Intracellular signalling, gene induction and histone modifications in *Drosophila melanogaster*



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Abstract

In eukaryotes, histone H3 residues at the promoter and transcriptional start site of immediate early genes are phosphorylated at serine 10, a process mediated by histone kinases downstream of the p38 and ERK cascades. However, this phosphorylation event has not yet been investigated in *Drosophila melanogaster,* and the existence of an inducible histone kinase is uncertain.

To identify the kinases involved in the nucleosomal response in *Drosophila*, I first investigated the stimuli inducing MAPK signal transduction pathways. In mouse cells, anisomycin at sub-inhibitory concentration is a strong activator of JNK and p38, but no activation of MAPKs was observed in *Drosophila*. Peptidoglycan treatment induced JNK activation without altering histone H3 phosphorylation, demonstrating that this part of signal transduction is conserved between vertebrate and insect cells. However, in contrast to mammalian cells, inducing ERK activity by stimulation with hydrogen peroxide, insulin, serum or heat shock does not lead to histone H3 phosphorylation. Osmotic stress resulted in a strong increase in phosphoacetylated histone H3, suggesting that p38 is upstream of the histone H3 kinase in *Drosophila*, but the presence of p38 inhibitor SB203580 did not decrease histone H3 phosphoacetylation. Further evidence suggesting that the nucleosomal response in *Drosophila* is not conserved includes the absence of induction of classical immediate early gene *jra* after stimulation with either sorbitol or sodium arsenite. In summary, these results indicate that histone phosphorylation and IE gene induction differ in mammals and *Drosophila* and provide the basis for further experiments aiming to identify the relationship between histone phosphorylation and transcription.

List of abbreviation

ActD	actinomycin D
AMP	antimicrobial peptide
AP1	activator protein 1
ASK	apoptosis signal-regulating kinase
ATF	activating transcription factor
ATP	adenosine triphosphate
AU	acid-urea
bFGF	basic fibroblast growth factor
BMK	big MAP kinase
bp	basepairs
BSA	bovine serum albumin
cAMP	cyclic AMP responsive element binding protein
CBP	CREB binding protein
ChIP	chromatin immunoprecipitation
CREB	cAMP response element-binding protein
Csw	corkscrew
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dpp	decapentaplegic
DRB	5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole
DREDD	death related ced-3/Nedd2-like
DRK	downstream of receptor kinase

ECL	electrochemiluminescent
EGTA	ethylene glycol tetraacetic acid
EGFR	epidermal growth factor receptor
EDTA	ethylenediaminetetraacetic acid
Eip75	ecdysone-inducible protein E75
ERK1/2	extracellular signal-regulated kinases 1 and 2
FADD	Fas-associated death-domain
FCS	foetal calf serum
Chris	chromodomain protein interacting with Z4
GCN5	general control of amino-acid synthesis
GGF	glial growth factor
Grb2	growth factor receptor-bound protein
HAT	histone acetyltransferase
HDAC	histone deacetylases
HMGN1	high mobility group protein
HOG	high osmolarity glycerol
HP1	heterochromatin protein
HSF	heat shock factor
hsp70	heat shock proteins
IE	immediate early
IGF	insulin/insulin-like growth factor
IGFBP	insulin-like growth factor-binding protein
JIP	JNK-interacting protein
JNK	c-Jun N-terminal kinases
Jra	Jun-related antigen

kDa	kilodalton
LPS	lipopolysaccharides
MAPK	mitogen-activated protein kinase
MAP2K	MAPK kinase
MAP3K	MAPK kinase kinase
MEKK	MEK kinase
MK2	MAPK-activated protein kinase
MLK	mixed lineage kinase
MOF	males absent on the first
MSK1/2	mitogen and stress-activated protein kinases 1 and 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDK	phosphoinositide-dependent kinase-1
PGN	peptidoglycan
PGRP-LC	peptidoglycan recognition protein receptor LC
PI3-K	phosphatidylinositol 3-kinases
PKB	protein kinase B
PVDF	polyvinylidene difluoride
REDD1	regulated in development and DNA damage responses
Rheb	Ras homolog enriched in brain
RNA	ribonucleic acid
ROS	reactive oxygen species
rp49	ribosomal protein 49
RSK	p90 ribosomal S6 kinase

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHP2	Src-homology 2 domain-containing phosphatase
SOS	son of sevenless
SRF	serum response factor
SSC	saline sodium citrate
TAK1	transforming growth factor β-activated kinase
TGF-β	transforming growth factor β1
TNF	tumour necrosis factor
TOR	target of rapamycin
TORC1	TOR complex 1
TSC1/2	tuberous sclerosis 1 and 2
VEGF	vascular endothelial growth factor

1 Introduction

Cells react to their environment by eliciting a specific response to coordinate basic cellular activities. For example external stimuli can induce proliferation, differentiation and apoptosis. In this thesis, I will first discuss cellular pathways that mediate a signal to activate a new pattern of gene expression, then I will address how a stimulus can change chromatin structure and which proteins are responsible for the post-translational modification of histones.

1.1 MAPK signalling

Signal transduction and mitogen-activated protein (MAP) kinase proteins, a family of serine threonine kinases, are conserved in eukaryotes. There are four major groups: extracellular signal-regulated kinases 1 and 2 (ERK1/2, p42/44), c-Jun N-terminal kinases (JNK, p46/54) and p38 and ERK5/big MAP kinase (BMK). Each pathway contains three protein classes that are serially activated by phosphorylation: MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAP kinases (MAPK) (Qi and Elion, 2005). In *Drosophila melanogaster (*hereafter referred to as *Drosophila)*, conserved kinases transduce an extracellular signal through each MAPK cascade (JNK, ERK and p38) to downstream effectors. A diverse range of stress stimuli activates specific and characteristic MAPK responses (Krishna and Narang, 2008).

MAPKs are activated by dual threonine/tyrosine phosphorylation in the activation loop of domain VIII. Different MAPKs can be activated by the same stimuli and can signal in parallel (Cano and Mahadevan, 1995). Since multiple MAP2Ks are able to activate multiple MAPKs, specificity is enhanced with scaffold proteins (Kolch, 2005). This cascade permits signal amplification and regulation at different levels.

MAPK mediated signal transduction pathways are key regulators for membrane, cytoplasmic and nuclear signalling networks and thus important for proliferation, differentiation, apoptosis and immune responses (Roux and Blenis, 2004). This underpins its relevance to diverse pathologies; aberrant MAPK pathways, especially ERKs, are implicated in uncontrolled proto-oncogene expression (Seger and Krebs, 1995). Furthermore, the p38 pathway plays a key role in the induction of pro-inflammatory cytokine genes, and has proven to be a useful pharmaceutical target for treating inflammation (Yang et al., 2014).

1.1.1 MAPKs in *Drosophila*

In *Drosophila*, there are fewer MAPK orthologues than in human or mouse, making it a good model to dissect MAPK pathways (Cargnello and Roux, 2011, Krishna and Narang, 2008, Edmunds et al., 2008). For example, all of the MAP3Ks, consisting of Slipper (also named mixed lineage kinase (MLK)), apoptosis signal-regulating kinase (D-ASK), transforming growth factor β-activated kinase (D-TAK1) and MEK kinase (D-MEKK1), are each the single orthologue of the MAP3K subfamilies (Hayakawa et al., 2006, Ryabinina et al., 2006, Takeda et al., 2008). Further, a specific set of MAP3Ks gets activated depending on the kind and strength of stimulus used.

1.1.1.1 p38 pathway

In yeast *Saccharomyces cerevisiae,* osmotic stress activates the high osmolarity glycerol (HOG) response (Brewster et al., 1993, Schuller et al., 1994). HOG1 is the p38 homologue in yeast (51% identity) and only get tyrosine phosphorylated, thus activated, upon osmotic stress (Brewster et al., 1993, Schuller et al., 1994). High external osmolarity causes glycerol accumulation in the cytoplasm in order to counteract osmotic stress. The HOG response genes were discovered by studying

mutants which failed to regulate glycerol concentration. This is the earliest reported function of the p38 pathway and is conserved from yeast to human.

There are three p38 orthologues in *Drosophila*; p38a, p38b (also known as mpk2) and p38c. The third gene, p38c, is a putative MAPK and lacks the characteristic dual regulation site threonine-glycine-tyrosine. Upon environmental stress, *Drosophila* p38a and p38b get activated (Craig et al., 2004, Han et al., 1998a, Han et al., 1998b). Tyrosine phosphorylation on their TGY sequence is mediated by the MAP2Ks D-MKK3 (licorne) and D-MKK4, although the necessity of D-MKK4 is controversial (Han et al., 1998a, Chen et al., 2010, Han et al., 1998b). In *Drosophila*, cellular stressors such as osmotic stress, UV radiation and pro-inflammatory cytokines activate p38 (Zhuang et al., 2006). In mammals, p38 activates mitogen and stress-activated protein kinases (MSK1/2).

In *Drosophila*, activating transcription factor (ATF2) and Jun-related antigen (Jra) are both downstream of stress-activated p38 (Sano et al., 2005, Han et al., 1998b). MAPK-activated protein kinase (MK2) is also a downstream effector of the p38 pathway. The MK2 orthologue has a MAPK phosphorylation site and a nuclear targeting sequence (Larochelle and Suter, 1995). MK2 is associated with p38b in the nucleus and translocates to the cytoplasm upon activation (Belozerov et al., 2012, Seisenbacher et al., 2011).

1.1.1.2 ERK pathway

In mammals, ERKs are differentiation and growth factor stimulated kinases. Most insight into the activation of the *Drosophila* ERK homologue Rolled has been gained from eye development research (Ciapponi et al., 2001, Ciapponi and Bohmann, 2002, Feller et al., 2002, Gabay et al., 1997). Downstream of receptor tyrosine kinases, adaptor protein downstream of receptor kinase (DRK, homologue of mammalian

growth factor receptor-bound protein (Grb2)) activates son of sevenless (SOS) which then binds to Ras to induce the ERK cascade (Feller et al., 2002). The primary MAP3K is Raf which phosphorylates MAP2K Dsor which in turn phosphorylates ERK (Clemens et al., 2000a, Avruch et al., 2001). Also, *Drosophila* ERK activates LK6 (Mnk1/2 in human) leading to eIF4E phosphorylation which is required for growth and development (Parra-Palau et al., 2005). ERK activates Fos homologue kayak which mediates transcriptional activation (Ciapponi et al., 2001). Transcription factors Jra and Fos are activator protein (AP1) family members (Shaulian and Karin, 2001).

Downstream of mammalian ERK are effector kinases such as MSK1/2 and p90 ribosomal S6 kinase (RSK) or transcription factors such as ETS domain-containing protein Elk-1, cyclic AMP responsive element binding protein (cAMP), and serum response factor (SRF) (Raman et al., 2007). In *Drosophila*, so far just one isoform of RSK has been identified and its knockout indicates the absence of crucial functions (Tangredi et al., 2012). The existence of a small C-terminal isoform is speculated, which could potentially mediate alternative effects (Putz et al., 2004). The MAPK phosphorylation site is conserved and RSK has been shown to be associated with p38b and cytoplasmic ERK (Kim et al., 2006, Tangredi et al., 2012, Belozerov et al., 2012).

1.1.1.3 JNK pathway

In mammals, JNK is a stress-activated kinase which responds to environmental stresses including heat shock, UV radiation, oxidative stress, DNA damage and inflammatory cytokines (Raman et al., 2007). In *Drosophila*, the JNK homologue Basket is activated in response to stress and can induce an anti-oxidant response or even apoptosis (Botella et al., 2001, Igaki, 2009). Classically, *Drosophila* JNK is

activated during the innate immune response (discussed in chapter 4.1.1.5) (Robertson and Postlethwait, 1986).

JNK mediates morphogenetic processes such as dorsal and adult thorax closure during embryonic development (Zeitlinger et al., 1997, Riesgo-Escovar et al., 1996). GTPase Rac activates MKK7 (hemipterous) which is upstream of JNK (Stronach and Perrimon, 1999). In cultured cells, D-MKK4 has been shown to regulate JNK activity as well (Geuking et al., 2009, Boutros et al., 2002, Han et al., 1998b). JNK induces *decapentaplegic* (*dpp*) gene expression to regulate dorsal closure. The prototypical target gene induced by JNK *via* Jra/Fos AP-1 is its phosphatase puckered, acting in a negative feedback loop to terminate JNK signalling (Martin-Blanco et al., 1998, Kockel et al., 1997).

A summary of the described pathways in *Drosophila* and mouse is illustrated in Figure 1. In mouse cells, growth factors mainly activate the ERK pathway, while cellular stress, inflammatory cytokines and anisomycin have been shown to activate the JNK and p38 pathways (Cano et al., 1994, Hazzalin et al., 1998, Hazzalin et al., 1997, Cano et al., 1995).

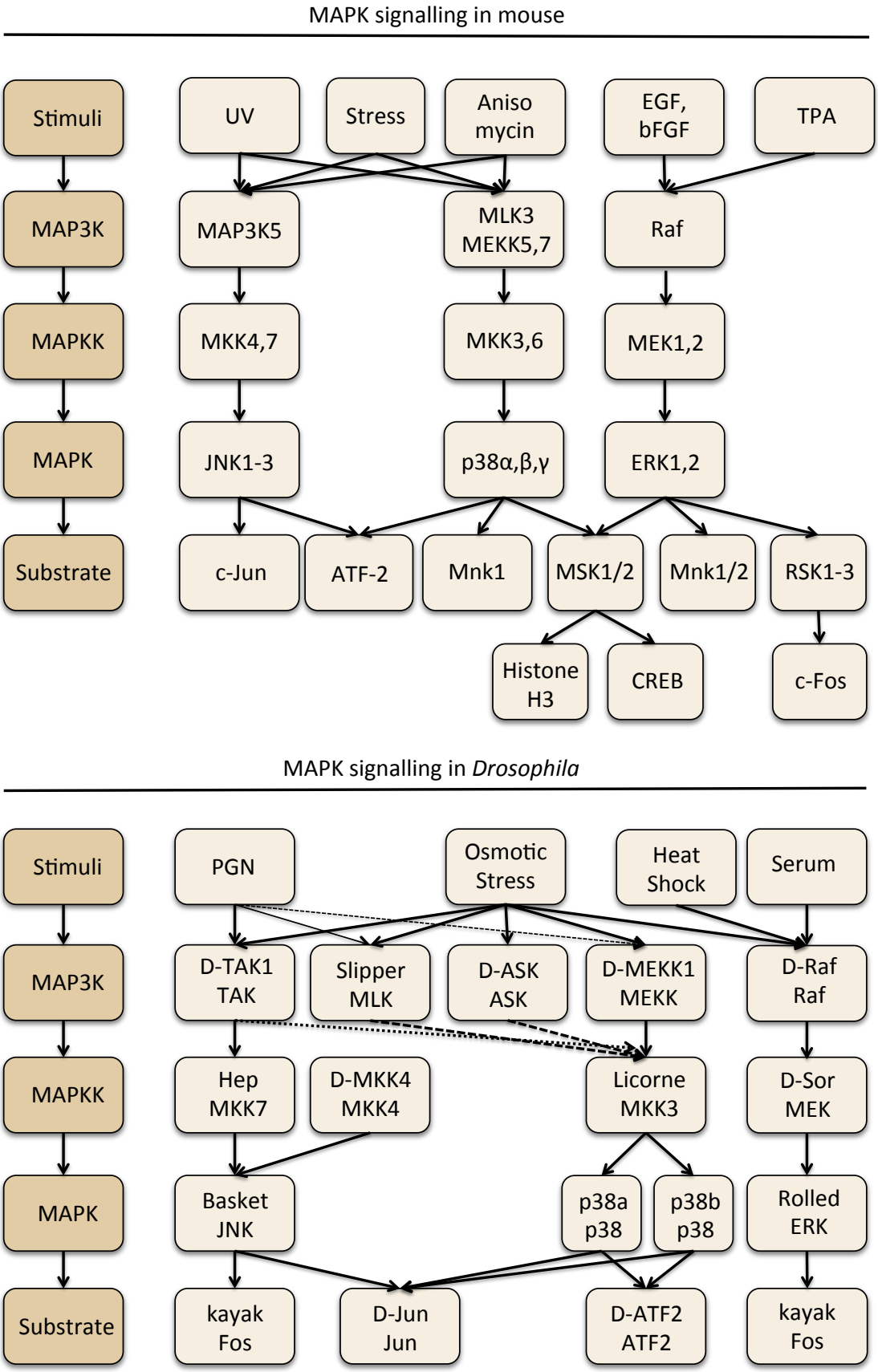


Figure 1: Comparison of MAPK signalling in mouse and *Drosophila.*

1.2 Chromatin

Chromatin, consisting of DNA wrapped around nuclear proteins, compacts eukaryotic genomic DNA in the nucleus. Heterochromatin refers to the compacted version of chromatin, in which dense folding of nucleosomes supresses gene activation. Euchromatin describes the accessible, open form of chromatin.

1.2.1 Nucleosome packing

Each nucleosome comprises approximately 147 bp of DNA wrapped around an octamer of histones in a left-handed superhelix (Luger et al., 1997). An octamer constitutes of a pair of each histone H2A, H2B, H3 and H4. Linker histone H1 stabilizes nucleosomes and linker DNA (Felsenfeld and Groudine, 2003). Nucleosome units are folded into higher order structures until compaction is more than 10,000 fold and hence roughly two metres of DNA fit in a nucleus which is only a few micrometres in size (Figure 2). Histones are extremely conserved from yeast to humans, with just 15 amino acids not conserved in histone H3.

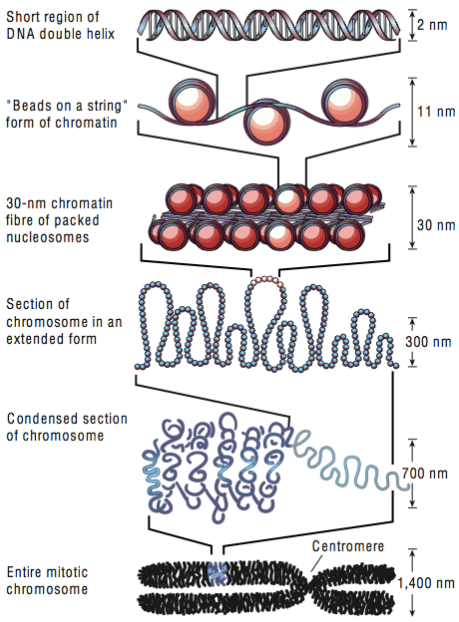


Figure 2: Chromatin and the organization of DNA within the cell. Adapted from Felsenfeld and Groudine (Felsenfeld and Groudine, 2003).

While the C-terminal region forms interactions between DNA and histones, the basic amino acid N-terminal tail of histones is subjected to post-translational modifications (Luger and Richmond, 1998). Histone modifications determine higher order chromatin structure and are responsible for DNA packaging. Chromatin and covalent modifications of histone tails also

determine and regulate mitosis, replication, transcription, DNA repair and apoptosis. To facilitate these processes, chromatin remodelling complexes can slide, relocate or exchange histones.

1.2.2 Histone variants in *Drosophila*

Histone variants cause heterogeneity in the nucleosome structure and alter nucleosome properties. Different histone variants can be found at specific regions in the chromosomes (Talbert and Henikoff, 2010). For example, histone H2A replacement by variant H2Av (H2AZ in humans) is a pre-requisite for heterochromatin establishment in *Drosophila* (Swaminathan et al., 2005). H2Av is also involved in DNA double strand repair (corresponding to H2AX in humans) (Madigan et al., 2002). The *Drosophila* genome encodes for three histone H3 forms: replication coupled H3, and two replication independent histones: a replacement variant H3.3 and centromeric histone H3 Cid (CENP-A in humans) (Ahmad and Henikoff, 2002b, Ahmad and Henikoff, 2002a). It has been proposed that in *Drosophila* histone variant H3.3 is incorporated during induction and is associated with transcription of highly expressed genes, for example ribosomal protein 49 (rp49) (Sakai et al., 2009).

1.2.3 Histone modifications

Post-translational modifications (acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP ribosylation) on the N-terminal histone tails play a pivotal role in regulating the accessibility of DNA and therefore gene expression. Especially arginine methylation, lysine acetylation or methylation and serine or threonine phosphorylation are important in regulating cell fate and the chromatin landscape because they integrate incoming signals (Kouzarides, 2007).

Histone modifications alter the chromatin structure and increase binding affinity of chromatin remodelling factors (Wolffe, 1998). A number of histone-binding proteins have been identified and shown to establish a repressive or active environment. Recruitment of other proteins occurs because domains detect and bind to their specific histone binding site. A domain within 14-3-3 proteins binds to phosphorylated serine, whereas bromodomains bind to monoacetylated and chromolike domains to methylated lysines (Kouzarides, 2007). See Figure 3 for histone modifications on a lysine or serine residue.

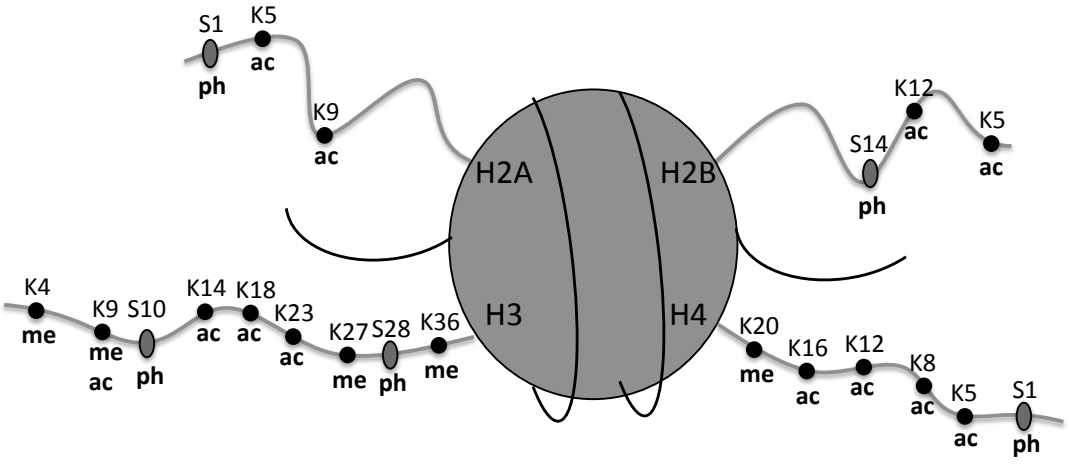


Figure 3: Schematic representation of the most common lysine acetylation/methylation and serine phosphorylation sites histone N-terminal tails.

