The influence of nutritional stress on growth and tissue homeostasis in Drosophila
“Life is no way to treat an animal”

To Kilgore Trout
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<tr>
<td>AEL</td>
<td>after egg laying</td>
</tr>
<tr>
<td>AMP</td>
<td>antimicrobial peptides</td>
</tr>
<tr>
<td>BDs</td>
<td>Black Dots</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn's disease or common docking domain in MAPKs</td>
</tr>
<tr>
<td>CW</td>
<td>critical weight</td>
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<tr>
<td>DILPs</td>
<td>Drosophila insulin-like peptides</td>
</tr>
<tr>
<td>ECs</td>
<td>enterocytes</td>
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<tr>
<td>FRT</td>
<td>flipase recognition target</td>
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<tr>
<td>IBD</td>
<td>inflammatory bowel diseases</td>
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<tr>
<td>IIS</td>
<td>insulin and insulin-like growth factors signalling</td>
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<tr>
<td>IPC</td>
<td>insulin producing cells</td>
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<tr>
<td>ISC</td>
<td>intestinal stem cells</td>
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<tr>
<td>MAPKAPK</td>
<td>Mitogen-activated protein kinase-activated protein kinase</td>
</tr>
<tr>
<td>MVW</td>
<td>minimal viable weight</td>
</tr>
<tr>
<td>NES/NLS</td>
<td>nuclear export/localisation signal</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-activated protein kinases</td>
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ZUSAMMENFASSUNG


Die SAPKs reagieren spezifisch auf verschiedene Stressoren und sind wichtig für eine korrekterlaufende Stressantwort. Die zwei Untergruppen p38 SAPKs und JNK SAPKs spielen auch eine Rolle in der Entstehung Krankheiten. Besonders p38 und MK2, ein Bindungspartner von p38, sind in verschiedene entzündliche Erkrankungen involviert.

Wir verwenden Drosophila melanogaster, um den Einfluss von Umweltfaktoren auf IIS und SAPKs zu studieren. Beide Signalwege kommen in der Fruchtfliege vor und haben dort auch eine ähnliche Funktion, sind aber wesentlich weniger komplex als bei Säugetieren.

IIS ist auch für die Regulierung des Zellwachstums und der Zellteilung in Drosophila wichtig. Wir konnten zeigen, dass Zellen, denen PTEN fehlt, weniger anfällig auf Hungerbedingungen reagieren und sogar normales
Gewebe im sich entwickelnden *Drosophila* Auge verdrängen können. Unsere Ergebnisse unterstützen und ergänzen eine kürzliche Studie, die gezeigt hat, dass Tumorzellen mit erhöhter PI3K Aktivität vor dem Verhungern geschützt sind.

Werden Komponenten des p38 SAPKs in *Drosophila* mutiert (p38a, p38b oder MK2), so zeigen die mutanten Fliegen unter normalen Laborbedingungen kaum Defekte, reagieren aber sehr empfindlich auf verschiedene Stresse. Wir konnten zeigen, dass MK2 einen Komplex mit p38b in *Drosophila* bildet. Dieser Komplex schützt die Darmzellen vor Stress. Fehlt MK2 oder p38b, so wird der JNK SAPK zu aktiv, was zu einem Absterben der Darmzellen führt. Diese Ergebnisse könnten bedeuten, dass auch beim Menschen, die an entzündlichen Darmerkrankungen wie Morbus Crohn oder Colitis ulcerosa leiden, die Funktion von p38 und JNK SAPK gestört ist.
SUMMARY

All organisms are not isolated entities but are in permanent crosstalk with their surrounding. To support growth, an integral part of life, nutrients have to be acquired. On the other hand, the environment can be harmful and organisms have to protect themselves against various stresses. Several pathways evolved that respond to environmental changes and help the organism to adapt to the new situation. Defects in those signalling pathways can lead to various diseases in humans. The aim of my work was to analyse the interplay between environment and the signalling pathways. We focused on insulin and insulin-like growth factor signalling (IIS) and the Stress-activated protein kinases (SAPKs).

IIS is found in higher eukaryotes and regulates growth, proliferation, metabolism and energy homeostasis. In humans, lack of IGF can lead to dwarfism. The “classical” insulin is not directly involved in growth but is an essential hormone that regulates the glucose metabolism, and defects in insulin signalling lead to diabetes. Overactivation of PI3K, a lipid kinase, and PKB/Akt, a protein kinase, as well as mutations in PTEN, a lipid phosphatase that counteracts PI3K, are involved in the development of cancer.

p38 SAPKs and JNK SAPK, the two branches of SAPK signalling, respond to various environmental stresses, but are also involved in many human diseases. Of special medical interest are p38 and its target MK2 since those two kinases are involved in various inflammatory diseases.

We use *Drosophila melanogaster* to study how environmental cues influence IIS and the SAPKs. Both signalling pathways are conserved and have similar functions from flies to humans. IIS and SAPKs circuits are much simpler in mammals and the fruitfly is amenable to genetic manipulation.

Here we show that IIS is important to regulate not only the size and weight of flies under normal but also under starvation conditions. Furthermore, *PTEN* mutant cells have a growth advantage over wild-type cells and can overtake the developing eye tissue in larvae reared on food that is reduced in yeast, the major source of amino acids. Our results support and amend a recently
published study where the authors showed that tumour cells with increased PI3K activity are less sensitive to starvation than cells with normal IIS signalling.

Mutating components of p38 SAPKs results in viable flies with almost no defects under normal laboratory conditions. If those flies are stressed, they are more sensitive than their wild-type flies. We show that MK2 forms a complex with p38b and that this complex is important for homeostasis of hindgut enterocytes. Flies deficient for p38b or MK2 have increased JNK activity and patches of apoptotic cells in their hindgut. Thus, MK2/p38b is required to respond to stress and to regulate the degree of JNK activation in the hindgut cells. Crohn’s disease (CD) and ulcerative colitis (UC) are the two most common forms of inflammatory bowel diseases (IBDs) in humans. Our results support the idea that deregulation of p38 SAPK and JNK SAPK in the enterocytes could be one aspect in the development of the IBDs.
PREFACE

My research focuses on how intracellular signalling pathways are modulated in a tissue-specific manner upon nutritional stress in *Drosophila melanogaster*. In the first part of the introduction I define what I refer to when talking about “nutritional stress” and how organisms respond to and avoid stress. The work is split in two parts designated

(A)  
**p38 stress-activated protein kinase signalling in *Drosophila***

(B)  
**Influence of yeast restriction on the tumor suppressor PTEN in *Drosophila***

In the discussion I am going to outline the similarities between the two parts, but for better understanding I will present the results in two separate sections. **Part (A)** gives a short evolutionarily perspective of stress response mechanism/stress-activated protein kinases (SAPK) from prokaryotes to higher eukaryotes. The main focus is on describing the evolutionary conserved SAPKs focusing on *Drosophila*. Furthermore I will give an overview on some effectors of eukaryotic SAPK signalling, especially on the p38 target Mitogen-activated protein kinase activated protein kinase 2 (MAPKAPK2/MK2). A quick perspective on the clinical relevance of MK2 will lead us to the open questions regarding p38/MK2 signalling. Finally I summarise what is known about stress and the intestinal system and the reasons why I think this organ is suitable to study the SAPKs in *Drosophila*. In the results part I present my manuscript on how *Drosophila* p38/MK2 signalling ensures the homeostasis of hindgut enterocytes (ECs). Additional results regarding *Drosophila* p38 SAPK signalling that are not in the manuscript are also presented in the results section for part A. The different requirements of *p38a* and *p38b* will be described genetically and biochemically as well as their impact on the third branch of SAPK signalling, the JNK signalling cascade. For comparative reasons I address the role of
these SAPK branches in other organs. In the discussion I am going to present a model how SAPK signalling ensures proper hindgut homeostasis under normal and stress conditions. Furthermore I will discuss those results and models in a disease specific context.

**Part (B)** will be kept short since I did the “founding work” inspired by the work of Jan Reiling and that is now continued together with Katarzyna Nowak. In this part I am going to describe the insulin and insulin-like growth factor signalling (IIS) system in *Drosophila* with special focus on the negative regulator PTEN. I am going to describe how starvation influences the growth and development of *Drosophila* larvae. For starvation we use reduce the yeast content in the food since yeast is the major amino acids source in the standard media. We study the effects of yeast-restriction in the developing eye disc, an example for a mitotically active tissue.
INTRODUCTION

What is “nutritional stress”?

From the moment first life forms emerged, their faith has always been coupled to their environment. Changes in the environment can be positive or negative, but in any case organisms need to respond in an appropriate way. Adaption from one state to the other involves potential risks and causes stress to the organism. Generally spoken stress can be seen as the organisms’ reaction to a change that requires a physical, behavioural – and in humans mental or emotional - adjustment or response. Thus stress and stress response is a very complex process to concert many different physiological responses and avoid abrupt changes in the inner milieu. In my work with Drosophila I focussed on the molecular and physiological aspects of the stress response. In our experimental setup stress is triggered by the change of reference (‘normal’ or ‘unstressed’) state to a stressed state that is marked by the following criteria:

(i) the change in one variable at the time (e.g.: reducing the yeast content or increasing the sodium chloride concentration in the food)

(ii) these changes involve a visible physiological response that is interpreted as a negative effect on the wild-type organism e.g: slowing down of developmental speed, reduced growth rates (decrease in size) or elevated lethality levels

(iii) ideally, mutants in components of the stress response pathway show a wild-type or close to wild-type behaviour under reference conditions, but a significantly altered response under stressed conditions. Generally a pathway is considered to be involved in a stress-relevant response when it changes its activity or function upon an alteration in the environmental state.

Thus when talking of stress we deal with situations that are not beneficial or even harmful to the organism, if their proper adaption response is inhibited. In addition “nutritional stress” means that the stressor affects the organism via
nutrition. This can be e.g. poor quality of the ingested food or the food can contain harmful substances.

**Mechanisms of stress protection and adaption**

**Aversion/Avoidance**

Avoidance is the first step in stress protection. It is a fast behavioural response to minimize and avoid the contact to a stressor that is found in all motile organisms. They constantly monitor their surroundings and upon the contact of a stressor avoidance response is initiated. Chemotaxis e.g. is a motility response to a concentration gradient of a chemical. Chemotaxis can initiate a positive movement towards an attractant or a negative movement away from a repellent. It involves chemosensing (the detection of a compound in the environment) that initiates signalling circuits that ultimately result into a directional movement of an organism. It has been mainly studied in bacteria, lower eukaryotes, such as *Dictyostelium*, but it is also found in mammalian cells (e.g. lymphocytes or in cell culture). Although the response is more complex in the aversion behaviour in higher eukaryotic organisms, the principle is the same: chemosensing leads to a behavioural response (via molecular, neural and hormonal circuits).

**Biomechanical Barriers**

Sensing a stressor implies that the organism has been in physical contact with it and thus might be exposed to a potential harmful situation. Moreover often avoidance is not possible. Therefore all organisms use biomechanical barriers to shed their inner milieu from the outside. This barriers range from simple lipid membranes, like in single cells or amoeba, to very complex cell walls made of carbohydrates, such as bacterial peptidoglycan walls or the chitinous exoskeleton of insects, and inorganic salt structures, the silica or calcium carbonate shells of diatoms and molluscs, respectively. The simplest barrier found in all living organism is the plasma membrane, composed of a lipid bilayer. Those membranes restrict the reaction volume for biochemical processes, act as selective barriers and help to stabilise an inner milieu. The more complex structures perform mainly mechanical protection.
Molecular mechanisms of stress adaption

Nevertheless those barriers are not a tight seal, can be damaged and cannot protect against every stress. Additionally stressors can originate within a cell (e.g. reactive oxygen species (ROS), toxic metabolites and miss/unfolded proteins). Therefore cells evolved molecular mechanisms that orchestrate the stress response. In theory the components that are required are

(i) a sensor molecule that monitors the outer and the inner milieu for changes

(ii) a signal transduction (and amplification) system that transmits the signal

(iii) effector molecules that mediate the stress/starvation response

(iv) regulatory molecules that ensure proper and correct response in time and space

Stresses from the environment such as heat, UV and osmolarity stress are the same for any organism. Intracellular stresses such as misfolded proteins and toxic metabolites are also often similar in different organisms. Therefore it is not surprising that stress response mechanisms are often evolutionarily conserved. There are some distinct mechanisms especially between bacteria, plants and fungi compared to higher eukaryotes. Nonetheless the above logic can be easily recognized in all the pathways that have been developed during evolution.
part A (introduction)

p38 SAPK signalling ensures hindgut homeostasis in *Drosophila* by modulating the strength of the JNK SAPK response under stress conditions

**Stress-sensing pathways/ Stress response signalling**

In the following section I will give an evolutionary overview of how molecular system have been employed in stress response signalling form bacteria and mammals. I start with prokaryotes and continue in chronological order according to their discovery and focus in the end on *Drosophila*.

**Stress signalling in prokaryotes: (gram-positive) bacteria**

Many bacteria can form spores or undergo “vegetative dormancy” under adverse condition and re-grow when the environment is more favourable. One of the best examples is the sporulation of *B. subtilis*. Due to its ability to sporulate and thereby outlive harmful conditions a general stress response has been ignored. In non-sporulating bacteria the core regulator of a general stress response is the sigma factor RpoS or $\sigma^S$. Present at low levels in non-stressed, growing bacteria, it gets strongly induced under stress conditions. This special subunit of the RNA polymerase controls directly hundreds of genes that mediate the bacterial stress response (Hengge, 2009). Similarly in *B. subtilis* the alternate transcription co-regulator sigB or $\sigma^B$ has been shown to regulate the transcriptional response to various stresses but is not required for sporulation. Thus, sigB is a true master regulator of a general stress response in *B. subtilis* (Hecker et al., 2007; Hecker and Volker, 2001). Those special, stress dependent regulons and their transcription factors act as effectors, but what acts upstream? In prokaryotes (fungi and plants) the two-component signal transduction systems (TCST) are the core signal transduction modules of environmental inputs (Stock et al., 2000; Whitworth and Cock, 2009). The basic TSCT consist of a histidine kinase (HK) that gets activated by the environmental signal and a response regulator (RR) that propagates the signal (Grebe and Stock, 1999). Recently it has been shown
that TSCT in \textit{B. cereus} directly impacts on $\sigma^B$ to modulate transcription (de Been et al., 2009). A plethora of membrane bound and soluble receptors and signal regulators have been identified in bacteria (Buelow and Raivio, 2009; Falke et al., 1997).

\textbf{Stress signalling in lower eukaryotes: \textit{Saccharomyces cerevisiae}}

Studies in \textit{S. cerevisiae} or Baker’s yeast significantly promoted our understanding of eukaryotic stress signalling. A novel osmosensing cascade was described in an article in \textit{Science} in 1993 (Brewster et al., 1993). The authors identified two members of MAPK signalling cascade, HOG1 and PBS2, which are required for high osmolarity response. One year later \textit{Maeda et al} identified two genes (SLN1 and SSK1) that show similarity to the prokaryotic two-component signalling system and are required for the high osmolarity response involving HOG1 and PBS2 (Maeda et al., 1994). The core high osmolarity signalling cascade was completed with the finding of another transmembrane osmosensing molecule sho1p (Maeda et al., 1995) and the MAP3K ste11p (Posas and Saito, 1997). At the same time it was realized that components of the high osmolarity signalling cascade are shared with the mating response (Bardwell et al., 1996; Zhou et al., 1993) and that mechanisms have to exist that prevent the crosstalk between those cascades (O’Rourke and Herskowitz, 1998; Posas and Saito, 1997). To cut a long story short, subsequent studies showed that the use of specific signalling scaffolds and feedback regulations as well as regulated crosstalk between the different signalling cascades that share common components prevent the miss-activation of those related pathways (Hall et al., 1996; O’Rourke and Herskowitz, 1998). Another level of regulation besides the signalling scaffold is the subcellular localisation and incorporation into localised signalling complexes. Under normal conditions hog1p is distributed equally in the cytoplasm and nucleus, but when stressed it gets rapidly enriched in the nucleus (Ferrigno et al., 1998; Reiser et al., 1999). In the nucleus it activates the transcription factor hot1p, the transcriptional activator msn2p and de-represses genes under the control of the transcriptional repressor sko1p to initiate high osmolarity dependent transcription (Capaldi et al., 2008;
Chellappan, 2001; Proft et al., 2005; Proft et al., 2001; Proft and Serrano, 1999).

Although the picture of how signalling specificity is ensured improved in the past two decades, several open questions remain. Most of the players have been identified and now sophisticated biochemical, structural and kinetic analyses as well modelling approaches are employed to figure out the fine-tuning of the HOG and related responses (Kaserer et al., 2009; Melamed et al., 2008; Rensing and Ruoff, 2009; Soufi et al., 2009; Xu et al., 2003).

The HOG pathway is probably the most prominent example of stress signalling in yeast, but a plethora of other signalling mechanisms exist that respond to various environmental stresses such as heat, cold, oxidative stress, heavy metals, unfolded proteins etc. Since the HOG pathway will be of central importance in my studies I do not want to go into more details of the other stress responses, but will introduced them if needed elsewhere.

**Stress signalling in higher eukaryots: mammalia**

From a historic perspective I start with mammalian stress signalling, when dealing with the *metazoa*, since stress signalling pathways have been found approximately at the same time as in yeast. In addition sequence and pathway analyses have revealed that those cascades are similar between mammals and yeast. They have been therefore classified as a novel branch of MAPK signalling termed SAPKs Since 1990 several groups identified proteins in the kDa range from 38 to 52 that had high homology to the recently discovered proline-directed MAP Kinases. Those new kinases were termed p46, p54\(\alpha\) and p55\(\beta\), c-Jun N-terminal Kinase/SAPK (JNK/SAPK), Reactive Kinase (RK) and cytokine-suppressive anti-inflammatory drug (CSAID) binding proteins 1 and 2 (CSBPs 1 & 2) (Ray and Sturgill, 1988a, b; Rossomando et al., 1989). In contrast to p42/44 (which were later termed ERK1/2) the novel kinases were only weakly induced by growth factors and phorbol esters, but strongly activated by cytokines and different cellular stressors. Furthermore they could be divided into two branches: one branch contains four true homologues to hog1p, termed p38\(\alpha-\delta\) and the second contains three similar genes termed JNK1-3 with at least 10 distinct isoforms.
In the following years several components that link the environmental stress to the SAPK, and downstream effectors have been identified. Direct activators of the SAPK are the SAPK Kinases (SKKs)/ MAPK Kinase (MKKs). Some of them are specific for the JNK branch (MKK7), some specific for p38 (MKK3/6) and some can activate both branches (MKK4). There are also reports that showed that activation of SAPKs could be in an MKK-independent fashion (Ge et al., 2002). Upstream of the MKKs a plethora of MKK Kinases/MKKKs has been identified. Of the 20 known MAP3K genes most have been shown to activate the SAPKs (MEKK1-4, MLK1-4, LZK, DLK, ZAK, TAK1, TAO1-3, and ASK1/2). Like MKKs, MKKKs can be specific for one of the SAPK branches or activate both. Until today it is hard to assign MKKKs or MKKs to a specific branch.

**Stress signalling in higher eukaryotes: other eukaryotes**

*S. cerevisiae*, mammalian cell lines and mice knock-outs have been the central models to study SAPK signalling. The elegant yeast genetics led to the discovery of various pathway components, structure-function analyses revealed many mechanistic aspects of HOG pathway regulation. Mice and human cell lines have been used to study the pathway activation to various stresses and cytokines and expanded the knowledge that has been acquired in yeast. With the availability of mice knock outs it became possible to study SAPK activation and regulation within a multicellular context. Gene redundancy and complex tissue specific regulation troubles the analysis in vivo and despite the broad mechanistic understanding from the yeast model a lot of open question remain unanswered in the multicellular models. Only recently SAPK signalling has been addressed in other organisms. Those studies shed light onto new mechanisms and evolutionary functions of stress signalling.

In marine sponges, one of the most basal metazoans, JNK and p38 have been cloned and are phosphorylated under hyperosmotic shock. Both SAPKs can complement HOG1 deficient yeast and sequence analysis indicates the conservation of upstream and downstream partners. The findings in *S. domuncula* have several interesting evolutionary aspects.
First, it supports the idea that SAPKs arose in the animal/fungus branch of metazoans (since MAPK, but not SAPKs are found in plants). Second, the branching of HOG1 into the two closely related genes, JNK and p38 occurred very early in the separation of animals from fungi. Third, although the long separation and the acquisition of distinct functions, the core function of JNK and p38 has been conserved throughout evolution since basically p38 and JNK of any organism can rescue HOG1 deficient yeast (Bohm et al., 2002; Bohm et al., 2000; Muller et al., 2002).

**Stress signalling in higher eukaryotes: *Drosophila***

*Drosophila* has a special role when discussing SAPK signaling. Three years after HOG1 was identified Riesgo-Escovar cloned the only *Drosophila* JNK homolog, DJNK. Although kinase activity of DJNK can be boosted by UV treatment the most prominent phenotype was the failure to undergo dorsal closure. Dorsal closure is an event that requires two epithelial sheets moving towards each other to enclose the embryo in its epidermis. Homozygous *bsk* and *hep* (a *Drosophila* JNK Kinase) mutants have been identified in the screen of Nüsslein Vollhard, and indeed DJNK is encoded by the *bsk* locus (Glise et al., 1995; Nüsslein-Volhard, 1984).

In the following years genes required for dorsal closure and belonging to the JNK signaling branch have been described (Martin-Blanco et al., 1999; Stronach and Perrimon, 2002; Su et al., 1998). At the same time a role for JNK in immune signaling has emerged and the role of JNK not only in morphogenesis but also in apoptosis and during stress signaling has been addressed (Goberdhan and Wilson, 1998; Sluss et al., 1996).

There are three homologues of p38 SAPKs in Drosophila, *p38a*, *p38b* and *p38c*, but there function has only been addressed by mutational studies recently. In a first attempt to study p38 signalling Adachi-Yamada and colleagues generated antisense RNA and dominant-negative constructs for *p38b* (Adachi-Yamada et al., 1999). They reported that *p38b* is phosphorylated by stress and by *dpp* signaling, indicating that, besides a potential role in stress response, *p38b* functions to modulate *dpp* signalling.
and thereby morphogenesis. In a genetic screen for immune tolerance $p38b$ has been identified as a crucial component. Immune tolerance is a mechanism that allows the host to cope with a pathogen burden without losing its fitness. In this process $p38b$ is required in phagocytic hemocytes to shed the bacterial burden by “cellular encapsulation” (Shinzawa et al., 2009). Megan Cully reported another deletion mutant for $p38b$ in 2010. In a screen for genes that suppress $TSC1$-knock down induced overgrowth $p38b$ has been identified as an activator of TORC1. Indeed, flies lacking $p38b$ are smaller and more sensitive to starvation, but also to other stresses (Cully et al., 2010). Although deletion mutants exist now, none of the studies addressed the potential role of $p38b$ in $dpp$ signalling.

