	2,1E-05	1,8	Papilin
CR32864	6,3E-05	1,8	
CG15534	5,1E-04	1,8	aSMase

CG11413	3,4E-05	1,8	
CG14047	3,3E-04	1,8	
CG31207	1,4E-04	1,8	CG7096
CG14259	1,7E-05	1,8	
CG12505	1,3E-04	1,7	
CG13962	1,8E-04	1,7	
CG2121	8,7E-05	1,7	CG8698
CG11378	1,3E-05	1,7	
CG1803	7,7E-05	1,7	regucalcin
CG5903	4,0E-05	1,7	
CG9338	6,7E-05	1,7	
CG2360	5,1E-05	1,7	cuticle cluster 8
CG14661	2,2E-04	1,7	
CG32209	6,6E-06	1,7	CG8748, CG8747
CG13504	4,0E-04	1,7	
CG10912	1,0E-05	1,7	
CG9672	4,7E-04	1,7	
CG5770	6,2E-04	1,7	
CG16705	3,4E-05	1,7	SP4
CG4962	2,4E-05	1,7	
CG12154	2,4E-04	1,7	orthodenticle
CG13117	1,1E-04	1,7	
CG4099	5,5E-05	1,7	SR-CI
CG14075	1,0E-04	1,7	
CG13377	1,9E-05	1,7	
CG2507	3,2E-05	1,7	stranded at second
CG9016	1,1E-04	1,6	
CG13321	2,5E-05	1,6	
CG2229	2,1E-05	1,6	Jon99F
CG16844	2,6E-04	1,6	Immune induced molecule 3
CG40251	4,5E-04	1,6	
CG6530	2,6E-04	1,6	methuselah-like 3
CG8736	2,5E-04	1,6	
CG3027	4,8E-05	1,6	
CG1873	2,6E-05	1,6	elongation factor 1-alpha F2
CG1919	1,3E-04	1,6	
CG7584	9,3E-04	1,6	Obp99b
CG10943	1,7E-04	1,6	
CG31199	3,8E-05	1,6	CG17837
CG2533	5,8E-05	1,6	
CG11841	1,1E-04	1,6	

CG30160	1,3E-04	1,6	
CG5506	3,2E-05	1,6	
CG13102	1,6E-04	1,6	
CG18106	9,3E-05	1,6	Immune induced molecule 2
CG14972	2,6E-04	1,6	
CG6357	4,0E-05	1,6	
CG8442	9,6E-04	1,6	dGluRI
CG6429	1,6E-04	1,6	
CG4734	4,6E-05	1,6	
CG8012	3,0E-04	1,6	
CG13067	1,2E-05	1,6	
CG6188	7,8E-04	1,6	
CG3006	3,7E-04	1,6	Flavin-containing monooxygenase 1
CG15316	2,5E-04	1,6	
CG3961	4,4E-05	1,6	
CG10178	1,4E-04	1,6	
CG6385	9,1E-04	1,6	
CG3168	5,9E-05	1,6	
CG14292	1,6E-04	1,6	
CG3066	2,8E-05	1,6	SP7
CG3132	3,4E-04	1,6	Ect3
CG1171	1,3E-04	1,6	Adipokinetic hormone-like
CG8756	1,7E-04	1,5	LDLa domain containing chitin binding protein 1
CG31747	5,2E-05	1,5	Gustatory receptor 36a
CG16885	2,5E-04	1,5	CG18634
CG18410	4,3E-05	1,5	
CG8281	1,4E-04	1,5	
CG15505	2,4E-04	1,5	Obp99c
CR33258	5,4E-05	1,5	
CG5254	1,8E-04	1,5	
CG40153	1,5E-04	1,5	
CG7267	2,6E-04	1,5	
CG17919	2,0E-04	1,5	
CG18778	1,3E-04	1,5	
CG15582	9,8E-04	1,5	Obp83c
CG4000	1,7E-04	1,5	
CG31148	1,7E-04	1,5	
CG13335	7,7E-05	1,5	
CG10911	1,6E-04	1,5	
CG4676	5,1E-04	1,5	
CG30219	1,3E-04	1,5	

CG11797	3,5E-04	1,5	Odorant-binding protein 56a
CG31004	7,7E-04	1,5	
CG14493	5,3E-04	1,5	Dpr-13
CG4312	5,3E-04	1,5	Metallothionein B
CG15690	2,6E-04	1,5	
CG32499	3,5E-04	1,5	
CG8615	6,8E-04	1,5	Ribosomal protein L18
CG10877	2,3E-04	1,5	
CG30026	4,7E-04	1,5	
CG31118	1,3E-04	1,5	RabX4
CG13510	6,6E-04	1,5	
CG6514	2,4E-04	1,5	TpnC II
CG9119	4,6E-04	1,5	
CG8300	3,3E-04	1,5	
CG11529	3,2E-04	1,5	SP84
CG14416	8,0E-04	1,5	
CG16858	4,6E-04	1,5	viking
CG5853	4,6E-04	1,5	
CG16771	5,8E-04	1,5	
CG15546	1,8E-04	1,5	
CG8629	2,8E-04	1,5	
CG13375	9,3E-05	1,5	
CG10250	4,4E-04	1,5	nautilus
CG15883	7,4E-04	1,5	Odorant-binding protein 18a
CG10246	2,7E-04	1,5	CYP6A9
CG32433	1,0E-04	1,5	Gustatory receptor 77E.1
CG9899	1,9E-04	1,5	
CG12023	1,9E-04	1,5	DIP1
CG15068	1,9E-04	1,5	
CG8561	1,3E-04	1,5	tartan/capricious-like
CG12817	2,2E-04	1,5	
CG4696	1,6E-04	1,5	Muscle protein 20
CG5873	4,9E-04	1,5	
CG18404	3,5E-04	1,5	
CG7106	1,6E-04	1,5	lectin-28C
CG4325	1,1E-04	1,5	
CG11961	1,3E-04	1,5	
CG8128	3,1E-04	1,5	
CG13467	2,7E-04	1,5	
CG12934	3,2E-04	1,5	
CG30016	2,3E-04	1,5	

CG17839	1,1E-04	1,5	
CG5390	1,2E-04	1,5	
CG1634	9,2E-04	1,5	Neuroglian
CG31749	1,5E-04	1,4	
CG8343	2,3E-04	1,4	Incilarin-like
CG40224	1,8E-04	1,4	
CG15422	8,3E-05	1,4	
CG30334	3,7E-04	1,4	
CG3088	4,2E-04	1,4	SPH188
CG10460	2,0E-04	1,4	crammer
CG2157	2,9E-04	1,4	
CG40486	3,8E-04	1,4	
CG8952	1,9E-04	1,4	SP52
CG3292	4,1E-04	1,4	
CG7442	4,2E-04	1,4	
CG31380	6,9E-04	1,4	
CG14125	3,7E-04	1,4	
CG12108	9,1E-04	1,4	PPT1
CG10039	7,3E-04	1,4	
CG31812	5,7E-04	1,4	
CG13930	2,8E-04	1,4	
CG5080	6,1E-04	1,4	
CG5778	1,6E-04	1,4	
CG5730	7,3E-04	1,4	Annexin IX
CG13155	6,5E-04	1,4	
CG32912	9,7E-04	1,4	Peptidoglycan recognition protein LD
CG12898	3,6E-04	1,4	
CG15625	9,5E-04	1,4	
CG4547	6,7E-04	1,4	
CG14479	6,2E-04	1,4	
CG6785	7,4E-04	1,4	
CG6579	2,8E-04	1,4	
CG10031	7,4E-04	1,4	
CG6678	9,5E-04	1,4	
CG6730	9,2E-04	1,4	Cyp4d21
CG7981	6,1E-04	1,4	
CG33473	9,6E-04	1,4	luna
CG14109	4,5E-04	1,4	
CG10617	2,1E-04	1,4	
CR32892	7,0E-04	1,4	
CG17086	7,1E-04	1,4	