In addition to serving as a signalling platform, histone phosphorylation or acetylation can change electrostatic interactions. While phosphorylation causes a negative charge, acetylation neutralizes the positive charge of lysine. A neutralized histone tail is less tightly associated with the underlying DNA, making it more accessible for transcription factors. Increased histone acetylation thus loosens histone-DNA interactions and could thus enhance nucleosomal remodelling (Varga-Weisz and Becker, 1995).

One of the most frequently occurring histone modifications is acetylation of lysine residues. Histone acetylation, mediated *via* a histone acetyltransferase (HAT), makes chromatin more accessible and has a positive effect on gene expression (Sterner and Berger, 2000). Conversely, histone deacetylases (HDAC) erases this acetylation mark, creating a more repressive chromatin environment.

Independent of transcriptional activation, histone H3 and H4 get acetylated and deacetylated by HATs and HDACs to mark transcriptional competence (Eberharter and Becker, 2002). In contrast to the previously existing paradigm, dynamic instead of stable acetylation of histone H3 occurs at actively transcribed genes (Hazzalin and Mahadevan, 2005). The half-life of acetylation is around two to three minutes (min) and rarely exceeds 30 min (Barth and Imhof, 2010). Acetylation occurs on all lysines on all histones in open chromatin, such as promoters, enhancers and insulators.

1.3 Nucleosomal response and immediate early genes

1.3.1 Nucleosomal response

Histones surrounding promoters are biochemically distinctly regulated to relax chromatin and create protein binding sites. It has been shown that all trimethylated histones H3 at lysine 4 (H3K4me3) are targeted for high turnover of acetylation, also in *Drosophila* (Crump et al., 2011). Histone modifications involved in the nucleosomal response are conserved in *Drosophila*. Up- and downstream of the transcription start site of immediate early genes, a small population of histone H3 becomes highly acetylated, including lysine 9, 14, 18, 23 and 27. Dynamic acetylation on H3K4me3 histone tails is mediated by CREB binding protein (CBP) and its paralogue p300, both which are evolutionary conserved (Hsu et al., 2012). To investigate dynamic acetylation, *Drosophila* S2 cells are incubated with Trichostatin A (TSA), an HDAC inhibitor, which promotes hyperacetylation of H3K4me3 (Crump et al., 2011).

In mammals, a small portion of acetylated histone H3 tails is also phosphorylated; this phosphoacetylation is associated with actively transcribed immediate early (IE) genes (Thomson et al., 2001b, Clayton et al., 2000). Histone phosphorylation is thus targeted to a much smaller fraction of histones coinciding with acetylation. Phosphoacetylation consists of lysine 9 acetylation and phosphorylation of the serine 10 residue of the N-terminal tail of histone H3 (H3K9acS10), and is very transient. The nucleosomal response is described as the phosphorylation of histone H3 concomitant with IE gene induction as a result of external stimulation (Clayton et al., 2000). As a part of the nucleosomal response, H3S10 phosphorylation is linked to transcriptional activation of proto-oncogenes and mitogen stimulated IE gene

response (Wolffe, 1998, Labrador and Corces, 2003b, Thomson et al., 2001a).

H3S10 is mainly phosphorylated by MSK2, but also by MSK1, as quickly as five min after stimulation (Soloaga et al., 2003). MSK1/2, which are now established to be the major downstream kinases of the MAPK signal transduction pathways, can also phosphorylate H3S28 and high mobility group protein (HMGN1) at S6. Both ERK and p38 pathways activate MSK1/2 and lead to phosphorylation of histone H3S10 permitting gene activation at inducible genes (Soloaga et al., 2003, Hazzalin et al., 1996). In mouse cells, ERK dependent RSK activation has also been proposed to lead to phosphorylation of H3S10, although this link has been more contentious (Sassone-Corsi et al., 1999, Soloaga et al., 2003). Gene activation is controlled by synergistic binding of transcription factors and other chromatin associated proteins (Nebert, 2002).

In yeast, histone H3S10 phosphorylation augments lysine 14 acetylation. Arginine at position 164 in HAT general control of amino-acid synthesis (GCN5) recognizes and binds to phosphorylated Ser10 (Lo et al., 2000). This is in contrast to mouse cells, where experiments have shown that acetylation and phosphorylation can occur independent of each other (Thomson et al., 2001b).

1.3.2 Immediate early genes

Immediate early or early response genes are defined as a set of genes which are the first line of response. All the required transcription machinery is already present in the cell or poised at the promoter, meaning that no translation is required to induce transcription.

RNA polymerase II may be stalled in the vicinity of IE promoters, ready to start transcription and waiting for incoming activating signal (Sterner and Berger, 2000). Promoters of IE genes, rich in transcription factor binding motifs and response

elements, are nucleosome free and DNaseI hypersensitive (Drobic et al., 2010, Thomson et al., 1999b, Moshkin et al., 2012). MAPKs and MAPK downstream kinases phosphorylate bound transcription factors which subsequently activate transcription of the according gene (Cheung et al., 2000). Upon a mitogenic stimulus 80-100 IE genes are induced rapidly and transiently, concomitant with hyperacetylation and phosphorylation of surrounding histones. IE genes, many of which are transcription factors themselves, therefore are able to give rise to a secondary transcriptional response. Included in this group of IE genes are members of the c*fos* and c*jun* proto-oncogene family, encoding for Fos and Jun. For example, at the c*fos* promoter, growth factor stimulation causes a three times increased DNaseI sensitivity and hence open active chromatin within 90 seconds after stimulation and c*fos* transcripts can already be detected 10 min after stimulation (Feng and Villeponteau, 1990, Cao et al., 1998).

In mammals, IE gene transcription is tightly controlled and transcripts are instable, due to an AU rich element in 3' UTR (Tourriere et al., 2002). This quick mRNA degradation ensures transient expression of a protein which regulates a lot of secondary responses that are crucial for cell fate (O'Donnell et al., 2012).

1.4 Histone H3 phosphorylation

Histone phosphorylation serves two opposing functions: chromatin condensation during mitosis, and chromatin relaxation associated with active genes.

1.4.1 Histone H3 phosphorylation during mitosis

During M phase, condensed chromatin is highly phosphorylated on histone H3 across the whole chromosome. At G_2/M phase, histone H3 gets phosphorylated at pericentromeric heterochromatin and phosphorylation extends to the ends of chromosomes concomitant with condensation (Crosio et al., 2002). This phosphorylation is mediated by Aurora kinases. Aurora kinases are highly conserved mitotic serine/threonine kinases with three kinases, Aurora A, B and C, identified in mammals.

In mice, Aurora A localizes at centrosomes during interphase and at spindle poles during metaphase (Dutertre et al., 2002). Aurora B accumulates first at centromeres and spreads to the central spindle and at the midbody during anaphase and can then be found at the post-mitotic bridge during telophase. Aurora B phosphorylates serine 10 and serine 28 on the same H3 tail during mitosis. Typically, Aurora C expression is restricted to meiosis and regulates chromosome segregation, similar to its family member Aurora B (Balboula and Schindler, 2014).

Two Aurora kinases, A and B, are present in *Drosophila* (Reich et al., 1999). *Drosophila* Aurora A does not phosphorylate histones but is essential for asymmetric protein localization and centrosomal organization (Giet and Glover, 2001, Berdnik and Knoblich, 2002). *Drosophila* Aurora B is 52% identical to murine Aurora B. In *Drosophila* Aurora B knock out cells, histone H3 phosphorylation is reduced and chromatin just partially condensates. In the absence of cytokinesis and metaphase

alignment, cells fail to undergo mitosis resulting in polyploidy (Giet and Glover, 2001).

The function of histone phosphorylation during mitosis is not completely elucidated, but might serve as a platform for protein binding or removal of heterochromatic marks. Heterochromatin protein (HP1) bound to H3K9me3 establishes and maintains heterochromatin. During mitosis, H3S10 phosphorylation by Aurora B disrupts HP1 interaction with H3K9me3 allowing the cell to proceed with chromatin condensation (Fischle et al., 2005, Hirota et al., 2005).

1.4.2 Histone H3 phosphorylation during interphase in *Drosophila*

In mammals, the role and regulation of H3S28 phosphorylation has been proposed to differ from H3S10 phosphorylation (Dyson et al., 2005a). In *Drosophila*, during interphase, no H3S28ph could be detected above baseline levels (Dyson et al., 2005, Wang et al., 2013). Although contrasting findings showed increased H3S28 phosphorylation in response to stimulation (Kellner et al., 2012), this thesis does not address H3S28 phosphorylation and will hence not discuss H3S28ph regulation.

H3S10ph can serve as a binding site for euchromatic factors and could cause the release of compacting factors. Nuclear tandem kinase JIL-1, a homologue of MSK1/2, is responsible for decondensed chromatin to allow gene expression (Regnard et al., 2011, Johansen and Johansen, 2006, Silva-Sousa and Casacuberta, 2013). JIL-1 mediates H3S10 phosphorylation and maintains an active chromatin state during interphase, although findings remain controversial whether phosphorylation is concomitant with transcriptional activation (Jin et al., 1999, Regnard et al., 2011, Wang et al., 2011, Kellner et al., 2012). It has been suggested that JIL-1 mediated histone H3S10 phosphorylation counteracts heterochromatin spreading by HP1 and

deposition of silencing mark H3K9me2 rather than activating transcription (Cai et al., 2008, Boeke et al., 2010, Girton et al., 2013, Cai et al., 2014).

Opposing results were also found for JIL-1 localization. In Regnard *et al.,* JIL-1 can mainly be found at the gene body concomitant with H3K36me3 and H4K16ac (Regnard et al., 2011). In two other papers, JIL-1 is said to phosphorylate histone H3 at promoters and enhancers correlating with transcription (Zielke and Saumweber, 2014, Kellner et al., 2012). H3S10ph would then recruit 14-3-3 serving as a scaffolding platform to mediate H3K9 acetylation (Karam et al., 2010).

Furthermore, JIL-1 knockout *Drosophila* show chromosomal abnormalities while mice lacking MSK1/2 do not show this phenotype (Jin et al., 1999, Wang et al., 2001). JIL-1 mutants show reduced levels of histone H3 phosphorylation and structural abnormalities (Cai et al., 2008, Deng et al., 2008). Although JIL-1 is the main H3 kinase during interphase, a link between inducible JIL-1 mediated histone phosphorylation and gene transcription is lacking (Bettencourt-Dias et al., 2004).

1.4.2.1 Heat shock

Heat shock causes a general shut-down of transcription concomitant with a rapid up-regulation of heat shock proteins (hsp). Heat shocked cells show a global loss of nucleosomes and hence down-regulation of H3 phosphorylation (Petesch and Lis, 2008). RNA polymerase II is paused at heat shock genes, and released by activation of heat shock factor (HSF) (Sandaltzopoulos and Becker, 1998, Lis and Wu, 1993, Boehm et al., 2003).

Drosophila salivary gland cells are post-mitotic polyploid cells with up to 1024 copies neatly aligned in one cell. Transcriptionally active, diffused uncoiled regions are visible as relaxed chromosome puffs on polytene chromosomes. In response to heat shock, puffs indicate underlying active heat shock responsive genes (Johansen et

al., 2009, Ashburner, 1967). It has been described that the chromatin of these heat shocked puffs is phosphorylated on histone H3 promoting transcription (Labrador and Corces, 2003a, Nowak and Corces, 2000b, Ivaldi et al., 2007). Nowak *et al*. state that H3S10 phosphorylation can be detected on heat shock puffs, while Johansen *et al.* argue that the anti H3S10ph antibody used in this study is cross-reacting with non-specific proteins present at heat shock puffs (Johansen et al., 2009, Nowak and Corces, 2000a, Cai et al., 2008). Supporting the latter statement, strongly transcribed genes are generally depleted of histones and it has been shown that histone kinase JIL-1 does not redistribute to heat shock puffs of actively transcribed genes (Wang et al., 2013, Deng et al., 2008, Johansen et al., 2009, Regnard et al., 2011). When JIL-1 is mutated, induction of hsp70 is decreased more than twofold, most likely because of the generally disrupted chromosome morphology (Cai et al., 2008). In *Drosophila*, histone H3 acetylation, which is generally considered to be associated with active chromatin, could not be detected in response at heat shock loci (Nowak and Corces, 2000b).

1.4.2.2 Dosage compensation

Drosophila have three homologous pairs of autosomes and male *Drosophila* carry sex chromosomes XY. In contrast to humans, the absence of a second X chromosome designates the male sex and female *Drosophila* do not shut down one of their sex chromosomes. To compensate for the lack of a second X chromosomes, male *Drosophila* epigenetically enhance transcription of their X chromosome twofold to normalize gene expression (Conrad and Akhtar, 2011).

In male *Drosophila* cells, H4K16 hyperacetylation by males absent on the first (MOF) found on the X chromosome prevents chromatin compaction for dosage compensation (Turner et al., 1992). H4K16ac is not a general mark of actively transcribed genes

since it cannot be detected on actively transcribed ecdysone-inducible protein E75 (Eip75) after ecdysone stimulation and can also be found in repressed chromatin (Turner et al., 1992, Zhou and Grummt, 2005). On the male X-chromosome, JIL-1 is enriched twofold to phosphorylate H3S10 at double transcribed chromatin, carrying also H3K36me3 (Jin et al., 2000).

To summarize, in *Drosophila,* histone phosphorylation occurs during mitosis mediated by Aurora kinases or dosage compensation and during interphase to counteract heterochromatin spreading mediated by HP-1. Currently, it is unknown how JIL-1 is activated. JIL-1 recruitment to polytene chromosomes was suggested to be mediated by chromodomain protein interacting with Z4 (Chriz), although further experiments are necessary to clarify this interaction (Gan et al., 2011, Rath et al., 2006). Targeting of JIL-1 correlates with and could be enhanced by H3K36me3 and H4K16ac (Regnard et al., 2011). However, histone phosphorylation downstream of MAPKs at inducible IE genes has not yet been reported.

1.5 Thesis aims and summary

MAPK targets include other kinases, transcription factors and nucleosomal proteins. In mouse cells, the ERK and p38 pathways phosphorylate and activate the downstream chromatin-associated kinase MSK1/2 which is responsible for the nucleosomal response, the phosphorylation of histone H3 at serine 10. Histone H3 phosphorylation is downstream of both these MAPK pathways and part of the nucleosomal response. In *Drosophila*, very little is known about nucleosomal response signalling.

If phosphorylation is transduced from MAPK to histone H3, an inducible histone kinase in *Drosophila* must mediate H3 phosphorylation. The kinases, JIL-1, MK2 and RSK are all potential candidates. So far, there has been no indication that JIL-1 phosphorylates H3 at promoters downstream of MAPK signalling. The hypothesis therefore is that, in *Drosophila,* a MAPK downstream kinase is responsible for histone H3 phosphorylation concomitant with gene induction.

Analysis of the existing literature reveals that there is a need for detailed research, especially in understanding the downstream effectors in the MAPK signalling pathways. Many studies analyse stimulus-responsive signalling in the context of embryonic development and thus at an organismic level, yet it remains unclear how nuclear signalling is regulated due to limited cell-based research. It is thus important to distinguish MAPK signalling and to identify stimuli specific cellular responses in *Drosophila*.

The aim of this thesis is to investigate extracellular stimuli which are able to alter histone modifications and gene expression in *Drosophila*. The experimental strategy is ultimately designed to identify the kinases responsible for this process and to compare these findings to those in the mammalian system. It is currently unknown

whether the nucleosomal response is an ancient or recent evolutionary development. Research in *Drosophila* has the potential to identify conserved pathways leading to histone modifications. My goal is to identify whether histone phosphorylation of underlying IE genes can be found in *Drosophila* and which pathway is responsible.

The following points highlight my main findings in *Drosophila* S2 cells:

- Anisomycin does not induce signalling in *Drosophila*
- Establishment of a number of systems to use stimuli as specific MAPK activators
    - p38 is activated by sorbitol and sodium arsenite
    - ERK is activated by sorbitol, sodium arsenite, hydrogen peroxide, heat shock, insulin and serum
    - JNK is activated by sorbitol, lipopolysaccharides and peptidoglycan
- Sorbitol and sodium arsenite induce histone H3K9acS10ph
- c*jun* homologue *jra* behaves like an immediate early gene
- Global histone H3 phosphoacetylation is not concomitant with *jra* induction
- Histone H3K9acS10ph does not appear to be downstream of a MAPK pathway

2 Materials and Methods

2.1 Cell culture and stimulation

Drosophila Schneider (S2) cells were grown in Lonza's Schneider's medium containing 10% foetal calf serum (FCS, Life Technologies) at 26°C in T-75 flasks and seeded for experiments in 10 cm dishes at a density of $2x10^7$ cells per dish. S2 cells were quiesced with 0.1% FCS for 48 hours before treatment for protein extraction and 24 hours for RNA analysis. To count cells, an aliquot of the cell suspension was diluted 1:1 with trypan blue, which stains dead cells. Cells were counted with a counting chamber. Mouse C3H10T$^1/_2$ fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS (37°C, 6% CO_2). Near-confluent plates of cells were quiesced overnight in DMEM containing 0.5% FCS.

Cells were pre-treated as needed with compound SB203580 (10 μM, NEB), PD0325901 (0.1 μM and 2 μM, Merck), SP600125 (10 μM, Sigma) and with rapamycin (20 nM, Sigma) for 30 min and then stimulated as indicated. Quiesced cells were treated with physiological stimuli such as FCS (15%, Life Technologies), lipopolysaccharides (50 μg/ml, LPS from E.coli 055:B5, Sigma), peptidoglycan (5 μg/ml, PGN from E.coli 0111:B4, Invivogen) and insulin (10 μg/ml, human recombinant insulin expressed in E.coli, I0259, Sigma). Stress stimuli were sodium arsenite (1 mM, Sigma), sorbitol (0.5 M, Sigma) and H_2O_2 (1, 5 and 10 mM, Sigma). S2 cells were subjected to instant heat shock at 37°C by adding an equal volume of 48°C warm quiescing medium (Boehm et al., 2003). Heat shock was maintained by transferring cells from a 26°C to a 37°C incubator. Quiescent S2 cells were irradiated with 400 J of UV radiation (254 nm) per m^2 UV in a 10 cm dish with 3 ml of Lonza medium using a Spectrolinker (XL-1000; Spectronics Corp.). Pharmacological

compounds included sodium fluoride (50 mM, Sigma), 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB, 25 μg/ml, Sigma), actinomycinD (ActD, 5 μg/ml, Enzo Lifesciences), anisomycin (25 ng/ml and 10 μg/ml, Sigma) and emetine (94 nM and 37.96 μM, Sigma).

2.2 Protein extraction and histone acid extraction

Cells were lysed by the addition of high salt lysis buffer consisting of 20 mM HEPES pH 7.6, 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 10% glycerol, 0.4% Triton X-100, 400 mM NaCl, 1 mM sodium orthovanadate (Sigma), 10 mM sodium butyrate, 20 mM α-glycerophosphate (Sigma), 1 μM microcystin (Enzo Lifesciences) and a protease inhibitor cocktail (200 μg/ml aprotinin, 500 μg/ml, leupeptin, 5 mg/ml soybean trypsin inhibitor, 5 mg/ml tosyl-lysyl-chlormethyl ketone, 10 mg/ml tosyl-phenylalanyl-chloromethyl ketone and 10 mg/ml phenylmethanesulphonyl fluoride). Extracts were put on ice for 20 min and centrifuged at 12,300 rcf at 4°C for ten min. The supernatant contained cytoplasmic and the pellet nuclear proteins. Histones were extracted from the pellet by adding 0.4 M HCl and precipitated by adding acetone. Acid soluble proteins were resuspended in 8 M urea and either added to Laemmli buffer for analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or added to 0.5% acetic acid and 0.5% β-mercaptoethanol for analysis on acid-urea (AU) gels. This protocol allowed the analysis of MAPKs, transcription factors and histones from the same sample which thus served as an ideal internal control.

2.3 SDS and AU PAGE

The high salt lysis fraction was separated by a 10% SDS-PAGE, 1/48th of a dish ($4.2x10^5$ cells) was loaded. Acid soluble proteins were resolved on AU or SDS-PAGE gels. For both gels, 1/10th of a dish was loaded. All SDS-PAGE gels were run at 150 V in SDS buffer. AU gels were pre-run overnight at 150 V, samples were loaded and run at 15 mA in 5% acetic acid. Before transfer, AU gels were equilibrated for 30 min in 10% methanol and 5% acetic acid. Proteins were transferred to polyvinylidene difluoride (PVDF, Immobilon, Merck Millipore) membranes by Western blotting. Before setting up the transfer, PVDF membranes were wetted in 100% methanol.

2.4 Western blotting

High salt lysis fractions on SDS-PAGE gels were transferred in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol (Towbin et al., 1979)) at 200 mA overnight and acid soluble proteins were transferred at 60 mA in 0.5x SDS Buffer and 10% methanol. Acid soluble proteins on AU gels were transferred at 200 mA in 10% methanol and 0.1% acetic acid. Following transfer, PVDF membranes were stained in Ponceau-S solution (0.5% Ponceau S, 1% acetic acid). Membranes were destained in 100% methanol. Dried membranes were incubated directly with the primary antibody (see Table 1) diluted in blocking buffer for one hour (PBS, 0.1% Tween-20, 5% skimmed milk powder). Membranes were washed three times for ten min. Following the washes, membranes were incubated with secondary antibody. Blots were washed with PBS-Tween three times for ten min and incubated with electrochemiluminescent (ECL) reagents for one min. Membranes were wrapped in Saran wrap and exposed to Fuji X-ray films.

