Defining functional specificity of stress responses in *Drosophila melanogaster*

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Abstract

Survival of an organism depends on its perception and response to external stressors such as infection, osmotic stress (ionic or desiccation) or xenobiotic stress. At the cellular level, stress is perceived and relayed via signal transduction pathways that alter transcription or establish new transcriptional programmes that modulate physiology at the whole-organism level to regain homeostasis and promote survival (Davies et al., 2012). The vital function of epithelial tissues (e.g., kidneys in vertebrates and Malpighian tubules in insects) is systemic balance of nutrient, solute and water levels (Dow and Davies, 2003). Additionally, epithelial tissues act in sensing stress and relaying signals for adaptation and tolerance to stress (McGettigan et al., 2005; Soderberg et al., 2011; Winther and Nassel, 2001) .

The PhD work presented here is to delineate the roles of Relish and cGMP-dependent kinases in mechanisms of epithelial stress handling using a genetically tractable epithelium, the *Drosophila* Malpighian tubule, as an *in vivo* model for stress sensing and response.

Relish, a transcription factor, is the insect orthologue of the mammalian NF-kB. It regulates the insect's innate immune pathway (Hedengren et al., 1999) and is highly expressed in *D. melanogaster* tubules (Chintapalli et al., 2007; Robinson et al., 2013). We show that Relish expressed in Malpighian tubules modulates organismal tolerance to osmotic stress caused due to a high salt diet (salt stress). In order to determine the genes that are involved in salt stress tolerance, Affymetrix *Drosophila* GeneChips (microarrays) were run with RNA isolated from the wild-type and Relish mutant tubules from flies fed either on normal food, or on 'salt food '. The transcriptomic data was analysed to find genes that were dependent and independent of Relish in response to stress. Additionally, the data also revealed that during salt stress, with the loss of Relish related signalling pathway, the other stress response pathways, in particular, the c-Jun kinase pathway is hyper-activated. This suggests (1) a potential cross-talk occurring between Relish and other stress response pathways, and (2) a redundancy in stress response pathways, for adapting to salt stress. These data demonstrate a novel role for Relish in salt tolerance in

Drosophila melanogaster. Moreover, under unstressed conditions, expression of 448 genes was significantly altered and a reduced basal fluid secretion rates were observed in Relish mutant tubules. This suggests that basal Relish activity is required for optimal working of the tissue.

In addition, a study to elucidate the immune and osmotic stress-associated roles of cyclic guanosine monophosphate (cGMP)-dependent kinases - Dg1 and Dg2 - in tubules was carried out. Salt stress and desiccation stress survival assays in flies with targeted knock down of each of Dg1 and Dg2 genes in tubule principal cells showed an opposing stress phenotype for these two stressors. No immune phenotype was observed on infection with non-lethal gram negative bacteria. This showed that the cGMP-associated osmotic stress response mechanisms were beneficial or detrimental to survival of organism, depending on the type of stressor and downstream effectors.

The understanding gained from this *in vivo* approach of studying stress pathways in *Drosophila* Malpighian tubules can be further explored through a systems biology approach .This, together with combinatorial gene knockdown studies to reveal stress network “hubs”, may be applied to development of potential targets of insecticides and in biomedical sciences.

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Author’s Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration, except where specifically indicated in the text.

Definitions/Abbreviations

°C	degree Celsius
µg	microgram
µl	microliter
µM	microMolar
AMP	antimicrobial peptides
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium ions
cAMP	3',5'-cyclic adenosine monophosphate
CAP2b	Cardioacceleratory peptide 2b
capa	capability
capaR	capa receptor
cA-PDE	cAMP-hydrolysing PDE
cDNA	complementary DNA
cGK	cGMP-dependent kinase
cGMP	3',5'-cyclic guanosine monophosphate
cG-PDE	cGMP-hydrolysing PDE
CNG	cyclic nucleotide gated (channels)
cRNA	complementary RNA
CYP	Cytochromes P450
DAPI	4',6-diamidino-2-phenylindole
Dg1	Pkg21D
Dg2	foraging
Dh	Diuretic hormone
Dh31	calcitonin-like neuropeptide
Dh44	CRF-related diuretic hormone (also known as Dh)
Dh44 R2	CRF-related diuretic hormone receptor type 2
dJNK	*Drosophila* Jun N-terminal kinase (basket)
dNOS	D. melanogaster calcium/calmodulin-sensitive nitric oxide synthase
dsDNA	double-stranded DNA
EPAC	exchange protein directly activated by cAMP
EtBr	Ethidium bromide
FC	fold change
FDR	False Discovery Rate

FOXO	forkhead
GC	guanylate cyclase
GO	Gene ontology
GST	Glutathione S-transferases
h	hour
H_2O_2	hydrogen peroxide
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IIS	Insulin and Insulin-like growth factor (IGF) Signalling
IMD	Immune Deficiency
ITP	Ion Transport Peptide
JAK	Janus kinase
JNK	Jun N-terminal kinase
Jra	*Drosophila* c-Jun
K^+	Potassium ions
Kay	*Drosophila* c-Fos
M	Molar
MAPK	Mitogen-activated protein kinase
min	minutes
mM	millimolar
mRNA	messenger RNA
MM	Mismatch
Na+	Sodium ions
NaCl	Sodium chloride
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NO	nitric oxide
OD	optical density
p	probabilty
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDE	phosphodiesterase
PGRP	peptidoglycan recognition protein
PGN	peptidoglycan

PKA	protein kinase A
PM	Perfect Match
qRT-PCR	quantitative reverse transcriptase PCR
Rel^{E20}	Relish mutant
rGC	receptor-guanylate cyclase
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT	room temperature
sec	second
sGC	soluble guanylate cyclase
SOD	superoxide dismutase
STAT	Signal Transducer and Activator of Transcription
TRPL	transient receptor potential-like (channel)
UAS	upstream activation sequence
V-ATPase	vacuolar H+-ATPase

Chapter 1
Introduction

1.1 Stress

Stress can be defined as “any external perturbation to the organism’s optimal homeostasis” (Dow, 2014). A response to stress is orchestrated through modulation of signal transduction pathways at the molecular level, leading to physiological changes at the tissue level that helps the organism to tolerate or adapt to stress (Chrousos and Gold, 1992; Dow, 2014). Many of the basic stress responses are evolutionarily conserved from bacteria to vertebrates, for example, expression of heat shock protein in response to heat stress or metal toxicity (Morimoto et al., 1994). There is a high degree of similarity in response to stress between invertebrates, vertebrates and plants. For example, in plants, the hormone abscisic acid (ABA) plays a role during drought stress to regulate water loss (Wilkinson and Davies, 2002). Similarly diuretic peptides and renal hormones are found in insects and mammals that adjust water-ion balance in the body during osmotic stress (Bahner et al., 2007; Della Penna et al., 2014; Terhzaz et al., 2015b).

There are also overlaps in molecular responses to stress. For example, a single signalling pathway can be activated by more than one stressor. The c-Jun N-terminal kinase cascade is activated by ultraviolet (UV) light and high osmolarity in mammalian cells (Rosette and Karin, 1996). Also, in *Drosophila melanogaster*, p38 mitogen-activated protein kinase plays a role in the innate immune response, the osmotic stress as well as in the oxidative stress response (Chakrabarti et al., 2014; Chen et al., 2010; Tian et al., 2014).