In 2004 a deletion mutant for $p38a$ was reported. Flies homozygous for the deletion are viable and do not show any obvious morphological defects. Exposed to stress, such as high temperature, oxidative stress and dry starvation, $p38a$ mutants do show higher lethality when compared to wild-type controls (Craig et al., 2004).

It is interesting to note that both $p38a$ and $p38b$ mutants are viable and seem to have partially different function. Nevertheless the double mutants are early larval lethal, indicating some redundant functions. From an evolutionary perspective this indicates that it is probably not possible to state that one of the $Drosophila$ $p38$ genes is the orthologue of $p38\alpha$, $\beta$, $\gamma$ or $\delta$. It is more likely that $p38a$ and $p38b$ are at least a mixture of $p38\alpha$ and $\beta$.

The function of $p38c$ remains disputed. Callie Craig reported to Flybase that $p38c$ is likely to be a pseudogene since otherwise conserved sites have been mutated, but a recent publication reported a function of $p38c$ in the control of female fertility and expression of the $DOPA$ decarboxylase in the fly (Davis et al., 2008).

Although mutants exist for all three putative $p38$ homologues little is known about their signalling specificity and in vivo function.

Three MAPK kinases are encoded in the $Drosophila$ genome: $licorne$/MKK3, MKK4 and $hemipterous$/MKK7. Mutants for MKK4 have been identified in a dominant suppressor screen for new components of $eiger$/TNF$\alpha$ signalling. It has been shown that MKK4, like $hep$, functions in the JNK signalling cascade
in a non-redundant fashion. Unlike hep, MKK4 homozygous mutants are viable and do not show a dorsal closure phenotype (Geuking et al., 2009).

The third SAP2K in Drosophila, licorne/MKK3, is the neighboring gene of hep. Lic is able to replace PBS2, the activator of HOG1 in yeast, and is able to phosphorylate p38 in kinase assays. In contrast to the DC phenotype of hep mutants, lic germ line mutants are ventralized and show a fusion of the egg shell appendages (therefore the name licorne, French for unicorn). During oogenesis lic is required for proper gurken localization and for the maintenance of oskar mRNA. This phenotype indicates a cross talk between SAPK and ERK signalling during oogenesis. A second mutant for lic, which does not affect the hep coding region, has been generated recently. Homozygous lic larvae are small and die in early L3 stage. This supports the idea that like p38b lic may act on TORC1 signalling. The early lethality of MKK3, similar to p38a and p38b mutants, indicates that p38a and p38b are redundant in regard to signal transduction from lic.

At the level of MAP3K one homologue to each class of mammalian MAP3Ks exist. Although fewer in number the regulation of the JNK and p38 branch at this level is far from understood. Especially how signalling specificity is assured is still puzzling. All identified MAP3K mutants have partially distinct phenotypes, although signalling through the same MAPK.

*Slipper*, a homologue of the mixed lineage kinases (MLK), has been identified to function as a MAP3K to regulate dorsal closure via hep/MKK7 and bsk/JNK (Stronach and Perrimon, 2002). On the other hand, *TAK1* has been identified in the same dominant suppressor screen as MKK4 and is not required for dorsal closure, but functions in *eiger/TNFα* induced bsk/JNK activation via MKK4 and hep/MKK7 (Geuking et al., 2005). Moreover TAK1 functions in the *IMD*-mediated immune response (Silverman et al., 2003; Vidal et al., 2001).

*Wallenda*, the DLK/LZK homologue, is required to regulate synaptic growth and bouton size in a JNK, but not p38 dependent fashion (Collins et al., 2006). So far no reports of a *wnd* function in the immune system, during dorsal closure or in stress response have been made.
Protein Kinase at 92B (PK92B) has been identified as a novel MAP3K in 1996, shortly before its homologue (MAPKKK5) in humans was found (Wang et al., 1996; Wassarman et al., 1996). Later MAPKKK5 was named Apoptosis signal-regulated kinase (ASK), and PK92B was shown to be the true homologue in Drosophila. Not much is known about PK92B, only that it is able to modulate the eiger/TNFα-induced apoptotic eye phenotype and mediates JNK activation through ROS (Owusu-Ansah et al., 2008).

A homologue of mammalian MEKK4, D-MEKK1/MEKK1, has been found in Drosophila to be required to phosphorylate p38 and to survive heat and salt stress conditions (Inoue et al., 2001). In S2 cells it has been shown that MEKK1 is also required to activate the JNK SAPK branch in response to stress (Ryabinina et al., 2006). A further function of MEKK1 is to regulate immune gene expression, thus creating a link between stress and immune response (Brun et al., 2006).

![Eukaryotic SAPKs](image)

**Figure 1. Eukaryotic SAPKs**

A simplified comparison of MAP3Ks, MAP2Ks and MAPKs found from yeast to mammals. For Drosophila a color code assigns them to either JNK SAPK (red) or p38 SAPK (yellow). Note that in yeast no separation between those two branches has occurred and that the MAP3K SSK2 and SSK22 are activated by a Two-component signaling system (not depicted).
**Effectors of SAPK signalling in higher eukaryotes**

In prokaryotes the TCST system activates *sigB* as master regulator of the stress response. In yeast I briefly mentioned the transcriptional regulators hot1p, msn2p and sko1p that are downstream targets of hog1p and are crucially involved in the regulation of the stress response. In higher eukaryotes (mammals, *Drosophila*) a plethora of SAPK downstream targets have been identified in the past years. *p38* and *JNK* targets involve transcription factors, other kinases, cytoskeletal proteins, chromatin proteins and many other proteins of various functions. Some targets are shared between those SAPK branches others are specific for either JNK or p38. Here I am going to mention a few prominent examples for downstream targets of SAPK signalling. More time will be spent on discussing *MK2*, a target of p38 SAPK.

**Transcription factor complexes targeted by SAPKs**

The protooncogene c-fos has been identified as a homologue to the viral gene product v-fos that can cause transformation of normal cells into tumour cells both in cell culture as well as in vivo (Verma and Graham, 1987). At the same time as c-fos was realized to be a transcription factor, several fos-associated proteins (FAPs) have been identified. One of the FAPs with a size of 39kDa turned out to be c-jun (Distel and Spiegelman, 1990). A cis-activating sequence had been identified in the SV40 early promoter, AP-1, that is bound by c-fos and c-jun. Today the heterodimer of c-Fos and c-jun is called AP-1 and its binding motif TRE (TPA responsive element). Both c-jun and c-fos are found at low expression levels in most cell types but are rapidly induced by stresses and mitogens and modified by protein phosphorylation. In a pulldown with GST tagged c-jun a serine/threonine kinase was co-precipitated (Hibi et al., 1993). This Jun N-terminal kinase (JNK) was proven to be an important regulator of c-Jun/AP-1 activity and linked the stress response to a transcriptional response (Ausserer et al., 1994; Dai et al., 1995; Derijard et al., 1994; Kyriakis et al., 1994). Another transcription factor that can heterodimerize with c-jun (but not with c-fos) is ATF-2 (Activating Transcription Factors 2/CREB II/ CREBP-1). In contrast to jun:fos complexes
the jun:ATF complex does not bind the TRE sites but interacts with the related CRE sequence (cAMP responsive element) (Angel and Karin, 1991; Benbrook and Jones, 1994; van Dam and Castellazzi, 2001; Wisdom, 1999). On the other hand JNK activates both transcriptional complexes, ATF-2 and AP-1, in response to stresses (Livingstone et al., 1995).

JNK signalling alone is not sufficient for stress-induced activation of ATF-2 and AP-1, but p38 SAPK activity is also required (Hazzalin et al., 1996). Thus both branches of the SAPK pathway, JNK and p38, act on a shared set of transcription factors (c-Fos, c-jun and ATF-2) with different, but partial overlapping preferences. Different heterodimeric combinations allow complex signalling combination. Therefore these transcription factors integrate diverse inputs by SAPK pathways as well as by other signalling modules upon stress response and during normal cellular processes. Oncogenic versions of c-jun and c-Fos indicate a link between stress signalling and tumour development. Furthermore ATF-2 and AP-1 have been implicated in various inflammatory and immunological diseases and represent potential drug targets (Vlahopoulos et al., 2008; Wagner; Zenz et al., 2008).

Another clinically relevant transcription factor family that is regulated by both SAPK branches are the Signal Transduction and Activators of Transcription transcription factors (STATs). p38 SAPK gets activated via the Type I Interferon response and phosphorylates STAT1 (Goh et al., 1999). This has been implicated in antiviral responses and regulation of hematopoiesis. Interestingly activation of p38α is apparently needed to mediate the growth inhibitory effects of IFNα treatment of primary leukemias (Platanias, 2003; Uddin et al., 1999). JNK SAPK on the other hand has been implicated in the activation of STAT3 in a parallel fashion to JAK (Liu et al., 2006; Miyazaki et al., 2008). Activation of STAT3 via the JNK might render cancer cells resistant to certain drug treatments such as doxorubin (Kim et al., 2009).

The family of ETS transcription factors (including ELK-1 and ETS1) controls various aspects of the cell including differentiation, cell cycle control, migration, proliferation and apoptosis (Jedlicka and Gutierrez-Hartmann, 2008; Randi et al., 2009). Many environmental signals converge on the ETS
transcription factors and both SAPK branches can target those transcription factors (Yordy and Muise-Helmericks, 2000). Interestingly it was shown that in Ewings sarcoma (caused by the fusion of EWS RNA-binding protein and Fli-1 ETS transcription factor due to a translocation between chromosomes 11 and 22) apoptosis is suppressed by inactivation of JNK signalling via NFκB (Javelaud and Besancon, 2001; Riggi and Stamenkovic, 2007).

Recently it was shown that p38 regulates various steps in muscle development. Two transcription factors that regulate the myogenic process are MyoD and MEF2. Both transcription factors can be phosphorylated by p38 and p38-dependent regulation ensures the correct chronological expression of muscle-specific genes (Keren et al., 2005; Keren et al., 2008; Keren et al., 2006). Thus p38 is required from early mesoderm specification to myoblast specification to muscle differentiation. At early steps p38 acts as a positive regulator and later during differentiation as a negative regulator.

**MAPK-activated protein kinases targeted by SAPKs**

The transcriptional response of SAPKs after stress stimulation, although by far not completely understood, has been studied well in the past years. Nevertheless not all targets of SAPK are nuclear proteins and MAPKAPKs/MKs are a group of kinases that expand the classical MAPK hierarchy by one kinase step (MAPKKK → MAPKK → MAPK → MAPKAPKs). MKs are targeted by the p38 SAPKs and ERK MAPKs (Roux and Blenis, 2004). The MK family is comprised of the Ribosomal S6 Kinases (RSKs), the MAP kinase signal-integrating kinases (MNKs), the Mitogen- and Stress-activated Kinases and the MAPKAPKs. The RSK group has 4 members (RSK1-4) that are activated by ERK1/2 and PDK1 and are involved in the control of cell growth, proliferation, survival and motility. At the domain level, RSKs are marked by two kinase domains, an N-terminal kinase domain (NTKD, PKC-like) and a C-terminal Kinase domain (CTKD, CaMK-like) (Anjum and Blenis, 2008).

The mammalian MSK group contains 2 members (MSK1 and MSK2) that are activated by ERK1/2 as well as by p38 SAPK. Like RSKs, MSK1/2 have a NTKD and a CTKD. MSKs are nuclear kinases that are involved in the
expression of immediate early genes, chromatin remodelling and the immune system (Arthur, 2008; Vermeulen et al., 2009).

MNK kinases control cellular survival, proliferation and the production of inflammatory cytokines and are activated by ERK1/2 and p38α and β. This group consists of two members (MNK1 and MNK2) with a single CaMK-like kinase domain. Each gene produces two alternative protein isoforms (1a/1b and 2a/2b, respectively) that differ in their C-terminal proportions. 1a and 2b are shorter and lack MAPK binding activity (Buxade et al., 2008).

The last group and the founding members of the MAPKAPKs are the three genes MK2, MK3 and MK5 (Gaestel, 2006). MK5/PRAK is bound and activated by the atypical MAPKs ERK3/4 (Aberg et al., 2009). MK2 and MK3 are structurally very closely related. Both can bind to p38α and β, they have similar targets and are involved in similar processes (Ronkina et al., 2008). MK2 is activated by p38α and has a plethora of nuclear and cytoplasmic targets. It is involved in actin cytoskeleton remodelling via the phosphorylation of small heat shock proteins and thereby regulates cellular motility. From a medical point of view MK2 got attention due to its crucial role in TNFα biosynthesis. Mice lacking MK2 have strongly reduced TNFα levels and are protected against endotoxic shock. It was shown that tristetraprolin (TTP), which destabilizes ARE (AU-rich element containing) mRNA, is phosphorylated by MK2. This phosphorylation inhibits the formation of a TTP-TNFα mRNA complex and thereby increases translation of TNFα (Kotlyarov and Gaestel, 2002; Kotlyarov et al., 2002; Neininger et al., 2002).

**Structure-function of MK2**

A hallmark of p38 is its stress-dependent shuttling between cytoplasm and nucleus. Similarly MK2 translocates from nucleus to the cytoplasm when cells are exposed to various stresses. This shuttling is dependent on the phosphorylation of MK2 by p38α. In an unstressed/non-phosphorylated state only the NLS is accessible, but when p38 phosphorylates MK2 the NES gets exposed and MK2 is exported from the nucleus in an exportin-dependent manner (Engel et al., 1998). Crystal structure analysis of the p38α/MK2
heterodimer revealed the molecular basis of the strong physical interaction of the two kinases and complex assembly has been structurally analysed (ter Haar et al., 2007; White et al., 2007). MK2 binds to the p38α docking groove and resides in the nucleus in an non-stimulated state. Upon p38α phosphorylation by MKK3/6, MK2 is released from the complex. The free docking groove of p38α is now able to either bind another nuclear substrate, or p38α gets inactivated by a phosphatase and re-assembles in an inactive MK2/p38α complex or binds MK2 and activates it. Activation of MK2 exposes its NES and allows the shuttling of the complex to the cytoplasm and allows targets there to be phosphorylated. Activation of MK2, but not its kinase activity, is required for the nuclear export (Ben-Levy et al., 1998; ter Haar et al., 2007).

Besides the cytoplasm/nucleus shuttling of p38, MK2 stabilises p38 on the protein level by binding to it. A splice variant of MK2 that lacks the C-terminal NES/NLS and the CD (C-terminal docking domain) MAPK docking domain is not able to rescue the p38 protein levels, underscoring the importance of complex formation (Kotlyarov et al., 2002). For this function the catalytic activity is not required.

Another prominent motif in MK2 that has been acquired later in evolution is the N-terminal SH3 binding domains. The SH3BD and the catalytic activity are required for cellular migration, which will be discussed in the following section (Gaestel, 2006; Kotlyarov et al., 2002).

**Biological functions of MK2**

**MK2 regulation of ARE containing TNF mRNA**

A mouse mutant for MK2 was first described in 1999. MK2 deficient mice are viable and fertile and more resistant to stress and endotoxic shock. Like p38 null mice, MK2-null mice show greatly reduced TNFα protein levels, although TNFα messenger and secretion are not affected (Kotlyarov et al., 1999). Mechanistically, activated MK2 stabilises tristetraprolin (TTP) mRNA and protein. TTP binds to the ARE of TNFα mRNA and inhibits its translation. Binding and phosphorylation of TTP by MK2 renders it inactive and TNF is
now strongly translated. When MK2 is subsequently inactivated, the protein levels of TTP are increased and can now efficiently block further translation of TNFα, leading to a recovery phase of the cytokine response (Brook et al., 2006; Hitti et al., 2006; Winzen et al., 1999).

**MK2 regulation of the cytoskeleton**

Another phenotype of cells deficient in MK2 is abnormal migration. MK2−/− neutrophils migrate faster but not directed in an fMLP (formyl-Methionyl-Leucyl-Phenylalanine) chemo-attractant gradient in an in vitro assay (Hannigan et al., 2001b). PTEN, which is excluded from the leading edge in migrating neutrophils is mislocalised in MK2−/− neutrophils, which might explain the loss of direction of the migrating cells (Wu et al., 2004). Although PTEN might be localised in an F-actin independent fashion, various studies showed the importance of MK2 in F-actin regulation, such as LSP1 (Lymphocyte-specific protein 1) being a phosphorylation target of MK2. P-LSP1 binds to the minus end of F-actin and thereby stabilizes the actin filament (Wu et al., 2007b). LSP1−/− neutrophils also show loss of migration directionality but, in contrast to MK2−/− deficient neutrophils, they have reduced migration speed. This indicates that MK2 also targets other factors that influence cellular migration (Hannigan et al., 2001a).

The mammalian small heat shock proteins for example are phosphorylated by MK2 (Stokoe et al., 1992). shsp25/27 can form large oligomers that get disaggregated by phosphorylation via MK2 into dimers or tetramers. The stabilisation of F-actin could be one function of the shsp25/27 dimers or tetramers to regulate migration. Surprisingly, MK2 mutant fibroblasts do not show any changes in actin cytoskeleton regulation unless confronted with harsh stress conditions (Vertii et al., 2006).

Another pathway that regulates the actin cytokeleteon and might be influenced by MK2 is VEGF/VEGFR signalling. VEGF stimulation of endothelial cells leads to LIMK activation and subsequently to phosphorylation of cofilin. Phosphorylated cofilin is no longer able to sever F-actin and thereby the filaments get stabilised. Inhibiting p38α/β or knocking down MK2 inhibits activation of LIMK1 and cofilin phosphorylation. Thus, MK2 might
influence the migration and cytoskeletal rearrangement via modulation of the VEGFR response (Kobayashi et al., 2006).

**MK2 regulation of the chromatin, the cell cycle and haematopoiesis**

In yeast two hybrid screens for novel MK2 interaction partners, the polycomb group (PcG) genes bmi1 and HPH2 have been identified (Neufeld, 2000; Yannoni et al., 2004). Recent evidence supports a biological function of the interaction of PcG with MK2. Mice lacking MK2 show a reduced number of hematopoietic stem cells (HSC) compared to wild-type littermates, and this HSC defect can only be rescued by a MK2 protein that is able to bind to the chromatin proteins (Schwermann et al., 2009). Besides the regulation of proliferation via chromatin regulation, MK2 acts as a cell cycle checkpoint kinase itself. Stressing cells results in a rapid block of mitosis. This arrest in G2/M is independent of the canonical ATM checkpoint. Thus p38/MK2 might act as a genuine cell cycle checkpoint that, although not required under normal conditions, becomes important when cells are stressed (Mikhailov et al., 2007; Mikhailov et al., 2005; Reinhardt et al., 2007; Reinhardt and Yaffe, 2009).

Mechanistically, the p38/MK2 checkpoint inhibits cdc25A/B and thereby stops the cell cycle. It was shown that MK2-dependent induction of an miRNA is needed to stop myc-dependent DNA replication in stressed cells (Cannell et al., 2010). Thus, MK2 might influence the proliferation and growth of cells at several levels during stressful conditions.

**Transcription factors targeted by MK2**

MK2 regulates gene expression not only at the level of translation but can directly bind and phosphorylate transcription factors. The SRF (Serum Response Factor) can be phosphorylated by MK2 on Ser-103. Although the biological function is not revealed yet binding studies showed that phosphorylation on this site reduces its binding to the CArG box (Heidenreich et al., 1999). Interestingly, SRF is not only a transcriptional regulator but it also regulates the actin dynamics, a process also regulated by MK2.
Another study showed that Heat Shock Factor 1 (HSF1) is phosphorylated on Ser-121 by MK2 and thereby inhibited. Phosphorylation of HSF1 on Ser-121 increases its binding to the chaperon hsp90. Thereby, it is kept in a monomeric and inactive state (Wang et al., 2006).

The basic helix-loop-helix transcription factor E47 has also been identified as a binding partner for MK2, and MK2 can phosphorylate E47 in vitro (Neufeld, 2000). Interestingly, among the various processes E47 is regulating it is important in various stress responses, hematopoiesis and recently implicated in immunological diseases as psoriasis (Fuxa and Skok, 2007; Shen et al., 2010; Smith and Sigvardsson, 2004).

![Figure 2. The different functions of mammalian MK2](image)

Mammalian MK2 has a plethora of functions in the cell in stressed and unstressed states. (1) regulation of actin cytoskeleton during migration, (2) phosphorylation of small heat shock proteins (sHSPs, purple stars) leads to dissolving of large oligomeric complexes, (3) binding to p38α and β via the docking domain (brown): this stabilises p38 on the protein level and further regulates its localisation, (4) posttranscriptional regulation of mRNA via phosphorylation of TTP and thereby regulation of TNFα production (red stars). In the nucleus MK2 regulates (5) transcription factors and (6) the chromatin, and (7) MK2 contains a bipartite NLS/NES that is required for correct subcellular localisation.
MK2, MK3 and MK5: (partially) redundant functions?

In the past years several interaction partners and biological roles besides the regulation of the cytoskeleton and of proinflammatory cytokines have been identified.

Although several other targets of MK2 are known, their biological role is far from understood. Since MK2 is activated by stress regulation of some targets might only become relevant under stress conditions. In addition many of the effects caused by MK2 might be masked by the presence of the closely related MK3 or even MK5 kinases (Ronkina et al., 2007; Ronkina et al., 2008; Shiryaev and Moens). Finally, MK2 may not regulate crucial steps in various cellular responses, but could be involved in the fine-tuning and optimisation of those pathways.

Other targets of SAPK signalling

Many other proteins and cellular processes are targeted by p38 and/or JNK, and there is no doubt that this list will be expanded in the future. One reason might be that classically most experiments were performed under standardised conditions. SAPK do mainly function in response to stress and their roles in diverse processes might not be uncovered under standard conditions. Furthermore often SAPKs only modulate a biological process and the changes might be subtle. It would be too much to report all known interactions of p38 or JNK thus I will briefly touch on a few examples.

The first report on Drosophila p38b was about a potential role in dpp/TGFβ signalling (Adachi-Yamada et al., 1999). Although deletion mutants did not confirm those results, examples from mammalian studies support a role for p38 and JNK in TGFβ signalling (Burch et al.; Fu et al., 2009).

Other pathways that are influenced by SAPK are for example myc and NFAT signalling (Cannell et al.; Liang et al., 2003; Ortega-Perez et al., 2005; Round et al., 2007).
p38 SAPK as therapeutic target

p38α is a major regulator of proinflammatory cytokine production and other inflammation mediators and it is involved in various inflammatory diseases. p38β-δ are not so well understood but there is cumulative evidence that those p38 isoforms also play a role in different disease states ranging from cancer and inflammation to diabetes. Therefore, p38 as a potential drug target has attracted medical interest, and some of the p38 inhibitors are in clinical trial.

However, p38 has other biological functions and inhibiting this kinase can have adverse effects. Furthermore the role of the different p38 isoforms and the complexes they form in diseases, tissues and organs have to be better understood since SAPKs are also regulated in a tissue specific manner. This is of great importance since some diseases involve various tissues. A complex cross influence between the immune system, the intestinal cells and the gut flora is happening in inflammatory bowels diseases for example.