CG3299	3,5E-04	1,4	Vinculin
CG17278	8,3E-04	1,4	
CR31379	2,8E-04	1,4	
CG18735	8,5E-04	1,4	SP22
CG4194	5,4E-04	1,4	
CG7291	4,6E-04	1,4	Niemann-Pick Type C-2
CG15152	9,6E-04	1,4	
CG14898	9,9E-04	1,4	
CG31421	8,8E-04	1,4	Tak1-like 1
CG2471	6,0E-04	1,4	
CG33080	4,7E-04	1,4	
CG5316	8,3E-04	0,7	
CG14061	6,4E-04	0,7	
CG6955	3,0E-04	0,7	DCP1
CG17090	6,2E-04	0,7	HIPK
CG3972	5,2E-04	0,7	Cytochrome P450-4g1
CG14811	7,9E-04	0,7	
CG13786	6,8E-04	0,7	
CG14365	8,2E-04	0,7	
CG17018	2,5E-04	0,7	
CG7962	2,9E-04	0,7	CDP diglyceride synthetase
CG17665	2,2E-04	0,7	
CG31280	6,5E-04	0,7	Gr94E1
CG7963	3,0E-04	0,7	
CG2668	2,5E-04	0,7	Protein ejaculatory bulb
CG12970	9,7E-04	0,7	
CG3159	3,5E-04	0,7	Excitatory amino acid transporter 2
CG10050	7,5E-04	0,7	
CG31832	5,5E-04	0,7	
CG17208	6,1E-04	0,7	
CG17097	7,9E-04	0,7	
CG12179	3,9E-04	0,7	
CG3295	7,1E-04	0,7	
CG14915	6,0E-04	0,7	
CG13738	1,9E-04	0,7	
CG18585	1,5E-04	0,7	
CG14309	4,1E-04	0,7	
CG8884	2,2E-04	0,7	Synapse-associated protein 47kD
CG32919	1,3E-04	0,7	
CG8087	8,3E-04	0,7	
CG3827	4,6E-04	0,7	scute-T4-transcript

CG32439	8,3E-04	0,7	
CG12535	6,0E-04	0,7	
CG18870	3,0E-04	0,7	
CG16827	8,2E-04	0,7	alphaPS4
CG18064	1,3E-04	0,7	Met75Cb
CG13759	2,0E-04	0,7	
CG12444	7,8E-04	0,7	
CG12593	4,0E-04	0,7	dpr4
CG18066	3,5E-04	0,7	
CG14030	1,0E-04	0,7	Bub1
CG7157	2,4E-04	0,7	Accessory gland peptide 36DE
CG31872	2,8E-04	0,7	
CG33495	6,0E-05	0,7	Ductus ejaculatorius peptide 99B
CG1154	6,7E-04	0,7	Osiris 12
CG8677	6,8E-04	0,7	
CG18609	3,8E-04	0,7	
CG4905	6,9E-04	0,7	Syntrophin-like 2
CG15002	9,2E-04	0,7	masquerade
CG31047	7,5E-05	0,7	
CG32774	1,8E-04	0,7	
CG8254	1,3E-04	0,7	hb9
CG6649	6,2E-05	0,7	ugt35b
CG4582	9,6E-04	0,6	
CG7693	3,4E-05	0,6	STE20-like/SPAK
CG15772	3,6E-04	0,6	
CG30071	1,4E-04	0,6	
CG15769	4,9E-05	0,6	
CG32602	9,9E-04	0,6	
CG6892	5,9E-04	0,6	Ets96B
CG11345	6,2E-04	0,6	
CG15741	9,4E-04	0,6	
CG3600	9,4E-05	0,6	
CG6214	1,7E-04	0,6	dMRP
CG11312	9,4E-04	0,6	Inscuteable
CG8807	2,0E-04	0,6	lush
CG14610	3,0E-04	0,6	
CG32596	4,8E-05	0,6	
CG10436	3,3E-04	0,6	Obp69a
CG32791	1,5E-05	0,6	
CG3474	3,5E-04	0,6	
CG32793	2,7E-04	0,6	

CG9786	2,0E-04	0,6	hunchback
CG31736	1,8E-05	0,6	
CG8533	8,1E-04	0,6	
CG18287	8,0E-04	0,6	pickpocket 19
CG7890	7,9E-04	0,6	
CG14678	4,4E-04	0,6	
CG2973	3,4E-04	0,6	
CG14069	3,1E-04	0,6	
CG13676	4,6E-04	0,6	
CG8046	2,5E-04	0,6	
CG40383	8,0E-05	0,6	
CG30103	4,5E-06	0,6	
CG14944	9,2E-04	0,6	
CG17601	8,5E-05	0,6	
CG1225	2,5E-05	0,6	DrhoGEF3
CG11281	1,9E-04	0,5	
CG14458	9,3E-05	0,5	
CG13196	2,6E-04	0,5	
CG14242	7,3E-04	0,5	
CG32256	9,6E-04	0,5	Gustatory receptor 64c
CG18030	2,1E-04	0,4	Jonah 99Fi
CG9668	9,9E-05	0,4	RH4
CG6788	9,0E-05	0,4	
CG32496	7,4E-05	0,4	

3.5 Materials and Methods

Drosophila stocks and microscopy

The *alph*$^{S-331}$, *alph*$^{S-355}$, *alph*$^{XS-88}$ (Baril and Therrien, 2006), *slpr*^{921} (Stronach and Perrimon, 2002), *slpr*^{BS06} (Polaski et al., 2006) and *hep*^{699} (Chou and Perrimon, 1996) alleles were described previously. The *PBac(PB)CG1906*^{c04528} (*alph*^{PBac}), *bsk*^{1}, *hep*^{G0107}, *lic*^{G0252}, *p38a*^{1}, *puc*$^{A251.1F3}$, *puc*^{E69} and *pnr-Gal4* alleles were obtained from the Bloomington Stock Center.

The *pGMR-Rac1* line was kindly provided by J. Settleman (Nolan et al., 1998). The UAS-Slpr^{WT} line has previously been described in (Polaski et al., 2006), whereas the *psE-hep*^{CA}, *psE-lic*^{CA}, *GMR-alph* and *GMR-alph*$^{S-331}$ lines were generated by P-element-mediated germline transformation as described in (Rubin and Spradling, 1982). Scanning electron microscopy was performed as described in (Wassarman et al., 2000) .

Plasmids

The vector used for transfection experiments (pRMHA-5) is a modified version of the copper-inducible pMet vector (Therrien et al., 1998) that contains an alternate multiple cloning site. psE is a pW8-derived P-element transformation vector containing two *sevenless* enhancer sequences upstream of the Drosophila hsp70 promoter (Dickson

et al., 1992). The pGMR vector has been described previously (Hay et al., 1994).

The *slpr* (Clone ID:GH26507), *ddlk* (Clone ID: LD14856) and *tak1* (Clone ID: LD42274) cDNAs were obtained from the Drosophila Genomics Resource Center collections. The cDNAs were PCR-amplified using a 5'-end oligonucleotide containing sequences encoding a V5 epitope (GKPIPNPLLGLDST) inserted in place of the first methionine and cloned into the pRMHA-5 expression vector. The hep^{CA} cDNA was amplified by PCR from genomic DNA of a transgenic line containing the *hep-RC* cDNA that has Ser-346, Thr-350 and Ser-352 changed to Asp residues (Adachi-Yamada et al., 1999a). The *lic* cDNA was amplified by PCR from an aliquot of the LD cDNA library (Berkeley Drosophila Genome Project) and mutagenized using the QuickChange™ kit (Stratagene) to replace Ser200 and Thr204 to Asp residues, thereby producing the lic^{CA} cDNA. The hep^{CA} and lic^{CA} cDNAs contain a Myc epitope (AEEQKLISEEDLL) at their N-terminus and were introduced into the *pRMHA-5* and *psE* expression vectors. The *alph* and $alph^{S-331}$ cDNAs, which have been described elsewhere (Baril and Therrien, 2006), were transferred into pRMHA-5 and pGMR. They do not contain an epitope tag as it inactivates catalytic function (unpublished observations). The *Act5C-Flag-Bsk* construct was kindly provided by T. Ip. New cDNA inserts produced by PCR were entirely sequenced.

Protein analyses

S2 cells were maintained in serum-free insect cell medium (Sigma) and transfected with Effectene™ (Qiagen). Protein expression was induced by adding $CuSO_4$ (0.7 mM) to the medium 24 h post-transfection. Sources for the antibodies are the following: α-Flag M2 mAb (Sigma); α-V5 mAb (Invitrogen); α-pJNK (Cell Signaling); α-Myc mAb (9E10; Santa Cruz Biotechnology). The rabbit α-Alph polyclonal antibodies have been described previously (Baril and Therrien, 2006).

Protein lysates were prepared using standard procedures. Briefly, cells were harvested 24 h post-induction and lysed (15 min; 4°C) in NP-40 lysis buffer (20 mM Tris at pH 8.0, 137 mM NaCl, 10% glycerol, 1% Igepal CA-630, 1 mM EDTA, 0.15U/mL aprotinin, 20 µM leupeptin and 1X Sigma phosphatase inhibitor cocktail). Cell debris were removed by centrifugation at 12,000g for 15 min (4°C).