Cross-tolerance is another feature manifested due to overlap of stress pathways; when exposure to one type of stress can help an organism to tolerate a second, different stressor; for example a variety of drought tolerant maize is resistant to the parasitic weed *Striga hermonthica* (Badu-Apraku and Yallou, 2009; Banziger et al., 2006). During an organism’s lifetime, it is exposed to multiple environmental stressors such as infection, osmotic stress, xenobiotic stress; or combination of stressors. As response to stress leads to rearranging of metabolic and energetic pathways (Ulrich-Lai and Ryan, 2014), having common strategies and pathways to cope with multiple stressors can be energetically favourable.

Epithelial tissues are key tissues that protect the organism's internal milieu from external environmental insults. Epithelial tissues express multiple stress associated cell-specific signalling pathways that use cyclic nucleotides and calcium ions as second messengers to transduce stress signals perceived at the cell surface, to sequentially modulate the physiology of the tissue resulting in the organism's ability to tolerate stress and maintain homeostasis. Therefore, an *in vivo* understanding on organism's stress tolerance can be gained by studying the molecular mechanisms of the epithelia (Davies et al., 2012; Davies et al., 2014).

1.2 *Drosophila melanogaster* as a model organism

Since the discovery made by Thomas Hunt Morgan of the *white* mutation in *Drosophila melanogaster* (Morgan, 1910),*Drosophila* has served as an important model for genetic studies to understand the underlying processes of developmental biology and physiology. The *Drosophila* genome contains ~130 Mb euchromatin that is equivalent to about the size of a single human chromosome (Dow and Romero, 2010).. Although evolutionarily diverged by 400 million years, at molecular level ~70% of Drosophila genes have human homologs with a high degree of conservation in the genetic mechanisms and signalling pathways that control developmental processes, and immunity, and maintain whole-organism homeostasis (Bier, 2005; Davies et al., 2012; Dow, 2009; Trinh and Boulianne, 2013).

Drosophila is also one of the most studied organisms in biological research because of ease in performing genetic experiments, short development life cycle (of ~2 weeks) and small body size, and high fecundity with low maintenance cost (Dow and Romero, 2010). An important advantage of using *Drosophila* for genetic studies is the time and cost-effective production of developing mutants and transgenics.

The P-element-mediated transformation is a powerful technique that revolutionised the generation of *Drosophila* transgenics (Spradling and Rubin, 1982). The first generation of *Drosophila* enhancer trap fly lines used *P*-element mediated transposition for identifying cell-type and tissue-specific patterns of

genomic enhancer elements. This enabled the development of the GAL4/UAS binary system aiding tissue-specific expression of transgenic constructs (reporter fusions, overexpressors, dominant negatives or RNAi) (Akbari et al., 2009; Boy et al., 2010; Brand and Perrimon, 1993). The two components of the GAL4/UAS system were derived from yeast (Duffy, 2002). In this heritable system, the expression of gene or gene-specific RNAi is induced when the GAL4 protein binds to the UAS (upstream activating sequence) promoter. The induction of GAL4 is under the control of a cell- or tissue-specific promoter. When flies expressing a transgene under control of a UAS promoter mate with flies expressing a cell- or tissue- specific GAL4 promoter, their progeny express both desired constructs. Therefore, the ectopic expression of the transgene in this progeny leads to either manipulation of the expression of the gene-of-interest, or expression of a foreign gene, in a cell- or tissue- specific manner (Figure 1.1). For example, using renal-specific GAL4 driver (Sozen et al., 1997), *Drosophila* was the first animal in which genetically encoded calcium reporters were expressed to study real-time renal calcium signalling (Rosay et al., 1997)

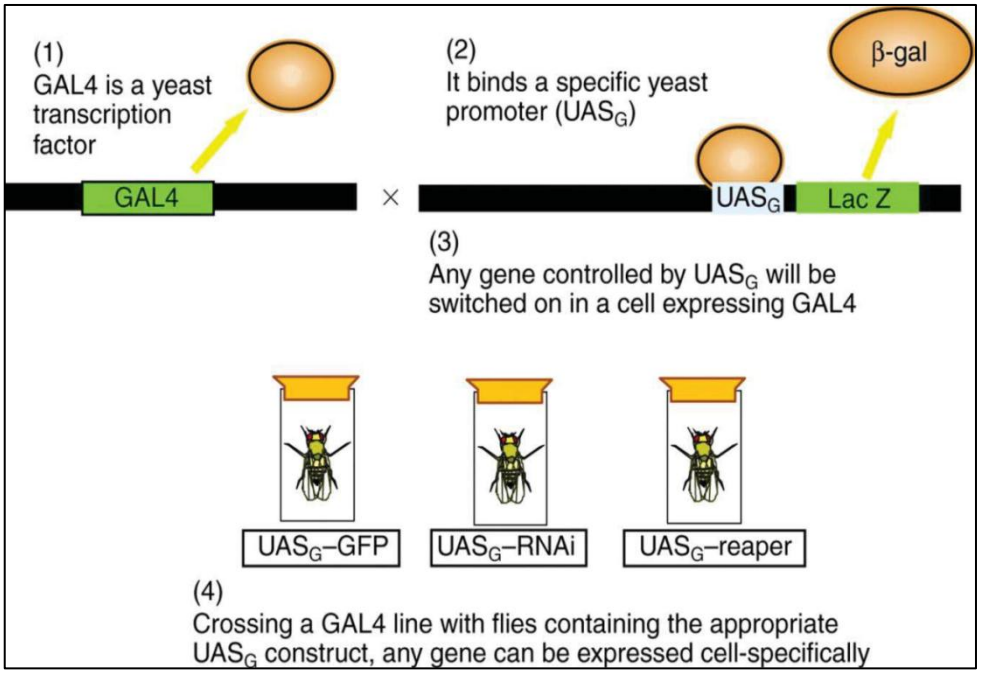


Figure 1-1 Schematic of heritable induction of transgenes using GAL4/UAS binary system. The induction of GAL4 is under the control of a cell- or tissue-specific promoter. Flies expressing UAS (upstream activating sequence) fused to a transgene sequence are mated to flies expressing cell- or tissue-specific GAL4 drivers. The F1 progeny contains both the constructs leading to either overexpression or repression of a gene of interest or expression a foreign gene in the cell- or tissue- specific manner. For example, a cell- specific GAL4 driven expression of UAS_G-GFP leads to production of Green Fluorescent Protein in that specific cell. Similarly, one can express UAS_G-RNAi to decrease expression of the gene, against which RNAi is made and express UAS_G-reaper for induction of cell death (Figure adapted from(Dow, 2007))

1.2.1 Functional genomic studies using *Drosophila*

The advent of whole genome sequences of human and model organisms augmented the 'omics technologies that provided data at the transcriptome, proteome and metabolome levels. This further helped in studies of comparative biology for analysis and functional annotation of genomes, mapping metabolic and signal transduction pathways, genetic interactions, and kinomes/photomes using both computational and experimental approaches (reviewed by (Joyce and Palsson, 2006))

The DNA microarray technology is a method for analysing transcriptomes at a massive scale and is part of the field of functional genomics (Bammler et al., 2005; Schena et al., 1995). The DNA microarray quantifies the expression of thousands of genes in a sample, producing the global picture of mRNA changes (Trevino et al., 2007). The mRNA levels is one of the indicators of changes at proteome level, thus studying the expression of genes at the transcript level is a powerful approach in understanding the relationship between genotype and phenotype (Tarca et al., 2006).