One attempt to target only a subgroup of p38 isoforms or complexes is to target a complex specific binding partner or downstream target. MK2 is a substrate of p38α and β that mediates most of the biosynthetic regulation of proinflammatory cytokines on the translational level. In contrast to p38α, MK2 mice are viable and fertile but are protected to endotoxic shock. Thus, the risk of side effects might be lower when targeting MK2, but still MK2 is widely expressed and has a plethora of other functions (Yong et al., 2009).

Open question on p38/MK2 signalling

Many players in the SAPK cascade have been identified in the past years. Due to their involvement in various disorders SAPKs caused attention in the medical world (Fang and Richardson, 2005; Feuerstein and Young, 2000; Milosso et al., 2008; Wagner and Nebreda, 2009; Wei and Feng). Inhibiting the activity of those kinases appeared to be the way to go in anti-inflammatory therapy (Salh, 2007; Schindler et al., 2007). Despite several encouraging results, concerns about side effects are growing larger. More data accumulate that clearly show that p38 and JNK do not only participate in diseases but have distinct roles in various biological processes. The dual role in muscle
development – in the beginning of differentiation acting as a positive and later as a negative regulator – should raise awareness about potential negative effects of inhibiting p38 function (Lassar, 2009). Another worrying example is the role of p38 in cytokine signalling. On one hand, p38α boosts the proinflammatory action of TNFα in diseases such as arthritis, on the other hand p38α activity is required to mediate the beneficial effects of IFNα therapy for certain leukemias (Platanias, 2003). To avoid two-sided sword scenarios, a better understanding of p38 and its complexes is needed. Targeting only one specific branch of p38 signalling or a specific binding partner is going to reduce potential negative effects. Nevertheless, as seen with MK2, this has also to be strictly evaluated. Moreover, due to the complex regulation, it is important to dissect what each kinase is actually mediating in the various responses. This ultimately leads us to the question of tissue-specific signalling complexes. Again, p38 has distinct functions, e.g. in the hematopoietic cells compared to the muscle or epithelial cells. Finally do the different SAPK branches modulate each other? The answer is clearly yes, but how they regulate each other and at which level remains to be understood.

To address these questions we argue that a model system of lower complexity will be helpful. Yeast genetics and biochemistry provides us with great details about signalling - mechanism, - feedback loops and – scaffold, but its unicellularity does not allow any tissue context studies. Mammalians, on the other hand, provide us with tissue-specific information but are of high complexity at the level of gene redundancy and protein isoforms. In addition diseases that involve SAPKs do affect more than one tissue at the same time. Arthritis, for example, involves immune cells, the joint cartilage as well as the synovium. Thus, experimental setups are often confined to cell culture studies and might not reflect the in vivo situation. SAPK modules behave differently depending on the environmental context, and a large amount of individuals and conditions would be desired.

Thus, others and we started to address the role of SAPKs in Drosophila melanogaster. JNK is a good example that Drosophila is a suitable model organism to gain new information on biological processes that might be
conserved in higher eukaryotes. *Drosophila* has remarkably lower redundancy within the SAPKs compared to mammals. Moreover the organs are much simpler but have to fulfil comparable functions. *Drosophila* is easy to genetically manipulate and offers good biochemistry, and in contrast to yeast, it allows studies in a tissue-specific context.

In a review on SAPK Sykiotis and Bohmann asked the following questions in 2007:

What are the physiological and/or developmental roles of D-p38b? Is this isoform functionally redundant with D-p38a?
Is D-p38 a mediator or a suppressor of the immune response?
Which MAP3K(s) and MAP2K(s) functions upstream of D-p38 in stress responses *in vivo*? Can D-p38 be activated by stress in a MAP2K-independent manner?
Is the developmental function of the MAP2K Licorne mediated by D-p38? Do the D-p38 kinases have roles in development under non-stressful conditions (like JNK does)?
Is there a D-p38-specific phosphatase (analogous to Puckered in the JNK branch)?

Another unresolved issue concerns the crosstalk between the different SAPK branches. Despite the extensive analysis of SAPK in the past, many open questions have remained. JNK and p38 kinases are activated simultaneously by similar stresses, but they do not necessarily act in a parallel fashion and can have opposing roles on each other e.g. in myocyte development and cardiomyocyte hypertrophy (Nemoto et al., 1998) (Perdiguero et al., 2007). An example in Drosophila is the development of sensory bristles from the SOP. *Ral* acts in a negative fashion on JNK and in a positive fashion on p38 to prevent apoptosis in the SOP(sensory organ precursors) (Balakireva et al., 2006). Most of the examples concern developmental functions. Do JNK and p38 also interfere with each other in their roles as stress-signalling kinases?

We wanted to address those outstanding questions, but we first needed to find a suitable tissue to analyse SAPK signalling in *Drosophila*. We chose the
intestinal system of Drosophila as a suitable organ to study different SAPK signalling aspects as outlined below.

**SAPK pathways in the Drosophila intestinal system**

As mentioned above dorsal closure during embryogenesis was the first processes a SAPK, JNK, has been implicated in. Very early during egg formation SAPKs are also required for morphogenesis. JNK and p38 are required for D-V axis formation in the future egg. Besides the developmental functions and stress response, SAPK is also required in the immune response and the hematopoietic lymph glands. Strikingly similar to the mammalian system, SAPK signalling strongly varies depending on the studied organ.

We chose to study the regulation of SAPKs in the Drosophila intestine for several reasons. First it is quite well studied how the intestinal system and its morphogenesis during development are orchestrated. At least 23 different domains have been identified by morphological features as well as by reporter gene expression. This allows to genetically manipulate different regions of the intestine. Although the developmental processes have been analysed in various studies not much is known about the functions of the different intestinal domains. Since embryonic gene expression is maintained it is likely that they have specific functions during larval stages (unpublished observation). Secondly, since the function of the gut is a very ancient one it shows similar features and structures as the mammalian gut, though much simpler in organisation. Like in mammals the gut is composed of enteroendocrine cells, enterocytes and stem cells. Thus, not only the proteins of signalling pathways are conserved it might be that the signalling mechanisms as a whole are conserved from the Drosophila to the mammalian system.

Finally, the gut and its enterocytes are the first line of defense against food-born pathogens and stresses. Due to the large nutrient turnover and the gut flora the intestinal system is permanently in a basal stress state and has to respond to the strength of a stress in a graded fashion. Moreover different stressor concentrations can be easily administered by feeding the larvae.
The structure of the intestinal system

Roughly the *Drosophila* gut consists of three regions: the fore-, the mid- and the hindgut. While the midgut is of endodermal, the fore- and hindgut are of ectodermal origin. Associated structures are the salivary glands and the Malpighian tubules. During metamorphosis the complete intestine gets destroyed (except the Malpighian tubules) and the adult gut arises from imaginal cells. The foregut consists of the pharynx, the esophagus and the proventriculus. The proventriculus is a folded structure formed by the fusion of the ectodermal fore- with the endodermal midgut and consists of ten compartments (pe – p9) (Fuss et al., 2004; Josten et al., 2004; Murakami R, 1993; Nakagoshi, 2005; Pankratz and Hoch, 1995). The midgut forms the largest portion of the intestinal system. It consists of at least seventeen tissue compartments (four gc (gastric cacaé) and m1 – m13, without those belonging to the midgut portion of the proventriculus) (Murakami R, 1993). During embryonic development the anterior and posterior midgut rudiment fuse to form a tube-like structure. This process is dependent on the visceral mesoderm that forms the midgut musculature (Tepass and Hartenstein, 1994). The most prominent cell types found in the midgut are the absorptive enterocytes. Other cell types are intestinal stem cells and enteroendocrine cells. At its posterior end the midgut fuses with the hindgut at the pyloric region. Close to the hindgut-midgut border, where the Malpighian tubules are connected to the intestine, the hindgut imaginal ring is found. The hindgut consist of nine domains (h1-4, h5d, h6d, by, hvm, and h7), and this organ’s development has been studied in greater detail (Lengyel and Iwaki, 2002; Murakami R, 1993). One remarkable feature of the hindgut is its dorso-ventral organisation. The dorsal h5d and h6d express *en grailed*. h4, also called boundary cells, forms a single cell row that separates the dorsal part from the ventral part and forms an anterior and posterior ring-like border between the pylorus and the rectum respectively. h4 is marked by the expression of *Crumbs*. hv (and hvm) form the ventral domain of the hindgut. *Delta* is expressed in hv/hvm and induces *Crb* expression in the h4 domain. *En* in h5d and h6d antagonises Notch signalling and thereby specifies the boundary cells (Iwaki and Lengyel, 2002). Posterior to the large intestine the rectum is
found. It is separated by a ring of border cells from the rest of the hindgut. The rectum is marked by Hh, Ser and Wg expression domains (Lengyel and Iwaki, 2002).

A special structure that is found in the intestinal system is the peritrophic membrane. It is a chitinous lining of the mid- and hindgut. Although its function is not completely understood it is assumed that it acts as a mechanical protection and as a kind of dialysis tube allowing the passage of smaller molecules but not of large entities like bacteria.

**The functions of the intestinal system**

The *Drosophila* intestine shares remarkably similar functions to the gut of humans and other higher eukaryotes. However it also differs at some important points. One of the principle functions is food uptake (pumped into the intestinal tract by the esophagus/proventriculus), digestion and absorption. Thus, distortion of the normal gut function affects the whole body. This is elegantly seen in *Drosophila* mutants for bonsai, a mitochondrial protein. *Bonsai* is required in the midgut and mutants are small due to impaired gut function (Galloni, 2003). Interestingly, in bonsai mutants, nutrient turnover and the pH of the midgut are not affected but mitochondrial activity is reduced. Another example of disturbed midgut function are mutants for the hsp90 chaperon, Gp93 (Maynard et al.). Gp93 mutant larvae are strongly reduced in weight and show signs of starvation. Midgut acidification, cell-cell junctions and nutrient turnover are affected in those larvae. Not only the digestive and absorptive functions of the gut are required for food processing. Also the transport through the gut is important. Hormones, such as tachykinins and allostatins, produced by endocrine cells of the midgut mainly regulate this process (Lenz et al., 2000a, b; Siviter et al., 2000). The gut and the Malpighian tubules also regulate ion homeostasis. Besides this primary function, the gut is in intensive crosstalk with the environment, the content of its lumen. Therefore, the gut forms a selective barrier that separates the outside from the inside. Gut homeostasis ensures the proper function of this barrier and the gut cells have to react in an appropriate way to signals from the outside.
Homeostasis of the intestinal system: control of the gut flora

Various bacteria populate the intestinal system, and new microbes constantly enter the gut with the food. The peritrophic membrane is synthesised by the proventriculus and forms a chitininos layer that restricts access to the gut cells (King, 1988). Antimicrobial peptides (AMPs) with bactericidal activity are not only expressed in the fatbody but also in various regions of the proventriculus and midgut to combat pathogens (Tzou et al., 2000). Another way to control the number and proliferation of bacteria in the gut is via the production of ROS (Ha et al., 2005a). Dual Oxidase (Duox) is a key enzyme in the production of ROS in the gut. A MEKK1/MKK3/p38/ATF2 pathway regulates the expression of Duox, and PLC-β regulates the activity of Duox (Ha et al., 2009a; Ha et al., 2009b). Thus, AMPs, regulated by NFκB, and ROS act together to limit the bacterial burden within the gut (Ryu et al., 2006). It is important to note that ROS, as well as high levels of AMPs, can destroy the beneficial gut flora composition. Thus, pathways have evolved to restrict their induction. In the case of ROS Drosophila has evolved a catalase system that maintains the redox balance of the gut during infection. If the immune-regulated catalase (IRC) is knocked down, individuals show high levels of lethality even when few bacteria enter the intestinal system. This lethality can be rescued by reducing the ROS-producing activity of Duox. Thus, this antioxidant response is needed to protect the gut cells from high levels of ROS and oxygen induced damage (Ha et al., 2005b).

It was tempting to assume that also a system regulating the expression of AMPs has to exist. This need becomes obvious since the NFκB pathway, the main inducers of AMPs, is constantly in an activated state in the gut, but the AMPs are only switched on if pathogens overtake. caudal has been identified as a major negative regulator that is responsible for the graded response to bacterial antigens (Ryu et al., 2008). The host-gut flora interaction is a very complex process even in an organism like Drosophila. Additional levels of regulation are the factors that are responsible for antigen processing (PGRCs). Not only in the immune system but also in the gut epithelial cells mediate the regulation of the gut flora. Most of the responses briefly
discussed above aim at anticipating damage to the gut cells by microbial components. What happens within the gut wall if it gets damaged or if it experiences other stresses than bacteria or fungi?

**Homeostasis of the intestinal system: intestinal stem cells**

In 2006 pluripotent cells with stem cell character have been identified in the posterior midgut of *Drosophila*. Those intestinal stem cells (ISCs) undergo mitosis and can produce all cell types of the midgut. Notch signalling is required for the differentiation into an enteroblast (EB), a stem cell offspring that can either differentiate into an enterocyte (EC) or an enteroendocrine (EE) cell. It has been shown that if Notch signalling is maintained in the EB it differentiates into an EC. If Notch signalling is lost Prospero expression in switched on and the EB differentiates into an EE (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007). In mammals the mean lifetime of a gut cell is about 8 days, and lost gut cells are replaced by new ones generated by the gut stem cells. The identification of ISC in *Drosophila* opened new questions about intestinal homeostasis: do ISC replenish the gut and ensure thereby gut homeostasis? What are the signals controlling the stem cells, and how do ISC behave in stressed or aged individuals?

Induction of apoptosis in the midgut by expressing the proapoptotic gene *reaper* in the EC induces the proliferation of the ISCs. This proliferation is dependent on the cytokine signalling system *upd/JAK/STAT*. Consistently, JNK activation in ECs triggers *upd* expression. Thus, compensatory ISC proliferation can be induced by damage to the intestinal tissue (Jiang et al., 2009). Indeed, in aged or stressed individuals, an increase in JNK activity has been observed in the ISCs. This increase in JNK signalling is associated with differentiation defects and contributes to the impaired regeneration ability of old/stressed midguts (Biteau et al., 2008). Moreover the gut flora and its bacterial composition impact on the ISCs in various ways. Bacteria (components) can directly activate JNK/JAK/STAT signalling, or the oxidative burst in response to pathogens induces JNK/JAK/STAT signalling and thereby induces compensatory proliferation of the ISCs. In case of lethal pathogens, it can lead to gut destruction and thereby death of the individual (Buchon et al.,
Besides JNK signalling, a potential role for p38 has been indicated (Park et al.; Park et al., 2009). Those studies are based on RNAi knock down and overexpression constructs, thus the precise role of p38 SAPK in the intestinal stem cells remains elusive.

There is no doubt that ISC have important roles in midgut homeostasis in Drosophila. Studying those stem cells will enhance our general knowledge of stem cells and their maintenance. Drosophila offers the advantage of straightforward genetic manipulations and thus is an excellent model system to analyse the interaction of different tissues in establishing a stem cell niche. For the midgut ISCs it was shown that Wg signalling from the muscle is absolutely critical for ISC homeostasis in the midgut. Another advantage is the relatively simple composition of gut flora in Drosophila.

Interestingly, besides the emerging knowledge of midgut ISCs, the topic of hindgut ISC remained untouched until recently. The presence of a hindgut imaginal ring that contains small immature cells that will form the adult hindgut has been recognized for a long time. Only recently Takashima et al. raised the possibility that those cells are hindgut ISCs that maintain the hindgut (Takashima et al., 2008). Gene expression analysis revealed an anterior-most row of cells that express Wg, followed by putative self-replicating hindgut ISC. Hh expression marks a region containing proliferating progenitors that give rise to differentiated ECs. Takashima et al. proposed a model that compares the Drosophila hindgut to a single crypt of the mammalian large intestine. Unfortunately, another study revealed that no constitutively active stem cells are present in the Drosophila hindgut (Fox and Spradling, 2009). The previously proposed model was based on a methodical mistake. Nevertheless, the gene expression analysis was confirmed by the second study. Fox and Spradling further show that – besides the absence of active stem cells – quiescent stem cells remain in the anterior-most region of the hindgut (pylorus). Additionally, stress and damage of the hindgut induce those quiescent ISC to proliferate, most likely to compensate for the tissue loss. Altogether further work based on these two studies will be needed to clarify the issue of hindgut stem cells.
Within a few years great progress has been made in elucidating signalling pathways and events that control the ISCs in the midgut. Less is known about the hindgut ISC, but at least from recent studies constitutively active stem cells appear to be not required for hindgut homeostasis. This raises the question of how the hindgut ensures homeostasis under normal and stressed conditions.

**Homeostasis of the intestinal system: the role of the enterocytes**

In the previous section I underscored the importance of the ISCs for gut homeostasis. Nonetheless, ECs are the first line of defense against stresses and damage. Only if ECs are lost ISCs are required to fill the gap. Surprisingly, our knowledge of signalling pathways that are required for EC homeostasis is only marginal.

Flies mutant for Myo1B, a structural component of midgut brush border cells, show an increase in apoptosis of midgut ECs and larvae are more susceptible of *Pseudomonas entomophila* oral infection (Hegan et al., 2007). Thus, keeping the proper cellular organisation intact is one aspect of intestinal ECs homeostasis. Although it has to be proven experimentally deletions of structural components like Singed and dMoesin are probably resulting in similar gut defects as Myo1B mutants.

On the level of signalling pathways, JNK activity is required to regulate stress induced autophagy in ECs and this in turn protects cells from undergoing apoptosis (Wu et al., 2009). It is apparent that JNK is an important regulator of gut homeostasis, but the levels of activation are determining the outcome. Whereas the lack of JNK activity renders the EC sensitive to stresses, too high activity results in JNK-mediated apoptosis. How other signalling pathways, like p38 SAPK, affect ECs is not known. Again these few examples focus on the midgut ECs. Whether the hindgut ECs evolved similar systems to ensure homeostasis has remained elusive.
part B (introduction)

PTEN loss-of-function confers a growth advantages to cells under dietary restriction in Drosophila melanogaster

Nutrition, Environment and Growth

One of the central definitions of life, which separates organisms from inorganic matter, is the ability to grow, reproduce and undergo metabolism. Thus growth and its regulation are central and ancient functions. Growth of an organism includes the increase in mass and size. In multicellular organism, growth can be achieved by the increase of size and mass of individual cells without changing the overall number of cells or by the increase in the number of individual cells without affecting the size or mass of the individual cells. Often these processes are coupled and cells that want to divide have to increase their size before division or re-grow to their desired size after the division is completed. It is obvious that these two processes have to be coordinated to achieve normal growth and proliferation. Furthermore all higher animals undergo different phases of growth during their lifetime. In a simplified view, every individual starts as a single cell (the egg) of relatively small size (compared to its final size), undergoes a phase of massive growth and divisions (accompanied by patterning) to reach a final size. After the final size has been reached growth and proliferation is reduced and only required for homeostasis e.g. to replace lost cells or regeneration of lost tissue parts and to produce germ cells. Thus, mechanisms exist that control not only the number and size of individual cells, but also the size of individual organs and the whole organism. Most of the cells in a body are not dividing and are differentiated to fulfil a certain function. Nevertheless, many cells retain their potential to proliferate. For example liver cells can re-grow a damaged liver to its original size within several days. Moreover, multipotent stem cells are found in many organs, divide slowly and one daughter cell retains the
stemness and the other one differentiates/or transiently divides to replace lost cells. Loss of growth and proliferation control can result in diseases such as cancer.

A prerequisite for growth is the availability of nutrients that supply the cells with energy, micronutrients (e.g. ions, vitamins) and building blocks (e.g. amino acids, lipids and sugars) to form its own structures. Nutrients have to be acquired from the environment by food uptake, have to be converted, absorbed and transported to the cells where they are needed. Underlying those processes are complex regulation circuits involving many organs, the hormone and the neuronal system. The availability of nutrients determines the extent cells and organisms can growth. Therefore, signalling pathways exist that link the information on available resources to the pathways that directly control growth and regulation. However, even in the presence of plenty of nutrients, unfavourable situations such as stresses or cellular damage restrict growth. This allows the cells to recover or to adapt to the adverse conditions and prevents additional stress.

**Systemic growth control by nutrition**

Nutrient uptake is controlled at the behavioral, cellular and molecular level, and the availability of nutrients influences growth in a cell-autonomous as well as in a cell-non-autonomous fashion. Mechanisms underlying the systemic growth control have been discussed in detail in my Diploma thesis (Seisenbacher, 2006). I will briefly summarise the systemic growth control by nutrition in *Drosophila* and I will subsequently focus on PTEN, a negative growth regulator of insulin signalling.

**Systemic growth control by nutrition in *Drosophila***

Within a short period of time *Drosophila* larvae have to increase their weight dramatically to store enough nutrients and energy to undergo metamorphosis. Yeast is the main amino acid/nutrient source for cultured *Drosophila*. Reduction in yeast prolongs development, the larvae pupariate later and at a smaller size, and the emerging adults are reduced in weight and size. The weight a *Drosophila* has to gain to successfully enter pupariation is called
“minimal viable weight”. If larvae are starved before they passed 70 hours after egg laying with nutrient supply they are dying and cannot undergo pupariation because they have not reached the MVW. A second parameter is the “critical weight”, which in *Drosophila* is also close to the MVW checkpoint. After larvae have fed for 70 hours they can undergo pupariation even in the absence of food, resulting in smaller individuals. Thus, how large a fly will be depends on how much nutrients they can acquire during the feeding period between CV and the onset of pupariation. Juvenile hormone titers, produced by the fat body, and ecdysone titers produced by the ring gland influence the time point of pupariation (Mirth and Riddiford, 2007).

On a systemic level IIS in the ring gland and the fat body has been shown to antagonise ecdysone signalling. Increasing insulin signalling via ring gland specific expression of PI3K reduces the size of the resulting fly, partially due to an increased ring gland, which results in higher levels of 20HE. 20HE activates its receptor in the fat body and reduces insulin pathway activity there, which results in reduced energy storage (Colombani et al., 2005).

Not only during metamorphosis IIS is required but it functions to control growth also on a systemic level. Together with TOR signaling, IIS forms a nutrient sensor mechanism that influences the organism’s growth. Fat body-specific knock down of the amino acid transporter *slif* results in larvae that are reminiscent of yeast-starved larvae. In their study, Colombani et al. showed that amino acid reduction reduces TOR activity in the fat body, and this subsequently results in downregulation of IIS in peripheral tissues and cessation of growth (Colombani et al., 2003).

A third example of how IIS influences growth in a systemic way are the medial neurosecretory cells (mNSCs) or insulin producing cells (IPCs). Seven IPCs are found in the upper part of the brain hemispheres of the larvae and extend their dendrites (personal communication with Irene Miguel-Aliaga) to the ring gland to potentially secrete DILPs into the circulating system. Indeed, when IPCs are ablated the larval development is slowed down and the resulting adults are reduced in weight and size (Brogiolo et al., 2001; Ikeya et al., 2002; Rulifson et al., 2002).
Overall systemic growth control in response to nutrition in *Drosophila* involves modulation of TOR and insulin signaling in various organs.