For immunoprecipitations, V5 antibodies (0.5 µl) and protein A/G Plus-agarose beads (20 µl; Santa Cruz Biotechnology) were added to cell lysates (~1 mg total proteins) and gently rocked for 4 h at 4°C. Immunoprecipitated proteins were then washed three times with cold NP-40 lysis buffer before analysis. Proteins were run onto 7-8% polyacrylamide gels and transferred to nitrocellulose membranes.

Microarray analyses

The *alph*$^{S-331}$ and *alph*^{PBac} lines were backcrossed six times in the w^{1118} isogenic background. In addition, flies were provided with food supplemented with a cocktail of ampicillin (50ug /ml)/ tetracyclin (500 ug/ml) and then penicillin/ streptomycin (Invitrogen Cat#; diluted 1:100) to get rid of any bacterial contamination that could alter the results. Isogenic and antibiotic treated flies were then raised on standard food for at least one generation before performing microarray experiments.

RNA from three independent cohorts of ~100 *alph*^{WT}, *alph*$^{S-331}$ and *alph*^{PBac} one-day-old flies was extracted with Trizol reagent (Invitrogen) using the Canadian Drosophila Microarray Center (CDMC) protocol (www.flyarrays.com; (Neal et al., 2003)). RNAs were then labeled and hybridized to the 14kv1 array at the CDMC platform (total of six array experiments). The probes on the 14kv1 array are long oligonucleotides from the International Drosophila Array Consortium (INDAC) and synthesized by Illumina. These probes are 65 to 69-mers and were designed against release 4.1 of the Drosophila Genome using a custom implementation of OligoArray2. There are 32448 spots on the array, including roughly 14 000 Drosophila transcript-specific probes spotted in duplicate, along with 13 Arabidopsis control spots that are spotted in duplicate in each of the 48 sub-arrays.

Array data were analyzed using the R and Bioconductor program. To uncover genes that are similarly modulated in both mutants compared to wild type, results from the six array experiments were normalized and analyzed simultaneously. Then, the selection of genes was based on the two following criteria: fold change of at least 1.3 and a maximal p-value of 0.001. This generated a list of 311 genes. Among these 311 hits, we searched for genes that were associated with either stress/immune/defense response, aging, detoxification or JNK/p38/Ras signaling using all attributed data found in Flybase (flybase.bio.indiana.edu). This results in a list of 58 genes shown in Table I.

3.6 Aknowledgements

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3.7 References

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Chapter 4: Discussion and perspectives

4.1 Preface

Results presented in this thesis highlight the involvement of Alph as a general negative regulator of the MAPK pathways. However, they also raise fundamental questions regarding the molecular and physiological functions of Alph. For instance, which protein(s) is/are targeted by Alph within the MAPK pathways? Since Alph is not required for normal development (loss-of-function mutants are homozygous viable with no apparent defects), what is the physiological relevance of this phosphatase? In the next section, I discuss results presented in Chapter 2 and 3 in a more general manner and I propose experiments to help answer these questions.

4.2 Strategies to find potential Alph substrates

Understanding how Alph negatively regulates the MAPK pathways, i.e. identifying potential substrates, is one crucial aspect concerning Alph molecular function. In the next sections, three strategies are discussed for the identification of Alph target proteins. The first one implies positioning more accurately Alph within the MAPK pathways. For example, if we could precisely narrow down the activity of Alph downstream of the small GTPases Ras and Rac and upstream of the MAPKKs, we could eliminate MAPKs and their downstream transcriptional targets as well as upstream

receptors as being potential substrates of Alph. A second approach would be to use forward genetic to find genes that interact genetically with *alph*. Finally, identification of Alph's interacting partners could also be done using affinity purification and mass spectrometry.

4.2.1 Determination of Alph position within the MAPK pathways

4.2.1.1 Using a genetic approach

The V12 mutation in Ras was shown to prevent GAPs from promoting the hydrolysis of GTP on Ras, and therefore cause Ras to be constitutively activated (Downward, 2003). The expression of the V12 activating variant of Ras in the Drosophila eye generates a rough eye phenotype (Karim et al., 1996), which is apparently not modified by mutations in upstream signaling molecules. Indeed, mutation in the Sev RTK or Boss ligand does not influence the rough eye phenotype generated by Ras^{V12} (Fortini et al., 1992). In addition, no mutation in DRK/Grb2, Sos or Gap1 was recovered in a genetic screen using the Ras^{V12} rough eye phenotype (Karim et al., 1996). Since *alph* mutants increased the Ras^{V12} rough eye phenotype (Chapter 2, Figure 1), its negative activity was therefore positioned downstream of Ras (Chapter 4, Figure 1).

Eye-specific expression of the Rac1 GTPase generates a rough eye phenotype, which is worsened by *alph* alleles and suppressed by

overexpression of Alph^{WT} (Chapter 3, Figure 3). Presumably, Rac1^{WT} is still sensitive to upstream signaling and the position of *alph* cannot be narrowed down with respect to this GTPase. As with Ras, the V12 mutation in Rac decreases its intrinsic GTPase activity and makes it unresponsive to GAPs (Diekmann et al., 1991). Overexpression of Rac^{V12} generates a rough eye phenotype, which is presumably due to defects in photoreceptor differentiation and misorientation of ommatidium (Fanto et al., 2000). Therefore, it would be interesting to test whether *alph* alleles increase Rac^{V12} rough eye phenotype, which would suggest that the phosphatase acts downstream of Rac1. Consistent with this model, preliminary data in S2 cells showed that overexpression of Alph suppresses pJNK generated by Rac^{V12} (data no shown) (Chapter 4, Figure1).

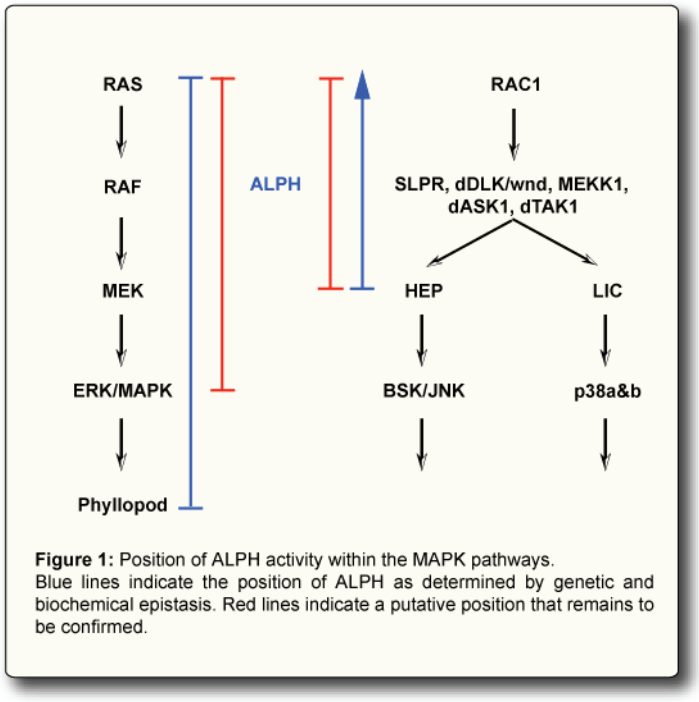

Figure 1: Position of ALPH activity within the MAPK pathways.
Blue lines indicate the position of ALPH as determined by genetic and biochemical epistasis. Red lines indicate a putative position that remains to be confirmed.

alph mutants also increased the rough eye phenotype generated by an activated form of Raf (Raf^{Act}; data not shown) and ERK/MAPK (MAPK^{SEM}; Chapter 2, Figure 1). Although this would suggest

that Alph activity resides downstream of ERK/MAPK, we cannot clearly rule out the possibility that Alph acts upstream of these two kinases. Indeed, the Raf transgene used in this genetic test (Dickson et al., 1992) is most likely responsive to upstream signaling since it still contains phosphorylation sites required for Raf activation. For example, the phosphoacceptor sites Thr571 and Thr574 are preserved in the Raf^{Act} construct, and have been shown to be critical for Raf activation (Douziech et al., 2006). Moreover, the *sevenmaker* (SEM) mutation introduced into ERK/MAPK transgene generates a kinase that still requires activation by RTKs, but renders the kinase less sensitive to downregulation by phosphatases (Bott et al., 1994). Our results at least imply that Alph acts upstream of Phyl (Chapter 2, Figure 1). In addition, the Alph-dependent diminution of pMAPK staining in Ras^{V12} expressing clones suggests that Alph activity resides upstream of, or on ERK/MAPK itself (Chapter 2, Figure 8). However, this decreased pMAPK staining may be due to unspecific effects of overexpressing a catalytically active phosphatase.