2.5 Antibodies

Anti phospho-ERK, -p38 and -JNK antibodies were purchased from Cell Signalling. Histone H3S10ph antibodies were raised against a peptide consisting of residue 7-20 of the N-terminal of histone H3. This antibody is not occluded by acetylation at lysine 9 or 14. Histone H3K9acS10ph antibodies were raised against residues 1-20 of the N-terminal H3 peptide antibodies acetylated at lysine 9 and phosphorylated at serine 10. Both histone antibodies were affinity purified, just recognize the indicated epitopes and are not occluded by surrounding acetylation. All secondary antibodies were coupled to horseradish-peroxidase. Primary and secondary antibodies were diluted in 5% skimmed milk powder (in 1xPBS and 0.1% Tween); dilutions can be found in Table 1. For detection, a homemade ECL reagent consisting of 1.25 mM luminol, 0.2 mM coumaric acid, 100 mM Tris pH 8.5 and 2.7 mM H_2O_2 was used.

Table 1: Antibody dilutions for Western blots. Dilution for primary and secondary antibodies are specified. For αhistone antibodies, dilutions needed for an SDS or AU gel are given.

Primary			**Secondary**	
αPhospho ERK	♯4337	1:1000	αRabbit	1:2500
αPhospho p38	♯9211	1:1000	αRabbit	1:2500
αPhospho JNK	♯9251	1:1000	αRabbit	1:2500
αPhospho p70	♯9206	1:5000	αMouse	1:2500
αH3S10ph	SDS	1:5000	αRabbit	1:10000
(R402)	AU	1:5000		1:5000
αH3K9acS10ph	SDS	1:5000	αSheep	1:5000
αH3K9acS10ph	AU	1:1500		1:5000

2.6 RNA extraction

Glassware was baked at 180°C for four hours. Water was treated with 0.1% diethylpyrocarbonate (DEPC, Sigma) overnight and autoclaved. Gel trays and electrophoresis tanks were treated with 5 M NaOH and rinsed with DEPC-treated water. Total RNA was isolated using the Solution D method described in Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). To harvest cells, 1.8 ml Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 0.1 M β-mercaptoethanol) was added to each 10 cm dish and cells were scraped into 15 ml Falcon tubes containing 200 µl sodium acetate (2 M, pH 4). Subsequently, 1.8 ml water-saturated phenol (MP Biomedicals) as well as 720 µl chloroform:ioamylalcohol (49:1) were added and incubated on ice for ten min. Tubes were vortexed in between each addition and at the end of the ten min incubation. To separate phases, the lysates were centrifuged for 15 min at 1,200 rcf. The upper aqueous phase was transferred to a new 15 ml Falcon tube containing 2.5 ml isopropanol. Samples were left at -20°C overnight and centrifuged for 15 min at 1,200 rcf. The pellet was resuspended in 300 µl Solution D and transferred to a 1.5 ml microfuge tube containing 200 µl isopropanol. Samples were incubated at -20°C for three hours and centrifuged for 15 min at 13,000 rcf at 4°C. Pellets were washed in 70% and 100% ethanol. RNA was air-dried and resuspended in 30 µl DEPC-treated water. Quantity and quality of the RNA were determined by 260/280 nm and 260/230 nm absorbance measurements, respectively, using a Nanodrop (Witec AG, Luzern, Switzerland).

2.7 Northern blotting

Gene expression was analysed by Northern blotting. Total RNA (5 µg) was resolved on denaturing formaldehyde/agarose gels and transferred onto nylon membranes (Hybond-N+, GE Healthcare). RNA was transferred from the gel to the membrane using capillary forces in 50 mM NaOH for four hours. Membranes were rinsed in 2x saline sodium citrate (SSC) buffer (0.3 M NaCl, 0.03 M sodium citrate pH 7). Probes were labelled with the random primed labelling method based on Feinberg and Vogelstein (Random Primed DNA Labelling kit, Roche) and spin columns were used for postreaction clean-up (Sigma) (Feinberg and Vogelstein, 1984). Blots were sequentially hybridised to α^{32}P-dATP (Perkin Elmer) labelled *jra* probes and then to the loading control *rp49*, as described in Church and Gilbert (Church and Gilbert, 1984). Primer sequences were *jra*F GAGAAGAAGGCCCCATGTCA, *jra*R CAGCTGCGCCACATGGT and *rp49*F GACGCTTCAAGGGACAGTATC and *rp49*R TTTACAAATGTGTATTCCGACCA. Northern blots were visualised by autoradiography.

3 Effect of anisomycin in *Drosophila*

3.1 Introduction

3.1.1 Translational inhibitor anisomycin

Anisomycin (2-p-methoxyphenylmethyl-3-acetoxy-4-hydroxypyrrolidine) is a pyrrolidine antibiotic with a range of possible applications (Figure 4). For instance, it has been shown to prevent the consolidation of long-term memory (Frey et al., 1988). It has also been suggested that anisomycin may have anticancer properties by sensitizing cells to anoikis (apoptosis of malignant anchorage-dependent cells detaching from the surrounding extracellular matrix) (Mawji et al., 2007).

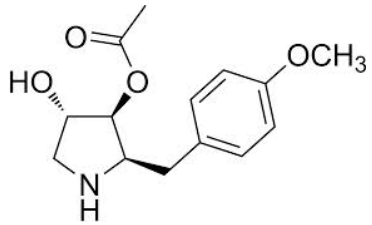


Figure 4: Structure of anisomycin.

Classically, anisomycin is a potent protein synthesis inhibitor isolated from *Streptomyces griseolus*. In yeast to mammals, anisomycin inhibits protein synthesis reversibly at high concentrations by binding to the 60S ribosomal subunit and blocking peptide bond formation (Barbacid et al., 1975). In the presence of anisomycin the release of polypeptides from the polyribosome is impaired. This inhibition of protein synthesis is very quick and can be observed after less than 30 seconds (Grollman, 1967).

3.1.2 Superinduction and anisomycin induced signalling

The translational inhibitor anisomycin in combination with a signalling agonist can prolong the normally transient induction of IE genes. This stronger and more sustained response of IE genes is referred to as superinduction, during which transcription termination is delayed and transcripts are stabilized. Augmented transcription frequency and the absence of labile repressors also contribute to superinduction. For example, EGF and anisomycin synergistically activate histone H3 and induce transcripts, like c*jun* and c*fos*. A similar, although less potent effect was reported with other translational inhibitors, cycloheximide and puromycin. Apart from superinduction observed with most protein synthesis inhibitors, anisomycin and cycloheximide have a signalling effect on their own.

Anisomycin activates stress-related JNK and p38 kinase cascades strongly, and also weakly activates ERK pathways, a process which is cell-type dependent (Dhawan et al., 1999, Bebien et al., 2003). Upon MSK1/2 activation, transcription factors (ATF1, CREB, SRF and Elk-1) get phosphorylated, enhancing transactivation potential and mediating anisomycin dependent IE gene expression (Bebien et al., 2003, Wiggin et al., 2002, Darragh et al., 2005). While p38 activation is essential for anisomycin dependent IE gene induction, JNK activation is not sufficient to mediate IE gene induction (Hazzalin et al., 1997, Hazzalin et al., 1996). In response to inhibitory anisomycin alone, c*fos* and c*jun* are more stable and the termination of their transcription is delayed correlating with more sustained histone H3 phosphorylation. Inhibitory anisomycin induced c*fos* and c*jun* for at least six hours. A comparable although less powerful gene inducing event was reported for cycloheximide (Edwards and Mahadevan, 1992). In addition, the levels of histone H3 phosphorylation were stronger than those observed with other stimuli, indicating that this phenomenon is

separate of its ability to inhibit translation.

At concentrations too low to affect translation, and thus independent of translational arrest, sub-inhibitory anisomycin can induce c*jun* to a similar extent as in cells stimulated with EGF. Sub-inhibitory anisomycin causes over-induction, but not stabilization, giving rise to a transient spike of RNA. Desensitization, a refractory period in which IE genes cannot get induced again by the same components, is a hallmark of true signalling agonists, and sub-inhibitory anisomycin has been shown desensitize c*jun*, c*fos*, *fos*B, *jun*B and *jun*D to UV radiation (Hazzalin et al., 1998). Sub-inhibitory anisomycin thus acts as a signalling agonist producing high levels of histone H3 phosphorylation and IE gene induction (Mahadevan and Edwards, 1991, Mahadevan et al., 1991). This is in contrast to other protein synthesis inhibitors, like emetine, which have not been shown to activate p38 and JNK or induce IE genes on their own at sub-inhibitory concentrations (Cano et al., 1994).

3.1.3 Anisomycin activates p70/p85^{S6kinase} in mammalian cells

The p70/85^{S6kinase} ribosomal S6 kinase is stimulated by a variety of mitogens and anisomycin. Anisomycin activated p70/p85 ribosomal S6 kinase (p70/p85^{S6kinase}) is independent of the nucleosomal response. Rapamycin treatment inhibits anisomycin activated p70/p85^{S6kinase}, but does not diminish p38 or histone phosphorylation and c*jun* induction. This demonstrates that the target of anisomycin lies upstream of p70/p85^{S6kinase} or at the bifurcation of histone phosphorylation and gene induction (Zhelev et al., 1993).

Phosphorylation of p70/p85^{S6kinase} by anisomycin is likely to be mediated by target of rapamycin (TOR) kinase. Activated p70/p85^{S6kinase} phosphorylates ribosomal S6 protein of the 40S subunit resulting in an increased translation of transcripts which are part of the translational machinery (Parrott and Templeton, 1999).

3.1.4 p70^{S6kinase} regulation in *Drosophila*

In *Drosophila*, the p70^{S6kinase} (dS6K), but not p85^{S6kinase} isoform is conserved. p70^{S6kinase} orthologue in *Drosophila* lacks a nuclear localizing signal and shows 78% identity to the catalytic domain in human p70^{S6kinase} (with an overall identity of 57%) (Stewart et al., 1996). Upstream signalling and most activating phosphorylation sites in the auto-inhibitory and linker region are conserved in *Drosophila*. p70^{S6kinase} is directly phosphorylated by TOR which is part of the TOR complex (TORC1). TORC1 in turn gets activated *via* the Phosphatidylinositol 3-kinases (PI3-K) - Phosphoinositide-dependent kinase-1 (PDK) / Protein kinase B (PKB/Akt) pathway (Kimball et al., 2008, Radimerski et al., 2002). PKB/Akt phosphorylates and inhibits tuberous sclerosis complex (TSC1/2) proteins hamartin and tuberin, relieving the inhibition of the small GTPase Ras homolog enriched in brain (Rheb), enabling it to activate TORC1 (Figure 2 A) (Stocker et al., 2003). Regulation of p70^{S6kinase} also takes place on a second level; regulated in development and DNA damage responses (REDD1), Scylla/Charybdis in *Drosophila*, represses p70^{S6kinase} by relieving PKB/Akt-dependent TSC1/2 inhibition (Abraham, 2005). An increase in TSC1/2 causes the shift from stimulating GTP-bound to inhibitory GDP-bound Rheb, which inhibits TORC and thus p70^{S6kinase} (Reiling and Hafen, 2004) (Figure 2 B).

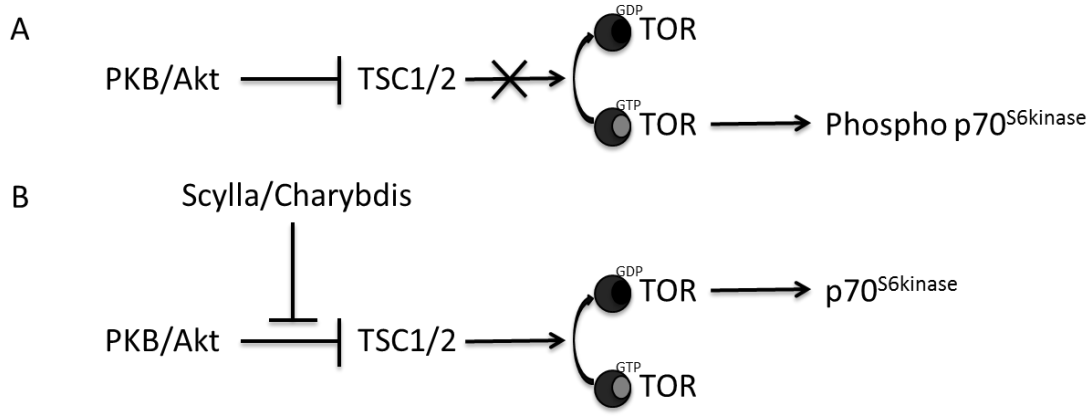


Figure 5: Schematic representation of p70^{s6k} activation. A) PKB/Akt inhibits TSC1/2 and enables TOR to phosphorylate p70^{S6kinase}. B) Scylla/Charybdis represses p70^{S6kinase} phosphorylation by relieving TSC1/2 inhibition and causing inactive TOR.

3.1.5 Mode of action of anisomycin

Currently, it is still unclear how anisomycin exerts its nuclear signalling and gene induction effect precisely. General cytoplasmic responses such as tyrosine phosphorylation are not affected by anisomycin (Zhelev et al., 1993). The involvement of reactive oxygen species (ROS) in conveying anisomycin induced signalling has been proposed, although clear evidence is lacking (Torocsik and Szeberenyi, 2000). Even though the target of anisomycin is unknown, protein kinase C, tyrosine kinases or ERK and their upstream kinases can be excluded. In fact, anisomycin is thought to act outside canonical signalling pathways and does not target receptor-mediated kinases (Hazzalin et al., 1998). In mammals, it has been considered that binding of anisomycin to the 60S subunit may activate unknown ribosome-associated intracellular signalling. Even though the ribosomal inhibition is not detectable, the stress signal might get amplified resulting in sufficient activation to produce gene induction.

In summary, in mouse cells, it is now established that, in addition to inhibiting translation at high concentrations (10 μg/ml), anisomycin is a strong selective inducer of JNK, p38 and S6 ribosomal protein kinase $p70^{S6kinase}$ at sub-inhibitory concentration too low to affect translation (25 ng/ml) (Thomson et al., 1999a, Cano et al., 1994). Anisomycin subsequently induces IE gene transcription. However, it is currently unknown how this signalling effect is achieved. It would therefore be interesting to establish whether the signalling activity of anisomycin is also present in other organisms, and in this chapter the effect of anisomycin will be investigated in *Drosophila*. If anisomycin acts on an evolutionary conserved target, the same signalling response would be expected.

3.2 Results

3.2.1 Anisomycin does not activate MAPK or histone H3 phosphorylation

In mouse cells, anisomycin is a potent activator of JNK and p38 leading to histone H3 phosphorylation and IE gene induction (Cano et al., 1994, Kardalinou et al., 1994). Considering the signalling activation in mouse cells, I first aimed to establish whether anisomycin also has an effect on MAPKs in addition to inhibiting translation in *Drosophila*. At concentrations under 70 ng/ml, anisomycin does not inhibit translation while 10 µg/ml anisomycin inhibits translation efficiently (Mahadevan and Edwards, 1991).

Therefore, I investigated the effects of anisomycin in *Drosophila* cells. Surprisingly, I did not observe any MAPK phosphorylation event (Figure 6 A). Additionally, increasing concentrations of anisomycin (sub-inhibitory and inhibitory) were analysed after 60 min of incubation, but histone H3 phosphoacetylation was not detectable above baseline levels (Figure 6 B). In both experiments, osmotic stress in the form of sorbitol stimulation was used as a positive control showing a strong phosphorylation signal for p38, JNK and MAPK, and a phosphoacetylation signal for histone H3, respectively (Figure 6 B).

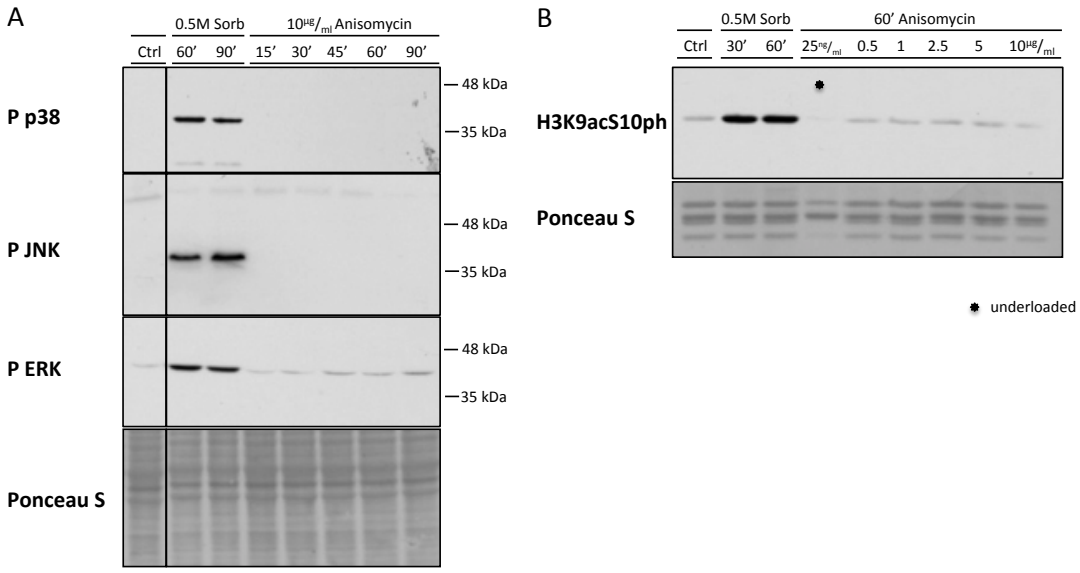


Figure 6: Lack of MAPK phosphorylation and histone H3 phosphoacetylation in response to anisomycin. A) Quiescent S2 cells were treated with 0.5 M sorbitol (60 and 90 min) and 10 µg/ml anisomycin for 15 until 60 min. High salt lysis extracts were analysed by immunoblotting with anti phospho-p38, anti phospho-JNK and anti phospho-ERK antibodies. B) Quiescent S2 cells were treated with 0.5 M sorbitol (30 and 60 min) and with 25 ng/ml and 0.5, 1, 2.5, 5 and 10 µg/ml anisomycin for 60 min. Acid-soluble proteins were extracted and separated on SDS-PAGE Western blots were carried out with anti phosphoacetyl-H3 antibodies recognizing H3 phosphorylated on serine 10 and acetylated on lysine 9. The star indicates that the decrease in intensity is due to under loading of the sample. Ctrl indicates the unstimulated control. Ponceau S staining was used as a loading control. Both experiments are replicates of two experiments.

3.2.2 Emetine induces p70^{s6kinase} phosphorylation to a similar degree as anisomycin

The absence of phosphorylation activation upon stimulation with anisomycin could be explained by a lack of signalling potential in *Drosophila*. To confirm the signalling potential of anisomycin as opposed to any other translational inhibitor, I compared phosphorylation levels after stimulation by anisomycin with stimulation by another translational inhibitor, emetine, in mouse cells. C3H10T$^{1}/_{2}$ mouse cells were treated with three concentrations of anisomycin and emetine for 30 and 60 min. Anisomycin, but not emetine, induced a strong phosphorylation of p70^{S6kinase} at a sub-inhibitory concentration of 94 nM (25 ng/ml) (Figure 7 A). At a concentration of 3.79 µM or 37.96 µM (10 µg/ml) anisomycin phosphorylates both isoforms, p70^{S6kinase} and also

p85^{S6kinase}, while emetine causes only a slight phosphorylation of p70^{S6kinase} at these concentrations.

Considering the signalling activation in mouse cells, I next wanted to establish whether anisomycin also stimulates p70^{S6kinase} phosphorylation in *Drosophila*. Quiescent S2 cells were treated with the same concentrations of anisomycin as in the previous experiment. Extracts obtained with high salt lysis were analysed by immunoblotting with an antibody recognizing Thr398 phosphorylation. A doublet at about 70 kDa was detected after stimulating cells with increasing concentrations of anisomycin (Figure 7 B). This doublet reflects p70^{S6kinase} as it is inhibited in presence of rapamycin (see appendix A.1) indicating phosphorylation at concentrations of 3.79 μM and 37.96 μM. The two bands may represent differentially post-translationally modified states, most likely resulting from phosphorylation (Watson et al., 1996). Similarly, treatment of the cells with these concentrations of the translational inhibitor emetine caused an increase of p70^{S6kinase} phosphorylation to a similar level as anisomycin. After treating cells with 94 nM anisomycin or emetine, which corresponds to the sub-inhibitory concentration for mouse cells, phosphorylation of p70^{S6kinase} was not increased above the base level seen in unstimulated cells. These results show that in response to inhibitory concentrations of anisomycin and emetine, p70^{S6kinase} is phosphorylated to the same extent.