**FIGURE 3. Systemic growth control in *Drosophila* larvae**

Amino acids (purple) are absorbed via the gut (grey) and act on TOR signalling (blue) in the fat body (yellow). Through an unknown mechanism, TOR signals to the brain (pink), and mNSCs (green) release insulin-like peptides (green) that act on peripheral organs. The ring gland (red) and its hormone ecdysone (the active form 20HE, red) are important for developmental timing and metamorphosis. IIS (light green) counteracts 20HE.

**Cell-autonomous growth control by IIS**

IIS in *Drosophila* is not only required for systemic growth control, but acts in a cell autonomous fashion. In screens for growth controlling genes several components of a conserved IIS cascade have been found in *Drosophila*. Mutations in pro-growth components such as *chico/IRS*, *Ink* or *InR* result in smaller and fewer cells in clones of homozygous mutant cells (Bohni et al., 1999; Oldham et al., 2002; Werz et al., 2009). Under fed conditions the levels of circulating DILPs are high and can activate the InR in target tissues (Wu and Brown, 2006). Activation of the InR results in a scaffold formation dependent on binding of Chico/IRS that recruits PI3K to the membrane. PI3K, a lipid kinase, phosphorylates PIP2 to PIP3, which acts as a binding molecule for PH-domain proteins, such as PKB/Akt. Recruitment of PKB to the membrane is required for its full activation. PTEN, a lipid kinase is the major negative regulator upstream of PKB. PTEN antagonises the function of PI3K and thereby reduces the membrane recruitment and activation of PKB, thus...
restricting growth in a cell-autonomous fashion (Gao et al., 2000; Goberdhan et al., 1999; Goberdhan and Wilson, 2003).

**Figure 4. PTEN antagonizes InR signaling (mammalian IIS)**

Simplified model of upstream events in the mammalian IIS growth signalling. Ligands (green) bind to a receptor tyrosine kinase (RTK, purple). Activation of the RTK recruits PI3K (orange) to the membrane and its activity catalyses the transformation of PIP2 to PIP3 (pink). PH domain-containing proteins are recruited to the membrane. PKB (brown) gets activated in a PDK1 dependent fashion and stimulates various pro-growth and anti-apoptotic processes. PTEN (blue) antagonizes PI3K by removing a phosphate of PIP3 to generate the lipid PIP2.

**PTEN functions as a tumor suppressor**

In 1997 *PTEN* was identified as a gene that is frequently mutated in several cancers (Dahia et al., 1997; Li et al., 1997; Rasheed et al., 1997; Tashiro et al., 1997; Wang et al., 1997). Subsequently, it was shown that the PTP catalytic domain is necessary to exert its tumour suppressive function, and that its phosphatase function is frequently lost in cancer. Proteins and RNA have been discussed as potential PTEN substrates, but also phosphoinositides have been considered to be dephosphorylated by PTEN (Myers and Tonks, 1997). A year later it was shown that the primary target of PTEN are lipids and that it has to be placed upstream of PKB/Akt (Li et al., 1998; Myers et al., 1998).
A hallmark of cancer is the ability to evade proliferation and growth control. Consistently, increasing PKB phosphorylation and its activity by eliminating PTEN results in a permanent pro-growth signal. PKB activation additionally suppresses apoptosis and thereby gives the tumour cells a further advantage (Duronio, 2008).

Recently another function has been added, how PTEN mutations and environmental signal-independent activation of the insulin signaling pathway can give tumour cells an advantage. The rapid growth of a tumour requires a lot of energy. To supply the tumour with nutrients and oxygen, tumours often induce angiogenesis. Nevertheless tumour cells face an environment that lacks nutrients and in the center of solid tumors a region with necrosis is often found. In a recent study, Kalaany and Sabatini showed that tumor xenografts could be distinguished by their sensitivity to dietary restriction. They showed that cancer cell lines with mutated PTEN or an activated version of PI3K are resistant to dietary restriction, whereas lines with wild-type PTEN or PI3K, but mutations in RAS remain sensitive to dietary restriction (Coffer, 2009; Kalaany and Sabatini, 2009). Thus, mutations in PTEN support cancer growth in several ways: by preventing apoptosis, promoting growth and helping the tumour cells to survive in an unfavourable environment.

**Rationale of the Project**

Insulin and TOR signalling have been mainly studied for their cell-autonomous growth function under standard conditions, which represent states of sufficient nutrient supply. On the other hand, the systemic growth has been addressed under several states, and it has been shown that the requirement of IIS or TOR varies between different conditions. During my diploma thesis I analysed in detail how reduction of yeast affects growth and development of Drosophila melanogaster. While wild-type flies got smaller under yeast reduction, flies mutant for PKB or chico grew to the same size. Here we analysed the response of PTEN mutant flies and cells under the same conditions and showed that moderate yeast reduction already leads to lethality of PTEN mutant flies. Surprisingly, when mitotic clones are induced in imaginal discs, starvation leads to overproliferation of the mutant tissue.
RESULTS

part A (results)

p38 SAPK signalling ensures hindgut homeostasis in *Drosophila* by modulating the strength of the JNK SAPK response under stress conditions

MK2 paper/manuscript

*MK2-dependent p38b Signalling Protects Drosophila Hindgut Enterocytes Against JNK-induced Apoptosis Under Chronic Stress Further Aspects of MK2*

*The results of an extensive analysis of p38/MK2 function in the larval hindgut are presented in a manuscript that is ready for submission.*
MK2-dependent p38b Signalling Protects *Drosophila* Hindgut Enterocytes Against JNK-induced Apoptosis Under Chronic Stress

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Running Head

Stress Protection in the *Drosophila* Hindgut

**Abbreviations**

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BD</td>
<td>Black dot, used as synonym for melanotic lesion</td>
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<tr>
<td>EC</td>
<td>Enterocyte</td>
</tr>
<tr>
<td>EE</td>
<td>Enteroendocrine cell</td>
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<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<tr>
<td>IEC</td>
<td>Intestinal epithelial cell</td>
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<tr>
<td>ISC</td>
<td>Intestinal stem cell</td>
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<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MEKK</td>
<td>MAPK kinase kinase</td>
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<tr>
<td>MK2</td>
<td>MAPK-activated protein kinase 2</td>
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<tr>
<td>MKK</td>
<td>MAPK kinase</td>
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<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
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<tr>
<td>TAK</td>
<td>TGF-beta activated kinase</td>
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Abstract

Homeostasis of the intestinal epithelium is crucial for the barrier function of the gut, and a loss of this barrier function results in intestinal diseases such as ulcerative colitis and Crohn’s disease. Replenishment of the gut epithelium by intestinal stem cells contributes to gut homeostasis but how the differentiated enterocytes are protected against stressors is less well understood. By performing a thorough genetic analysis in the model system Drosophila, we demonstrate that a signalling complex consisting of p38b and MK2 forms a branch of SAPK signalling that is required in the larval hindgut to prevent stress-dependent damage to the enterocytes. Impaired p38b/MK2 signalling leads to apoptosis of the enterocytes and a subsequent loss of hindgut epithelial integrity, as manifested by the deterioration of the overlying muscle layer. Damaged hindguts show increased JNK activity, and removing upstream activators of JNK suppresses the loss of hindgut homeostasis. Thus, the p38/MK2 complex ensures homeostasis of the hindgut epithelium by counteracting JNK-mediated apoptosis of the enterocytes upon chronic stress. Our findings may contribute to a better understanding of early steps in the development of inflammatory bowel diseases.
Author Summary

The gut epithelium forms the first barrier against pathogens and stressors in the gut lumen. Malfunction of the gut epithelium can result in inflammatory bowel diseases like ulcerative colitis and Crohn’s disease. It is has been shown that damage in the gut epithelium triggers the proliferation of intestinal stem cells to replenish the epithelium. However, little is known about how the differentiated cells of the gut epithelium (mostly enterocytes) are protecting themselves against stressors. We used the model system Drosophila to address the function of stress-activated protein kinase (SAPK) signal transduction pathways in the larval gut. Our study revealed that a particular module of the p38 SAPK signal cascade is specifically required in the larval hindgut epithelium to protect the larvae against chronic salt stress. We identified the two kinases p38b and MK2 as key components of this protective signal. In the absence of p38b or MK2, another stress-activated signal transduction pathway (JNK) is locally upregulated and eventually induces apoptosis. Although the function of the p38b/MK2 module is only required in the enterocytes, the elimination of the affected cells results in atrophy of the overlaying muscle layer and subsequent systemic defects in the larvae (e.g. induction of antimicrobial peptides). We hope that our findings will contribute to a better understanding of early (i.e. pre-inflammatory) events in the development of human diseases of the intestine.
Introduction

The gut epithelium forms a protective barrier that separates the body cavity from the outside [1], and the proper function of this barrier is ensured by complex interactions between the gut microbes, the immune system and the epithelium. The intestinal epithelium is exposed to the gut flora, a potential source of antigens and pathogens, as well as to various environmental stressors. Imbalance of gut homeostasis may result in various diseases, reflecting the importance of this barrier [2–4]. Inflammatory bowel diseases (IBDs), including ulcerative colitis and Crohn’s disease, are well known examples for diseases of the intestine [5,6]. Various genetic and environmental factors have been linked to IBDs [7–12]. An alteration in mucosal immunity is a central theme in the development of IBD [13–15] and thus therapeutic approaches focused on counteracting the inflammation [16]. More recently, the increasing understanding of the interactions between the intestine and the gut flora has enabled novel strategies targeting the host-microbe interactions [7,17]. However, the intestinal epithelium itself as the first barrier has received less attention.

The intestinal epithelium consists of differentiated cells, the intestinal epithelial cells (IECs) and the intestinal stem cells (ISCs). ISCs have the potential to replenish lost and dying cells and thus impact on the ability to seal lesions in the gut epithelium [18–20]. Deregulation of ISCs can lead to defects in intestinal regeneration and to cancer. Most differentiated IECs are large absorptive cells, the enterocytes (ECs). In addition to their absorptive function, they form the first line of defense against pathogens and stresses and act as mediator between the luminal microbiota and stimuli and the immune system. NFκB signalling has been shown to be essential for survival of ECs, and mice with gut-specific knockout of NEMO spontaneously develop intestinal lesions reminiscent of those in IBDs [21,22]. Recently, ER stress has also been found to influence epithelial homeostasis, and mutations in XBP1 are sufficient to trigger an IBD-like phenotype [5].

Studies in the model organism Drosophila have provided new insights in how intestinal homeostasis is maintained. ISCs have been identified in the mid- and hindgut. In the midgut, stem cells are scattered between differentiated enterocytes (ECs) and enteroendocrine cells (EEs) and located at the basal side of the organ [23]. ISCs are required for normal gut homeostasis, but in aged and/or stressed individuals the number of midgut ISCs is increased and differentiation is disturbed [24,25]. In the hindgut, stem cells are found at the border between posterior midgut and hindgut in the pylorus [26]. In contrast to the situation in the midgut, the hindgut...
stem cells do not appear to be required for hindgut homeostasis in the larva and the adult fly. The hindgut ISCs are rather needed during the shift from larval to adult hindgut, and stress induces proliferation and cell migration in the pylorus region [27]. Although this offers a good experimental model for the analysis of EC replenishment upon damage, the mechanisms governing a proper stress response have remained elusive.

p38 SAPK signalling plays a versatile role in various stress responses and inflammatory processes, and it has also been considered in the context of IBDs [28]. In vivo and in vitro studies of p38 inhibitors have yielded promising results but clinical trials have not been successful so far [29,30]. The inhibition of p38 might lead to harmful side effects due to the plethora of p38 functions. Furthermore, the roles of p38 and other stress-activated protein kinases in IBD are still controversially discussed [31,32]. Thus, it is crucial to understand the biological functions of SAPK in the intestinal system.

SAPK signalling also plays a role in gut homeostasis in Drosophila. In the midgut JNK is required in the ECs to induce autophagy and thereby ensure survival of the ECs during oxidative stress [33]. In the ISCs, JNK is required for proper stress response but strong activation of JNK leads to differentiation defects and loss of gut homeostasis [24]. Similarly, p38 SAPK has been shown to be required in the ECs for DUOX expression and in the ISCs for normal differentiation [34,35]. A recent study showed that the intestinal epithelium is more susceptible to damage by pathogens in the absence of p38 function [36]. However, the mechanism of p38 action within the ECs remains unclear.

In this study, we investigate how the Drosophila larval hindgut is enabled to maintain homeostasis under stress conditions. Our results reveal that the ECs of the larval hindgut epithelium are protected against chronic stress by a p38b/MK2 signalling complex. In the absence of this p38b/MK2 complex, patches of ECs undergo JNK-dependent apoptosis, epithelial organisation is lost and the musculature of the hindgut gets severely damaged. Thus, we identify a specific SAPK signalling module required to maintain hindgut epithelial integrity upon stress.
Results

*Drosophila MK2* is required to protect the hindgut from stress-induced apoptosis

Raising *Drosophila* larvae on food containing high levels of sodium chloride impairs their growth rate but only marginally increases mortality. In contrast, several mutants for SAPK pathway components are sensitive to stresses including high osmolarity [37,38] but the cause of lethality and the tissue and cell types requiring SAPK function are unknown. To generate deletion mutants for MK2, encoding the orthologue of mammalian MAPKAP-K2 that is known to be a downstream kinase of the p38 branch of SAPKs [39], we mobilised a P-element insertion located in the MK2 locus (Figure 1A). Whereas Δ43 is a null allele as judged by the absence of MK2 protein and by the failure of Δ43 larval lysates to phosphorylate mammalian small heat shock protein 25 in a kinase assay (Figure S1), the alleles Δ41 and Δ12 are likely to represent hypomorphic alleles. Δ38 is probably also a null allele although the generation of a truncated protein (initiated from alternative Methionine codons) cannot be excluded (Figure 1A). A precise excision allele of the same P-element (Δ1A) was used as control throughout this study. MK2 mutant larvae displayed elevated mortality rates when reared on food containing high levels of sodium chloride (0.2M). At this salt concentration, the population of MK2 mutant larvae was heterogeneous, with some larvae displaying no defects and others appearing small and weak. 35 to 45 % of the MK2 mutant larvae developed a melanisation in a specific posterior location that we called “black dot” (BD; Figure 1B). Closer examination revealed that the BD localised to the posterior hindgut, and the affected hindgut epithelium appeared pathologically altered (Figure 1C). All MK2 alleles were analysed for the appearance of BDs and survival at three conditions: normal food, weak salt stress (0.1M NaCl) and strong salt stress (0.2M NaCl) (Figure 1D). The behaviour of MK2 hypomorphic larvae indicated that the levels of MK2 get more important with increasing osmolarity. Introducing a genomic rescue construct completely rescued the BD phenotype and the lethality of Δ43 mutants on 0.2M NaCl food. Thus, MK2 is not essential at normal conditions but is required when larvae are reared on a high sodium chloride diet.

In sections of the affected hindguts, the hindgut ECs were ruptured and the gut content appeared compressed (Figure 2A). The melanised patches localised to the apical surface of the ECs (Figure 2Aiii). TUNEL staining revealed an increase in apoptotic hindgut ECs of MK2 mutant larvae but not of wild-type larvae reared on
0.2M NaCl (Figure 2B). Loss of epithelial integrity was also evident from the mislocalisation of Neuroglian. Instead of the normal localisation at the lateral cell-to-cell contacts, Neuroglian was diffusely localised in Δ43 mutant ECs at the melanisation site (Figure 2Ci, 2Cii). Disturbed hindgut musculature indicated that the structure of the entire organ was affected (Figure 2Ciii).

To determine the tissue where MK2 function is required under high osmolarity, we performed a series of rescue experiments. The BD phenotype was rescued by ubiquitous and hindgut-specific but not by midgut-specific expression of MK2 (Figure S2). Moreover, only wild-type MK2 but not a kinase dead version of MK2 rescued the BD phenotype. The largest domains of the larval hindgut are a dorsal engrailed-positive and a ventral Delta-positive domain. BDs were only observed in the engrailed-positive domain (Figure 2D). Consistently, engrailed-Gal4 driven expression of a wild-type Drosophila MK2 or a wild-type murine MK2 but not of a kinase dead Drosophila MK2 rescued the BD phenotype (Figure 2E). Thus, Drosophila MK2 kinase function is required in the engrailed-positive hindgut compartment to protect hindgut ECs from stress-induced apoptosis upon salt stress.

**MK2 genetically interacts with p38a and p38b**

Mutations in MEKK1 and p38b but not in MKK3/ lic and p38a resulted in an even stronger BD phenotype than observed in MK2 mutants (Figure 3A). To define the roles of p38a and p38b with respect to MK2, we next tested these kinases genetically for the behaviour at normal conditions and under salt stress (Figure 3B). At unstressed conditions, MK2 was required neither for hindgut homeostasis nor for survival. Similarly, p38a mutants did not display BDs or elevated mortality rates. In contrast, MK2; p38a double mutants developed a weak BD phenotype, indicating that p38a and MK2 act in two parallel stress signalling pathways. p38b mutants had a decreased survival rate, consistent with published findings [38]. p38b is required for hindgut homeostasis even under normal conditions, because larvae lacking p38b function developed BDs on normal food. Interestingly, MK2; p38b double mutants displayed a slight increase in BDs but no increase in lethality rates compared to p38b single mutants. Thus, p38b and MK2 are likely to function in the same pathway but both kinases may have additional independent functions in the hindgut or in other tissues.
Consistent with a common MK2/p38b pathway, p38b single mutants and MK2; p38b double mutants displayed the same phenotype at both weak (0.1M NaCl) and strong (0.2M NaCl) stress conditions. At weak stress conditions, p38a was not crucial for survival and hindgut homeostasis. In contrast, MK2 mutants displayed hindgut defects but no increase in mortality. In agreement with MK2 and p38a acting in two parallel stress signalling pathways, BD formation was only slightly increased in the MK2; p38a double mutants but the absence of MK2 significantly enhanced the mortality of p38a mutants. This lethality increase of MK2; p38a double mutants was also observed at strong salt stress. At this condition, both MK2 and p38a mutants resulted in increased mortality rates, indicating that both branches of stress signalling are required for survival. Whereas MK2 is specifically required in the hindgut, p38a might be needed in other organs since p38a mutants hardly developed BDs.

Taken together, our genetic data suggest the existence of two major p38 branches in Drosophila that are required to varying extents during normal and different salt stress conditions. The p38a branch is not essential at normal or weak salt stress conditions but required at strong salt stress conditions. In contrast, the p38b branch is required at both normal and salt stress conditions to protect the hindgut. Moreover, the lethality of p38a; p38b double mutants [38] suggests the existence of a third general (stress-protective) p38 SAPK signalling pathway. MK2 forms a sub-branch of the p38b branch and appears to be the major downstream target of p38b in the hindgut. MK2 becomes more important with increasing stress conditions specifically in this tissue.

**p38b phosphorylation is increased in melanised larval hindguts**

We next checked the activation status of p38 by Western analysis (Figure 3C). Chronic exposure (from L1 to L3) to 0.2M NaCl did not increase p38 phosphorylation in total and hindgut lysates of wild-type larvae. In contrast, stronger p38 activation was observed in total larval lysates of MK2 mutants under stress conditions. This strong activation was also apparent in the hindgut but only in larvae with BDs. Since we were not able to distinguish the endogenous p38a and p38b, we overexpressed GST-tagged versions of p38 and analysed their activation status in the hindgut (Figure 3D). GST-p38a was strongly activated even under normal conditions, and the activation was slightly increased under stress conditions. In MK2 mutants, GST-p38a was more strongly activated under all conditions and especially in hindguts with BDs. In contrast, GST-p38b was only weakly phosphorylated in wild-type and MK2 mutant
hindguts at all conditions, except in hindguts of MK2 mutant larvae with BDs where a boost in GST-p38b phosphorylation was seen. Thus, the increase in endogenous p38 phosphorylation observed in MK2 mutant larval hindguts is probably due to p38b phosphorylation, suggesting that a negative feedback loop operates from MK2 to the upstream signalling components.

**MK2 protein abundance depends on p38b**

We wondered whether the protein levels of MK2 would depend on the presence of the upstream components. No change in MK2 expression was observed in p38a mutants, and a slight increase was detected in MEKK1 mutants. In sharp contrast, MK2 protein levels were reduced in p38b mutants (Figure 3E). Using a genomic gMK2 rescue construct and a genomic gMK2-GFP reporter in an MK2 null mutant background, we found that transcription from the MK2 locus was unchanged but protein levels were reduced, probably due to destabilisation of MK2 in the absence of p38b (Figure 3F).

**MK2 physically interacts with p38b**

The facts that MK2 and p38b genetically interact and that MK2 protein levels depend on the presence of p38b suggest a close physical interaction of the two kinases. We expressed tagged versions of p38a, p38b and MK2 in cultured S2 cells and checked for co-localizations of these kinases. In mammalian cells, MK2 is cytoplasmic under normal conditions and translocates to the nucleus upon stress [40]. In contrast, Drosophila MK2 was mainly found in the nucleus, whereas p38a and p38b were largely cytoplasmic (Figure 4A). Co-overexpression of p38b and MK2 (but not of p38a and MK2) resulted in a nuclear-to-cytoplasmic translocation of GFP-tagged MK2 (Figure 4A). Consistently, MK2 was found to bind p38b but not p38a in pull down experiments (Figure 4B). This interaction as well as the nuclear-to-cytoplasmic shuttling depended on a four amino acid motif (DPTD) in p38b. In p38a, the respective four amino acids are EPSV. Exchanging these motifs revealed that the DPTD motif is necessary and sufficient to dock MK2 to p38 proteins (Figure 4A and 4B).

The expression of GST-p38b completely rescued the BD phenotype of p38b deficient flies, whereas expression of GST-p38a had no influence on the BD phenotype.
Expressing either GST-p38a→b (p38a with docking motif of p38b) or GST-p38b→a (p38b with respective sequences of p38a) resulted in a partial rescue of the BD phenotype. On normal food, both versions rescued partially, indicating that although not essential, binding to MK2 is required for a complete rescue under normal conditions. On 0.1M and 0.2M NaCl, GST-p38a→b resulted in substantial but incomplete rescue, suggesting that other aspects of p38b function not mediated by binding to MK2 are required for a complete rescue. Consistently, a GST-p38b→a protein that is not able to bind MK2 also partially rescued the p38b phenotype but to a lesser extent than p38a→b (Figure 4C). MK2 protein levels in the hindgut were restored by expressing p38b or p38a→b but not by p38a or p38b→a (Figure 4D). Thus, binding of p38 to MK2 is required to localise and stabilise MK2.