Having transgenic flies that express constitutively activated forms of Raf, MEK or ERK/MAPK that are insensitive to upstream signaling would be great tools to investigate the position of Alph within the Ras/MAPK pathway. No activating modifications of Raf or ERK/MAPK have been shown to have these properties. However, a gain-of-function variant of MEK (MEK^{EE}), which contains a substitution of two serine residues for glutamic acid in its activation loop, is insensitive to upstream signaling

(Huang and Erikson, 1994; Huang et al., 1995). Therefore, transgenic flies expressing MEK^{EE} during eye development should help position the activity of Alph with respect to MEK. The Hep^{CA} and Lic^{CA} transgenes both have these phosphomimetic mutations that render the kinase constitutively activated and insensitive to upstream signaling. Since *alph* alleles as well as overexpression of $Alph^{WT}$ do not modify this phenotype, it suggests that the phosphatase is acting upstream of both SAPKKs (Chapter 3, Figure 3). Biochemical experiments in S2 cells also support this view, since overexpression of $Alph^{WT}$ has no impact on the pJNK (Chapter 3, figure 4) or pp38 (data not shown) generated by the overexpression of Hep^{CA} or Lic^{CA}, respectively.

4.2.1.2 Using a biochemical approach

The RNAi technology was also used to position the activity of Alph using a biochemical approach in S2 cells. We expected that the knockdown of Alph expression would enhance ERK/MAPK or SAPK signaling following various activating signals. As a readout for pathway activation, we evaluated the amount of dually phosphorylated ERK/MAPK or JNK (pMAPK or pJNK) using a phospho-specific antibody and immunoblotting strategy. Even though Alph RNAi was strongly depleting Alph proteins, no increase in pMAPK was detected following activation by Sev, DER or activated Ras, Raf or MEK (data not shown). The effect of

Alph RNAi was also evaluated on the level of pJNK generated by overexpression of Slpr, dDLK, Tak1 and Hep^{CA} as well as by treatment with bacterial lipopolysaccharide (LPS), H_2O_2 or NaCl. Again, in all conditions tested, Alph RNAi had no impact (data not shown). In brief, all activating conditions of the ERK/MAPK or JNK pathway tested so far were unaffected by Alph depletion.

Interestingly, a high throughput RNAi screen designed to isolate regulators of the RTK/Ras/MAPK pathway was recently published (Friedman and Perrimon, 2006). The authors used various ERK/MAPK pathway activators (PDGF, DER ligand Spitz and Insulin) to activate the pathway in S2R+ cells (S2 cells that express the wingless receptor and which are more strongly adherent than regular S2 cells) and Drosophila embryonic KC167 cells. They screened the whole genome for genes that, when silenced by RNAi, would modify the level of pMAPK detected by immunofluorescence. The screen was validated by pulling out known regulators of the Ras/MAPK pathway such as Ras, KSR, CNK and PTP-ER. However, in accordance with our own results, Alph depletion had no effect on pMAPK levels in any condition tested.

Although it is possible that embryonic cell lines (S2 and KC167) do not depend on Alph, functional redundancy among specific PP2C phosphatases in the Drosophila genome most likely explains these negative results. Indeed, this phenomenon has been observed in *S. cerevisiae*. The yeast genome comprises 32 phosphatases and only two

single disruptants showed a lethal phenotype. In addition, double disruptants of the 30 non-essential phosphatases in all possible combinations showed no synthetic lethality (Sakumoto et al., 2002). More closely related to my work, the yeast Ptc1, Ptc2 and Ptc3 PP2C phosphatases are all implicated in dephosphorylating the Thr residue in the activation loop of Hog1, the p38/JNK homolog. However, extensive analyses of Ptc functions revealed that while Ptc1 is required to maintain a low basal Hog1 activity, Ptc2 and Ptc3 set the maximal limit to which stress can activate Hog1 (Saito and Tatebayashi, 2004). We identified 15 PP2C phosphatases in the Drosophila genome, among which three have high sequence homology to Alph (Chapter 2, suppl. Figure 1 and 2). Indeed, CG6036, CG12169 and CG17746 share 53%, 35% and 38% amino acid identity to Alph respectively and could thus perform similar molecular functions. In support of this view, the genome wide RNAi screen designed to isolate new regulators of the RTK/Ras/MAPK pathway identified CG12169 (renamed dPPM1) as a potential negative regulator of the pathway (Friedman and Perrimon, 2006). They showed that dPPM1 RNAi increased pMAPK staining intensity by up to 25% following Insulin and EGF receptor activation. In addition, by co-immunoprecipitation assays using overexpressed proteins, they also demonstrated that dPPM1 physically interacts with ERK/MAPK, which suggests that ERK/MAPK itself is a dPPM1 target. However, the effect of dPPM1 depletion is very modest when ERK/MAPK is activated by Ras^{V12} in S2R+ cells (less than 10% increase).

The ability of the cell to maintain normal levels of pMAPK or pJNK in the absence of Alph may thus rely on a backup phosphatase that performs similar functions. The identification of this redundant phosphatase is probably crucial to reveal the true function of Alph and also to help positioning its activity within the MAPK pathways in our cell-based assay. A systematic approach using high throughput RNAi screening could achieve such goal. Single disruption of the 86 phosphatases present in the Drosophila genome (Morrison et al., 2000) could first be tested on the level of pMAPK, pJNK and pp38 generated by various activating stimuli in S2 cells. Then, the impact of double, triple or quadruple disruptants (or even more!) could be assessed using the same conditions. Another possibility would be to block the availability of Alph substrate to a potential redundant phosphatase. It has been shown that the substitution of an arginine residue for alanine in human PP2Cα and *C. elegans* FEM-2 PP2C domain impairs catalytic function without affecting substrate binding (Chin-Sang and Spence, 1996; Takekawa et al., 1998). This particular amino acid substitution, which could be performed in Alph (Arg189), may therefore protect Alph phosphorylated substrate by limiting the access to redundant phosphatase. Therefore, we could deplete the endogenous contribution of the wild type Alph phosphatase using a UTR-targeting RNAi and concomitantly restore it exogenously using a non-targettable construct that expresses $Alph^{Arg189Ala}$ proteins (a rescue strategy previously used in (Douziech et al., 2003)). Using this rescue strategy, the

effect of Alph^{Arg189Ala} expression could then be evaluated on the activation of the ERK/MAPK and SAPK pathways in S2 cells.

4.2.2 Forward genetics

As mentioned earlier in the Introduction, forward genetics in Drosophila has been widely employed to dissect signaling pathways or to find genetic interactors. A strategy to gain insight about Alph would be to perform an enhancer screen using a homozygous viable *alph* allele. We would probably recover mutations in known negative regulators of the Ras/MAPK pathway, since it has already been shown that removing Gap1, PTP-ER or Yan in the *alph*$^{-331}$ or *alph*^{PBAC} background produces a mild rough eye phenotype or extra wing vein materials (Chapter 1, Figure 6). In addition, this type of screen could also identify new negative regulators of the MAPK pathways as well as putative phosphatases having redundant functions with Alph. Similar screens in yeast have successfully identified genetic interactions between PP2C family member Ptc1 and the tyrosine phosphatase Ptp2 (Maeda et al., 1993). Ptc1 and Ptp2 were then shown to dephosphorylate respectively the Thr and the Tyr residue in the activation loop of Hog1, the p38/JNK yeast homolog (Young et al., 2002).

Overexpression of Alph^{WT} during Drosophila eye development had no effect on the external and internal architecture of the eye (data not shown and Chapter 2, Table 2). Using the UAS/GAL4 system, we tested

whether overexpression of Alph^{WT} would have an effect in other tissues. Although Alph^{WT} generated no visible phenotype when overexpressed in the brain, hemocytes, fat bodies and postmitotic neurons, ubiquitous expression of Alph^{WT} (but not a catalytically dead mutant) was lethal (data not shown). The developmental defect that caused lethality is not known. However, this phenotype could be used to screen for mutations in genes that would restore viability. These mutations could possibly be in putative Alph substrates if, for example, they inhibit the interaction of the phosphatase with its substrate(s). We could also uncover mutations in genes critically required to generate Alph-dependent lethality phenotype such as in Alph regulatory subunits or cofactors as well as downstream targets.