The Affymetrix DNA microarrays also called GeneChips® are widely used for microarray studies of tissues or whole organism under different experimental conditions (www.Affymetrix.com). The GeneChip of *Drosophila* Genome v2.0 designed on the information from *D. melanogaster* genome on Flybase (www.flybase.org) and the Berkeley *Drosophila* Genome Project (BDGP) (www.fruitfly.org), provides comprehensive coverage of the *Drosophila melanogaster* genome for surveying gene expression of ~13,500 genes.

Flyatlas (www.Flyatlas.org) is a resource of tissue-specific transcriptome information developed from the information gained from these arrays. This has increased the understanding of tissue function and stand-alone expression of genes (Chintapalli et al., 2007; Wang et al., 2004). It has also helped in recognising the suitability of *Drosophila* to study human disease in a cognate fly tissue.(Dow, 2009; Wang et al., 2004).

At present, the i5k initiative (https://www.hgsc.bcm.edu/i5k-pilot-project-summary) aims to sequence the genomes of 5,000 arthropod species. Included in this project are the genomes of insects often seen as pests, such as *Aedes aegypti* and *Tribolium castaneum*. Thus by orthologue mapping of the gene sequences of these insects with the widely studied *Drosophila melanogaster* genes, tissue specific- and functional information of the genes can be gained for those insects.

The relative ease of carrying out organotypic studies of gene function using *Drosophila* transgenics, with the wealth of functional and comparative information available on integrated online genetic and genomic resources (such as Flybase.org, Flymine.org , DIOPT.org) and availability of stock centres that have gene knockouts and hypomorphs or mutants for nearly all Drosophila genes available at nominal cost, makes *Drosophila melanogaster* an excellent model organism for carrying out functional genomic research.

1.2.2 Malpighian tubules as a model epithelium

Insect tubules, like human kidneys are involved in urine formation, performing the basic task of transport, excretion and osmoregulation. *D. melanogaster* have four Malpighian tubules, an anterior pair and posterior pair, which float freely in the haemolymph of the insect body. The tubules are blind-ended and open into the hindgut of the alimentary canal. The two tubules in a pair are joined at a common ureter. A pair of tubules consists of ~150 cells. Based on morphology and function each tubule can be divided into four domains: initial segment, distinctly narrower transitional segment, fluid- secreting main segment and lower tubules (Figure 1-2 A). In contrast to the smaller initial segment seen for the posterior tubules, the anterior tubules have an enlarged initial segment that balance the haemolymph Ca^{2+} (Dube et al., 2000). Enhancer trapping method provides information of spatial patterning of gene expression helping in understanding tissue's genetic organisation (Sozen et al., 1997). The GAL4 enhancer trap analysis shows that there are six distinct genetic boundaries in tubules (Sozen et al., 1997). In addition, there is recent evidence showing a

molecular and functional asymmetry between the anterior and posterior tubules (Chintapalli et al., 2012).

The tubules are primarily composed of two cell-types: the columnar principal cells (~77 in number in each tubule) and star-shaped stellate cells (~15 in number in each tubule) (Figure 1-2 B). The principal cells have deep basal infoldings and long microvilli, whereas stellate cells have less basal infoldings with shorter apical microvilli. There is a distinct compartmentalization for ion transport and cell signalling pathways between the two cell types (Dow and Davies, 2003). The functional studies so far have shown the stress mediated signalling pathways in only the principal cells. The role of the stellate cells during stress is yet to be characterised. Other type of cells, include the stem cells, probable neuroendocrine cells, and mechano-sensory or chemosensory cells that have been detected using lineage tracing and molecular marker analysis and the enhancer trap analysis, respectively (Singh et al., 2007; Sozen et al., 1997).

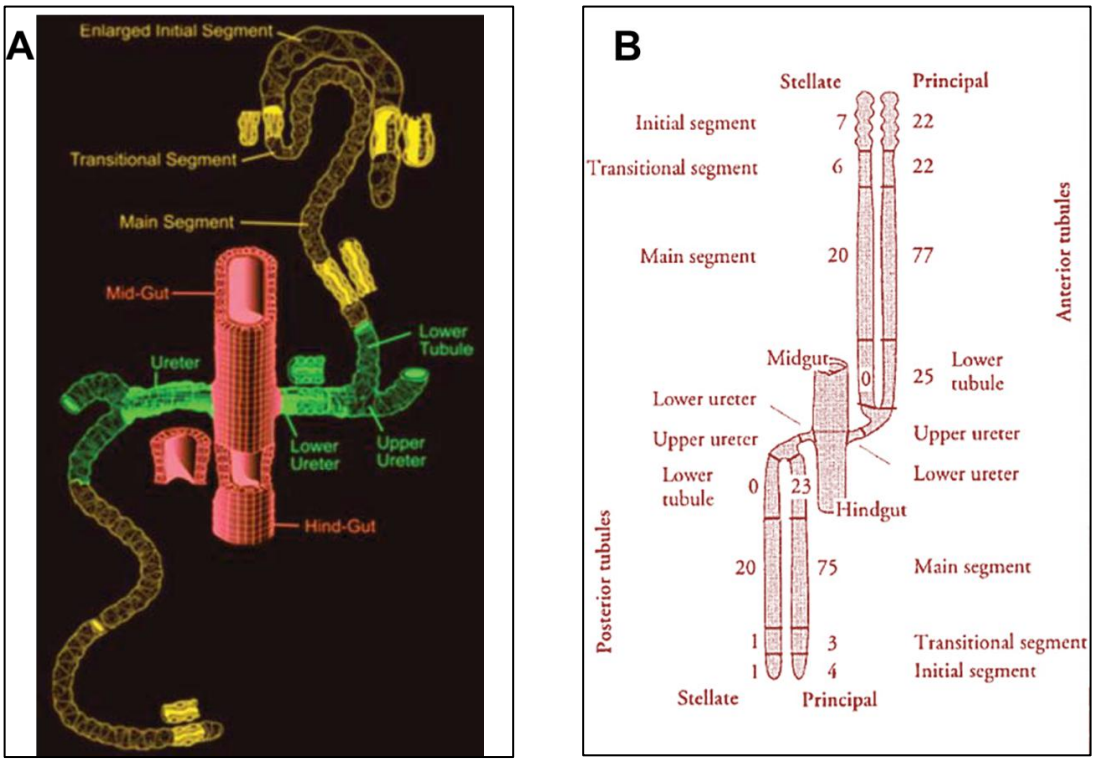


Figure 1-2: Tubules of Drosophila melanogaster. (A) The two pairs of tubules, the anterior and posterior tubules that ramify through haemolymph of the insect body. The genetic analysis using enhancer trapping technique revealed the morphologically and functionally distinct domains (adopted from Sozen, 1997) that are labelled for anterior tubules; the anterior and posterior tubules have similar domains except the latter has no enlarged initial segment (adapted from (Wessing and Eichelberg, 1978) and Chintapalli thesis, 2012) **(B)** The number of stellate and principal cells in each of the distinct domain is shown (adapted from Sozen, 1997).