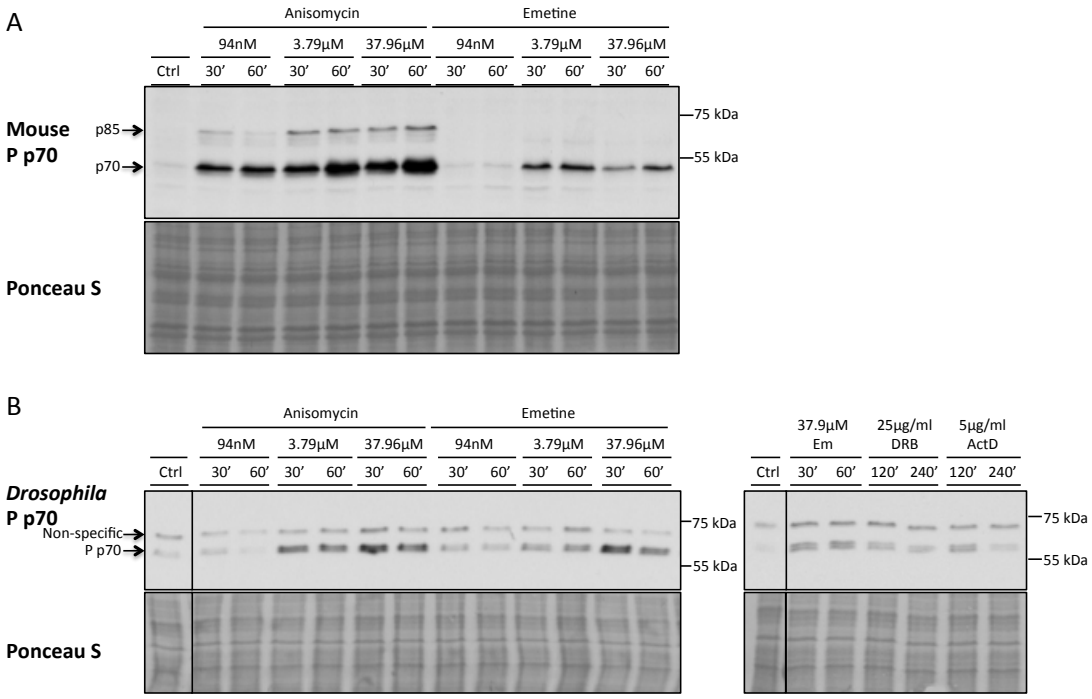


Figure 7: A) Effects of anisomycin and emetine on p70/p85^{S6kinase} phosphorylation on mouse C3H 10T½ cells. Cells were not treated (Ctrl) or treated with sub-inhibitory anisomycin (94 nM = 25 ng/ml), intermediate anisomycin (3.79 µM) and inhibitory anisomycin (37.96 µM = 10 µg/ml) for 30 and 60 min as indicated. The same treatment was performed with emetine at 94 nM, 3.79 µM and 37.96 µM. High salt lysis extracts were probed using an anti p70/p85^{S6kinase} antibody. **B) Effects of translational and transcriptional inhibitor on p70^{S6kinase} phosphorylation on *Drosophila* S2 cells.** Cells were treated as in A). In the second blot, cells were treated with emetine at 37.96 µM for 30 and 60 min and with transcriptional inhibitors (25 µg/ml DRB and 5 µg/ml actinomycin D) for two and four hours. High salt lysis extracts were analysed by immunoblotting with anti-phosphorylated p70^{S6kinase} antibodies. Ponceau S staining was used as a loading control. This experiment is a replicate of ten experiments.

In order to verify whether p70^{S6kinase} phosphorylation is caused by reduced translation or a secondary effect of stress resulting from translational inhibition, I also analysed p70^{S6kinase} phosphorylation upon stimulation with two transcriptional inhibitors, actinomycin D (ActD) which preferentially intercalates into GC rich sequences and prevents RNA polymerase progression, and 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB) which prevents activating phosphorylation of RNA Pol II (Yankulov et al., 1995, Sobell, 1985). The right panel of Figure 7 B illustrates that both transcriptional inhibitors DRB and ActD induced p70^{S6kinase} phosphorylation after two hours to a similar level as seen after 30 min of treatment

with emetine. Thus, both translational inhibition for 30 min and transcriptional inhibition for two hours lead to phosphorylation of p70^{S6kinase} in *Drosophila*.

3.2.3 Strong hydrogen peroxide dependent p70^{S6kinase} phosphorylation

The increase in p70^{S6kinase} phosphorylation by transcriptional or translational inhibition was moderate, so a variety of stimuli were tested to achieve a more pronounced phosphorylation event under physiological conditions.

After stimulating S2 cells either with 1 or 5 mM hydrogen peroxide (Figure 8) a very strong p70^{S6kinase} phosphorylation was seen after only five min. Sorbitol treatment caused decrease in p70^{S6kinase} phosphorylation. When treating with UV light for 30 min or anisomycin at 10 µg/ml for one hour, only a weak phosphorylation of p70^{S6kinase} was observed.

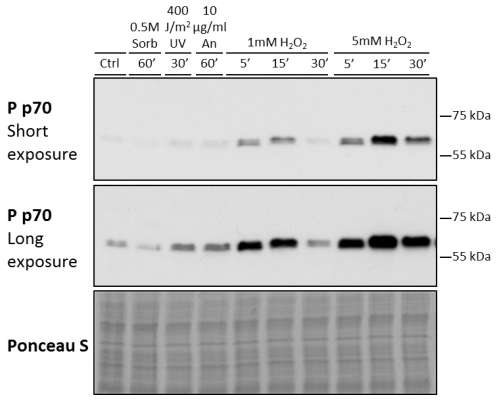


Figure 8: Hydrogen peroxide induces strong p70^{S6kinase} phosphorylation in *Drosophila*, while anisomycin and UV exposure only have a small effect. Quiescent S2 *Drosophila* cells were treated with 0.5 M sorbitol (60 min), 400 J/m^2 UV (30 min), 10 µg/ml anisomycin (60 min) and 1 or 5 mM hydrogen peroxide (5, 15 and 30 min). High salt lysis extracts were probed against phosphorylated p70^{S6kinase}. Ctrl indicates the unstimulated control. Ponceau S staining was used as a loading control. Results are representative of three independent experiments.

3.3 Discussion

3.3.1 Lack of signalling activity of anisomycin in *Drosophila*

Extracellular stimuli may trigger quiescent cells in G_0 to undergo mitosis. This reprogramming is dependent on the rapid transcription of IE genes. In mammalian cells, one of those stimuli is sub-inhibitory anisomycin, causing JNK, p38 and subsequent histone H3 phosphorylation which ultimately results in IE gene induction. Despite the strong effect in mouse cells, there was no MAPK or histone H3 activation with anisomycin treatment in *Drosophila* cells (Figure 6). It has been hypothesized that anisomycin is able to activate p38 in S2 cells which leads to the phosphorylation of the TOR complex, upstream of $p70^{S6kinase}$. However, p38 phosphorylation in Cully *et al.* was not clear and is contradicted by my findings (Cully et al., 2010).

It was previously thought that the signalling properties of anisomycin may be a result of the stress caused by translational arrest. As Figure 7 A has shown, anisomycin signalling activity is achieved at sub-inhibitory concentration while emetine fails to activate $p70/p85^{S6kinase}$ in mouse cells. At inhibitory concentration, emetine causes a slight phosphorylation of $p70^{S6kinase}$ although it is much weaker than the one caused by anisomycin.

In *Drosophila*, both emetine and anisomycin cause $p70^{S6kinase}$ phosphorylation to the same extent, but not at sub-inhibitory concentrations (Figure 7 B, first blot). The observed $p70^{S6kinase}$ phosphorylation event could be caused by a secondary effect of stress resulting from translational inhibition or by a reduced translation of the repressor of $p70^{S6kinase}$. If the latter hypothesis were true, transcriptional inhibitors would show the same effect as translational inhibitors. Indeed, transcriptional inhibitors DRB and ActD also induce $p70^{S6kinase}$ phosphorylation, which supports the

repressor hypothesis. It is of note that both transcriptional inhibitors cause p70^{S6kinase} phosphorylation after two hours, while translational inhibitors already show an increase after 30 min (Figure 7 B, right panel). This indicates that the RNA levels of a repressor, presumably Scylla/Charybdis (REDD1 in human), are more stable than protein levels (Shah et al., 2000, Reiling and Hafen, 2004). In fact, human REDD1 protein is very labile with a protein half-life of less than five min (Loreni et al., 2000). In presence of transcriptional or translational inhibitors levels of repressors Scylla/Charybdis are reduced, causing a release of inhibition of PKB/Akt dependent TSC inhibition. In the absence of TSC dependent TOR inhibition, p70^{S6kinase} gets phosphorylated.

Although p70^{S6kinase} phosphorylation increased with all inhibitors tested, it still has to be shown how far this level of p70^{S6kinase} phosphorylation would compare to stimulation with a physiological proliferation-inducing stimulus. Hydrogen peroxide strongly activates p70^{S6kinase} phosphorylation within 15 min, an effect far more pronounced than any inhibitor tested (Figure 8). In comparison, emetine, DRB, ActD and anisomycin dependent p70^{S6kinase} phosphorylation was not representative of distinctive phosphorylated p70^{S6kinase}. As expected from the effect of anisomycin as a protein synthesis inhibitor, just a slight increase of phosphorylated p70^{S6kinase} was seen at the protein level. Hydrogen peroxide, on the other hand, triggers a clear p70^{S6kinase} phosphorylation event. The slight decrease in p70^{S6kinase} in response to sorbitol has previously been suggested to be mediated *via* activation of a phosphatase causing p70^{S6kinase} dephosphorylation (Parrott and Templeton, 1999).

These experiments represent the first demonstration that anisomycin does not elicit a signalling response in *Drosophila*. Anisomycin targets the universally conserved A-site cleft in the peptidyl transferase centre of 28S rRNA (Anger et al., 2013, Blaha et

al., 2008). This does not dismiss ribosomes being upstream of anisomycin signalling but might point towards other targets. If the signalling activity of anisomycin is not caused by ribosomal stress, the divergence in signalling kinases between *Drosophila* and mammals might give a clue as to where anisomycin targets the cascade.

4 Stimulus-specific MAPK, histone H3 activation and gene induction in *Drosophila*

4.1 Introduction

4.1.1 MAPK signalling agonists in *Drosophila*

In mammals, the nucleosomal response is mediated *via* MSK1/2, which are themselves activated by the MAPKs p38 and ERK. However, the kinases responsible for histone H3 phosphorylation remain unclear in *Drosophila*. Specific emphasis lies on identifying whether a putative MAPK dependent histone H3 kinase exists, as there is no direct functional equivalent of mammalian MSK1/2 within *Drosophila* MAPK pathways. To study this, I first need to find stimuli which can induce the MAPK signal transduction pathways.

4.1.1.1 Osmotic stressor sorbitol

Cells are sensitive to changes in osmolarity. In hyperosmolar conditions, cells acutely arrest in all stages of cell cycle and try to adapt by accumulating osmolytes, increasing ionic strength, water efflux and cell shrinkage (Burg et al., 2007). Osmotic stress can be accompanied by cytoskeletal rearrangements, DNA damage and oxidative stress ultimately resulting in apoptosis when a certain threshold is exceeded (Alfieri and Petronini, 2007). Sorbitol poorly permeates membranes and hence produces hypertonic stress. Hypertonicity is described as the effect on cells of non-penetrating solutes. For example with non-penetrating solute sorbitol, osmosis of water must occur for the cell to reach equilibrium. Unlike in yeast, no osmosensing protein has been identified in higher organisms and upstream effectors remain uncharacterized. Apart from the general adaptions to hyperosmolarity and

hypertonicity common to all osmotic stressors, sorbitol has been implicated in the phosphorylation of epidermal growth factor receptor (EGFR) (Rosette and Karin, 1996, Fischer et al., 2004). This classic EGFR activation has been postulated to result in the active GTP bound state of small G protein Ras, activating JNK, p38 and ERK (de Nadal et al., 2002, Cheng et al., 2002). In *Drosophila*, downstream of Ras all four MAP3Ks (D-MEKK1, D-ASK, D-TAK1 and slipper) are activated by sorbitol and in turn lead to p38 and JNK activation (Inoue et al., 2001, Zhuang et al., 2006, Chen et al., 2002).

4.1.1.2 Cellular stressor sodium arsenite

Arsenic is a carcinogenic environmental toxin and its main activity comes from the trivalent form, arsenite (Tinwell et al., 1991). Sodium arsenite is a potent tumour progressor causing genetic and epigenetic changes (Singh and DuMond, 2007). Despite being carcinogenic, it has also been used as a therapeutic agent against acute promyelocytic leukaemia due to its capacity to arrest mitosis and induce apoptosis (McCollum et al., 2005, Cai and Xia, 2008).

In mouse cells, sodium arsenite was shown to induce p38, ERK and JNK phosphorylation (Chen et al., 1998, Bebien et al., 2003). Arsenite results in p38 dependent CREB and MSK1/2 activation, which ultimately leads to transcription of IE genes, such as c*fos* or *mkp-1* (Mitogen-activated protein kinase phosphatase-1), concomitant with histone H3 phosphoacetylation (Li et al., 2001, Deak et al., 1998). In *Drosophila* S2 cells, strong phosphorylation of all MAPKs in response to sodium arsenite was shown (Ryabinina et al., 2006, Botella et al., 2001).

4.1.1.3 Oxidative stressor hydrogen peroxide

Hydrogen peroxide, superoxide anion and hydroxyl radical are all part of ROS. At low concentrations, endogenous hydrogen peroxide acts as a signalling molecule to

promote proliferation. At high levels of hydrogen peroxide, apoptosis is induced (Lambeth, 2007). Depending on the cell type and administered dose, ERK activation can protect the cell from hydrogen peroxide by enhancing survival, but it can also mediate the pathway leading to apoptosis (Song et al., 2005, Abe et al., 1998, Meng et al., 2002).

The mechanism of ROS dependent ERK activation has been hypothesized to be a result of phosphatase inhibition. Protein tyrosine phosphatases get oxidised by hydrogen peroxide. Inactivation of phosphatases occurs *via* transformation of a cysteine residue in the active site, which is no longer able to accept a phosphate group (Meng et al., 2002). In rat cells, tyrosine phosphatase Src-homology 2 domain-containing phosphatase (SHP-2) is inhibited by hydrogen peroxide, resulting in ERK phosphorylation (Meng et al., 2002). SHP-2 has a dual function: although it has been shown that it can enhance signalling upstream of ERK at the cell surface, it also has an inhibitory effect acting as a phosphatase. In *Drosophila*, the SHP-2 homologue corkscrew (csw) is targeting ERK signalling downstream of Raf which could turn hydrogen peroxide into a potent proliferative stimulus (Allard et al., 1996).

4.1.1.4 Insulin as a proliferative stimulus

Insulin is a major energy controlling hormone which activates glucose transport, gene expression and changes in cell morphology. The insulin receptor is a member of the receptor tyrosine kinase family and upstream of a plethora of pathways. The insulin/insulin-like growth factor (IGF) pathway is conserved from yeast to human. Hence, human recombinant insulin can be used to dissect the ERK pathway in *Drosophila* (Swanik, 2006). In *Drosophila* there is no endogenous insulin, but insulin-like growth factors and insulin-like growth factor receptors. Despite this, their receptors are able to react with human insulin to activate proliferation *via* ERK and

p70^{S6kinase} (Anselmo et al., 2002b, Kwon et al., 2002, Miron et al., 2003, Lizcano et al., 2003). In a similar manner, insulin receptors bind insulin-like growth factors with a lower affinity in human (Kido et al., 2001).

In *Drosophila*, upon activation and autophosphorylation of the insulin receptor, SHP-2 binds to the receptor, recruiting the transducing adapter DRK. DRK subsequently recruits in turn the guanine-nucleotide exchange factor SOS. SOS then activates Ras which leads to an activation of the ERK cascade (Anselmo et al., 2002b, Kwon et al., 2002, Clemens et al., 2000b, Matheny et al., 2004). Unlike in human, ERK cross-activation *via* PI-3K and PKB/Akt has been proposed (Kim et al., 2004).

4.1.1.5 Immune response activator peptidoglycan

Drosophila's host defence resembles the mammalian innate immune system (Lemaitre and Hoffmann, 2007). Depending on the challenging pathogens, two pathways get activated, the Toll and immune deficiency (IMD) pathway (Kounatidis and Ligoxygakis, 2012). While the Toll pathway is primarily activated by fungi and gram-positive bacteria, the IMD pathway is mainly activated by gram-negative bacteria, resembling the mammalian tumour necrosis factor (TNF) receptor pathway (Wang and Ligoxygakis, 2006, Lemaitre and Hoffmann, 2007). *Drosophila* JNK gets activated as part of the immune response after infection with gram-negative bacteria, for example bacterial cell wall components lipopolysaccharide (LPS) and gram-negative peptidoglycan (PGN) (Sluss et al., 1996, Stronach and Perrimon, 1999, Leulier et al., 2002, Kaneko et al., 2004). Diaminopimelic acid, the active compound of gram-negative peptidoglycan, is recognized by the transmembrane receptor peptidoglycan recognition protein receptor (PGRP) LC (Stenbak et al., 2004, Royet and Dziarski, 2007). Upon dimerization, the signal is relayed *via* IMD, *Drosophila* Fas-associated death-domain (FADD) and death related ced-3/Nedd2-like (DREDD)

to activate the MAP3K D-TAK1 (Takatsu et al., 2000, Naitza et al., 2002). Downstream of D-TAK1 are the MAP2Ks D-MKK4 and MKK7 homologue hemipterous which activate JNK (Riesgo-Escovar and Hafen, 1997, Ryabinina et al., 2006). JNK induces a gene expression programme including wound healing genes and its phosphatase *puckered* (Wang et al., 2003). Thereafter, Relish, a NF-κB-like protein, gets cleaved and translocates to the nucleus, inducing antimicrobial peptide (AMP) gene transcription in the fat body (Boutros et al., 2002, Park et al., 2004, Erturk-Hasdemir et al., 2009). AMPs are then released into the hemolymph and target invading microorganisms (Lemaitre and Hoffmann, 2007).

4.2 Results

4.2.1 Stimulus-specific MAPK activation and histone H3 phosphoacetylation in *Drosophila*

Drosophila S2 cells were used to study the conservation of mammalian nuclear signalling and histone modification in *Drosophila*. S2 cells were isolated by Schneider in 1972 from embryos on the verge of hatching. S2 cells are a heterogeneous population of male cells with a range of karyotypes. Even though S2 cells do not represent a specific cell type, similarities exist between the gene expression profile of S2 cells and macrophages, crystal cells and plasmatocytes (Schneider, 1972).

I sought to characterize a specific stimulus for each MAPK, and to correlate it with histone H3 phosphorylation. To establish the conditions in which MAPKs are stimulated, I consequently tested a variety of physiological and stress stimuli and analysed MAPK phosphorylation on Western blots.

4.2.1.1 Sorbitol and sodium fluoride activate MAPK phosphorylation and histone H3 phosphoacetylation

Osmotic stress, which can be artificially created by incubation of cells with sorbitol, induces a stress-signalling response in cells (Ryabinina et al., 2006, Chen et al., 2002, Seong et al., 2011, Sano et al., 2005, Kultz and Burg, 1998). To identify, which MAPKs are activated upon stress, quiescent S2 *Drosophila* cells were incubated with sorbitol for five min until up to three hours. Initially, different quiescence conditions for S2 cells were tested (24 hours versus 48 hours and 0.1% versus 0.5% FBS, data not shown). With 48 hours of quiescence I obtained the lowest phosphorylation background, while still achieving inducible histone phosphorylation without causing

apoptosis due to the limitation of growth factors. Hence, cells were quiesced for 48 hours in 0.1% FBS for all following experiments.

Osmotic stress induced a strong and prolonged phosphorylation of p38, JNK and histone H3 (Figure 9 A). Phosphorylation of p38 was observed after five min and did not decline within the three hour time-course. JNK phosphorylation was detectable after 15 min. The anti phospho-JNK antibody detects a doublet indicative of phospho-JNK and phospho-p38 due to cross-reactivity between the phospho-JNK antibody and p38. ERK phosphorylation occurred after five min, peaked at 15 min and subsequently decreased again. As the available antibodies detecting total p38, JNK or ERK were raised against the non-conserved peptide of the human orthologue, they did not detect MAPKs in *Drosophila*. Using antibodies recognizing the phosphorylated form of MAPKs impedes analysis and it is to note that detected decreased/increased MAPK phosphorylation could either be a result of true phosphorylation events or due to changes in the total level of the respective MAPK.

The anti phosphoacetyl-H3 antibody recognizes H3 phosphorylated on serine 10 and acetylated on lysine 9. Histone H3 phosphoacetylation resembled the induction pattern of the three MAPKs, increasing after 15 min and maintaining that level even after three hours. On AU gels, histones get separated according to molecular weight and charge; phosphorylation and acetylation of histone H3 retard migration. This enables identification of each additional modification by an upward shift of the "ladder". Western blotting of an acid urea gel resulted in an upshift of the H3 signal indicating penta-acetylation and phosphorylation of histone H3 in response to sorbitol treatment.

In *Drosophila*, cells escaping quiescence and undergoing mitosis cause high background phosphorylation of histone H3. It is thus difficult to distinguish inducible

from background phosphorylation. Hence, just a weak sorbitol dependent increase in H3S10 phosphorylation compared to unstimulated cells was observed, increasing steadily until the three hour time-point (Figure 9 B).

Western blotting of the samples of this time-course with H3K9ac antibodies showed no difference between unstimulated and stimulated cells, indicating that the increase in H3 phosphoacetylation is not caused by global H3K9 acetylation (data not shown).

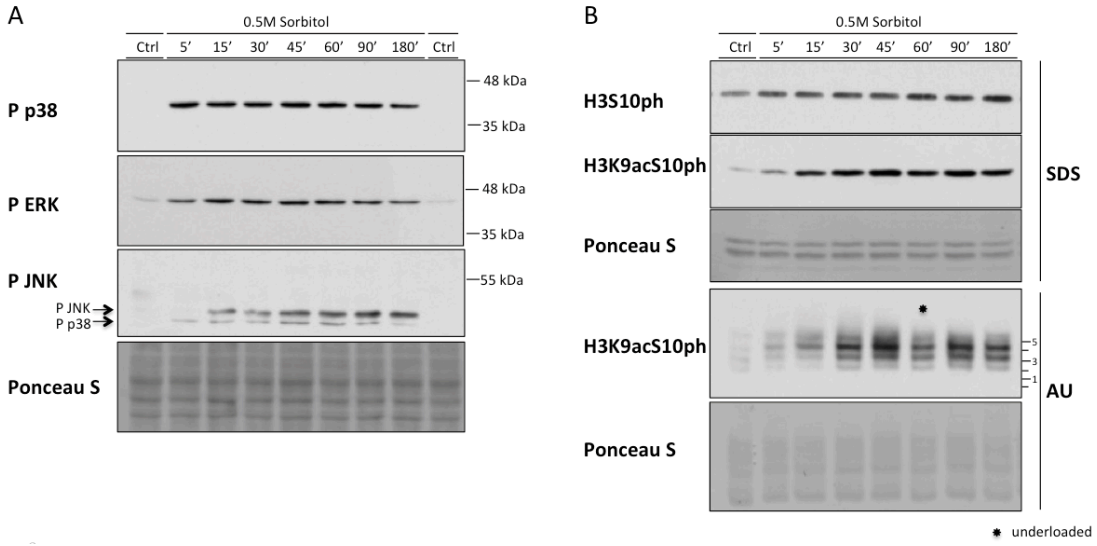


Figure 9: Sorbitol treatment leads to strong phosphorylation of JNK and p38 and phosphoacetylation of histone H3. Quiescent S2 *Drosophila* cells were treated with 0.5 M sorbitol (5 to 180 min). A) High salt lysis extracts were analysed by immunoblotting with anti phospho-p38, -JNK and -ERK antibodies. B) Acid-soluble proteins were extracted and separated on SDS-PAGE or acid-urea (AU) gels. The star indicates that the decrease in intensity is likely to result from under loading of the sample. Western blots were carried out with anti phosphoacetyl-H3 antibodies recognizing H3 phosphorylated on serine 10 and acetylated on lysine 9 or anti phospho-H3 recognizing H3 phosphorylated on serine 10. Positions of histone isoforms are shown on the right with zero being unmodified histone H3. Ctrl indicates the unstimulated control. Ponceau S staining was used as a loading control. All results are representative of at least ten independent experiments.