4.2.3 Proteomics

The systematic study of the complete protein complement of organisms was greatly facilitated by mass spectrometry (MS) analyses. For example, many protein/protein interactions as well as post-translational modifications on particular proteins were discovered using MS. Mapping of protein-protein interactions involves an affinity purification step of protein complexes which is usually the limiting and challenging part of the experiment rather than protein identification. This is due to the fact that interactions are transient in nature and interacting proteins can be lost during sample preparation. In addition, protein complexes may also be low in abundance, which render enrichment highly difficult. The use of cross-

linking agents, which “freeze” protein–protein interactions in their in vivo state and stabilizes complexes for subsequent purification, is an effective tool used to circumvent the labile nature of protein complexes. In addition, the fusion of tags, peptides, or protein domains to protein of interest has facilitated the purification of protein complexes to near homogeneity (Vasilescu and Figeys, 2006).

The identification of proteins associated with Alph should help elucidate its function and also shed light on putative substrates. However, possible problems may interfere with this strategy. Indeed, earlier attempts to fuse an epitope tag to the N-terminal or C-terminal part of the protein were found to inactivate the enzyme. We have shown that overexpression of $Alph^{WT}$ suppressed the pMAPK generated by activated Ras using an immunoblotting approach in S2 cells (data not shown). However, the fusion of a Polyoma (PYO) epitope to either the 5’ or 3’ extremities of Alph generated a catalytically dead enzyme as shown by its inability to suppress the pMAPK generated by Ras^{V12} in S2 cells (data not shown). Using a similar assay, the insertion of a Glutathione-S-Transferase (GST) tag to the 5’ end of the protein also inactivated the enzyme (data not shown). These results suggest that adding a tag either to the N-terminal or C-terminal part of Alph interferes with its enzymatic activity *in vivo* or with its ability to bind its substrates. It is also possible that this effect is specific to the PYO and GST epitopes and therefore, other tags must be tested. For example, the Tandem Affinity Purification (TAP) method, which

involves the fusion of a bipartite tag (ProtA and CBP) to the protein of interest, could be assayed in S2 cells (Puig et al., 2001). In addition, modified versions of the TAP tag, which include a peptide that binds a derivative of streptavidin or a hexahistidine peptide that binds Nickel column, could also be tested (Vasilescu and Figeys, 2006). As an alternative purification step, the polyclonal antibody raised against the full-length Alph protein could be used to immunoprecipitate endogenous Alph and its associated proteins in S2 cells or WT adult flies. As mentioned earlier, the substitution of a conserved Arg for Ala in the PP2C catalytic domain inactivates the phosphatase without affecting substrate binding (Chin-Sang and Spence, 1996; Takekawa et al., 1998). In addition, this mutation was shown to generate a "substrate-trapping"-like phosphatase (Takekawa et al., 1998). Therefore, the expression of this particular Alph mutant (Arg189Ala) in S2 cells or in transgenic flies would probably be more efficient to purify proteins transiently associated with Alph.

4.3 Is Alph mediating crosstalk between ERK/MAPK and SAPK pathways?

The work presented in this thesis showed that Alph acts as a general negative regulator of ERK/MAPK and SAPK pathways. In other organisms, PP2C phosphatases have been implicated in the dephosphorylation of the cell cycle regulators CDK2 and CDK6 (Cheng et

al., 2000); the cell cycle arrest most probably via p53 activation (Ofek et al., 2003); the regulation of glucose uptake via the insulin/PI3K pathway (Yoshizaki et al., 2004); the positive regulation of the Wingless pathway by dephosphorylating axin (Strovel et al., 2000); the downregulation of the NFκB pathway through the dephosphorylation of IκB (Prajapati et al., 2004); and the inhibition of the SAPK (JNK and p38) pathway (Hanada et al., 1998; Hanada et al., 2001; Takekawa et al., 1998). Since PP2C phosphatases dephosphorylate a broad range of substrates implicated in a diversity of biological processes, it would not be surprising to find that Alph has multiple targets within the ERK/MAPK-SAPK cascades (Chapter 4, Figure 2A) or that it modulates these signaling pathways through a third pathway (Chapter 4, Figure 2B). Crosstalk may also exist between ERK/MAPK and SAPK pathways and Alph activity could reside at this interconnecting point. For example, Alph could directly dephosphorylate/inactivate a common constituent required positively in both pathways (Chapter 4, Figure 2C). Moreover, Alph could be involved in the inactivation of the SAPK pathways, which then influences signaling through the ERK/MAPK cascade or vice versa (Chapter 4, Figure 2D). These two latter possibilities are discussed in the next sections.

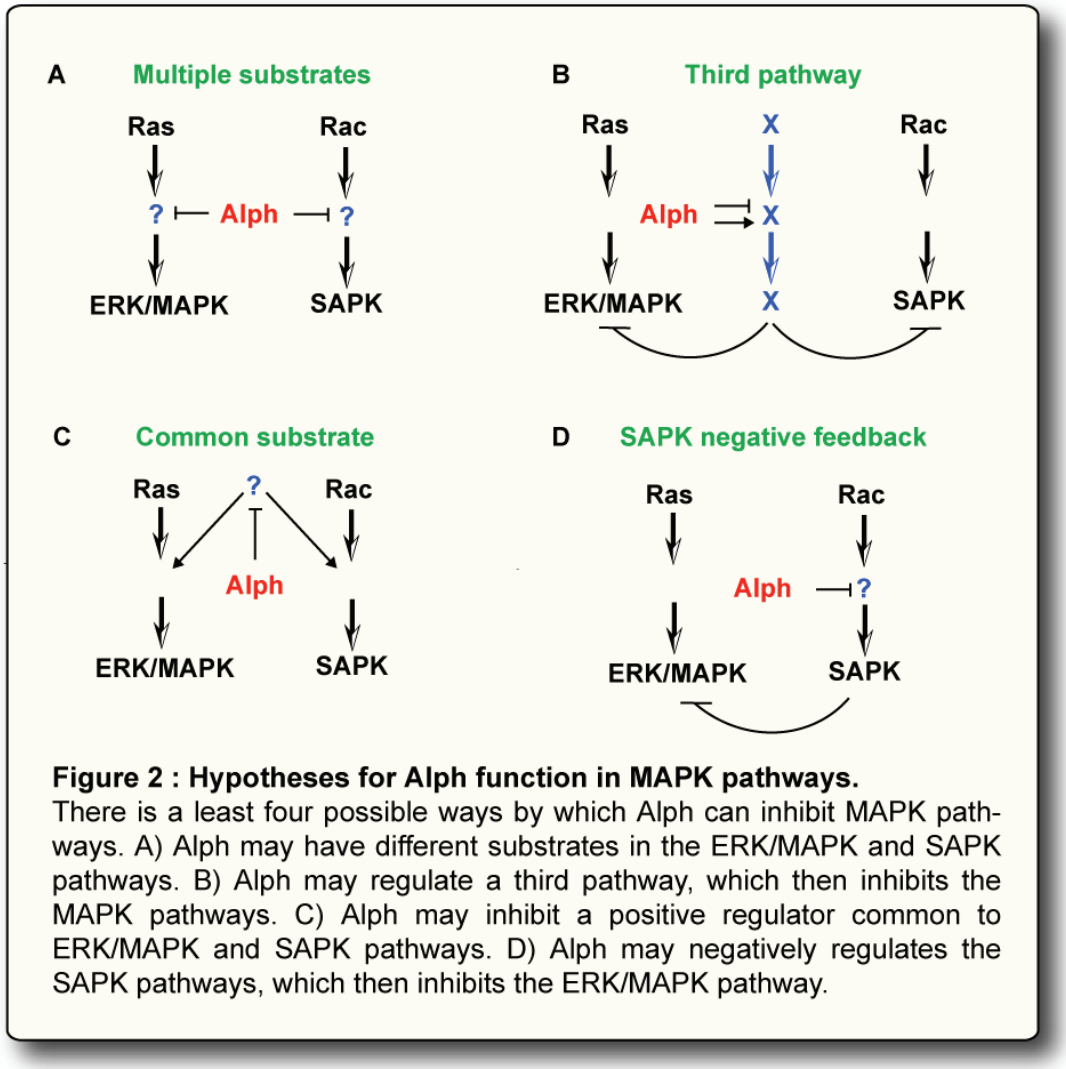

Figure 2 : Hypotheses for Alph function in MAPK pathways.
There is a least four possible ways by which Alph can inhibit MAPK pathways. A) Alph may have different substrates in the ERK/MAPK and SAPK pathways. B) Alph may regulate a third pathway, which then inhibits the MAPK pathways. C) Alph may inhibit a positive regulator common to ERK/MAPK and SAPK pathways. D) Alph may negatively regulates the SAPK pathways, which then inhibits the ERK/MAPK pathway.