D. melanogaster tubules are the fastest fluid-transporting epithelia known in biology (Dow et al., 1994b). The primary urine formation in tubules is based on active transport and this primary urine contains ~120 mM KCl and ~30 mM NaCl (O'Donnell and Maddrell, 1995). The lower tubule segment reabsorbs ~30% of the KCl and fluid secreted by the main segment before it enters the hindgut for final formation of urine (O'Donnell and Maddrell, 1995).

The main segment secretes fluid using active transport of K^+ as the main driving force whereas most other substances and water follow passively as a consequence of the electrochemical gradient (Beyenbach and Liu, 1996; Ianowski and O'Donnell, 2004; O'Donnell and Maddrell, 1995). The active K^+ transport from the haemolymph (basolateral surface) to tubule lumen (apical surface) occurs through principal cells. The classes of the transporters and channels that facilitate this transport are: a basolateral Na^+-K^+-ATPase (Torrie et al., 2004), basolateral inward-rectifier K^+ channels (Kir) (Evans et al., 2005),an apical plasma membrane V-ATPase (Davies et al., 1996), and the apical alkali-metal/proton exchanger (NHA) (Day et al., 2008). The chloride channels and aquaporins in stellate cells provide the anion flux and water passage, respectively (O'Donnell et al., 1998). A paracellular movement through the tight (in insects, septate) junctions of chloride is also thought to occur (Beyenbach, 2003). In addition, the tubules also express Na^+driven Cl^-/HCO_3^-exchanger (NDAE1) (Sciortino et al., 2001)and Na^+-K^+-$2Cl^-$cotransporter (NKCC) (Ianowski and O'Donnell, 2004) which may be contributing to transepithelial chloride flux.

Tubule fluid secretion and transepithelial cation transport have been shown to be modulated by 3′,5′-cyclic adenosine monophosphate (cAMP) and, 3′,5′-cyclic guanosine monophosphate (cGMP) (Bijelic and O'Donnell, 2005; Davies et al., 1995; Dow et al., 1994b). The V-ATPase localised at the apical membrane of the tubule principal cell energizes fluid secretion into the tubule lumen (Allan et al., 2005; Davies et al., 1996; Dow, 1999). This V-ATPase is suggested to be the main downstream effector of the cyclic nucleotide signalling in the tubule principal cell (Davies et al., 2014). Recent evidence shows that exogenous applied cGMP is transported only into tubule principal cells (Riegel et al., 1998), most probably via an ABC transporter (*Drosophila* ABC transporter white) (Evans et al., 2008),

which leads to an increase in ATP concentration (Davies et al., 2013). The availability of this ATP pool may then lead to the increase in V-ATPase activity (Davies et al., 2014). Studies using photoactive adenylate cyclase transgene (bPAC) targeted to express in either tubule principal or stellate cells showed differential roles of protein kinase A (PKA) in both cell types, as well as unravelled a novel role of cAMP exchange protein, EPAC, in tubules. This demonstrated the existence of compartmentalisation of cAMP signalling for both basal and stimulated (by neuropeptide) fluid secretion rates in *Drosophila* tubules (Efetova et al., 2013).

Additionally, nitric oxide (NO)-modulated renal function was first demonstrated in tubules of *Drosophila* (Dow et al., 1994a). Moreover, using genetically encoded aequorin-based luminescent Ca^{2+} reporters it was shown that a rise in intracellular calcium ions through internal calcium stores (e.g. SERCA), L-type and cyclic nucleotide gated calcium channels can affect the fluid secretion phenotype of tubules, V-ATPase activity, and downstream signalling and ion transport events (Davies and Terhzaz, 2009).

The signalling pathways of four neuropeptides and a biogenic amine, that use cAMP /cGMP or Ca^{2+} as second messengers have been well-studied in *Drosophila* tubules (Summarised in Figure 1-3). Neuropeptide signalling via the corticotropin-releasing factor (CRF)-related diuretic hormone (CRF-related DH), also known as Dh44, and calcitonin-like neuropeptide, Dh31 activates cAMP signalling in tubule principal cells (Cabrero et al., 2002; Coast et al., 2001). In tubules, Dh44 binds to Dh44-R2 on the tubule principal cell and increases fluid secretion rates (Cabrero et al., 2002)(Cannell E. unpublished). In addition, it also increases total cAMP hydrolysing activity by cAMP phosphodiesterases (PDEs) (Cabrero et al., 2002). This suggests that action of Dh44 requires a fine tuning between generation and targeted breakdown of cAMP. It has been proposed that though cAMP transduces both Dh44 and Dh31 signals, the downstream effectors may be different; as in tubules of the malarial mosquito *Anopheles gambiae* an increase in basolateral Na^{+} conductance is seen on stimulation with Dh31 and not Dh44, (Coast et al., 2005)