Based on the previous findings, I suspected that osmotic stress might not cause maximal histone H3 phosphorylation. Therefore, the phosphatase inhibitor sodium fluoride was used to artificially enhance phosphorylation, and thus activity of histone upstream kinases. The maximal histone H3 phosphorylation was observed when cells were treated with 50 mM sodium fluoride for 30 min. To compare the extent of phosphorylation between this artificial stimulus and osmotic stress, S2 cells were

stimulated with 0.5 M sorbitol for 5 to 60 min. On an SDS gel, sodium fluoride treatment caused a greater increase in H3S10ph intensity than sorbitol after one hour, whilst the increase in H3K9acS10ph was similar for both treatments (Figure 10).

On an AU gel, which retards the migration of phosphorylated and acetylated histone H3, the difference between the two treatments becomes clearer. Upon sorbitol treatment for 15 to 60 min, the H3S10ph antibody detected less acetylated histone H3 (band one and two) as well as more acetylated histone H3 at a lower intensity (band three). In response to sodium fluoride, band one and two (less modified histones) increased in intensity without shifting to a higher level on the ladder. When probing for H3K9acS10ph, sorbitol caused an increased retention (shift up to band five), whereas sodium fluoride retention was weaker (shift up to band four). This shows that sorbitol acts on a small fraction of phosphorylated histones which also get acetylated, without affecting global phosphorylation levels.

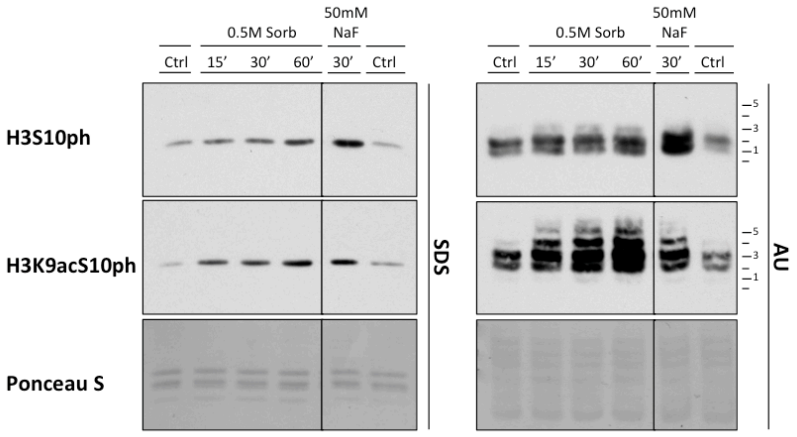


Figure 10: Sodium fluoride treatment activates phosphorylation of histone H3. Quiescent S2 *Drosophila* cells were treated with 0.5 M sorbitol (15 to 60 min) or 50 mM sodium fluoride (30 min). Acid-soluble proteins were extracted and separated on SDS-PAGE (first panel) or AU gels (second panel). Western blots were carried out with anti phosphoacetyl-H3 (H3K9acS10ph) or anti phospho-H3 (H3S10ph) antibodies. Positions of histone isoforms are shown on the right with zero being unmodified histone H3. Ctrl indicates the unstimulated control. Ponceau S staining was used as a loading control. Results are representative of three independent experiments.

The analysis of histone phosphorylation was hindered by high background phosphorylation levels and by the fact that sorbitol does not increase global H3 phosphorylation to a maximum level. However, sorbitol treatment leads to strong phosphoacetylation of histone H3. Therefore, histone H3 phosphoacetylation was used as a read-out for targeted histone H3 phosphorylation and acetylation in the following experiments.

4.2.1.2 MAPK activation in response to sodium arsenite and hydrogen peroxide

As discussed in the introduction, hydrogen peroxide is an oxidative stimulus activating ERK in mammalian cells. The cellular stressor sodium arsenite causes MAPK activation in *Drosophila* cells. To investigate the involvement of MAPK pathways on H3 phosphoacetylation, a specific stimulus for each MAPK had to be found. I therefore considered hydrogen peroxide and sodium arsenite a potential MAPK and histone H3 stimulus. S2 cells were treated with sorbitol for 15 to 90 min, hydrogen peroxide for 15 to 120 min inducing oxidative stress, or sodium arsenite for 30 to 120 min.

In response to hydrogen peroxide, JNK phosphorylation was absent, and very little p38 phosphorylation was detected at 15 and 30 min (Figure 11 A). Both hydrogen peroxide and sodium arsenite are potent ERK activators. Although the effect of hydrogen peroxide on p38 is weak, the activation of ERK at 15 and 30 min was evident. In contrast, sodium arsenite caused strong ERK and p38 phosphorylation at 30 and 60 min. JNK got very weakly phosphorylated after 30 min of sodium arsenite treatment. Moreover, a non-specific JNK band was detected at around 70 kDa which was not detected when using another batch of anti phospho-JNK antibody (see Figure 9).

To analyse the effect of hydrogen peroxide and sodium arsenite on histone H3 phosphoacetylation, blots were probed with an anti H3K9acS10ph antibody. On an SDS gel, strong phosphoacetylation by sorbitol can be seen (Figure 11 B). Hydrogen peroxide treated samples however were not phosphoacetylated above control levels. The increase by sodium arsenite was strong, but less intense than the sorbitol dependent response.

On an AU gel the difference between the stimuli becomes clearer. With hydrogen peroxide there was no increase above control levels, with sorbitol, the bands shifted up to band five, whereas sodium arsenite caused a shift up to tetra-modified histones (band four).

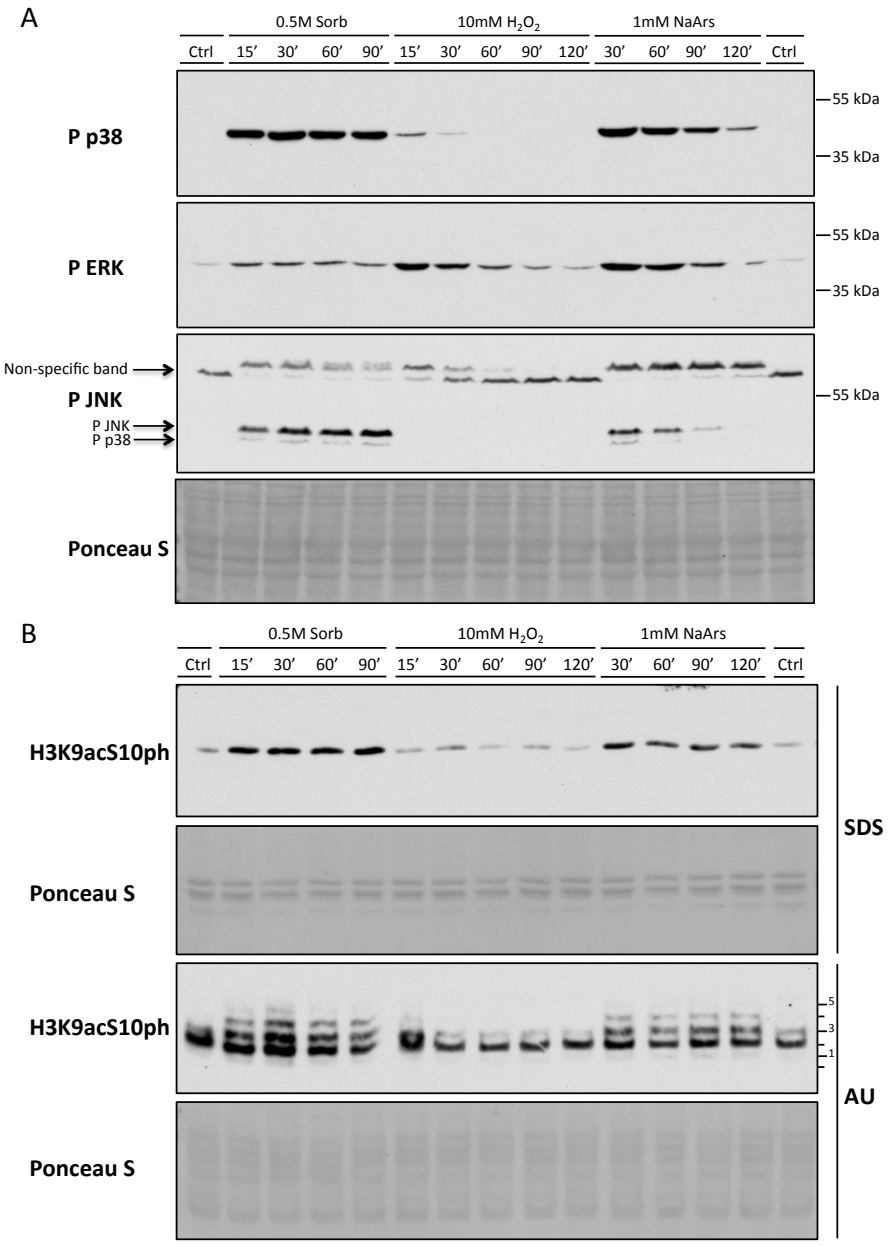


Figure 11: A) In *Drosophila*, hydrogen peroxide treatment leads to ERK phosphorylation, while sodium arsenite leads to ERK and p38 activation. Quiescent S2 *Drosophila* cells were treated with 0.5 M sorbitol (15 to 90 min), 10 mM hydrogen peroxide (15 to 120 min) and 1 mM sodium arsenite (30 to 120 min). High salt lysis extracts were analysed by immunoblotting with anti phospho-p38, -ERK and -JNK antibodies. All results are representative of eight experiments. **B) Sodium arsenite treatment leads to phosphoacetylation of histone H3.** Acid-soluble proteins were extracted and separated on SDS-PAGE or AU gels. Western blots were carried out with anti H3K9acS10ph antibodies. Ctrl indicates the unstimulated control. Ponceau S staining was used as a loading control.

The results for sodium arsenite-induced histone H3 phosphoacetylation were less reproducible than for sorbitol and phosphoacetylation was not always detectable (Figure 12). This experiment was repeated twelve times and an increase in histone H3 phosphoacetylation by sodium arsenite on an AU gel was just observed five times. The results were inconsistent, as a partial increase (peaking at 60 or 90 min) was found twice. A very weak increase of H3 phosphoacetylation, as seen in Figure 12, was detected five times.

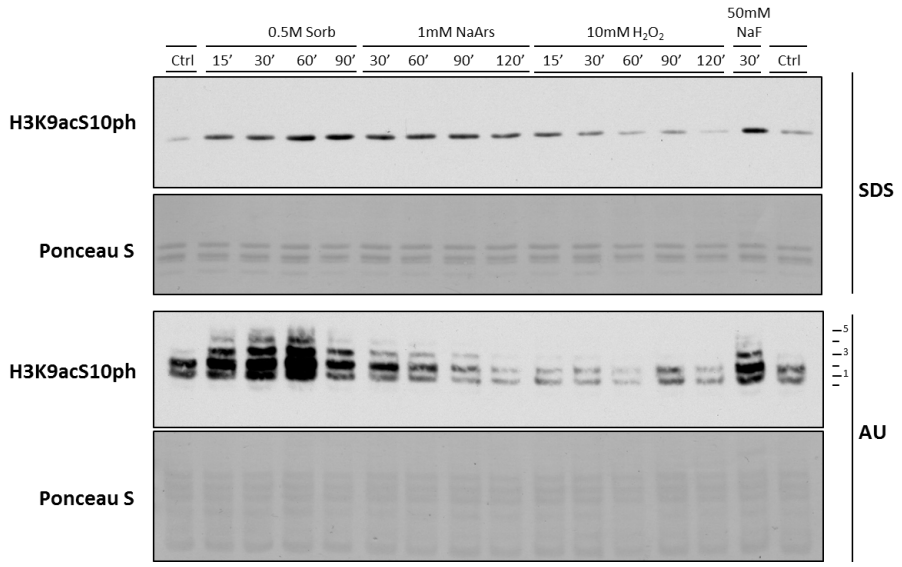


Figure 12: Histone H3 phosphoacetylation in response to sodium arsenite is not consistent. Quiescent S2 *Drosophila* cells were treated with 0.5 M sorbitol (15 to 90 min), 1 mM sodium arsenite (30 to 120 min) or 10 mM hydrogen peroxide (15 to 120 min). Acid-soluble proteins were extracted and separated on SDS-PAGE or AU gels. Western blots were carried out with anti H3K9acS10ph antibodies. Ctrl indicates the unstimulated control. Ponceau S staining was used as a loading control.

4.2.1.3 Insulin dependent ERK activation

Next, I aimed to find an ERK specific stimulus to see whether histone H3 phosphoacetylation is downstream of ERK in *Drosophila*. Insulin possesses growth-promoting activity and is a potent mitogen for cultured cells *in vitro* making it a potential ERK stimulus. *Drosophila* S2 cells were stimulated with 10 µg/ml insulin. In S2 cells, similar to sorbitol, insulin was a weak activator of ERK. ERK phosphorylation was transient, peaking at five and ten min (Figure 13). Insulin did not activate p38 or JNK phosphorylation or histone H3 phosphoacetylation.

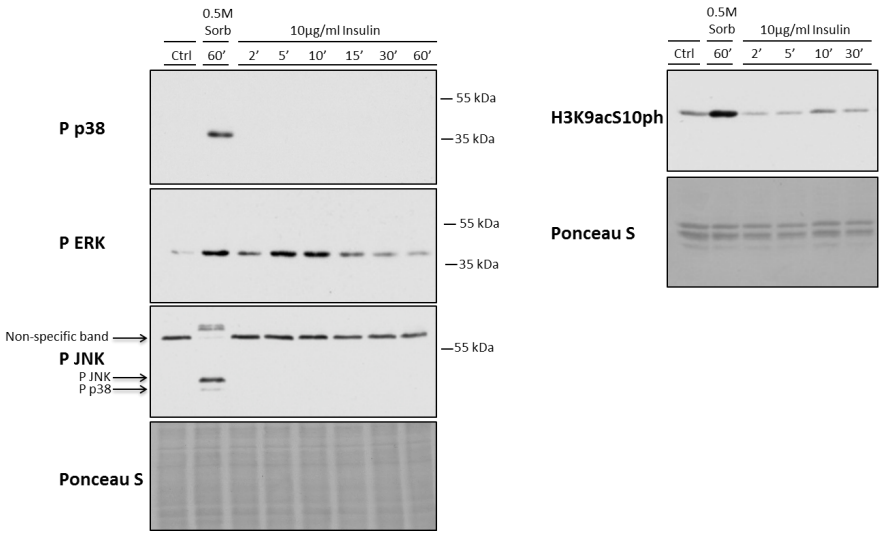


Figure 13: Insulin induces phosphorylation of ERK but not p38, JNK or histone H3. Quiescent S2 *Drosophila* cells were treated with 0.5 M sorbitol (60 min) and 10 µg/ml insulin (2 to 60 min). High salt lysis extracts were analysed by immunoblotting with anti phospho-p38, anti phospho-ERK and anti phospho-JNK antibody (left). Acid-soluble proteins were extracted and separated on SDS-PAGE Western blots were carried out with anti H3K9acS10ph antibodies (right). Ctrl indicates the unstimulated control. Ponceau S staining was used as a loading control. All results are representative of three experiments.

4.2.1.4 ERK activation by alternative stimuli, serum and heat shock

To confirm the lack of histone H3 phosphoacetylation despite ERK activation, I tested two alternative ERK stimuli, foetal calf serum and heat shock.

Foetal calf serum contains a variety of components including inorganic salts, carbohydrates, amino acids, growth factors and bovine serum albumin (BSA). The following growth factors are expected to be present in any serum; IGF, insulin-like growth factor-binding protein (IGFBP), transforming growth factor β1 (TGF-β), basic fibroblast growth factor (bFGF) and glial growth factor (GGF) (Zheng et al., 2006). According to Cherbas *et al.* EGF receptors are expressed in S2 cells, enabling them to respond to growth factor mediated activation (Cherbas et al., 2011). Serum can thus be used as a generic growth factor stimulus and *Drosophila* cells were treated with 15% serum for 5 to 60 min. Serum induced ERK, but not p38, JNK and histone H3 phosphorylation (Figure 14 A). Serum changes the physiochemical properties of the cell culture media, such as viscosity, osmolality and buffering capacity. When cells were treated with an equivalent concentration of BSA that was present in the serum used (0.3% BSA), no response was observed; indicating that serum dependent ERK activation was not caused by BSA (data not shown).

Based on the contradicting data discussed in the introduction, I was intrigued to analyse the effect of heat shock in S2 cells. S2 cells were subjected to instant heat shock and transferred from a 26°C to a 37°C incubator for 15 to 60 min. This heat shock caused specific phosphorylation of ERK but did not markedly activate any other MAPK or histone H3 phosphoacetylation (Figure 14 B).

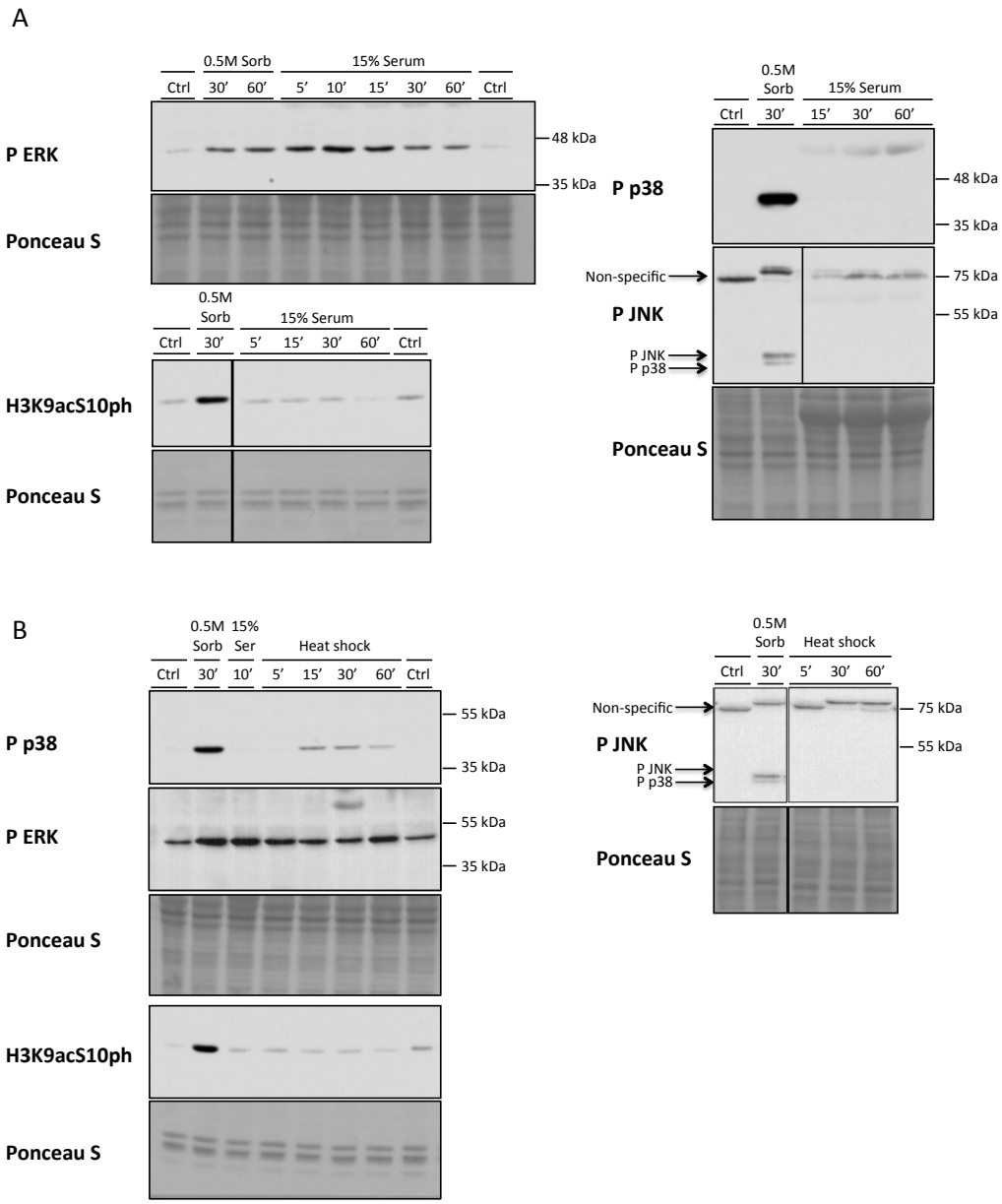


Figure 14: A) ERK phosphorylation is activated by 15% serum. Quiescent S2 *Drosophila* cells were treated with 0.5 M sorbitol (30 and 60 min) and 15% serum (5 to 60 min) as indicated. **B) ERK phosphorylation in response to heat shock at 37°C.** Quiescent S2 *Drosophila* cells were treated with 0.5 M sorbitol (30 min), 15% serum (ten min) and heat shock at 37°C (5 to 60 min). High salt lysis extracts were analysed by immunoblotting with anti phospho-p38, anti phospho-JNK and anti phospho-ERK antibodies. Acid-soluble proteins were extracted and separated on SDS-PAGE Western blots were carried out with anti H3K9acS10ph antibodies. Ctrl indicates the unstimulated control. Ponceau S staining was used as a loading control. Figure A is representative of at least five independent experiments. Figure B is representative of three independent replicates.