4.3.1 One substrate, two pathways: The SAPKKK theory

According to our genetic and biochemical data, Alph negative activity resides upstream of Hep and Lic and possibly downstream of Rac1. Therefore, SAPKKKs (Slpr/dMLK, Wnd/dDLK, Tak1, Mekk1 or Pk92b/dASK1), which are positively required for JNK signaling at a step upstream of Hep/Lic and necessitate phosphorylation events for full catalytic activation, are candidate Alph substrates. Furthermore, mammalian PP2Cα/β/ε were shown to specifically dephosphorylate the

SAPKKK Tak1 and inactivate IL1-induced JNK pathways in mammalian cells (Hanada et al., 2001; Li et al., 2003). Interestingly, SAPKKKs have been implicated in crosstalk between SAPK and ERK/MAPK. For instance, MLK3 is required for Ras-induced proliferation and mitogen activation of ERK (Chadee and Kyriakis, 2004a; Chadee and Kyriakis, 2004b). The effect of MLK3 on ERK pathway is independent of MLK3 kinase activity since kinase-dead mutants are still capable of ERK activation. Instead, MLK3 is implicated in the maintenance of a heterotrimeric complex containing Raf-1 and B-Raf (Chadee et al., 2006). It was also demonstrated that activation of ERK by serum factors is strongly diminished in *mekk1-/-* mouse embryonic fibroblast (Yujiri et al., 1998) and that Mekk1 serves as a platform for Raf/MEK/ERK proteins (Karandikar et al., 2000). Mekk1 interacts with KSR and this interaction may facilitate the activation of ERK by proinflammatory stimuli such as TNF, IL-1, and LPS (Fusello et al., 2006). Since SAPKKKs appear to be required for activation of the SAPK pathways as well as the ERK/MAPK module, they are good candidate substrates for Alph. The SAPKKK theory obviously raises many questions concerning the potential connection between ERK/MAPK and SAPK pathways. Are SAPKKKs directly implicated in the activation of ERK/MAPK by usual RTKs (EGF and Sevenless receptors) or by other receptors? Do SAPKKKs interact with known components of the RTK/Ras/ERK pathway? Are SAPKKKs true Alph substrates and what is the contribution of Alph to SAPKKKs-mediated ERK/MAPK regulation?

4.3.1.1 Are SAPKKKs capable of activating the ERK/MAPK pathway?

Preliminary data in S2 cells showed that overexpression of Slpr, dDLK and Tak1 leads to increased level of pMAPK as revealed by Western blot (data not shown). The RNAi technology should help elucidate whether intrinsic ERK/MAPK cascade components such as Ras, Raf, KSR, CNK or MEK are required for SAPKKKs-mediated activation of ERK/MAPK. In addition, physical interaction between SAPKKKs and components of the ERK/MAPK pathway could be evaluated using immunoprecipitation and immunoblotting techniques. Interestingly, MLK2 as well as MKK7 and two RhoGEFs have been reported to interact with CNK in mammalian cells. However, this interaction appears to be required for JNK pathway activation (Jaffe et al., 2005).

Since Slpr, dDLK and Tak1 UAS transgenic lines are available, we could also test whether clonal expression of these kinases during Drosophila eye development increases pMAPK staining as has been shown for activated Ras (Chapter 2, Figure 8). Moreover, the impact of loss-of-function mutations in components of the Ras/MAPK pathway could be evaluated on the phenotype generated by the overexpression of SAPKKKs. For example, expression of Tak1 during Drosophila eye development generates a rough eye phenotype, which is suppressed by mutation in Bsk (Takatsu et al., 2000). We could also verify whether

ERK/MAPK or MEK inactivation modify the Tak1-dependent rough eye phenotype.

4.3.1.2 Are SAPKKKs activating ERK/MAPK signalling via usual RTKs?

At this point, we do not know if SAPKKKs are required for activation of the Ras/MAPK module by cognate RTKs (DER or Sev) or by other receptors known to activate the SAPK pathways. Interestingly, RTK/Ras/MAPK-dependent screens in the Drosophila eye did not uncover mutations in SAPKKKs (Dickson et al., 1996; Huang and Rubin, 2000; Karim et al., 1996; Pagliarini and Xu, 2003; Rebay et al., 2000; Therrien et al., 2000). These results suggest that SAPKKKs are not intrinsically required for RTK/Ras/MAPK pathway activation. However, functional redundancy may also explain the failure to recover SAPKKKs alleles. It would therefore be interesting to test the ability of *tak1*, *ddlk*, *slpr* and *mekk1* alleles (*ask1* alleles are presently not available) to modify the rough eye phenotype generated by activated RTKs or Ras, alone or in combination.

If SAPKKKs are not intrinsically required for activation of the ERK/MAPK module by DER or Sev, they may be used by proinflammatory cytokines to signal via the ERK/MAPK cassette. Indeed activation of ERK has been reported following treatment of cells with TNF or IL-1 (Lu et al.,

1997; McLeish et al., 1998). In Drosophila, the eye-specific expression of Eiger, the sole TNF superfamily ligand encoded in the genome, induces massive apoptosis and generates flies with no eyes. In addition, Eiger-dependent apoptotic program relies on the activation of Tak1, Hep and Bsk (Igaki et al., 2002). Mutations in components of the ERK/MAPK pathway could be tested to verify if they dominantly modify the Eiger-dependent phenotype.

4.3.1.3 Are SAPKKKs true substrates for Alph?

Biochemical experiments in S2 cells showed that overexpression of $Alph^{WT}$ suppressed pJNK induced by Slpr, dDLK and Tak1 but not by activated Hep (Chapter 3, Figure 4). In addition, Tak1 is a PP2C phosphatase substrate in other model organisms (Hanada et al., 2001; Li et al., 2003). Hence, we investigated whether Alph would act in a similar way in Drosophila. Using an overexpression system, we showed that $Alph^{WT}$ physically interacts with Tak1 but not with dDLK or Slpr (Chapter 3, Figure 4). The interaction of $Alph^{WT}$ with dDLK or Slpr may be weaker or more labile than with Tak1. Interestingly, catalytically dead *alph* mutants (XS-88, S-331 and S-355) are found in a complex with all three SAPKKKs (Chapter 3, Figure 4 and data not shown). These results suggest that the mutants recovered in the screen could possibly act as natural "substrate-trapping" molecules. To validate these interactions in an endogenous context, protein extracts from wild type versus $alph^{S-331}$ mutant flies could

be used for coimmunoprecipitation studies. Physical interactions between Alph and Tak1 (and possibly Slpr and dDLK) suggest that these kinases are potential Alph substrates. However, we do not know if Alph has the capacity to directly dephosphorylate SAPKKKs. The chemical shift of Slpr, dDLK and Tak1 shown by Western blot analyses suggests that these kinases are postranslationally modified (Chapter 3, Figure 4). Phosphorylation is likely to be responsible for SAPKKKs slow migrating forms since treatment of immunoprecipitated Slpr, dDLK and Tak1 with alkaline phosphatase abolishes this shift (data not shown). We could therefore address whether bacterially produced Alph behaves similarly, which would suggest a direct effect of Alph on these kinases.

The SAPKKKs Mekk1 and Pk92b/dASK1 may also be implicated in the activation of JNK at a step upstream of Hep (Stronach, 2005). We should therefore test the ability of Alph to suppress pJNK generated by Mekk1 and Pk92b. Based on their primary amino acid sequence, Mekk1 and Pk92b belong to a different family of kinases (Manning et al., 2002). Indeed, Tak1, dDLK and Slpr are part of the Tyrosine-Kinase Like (TKL) family while Mekk1 and Pk92b are included in the Sterile Kinase (STE) family. If Alph overexpression has no impact on the pJNK induced by Mekk1 and Pk92b, this would suggest that the phosphatase is a specific inhibitor of TKL but not of STE family members.