**The catalytic activity of p38b is required to localise MK2**

The catalytic activity of MK2 and MK2 binding to p38b are required to protect the hindgut epithelial cells upon salt stress. To address whether the catalytic activity of p38b is also necessary, we used GST-tagged non-activatable p38bAGF and kinase dead p38bKR protein mutants. Whereas co-expression of wild-type GST-p38b and GFP-MK2 led to a nuclear export (> 70%) of MK2, the localisation of GFP-MK2 was random when GST-p38bAGF or GST-p38bKR were co-expressed (Figure 5A and 5B). Consistently, the BD phenotype of p38b null mutants was not rescued by re-expression of the kinase dead or of the non-activatable p38b protein version (Figure 5C). Moreover, at high NaCl stress, the p38bAGF and p38bKR expressing larvae died. This could be explained by the titration of an upstream kinase of p38b, which might impinge on the activation of other downstream effectors. Thus, the catalytic activity of p38b is required to impact on the subcellular localisation of MK2.

**JNK signalling is induced in MK2 mutants and is linked to apoptosis**

JNK signalling has been implicated in triggering apoptosis [41]. Furthermore, a JNK antagonizing activity of p38 signalling has been observed in developmental processes [42] and at the systemic level during infection [36]. Thus, we tested whether the cell death observed in MK2 mutant larvae reared on high salt correlated with JNK activation. As readouts for JNK signalling activity, we used reporters for misshapen and puckered [43,44]. We detected an induction of both msn>lacZ and
puc>lacZ in MK2 mutant larval hindguts, with highest levels adjacent to the BDs (Figure 6A). Strong puc-GFP reporter activity co-localised with TUNEL positive cells close to the BDs (Figure 6B). In addition, the levels of activated JNK in the hindgut were higher in MK2 mutant larvae with BDs (Figure 6C). Removing the JNK upstream components TAK1 and MKK4, respectively, in an MK2 mutant background partially suppressed the BD phenotype, indicating that the hindgut epithelial cells are dying due to JNK-induced apoptosis (Figure 6D). To exclude that the induction of JNK signalling is a secondary consequence of wound healing, we checked for puc-GFP activity in stress challenged MK2 mutant larvae before BD formation. Interestingly, patches with puc-GFP signal were readily detected upon stress in larvae devoid of BDs, and the area of those patches correlated with the strength of the stress (Figure 6E). We noted that puc-GFP was activated in a graded fashion, with highest activity where ECs undergo apoptosis and a BD will ultimately form (Figure 6F). Thus, deregulated JNK activation in the hindgut of MK2 mutants precedes and probably causes cell death and BD formation.
**Discussion**

Gut homeostasis needs to be maintained under stress conditions. Replenishment of the gut epithelium by ISCs clearly contributes to epithelial homeostasis but how the differentiated ECs are protected against stressors has remained largely unknown. Here we addressed the question how *Drosophila* hindgut homeostasis is sustained in the absence of constitutive stem cells. We identify the p38b/MK2 signalling module as a critical component in the protection of hindgut ECs against salt stress.

We propose a model that puts a p38b/MK2 complex in the centre of stress-protection of the hindgut ECs (Figure 7). In the absence of this signalling module, cells are undergoing JNK-dependent apoptosis upon stress. The lesion in the EC monolayer results in the damage of the overlying hindgut musculature. This regional loss of the barrier function leads to systemic defects in the larvae (Figure S4), further weakening the larvae and impairing growth under stress conditions. As a consequence, pathogens and toxic substances might enter the body cavity, eventually resulting in the melanisation of pericardial cells and the induction of cecropin in the midgut (Figure S4).

Interestingly, JNK activation in MK2 mutant hindguts precedes the tissue damages and it consistently occurs in patches. Within these areas, some cells acquire highest amounts of JNK activity and eventually undergo apoptosis (Figure 7B). The surrounding cells maintain JNK activity, forming a rim around the scar in the tissue. The size of the areas with high JNK activity does not expand during the course of development; it rather correlates with the strength of the stress and with the developmental stage at which the larvae are exposed to the stressor. The latter effect probably reflects a direct correlation with cell size. As larvae develop, the ECs enlarge due to polyploidisation. The number of affected ECs remains roughly constant for a given stress (Figure 7C). Presently we do not know what determines the patches with high JNK activity within the tissue. Although all ECs of the hindgut epithelium are genetically identical and are facing the same stressor, JNK signalling is only induced in clusters of a certain size but not in surrounding cells. We note that a similar patchy pattern of ulcerations is observed in IBDs [45].
Several lines of evidence support the notion that the p38b/MK2 signalling complex is key to EC protection against chronic salt stress. (1) p38b and MK2 mutant larvae both develop BDs upon stress conditions. (2) The severity of the p38b; MK2 double mutant phenotype upon stress is similar to the p38b single mutant phenotype, suggesting that they act in the same signalling pathway. (3) p38b but not p38a physically associates with MK2 via its C-terminal DPTD motif. (4) The binding of p38b to MK2 stabilises MK2. (5) Upon co-expression, p38b but not p38a redirects MK2 to the cytoplasm. (6) Both the activation and the catalytic activity of p38b are required to efficiently relocalise MK2. (7) The binding of MK2 to p38 and the catalytic activity of MK2 are essential to protect the ECs of the larval hindgut upon salt stress. Taken together, stabilisation, localisation and activation of MK2 by p38b is required for a proper stress response.

Our genetic analysis also revealed that p38 SAPK signalling is contributing to stress protection in different ways in addition to the pivotal role of the p38b/MK2 complex. First, p38b impacts on hindgut homeostasis in an MK2-independent manner. This is apparent from the p38b mutant larvae that, in contrast to MK2 mutant larvae, develop BDs even at normal conditions. Consistently, a p38b protein version that no longer binds MK2 partially rescues the p38b mutant phenotype. Second, p38a is also required for full stress protection. The strong phenotype of MK2; p38a double mutants underscores the importance of the p38a SAPK pathway upon severe salt stress. However, the double mutants do not display an increase in BD formation but rather a decrease in viability. Thus, p38a may be involved in general stress protection that is not specific to the hindgut tissue. Third, a common p38 SAPK branch, encompassing p38a, p38b and potentially also p38c, is essential as the p38a; p38b double mutants die. Since the p38a\textsuperscript{1} allele affects the coding sequences of both p38a and p38c, the p38a\textsuperscript{1}; p38b\textsuperscript{27IP} double mutants may even represent p38a; p38b; p38c triple mutants. However, it is unclear to date whether p38c is a pseudogene. Recent studies have suggested an involvement of p38c function in immune gene regulation, early larval survival, and fertility [36,46]. Thus, further studies will be required to clarify whether p38c contributes to p38 signalling subbranches. Finally, the slight increase in BDs seen in MK2; p38b double mutants under normal conditions suggests that MK2 also performs a p38b-independent function in the hindgut. p38b mutants always impact on MK2 signalling since the MK2 protein is not stable and probably not correctly localised if not bound to p38b. A negative feedback regulation acting from MK2 on the activation of p38b further complicates the SAPK signalling network.
What are the upstream components regulating the p38b/MK2 complex? To our surprise, MKK3/lic does not appear to play a role in the hindgut function of the p38b/MK2 branch. MKK3 but not MKK4 can activate p38 proteins in cell culture [47]. On the other hand, siRNA mediated knock down of both MKK4 and MKK3 is required to fully block the activation of p38 under certain stresses in S2 cells [48]. Both p38b and MKK3/lic mutants show a strong reduction in p38 activation but no BDs are observed in MKK3/lic mutants. In contrast, mutants for MEKK1, which acts upstream of MKK3, do develop BDs similar to p38b and MK2 mutants. In mammalian cells, it has been shown that p38 can be activated independently of a MAP2 kinase (Rudd, 2005 #202). Since MKK4 is a suppressor of the BD phenotype and MKK7 most likely does not activate p38, such a scenario could also apply for the p38b/MK2 signalling branch in the Drosophila hindgut.

Interestingly, overexpression of the kinase dead or of the non-activatable p38b protein version results in an even stronger phenotype than the deletion of p38b, probably by titrating upstream partners that would have additional functions besides activating p38b. A common p38a/p38b activator would be a strong candidate for such an upstream component. Saturating this common p38 activator with p38bKR or p38bAGF would essentially result in a p38a/p38b double mutant situation and therefore would explain the strong phenotype, especially at high salt conditions.

Our analysis of the p38b/MK2 signalling module in hindgut ECs reveals how deletion of SAPK members results in increased sensitivity towards a particular stressor from the molecular level to the level of the whole organism. These findings provide a new model of how hindgut homeostasis is maintained and how different SAPK branches act together in vivo to ensure cellular survival upon stress. Our results might also be important in the context of various diseases of the intestinal system. A variety of different signalling pathways have been implicated in IBDs, underscoring the complex nature of these diseases. p38 and MK2 are critical regulators of TNFα production and are thereby associated with IBDs [28] but the role of p38 SAPK in IBDs has remained controversial [31,32]. Our study identifies a crucial role of p38b/MK2 signalling in the first line of defense against a particular stressor in a model system devoid of an adaptive immune system. The consequences of lacking
this immune system-independent protective function of a SAPK branch might parallel early steps in the development of IBDs.
Materials and Methods

Fly media and stock keeping

1 litre *Drosophila* media contains 100g fresh yeast, 55g cornmeal, 10g wheat flour, 75g sugar and 8g bacto-agar. For stress medium, *Drosophila* media were boiled and sodium chloride was added from a 5M stock solution. 15ml/l of a stock solution containing 33g/L nipagin and 66g/L nipasol in 96% EtOH were added to prevent growth of mould and bacteria. All crosses and experiments were performed at 25°C.

Fly stocks

*w* GE3296 was remobilised to generate the MK2 deletion mutants. *y w; gMK2* (genomic rescue), *y w; gMK2-GFP* (genomic GFP reporter); *y w; 86Fb pAttB [GST-X] (X…p38a, p38b, p38a>b, p38b<a, p38bKR, p38bAGF*), *y w; 51D [MK2], y w; 51D [MK2KD], y w; 51D [mouseMK2], y w; nbyn2-GAL4 were generated in this study. For overexpression analyses, the following lines were used: *y w; cad-Gal4/CyO y+, y w; byn-Gal4/TM6B, y w; en-Gal4/CyO y+, y w; NP1-Gal4/CyO y+, y w; da-Gal4 and y w; act-Gal4*. For genetic interactions, the following lines were used: *y w; FRT82B D-p38a7/TM6B [49], y w; p38b27P/CyO y+ [38], y w licome413/Binsn [38], y w; MKK4414/TM6B, y w; MKK4585/TM6B [50]; w dTAK12, w dTAK14 [51], y w; MEKK1Ur36/TM6B [37]. The following reporter lines have been used: *nrgG00305* [52], *y w; cecA1-lacZ* [53]; *y w; pucE69*, *y w; UAS-EGFP; puc-GAL4/TM6b* (gift from K. Basler), and *y w; msn-lacZ* [44].

Stress experiments

Females were allowed to lay eggs overnight on apple agar plates. Eggs were collected and 80 – 120 eggs were transferred to the different food vials. For BD quantification, larvae were analysed before reaching L3 wandering stage. For survival quantification, dead embryos were counted 24h after seeding to the food and survival to pupae was recorded, respectively.

Plasmid constructs and transgenic animals
For the gMK2 genomic rescue construct, the genomic region between CG15771 and CG15770 was PCR-amplified and cloned into \textit{pCasper3}. For the genomic gMK2-GFP reporter, the same region was used but the \textit{MK2} coding sequence was replaced by the \textit{GFP} coding sequence.

For overexpression constructs, the \textit{MK2} coding sequence was amplified and cloned into \textit{pENTR/D-TOPO} (Invitrogen). For the kinase dead MK2 protein, the mutation leading to the K49A substitution was introduced by PCR mutagenesis. The inserts were shuttled into the destination vector pTGW (http://www.ciwemb.edu/labs/murphy/Gateway\textasciitilde20vectors.html) for N-terminal GFP tagging. To express untagged MK2, the \textit{MK2} coding sequence was cut from the \textit{pENTR/D-TOPO} and ligated into a \textit{pUAST} vector.

GST-tagged p38a and p38b overexpression constructs were generated by ligating the GST coding sequence in frame to the p38a or p38b coding sequence, and the resulting fusion sequences were cloned into a \textit{pUAST} vector. The constructs encoding the p38a or p38b protein mutants were generated by PCR mutagenesis. In the p38a\rightarrow b and the p38b\rightarrow a protein mutants, the EPSV motif was changed to DPTD and vice versa, respectively. The kinase dead (KD) or non-activatable p38b (NA) protein mutants were generated by introducing mutations in the coding sequence that result in the K53R substitution and in the replacement of the TGY dual phosphorylation motif by AGF, respectively.

\textit{pUAST} based constructs were injected into embryos carrying a landing site (\textit{vas-φC31-zh2A}; \textit{ZH-attP-51D} for chromosome II and \textit{vas-φC31-zh2A}; \textit{ZH-attP-86Fb} for chromosome III, [54]). The \textit{gMK2} genomic rescue and reporter lines were generated by co-injecting the respective plasmid with \textit{Δ2-3} helper plasmid into \textit{y w} embryos.

**Cell transfection and pull downs**

Transfection of S2 cells was done using the Effectene Transfection Reagent (QUIAGEN) according to the manufacturer’s protocol. After four days of protein expression, cells were harvested, lysed and pull down of GST-p38 proteins was performed using glutathione sepharose beads (Pharmacia Biotech AB). 10\% of the lysates was loaded as input and the complete pull down sample was loaded onto SDS PAGE.
Dissection and fixation of larval tissues

Larvae were collected in 1xPBS on ice. Three to five larvae were transferred to new vials containing 400µl ice-cold 1xPBS. Larvae were dissected with two fine forceps and the desired organs were transferred into a microfuge tube containing 500µl of ice-cold 4% paraformaldehyde in 1xPBS. Imaginal discs were fixed for 20-30’ at room temperature under mild shaking. Hindguts/midguts were fixed for 40-50’ at 4°C. Subsequently, the fixative was removed by three washing steps with cold 1xPBS. Fixed preparations were stored in 1xPBS at 4°C until further use.

Preparation of cells for IHC

Cover slips were washed by dipping into 100% EtOH and air-dried. They were then incubated in 0.15% ConA solution (in ddH$_2$O) for 1-2h, washed with ddH$_2$O and air dried overnight.

ConA slides were placed into a small Petri dish and covered with Schneider’s medium (approx. 1ml). 200µl S2 cells were added and allowed to settle onto the discs for 45’ to max. 90’. Cover slips were then washed once with ice cold 1xPBS, and 1ml 4%PFA was added for 5’ fixation on ice followed by 10’ fixation at room temperature. Cover slips were washed 3x with 1xPBS, cells were permeabilised with 1xPBT for 10’ and stored till further processing in 1xPBS.

Antibody stainings

Specimens were blocked by incubating in 2% NDS in 1xPBS 0.2% Triton X-100 (or 0.3% Triton X-100 for hindguts) for 1h (larval tissues) or 30’ (S2 cells), respectively. Primary antibodies were added in 2% NDS in 1xPBS 0.2% Triton X-100 for 1h (S2 cells) or overnight (larval tissues), respectively. Before secondary antibodies were added, samples were washed 3x in 1xPBS 0.2% Triton X-100. Secondary antibodies were added in 2% NDS in 1xPBS 0.2% Triton X-100 for 1h (S2 cells and larval tissues).

Western blot (WB) membranes were blocked in 3% membrane blocking agent (GE Healthcare) for one hour. Membranes were incubated with the primary antibodies
overnight and one hour with the secondary antibodies in 3% membrane blocking agent.

Primary antibodies: rabbit anti-GST (1:5,000 WB or 1:500 IHC, SIGMA G7781); rabbit anti-activated JNK (1:1000 WB, Promega V793A); rabbit anti-pTGpY-p38 (1:1000 WB, Cell Signaling 4631); mouse anti-tubulin (1:10,000 WB, SIGMA T9026) and rabbit anti-D-p38b (1:1000 WB,[55]). The anti-Drosophila MK2 antibody was generated by EUROGENTEC by immunising a rabbit with the peptide H$_2$O-QPKTPTPTDDYVTSN-COOH, and the final bleed was used 1:500 on WB.

Secondary antibodies: HRP coupled anti-mouse IgG (Jackson ImmunoResearch) and HRP coupled anti-rabbit IgG (Jackson ImmunoResearch) (1:10,000; WB), Cy3 coupled anti-rabbit antibody (1:500; IHC).

Other histological stainings

Larvae were dissected and fixed by standard procedure. After washing with 1xPBS, 500µl X-gal staining solution was added and the samples were incubated at 37°C in the dark. The staining progress was observed every 10’, and the staining reaction was stopped by two washes with 1xPBT.

Alexa Fluor 594 conjugated phalloidin (Molecular Probes) was used to stain muscles. For apoptosis detection, the TUNEL assay kit ApopTag RED In Situ Detection Kit (Millipore S7165) was used.

Hindgut sections

Larvae were dissected on ice and hindguts were immediately fixed in 2.5% glutaraldehyde, 1% paraformaldehyde, 1% potassium ferrocyanide, 0.1M cacodylate buffer for 80’. After 3x washing in 0.1M cacodylate buffer hindguts were postfixed in 1% osmium tetroxide, 1% potassium ferrocyanide, 0.1M cacodylate buffer (pH7.4) for 60’. Hindguts were then dehydrated in an ascending acetone series (30% > 50% > 70% > 90% > 100% 3’ each and 5’ 100%). The samples were incubated overnight in a 1:1 acetone:Spurr solution. After equilibration in Spurr solution for 4h, samples were embedded in Spurr solution and hardened at 65°C overnight. 2µm sections were made with a Supercut Reichert-Jung 2050 microtome, and sections were mounted in DPX Mountant for histology (Fluka).
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Figure Legends

Figure 1. MK2 deficient larvae are sensitive to salt stress

(A) Deletion alleles of MK2 generated by imprecise excision of the P-element GE3296. (B) MK2 null mutants but not wild-type larvae develop melanisations in the posterior part of the body (BDs) when reared on 0.2M NaCl food. (C) Bright field pictures of wild-type (Δ1A) and MK2 mutant (Δ43) hindguts of larvae reared on 0.2M NaCl food. Rectum points to the left. (D) Different MK2 deletion alleles reared on normal food, 0.1M and 0.2M NaCl food, respectively, revealed an increase in BDs with the severity of the stress.

Figure 2. Increased apoptosis and tissue damage in hindguts of MK2 mutants

(A) Bright field pictures of sections of hindgut tissue. MK2 mutants show ruptures of ECs when reared on high salt diet. (A1) wild-type, (A2, A3) Δ43 MK2 mutant. (B) Apoptosis is increased in the hindguts of MK2 mutant larvae reared on high salt conditions. (B1) wild-type, (B2) Δ43 MK2 mutant. (C) Epithelial organisation is lost in MK2 mutants. (C1) Wild-type flies show normal cell-cell junction localisation of Neuroglian-GFP and hindgut musculature is intact. (C2) In Δ43 mutants, Neuroglian-GFP is diffusely localised and the musculature is ruptured at the sites of BDs. (C3) Ruptured musculature in Δ43 mutants reared on high salt diet. (D) BDs are always found in the engrailed-positive compartment (green) of the hindgut. (E) MK2 cDNA constructs were expressed in the engrailed-positive compartment and L3 larvae were analysed for BD appearance.

Figure 3. Interactions of MK2 with the p38 SAPK pathway

(A) Members of the p38 SAPK were tested for a BD phenotype (normal food, 0.1M NaCl, 0.2M NaCl). MEKK1 and p38b but not MKK3 or p38a show a BD phenotype similar to MK2. (B) p38a, p38b and combinations with MK2 Δ43 were analysed in detail for survival and BD appearance on normal food, 0.1M NaCl and 0.2M NaCl. (C) The activating phosphorylation of p38 is increased in MK2 mutants under stress, especially under 0.2M NaCl in larvae with BDs. (D) GST-tagged p38a or p38b was overexpressed in wild-type or MK2 mutant larvae to distinguish the activation of the two p38 kinases. (E) MK2 protein levels were analysed on Western blot of different SAPK mutants. (F) Stabilisation of MK2 happens at the level of protein or translation
and not at the level of transcription, because GFP expressed under the control of the genomic MK2 locus is unaltered.

**Figure 4. A p38b/MK2 complex is required for hindgut EC stress protection**

(A) GFP-MK2 localises to the nucleus. GST-p38a and GST-p38b show a similar broad distribution. GST-p38b co-expression shuttles GFP-MK2 to the cytoplasm, which is dependent on the C-terminal DPTD motif. GST-p38a or GST-p38b→a (DPTD changed to EPSV) do not change the localisation of MK2, but a GST-p38a→b protein mutant can export GFP-MK2 from the nucleus. (B) In pull down experiments, GST-p38b co-precipitates MK2, whereas p38a and the docking mutant p38b→a can not bind MK2. The DPTP motif introduced into p38 (p38a→b) is sufficient for binding to MK2. (C) Whereas overexpression of GST-p38a fails to rescue the BD phenotype of p38b mutants, GST-p38b rescues completely. GST-p38a→b and GST-p38b→a partially rescue the BD phenotype with p38a→b performing better. (D) Docking of MK2 to p38 that harbours a DPTD motif is sufficient to restore wild-type levels of MK2 protein.

**Figure 5. p38b activation and kinase activity are required to protect the hindgut**

(A) Co-expression of GST-p38b and MK2 results in cytoplasmic localisation of GFP-MK2. When a kinase dead (KR) or non-activatable (AGF) version of p38b was expressed, the nuclear export was not efficient. (B) Quantification of GFP-MK2 localisation when GST-p38b, GST-p38bAGF and GST-p38bKR are co-expressed. Localisation was assigned to three classes: cytoplasmic (c), equal distribution between nucleus and cytoplasm (e), and nuclear localisation (n). (C) Activation and kinase function are required for proper function of p38b in the hindgut. Percent BDs of p38b mutants was quantified when p38b protein mutants were re-expressed using Act-Gal4.

**Figure 6. JNK activation leads to EC apoptosis in MK2 deficient hindguts**

(A) Wild-type control *msn-lacZ, Δ43 msn-lacZ* and *Δ43 puc-lacZ* in larvae reared on 0.2M NaCl. In Δ43 mutants, the JNK activity reporters get induced, especially close to the BDs. (B) In Δ43 mutants on 0.2M NaCl, highest puc-GFP (green) and TUNEL
staining (red) co-localise to the BD border region. (C) JNK phosphorylation is strongly increased in hindguts of MK2 mutant larvae with BDs reared on 0.2M NaCl food. (D) Removing two copies of TAK1 or of MKK4 partially suppresses the BD phenotype of MK2 mutants. (E) Activation of puc-GFP in Δ43 mutants prior to BD appearance. Whereas no activation of puc-GFP occurs on normal food (E1), fields of increased puc-GFP appear in the hindgut epithelium on 0.1M NaCl (E2) and 0.2M NaCl (E3). The area of the patches and the number of affected ECs correspond to the strength of the stress. (F) puc-GFP is activated in a graded fashion. It is expressed highest surrounding the site of the lesion and decreases in ECs further away. (F1) hindgut of Δ43 mutants at the initiation of BD, (F2) Δ43 mutants after BD appearance on 0.1M NaCl food.