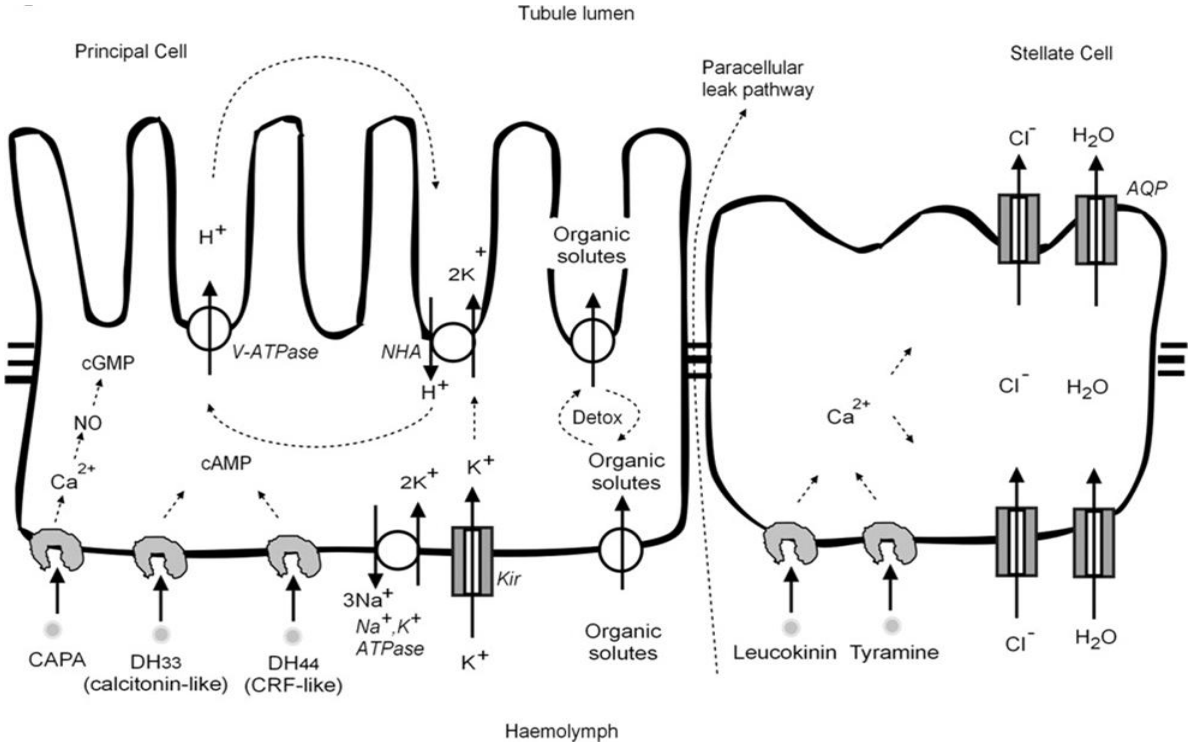


Figure 1-3: Ion transport and signalling pathways of the *Drosophila melanogaster* tubule. The active transport of cations is carried out in the principal cells by basolateral Na^+-K^+-ATPase and basolateral inward-rectifier K^+ channels (Kir), and apical plasma membrane V-ATPase, and an alkali-metal/proton exchanger of the NHA. The neuropeptides Dh31 and Dh44 bind to their respective receptors to initiate signalling through cAMP. The signalling of capa peptide in the principal cells occurs through Ca^{2+}/NO/cGMP pathway. The signalling of peptide NPLP1-VQQ (not shown in the diagram) is also through the receptor on the basolateral membrane of the principal tubule cells and produces an increase in cGMP and intracellular Ca^{2+}. The passive transport of anions (chloride ions) and water (through aquaporins) occurs in stellate cells. The *Drosophila* neuropeptide Leucokinin (Drosokinin) and biogenic amine Tyramine act through increase in Ca^{2+} to produce chloride flux (adopted from (Dow and Romero, 2010)).

The mechanistic understanding of the *Drosophila* capa peptides, capa-1 and capa-2, was originally from studies of *Manduca sexta* Cap2b on tubules of *Drosophila melanogaster* (Davies et al., 1995). The signalling of capa peptide across *Dipterian* species is similar and have shown to work through a complex Ca^{2+}/NO/cGMP signalling pathway (Broderick et al., 2003; Pollock et al., 2004). The stimulation of the principal cells with capa peptide produces a distinct calcium signature (Rosay et al., 1997). The signature shows two distinct calcium peaks denoting the rise in intracellular Ca^{2+} ions. The first peak is produced by the quick rise in Ca^{2+} ions occurring via phospholipase Cß and inositol 1,4,5-trisphosphate receptor(Pollock et al., 2003), and also involves Ca^{2+}release via the Golgi sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) channel (Davies and Terhzaz, 2009). The second peak denotes the slow rise in intracellular Ca^{2+} by

Ca^{2+} influx through principal cell plasma membrane Ca^{2+} channels – transient receptor-like (TRPL), L-type and cyclic nucleotide-gated (CNG) channels which are stimulated by the cGMP produced through the activation of soluble guanylate cyclase by NO produced by nitric oxide synthase (Broderick et al., 2003; MacPherson et al., 2001; MacPherson et al., 2005).

The neuropeptide Leucokinin (*Drosophila* specific Leucokinin is known as Drosokinin) and biogenic amine tyramine both work through calcium signalling in the stellate cells to produce a chloride shunt across the membrane (Cabrero et al., 2013; O'Donnell et al., 1998; Terhzaz et al., 1999).
Recently a neuropeptide, NPLP1-VQQ has been characterised. It binds to the receptor, Gyc76C, at the basolateral membrane of the principal tubule cells and produces an increase in cGMP and intracellular Ca^{2+}. It has shown to be a partial agonist to fluid secretion phenotype of the tubules (Overend et al., 2012).

The neuropeptide studies in tubules have been typically focused on characterising its effect on water-ion transport. Recent studies have shown that peptides can have other downstream targets that may play a role apart from directly contributing to osmoregulation (Overend et al., 2012; Terhzaz et al., 2014). In addition, a number of stress studies show that by altering expression of genes in only the tubule principal cells (that account for about ~77 cells per tubule),organism's ability to adapt to a stressor can be modulated (Davies and Dow, 2009; Davies et al., 2012; Overend et al., 2012; Soderberg et al., 2011; Terhzaz et al., 2010; Terhzaz et al., 2014; Yang et al., 2007). For example, overexpression of NOS gene in tubule principal cells confers tolerance to septic infection (McGettigan et al., 2005). The other examples are tolerance to desiccation observed on targeted knockdown of either capa neuropeptide receptor (CapaR)(Terhzaz et al., 2015b) or insulin receptor (InR) (Soderberg et al., 2011).

In addition to osmoregulation, tubules are key tissues for detoxification and defence. The *Drosophila* tubules express an array of detoxifying genes belonging to various families of cytochrome *P*-450s and glutathione-*S*-transferases, some of which are abundantly expressed and highly enriched in the tubules (Chintapalli

et al., 2007; Wang et al., 2004). For example *Cyp6g1* is very highly expressed and enriched in the tubules and alteration in its expression specifically in the tubules leads to sensitivity of the organism to insecticide DDT (Yang et al., 2007).

The anterior tubules are on the right-side of the insect's body, spreading throughout the thorax and are in the vicinity of the midgut. The posterior tubules are on the left-hand side of the body and are in proximity to the hindgut. Thus the tubules ramify throughout the body cavity and efficiently regulate the haemolymph. Recent study by Chintapalli et. al (2012) demonstrated a functional asymmetry and molecular difference between the pair of tubules, for example chloride channel *Bestrophin 2* is enriched in the anterior tubule over the posterior. They suggested that the basic function of transport and signalling was similar for the tubules but the positional difference between the two pair of tubules resulted in functional difference. The anterior tubules being positioned close to the midgut functioned in sequestering excessive solutes (like calcium), detoxifying dietary toxins, and received signals from the neuroendocrine cells of the midgut. The proximity of posterior tubules to the hindgut perhaps protected the insect by neutralising the excess of ammonia being processed in the hindgut (Chintapalli et al., 2012) .

Thus the strategic location of the tubules allows them to communicate to other tissues and help to sense and respond to different types of stressors. Hence, the Malpighian tubules are an excellent *in vivo* model to study mechanisms of epithelial stress handling

1.3 Rationale of the PhD

In a microarray study of whole flies that were fed on a diet of 4% sodium chloride salt (salt-stressed), it was observed that immune response genes previously shown to be transcriptionally activated by immune-related transcription factor Relish (a Drosophila orthologue of human NF-kB), were upregulated when flies were exposed to salt stress for a short time (4 h; 8 h), but were down-regulated when salt stress was continued over longer periods

(Stergiopoulos et al., 2009). These responses suggest that Relish-controlled gene expression is required for an appropriate response to salt stress in the whole fly over shorter timespans. Outside of the immune response genes, other genes that showed significant differentiation in expression (either up- or down- regulated) in this microarray dataset were highly enriched in the tubules and/or the hindgut, suggesting that these tissues play a role in responses to salt stress (Stergiopoulos et al., 2009).

Subsequently, it was shown that the expression of Relish in tubules was localised to the nucleus of tubule principal cells when flies were fed for 24 h on a diet of 2-4% sodium chloride (Overend et al., 2012). Furthermore, in tubules of salt-stressed flies, an upregulation of Diptericin (the signature gene activated by Relish) was observed, which further implies the activation of Relish and its downstream transcriptional effects in the response to salt stress in Drosophila. An increase in Diptericin expression was also detected when tubules were stressed with bacterial lipopolysaccharide (LPS) (McGettigan et al., 2005). This upregulation was a consequence of the increased activity of nitric oxide (NO) synthase, a component of the NO signalling pathway (McGettigan et al., 2005). In addition, Relish in the Drosophila tubule was observed as being activated by cGMP in a dose-dependent manner (Davies et al., 2014).