4.3.2 One substrate, one pathway and a negative feedback

4.3.2.1 Alph inhibits SAPK signaling, which results in ERK/MAPK signaling attenuation

Three independent studies in Drosophila recently reported that JNK signaling influences ERK/MAPK pathway activation. A first study showed that the clonal overgrowth of cells overexpressing oncogenic Raf was suppressed by JNK activation during Drosophila eye development. However, these clones expressing activated Raf and JNK mediated hyperproliferation of adjacent wild type tissues in a non-cell autonomous manner (Uhlirova et al., 2005). In a second study, loss of polarity genes (such as *scribble*, *discs large* and *lethal (2) giant larvae*) in Ras^{V12}-expressing clones drove overgrowth and metastatic behavior in a JNK-dependent manner (Igaki et al., 2006; Pagliarini and Xu, 2003). The invasiveness of these cells was subsequently explained by the upregulation of matrix metalloprotease gene *mmp1* downstream of JNK (Uhlirova and Bohmann, 2006). Finally, the high throughput RNAi screen designed to identify genes implicated in RTK/Ras/MAPK signaling showed that JNK activation consistenly negatively affected this pathway (Friedman and Perrimon, 2006). Indeed, gene silencing of two downstream transcriptional targets of the JNK pathway, DJun and DFos, as well as Rac and Rho GTPases led to increased pMAPK staining generated by activation of DER, InR or PDGF-R. On the other hand, inactivation of negative regulators of the JNK pathway such as Puc and RhoGAPs

suppressed pMAPK staining generated by these RTKs. Taken together, these results suggest that, depending on the cellular context, the JNK pathway either cooperates with or antagonizes ERK/MAPK signalling to generate a specific biological response. It is thus possible that Alph negative outcome on the Ras/MAPK pathway is mediated by an indirect effect on the JNK pathway.

Increased JNK signaling in Ras^{V12}-expressing clones results in tissue overgrowth and metastasis (Igaki et al., 2006; Pagliarini and Xu, 2003). Since *alph* inactivation leads to increase JNK signaling, one could test whether *alph* mutants promote overgrowth and/or metastatic behavior of Ras^{V12}-expressing clones. In a more simplistic manner, we could also test whether mutations in *bsk*, *hep* or SAPKKKs suppress the increased rough eye phenotype generated by *alph* alleles in the Ras^{V12} background (Chapter 2, Figure 1). Although heterozygous mutations in JNK pathway components have no effect on the rough eye phenotype generated by Ras^{V12} (data not shown), a slight increase in JNK signaling, which is provided by *alph* inactivation, may cooperate with Ras. This would explain why *alph* alleles were pulled out of a Ras-dependent screen. In the next section, I describe one way by which *alph* alleles could increase SAPK signaling and modulate indirectly the ERK/MAPK pathway at the same time.

4.3.2.2 The TAB1 theory

Tak1 was originally identified as a TGF-β-activated SAPKKK and was subsequently shown to play an important role in intracellular responses to proinflammatory cytokines, such as IL-1 and TNF (Chen et al., 2006b). In mammalian cells, Tak1 is interacting with two other proteins, namely Tak1-binding protein 1 (TAB1) and either TAB2 or the structurally related TAB3 (Cheung et al., 2004). TAB1 is apparently required for Tak1 activation (Shibuya et al., 1996), while TAB2/TAB3 mediate interactions with upstream signaling components (Cheung et al., 2004; Takaesu et al., 2000). Interestingly, sequence alignment and crystal structure analyses revealed that the TAB1 N-terminal portion is related to PP2Cα (Conner et al., 2006; Ge et al., 2003). However, this PP2C domain is not functional due to the non-conservation of critical amino acid residues required for catalysis. It has thus been hypothesized that TAB1 acts as a pseudo-phosphatase regulating the availability of phosphorylated residues on downstream targets of Tak1 or on Tak1 itself (Conner et al., 2006). No TAB1-like gene was identified in Drosophila. However, Drosophila TAB2 was recently reported to bind Tak1 and have a role in innate immunity induced by the JNK and NF-κB pathways as well as in apoptosis induced by Eiger (Geuking et al., 2005; Zhuang et al., 2006a). Interestingly, the closest Drosophila homolog of human TAB1 protein is Alph. In addition, the three *alph* alleles recovered in the genetic screen (XS-88, S-331 and S-355) possess point mutations in highly conserved residues within the

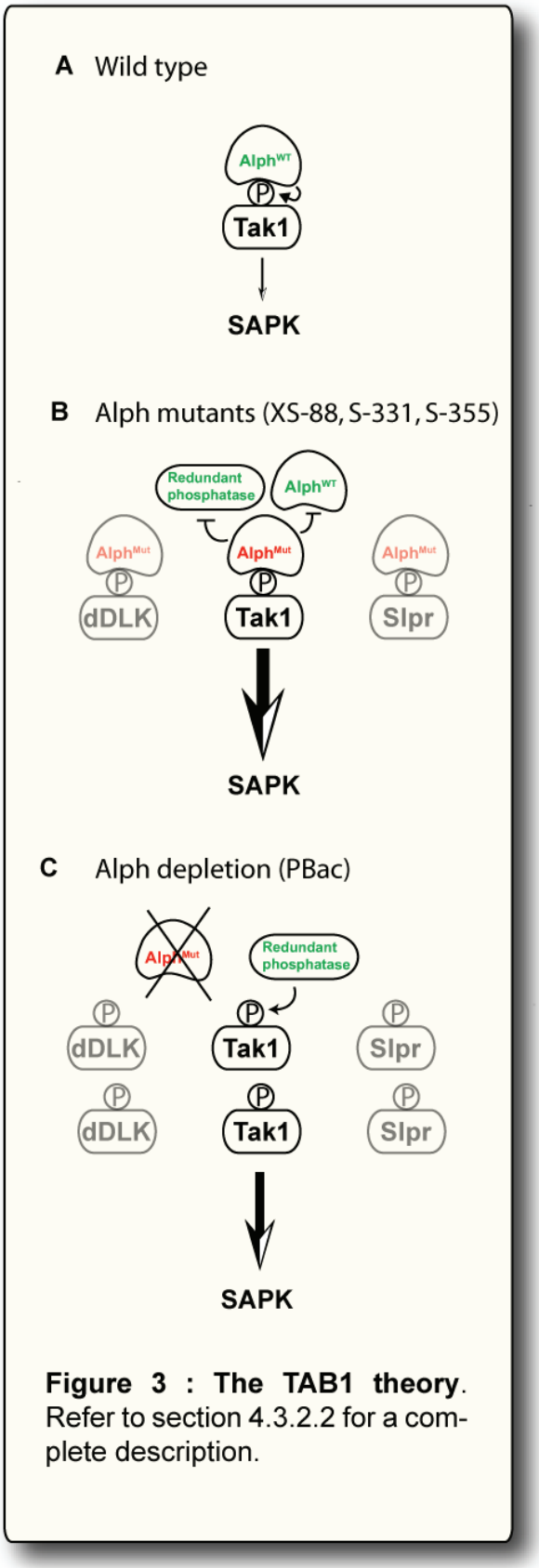

Figure 3 : The TAB1 theory. Refer to section 4.3.2.2 for a complete description.

catalytic domain, which impaired phosphatase activity *in vitro* (Chapter 2, Figure 4). It is thus possible that *alph* alleles behave like a TAB1 molecule. In a normal cellular context, Alph would dephosphorylate and inactivate Tak1 (Chapter 4, Figure 3A). In the case were Alph is catalytically inactive, it would somehow protect Tak1 from being dephosphorylated by $Alph^{WT}$ or another redundant phosphatase. Inactivating mutations in *alph* could also render the phosphatase capable of binding and protecting other SAPKKKs such as dDLK or Slpr. These two latter scenarios would lead to increased SAPK

signaling (Chapter 4, Figure 3B). Finally, in the case where Alph is depleted, more Tak1 molecules (and/or other SAPKKKs) would be phosphorylated or would be maintained phosphorylated for a longer period of time. However, SAPK signaling may not be as high as in the case of mutant Alph since redundant phosphatases may compensate (Chapter 4, Figure 3C).

If the mutated versions of *alph* recovered in the screen act as TAB1-like molecules, how come their overexpression in S2 cells does not lead to increased pJNK when the pathway is induced by Slpr, dDLK and Tak1 (Chapter 3, Figure 4)? It has been proposed that the simple overexpression of these SAPKKKs bypasses the requirement for upstream activating signaling. The mechanism by which these kinases are activated is not fully understood but seems to involve autophosphorylation events (Gallo and Johnson, 2002). Thus, in our assay, it is likely that SAPKKKs are always fully activated and no longer sensitive to mutant Alph. To circumvent this problem, we should use a more upstream activator of the JNK pathway such as Rac1 or Eiger and test whether overexpression of mutant Alph increases the level of pJNK. Another experiment would be to look whether Alph mutant proteins have dominant-negative activity toward $Alph^{WT}$. To do so, one could express $Alph^{WT}$ together with increasing amounts of mutated Alph and see whether it influences the level of pJNK generated by the overexpression of Slpr, dDLK and Tak1. Finally, pJNK may not be a suitable readout to evaluate

the TAB1-like effect of Alph mutants. Indeed, in certain biological contexts, activation of p38 relies on an autophosphorylation mechanism, which is independent of usual SAPKKs/SAPKKKs but dependent on TAB1. In this particular situation, TAB1 appears to act as an adaptor molecule for p38 and upstream TNF-associated signaling proteins such as TRAFs (Ge et al., 2002). More recently, TAB1 was proposed to sequester p38 in the cytoplasmic fraction and to antagonize the downstream activity of p38 induced by MKK3 (Lu et al., 2006). It is therefore possible that phosphorylated p38 is a more appropriate readout to evaluate the TAB1-like function of mutant Alph.