In conclusion, these findings show that ERK phosphorylation is specifically stimulated by insulin, serum and heat shock. However, none of these stimuli resulted

in H3 phosphoacetylation. I therefore aimed to investigate the activation of JNK further.

4.2.1.5 Peptidoglycan and lipopolysaccharide dependent JNK activation

To confirm JNK activation in response to an immune challenge and to establish if histone H3 gets phosphoacetylated, quiescent S2 cells were stimulated with LPS and PGN (Figure 15). Sorbitol stimulation was used as a control to compare the extent of phosphorylation.

Upon sorbitol stimulation, which activates all MAPKs, a doublet indicative of phospho-JNK and phospho-p38 was observed due to cross-reactivity between the anti phospho-JNK antibody and p38. Only a single band was seen after treatment with LPS or PGN. This allowed the identification of the upper band as JNK, because this protein was phosphorylated upon treatment with LPS or PGN. In addition, according to their molecular weight, p38a and p38b are expected to run at around 42 kDa while JNK should run at 43 kDa.

The induction of JNK in response to LPS and PGN was rapid and transient, with a peak at 15 min and a loss of JNK phosphorylation after 60 min. LPS and PGN induced specific phosphorylation of JNK, since neither ERK, p38 nor histone H3 were phosphorylated.

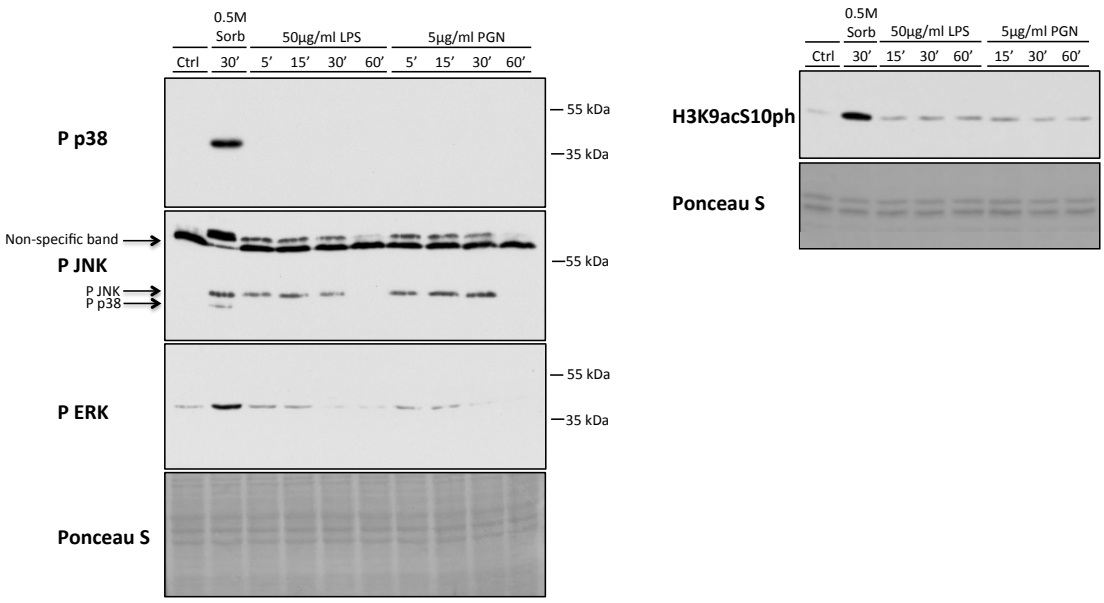


Figure 15: Lipopolysaccharide and peptidoglycan induce JNK phosphorylation transiently in *Drosophila*. p38 and ERK phosphorylation as well as histone H3 phosphoacetylation were not activated by LPS or PGN. Quiescent S2 *Drosophila* cells were treated with 0.5 M sorbitol (30 min), 50 µg/ml lipopolysaccharide or 5 µg/ml peptidoglycan (5 to 60 min). High salt lysis extracts were analysed by immunoblotting with anti phospho-p38, anti phospho-JNK and anti phospho-ERK antibody. Acid-soluble proteins were extracted and separated on SDS-PAGE. Western blots were carried out with anti H3K9acS10ph antibodies. Ctrl indicates the unstimulated control and Ponceau S staining was used as a loading control. All results are representative of five independent experiments.

In conclusion, the experiments presented here showed that p38 was activated by sorbitol and sodium arsenite, JNK was activated by sorbitol and peptidoglycan whereas ERK was activated by sorbitol, sodium arsenite, hydrogen peroxide, serum, insulin and heat shock. Histone H3 phosphoacetylation was activated by sorbitol and sodium arsenite. Table 2 summarizes the performed experiments and indicates the phosphorylation level of MAPKs and histone H3 in response to the tested stimuli.

Table 2: Summary of MAPK and histone H3 phosphoacetylation in response to the tested stimuli. A black tick indicates a strong, while a grey tick represents a weak phosphorylation event. A cross illustrates the absence of any phosphorylation above background levels.

	Sorbitol	Sodium Arsenite	H_2O_2	Insulin/ Serum	Heat shock	PGN/ LPS
p38	✔	✔	✔	✗	✔	✗
JNK	✔	✔	✗	✗	✗	✔
ERK	✔	✔	✔	✔	✔	✗
Histone H3	✔	✔	✗	✗	✗	✗

4.2.2 Characterisation of immediate early gene induction in *Drosophila*

4.2.2.1 Hydrogen peroxide and peptidoglycan dependent *jra* induction

A RNA probe was designed against *jra,* and its expression was analysed by Northern blot. Similar to the previously performed time-course for protein extraction, I treated S2 cells after 24 hours quiescence with the strongest MAPK stimuli (sorbitol, sodium arsenite, hydrogen peroxide, insulin and peptidoglycan).

In *Drosophila* S2 cells, hydrogen peroxide (a strong ERK activator) and peptidoglycan (a strong JNK activator) induced *jra* gene expression after only 30 min of treatment (Figure 16). Neither insulin nor sodium arsenite induced *jra*. The strongest histone H3 stimulus I previously identified, sorbitol, did not show any *jra* induction at 15 to 120 min (data not shown). It is to note that in this blot, the *jra* probe detects a presumably non-specific band just above *jra*. Ribosomal protein *rp49* was used as a loading control and showed equal loading for all conditions.

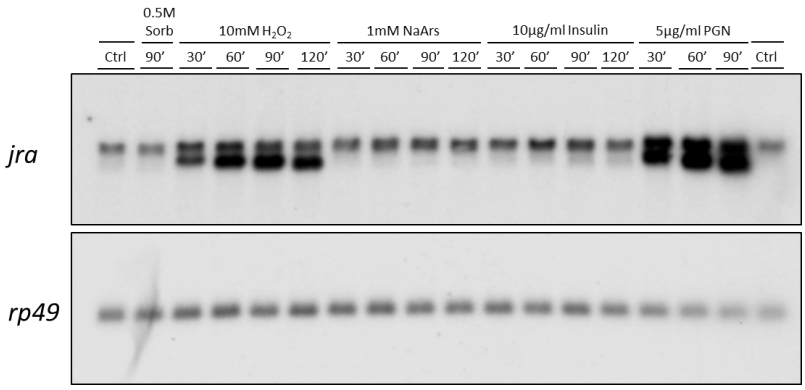


Figure 16: *Jra* induction in response to hydrogen peroxide and peptidoglycan treatments. Quiescent S2 *Drosophila* cells were treated with 0.5 M sorbitol (90 min), 10 mM H_2O_2 (30 to 120 min), 1 mM sodium arsenite (30 to 120 min), 10 µg/ml insulin (30 to 120 min) or 5 µg/ml PGN (30 to 90 min). Total RNA was extracted from *Drosophila* S2 cells and analysed by Northern blotting using a *jra* probe. Stripping and rehybridization of the membrane with a *rp49* probe showed equal loading in each lane. Ctrl indicates the unstimulated control. This figure is representative of three independent replicates.

The dsRNA probe used in this study might also detect antisense non-coding RNA instead or in addition of *jra* mRNA.

4.2.2.2 Expression of *jra* in presence of translational inhibitor emetine

To confirm *jra* is an immediate early gene in *Drosophila*, cells were treated with either hydrogen peroxide or peptidoglycan in the presence of the translational inhibitor emetine. Emetine binds to the 40S subunit and stabilizes polysomes. Emetine is a fast-acting translational inhibitor and it is thought that simultaneous treatment with a stimulus is enough to block *de novo* protein synthesis. Figure 17 shows that *jra* was induced, even when cells were treated with emetine just before adding the stimulus for 30 or 60 min. For peptidoglycan stimulated cells, the addition of emetine caused an enhanced *jra* induction. *jra,* a putative IE gene in *Drosophila*, is induced by hydrogen peroxide and peptidoglycan and is still transcribed in the presence of translational inhibitor emetine.

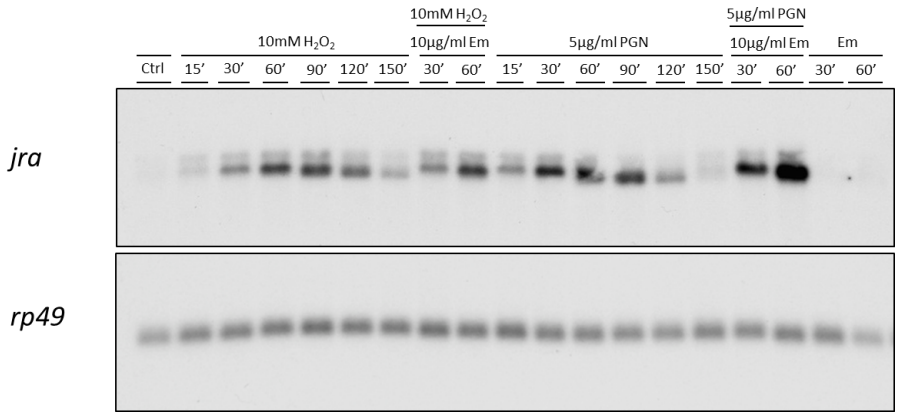


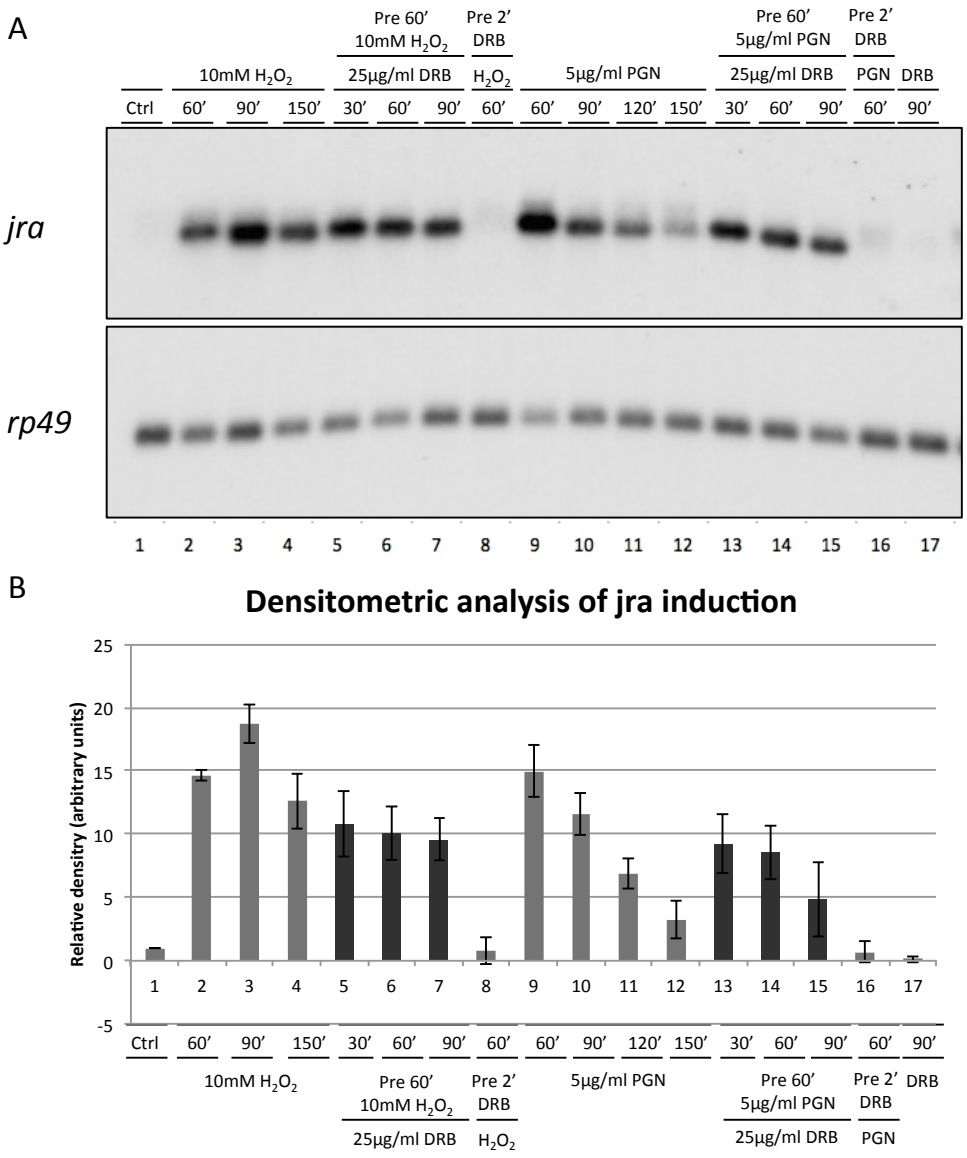
Figure 17: *Jra* in presence of protein synthesis inhibitor emetine: 10 mM hydrogen peroxide and 5 µg/ml PGN induce *jra* expression, independent of pre-treatment with emetine. Quiescent S2 *Drosophila* cells were treated with 10 mM H_2O_2 (15 to 150 min) or 5 µg/ml PGN (15 to 150 min) Where indicated, cells were simultaneously treated with 10 µg/ml translational inhibitor emetine for 30 and 60 min. Total RNA was extracted from *Drosophila* S2 cells and analysed by Northern blotting using the indicated probes. Stripping and rehybridization of the membrane with a rp49 probe showed equal loading in each lane. Ctrl indicates the unstimulated control. This figure is representative of three independent replicates.

4.2.2.3 Half-life and stability of *jra* mRNA

For a more detailed characterization of *jra*, its half-life and stability were investigated. *Drosophila* cells were treated with hydrogen peroxide for 60, 90 and 150 min (Figure 18 A). After one hour of pre-treatment with hydrogen peroxide, DRB was added to inhibit any further transcription and cells were harvested at several time-points thereafter (30, 60 and 90 min; summing up to a total of 60, 90 and 120 min of hydrogen peroxide treatment). Despite the absence of newly formed transcripts, *jra* was still detected 90 min after DRB was added. When cells were pre-treated with DRB for two min prior to adding hydrogen peroxide for another 60 min, *jra* was not transcribed. Hence, the used concentration of DRB blocked transcription completely.

The same experimental set-up was applied for treatment with peptidoglycan and similarly, addition of DRB after 60 min of peptidoglycan stimulation did not ablate *jra* mRNA. On the contrary, peptidoglycan induced *jra* seemed to be more stable in the presence of DRB.

To determine the half-life of *jra,* a densitometric analysis was performed (Figure 18 B). Band intensities were quantified using Image J and the half-life of *jra* was calculated. With a linear regression analysis, the decay rate constant was obtained from the slope of a semilogarithmic plot of mRNA concentration as a function of time. The median half-life ($t^1/_2$) of *jra* mRNA was 138 min.



$T_{1/2m}$ = 138min (calculated *jra* half-life based on densitometric analysis)

Figure 18: A) *jra* mRNA expression after treatment with transcriptional inhibitor DRB and stimulation with hydrogen peroxide or peptidoglycan. Quiescent S2 *Drosophila* cells were treated with 10 mM H_2O_2 (60 to 150 min, lane 2-4) and 5 µg/ml PGN (60 to150 min, lane 9-12). Where indicated, cells were pre-treated for 60 min with 10 mM H_2O_2 (lane 5-7) or 5 µg/ml PGN (lane 13-15). After pre-treatment, cells were inhibited with 25 µg/ml DRB for a further 30, 60 and 90 min. Cells were also pre-treated with 25 µg/ml DRB for 2 min before stimulating either with 10 mM H_2O_2 (lane 8) or 5 µg/ml PGN (lane 16) for 60 min. In lane 17, cells were treated with 25 µg/ml DRB for 90 min alone. Total RNA was extracted from *Drosophila* S2 cells and analysed by Northern blotting using a *jra* probe. Stripping and rehybridization of the membrane with a rp49 probe showed equal loading in each lane. Ctrl indicates the unstimulated control (lane 1). This figure is representative of three independent experiments. **B) Densitometric analysis and half-life of *jra* in *Drosophila* S2 cells.** mRNA levels were assessed by Northern blot using *jra* probes and stability of *jra* mRNAs was evaluated by measuring steady-state mRNA levels after the addition of 25 µg/ml DRB. Band intensities were quantified using Image J. The half-life of *jra* was calculated by plotting densitometric data from lanes 2,5,6,7 and 9,13,14,15 (arbitrary units). With linear regression analysis, the decay rate constant was obtained from the slope of a semilogarithmic plot of mRNA concentration as a function of time. The mean and standard error (error bars) were calculated based on three separate experiments and the median half-life of *jra* mRNA was calculated with the following equation $t_{1/2}=\ln 2/k_{decay}$ (Chen et al., 2008).

4.2.3 Identification of MAPK pathway upstream of histone H3 and *jra* gene induction by the use of inhibitors

So far only p38 phosphorylation parallels histone H3 phosphoacetylation, indicating that these two events are mechanistically linked. With the availability of small kinase inhibitors, it is straightforward to investigate the relationship between MAPK, histone H3 and *jra* induction. Inhibitor SB203580 binds to and blocks p38, PD0325901 specifically blocks the activation of MEK1/2/5 and hence the ERK cascade, while SP600125 inhibits JNK (Figure 19).

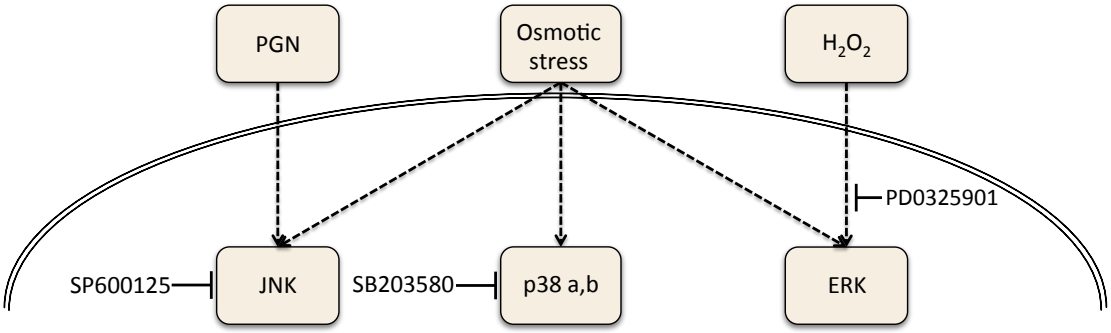


Figure 19: Schematic representation of MAPK inhibitors SP600125, SB203580 and PD0325901 and their targets. Osmotic stress activates all three MAPKs, peptidoglycan specifically activates JNK and hydrogen peroxide specifically activates ERK. For simplicity the upstream kinases such as MAP2Ks and MAP3Ks are not represented. The inhibitors used in this study are indicated: PD0325901 and SP600125 prevent the phosphorylation of MEK1/2/5 and JNK, respectively. SB203580 blocks the kinase activity of p38.

4.2.3.1 Histone H3 phosphoacetylation in presence of MAPK inhibitors

Drosophila cells were incubated with sorbitol for 60 min and as indicated, cells were pre-treated for 30 min either with SB203580, PD0325901 or SP600125 (Figure 20). To show that inhibitors alone do not alter phosphorylation states, cells were incubated in the presence of inhibitors without stimulation.

Phosphorylation levels of p38 and JNK did not change in the presence of any inhibitor. Both tested concentrations of PD0325901 (0.1 µM and 2 µM) inhibited phosphorylation of ERK completely. Histone H3 phosphoacetylation decreased in intensity and shifted down from band five to band four in SB203580 and PD0325901 treated cells. SP600125 caused a major loss of histone H3 phosphoacetylation.