Although Drosophila is not normally exposed to a high level of salt stress, its genetic amenability and usefulness in enabling comparative genomic studies with humans and other insects, makes it an excellent model for studies in salt stress ((Dow, 2003; Dow and Davies, 2006)). In a study of the gene known as "*inebriated*" (putative neurotransmitters/osmolyte transporter (ine)), it was observed that the *ine* mutants were sensitive to high dietary salt (Huang et al., 2002). It was suggested that this phenotype was a consequence of an inability to accumulate osmolytes (compounds that maintain cell volume and fluid balance) within the tubules and hindgut (Huang et al., 2002). Osmotic stress can be induced by a high salt diet, as well as by desiccation and rehydration (Stergiopoulos et al., 2009). The tubules are key osmoregulatory tissues in which NO/cGMP signalling is an important regulator of tubule function ((Davies, 2000) (Dow and Davies, 2001)).

1.4 Aim of the PhD

The PhD work presented here aims to understand the stress mediated- survival mechanisms, primarily focused on stress-response mechanism of osmotic stress caused due to high dietary salt; with respect to the transcription factor Relish and c-GMP dependent kinases, in the extensively studied epithelium, the *Drosophila* Malpighian tubule. The aims of the study were investigated predominately by carrying out survival assays with the transgenic flies and studying the gene expression in the tubules by qRT-PCR and Affymetrix microarray.

Chapter 2
Material and Methods

2.1 Materials

2.1.1 *Drosophila* stocks

The below list describes the various *Drosophila* lines used in this study, their genotypes and the application. All the fly lines are homozygous unless indicated otherwise in the description. The source of the lines and where applicable the reference to the line is listed.

Table 2-1 *Drosophila melanogaster* lines used in this study

Fly line	Genotype	Description	Source
Canton S	w^+;+/+;+/+	Wild type	Dow/Davies lab stock
Oregon R	w^+;+/+;+/+	Wild type	Dow/Davies lab stock
Rel^{E20}	w^+;+/+;[Rel^{E20}]	Relish mutant (E20 indicates the line number which was found to be a homozygous mutant in the original screening process)	Gift from Prof. B. Lemaitre, EPFL, Switzerland and Prof. S. Kurata, Tohoku University.
UAS-Control RNAi	*y^-w^-;(PKC26)VIE;+;+*	UAS line expressing an empty *KC26* plasmid	Gift from Dr. Edward Green, German Cancer Research Center, Heidelberg
UAS-Relish RNAi	*y^-w^-; P(KK109851)VIE-260B*;+/+	UAS line driving Relish RNAi	Vienna Drosophila Resource Center
UAS–Dg1 RNAi	*y^-w^-; P(KK112484)VIE-260B*, +/+	UAS line driving Dg1 RNAi	
UAS-Dg2 RNAi	*y^-w^-; P(KK112484)VIE-260B*, +/+	UAS line driving Dg2 RNAi	
UAS-Dh44-R2 RNAi	*y^-w^-; P(KK111461)VIE-260B*, +/+	UAS line driving Dh44-R2 RNAi	
Actin-GAL4	*w-; actin-GAL4/cyo;* +/+	GAL4 driver line expressed in all the tissues (Heterozygous)	Dow/Davies lab stock

Cont. Table 2-2 *Drosophila melanogaster* lines used in this study

Fly line	Genotype	Description	Source
c42-GAl4	*w⁻; +/+; c42-GAL4/c42-GAL4*	GAL4 enhancer trap specific to the tubule principal cells.	Dow/Davies lab stock
CapaR-GAL4	*w⁻; +/+;CapaR-GAL4/CapaR-GAL4*	GAL4 driver line expressed in the tubule principal cells	Generated by Dr.Selim Terhzaz (Dow/Davies lab)
c564-GAL4	*w⁻; +/+;c564-GAL4/c564-GAL4*	GAL4 enhancer trap specific to the fat body	Gift from Dr Prof. S. Kurata, Tohoku University.
Alka-GAL4	*w⁻; +/+; Alka-GAL4/Alka-GAL4*	GAL4 driver line expressed in the posterior midgut	Generated by Dr. Gayle Overend (Dow/Davies lab)
Tsp42-GAl4	*w⁻; +/+;Tsp42-GAL4/ Tsp42-GAL4*	GAL4 driver line expressed in the anterior midgut	Generated by Dr.Selim Terhzaz (Dow/Davies lab)
c42-GAL4>UAS-Relish.His6	*w- ;Relish.his6/Cyo;c42-Gal4/+*	Expressing histidine tagged Relish in the tubule principal cell	Generated by Dr. Jon Day (Dow/Davies lab)

2.1.2 Bacterial strain

The below list describes the different bacteria strains used for different experimental studies.

Table 2-2 List of bacterial strain describing their source and use

Bacteria strain	Description and Source
Subcloning Efficiency DH5α Competent Cells	Genotype: F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ. Source Invitrogen.
Erwinia caratovora	Phytopathogen that kills immunocomprised fly. This particular strain is resistant to rifampicin antibiotic. Gift from Prof. B. Lemaitre, EPFL, Switzerland and Prof. S. Kurata, Tohoku University.

The bacterial cultures grown on plates of LB-agar medium containing specific antibiotic were sealed with paraffin tape and stored at 4˚C for a month. For long term storage, 800 µl of bacterial culture was added to 1 ml of 2% peptone, 40% glycerol solution and stored at -80˚C.

2.1.3 Antibodies

The below table describes the primary and secondary antibodies used for the immunohistochemistry, immunocytochemistry and immunoblotting, along with the dilutions at which they were used.

Table 2-3 Antibodies used for immunoblotting immunohistochemistry and immunohistochemistry

Antibody and Source	Concentration and Use
Anti-his(mouse) (Invitrogen)	1:50 (IHC)
Anti-Relish C21F3 (Hybridoma bank, U.S.A) (mouse)	1:100 (Immunoblotting) 1:500 (ICC)
Anti-Rabbit-HRP (Amersham)	1:2000 (Immunoblotting)
Anti-Tubulin(Sigma)	1:2000 (Immunoblotting)
Anti-Mouse-HRP (Amersham)	1:2500 (Immunoblotting)
Anti-Rabbit-cmyc(Sigma)	1:100 (ICC)
Anti-Rabbit-Alexa Fluor 488 (Sigma)	1:600 (ICC)
Anti-V5(mouse) (Sigma)	1:500 (ICC)
Anti-Rabbit- Alexa Fluor 633 (Sigma)	1:600 (ICC)
Anti-ß galactosidase (rabbit) (Sigma)	1:100 (IHC)
Anti-Rabbit-Alexa Fluor 540 (Sigma)	1:600 (IHC)

2.1.4 Recipes of media and solutions

The below section is a list of recipes of media and solutions used in this study for various experiments.

Table 2-4 Recipes of media and solutions

A. Fly food

Mix the contents in 1 litre of H_2O in the below order of preference.