4.4 Alph physiological functions

Although the three *alph* alleles recovered in the screen have impaired phosphatase activity *in vitro*, homozygous *alph* mutants are viable with no external defects. Hence, Alph activity is apparently dispensable during Drosophila development but may be required for the maintenance of homeostasis in adult organism. While the role of Ras/MAPK signaling in adult Drosophila is currently unknown, signaling via the SAPK pathways is positively required for immune and stress response. We therefore hypothesize that Alph may negatively regulate these two responses in adult Drosophila.

4.4.1 Immune response

Humoral innate immunity in Drosophila relies on the production and secretion of a set of antimicrobial peptides in the hemolymph. Specific signaling pathways govern the production of these peptides, which are induced upon infection by bacteria and fungus. For instance, Gram- bacteria activate the Immune Deficiency (IMD) pathway, which is homologous to the TNF pathway in mammals. Upon infection, the death domain containing IMD protein will initiate the sequential activation of the SAPKKK Tak1 and NFκB-like molecule Rel. Activated Rel will then enter the nucleus and act as a transcriptional activator of antimicrobial peptide genes (Pinheiro and Ellar, 2006). Although Tak1 is clearly implicated in the humoral response to Gram- bacteria, conflicting results exist concerning its mode of action in the IMD pathway. Indeed, microarray experiments in S2 cells and genetic analyses showed that Tak1 regulates antimicrobial peptide production via the phosphorylation and activation of Rel (Boutros et al., 2002; Vidal et al., 2001) and/or the direct activation of the JNK pathway (Delaney et al., 2006; Kallio et al., 2005). In light of these studies, it is thus possible that Tak1 mediates part of its effect on innate immune response via direct activation of the JNK pathway.

Wild type flies normally recover from infection with Gram- bacteria. However, *tak1-/-* flies (as well as most l-o-f mutants of the IMD pathway, such as *imd-/-* and *rel-/-* flies) are highly sensitive to Gram- bacterial

infection and die two to three days post-infection (Hedengren et al., 1999; Lemaitre et al., 1995; Vidal et al., 2001). In addition, inactivation of the JNK pathway was reported to block antimicrobial peptide production in larval fat body tissue (Delaney et al., 2006). In our assays, *alph* inactivation increased JNK signaling possibly via Tak1. Moreover, microarray data showed that *alph* alleles generate a pattern of gene expression that would predict immune resistance (Chapter 3, Table I). Therefore, insertion of *alph* alleles into the *tak1-/-* or *imd-/-* mutant background could possibly increase resistance to Gram- bacterial infection. On the other hand, the expression of $Alph^{WT}$ in adult fat bodies may decrease the production of antimicrobial peptides and sensitize flies to Gram- bacterial infection.

4.4.2 Stress response

In nature, most organisms have to cope with stressful conditions, such as temperature extremes, food deprivation and infection. These environmental factors coupled with specific genetic traits often determine the lifespan expectancy of these organisms. Therefore, it was proposed that organisms resistant to various stressful conditions are more likely to live longer. The molecular mechanisms underlying stress resistance/lifespan extension are very poorly characterized (Vermeulen and Loeschcke, 2006). The SAPK pathways are one example of signaling modules implicated in such responses. For example, in Drosophila,

elevation of JNK signaling in *puc+/-* mutant flies increased lifespan and resistance to oxidative stress generated by paraquat, while an inactivating allele of p38a had the opposite effect (Craig et al., 2004; Wang et al., 2003a). These phenotypes are most likely explained by the fact that SAPK signaling increases the level of protein implicated in stress response. These include Heat Shock Proteins (HSPs), DNA damage response proteins and detoxifying enzymes such as Catalase, Superoxide Dismutase (SOD), Cytochrome p450 and Gluthathione S Transferase (GST) (Vermeulen and Loeschcke, 2006). Indeed, overexpression of hep^{CA} in Drosophila embryos enhanced the transcription of genes coding for HSPs and GST (Jasper et al., 2001).

We have shown that inactivation of *alph* increases SAPK signaling during Drosophila development (Chapter 3, Figure 1 and 3). In addition, microarray experiments using RNA extracted from *alph* mutant flies revealed that many genes related to stress response and detoxification are modulated in a way that would predict stress resistance (Chapter 3, Table I). Therefore, *alph* mutants could be resistant to various environmental stresses and possibly have an extended lifespan compare to wild type flies. However, survival curve experiments comparing homozygous $alph^{XS-88}$, $alph^{S-331}$ and $alph^{PBAC}$ alleles to wild type flies revealed that mutants have a shorter lifespan (data not shown). This decrease in lifespan may be due to internal developmental defects that have yet to be discovered. To circumvent this problem, we could look at

the survival of heterozygous *alph* mutants. The effect of various environmental stresses such as paraquat, rotenone or H_2O_2 treatment (oxidative stress), osmotic shock (NaCl feeding), heat shock and starvation will also be evaluated on *alph* mutants and compared to wild type flies.

The central nervous system is highly prone to oxidative stress-induced damages due to its high oxygen consumption. Accordingly, certain neurodegenerative diseases have been associtated with increased reactive oxygen species (ROS) production and subsequent neuronal cell death (Halliwell, 2006). For example, loss of dopaminergic (DA) neurons from the substantia nigra in patients with Parkinson's disease (PD) is associated with increased oxidative stress due to mitochondrial dysfunction (Michel et al., 2006). Substantial evidence suggests that environmental as well as genetic factors contribute to PD. Indeed, certain ROS-producing agents such as rotenone, paraquat and MPTP are selectively killing DA neurons and generate PD-like phenotype in vertebrate model systems (Bove et al., 2005). Linkage studies have also identified nine loci that are responsible for simple Mendelian forms of PD, of which six have been cloned (α-synuclein, Parkin, UCH-L1, DJ-1, PINK, LRRK2) (Farrer, 2006). Insight into the mechanism by which alteration of these genes causes PD requires detailed knowledge of the biological function of the corresponding genes and the pathways they regulate. Since five out of the six PD-related genes have homologs in Drosophila

(no fly α-synuclein homolog), the use of this vertebrate model system should be useful to elucidate the function of PD-related genes.

Of particular interest to my work, Parkin, DJ-1 and PINK l-o-f mutants are sensitive to ROS-generating agents such as paraquat and rotenone (Whitworth et al., 2006). In addition, some of the parkin mutant phenotypes, which include semi-lethality, loss of DA neurons, locomotor defects and male sterility, are rescued by overexpression of GstS1, an enzyme implicated in the cellular detoxification of ROS (Whitworth et al., 2005). We have shown that *alph* mutants have increased expression of genes implicated in detoxification (Cyp4e3, GstD2, Cyp4d21, Cyp6a9, CG5873). Therefore, as a first set of experiments, we would like to evalute the ability of *alph* alleles to suppress *parkin*, *DJ-1* and *pink* sensitivity to paraquat and rotenone (Greene et al., 2005; Pesah et al., 2004). In addition, we will also address whether *alph* alleles rescue certain phenotypes associated with *parkin* l-o-f. Finally, as feeding rotenone to wild type flies induces PD-like phenotypes such as locomotor defects (Coulom and Birman, 2004), we would like to test whether *alph* alleles suppress this phenotype.

4.5 Conclusion

Misregulation of MAPK (ERK, JNK and p38) pathways is a major cause of diseases. A better understanding of the molecular mechanisms

underlying the transmission of signals within these kinase cascades should ultimately result in the design of more effective drugs. In the present thesis, I am reporting the identification of a new general negative regulator of MAPK pathways in Drosophila. This regulator, called Alphabet, is a Ser/Thr phosphatase of the PP2C family, which is highly conserved throughout evolution. Genetic and biochemical evidence suggests that Alph acts through SAPKKKs at least for inactivation of the SAPK pathways. Further analyses are required to identify how Alph regulates the ERK/MAPK pathway. The *alph* locus is apparently dispensible for Drosophila development. However, the characterization of its function in adult flies may shed light on the physiological importance of MAPK pathways in adult organisms.

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