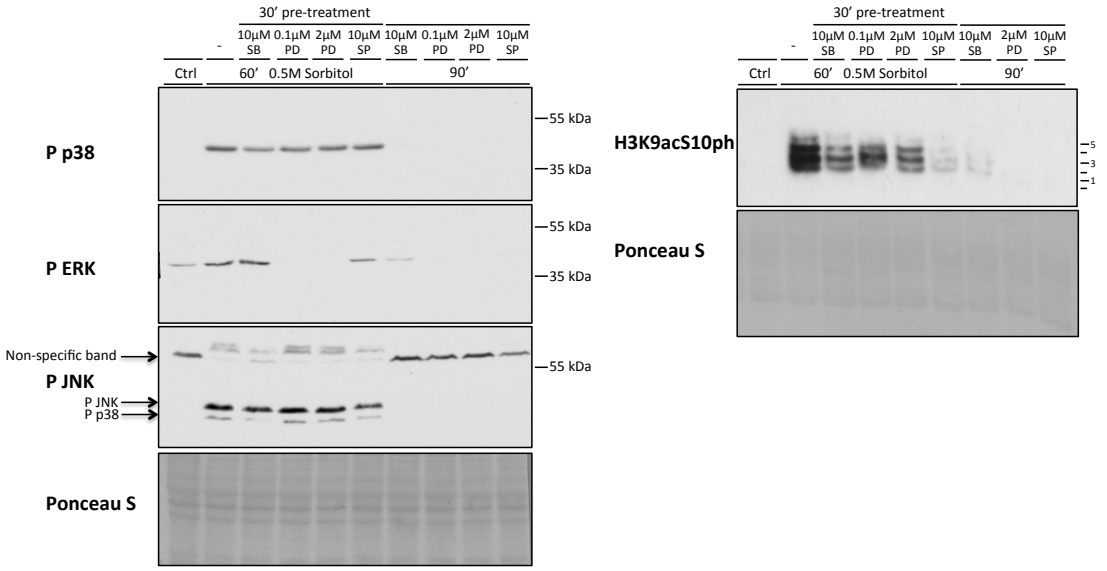


Figure 20: Sorbitol induced ERK phosphorylation is inhibited by PD0325901, while SP600125 reduced histone H3 phosphoacetylation. Quiescent S2 *Drosophila* cells were pre-treated for 30 min with 10 µM p38 inhibitor SB203580, 0.1 µM or 2 µM MEK inhibitor PD0325901, or 10 µM JNK inhibitor SP600125 followed by treatment with 0.5 M sorbitol (60 min). Cells were also treated for 90 min with 10 µM SB203580, 0.1 µM or 2 µM PD0325901 or 10 µM SP600125. High salt lysis extracts were analysed by immunoblotting with anti phospho-p38, anti phospho-ERK and anti phospho-JNK antibodies. Acid-soluble proteins were extracted and separated on SDS-PAGE gels. Western blots were carried out with anti H3K9acS10ph antibodies. Ctrl indicates the unstimulated control. Ponceau S staining was used as a loading control. This figure is representative of four experiments.

4.2.3.2 Dissecting MAPK pathways leading to *jra* induction

In order to identify the MAPK pathway leading to *jra* induction by peptidoglycan and hydrogen peroxide, all three MAPKs in presence of either stimulus were inhibited. *Drosophila* cells were stimulated with 10 mM hydrogen peroxide for 15 min and either pre-treated with SB203580, PD0325901 or SP600125 (Figure 21 A). Similarly, 5 µg/ml peptidoglycan were incubated for 15 min with one of the three inhibitors, respectively.

Western blot analysis revealed that despite generally low phosphorylation levels of p38 in response to hydrogen peroxide, p38 phosphorylation is increased upon inhibition with SB203580 or SP600125. Peptidoglycan does not usually activate p38 (Figure 15). However, pre-treatment with SB203580 and subsequent stimulation with peptidoglycan resulted in a slight p38 phosphorylation. In peptidoglycan stimulated cells, JNK phosphorylation was inhibited almost completely by JNK inhibitor SP600125, while the other inhibitors had no effect. MEK inhibitor PD0325901 specifically inhibited ERK in presence of hydrogen peroxide. However, no significant change in ERK phosphorylation was observed with SB203580 or SP600125. For all three MAPKs, the sorbitol response was unaltered, while no phosphorylation was caused by inhibitor treatment alone.

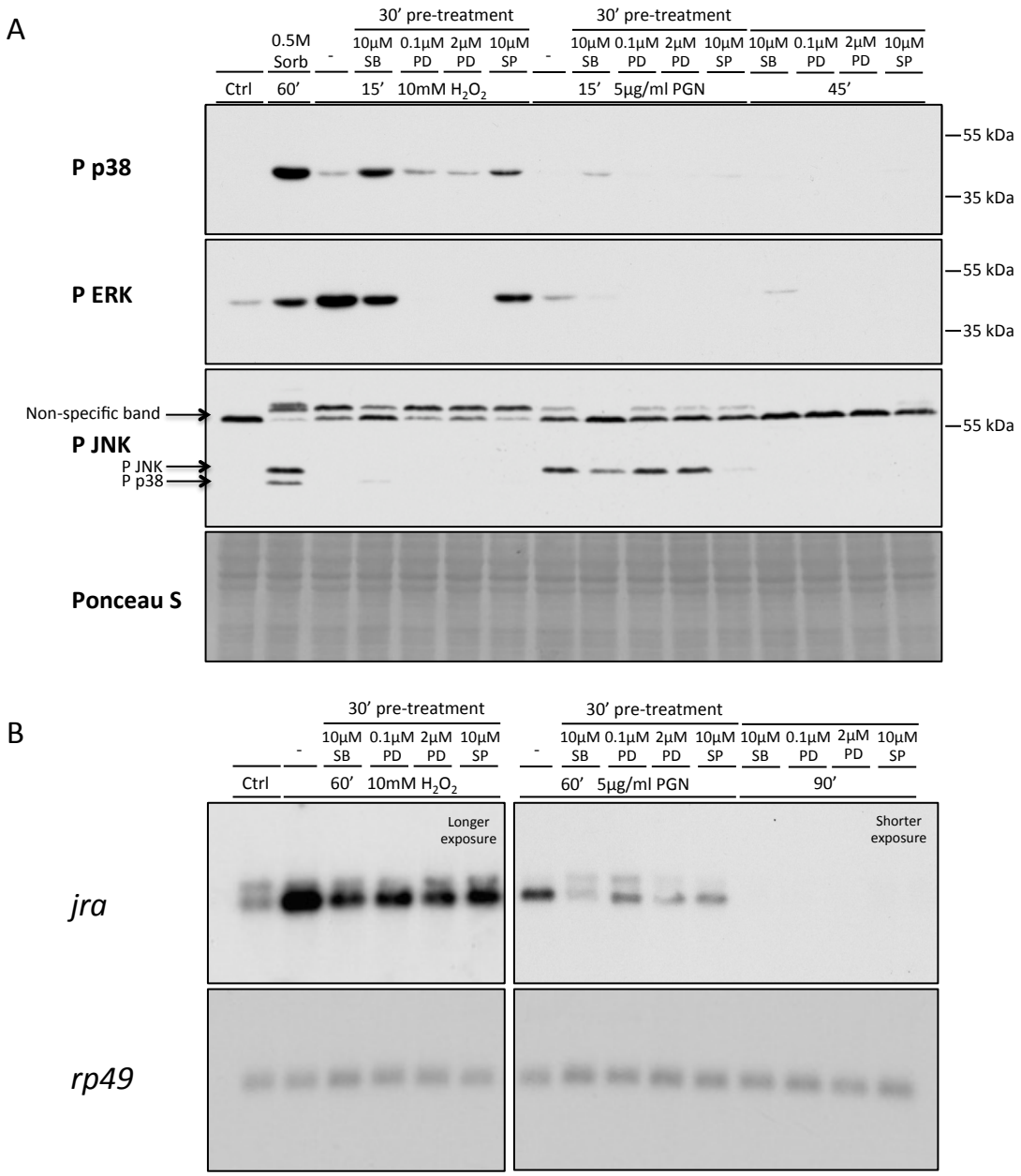


Figure 21: A) Hydrogen peroxide induced ERK phosphorylation is inhibited by PD0325901 and peptidoglycan induced JNK phosphorylation is inhibited by SP600125. Quiescent S2 *Drosophila* cells were treated for 60 min with 0.5 M sorbitol. As indicated, cells were pre-treated for 30 min with 10 μM p38 inhibitor SB203580, 0.1 μM or 2 μM MEK inhibitor PD0325901, 10 μM JNK inhibitor SP600125 followed by treatment with 10 mM H_2O_2 (15 min) or 5 μg/ml PGN (15 min). Cells were also treated for 45 min with 10 μM SB203580, 0.1 μM or 2 μM PD0325901 or 10 μM SP600125 alone. High salt lysis extracts were probed using antibodies against phospho-p38, phospho-ERK and phospho-JNK. Ctrl indicates the unstimulated control and Ponceau S staining was used as a loading control. **B) All MAPK inhibitors decrease *jra* gene induction.** 24h quiesced S2 *Drosophila* cells were pre-treated for 30 min with 10 μM p38 inhibitor SB203580, 0.1 μM or 2 μM MEK inhibitor PD0325901, 10 μM JNK inhibitor SP600125 followed by treatment with 10 mM H_2O_2 (60 min) or 5 μg/ml PGN (60 min). In addition, control cells were treated with MAPK inhibitors for 90 min with 10 μM SB203580, 0.1 μM or 2 μM PD0325901 or 10 μM SP600125 alone. Total RNA was extracted from *Drosophila* S2 cells and analysed by Northern blotting using a *jra* probe. Stripping and rehybridization of the membrane with a *rp49* probe showed equal loading in each lane. Two different exposures are shown for *jra* in response to H_2O_2 and PGN due to different strengths of induction. This figure is representative of two independent experiments.

In a similar time-course RNA, instead of protein, was harvested (Figure 21 B). Northern blot analysis showed a robust induction of *jra* mRNA in response to both stimuli after 60 min. For hydrogen peroxide treated cells a longer and hence darker exposure is shown, while for peptidoglycan a shorter exposure was sufficient to show induction. Whereas MAPK inhibitors SB203580, PD0325901 or SP600125 just caused a slight decrease of hydrogen peroxide-induced *jra* expression in all conditions, peptidoglycan induced *jra* was severely impaired in the presence of all inhibitors. Inhibitors show a stronger effect on peptidoglycan induced *jra* than on hydrogen peroxide induced *jra* expression.

4.3 Discussion

4.3.1 Specific MAPK activation

The objective of this study was to determine if a relationship exists between an extracellular stimulus, MAPK activation, histone H3 phosphorylation and IE gene induction. Stimuli were mainly used as a mechanism to activate MAPKs individually and to dissect the pathways leading to histone phosphorylation. Consequently, the detailed mode of action of each stimulus will not be discussed extensively.

4.3.1.1 Activation of all three MAPKs and histone H3 phosphoacetylation by sorbitol

As discussed in the introduction, it has been reported that p38 and JNK are activated in hyperosmolar conditions (Inoue et al., 2001, Zhuang et al., 2006, Chen et al., 2002). When treating with 0.5 M sorbitol, ERK and histone H3 get phosphorylated in addition to JNK and p38 (Figure 9). For all MAPKs, both the threonine as well as the tyrosine residue need to be phosphorylated for activation. In this study, phosphorylated MAPKs are thus representative of their activated forms.

In *Drosophila*, three p38 genes exist (p38a, b and c). The anti phospho-p38 antibody detects the dual phosphorylation site on threonine and tyrosine. In *Drosophila* these phosphorylation sites are present on gene p38a and p38b, but not on p38c which is not detected by Western blot in this study. It is not possible to distinguish between p38a and p38b due to their similar size. When probing for *p38a* and *p38b* mRNA in *Drosophila*, only *p38b* could be detected suggesting that the band picked up by Western blot might be p38b (appendix A.2). Confirming this assumption, according to Craig *et al.* the main kinase mediating the osmotic stress response might be p38b (Craig et al., 2004, Belozerov et al., 2012, Han et al., 1998a).

The activation of JNK in response to osmotic stress has been reported previously and its stimulation in *Drosophila* was confirmed by my results (Rosette and Karin, 1996, Chen et al., 2002). Three out of four JNK isoforms could potentially be detected by the phospho-specific antibody, as their size matches the detected protein and they have the Thr-Pro-Tyr phosphorylation site.

All six transcripts of ERK have a Thr-Glu-Tyr site and could theoretically get detected by the anti phospho-ERK antibody, but just three isoforms have a molecular weight that correlates with the size of the band picked up by the antibody. It is not published whether the other three isoforms are actually translated and functional. ERK phosphorylation occurred after five min and increased until 15 min, although activation is weak compared to hydrogen peroxide or sodium arsenite stimulation (Figure 11). A mildly activated ERK pathway could still be essential in controlling histone phosphoacetylation, independent of the strength of MAPK activation. In *Drosophila*, ERK phosphorylation upon osmotic stress has not been reported hitherto. However, in human epithelial cells, ERK is involved in the osmotic stress response and able to induce IE gene induction (Cohen, 1996, Burg et al., 2007).

Even though sorbitol is a strong stimulus for MAPKs and histone H3 phosphoacetylation, the level of H3 phosphorylation did not increase substantially. Background phosphorylation was high in in quiescent *Drosophila* S2 cells, making it difficult to analyse inducible histone H3 phosphorylation. After serum reduction (0.1% FBS) for 48 hours, around 5% of cells were still able to escape quiescence. As previously reported, the different background level of H3 phosphorylation and phosphoacetylation was a result of the high mitotic background phosphorylation in *Drosophila* S2 cells (Regnard et al., 2011).

The population of histones targeted for phosphoacetylation was smaller, more specific and thus easier to detect by Western blot than the histones that are also targeted for global mitotic histone H3 phosphorylation. The increase in sorbitol dependent histone H3 phosphoacetylation indicates activity of both a histone kinase and a HAT. The dynamic model suggests independent regulation of phosphorylation and acetylation. It would be plausible that, as previously suggested, histone H3 phosphorylation recruits a HAT *via* 14-3-3 binding to H3S10ph (Karam et al., 2010). Alternatively, a sorbitol dependent kinase activation could lead to an increase in HAT recruitment, for example CREB could activate p300/CBP to target H3.

Sodium fluoride, a non-specific phosphoseryl and phosphothreonyl phosphatase inhibitor, is able to cause an increase in histone H3 phosphorylation. On H3K9acS10ph blots, sorbitol stimulation causes one more acetylation event (shift to band five) than sodium fluoride (shift to band four). The different phosphoacetylation pattern caused by sodium fluoride suggests that the sorbitol induced phosphoacetylation event is, if it is, not solely mediated by phosphatase inactivation. Despite the information obtained from AU gels (a shift corresponding to phosphorylation and acetylation), it is not possible to identify which band correlates to which modification (Figure 10).

4.3.1.2 Sodium arsenite dependent p38, ERK phosphorylation and histone H3 phosphoacetylation

In S2 cells, p38, ERK and histone H3 were phosphorylated in response to sodium arsenite. Unlike with sorbitol stimulation, sodium arsenite induced histone H3 phosphoacetylation was inconsistent and hence not an ideal stimulus for *Drosophila* S2 cells. The cause of this variability has not been identified, but might be related to quiescence time or extraction method of histones. Perhaps sodium arsenite only activates a small fraction of a putative histone kinase, for example JIL-1, while sorbitol activates the putative histone kinases strongly, ensuing consistently strong histone H3 phosphoacetylation.

Regardless of consistently strong p38 and ERK phosphorylation, H3 phosphoacetylation was not always reproducible. If the inconsistency was biologically relevant and not due to methodological limitations, it would point against the existence of a MAPK regulated histone kinase in *Drosophila.* Moreover, although p38 and ERK are strongly induced by sodium arsenite, it was impossible to deduce whether histone H3 phosphoacetylation is downstream of one of the two MAPKs. An ERK specific stimulus was thus missing.

4.3.1.3 ERK activation with no effect on histone H3 phosphoacetylation

4.3.1.3.1 Hydrogen peroxide dependent ERK activation

As previously published, hydrogen peroxide activates ERK in *Drosophila* cells although no effect on histone H3 was observed. ROS is classically known as an activator of MAP3K (Hayakawa et al., 2006). Although *Drosophila* ASK1 is also upstream of p38 and JNK, I could not detect JNK phosphorylation and just very little p38 phosphorylation (Figure 10).

In *Drosophila* S2 cells, exogenously administered hydrogen peroxide is less effective than it would be expected considering the high concentration (10 mM) used in this study. The actual concentration of hydrogen peroxide in S2 cells cannot be directly measured. Final intracellular concentration of hydrogen peroxide is estimated to be lower than the 10 mM H_2O_2 that were added to the dish. Hydrogen peroxide is a nonpolar molecule and, although it can diffuse across membranes, it might diffuse slowly (Gough and Cotter, 2011). Moreover, antioxidants can decrease hydrogen peroxide abundance, such as intrinsic catalase, degrading H_2O_2 into H_2O and O_2 (Veal et al., 2007). This statement was supported by the finding that addition of the catalase inhibitor aminotriazole enhances the effect of H_2O_2 in *Drosophila* Kc167 cells (Courgeon et al., 1988).

4.3.1.3.2 Insulin dependent ERK activation

Even though hydrogen peroxide is a strong ERK stimulus, it is not solely activating ERK as a small amount of p38 also gets activated. My objective in this chapter was to dissect the MAPK signalling pathway down to histone phosphorylation, so I searched for a stimulus specifically activating ERK. Previous studies have shown that H_2O_2 might act as an insulin-mimetic (Czech et al., 1974, Lee et al., 1996). Vice versa, it

has been reported that insulin might stimulate endogenous ROS production activating ERK.

Analogous to mammalian cells, insulin-like growth factor activates MAPK and PI-3K pathways in *Drosophila* (Anselmo et al., 2002a, Friedman and Perrimon, 2006). ERK is downstream of at least five receptor tyrosine kinases. Most of the ligands have to be cleaved or post-translationally modified and are not commercially available. Hence, insulin was used as a ligand upstream of receptor tyrosine kinases to stimulate ERK phosphorylation specifically. ERK phosphorylation was greatest at 10 to 15 min, while histone H3 and other MAPKs are unaffected by insulin stimulation (Figure 13).

4.3.1.3.3 Serum and heat shock dependent ERK activation

Stimulation with serum caused ERK, but not p38, JNK phosphorylation or histone H3 phosphoacetylation (Figure 14 A). Which factor caused ERK phosphorylation cannot be determined, but it is likely that the combination of growth factors activates the ERK pathway. Independent of the mechanism, it can be concluded that there is no histone H3 phosphoacetylation in presence of activated ERK (Figure 14 A).

Heat shock mainly caused ERK phosphorylation in waves at 5 and 60 min. This phosphorylation was comparable to sorbitol and serum stimulation in strength. In murine cells, heat shock has been shown to activate ERK and JNK just after 60 min (Ng and Bogoyevitch, 2000). As for hydrogen peroxide, there was weak p38 activation, but no effect on JNK or histone H3 (Figure 14 B). Based on my findings and non-specific cross-reactivity of the antibody used in Nowak *et al.*, it can be assumed that H3K9acS10ph does not accumulate at actively transcribing heat shock puffs (Johansen et al., 2009, Nowak and Corces, 2000a). Also, despite global H3

dephosphorylation, I could not observe a decrease below base levels of histone H3 phosphoacetylation as this modification targets a specific small fraction of histones.

4.3.1.4 Peptidoglycan dependent JNK activation with no effect on histone H3 phosphoacetylation

Treating *Drosophila* S2 cells with LPS and gram-negative PGN activated JNK phosphorylation transiently, but no other MAPKs or histone H3 (Figure 15). A ten times higher concentration of LPS elicits the same JNK response as 5 μg/ml PGN. This is in agreement with Kaneko *et al.*, who have shown that LPS is inactive in *Drosophila* and that IMD pathway activation is due to contaminating PGN present in commercial LPS (Kaneko et al., 2004). JNK phosphorylation is transient, peaking at 15 min and declining at 60 min, most likely due to increased expression of phosphatase puckered (Martin-Blanco et al., 1998). Activation of p38 was reported earlier upon immune challenge (Seong et al., 2012, Chen et al., 2010). This discrepancy of variable p38 activation in S2 cells in response to PGN was attributed to the different properties S2 cells acquired since their isolation in 1972 (Han et al., 1998b).

The observation that neither JNK nor ERK activation correlates with histone H3 phosphoacetylation strengthens the link between p38 activation and histone H3. Although, alternatively, the fraction of histones surrounding ERK or JNK induced IE genes could be too small to be detected by Western blot.

4.3.2 Immediate early gene *jra* in *Drosophila*

4.3.2.1 Stress stimulus hydrogen peroxide and immune challenge with peptidoglycan induce *jra*

Based on the assumption that an extracellular stimulus activates a MAPK pathway leading to histone H3 phosphoacetylation at the promoter of an IE gene concomitant with transcription, I was curious which stimulus would cause IE gene induction. In *Drosophila* no IE genes have been identified and it is unknown whether this paradigm, the nucleosomal response, holds true. In mammals, the most well known and best studied IE gene is c*jun*. I therefore aimed to study the behaviour of its orthologue *jra* in *Drosophila.*

Sorbitol activates p38, JNK, ERK and histone H3 phosphoacetylation stronger than any other stimulus. If *jra* transcription were regulated by MAPKs like c*jun*, an increase of *jra* transcripts in presence of sorbitol would have been expected. But despite strong p38 and histone H3 phosphoacetylation, no *jra* transcripts were detected. Moreover, sorbitol activates JNK and would hence be expected to stabilize Jra by phosphorylation, which would then induce *jra* transcription in a positive feedback loop. Sorbitol treatment does not result in *jra* transcription, which has been confirmed by Zhang *et al.* who state that osmotic stress causes ubiquitin mediated degradation of *jra* (Zhang et al., 2013). This *jra* down-regulation could promote sorbitol-induced apoptosis (Xia et al., 2007).

The same scenario occurs when treating with 1 mM sodium arsenite, which also activated p38, ERK and less consistently histone H3 phosphoacetylation, while induction of *jra* transcripts was absent. In mammalian cells, on the other hand, arsenite induces c*jun* and c*fos* transcription (Chen et al., 1998). At least for the

putative IE gene *jra*, there is no correlation between global H3 phosphoacetylation and transcription.

This absence of *jra* induction despite global histone H3 phosphoacetylation must be interpreted under the following presumption. Sorbitol and sodium arsenite could induce a different set of genes than *jra*. In a recent study, S2 *Drosophila* cells were stimulated with 0.4 M sorbitol for 15 min and harvested three hours thereafter for microarray analysis. *jra* was not one of the 108 up-regulated genes, with the most up-regulated genes being AMPs, cell surface proteins, transporters and endopeptidases (Sano et al., 2005). It is thus a possibility that a MAPK downstream kinase phosphorylates histone H3 not at the *jra* promoter, but at promoters of other IE genes. As H3K9acS10ph chromatin immunoprecipitation was not performed, it cannot be addressed whether histones at the *jra* promoter were or were not phosphorylated in response to sorbitol or sodium arsenite. On the modENCODE database, chromatin modifications of unstimulated *Drosophila* S2 are annotated for each gene. At the *jra* promoter is a comparable small peak of H3K9acS10ph, while H3K4me3, H3K9ac and K27ac seems to be more abundant. Even if histone H3 were phosphorylated at the *jra* promoter without correlation to transcription, it could be explained by the absence of this mechanism of gene induction, the nucleosomal response, in *Drosophila*.