**Figure 7. Model for hindgut EC homeostasis by MK2/p38 SAPK signalling**

(A1) A high sodium chloride diet directly imposes stress on the hindgut ECs and activates both SAPK branches, p38 and JNK, leading to the survival of ECs and proper gut homeostasis. (A2) In the absence of MK2, JNK activation is no longer kept in check but increases beyond a threshold level, resulting in the death of ECs and a loss of hindgut homeostasis. (B) On the tissue level, hindgut ECs increase JNK signalling in a patchy pattern. The area of a cell cluster that increases JNK signalling depends on the strength of the stress. Within the patch of high JNK activity, cells undergo apoptosis, leaving a melanised scar in the hindgut tissue (BD). (C) The size of the clusters of increased JNK signalling mainly depends on the strength on the stress. The earlier a larva is affected during development the smaller the BD will be. This effect is mainly due to the smaller size of the affected cells and not due to the involvement of fewer cells.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
References


Supporting Information

Figure S1. *MK2* null mutants and development of BDs

(A) Larval extracts of wild-type larvae but not of Δ43 mutants have *in vitro* kinase activity towards small heat shock protein 25. Overexpression of wild-type MK2 but not of kinase dead MK2 boosts small heat shock protein 25 phosphorylation. Antibodies against *Drosophila* MK2 do not recognize a band in Western analysis on Δ43 total lysates. (B) MK2 Δ43 larvae reared on 0.2M NaCl food were analysed for the presence of BDs at the indicated time points. (C) Representative pictures of Δ43 larvae used for BD quantification in (B). (D) Size of BDs depends on the strength of the stress. Δ43 larvae were reared on 0.1M or 0.2M NaCl food, respectively, and BDs were quantified in L3 before wandering stage.

Figure S2. Hindgut-specific rescue of *MK2* mutants

(A) Various Gal4 lines were used to drive MK2 expression from a wild-type UAS-MK2 cDNA construct in a Δ43 mutant background, and the ability to rescue the BD phenotype was scored. Only ubiquitous and hindgut-specific expression of catalytically active but not of a kinase dead MK2 rescues the BD phenotype.

Figure S3. *p38b* BD phenotype rescued by *p38* expression

Homozygous *p38b* mutants were reared on 0.2M NaCl food. Rescue of the BD phenotype by *p38a* and *p38b* was quantified (Figure 4). Representative examples of larvae that were quantified for their BD appearance are shown.

Figure S4. Systemic effects observed in *MK2* mutants

(A) *MK2* mutants reared on 0.2M NaCl food often display a severely ruptured hindgut musculature resulting in a local gut barrier breakdown. (B) In such strongly affected larvae, the antimicrobial peptide CecA1 is induced in the midgut, indicative of a systemic response. (C) This systemic disturbance is underscored by the appearance of melanised pericardial cells.
Figure S1
Figure S2

A

B
gut expression pattern

da>GAL4  byn>GAL4  NP1>GAL4  cad>GAL4
Figure S3
Figure S4
Further Aspects of MK2

Details on MK2 alleles

We used the largest deletion, Δ43, as null allele in most of our studies. Nonetheless our allelic series represents a valuable toolkit for further analysis of the MK2 locus. The second largest deletion, Δ38, appears to be a null allele since it shows a similar phenotype as Δ43. There might be some background problems since homozygous Δ38 flies display a slightly stronger BD phenotype than Δ43 and the heteroallelic combination of Δ38/Δ43 behaves like homozygous Δ43 mutants. Nevertheless we cannot rule out that this MK2 deletion locus gives rise to a truncated protein version since potential in frame start codons follow the deletion. The two other deletions, Δ12 and Δ41, do not affect the coding sequence. Δ12 affects only exon 1b and Δ41 only exon1a and 1a’. Under moderate salt stress (0.1M NaCl) both alleles give the same weak BD phenotype, indicating that the expression of MK2 reaches sufficient levels to protect the hindgut. Increasing the stress to 0.2M NaCl increases the BD in both alleles but Δ41 is twice as strong as Δ12. This indicates that either exon 1a is responsible for the hindgut expression of MK2 or that generally exon 1a is the major MK2 transcript in Drosophila and the overall MK2 protein level is reduced.

Expression analysis using a genomic MK2 GFP reporter

One of the highest expressions of the MK2 reporter has been observed throughout the gut epithelium. However we refer to MK2 as a ubiquitously expressed gene since lower expression levels are found in other organs. This is supported by Western blot analysis (not shown). In the egg MK2 is highly maternally distributed as judged by in situ hybridisation. During early larval stages MK2 is observed throughout the body. In L3 larvae we primarily found MK2 in the intestinal system with high levels in the hindgut and the proventriculus; the levels in the midgut are slightly lower. The Malpighian
tubules also show considerable MK2 expression whereas imaginal discs and the brain show low levels and the fat body and muscle very low levels. Interestingly, the ring gland shows very high levels of MK2 expression.

In the adults MK2 expression is also found in the gut and the testis and ovaries, but other tissues have not been analysed.

**Figure 4. genomic MK2 GFP reporter in larval (L3) tissues**

Different organs of L3 larvae expressing GFP under the control of the MK2 genomic region have been stained with phallodin (red) to visualise actin and DAPI (blue) to outline the nuclei. hg (hindgut), lg (lymph glands), mt (Malpighian tubules), fb (fat body), sg (salivary glands) eid (eye imaginal disc), wid (wing imaginal disc), pv (proventriculus), mg (midgut), br (brain) and ms (muscles).

**Development of BDs**

No BDs are detected in Δ43 mutants within the first 24hrs when larvae are reared on high salt stress conditions. Maternal contribution can be ruled out since Δ43 flies are kept as a homozygous stock. We rather speculate that it takes some time before the first cells are dying and melanisation of the wound takes place. Within the following 40 hrs a steep increase is seen in Δ43 larvae and after 90hrs AEL the level of BDs reaches its plateau. The size of the BDs
is in a narrow range and depends on the concentration of salt stress: the higher the stress, the larger the BDs. It remains puzzling whether (1) the BDs are first small and increase with time or (2) if they arise in a short period of time and the later they arise the larger they are. The following results speak in favour of possibility (1): Younger larvae have smaller BDs than older larvae and the total number of larvae with BD is not dramatically changing (e.g. 69 vs. 113hrs). Next puc-lacZ and msn-lacZ are activated around the BD in a graded fashion and TUNEL positive cells merge with cells of strong pucker expression. Thus, cells with highest JNK activity are dying and then melanise and thereby temporally contribute to a growing BD.

Possibility (2) is supported by single larva studies. To this aim single Δ43 larvae are transferred to stress food after they hatched and observed for BD occurrence over time. According to this experiment, BDs arise within a short period of time (less than 8hrs) and then do not change their size. Sometimes small BDs even disappear from the gut. This could explain the observation that, depending on the time point of BD analysis, small BDs might have disappeared and large one arose recently. Nevertheless, it has to be noted that those experiments are in their initial phase and expose larvae to a lot of mechanical stress and thereby might not reflect the correct rearing situation.

**BDs and the gut flora**

We did not perform detailed experiments on infections, but MK2 mutants do not appear to be more sensitive towards bacterial infection via pricking (E.coli) or via natural infection (P. entomophila). Consistently, damage to the gut via P. entomophila is occurring mainly in the proventriculus and midgut region, but not in the hindgut. Further manipulating the NfkB pathway using Toll and relish mutants or overexpressing IMD does not alter the BD phenotype tremendously. Still there appears to be a connection between the development of the BD phenotype and the gut flora. Using 0.2M sodium chloride food without nipagin/nipasol does not result in BD, and supplying the food with tetracycline does increase the number of BDs. Consistently, initial experiments with germ-free larvae suggest an increase in BDs under stress when the gut flora is ablated. Analysing the gut flora composition will provide
more evidence. Another link to the immune system is the activation of *CecA10-lacZ* in the midgut region of *MK2* mutant larvae having BDs. This is interesting since no obvious damage happens in the midgut nor do the larvae seem to be infected. Experiments with germ-free animals will show whether the increase *CecA10-lacZ* is seen even in the absence of potential bacterial antigens.

![Figure 5. Gut flora and immune response in MK2 mutants](image)

(A) MK2 mutant larvae were reared on 0.1M NaCl without (CV, conventional) or with tetracycline (+tet) (B) MK2 mutant larvae were reared on 0.2M NaCl under normal conditions (CV, conventional) or as germ-free animals (GF, germ free) (C) *cecA10-lacZ* expression was analysed in MK2 mutants reared on 0.2M NaCl (D) BD were quantified in larvae reared on 0.2M NaCl. *relE38* and *relE20* are mutants for relish, *Tlr64* is an loss of function allele allele for *Toll* and *relspez* are a recombined mutations for relish and spätzle.

**BDs and brachyenteron**

To express transgenes in the hindgut *brachyenteron* GAL4 (*byn-GAL4*) is routinely used. It is a P{GawB} element insertion in the *byn* genomic locus and causes a *byn* mutation. We tried to use *byn-GAL4* to express *MK2* in the
hindgut. Indeed, using *byn-GAL4* to express *bsk^{DN}* or *MK2* we achieved a complete rescue. Unfortunately, control GFP expression was also able to almost completely rescue the BD phenotype. We tested a deletion uncovering *byn* and indeed this deletion strongly suppressed the *MK2* mutant phenotype in a dominant fashion. Thus, *byn* is a dominant suppressor of the *MK2* hindgut phenotype. In order to generate a *byn-GAL4* line, we cloned a part of the *byn* promoter and placed it in a pChs-GAL4 vector. After injection we recovered two independent insertions on the 3rd and one insertion on the second chromosome. All lines strongly express GAL4 in the hindgut but to a weaker extent in the body wall and the midgut and other epithelial organs. Combining the GAL4 lines with a temperature-sensitive tubulin-driven GAL80 reduced some of the misexpression in the other tissues. To our surprise these lines also partially suppressed the BD phenotype of *MK2* mutants. In agreement with that, GAL4 expression itself can modulate the function of the hindgut (personal communication with Irene Miguel-Aliaga). Further experiments are currently performed to determine the function of *byn* in the homeostasis of the hindgut.

**Figure 6. Influence of *byn* on BDs**

*Byn-GAL4* is commonly used to drive hindgut-specific expression but it is also a mutant allele for *byn*. *Byn-GAL4* is a strong suppressor of the BD phenotype. A similar result is seen when one copy of *byn* is removed using a deficiency uncovering the *byn* locus.

**BDs and engrailed**

*engrailed* GAL4 turned out to be a useful line for transgene expression in the hindgut. It is expressed in the dorsal h5d and h6d compartments of the hindgut. GAL4 expression by *en>GAL4* only slightly suppresses the BD phenotype and thus was useful for the rescue analysis. During the mapping of the BD to the *en* domain in the hindgut we realised that frequently *en* expression gets patchy and eventually lost close to BDs in h6d. Interestingly, mutants for *Cg4749*, a methyltransferase and orthologue of human p120, do
give BDs in the hindgut and completely lose *engrailed* expression. I am not postulating that *MK2* is directly involved in maintaining *en* expression, rather suggest that *en* is required for maintaining homeostasis of the hindgut after its patterning function of the hindgut.

**Figure 7: en-GFP in MK2 mutants with BDs**

*en* expression is seen in the dorsal compartments of the hindgut. Using an *en* GFP reporter we could showed that in *MK2* mutants *en* expression is lost in affected areas and surrounding BD.

**SAPK activation in Drosophila cells**

In order to choose the best concentrations of salt stress we tested the activation of JNK and p38 in response to varying NaCl concentrations and different lengths of stress exposure in *Drosophila* S2 cells.

**Time- and concentration-dependent activation of JNK and p38**

When 0.2M NaCl was used an increase in P-JNK and P-p38 was observed. This increase started after 5 minutes and gradually increased. A peak was observed at the last time point (100’) and after an overnight exposure to 0.2M NaCl no more P-JNK/P-p38 was observed (data not shown).

We tested varying concentration of NaCl at an exposure time of 100’. Up to 0.05M NaCl no change compared to the basal activation of either P-p38 or P-JNK was observed. At 0.1M a clear, but weak, increase was observed in P-JNK and, to a lesser extent in P-p38. At 0.2M a strong increase in P-p38 and P-JNK was observed. This indicates that activation of JNK and p38 at this setting is not just a graded response, but resembles more a switch over a short concentration range. Thus, for all experiments with flies and larvae we
used normal food as a control (no stress), 0.1M NaCl as a moderate salt stress (weak stress) and 0.2M as a salt stress condition that strongly activates the SAPKs (strong stress).

![Figure 8. Activation of SAPK in S2 cells in response to varying NaCl concentrations after 100' of stimulation](image)

S2 cells were stressed for 100’ with the indicated concentrations of sodium chloride in Schneider’s medium. 50µg of protein was loaded, and the blot was probed for JNK and p38 phosphorylation. Total JNK (t-JNK) and tubulin were used as loading controls.

**SAPK complexes and activation of JNK and p38**

Genetically we showed that lack of MK2 or p38 results in JNK dependent apoptosis. Furthermore co-expression of p38a or p38b in the Drosophila eye using GMR>GAL4 suppresses or enhances the necrotic GMR>eiger eye phenotype, respectively (figure 9). We expressed high levels of flag-tagged JNK in S2 cells and co-expressed p38a, p38b and MK2. Cells were not stressed or stressed with 0.1M and 0.2M NaCl, and P-p38 and P-JNK levels were analysed. No influence on JNK phosphorylation was observed in any combination of p38a or p38b with or without MK2 co-expression. At the level of p38 phosphorylation it appears that co-expression of MK2 enhances the phosphorylation of p38b but not of p38a.
Flag-tagged JNK was expressed in S2 cells, and its phosphorylation was assessed in the absence or presence of GST-p38a or GST-p38b with or without MK2 co-overexpression. 50µg was loaded and tubulin was used as loading control.

Those data suggest that p38b/MK2 do not directly influence the activation of JNK in S2 cells. One has to keep in mind that the situation in the hindgut might be completely different and that in those experiments the endogenous levels of the SAPK were present. Further experiments with knocking-down either p38a, p38b or MK2 will be performed to address their requirements in regulating the activation of JNK.

**p38b kinase activity, activation and MK2 localisation**

In our manuscript we show that the MK2 kinase activity is needed to rescue the BD phenotype in the hindgut. Currently we are investigating whether the kinase activity of p38b is required for the interaction with MK2 and for the rescue of the BD phenotype of p38b deficient larvae. To this goal we generated two constructs: (1) a non-activation version of p38b (TGY→AGF) and (2) a kinase-dead version of p38b.
Figure 10. MK2 nuclear export is partially dependent on p38b activity

(A) z-sections of S2 cells expressing GFP-MK2 (green) and GST-tagged (red) p38bw1, p38bAGF or p38bKR in S2 cells, DAPI (blue) for nuclear staining

(B) three classes of GFP-localisations: either cytoplasmic, equally distributed between cytoplasm and nucleus and nuclear localisation

(C) distribution of GFP-tagged MK2 alone or with GST-tagged p38bw1, p38bAGF or p38bKR in S2 cells, n=100

Figure 11. MK2 binding and p38b activity

(A) S2 cells were transfected with either GST-tagged p38bw1, p38bAGF or p38bKR, cells were unstressed or stressed with salt for 25' and phosphorylation of p38 was assayed

(B) S2 cells were transfected with either GST-tagged p38bw1, p38bAGF or p38bKR, GST-PD were performed and binding to p38 was assayed on western blot by probing with MK2 antibody
The kinase-dead or non-activatable version of p38b is still able to re-locate GFP-MK2 from the nucleus to the cytoplasm (figure 10A). In contrast to wild-type p38b the mutated p38b versions are less efficient in their ability to export MK2 (figure 10B and 10C). On western blot the p38b\textsuperscript{AGF} and p38b\textsuperscript{KR} version are expressed at lower levels than the wild-type protein (figure 11A). As expected activation of p38b is no longer detected in p38b\textsuperscript{AGF} but p38b\textsuperscript{KR} is still phosphorylated at high salt stress (figure 11A). MK2 still binds to p38b\textsuperscript{AGF} but p38b\textsuperscript{KR}, but affinity appears to be reduced (figure 11B). Thus the low efficiency of GFP-MK2 nuclear export could be due to (1) reduced p38b\textsuperscript{AGF} but p38b\textsuperscript{KR} levels, (2) reduced binding affinity of MK2 to p38b\textsuperscript{AGF} but p38b\textsuperscript{KR} or (3) both phenomenon’s.

**p38 SAPK interaction with GMR-eiger**

Overexpression of eiger, the orthologue of TNF\(\alpha\), in the eye using GMR-GAL4 leads to a severe necrotic eye phenotype that is sensitive to alterations in JNK signalling. This setup has been a valuable tool to screen for components of the JNK signalling cascade. Since MK2/p38 signalling plays a crucial role in TNF\(\alpha\) biosynthesis, we used the GMR-GAL4 UAS-eiger system to test p38 pathway components. None of the mutations in p38a, p38b or MK2 resulted in a dominant suppression of the necrotic phenotype (personal communication with Peter Geuking). On the other hand, p38a overexpression suppressed and p38b overexpression enhanced the necrotic eye phenotype (figure 12). Interestingly, MK2 overexpression did not influence the phenotype, supporting the idea that the enhancement observed with p38b is independent of the interaction with MK2. This is further supported by the fact that the p38b\(\rightarrow\)a protein mutant still results in an enhancement. An alternative explanation would be that p38a titrates away binding partners that are required to act in the JNK pathway.
Figure 12. Overexpression of MK2 and p38 in an GMR-eiger background

GFP OE in a GMR-GAL4 UAS-eiger background does not result in any alteration in the necrosis. Co-overexpression of MK2 does not alter the phenotype. p38a OE suppresses and p38b OE enhances the phenotype.

In the hindgut we did not observe an interaction between MKK3/lic and p38/MK2 signalling. MKK4 is a known suppressor of JNK signalling. Thus, we asked whether we could further modulate the by p38a overexpression rescued GMR-GAL4 UAS-eiger eye. To this end we used the line GMR-GAL4 UAS-eiger UAS-GST-p38a and expressed dsRNA against MKK3 and MKK4 in the eye. As expected the positive control UAS-JNK\textsuperscript{DN} further enhanced the rescue by p38a overexpression, and knocking down p38a dampened the rescued eye (in some cases back to the necrotic GMR-eiger phenotype) (figure 13). It was no surprise that \textit{MKK4} knock down also enhanced the p38a overexpression-mediated rescue. \textit{MKK3} knock down, which was shown to act on p38 in cell culture, also enhanced the rescue of the necrotic eye. Thus, the possibility remains that (1) \textit{MKK3} also acts on JNK signalling or (2) that p38b counteracts p38a in the developing eye. Indeed, recent results show that moderate overexpression of \textit{MKK3} using a direct \textit{ub-lic} transgene does enhance the \textit{GMR-eiger} phenotype similarly to p38b overexpression (data not shown).
Figure 13. MKK3 acts on p38 signalling

GMR-GAL4 UAS-eiger UAS-p38a was used as a tester line. Either UAS-JNK$^{DN}$, UAS-GFP or VDRC RNAi lines were co-expressed in this tester line. Modulation of the UAS-p38a-mediated rescue was used as readout. Showing a picture of the strongest and of the weakest phenotype, respectively, reflects the variance in the expressivity of the phenotype.
part B (results)

**PTEN loss-of-function confers a growth advantages to cells under dietary restriction in *Drosophila melanogaster***

**PTEN homozygous mutants are sensitive to starvation**

Previously we showed that *PKB* or *chico* mutants are not further reduced in size and weight when reared at reduced yeast content. Below 10% of the original yeast concentration *chico* and *PKB* do not survive to pupariation, suggesting that they cannot acquire the minimal viable weight. We next used a heteroallelic combination of a *PTEN* null and a hypomorphic mutation. Under normal conditions those flies are larger and heavier. To our surprise homozygous *PTEN* mutants are very sensitive to starvation and even moderate starvation at 30% yeast resulted in the failure to pupariate and death of *PTEN* mutant larvae in L3.
Figure 14. Effect on yeast starvation on growth and development of *Drosophila*. (A) dry weight (µg) of *yw* flies reared at the indicated yeast concentrations, (blue) male, (red) female; (B) egg to pupae survival of *yw* larvae was quantified at the indicated yeast concentrations; (C) time required for *yw* larvae to enter pupariation at indicated yeast concentrations; (D) the relation of weight loss of male (blue) and female (red) *yw* flies reared at the indicated yeast concentrations; (E) three different phases of starvation with respect to survival, weight loss and developmental delay were distinguished; (F) dry weight (µg) of *chico*¹, *PKB*² and *PTEN*¹⁷/¹⁰⁰ mutants reared at 100% yeast or 10% yeast. Heterozygous animals were used as controls, weight of females (*chico*¹, *PKB*¹) or males (*PTEN*¹⁷/¹⁰⁰) was assessed.

**PTEN** mutant clones under starvation condition

Since homozygous *PTEN* mutants were extremely sensitive to starvation we wanted to know whether a similar effect is observed at the cellular level. To this end we induced patches of homozygous mutant tissue using the technique of somatic recombination. We crossed wild-type controls (*FRT40 isogenic*) or *PTEN* mutants (*FRT40 isogenic PTEN*¹⁷) to a heat shock flipase line (*yw hsFLP; FRT40 ubiquitin-GFP*). Flies were allowed to lay eggs overnight and a heat shock was given 48hrs AEL. Initially, we used a long heat shock (25’) but then we switched to short heat shock condition to keep the number of clones low (<10/eye). After heat shock larvae were split in two populations: one was transferred to normal food, the other was transferred to yeast-reduced food. As found in the literature *PTEN* mutant clones are larger compared to wild-type twin spots. Eyes with a high number of clones (long heat shock) increase the total size of the head (figure 15A) whereas heads with few *PTEN* mutant clones (short heat shock) had no influence on the overall head size (figure 15B). This is mainly due to a cell size increase, and to a lesser extent, to a number increase. Astonishingly, when larvae were reared under dietary restricted conditions, the mutant tissue did not die but rather expanded to fill large areas of the eye, and the wild-type tissue appeared to be reduced. Furthermore the overall size of the eyes was increased regardless of the initial number of *PTEN* mutant clones. Although heat shock flipase induced clones arise in all imaginal tissues only in the eye
an overgrowth phenotype was observed and no obvious alterations were observed in other tissues.

Figure 15. Heads of flies with hsFLP induced PTEN\textsuperscript{117} mutant clones

(A) long hs (25') leads to larger heads due to large PTEN\textsuperscript{117} clones. This effect is strongly enhanced when the larvae are reared at low yeast (20%) conditions. Clones for an isogenic FRT40 chromosome do not alter the eye at any condition; (B) short hs (5') leads to few isolated PTEN\textsuperscript{117} clones. Although the clones are enlarged the total eye size is not altered under 100% yeast conditions. Again, when starved, the PTEN\textsuperscript{117} mutant clones take over most of the eye and the total head size is enlarged.
Figure 16. Eye discs with hsFLP induced PTEN\textsuperscript{117} clones (I)
Under normal conditions no total disc size alteration is seen between iso and PTEN\textsuperscript{117} clones. At the level of clone size the PTEN\textsuperscript{117} mutant clones are larger than the wild-type twin spots. Already early during development the overgrowth phenotype by PTEN\textsuperscript{117} mutant tissue is obvious. When L3 wandering (L3W) is reached the eye disc is massively increased in size compared to the disc with control clones.