- 10 g Tayo agar
- 1 tbsp Soya fluor
- 15 g Sucrose
- 33 g Glucose
- 15 g Maize meal
- 10 g Wheat germ

Cont. A. Fly Food

- 30 g Treacle
- 35 g Yeast

Bring to boil, stirring constantly; cool to about 70 ºC add below contents:

- 10 ml Nipagin [Nipagin = 25 g. Nipagin M (Tegosept M, p-hydroxybenzoic acid methyl ester) in 250 ml Ethanol]
- 5 ml Propionic acid

Dispense: Fly Vials = 8 ml

B. *Drosophila* Schneiders' & Saline

Amino Acids

- Glycine
- L-Arginine
- L-Aspartic acid
- L-Cysteine
- L-Cystine
- L-Glutamic Acid
- L-Glutamine
- L-Histidine
- L-Isoleucine
- L-Leucine
- L-Lysine hydrochloride
- L-Methionine
- L-Phenylalanine
- L-Proline
- L-Serine
- L-Threonine
- L-Tryptophan
- L-Tyrosine
- L-Valine
- beta-Alanine

Inorganic Salts

- Calcium Chloride ($CaCl_2$-2H2O)
- Magnesium Sulfate ($MgSO_4$-$7H_2O$)
- Potassium Chloride (KCl)
- Potassium Phosphate monobasic (KH_2PO_4)
- Sodium Bicarbonate ($NaHCO_3$)
- Sodium Chloride (NaCl)
- Sodium Phosphate monobasic (NaH_2PO_4-$2H_2O$)

Other Components

- Alpha-Ketoglutaric acid
- D-Glucose (Dextrose)
- Fumaric acid
- Malic acid
- Succinic acid
- Trehalose
- Yeastolate

***Drosophila* Saline'(in 1 litre of H_2O)**

- 6.86g NaCl
- 1.49g KCl
- 0.41g $MgCl_2$
- 0.29g $CaCl_2$
- 0.86g $NaHCO_3$
- 0.7g NaH_2PO_4
- 2.05g HEPES

3.96g Glucose (to be added just before the start of secretion assay)

C. Loading dye

- 0.25% (w/v) bromophenol blue
- 0.25% (w/v) xylene cyanol
- 30% (v/v) glycerol in water

D. Buffers

Phosphate Buffer Saline (PBS) (in H_2O)

- 137 mM NaCl
- 2.7 mM KCl
- 10 mM Na_2PO4
- 2 mM KH_2PO4 (pH 7.4)

Lysis RIPA Buffer (in H_2O)

- 100mM Tris-Cl (pH 7.4), 300mM NaCl
- 10% Triton®X100
- 10% Na deoxycholate
- 200mM Phenylmethanesulfonyl fluoride (in isopropranol)
- 10% SDS
- Pierce® protease and phosphatase inhibitor (Thermo)
- 0.01M EDTA (pH 7.4)

Transfer Buffer (in 1 litre of H_2O)

- 20 % (v/v) Methanol
- 14.4 g Glycine
- 3 g Tris Base

Tris/Borate/EDTA Buffer

- 90 mM Tris
- 90 mM boric acid (pH 8.3)
- 2 mM EDTA

E. Ponceau Stain

- 1.5 g TCA
- 0.5 g Ponceau S stain

F. Bacterial growth media

Component	grams/litre
LB-agar	10
Dried yeast	5
NaCl	10
Bacto-agar	15
SOC broth	2 % (w/v)
Bacto-tryptone	0.5 % (w/v)
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	20 mM
Bacto-tryptone	

2.1.5 Oligonucleotide sequences

The table below is lists of the oligonucleotide sequences (primers) and its application in this study.

Table 2-5 Primer sequences used in this study

Primer name	Sequence (5'→3')
***Pfu*-based Herculase II Fusion polymerase PCR primers**	
Rel-KpnI-FLAG (Forward)	5' – GCACGGTACCATGGA TTACAAGGATGACGACGATAA GATGAA CATGAATCA GTACTACG – 3'
Rel-AgeI (Reverse)	5' – GCACACCGGTTTATCAAGTTGGGTTAACCAGTA GG – 3'
TaqMan-based qRT-PCR primers 56-FAM – Fluorophore with emission wavelength 520nm ZEN-3IABkFQ (3' Iowa Black® FQ)- Quencher with broad absorbance spectra ranging from 420 to 620 nm with peak absorbance at 531 nm	
Rpl32 (Forward)	5' – CGCTTCAAGGGACAGTATCTG – 3'
Rpl32 (Reverse)	5' – TTCTTGAATCCGGTGGGC– 3'
Rpl32 Probe	5' – /56-FAM/ TGTTCGATC/ZEN/CGTAACCGATGTTGGG/3IABkFQ – 3'
Dg1 (Forward)	5' – AGCAATCCTACAGCATACCG– 3'
Dg1 (Reverse)	5' – TTGAATCTACCAGTTCCCGC– 3'
Dg1 Probe	5' – /56-FAM/CCTGCGATG/ZEN/CGTCTATGTTCTTTAGGA/3IABkFQ – 3'
Dg2 (Forward)	5' – CGTCAGTTGTCTGGTCATCG– 3'
Dg2 (Reverse)	5' – GATCTGTGAGGTTAATGTCCCG– 3'
Dg2 Probe	5' – /56-FAM/TTGATCTTT/ZEN/CTGCGTTCCATGGCG/3IABkFQ/– 3'
Relish (Forward)	5' – ATCGCCTGGTCT TTCAGA TG– 3'
Relish (Reverse)	5' – CGCCGTTGTCCTCAATTTTAA AG– 3'
Relish Probe	5' –/56-FAM/ CGCACCTGG/ZEN/TTCAAGTTCATGTCCT/3IABkFQ/– 3'
Diptericin A (Forward)	5' –CAGTACCCACTCAATCTTCAGG– 3'
Diptericin A (Reverse)	5' –CCTCCATTCAGTCCAATCTCG– 3'
Diptericin A Probe	5' –/56-FAM/ TGCAAAGCC/ZEN/AAAACCATCGCCG /3IABkFQ -3'

Cont.:- Table 2-5 Primer sequences used in this study

Drosomycin (Forward)	5’ – ACAAGTCGCTGATAATTCAAACAG – 3’
Drosomycin (Reverse)	5’ – CGAAGAGGGCGAACAAGTAC – 3’
Drosomycin Probe	5’ –/56-FAM/ TGGAAAAGG/ZEN/–TTCTCACGGAGCTTGG /3IABkFQ-3’
Sybr-based qRT-PCR primers	
Diptericin A (Forward)	5’ – TTGCCGTCGCCTTACTTTGCT G – 3’
Diptericin A (Reverse)	5’ – TCCATTCAGTCCAAT CTCGTG G – 3’
Rpl32 (Forward)	5’ – TGACCATCCGCCCAGCATAC – 3’
Rpl32 (Reverse)	5’ – TTCTTGGAGGAGACGCCG TG – 3’
Diptericin B (Forward)	5’ - CTCGAGTGCCTGGGCTTATC - 3’
Diptericin B (Reverse)	5’ - AAGGTGCTGGGCATACGATC - 3’
Sugarbabe (Forward)	5’ – GCGATAGAGTTTTCGACACCTTG – 3’
Sugarbabe (Reverse)	5’ – TCGAAGCTGCACTTGTAGGG – 3’
CG17751 (Forward)	5’ - GCGATAGCAGCAGTTTTGTGT - 3’
CG17751(Reverse)	5’- TCTGGCCGAGAACATCTGTG -3’
CG10621 (Forward)	5’ – ATGTGGGTGACTCTGTGGATG – 3’
CG10621 (Reverse)	5’ – GAGATAGCGTTCCTTGGCGA – 3’
CG6293 (Forward)	5’ – GCCATTGAGTATCGGGGCTT - 3’
CG6293 (Reverse)	5’ – CTTGGAAGCATCTGTGGCGT - 3’
CG11407 (Forward)	5’ – GAGAAGGTCCGATCTGCCAC – 3’
CG11407 (Reverse)	5’ – TGGTGGTTAAGGAATGACTGGA– 3’
CG7968 (Forward)	5’- ACTGAGAACTTTGGTTGCAATGG - 3’
CG7968 (Reverse)	5’ – CAACTCCAGACTGAGGGCAC – 3’
Cyp4e1 (Forward)	5’ – AGTACGGCAAGGATAACTTTCG – 3’