More importantly, histone phosphorylation does not always correlate with transcription in mouse cells. In Thomson *et al.*, heat shock causes H3 phosphorylation at the hsp70 promoter, independent of transcription (Thomson et al., 2004). It has been suggested that activation of p38 or ERK and MSK1/2 in mammalian cells might induce transcription based on CREB phosphorylation more than based on histone modifications.

Conversely, *jra* is induced in response to peptidoglycan and hydrogen peroxide, but not insulin (Figure 15). Although insulin and hydrogen peroxide both activate ERK, not the same gene induction event was observed. Different stimuli can activate the same MAPK pathway inducing the same genes, and yet, the physiological outcome may vary for each stimulus. This phenomenon may be explained by the duration of the stimulus as well as the sensitive regulation of each gene. Previously, both hydrogen peroxide and insulin have been shown to activate c*jun* expression in mammalian cells, while no such connection was reported for *Drosophila* (Morino et al., 2001, Abe et al., 1998). A study in *Drosophila* S2 cells investigated JNK dependent gene expression in presence of gram-negative bacterial challenge and 53 genes, but not *jra*, were found to be induced (Kallio et al., 2005). Additionally, in a screen attempting to identify all *Drosophila* JNK target genes during development, *jra* was not identified (Jasper et al., 2001). As my experiments took place in cell culture in the context of immune challenge, but not development, it is maybe not surprising to find *jra* up-regulated.

Naturally, each stimulus elicits a distinctive temporal pattern of MAPK activation resulting in a distinctive downstream transcription event. Even though hydrogen peroxide and peptidoglycan (a strong JNK activator) induce the same gene, the rate of transcription/quantity of transcript is specific for a stimulus used. Peptidoglycan strongly induces *jra* already after 30 min, whereas the response with hydrogen peroxide is slightly delayed. Both stimuli transiently activate *jra* and induction is terminated 150 min after stimulation (Figure 17). Mammalian c*jun* returns to baseline levels one hour after EGF stimulation, however in my *Drosophila* experiment *jra* induction is more prolonged (Edwards and Mahadevan, 1992). To conclude this

section, I can postulate that stimuli inducing global histone H3 phosphoacetylation do not correlate with *jra* gene induction.

4.3.2.2 Characterization of immediate early gene *jra*

Immediate early genes are genes which are transiently and rapidly induced. This statement is true for hydrogen peroxide or peptidoglycan induced *jra*. Before any new proteins are synthesized, IE genes are activated at the transcription level as a primary response to external stimuli. *jra* also fulfils this criterion and is still induced in the absence of any newly formed proteins (Figure 17).

When cells are incubated with a translational inhibitor and a stimulus at the same time, the responsive IE gene is delayed in shutting off transcription and has an increased stability (which is different from the phenomenon with signalling agonist anisomycin). *jra* mRNA is stable, as it can still be detected after 90 min of inhibition with DRB when pre-treating with either hydrogen peroxide or peptidoglycan. I can especially observe this enhanced transcription of *jra* in response to peptidoglycan (Figure 17). This enhanced induction might result from failed termination of transcription or from a lack of mRNA degradation due to the disappearance of a labile repressor. Another explanation would be that *jra* mRNA gets stuck in polysomes due the mechanism of action of emetine and is protected from degradation by nucleases.

Following stimulation with hydrogen peroxide or peptidoglycan for 15 min, any further transcription was arrested by the addition of DRB to characterize *jra* stability and to determine its half-life (Figure 18). In my experiments, *jra* was very stable and had a half–life of 138 min. A shorter half-life (10-60 min) has generally been reported for IE genes and proto-oncogenes. Depending on the stimulus used, c*fos* has a half-life of around 9 min and c*jun* has one of 20 min (although it has been claimed to be as long as 100 min) (Rahmsdorf et al., 1987, Kayahara et al., 2005, Spruill and McDermott, 2006).

This mRNA instability of IE genes has been explained by their AU-rich elements in

the 3' UTR which promotes rapid Poly(A) degradation and subsequent mRNA decay (Veyrune et al., 1997). Other processes like 5' decapping and endo/exonuclease mediated degradation also contribute to the mRNA decay pathway (Tourriere et al., 2002). Unlike its mammalian orthologue c*jun, jra* does not have a classic AUUUA element in its 3'UTR which may partially account for its long half-life (Cairrao et al., 2009).

Normally, IE gene induction is transient and correlates with histone H3 serine 10 and possibly serine 28 phosphorylation. I could not detect histone phosphoacetylation by Western blot in the samples treated with hydrogen peroxide or peptidoglycan. Either the fraction of phosphorylated histones by H_2O_2 or PGN was too small to be detected by Western blot, or histone phosphorylation did not take place at the *jra* promoter.

Figure 22 summarizes the performed experiments and shows the phosphorylation of MAPKs and *jra* induction in response to the tested stimuli. So far, a connection between MAPK activation histone H3 phosphoacetylation and *jra* IE gene induction could not be verified. This emphasized the need to study whether histone H3 phosphoacetylation and *jra* induction get abolished when MAPKs are inactivated.

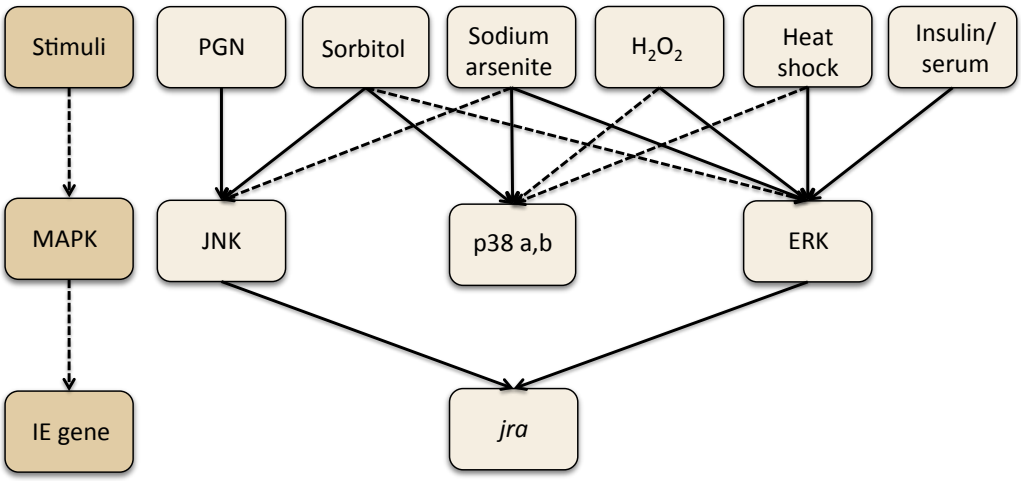


Figure 22: Summary of MAPK and *jra* induction response to the tested stimuli. A thick black line indicates a strong, while a dotted line represents a weak correlation.

4.3.3 Histone H3 phosphorylation occurs independent of MAPK signalling

The ability of MAPK inhibitors to decrease histone H3 phosphorylation was assessed by Western blot and their effect on *jra* was assessed by Northern blot.

At the used concentrations, p38 inhibitor SB203580 and MEK inhibitor PD0325901 have no non-specific effects on other kinases in mammalian cells (Cuenda et al., 1995, Alessi et al., 1995, Davies et al., 2000, Barrett et al., 2008). Previous work has shown that 0.1 μM PD0325901 inhibits the activation of ERK1 and 2 while 2 μM PD0325901 additionally inhibits activation of ERK5 (Bain et al., 2007). An ERK5 homologue in *Drosophila* has not yet been identified and the highest sequence similarity can be found within the ATP binding site (achieving a total of around 28% identity with segment polarity serine threonine kinase Fused). Both tested concentrations of MEK inhibitor PD0325901 inhibit sorbitol or hydrogen peroxide induced ERK completely, verifying their inhibitory potential (Figure 20 and Figure 21A).

SP600125 on the other hand does not selectively inhibit JNK, but also inhibits at least 22 other kinases, including mitotic kinases Aurora A and B (Bain et al., 2003, Colombo et al., 2010, Bain et al., 2007). Han *et al.* published that SB203580 is efficient in inhibiting both p38a and p38b in *Drosophila*. SB203580 is a commonly used inhibitor in *Drosophila* cells and it was shown that 10 μM of SB203580 ablates p38a and p38b kinase activity almost completely (Han et al., 1998b, Sano et al., 2005). Ideally, I would have liked to verify functionality of JNK and p38 inhibitors by the lack of phosphorylation of known downstream targets, but antibodies were not available. Carrier dimethyl sulfoxide (DMSO) did not affect MAPK or histone phosphorylation (data not shown).

In sorbitol stimulated cells, histone H3 phosphoacetylation decreased a little in intensity when inhibiting p38 or ERK, but decreased majorly when treating with JNK inhibitor SP600125. A plausible explanation for reduced histone phosphoacetylation in presence of SP600125 is that mitotic histone phosphorylation was inhibited, independent of its effect on JNK (Giet and Glover, 2001, Oktay et al., 2008, Kim et al., 2010). Given that PGN or LPS stimulation failed to activate histone H3 phosphoacetylation and that S2 cells escape quiescence, it is likely that inhibition of another kinase, for example Aurora B, is responsible for reduced H3 phosphorylation. Northern blot analysis shows that all three MAPK inhibitors reduce, but do not abolish, hydrogen peroxide or peptidoglycan induced *jra* (Figure 21). Presuming that inhibitors are functional in *Drosophila*, neither JNK nor ERK are essential for *jra* induction. For example, *jra* induced by hydrogen peroxide is slightly decreased in presence of all inhibitors, which would imply that no MAPK is crucial for transcription. In mouse cells, each MAPK subtype is capable, but not required for IE gene induction (Cano et al., 1995).

It is unclear why all inhibitors attenuate *jra* induction, but it could be attributed to the lack of a linear causal relationship between MAPKs and *jra* transcription. In Figure 19 A hydrogen peroxide induced p38 was decreased by ERK inhibitors, suggesting a cross regulation between ERK and p38. This cross-regulation of MAPKs precludes a clear interpretation.

Peptidoglycan dependent JNK phosphorylation was abolished in the presence of SP600125, hinting towards a JNK regulated positive feedback loop. Interestingly, p38 got slightly phosphorylated in presence of SB203580, although no p38 activation was observed in other peptidoglycan stimulated cells. Peptidoglycan induced *jra* was down-regulated the most with SB203580. This might indicate that p38 and JNK

interact to enhance *jra* transcription upon immune challenge. According to my findings and the literature, I would have expected to find a greater *jra* inhibition with SP600125 as JNK is upstream of *jra* (Sluss et al., 1996, Chen et al., 2002). The lack of potency of SP600125 in inhibiting JNK might partially attribute for the absence of clear reduction in *jra* transcript. I suggest discontinuing the use of SP600125 as a tool to investigate JNK signalling. Unfortunately there is no thoroughly tested alternative inhibitor for JNK. For example, the MLK inhibitor CEP-1347 inhibits the JNK pathway but also inhibits p38 (Falsig et al., 2004). A small molecule inhibitor targeting the JNK scaffolding protein, JNK-interacting protein (JIP), has been described to inhibit mammalian JNK activity *in vitro* (Barr et al., 2002). Even though an orthologue of JIP exists in *Drosophila*, the 20 amino acid sequence which is targeted by the inhibitor, is not conserved.

To conclude, I investigated the effect of MAPK inhibitors and showed that histone H3 phosphoacetylation is only reduced in presence of JNK and Aurora B inhibitor SP600125. Despite my extensive attempts, I was unable to associate MAPK signalling with histone H3 phosphorylation. Although p38 did initially appear to be a good candidate to be an upstream histone kinase, this failed to be proven. It is tempting to speculate that there is no MAPK inducible histone kinase in *Drosophila*, and that observed H3 phosphoacetylation was mediated by JIL-1. However, specificity of inhibitors in *Drosophila* is not yet clear and knockout experiments will elucidate whether p38 is involved in H3 phosphorylation. The hypothesis that there is no MAPK inducible histone kinase in *Drosophila* is a departure from established thinking and would confirm the divergence between mammalian and invertebrate gene regulation.

5 Final discussion and further directions

5.1 Inducible H3 phosphoacetylation in *Drosophila*

In *Drosophila* S2 cells, inducible H3 phosphoacetylation has not been shown previously and evidence against a correlation between histone phosphorylation and transcriptional activation is accumulating (Wang et al., 2013, Wang et al., 2011). Despite a strong correlation between p38 activation and histone H3 phosphoacetylation, inhibitor SB203580 did not reduce histone phosphoacetylation. Additionally, I have not detected concomitant *jra* induction. One explanation could be that sorbitol and sodium arsenite activate transcription of a different gene or, alternatively, that the nucleosomal response does not exist in *Drosophila*. Regnard *et al.* show that in *Drosophila,* histone H3 phosphorylation is not inducible at promoters and that JIL-1 mediated phosphorylation at the gene body mainly counteracts silencing (Regnard et al., 2011). However, H3K9acS10ph was observed genome wide and H3S10ph was suggested to be induced at Eip75 promoters in response to ecdysone stimulation (Kellner et al., 2012). My results show that inducible histone phosphorylation exists in *Drosophila* independent of MAPK signalling. It would be interesting to investigate how the observed increase in H3 phosphoacetylation is mediated in response to cellular stress and whether it correlates with transcriptional activation.

There is an alternative biological event which could explain histone H3 phosphoacetylation in response to sorbitol and sodium arsenite. For example, one could conclude that instead of stimulating the cells, sorbitol and sodium arsenite stressed and programmed the cells to undergo apoptosis. Chromatin condensation mediated by histone H3S10 and H2B phosphorylation have been shown to be a

hallmark of apoptosis (Park and Kim, 2012). Moreover, after a three hour sorbitol time-course, cells were stained with trypan blue. Quantification of the stained cells revealed that 20% of sorbitol treated cells did undergo apoptosis (data not shown).

5.2 Future experiments

5.2.1 Alternative *Drosophila* cell model to study histone phosphorylation

For further experiments, I would recommend using Kc167 cells. Even though derived from different embryonic stages, Kc167 cells are similar to S2 cells in gene expression profiles (Cherbas et al., 2011). Additionally, Kc167 cells secrete PDGF- and VEGF-related factor isoforms, indicating the ability to respond to classical MAPK stimuli (Bond and Foley, 2009, Friedman and Perrimon, 2006). As they are derived from female flies, Kc167 cells have the benefit of not having a JIL-1 dependent histone phosphorylated X chromosome which would avoid interfering with the analysis of inducible histone H3 phosphorylation (Echalier and Ohanessian, 1969, Liu et al., 2009). In male flies, the X chromosome accounts for 13.7% of the total genome and hence for 13.7% of all putative phosphorylated histones. This compensatory histone phosphorylation impedes analysis of the remaining inducible fraction of 86.3% of histones, which is most likely to be smaller than the "background" X-chromosome phosphorylation of 13.7%. The assumption is that in Kc167 cells, inducible H3 phosphoacetylation and H3 phosphorylation could be studied directly.

5.2.2 Knockdown of p38 and putative histone kinases

It is theoretically possible that *Drosophila* cells respond to an extracellular stimulus in the same way as mammalian cells respond; MAPK pathway activation leading to activation of a histone kinase and subsequent histone phosphorylation. However, the small molecule p38 inhibitor SB203580 did not decrease histone H3 phosphoacetylation. Those results demonstrate the need for MAPK inhibition with

p38 RNAi to verify the lack of MAPK involvement in phosphorylating histone H3. *Drosophila* cells do not provoke an immune reaction against RNA and are hence suitable for knockdown.

Additionally, I would like to verify that JIL-1 is responsible for sorbitol and sodium arsenite induced histone phosphorylation. To do this, putative histone kinases Aurora B, MK2, RSK and JIL-1 would be silenced and, upon stimulation, lysates would be probed for H3K9acS10ph. To verify that the identified histone kinase preferentially binds to and phosphorylates histone H3 peptides, nuclear extracts from *Drosophila* cells would be immunoprecipitated with a specific antibody targeted against the identified kinase. Moreover, associated pulled-down proteins could be identified by mass spectrometry as described in Wysocka *et al.,* which might also give an insight into the upstream regulators (Wysocka et al., 2006).

5.2.3 Analysis of putative IE genes in *Drosophila*

Until now, IE genes are less characterized in *Drosophila* than in mammalian cells and further experiments would aim to identify a set of IE genes in *Drosophila*. Most notably is the rapid activation of hsp70 in response to heat shock (Boehm et al., 2003). Heat shock factor HSF binds to HSEs in the promoter region within five seconds and hsp70 RNA transcription peaks after five min of heat shock. Immune defensive AMP gene expression is rapid and transient and the primary response of *Drosophila*. Although AMP expression is extensively studied in *Drosophila*, it still has to be determined whether AMPs act as IE genes (Lemaitre and Hoffmann, 2007). Based on the rapid activation of the immune system in *Drosophila*, it could be possible that peptidoglycan induces AMPs in a similar manner as it induced *jra.* Additionally, it has been proposed that ecdysone induced *Eip75* is an IE gene, although clear evidence is lacking. A correlation between histone H3K9 or H3K23

acetylation in the promoter and Eip75B transcription was reported (Bodai et al., 2012, Kellner et al., 2012). I would thus analyse the induction profile of developmental *Eip75,* heat shock responsive and AMP genes.

5.2.4 H3S10ph and H3K9acS10ph ChIP

To analyse distribution of histone marks on identified IE genes in *Drosophila*, promoters and coding regions would be analysed by chromatin immunoprecipitation (ChIP) with H3S10ph or H3K9acS10ph antibodies. In presence or absence of stimuli, immunoprecipitated DNA would be analysed by qRT-PCR. This would elucidate whether histone H3 phosphoacetylation correlates with IE gene induction in *Drosophila*.

A Appendix

A.1 Specificity of anti phospho-p70^{S6kinase} antibody

The anti phospho-p70^{S6kinase} antibody detects a doublet band as well as an additional single band at higher molecular weight in Western blots. The antibody thus appears to cross-react with another protein, so the correct p70^{S6kinase} band needed to be determined.

In *Drosophila*, p70^{S6kinase} phosphorylation was induced by 15% serum, 37 μM anisomycin or 400 J/m^2 UV exposure (Figure 23). Cells were pre-treated with the macrolide immunosuppressant rapamycin which inhibits p70^{S6kinase} activation by binding to TORC1 (29). Pre-treatment with rapamycin caused a loss of the doublet while the upper band remains unchanged (Figure 23). This result indicates that the doublet represents p70^{S6kinase} as it is sensitive to ablation in presence of rapamycin. Moreover, the non-specific protein detected by the phospho-p70^{S6kinase} antibody did not respond to any stimulus.

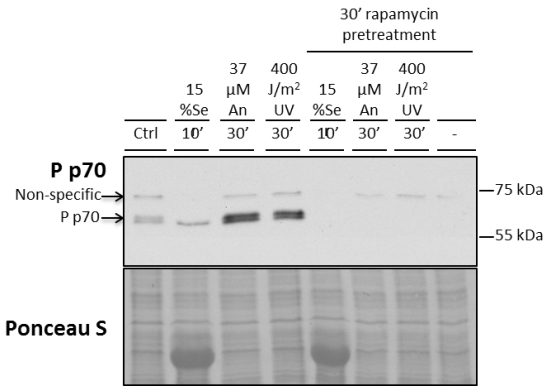


Figure 23: The doublet representing p70^{S6kinase} phosphorylation is inhibited by rapamycin. Quiescent S2 *Drosophila* were treated with 15% serum (ten min), 37 μM anisomycin and 400 J/m^2 UV for 30 min. Cells were pre-treated for 30 min with 20 nM rapamycin followed by the previous specified treatment. High salt lysis extracts were analysed by immunoblotting with anti phospho-p70^{S6kinase} antibody. Ctrl indicates the unstimulated control. Ponceau S staining was used as a loading control. Note that in the serum treated samples, a decrease in the upper non-specific band and a squashing of the p70^{S6kinase} doublet was observed because serum runs right above the p70^{S6kinase} doublet.

A.2 p38b, but not p38a expression in *Drosophila* S2 cells

According to the gene expression FlyAtlas, both *p38a* and *p38b* are expressed in *Drosophila* S2 cells. Expression of both isoforms is said to be lower in male flies and hence male S2 cells. I designed specific RNA probes against *p38a* and *p38b,* and even after long exposures, *p38a* expression could not be detected, while *p38b* was present. The lack of *p38a* might represent a true biological absence in *Drosophila* S2 cells. However, the possibility that the probe was not functional and failed to detect *p38a* could not be excluded.

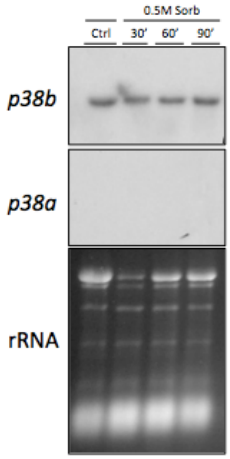


Figure 24: *p38b* expression in *Drosophila* S2 cells. Quiescent S2 *Drosophila* cells were treated with 0.5 M sorbitol (30 to 90 min). Ctrl indicates the unstimulated control. Total RNA was extracted from *Drosophila* S2 cells and analysed by Northern blotting using *p38a* and *p38b* probes. Ribosomal RNA showed more or less equal loading in each lane.

In the literature, p38b is reported to be functionally more important for the signalling response to stress. Seisenbacher *et al.* observed a decrease in cytoplasmic MK2 localisation in p38b knock out flies in the context of hindgut homeostasis (Seisenbacher et al., 2011). Chen *et al.* analysed the role of p38 orthologues in the immune response and concluded that the puckered mediated JNK shut off is p38b-dependent. Also, it was found that p38a plays a minor role in the host defence (Chen et al., 2010). Therefore, it is in agreement with the described literature that p38b plays a greater role in the context of stress activation, independent of whether p38a is present or not in *Drosophila* S2 cells.

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