Figure 17. Eye discs with hsFLP induced PTEN\textsuperscript{117} clones (II)
(A) Several examples of overgrowth of PTEN\textsuperscript{117} mutant tissue in eye discs of L3 wandering; (B) bright field picture of a disc harbouring PTEN\textsuperscript{117} clones; (C) close up of a disc with PTEN\textsuperscript{117} clones, the morphogenetic furrow is marked by a dashed white line; heterozygous or wild-type tissue is marked by the presence of one or two
copies of GFP, respectively; all larvae were reared on 10% yeast food and clones were induced by 5’ heat shock at 37°C.

**PTEN mutant ommatida decrease in size but increase in number under starvation**

Using the FRT/FLP system and a cell lethal chromosome we generated eyes that were largely composed of *PTEN* mutant tissue. Under standard rearing conditions homozygous mutant heads are larger compared to isogenic controls (figure 18A). This bighead is due to a strong increase in ommatidia size (+30% compared to wildtype) and partially due to a moderate increase in ommatidia number (+10% compared to wildtype) (figure 18B). When starved at 10% yeast wild-type heads got smaller due to a decrease in ommatidia size (-10%) and number (-6%). Altogether the proportion to the rest of the body remained constant. As expected *PTEN* mutant heads did not get smaller and even got absolutely larger under starvation conditions. Ommatidia size measurements revealed that the ommatidia get reduced in size (-14% to *PTEN* ommatidia size at 100% yeast) to a similar extent as wild-type ones but the number of ommatidia per eye was strongly increased (+44% compared to wild-type at 100% yeast at +50% compared to wildtype at 10% yeast or +30% compared to *PTEN* at 100% yeast). Thus, *PTEN* mutant cells can sense certain aspects of starvation resulting in a reduced cell size but proliferation control is disturbed under those dietary restricted conditions.
Figure 18. Eyes with eyFLP induced PTEN mutant clones

(A) SEM pictures of homozygous PTEN\textsuperscript{117} or iso heads; (B) quantification of ommatidia number and ommatidia size using homozygous PTEN\textsuperscript{117} or iso heads, the percentage indicates the in- or decrease in size and number using wild-type heads (reared at 100% yeast as a reference). n indicates the number of analysed eyes. An eyFLP; FRT40 cell lethal chromosome was used to get largely homozygous mutant heads.

**Proliferation is increased during starvation in PTEN mutant clones**

The results observed in adult heads bearing clones for PTEN suggest that PTEN mutant tissue proliferates faster under starvation conditions compared to wild-type tissue. We performed Phospho-Histone 3 staining (not shown) and BrdU incorporation to analysis proliferation in the eye discs. We checked the eye discs at two time points: First, when larvae reached L3 wandering and second, eye discs at 10% yeast at the time point when wild-type larvae reach L3 wandering at normal food. At the latter condition, the clones in starved individuals are the same age and had exactly the same time to grow as clones on normal food. The overgrowth effect of PTEN mutant tissue is already observed at the very early stages of starvation. This excludes the
possibility that PTEN mutant tissue has more time to grow since starved larvae are delayed. At the level of BrdU incorporation there appears to be a slight increase in PTEN mutant clones (figure 19). Nevertheless, a small increase in proliferation already at early stages would allow such a massive overgrowth. Pulse chase experiments with BrdU feeding will help to elucidate the proliferation phenotype of starved PTEN mutant cells.

![Figure 19. BrdU incorporation in young eye disc containing PTEN mutant clones](image)

Eye discs were dissected and incubated for 30’ in a BrdU/Schneiders medium solution prior to fixation. (blue) DAPI nuclear staining, (red) BrdU-positive cells, (green) GFP marks heterozygous and wild-type tissue.

**Apoptosis is increased during starvation in PTEN mutant clones**

In contrast to Myc level alterations, no cell competition has been observed with PTEN or the IIS pathway (de la Cova et al., 2004; Gallant, 2005; Moreno and Basler, 2004). Nevertheless those studies have been performed at standard conditions and the behaviour of PTEN mutant cells might be
different under starvation conditions. The overrepresentation of PTEN mutant tissue and the strong reduction of wild-type tissue tempted us to speculate that a cell competition phenomenon might take place in those discs or that wild-type tissue does not survive the competition of fast growing PTEN cells and undergo apoptosis. Surprisingly, TUNEL and cleaved caspase 3 staining revealed an opposite picture: PTEN mutant clones have higher levels of apoptosis than wild-type tissue (figure 20 and 21). This means that although the PTEN mutant cells have a growth advantage they are prone to apoptosis.
Figure 20. Apoptosis of PTEN mutant cells (I)

*PTEN* mutant clones show increased cleaved caspase 3 staining at all conditions. Left panel shows wild-type control; (red) α-cleaved caspase 3 staining, (green) heterozygous or wild-type tissue
Figure 21. Apoptosis of *PTEN* mutant cells (II)

*PTEN* mutant clones show an increase in TUNEL staining at all conditions, very little TUNEL positive cells are observed in the wild-type or heterozygous tissue; (red) TUNEL positive cells, (green) heterozygous or wild-type tissue, (blue) DAPI nuclear staining
DISCUSSION

part A (discussion)

p38 SAPK signalling ensures hindgut homeostasis in Drosophila by modulating the strength of the JNK SAPK response under stress conditions

The function(s) of Drosophila p38 SAPK

Using the intestinal system we could indeed answer several open questions regarding p38 signalling in Drosophila. Before going into detail, I will quickly repeat the questions and give short answers to them. In the manuscript added to my thesis we specifically discuss how the hindgut is maintained in the absence of proliferative stem cells. We postulate a model that requires an intact p38 SAPK system to prevent overactivation of JNK SAPK that would lead to induction of apoptosis. We discuss how our results in Drosophila could (1) impact using p38/MK2 as a drug target and (2) influence our understanding of early steps in inflammatory diseases. Here I discuss how the other results could fit into our postulated model and explain certain aspects of the MK2 phenotype in Drosophila.

What are the physiological and/or developmental roles of p38b? Is this isoform functionally redundant with p38a?

Others and we show that although p38a and p38b mutants are each viable, the double mutants are dying early in development (Craig et al., 2004; Cully et al., 2010). Therefore p38a and p38b have redundant functions. On the other hand a role specific to p38b is to ensure the homeostasis of hindgut enterocytes even in the absence of stress.

Is p38 a mediator or a suppressor of the immune response?

We did not address this question but our results strongly advice to specifically
modulate p38 function in the immune tissue that is studied. Otherwise the phenotype might be masked since p38 function varies from organ to organ. In addition several publications found a link between immune response and p38 SAPK in *Drosophila* (Ha et al., 2009b; Han et al., 1998; Shinzawa et al., 2009).

*Which MAP3K(s) and MAP2K(s) functions upstream of p38 in stress responses in vivo? Can p38 be activated by stress in a MAP2K-independent manner?*

We show that MEKK1 has to be placed upstream of at least p38b. To our surprise MKK3/lic does not show a genetic interaction with p38b in the hindgut. Thus in the hindgut enterocytes a signaling mechanism might exist that is independent of a MAP2K.

*Is the developmental function of the MAP2K Licorne mediated by p38? Do the p38 kinases have roles in development under non-stressful conditions (like JNK does)?*

We are currently testing if lic acts upstream of the common p38 complex. This is very likely since MKK3/lic can activate *Drosophila* p38 in vitro (Han et al., 1998). Although p38a or p38b is dispensable during normal development we are curious to see if there is some developmental phenotype in p38a/p38b double mutant germline clones.

Finally our results in the paper strongly favour a crosstalk between p38 SAPK and JNK SAPK in the hindgut.

**Formation of BDs?**

Although we have thoroughly described what the BDs are and what signalling pathway defects lead to them we still have no clear picture how the BDs arise in time. Clearly, the strength of stress and the time the larvae spent on the stress influence the incident and size of the BDs. In figure 22B we illustrated different models how BDs can arise. In the chronic model a random cell exceeds the activation of JNK and enters apoptosis. Neighbouring cells
respond to the damage and increase JNK themselves, which would lead to an expansion of the BDs. This is supported by the fact that the longer larvae are on the stress the more individuals with large BDs are found. Additionally, we observe a gradient in puc-lacZ and msn-lacZ surrounding the BD. In the “affected area” model (figure 22C) a larger area would die within a short period of time leaving a scar that is melanised and “circle” of JNK “active” cells. These cells would have increased JNK activity rather due to a wound healing process than an apoptosis process. This is unlikely since removing JNK activators suppress the BDs. In figure 22D we depict a fusion of the two models presented in figure 22B and figure 22D. Here again a patch of cells would spontaneously undergo apoptosis (dependent on the strength on stress). In contrast to the “affected area model” this BD would still grow like in the “chronic model” but at much lower speed. This would explain why in the surrounding of BDs puc-GFP+ TUNEL+ cells are still found without completely dismissing the “affected area” model that is supported by single larva analysis. Why do the BDs not expand indefinitely and destroy the complete hindgut? In figure 22E we add an additional level of BD size regulation. In the “counteraction model” some factors that would counteract the events in the BDs would regulate the size of the BD. This could further explain why the size of BD is relatively homogenous in size at a given stress: the milder the stress the better the counteractor would be. It is not necessary that the counteractor acts in the cells surrounding the BDs, but it can also act within the affected area or it even increases further away of the BD. In this light the MK2-independent role of p38b and p38a are counteractors since they negatively influence the BD size.
Figure 22. Theoretical model of BD development

(A) undamaged hindgut tissue; (B) the “chronic model”; (C) the “affected area” model

(D) modified “affected area” model; (E) the “counteraction model”

MK2, black dots and the gut flora

The fact that the gut flora apparently positively regulates the gut function in Drosophila might explain how the BD phenotype arises. It is possible that constant stress changes the gut function and the gut flora and thereby stresses the gut epithelial cells. We observed that in MK2 mutants cecA expression gets upregulated. It has been shown that different bacteria have different sensitivities towards antimicrobial peptides (AMPs) and high AMP production in the gut can affect the composition of the gut flora (Ryu et al., 2008). This in turn stresses gut ECs and in the absence of MK2 might overactivate JNK and thereby lead to further gut damage. Thus a vicious cycle might arise that involves gut damage-dependent expression of AMPs, which alters the gut flora and subsequently stresses the cells even more. But how do the AMPs get induced in the midgut when the damage is happening in the hindgut? (1) Although MK2 deficient midgut cells do not show any obvious phenotype the response to stress might be altered. This could lead to the induction of AMPs when MK2 mutants are stressed without damaging the cells. (2) The gut musculature gets ruptured when the underlying ECs are lost
by apoptosis. This could allow bacteria to reach the body cavity and AMPs might be induced in various organs. A closer analysis of AMP expression in SAPK mutants will answer that question. We have to closely follow the changes in gut performance and its interaction with the commensal bacteria to fully understand the function of the hindgut and the pathways acting there when studying stress signalling.

**Hindgut organisation: from developmental functions to homeostasis**

Another puzzling observation is the dorsal-ventral organisation of the larval hindgut. In contrast this DV organisation is lost in the adults. But why is the dorsal side sensitive to overactivation and apoptosis induction by JNK? We assume that different functions of the gut are associated with the different expression domains of patterning genes. Like in the midgut, where caudal is first required for patterning and then plays a critical immune modulator role, byn might have a similar role in the hindgut. This assumption is supported by the strong dominant interaction with the $MK2$ BD phenotype. It will be interesting to check the activation of JNK and p38 in byn mutants. Byn might influence the gut phenotype more indirectly. One copy of byn might be sufficient for embryonic patterning but could be insufficient to “molecularly pattern” the larval hindgut. Additionally engrailed might be an important homeostasis regulator in the dorsal compartment of the gut. A very speculative idea involves changes in the hindgut during metamorphosis. During metamorphosis the hindgut epithelial cells get destructed and the adult hindgut arises by cells form the pylorus region. Thus the larval hindgut has to be competent to undergo developmental apoptosis. The mechanisms that govern this process are not known at all. It is tempting to speculate that JNK might be involved in this process. Thus the dorsal side of the hindgut and its ability to strongly activate JNK could be some kind of trigger in the destruction process. In the absence of $MK2$ and under stress JNK might accidentally reach the levels of JNK that are needed for cell destruction. Indeed, $PK92B$ knock down or mutants for misshapen result in relatively normal larval growth but die when they enter pupariation. Moreover autophagy that is regulated by
JNK is also required for metamorphosis. Thus, although it does not appear logical at the first sight, retaining a potent apoptosis machinery might be useful at some developmental stages.

**Implications for MK2 as a drug target**

Due to its role in inflammation p38 SAPK has been chosen as an ideal target to treat inflammatory diseases and other diseases ranging from Alzheimer to cancer (Feng et al., 2009; Munoz and Ammit; Schieven, 2009; Schindler et al., 2007). Since p38α mutant mice die during embryonic development and various other biological functions rely on one of the p38 isoforms worries about side effects emerged. In addition the various p38 inhibitors existing did not perform that well in clinical trials or model systems and due to the high structure and sequence homology targeting the activity of this kinase has been difficult (Kumar et al., 2003; Margutti and Laufer, 2007; Yong et al., 2009). Targeting a sub-branch of p38 would bear less danger and MK2 appears to be the ideal candidate, since MK2 null mutant mice are viable and do also show a strong reduction in TNFα (Duraisamy et al., 2008; Gaestel et al., 2007). Here we show that MK2 is required to protect the hindgut epithelium from stress. Thus targeting MK2 might weaken the epithelial barrier function although the inflammatory response might be suppressed. Bearing this in mind using p38/MK2 inhibitors over a longer period of time might be harmful. Inhibiting p38 or MK2 might be the method of choice in acute inflammation or if only applied locally, but might lead to strong side effects if systemically used over a longer period, e.g. in cases of chronic diseases.
part B (discussion)

**PTEN loss-of-function confers a growth advantages to cells under dietary restriction in Drosophila melanogaster**

We show that reduction in yeast influences the growth and development in at least three obvious ways. (i) Development slows down, (ii) the resulting adults are reduced in weight and size and (iii) mortality rate increases with the strength of starvation. The work of several groups has shown that insulin and TOR signalling impacts on the growth and development of *Drosophila* (Bohni et al., 1999; Colombani et al., 2005; Colombani et al., 2003; Gao and Pan, 2001; Oldham et al., 2002). Consistently *chico* and *PKB* homozygous mutants are smaller and delayed when compared to wild-type or heterozygous controls. Previously we reported that yeast reduction does not further decrease the size of *PKB* or *chico* mutant flies although their development is delayed to a similar extent as in wild-type flies. We assumed that ablating positive components of IIS makes the larvae permanently starved and thus they do not respond to increasing yeast concentration rather than not responding to yeast reduction. This is just judged by size and weight since there is a clear response to starvation at the level of developmental speed. It is tempting to speculate that *chico* or *PKB* mutant larvae need more time to accumulate energy for going into pupariation. More experimental data on that will be required before we dare to discuss the term minimal viable weight (MVW) or critical weight (CW) in this context for the following reason(s): (i) wild-type flies can get smaller than *PKB* or *chico* mutants and do survive yeast starvation down to 2% yeast. Thus *chico* and *PKB* mutants are above the minimal viable weight. (ii) It is interesting that *chico* and *PKB* mutants do respond to starvation on the level of developmental speed. We cannot completely rule out any cell size/number changes in those mutants but the weight stays the same on different starvation levels. Thus a parameter of “Maximal Reachable Weight (MRW)” defined by the insulin signalling pathway might exist in *Drosophila*. Lack of insulin signalling allows the flies to grow
only to a certain small size and under starvation this final size is not changed but it takes them longer to reach that size. This MRW appears to be tightly linked to the metabolic function of the flies, but not to the developmental (timing) function of IIS.

Increased insulin signalling should consequently increase the upper border of the MRW. *PTEN* mutants are larger than wild-type flies. Whether they also develop faster has not been addressed here, but I would assume that they are not. We tested them under starvation conditions and would have predicted that they now develop more slowly but are still increased in size. Due to increased IIS the nutritional demands are higher than in wild-type flies. They would need to feed longer to gain the same weight as on normal food when starved. To our surprise PTEN mutants did not survive moderate starvation below 50%, the yeast concentration where first changes are seen in wild-type flies. Dead L3 larvae are found in the food, but no pupae are observed. This indicates that although the minimal viable weight is easily reached the *PTEN* larvae fail to reach the critical weight. PTEN mutant flies are larger than wild-type flies. Thus they should have gained the CW unless some other mechanism is acting. Here we offer several speculations that might be true to some extent but still await experimental verification.

One clear aspect is the metabolism that is higher in *PTEN* mutants. When those flies are starved they cannot meet their energy need since PTEN larvae “feel” non-starved but the ingested food is of poor nutritional value. Here behavioural tests will be helpful to see if the feeding rate is changed in wild-type and IIS mutant larvae. It could also be that the MVW and the CW that are normally close in *Drosophila* are now separated and the CW is increased and can therefore not be reached under starvation conditions. A third possibility is that ecdyson signalling is disturbed in starved PTEN mutants and although MVW and CW are reached pupariation is not initiated. Regarding a MRW hypothesis this would again mean that the metabolism would define a MRW and that CW and MRW are coupled.

On the cellular level *PTEN* acts autonomously. *PTEN* mitotic clones in imaginal discs are larger, mainly due to cell size increase and, to a lesser
extent, due to an increase in the number of cells. Would PTEN mutant cells die or would they survive when starved? In contrast to homozygous mutants we expected that larvae with induced PTEN mutant cells behave largely like wild-type larvae. Thus on a systemic level the larvae respond to starvation and PTEN mutant cells would be exposed to a starved milieu. First we assumed that the high nutrient need of those PTEN mutant cells would not be met in the starved environment and PTEN mutant clones would die. To our surprise the opposite was the case and PTEN mutant clones overtook the eye. In contrast to normal condition the observed effect was mainly due to an increase in cell number.

Figure 23. Model for PTEN mutant tissue overgrowth under starvation conditions

Different explanations are offered in this model involving (1) competition for growth factors, (2) competition for nutrients, (3) mechanical stress and (4) developmental timing. See text for detailed explanation. (green) wild-type cells, (red) PTEN mutants, (white dashed line) morphogenetic furrow. The models are not mutually exclusive.

An easy explanation is that PTEN mutant clones do have more time to grow since larvae develop more slowly on reduced yeast conditions. Indeed, clones for PTEN induced late are smaller and the overgrowth phenotype is not observed. On the other hand protein persistence could mask the effect in late induced clones. Thus we decided to look at PTEN mutant clones in eye discs under ad libitum fed and yeast starved conditions at the same time point. When clones were analysed at 4.4 days after induction the overgrowth phenotype was already observable in PTEN mutant clones at 10% yeast. This indicates that the cell-autonomous phenotype of PTEN is not only due to an extended period of growth. Also the shift from cell size phenotype to cell
number phenotype argues against a pure timing phenotype. Increased BrdU staining in PTEN mutant clones at early stages supports this notion. Are PTEN mutant cells supercompetitors under starved but not fed conditions? Cell competition was observed in neighbouring cells that express different levels of myc. This quantitative difference in proliferation allows the supercompetitor to overtake the tissue by actively eliminating the cells with lower growth potential. The higher proliferation rate of PTEN mutant cells tempted us to test this hypothesis. At the same time we were reluctant to favour this hypothesis since cell competition does normally not alter the final size of an organ and PTEN mutant heads are enormously increased in size. One hallmark of cell competition is the induction of apoptosis in the cells that are eliminated. Indeed, increased apoptosis in eye disc with PTEN mutant clones was observed. Surprisingly not in the wild-type tissue that is reduced, but in the PTEN mutants clone themselves. It is puzzling to find an explanation how clones that have increased level of apoptosis and proliferation still have an advantage over wild-type cells. What we think is that PTEN mutant cells under starvation are at the border to apoptosis. They have a high nutritional demand and in starved larvae nutrients are scarce. In contrast to a complete mutant organism, mutant cells might take up nutrients more efficiently from the surrounding than wild-type cells. One hypothesis is therefore that PTEN mutant cells take up more nutrients and therefore further starve their environment (figure X (2)). Wild-type cells can cope with that since they are able to adjust their metabolism correctly. In other words under 10% starvation wild-type cells in the disc might “feel” starved to e.g. 5% and therefore downregulate their metabolism. The result would be that PTEN mutant cells proliferate fast and wild-type cells proliferate as if they were reared at very low concentrations of yeast, therefore the resulting organ is mostly composed of PTEN mutant cells and wild-type cells are underrepresented and less then would be expected from the starvation conditions. A similar model would be the competition to growth factors (figure X (1)). During starvation TOR activity in the fat body is going done and this should lead – according to the nutrient sensor model by Colombani – to a reduction in circulating DILPs (Colombani et al., 2004; Colombani et al., 2003). The circulating DILPs might be titrated by the fast growing PTEN
mutant cells. Staining for InR levels on PTEN mutant cells should give an answer to that question. Moreover it is possible that both competitions (nutritional and for growth factors) result in the overgrowth phenotype.

A hypothesis that is based on mechanical stress (figure X (3)) would assume that the fast growing PTEN mutant cells mechanically stress the wild-type cells and this would lead to a growth reduction in the wild-type cells. This model is possible since the wild-type tissue appears stretched in starved discs with large PTEN mutant clones. Again hypothesis (1) and (2) can be additionally applying.

Finally model (4) might explain some phenotypical features of the overgrowth. In starved discs with large PTEN mutant clones the morphogenetic furrow appears to be shifted forwards more anterior (Figure X). This led us to speculate whether the morphogenetic furrow is initiated earlier in PTEN mutant tissue. Although experimental testing is still underway we tend to exclude this hypothesis. Rather we favour again a mechanical explanation for this phenotype. Since PTEN mutant photoreceptors are larger and need more space the region ahead of the furrow might appear compressed. Indeed, bright field pictures of starved discs with PTEN mutant clones show various foldings that appear to arise to relax tension from the tissue.

Due to the nutritional stress PTEN mutant cells are more prone to apoptosis. In fact preliminary results of larvae reared below 10% yeast show that often PTEN mutant clones are completely lost and only the twin spot remains, indicating that the nutritional demands of the PTEN mutant cells were not met. Further the resulting eye appears to be as the expected size on the respective yeast concentration, suggesting that when the PTEN mutant clone is lost (most likely an early event) the surrounding tissue is freed from nutritional competition or mechanical stress and “repopulates” the eye. Otherwise a deformed or even smaller eye would be expected. Another consequence of the loss of PTEN mutant cells could be compensatory proliferation of the wild-type tissue. To clarify this hypothesis we are currently performing further experiments at < 10% yeast concentration or we genetically kill PTEN mutant clones at various time points. Thus besides direct cell competition a sort of
nutritional competition might exist in *Drosophila* that does not require to kill the cells with lower growth rate but rather imposes a brake on their proliferation.

Recently it was shown that tumours can be classified by their behaviour towards dietary restriction (Kalaany and Sabatini, 2009). Cancer cells with constitutively active PI3K are not sensitive to DR. Often tumours are in a milieu that lacks nutrients and therefore starvation resistance gives them a survival advantage. These results are in agreement with what we have found and prove that *Drosophila* is a valuable model to study tumour suppressors under different environmental conditions.

**Common themes in nutritional stress response**

The main focus of my PhD thesis was to analyse how signalling pathways react to nutritional stress. We have chosen two pathways that respond primarily to different environmental stimuli. The IIS responds to nutrients and SAPKs to various environmental stresses. In both cases, we analysed what happens to the larvae when they are exposed to nutritional stress during their development. In case of IIS, we reduced the yeast content and thereby mainly affected the levels of amino acids in the food. For SAPK, we used normal food that was supplied with different levels of sodium chloride. In both cases, the effect on development and growth was the same: larval development was prolonged and the resulting adult flies were reduced in size and weight. Thus, at some point these two different pathways might converge. In mammals it was shown that JNK directly phosphorylates IRS and a crosstalk between IIS and JNK SAPK signalling can also happen at the level of the JNK scaffold protein JIP (Lee et al., 2003; Standen et al., 2009). p38 SAPK can also influence IIS and insulin secretion at the level of PDK1 phosphorylation (Sumara et al., 2009). p38b can influence the activity of TORC1 in *Drosophila* and p38b mutant flies are smaller (Cully et al., 2010). JNK also antagonizes IIS in *Drosophila* on a systemic level in the insulin producing cells (IPC) in the brain and thereby regulates homeostasis under stress conditions (Karpac et al., 2009). Thus, starvation acts directly at the core growth signalling pathways (IIS and TOR) whereas stress modulates growth in rather indirect ways. The more direct way would be by SAPKs acting on components of the
IIS or TOR like in the examples mentioned above. Another way to impact
growth is in a more tissue specific manner. In our experimental setups, plenty
of nutrients are in the high sodium chloride food, but growth is still decreased.
This could be due to a decreased gut function. Absorptive cells of the gut are
directly exposed to the high salt environment and thereby SAPKs are
activated, which could result in a reduced uptake of nutrients. The lack of
nutrients subsequently activates the nutrient sensor in the fat body to mediate
a systemic growth reduction. Similar mechanisms could exist for various
organs. Thus, although only a subset of organs is affected, growth is
coordinated on the systemic level to ensure proper organs and organism size.

It makes sense that SAPK signalling superimposes on growth signalling since
although nutrients are available the coexistence of stressors would lead to
tissue damage if growth was not reduced. This is obvious in the case of PTEN
mutant clones. Although starvation triggers a “slow down” in growth, PTEN
mutant cells still proliferate, leading to an enlarged organ and a reduction of
wild-type tissue. In case of PTEN homozygous mutant flies, this creates a
lethal situation. Flies with heads bearing very large PTEN mutant clones also
die before or during pupariation. Although PTEN mutant tissue does
overproliferate it respects the tissue boundaries and do not show metastatic
behaviour. Nevertheless, the switch from “major cell size – minor cell number”
phenotype under normal conditions to “minor cell size – major cell number”
indicates a first step in neoplasm formation. Preliminary results show that
PTEN mutant clones also do not slow down growth under stress conditions.
This could explain how loss of PTEN function or PI3K overactivation helps
tumour cells to grow in a non-supportive microenvironment.

Thus, the definition of stress-responsive pathways is very vague and depends
on the tissue and cellular context. We conclude that a future challenge will be
to understand all pathways that integrate diverse environmental signals and
their connections to understand the nature of complex diseases. Systems
biology offers great tools to understand metabolic and signalling networks. We
started to perform pulldown experiments with tagged SAPKs under different
environmental conditions. We hope to identify new interactors and scaffolding
molecules to understand the changes that happen to the signalling networks.
during stress. Nevertheless, the power of genetic models must not be neglected since every signalling circuit behaves in a tissue-dependent manner. Finally I hope we could convincingly show that simple experimental setups (e.g. adding less yeast or a bit more salt) can aid to find new functions of pathways that have been studied for ages.
MATERIAL AND METHODS

Fly work

Fly media and stock keeping

Fly stocks were maintained on standard media (10ml per culture vial or 50ml per bottle) at 25°C or 18°C at 70% relative humidity. Composition of standard/normal food per liter: 100g fresh yeast, 75g sugar, 55g corn 10g flour, 8g agar and nipagin and nipasol as antifungal/bacterial agents.

Fly stocks

Part (A)

MK2 stocks

w GE3296  GenExel P-element insertion in genomic region of MK2
yw GE3296  GenExel P-element insertion in genomic region of MK2 recombined into yw background
yw MK2 Δ43 (Δ43)  MK2 deletion line
yw MK2 Δ38 (Δ38)  MK2 deletion line
yw MK2 Δ41(Δ41)  MK2 deletion line
yw MK2 Δ12(Δ12)  MK2 deletion line
yw ; pCasper4[MK2:5’-MK2-3’] (SRR1) genomic rescue of MK2
yw ; ; pUAST [MK2 cDNA] cDNA rescue of MK2
yw ; 51D pAttB[MK2] cDNA rescue of MK2
yw ; 51D pAttB[MK2KD] cDNA rescue of MK2KD

p38 stocks

yw ; ; FRT82B D-p38a+/TM6B p38a deletion mutant (Craig et al., 2004)
yw ; p38b27IP/Cyo y+ p38b deletion mutant (Cully et al. 2009)
yw ; ; 86Fb pAttB[GST-X]/TM6B GST-tagged p38 overexpression lines
X...p38a, p38b, p38a>b, p38b>a, p38bAGF, p38bKR

Other SAPK stocks

yw licorne13/Binsn MKK3/lic deletion mutant (Cully et al., 2010)
y w ; ; MKK4^{414} and^{589}/TM6B MKK4 mutants (Geuking et al., 2009)
dTAK1^{2} and^{4} TAK1 mutants (Geuking et al., 2005)
y w ; ; wnd^{1}eb^{3}/TM6B wallenda/dDLK mutants (Wu et al., 2007a)
y w ; ; MEKK1^{U_r36}/TM6B MEKK1 deletion mutant (Inoue et al., 2001)

**Reporter lines**

y w ; pCasper4[MK2:5'-GFP-3'] genomic MK2 GFP reporter
y w ; ; cec-lacZ a gift from Ylva Engstrom
y w ; ; msn-lacZ/TM6B a gift from Dan Hultmark
y w ; ; puc^{E69} (lacZ)/TM6B a gift from Koni Basler
y w ; ; UAS-GFP ; puc-GAL4 a gift from Koni Basler

GAL4 lines

y w ; ; nbyn1-GAL4 hindgut GAL4 expression
y w ; ; byn-GAL4/TM6B hindgut GAL4 expression
y w ; ; en-GAL4/CyO y+ dorsal hindgut expression
y w ; ; NP1-GAL4 midgut expression
y w ; ; cad-GAL4/CyO y+ posterior midgut expression

**Deficiencies**


**Part (B)**

y w ; FRT40 PTEN^{117}/CyO y+

y w ; FRT40 PTEN^{100A1}/CyO y+

y w hsFLP ; FRT40 iso 4

y w eyFLP ; FRT40 cl

**Starvation and stress media**

High NaCl food: normal food was boiled and supplied with NaCl from a 5M stock solution to achieve the desired final solution.
Starvation food: 100g yeast/L water was set as 100%. All other components stayed the same only yeast was reduced.

Other stresses see (Seisenbacher, 2006).

**Egg harvesting**

20-30 female and 10-15 male flies were crossed in a nf vial for 2 days and then transferred to egg laying cages and were allowed to lay eggs on apple agar plates. After two days in the cages eggs were collected on membrane filters (0.6 µm pores, SCHLEICHER & SCHUELL) using a water jet pump. Similar number of eggs (~ 80) was transferred to the different media. For quantification purposes the exact number of transferred and non hatched eggs was counted.

**Fly weighing**

Dry weight of flies was acquired using a microbalance (METTLER TOLEDO M5X). At least 10 flies were weighted per sex and genotype. For technical details see (Seisenbacher, 2006).

**SEM microscopy**

A Jeol JSM-6360LV microscope was used for scanning electron microscope pictures. Flies were killed in ether (6’) and put onto a sample holder. Scanning pictures were taken under high vacuum and 180x magnification. The accelerating voltage was 5kV and the spot size was set to 42.

**Ommatidia number and size measurement**

At least 7 eyes per genotype have been used to count the number of individual ommatidia in SEM pictures. For the size at least 5 eyes have been used to measure the area of the seven central ommatidia.
Immunohistochemistry

Dissection and fixation of larval tissues

Larvae were collected in 1xPBS on ice. 3-5 larvae were transferred to new vials containing 400µl ice-cold 1xPBS. Larvae were dissected with two fine forceps and the desired organs were transferred into a microfuge tube containing 500ul of ice cold 4% Paraformaldehyde in 1xPBS. Imaginal discs were fixed for 20-30’ at room temperature under mild shaking. Hindguts/midguts were fixed for 40-50’ at 4°C. Subsequently the fixative was removed by three washing steps with cold 1xPBS. Fixed preparations were stored in 1xPBS at 4°C until further use.

Preparation of cells for IHC

Concavalin coverslips

Cover slips were washed by dipping into 100% EtOH and air dried. Than cover slips were incubated in 0.15% ConA solution (in ddH2O) for 1-2h, washed with ddH2O and air dried over night.

ConA slides were placed into a small Petri dish and covered with Schneiders medium (approx 1ml). 200ul S2 cells were added and allowed to settle onto the discs for 45’ to max. 90’. Cover slips were washed once with ice cold 1xPBS and 1ml 4%PFA was added for 5’ fixation on ice followed by 10’ fixation at room temperature. Then slips were washed 3x with 1xPBS, permeabilised with 1XPBT for 10’ and stored till further processing in 1xPBS.

Antibody staining of S2 cells and larval tissues

Specimen were blocked by incubating in 2% NDS in 1xPBS 0.2% Triton X-100 (or 0.3% Triton X-100 for hindguts) for 1h (larval tissues) or 30’ S2 cells.

Primary antibody was added in 2% NDS in 1xPBS 0.2% Triton X-100 for 1h (S2 cells) or over night incubation (larval tissues). Before secondary antibodies were added samples were washed 3x in 1xPBS 0.2% Triton X-100.

Secondary antibody was added in 2% NDS in 1xPBS 0.2% Triton X-100 for 1h (S2 cells and larval tissues).
**Antibodies for IHC**

**Primary antibodies**

- rabbit $\alpha$ cleaved casp3 (asp175) Cell signalling 1:300
- rabbit $\alpha$ GST 1:300
- $\alpha$ phalloidin conjugated to 1:1000

**Secondary antibodies**

- $\alpha$ rabbit Cy3 1:300

**DAPI staining**

1:5000 form a 0.2mg/ml in 1xPBS 0.2% Triton X-100 for 10’

**X-gal staining**

Larvae were dissected and fixed by standard procedure. After washing with 1xPBS 500µl X-gal staining solution was added and the sample was incubated at 37°C in the dark. The staining progress was observed every 10’ and staining reaction was stopped by two washes with 1xPBT.

**TUNEL staining**

Was performed according to manufacturers manual ApoTAG red.

**DNA/RNA preparation and cDNA synthesis**

See (Seisenbacher, 2006).

**PCR protocol and primer for MK2 mutant recovery**

See (Seisenbacher, 2006).

**Plasmid constructs**

**cDNA constructs**

All protein coding sequences were amplified from a cDNA library made of y w embryos and L1 larvae using Phusion High-Fidelity DNA Polymerase
Amplified sequences were subcloned into pCRII TOPO (Invitrogen) or pENTR/TOPO (Invitrogen) vector and sequenced. Overexpression constructs were cloned into a pUASattB vector and tested in S2 cells for their expression. Injecting pattB (UAS) constructs into embryos with an attP landing site and expressing the phi integrase generated transgenic animals. For transgenes on the second chromosome 51D and for the third 86FB was used as a landing site. For generation of transgenes with the classical transposase method see (Seisenbacher, 2006).

**MK2 genomic rescue construct and MK2 GFP reporter**

See (Seisenbacher, 2006)

**2um sections of hindgut for light microscope**

After standard dissection the hindgut gets immediately fixed with 2.5% glutaraldehyde, 1% paraformaldehyde, 1% potassium ferrocyanide*. 0.1M cacodylate buffer (pH7.4) at room temperature for 80’. Then the samples are rinsed three times with 1xcacodylate buffer and all following steps are performed in the hood. After 60’ postfixation (1% osmium tetroxide, 1% potassium ferrocyanide in 0.1M cacodylate buffer (pH7.4) a ascending acetone series is started to dehydrate the tissue (30% > 50% > 70% > 90% > 100% each 3’ and 1x 100% 5’). Samples are then incubated in 1:1=100% acetone:Spurr overnight. On the next day the 1:1 Spurr solution is replaced by pure Spurr solution and samples are incubated at least for 4h. Finally samples are embedded into sections cubes (in Spurr). The samples were hardened at 65°C for two days.

**Cell culture**

*Drosophila* S2 cells were grown at 25°C in Schneider’s *Drosophila* medium (Gibco/Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS) and Penicillin and Streptomycin. Cells were split every week 1:3.

**Transient of S2 cells**

Transient transfection of S2 cells was done according to the Effectene transfection protocol for adherent cells (Qiagen). Cells were harvested 3-4 days after transfection.
Mounting
All samples were mounted in Vectashield Mountig Medium for Fluorescence and cover slips were sealed using red, green or colorless nail polish.

Western Blot of larval tissues and S2 cells
Lysis and sample preparation
Cells were harvested by centrifugation at 2500rpm for 3’ at 4°C and once washed with ice-cold 1xPBs. Liquid was removed thoroughly and cells were resuspended in 60ul complete lysis buffer (120mM NaCl, 50mM Tris-HCl, 1mM EDTA, 6mM EGTA, 1% Nonidet P- 40 containing protease inhibitors: 20mM NaF, 1mM Benzamidine, 15mM Na4P2O7 and Mini Protease Inhibitor Cocktail Tablets (Roche #11873580001) supplemented with phosphatase inhibitors before use (30mM β-glycerolphosphate, 30mM pNPP, 2mM Pefabloc (Roche), 4uM pepstatin, 2uM aprotinin, 4uM leupeptin, 100uM PMSF). After cells were allowed to lyse on a shaker at 4°C for 15’ insoluble fragments were pelleted at full speed centrifugation at 4°C for 20’. Protein concentrations were determined using the RC DC Protein Assay kit (BioRad) and probes were diluted to the desired concentration (20ug -50 ug protein) with lysis buffer and 4xLoading Buffer was added. Before loading onto the SDS page samples were boil at 95°C for 5’.

SDS page
For MK2 detection in larval tissues a 14% SDS PAGE gel was used. For all other purposes pre-casted 10% SDS PAGE gel was used. Runs thorugh the stackking gel were done at 120V, and through the separation gel at 130V until the leading edge of the Loading Buffer left the gel.

Western Blotting
For protein transfer to a Hybond ECL Nitrocellulose membrane (Amersham) a wet blot set up was used. Blotting was performed at 150mA for 90’ to 120’ at 4°C

Membranes were stained with ponceau red to control for loading and then blocked using blocking reagent dissolved to a final weight/volumne
concentration of 3%. The membrane was then incubated in the primary antibody dissolved in 3% blocking solution for the recommended time points, washed 3-4x (at total 1h) with 1xTBST and than incubated in secondary antibody for 1h at room temperature.

**Detection**
For detection of bands the membrane was incubated with Immobilon Western HRP Substrate (Millipore) for 5’ and exposed to an X-ray film (Fuji) for initially 5” up to 1’.

**Stripping**
To re-probe the membrane with another antibody it was stripped using following stripping buffer: . First the membrane was incubated for 5’ in Stripping buffer and then with fresh stripping buffer for further 25’ at room temperature. Before re-probing the membrane wash washed 4x in 1XTBST for 5min and blocked for 15’ in 0.15% blocking solution for 15’.

**Antibodies for Western blot**
**Primary antibodies**
- mouse $\alpha$ tubulin (1:10000 -1:20000, Sigma T9026) 1h RT
- rabbit $\alpha$ GST (1:20000 – 1:50000) 1h RT
- rabbit $\alpha$ activated JNK (1:1000) ON 4°C
- rabbit $\alpha$ JNK (1:1000) ON 4°C
- rabbit $\alpha$ PP-p38 (1:1000) ON 4°C
- rabbit $\alpha$ D-p38b (1:1000) ON 4°C
- rabbit $\alpha$ MK2 (1:500) ON 4°C
  was generated by immunizing a bunny with the peptide (Eurogentec, Liege Science Park, Seraing, Belgium). The serum from the final bleed was affinity purified against the peptide coupled to a column.

**Secondary antibodies**
- mouse $\alpha$ rabbit HRP (1:5000-30000) Jackson 111-035-003 1h RT
- sheep $\alpha$ mouse HRP (1:5000 – 30000) Jackson 115-035-174 1h RT
Imaging

For simple fluorescent and bright field picture a Zeiss Axiophot fluorescent microscope connected to a Leica DFC420C camera was used. For confocal images a Leica SP2 confocal laser scanning microscope was used.
DANKSAGUNG


Als nächstes möchte ich mich gleich bei meinem Doktorvater Ernst Hafen bedanken: Viele Dank auch für deine Unterstützung und Begleitung während meiner Diss! Vielen Dank für die vielen Freiheiten die ich hatte und habe, für die Tipps die du mir gabst und für die Nachsichtigkeit wenn ich ab und zu etwas dickköpfig und nicht ganz umgänglich war!

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CURRICULUM VITAE

Gerhard Rudolf Seisenbacher

- born in Vienna (Austria) on April 23rd 1982
- 1986 – 1987: kindergarten

Education/school:

- 1992 – 2000: grammar school BRG/BORG 1100 WIEN Pichelmayergasse 1
  
  Abitur: Juni 2000, mit gutem Erfolg bestanden

  Subjects: English, German, Latin, Mathematics, Biology, Chemistry

Education/university:

- starting 1. 10. 2000: Molecular Biology, University of Vienna/Austria
- 1. 10. 02 – 30. 5. 03: ERASMUS-exchange student in Zurich/Switzerland
- 22. 8. 2003: finished the first part of undergraduate studies
- September 04 - October 05 Diploma thesis as an exchange student in Zurich/Switzerland in the lab of Ernst Hafen
- January 2006 – June 2010: PhD in the lab of Ernst Hafen, Institute for molecular systems biology, ETH Zurich, Switzerland

**scholarships/ awards:**

- Semester-award 2003 of the university of Zurich:
  "Heavy metal homeostasis in Drosophila: food choice experiments"
- „Leistungs“-scholarship for the years 2003 and 2004
- Scholarship for short study visits abroad (September 04 – November 04)
- „Förderungs“-scholarshipstipendium of the university of Vienna (04)
- Roche Research Foundation

**Practical training**

- 14.04.03 – 31.05.03:
  "Heavy metal homeostasis in Drosophila: food choice experiments"
  Supervisor: Prof Dr Walter Schaffner, Institute for Molecularbiology,
  University of Zurich, Switzerland

- 02.06.03 – 11.07.03:
  "Expression analysis of BMP and Ectodysplasin signalling components
  in the developing chicken skin by in situ hybridisation"
  Supervisor: Dr Anette Neubüser, research institute for molecular
  pathologies, IMP Vienna

- 08.09.03 – 03.10.03:
  "The actin stress fiber phenotype of plectin +/- keratinocytes"
Supervisor: Prof Dr Gerhard Wiche, institute for cell biology, University of Vienna

- 26.01.04 – 12.03.04: “VEGFR-2 signaling and inhibition of tumour angiogenesis”
  Supervisor: Dr Frank Hilberg, Boehringer Ingelheim Austria

- 15.03.04 – 15.06.04: “ChIP on chip analysis of histone methylation states”
  Supervisor: Prof Dr Thomas Jenuwein, research institute for molecular pathologies, IMP Vienna

Scientific programs

- September 2004 – November 2006: member of the research training network (RTN): Modulation of kinase signalling; Coordinator: Matthias Gaestel (medical high school of Hannover)

- January 2006 – June 2010 PhD student of the CC-SPMD programm (Competence Center for Systems Biology and Metabolic Diseases

Meetings

- 8.04.05 – 10.04.05 RTN-meeting „Modulation of Signalling” Tübingen (D) talk “Genetic characterization of the Drosophila MAPKAPK2 kinase”
- 15.06.05 – 17.06.05 NCCR Frontiers in Genetics meeting Saas Fee (CH)
  poster “Influence of diet on development and growth of D. melanogaster”

- 11.07.05 University College London (GB)
  talk “Influence of diet on development and growth of Drosophila melanogaster” invited by Prof Linda Partridge

- 8.08.05 – 19.08.05 collaboration with Prof Matthias Gaestel at Medizinischen Hochschule Hannover (D)

- 31.08.05 – 3.09.05 19th European Drosophila Research Conference, Eger (HU) poster “Influence of diet on development and growth of D. melanogaster”

- 11.11.05 – 13.11.05 RTN-meeting „Modulation of Signalling“ Utrecht, (NL) talk “Genetic characterization of the Drosophila MAPKAPK2 kinase”

14.06.06 – 17.06.06 3rd Drosophila cell cycle workshop, Porto (P),
poster “Influence of diet on development and growth of D. melanogaster”
- 30.06.06 – 02.07.06 RTN-meeting „Modulation of Signalling“ Dundee, (UK) talk “Genetic characterization of the *Drosophila* MAPKAPK2 kinase”

- 25.11.06 – 27.11.06 RTN-meeting „Modulation of Signalling“ London, (UK) talk “Genetic characterization of the *Drosophila* MAPKAPK2 kinase”


- 26.04.08 Annual Swiss Drosophila meeting, Bern (CH) talk “Black dots and inflammation: Analysis of a chronic inflammation-like phenotype in Drosophila”

- 13.10.08 – 15.10.08 CNIO Special Workshop: Stress Signalling and Cancer Madrid (E), poster “*Drosophila* p38 SAPK regulates inflammation and apoptosis upon chronic stress”

Other activities and trainings

**Sports**

November 2008 – (ongoing)

kayak teacher/coach at ASVZ

May 2008 – (ongoing)

Swift water rescue technician SRT1
December 2008 – (ongoing)

Lifeguard brevet1 (Schweizer Lebensrettungs-Gesellschaft SLRG)

J+S squad programm

April 2009 “Hilfsleiter” exam for white water kayak

May 2010 “Leiter” exam for white water kayak

Arts

EMBO Journal Cover Contest 2009

rank 26 in category “science” (out of > 700 submissions)


Contributed picture of the Drosophila gut

Life Science Art calendar

Contributed cover