Cyp4e1 (Reverse)	5’ – GAAGTGGAAGGCTGGGGTT - 3’
Cyp4e3 (Forward)	5’ - TTTGAACGGAAGGCATCGC - 3’
Cyp4e3 (Reverse)	5’ – ATTGCTTGGGATTGGTCATCAC – 3’
NPFR1 (Forward)	5’ – ACCTGGCCATATCGGACCT – 3’
NPFR1 (Reverse)	5’ – GTGGGGTACACGATCACCTG - 3’
CG12290 (Forward)	5’- TTCCCTGGTCAGTCAAGTGC - 3’
CG12290 (Reverse)	5’ – CTTTTGGGGCATGGTTGGTG - 3’
Ndae1 (Forward)	5’ – TTTTCGGAAATGGGGATGCTG - 3’
Ndae1 (Reverse)	5’ – ACCATGTGATCGCACACGAG - 3’

2.2 *Drosophila* rearing

Flies were maintained at 55% humidity on a standard Drosophila diet (recipe in Table 2-4.A) during 12:12 h light-dark cycle. Unless specified all flies were reared 22°C. To avoid the possible stressful effects caused by CO_2 that was used for anesthetizing flies, CO_2-anaesthetized flies were segregated into males and females and grouped in number of 30-35 flies and allowed to recover for 24 h on normal food before performing any experiments. All the experiments performed in this thesis work are carried out in adult male flies only unless specified otherwise.

2.3 Survival assay experiments

7- to 12-day-old adult were subjected to stress in groups of 30-35 with 2-5 biological replicates for each line. The assay was terminated once 100% mortality was reached. The mortality data were curated using Perl script (written by me) and expressed as percentage survival ± S.E.M, plotted in form of Kaplan-Meier survival curves using Graph Pad Prism 6.0 software. The curve was plotted in form of trend line. Data were assessed for statistical significance by the Log Rank (Mantel-Cox) test. The *p*-value calculated is two-tailed and *p*-value ≤ 0.05 was considered significant. Each fly death on a certain time point was

inputted and the graph generated had the mean of the deaths at each time point with error bars showing the ± S.E.M. The number of flies (n) was sufficiently high to allow for statistical significant differences in survival.

2.3.1 Salt and Desiccation stress

For salt stress assay, flies were kept in vials without food or water for 5 h in the afternoon and then fed in the evening with normal food supplemented by 4% NaCl (Stergiopoulos et al., 2009) and mortality rate was counted every 24 h. For desiccation assay, flies were kept in vials without food or water. The mortality rate was determined ever hour after 12 h. All flies were maintained at 22°C.

2.3.2 Immune stress assays

One colony of rifampicin resistant *Erwinia caratovora* (Basset et al., 2000) were grown in LB broth (Table-2-4.F) containing 50 µg/ml of rifampicin in an incubating shaker at 29°C for 8 h. A 1:200 dilution of the culture was made and the diluted culture was grown for a further 16 h while shaking at 29°C. The bacteria were concentrated to OD_{600}=1 (containing ~10^{10} CFU/ml) in 1X PBS (Table 2-4.C). Flies were gently pricked in the abdomen with a thin needle (BD MicrolanceTM 3, 26G x 5/8) dipped in either 1X PBS or concentrated bacteria (Basset et al., 2000). Following the infection, flies were transferred into clean vials, incubated at 26°C depending on the temperature at which the flies were reared. The flies' survival was monitored over a time course of days depending on the lethality of the bacteria.

2.4 *Drosophila* tissue dissection

Tissue dissections were performed on flies anaesthetised on ice. Tissues were dissected in *Drosophila* Schneider's medium (Gibco) with two pairs of watchmaker's forceps (No.55 Biologie,U.K.). The tubules were kept in this medium for no more than 30 min and were used in various experiments: fluid secretion assay, microarray experiment, and immunohistochemistry.

2.5 Ramsay's Fluid secretion assay for *D.melanogaster* tubules

The fluid secretion assays were performed as described previously (Dow et al., 1994b). A petri dish filled with paraffin wax having depressions filled with 9 µl bathing medium drops (1:1 Schneider's and *Drosophila* saline/glucose (396 mg of glucose per 100 ml). The dish was filled with mineral oil (to prevent evaporation). The bathing medium contained trace amounts of the red dye, Amaranth, to view the secreted fluid. A pair of Malpighian tubules linked by ureter were dissected as above, was placed in the drop using a fine glass rod. In the pair of tubule, one tubule end was inside the drop while the other tubule was wrapped around a thin steel pin outside the drop, to which it adhere by surface tension (Figure 2-1).

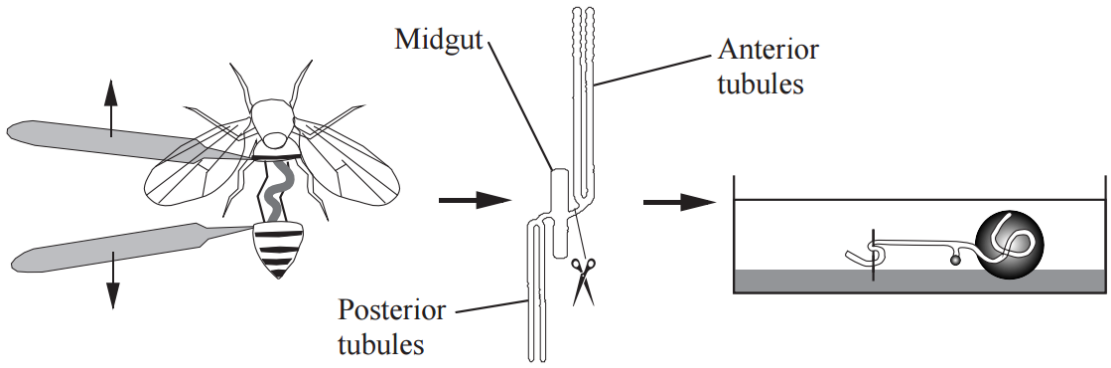


Figure 2-1: Schematic diagram of fluid secretion assay ((Dow et al., 1994b)). Description in text.

Every 10 min, the secreted fluid emerging at the cut end of the ureter was removed and measured for its diameter. The measurement was performed using an ocular micrometer and the volume of the secreted fluid was calculated in nl/min using the equation $4/3(\pi r^3)$. The data was analysed in Microsoft Window Excel version 14.0 and processed data was plotted as mean ±S.E.M using Graph Pad Prism 6.0 software. Student's t-test for unpaired samples (in a group of at least 8 tubules per genotype), taking the critical p-value as 0.05 (two tailed) was used for assessing statistical significance between the fluid secretion rates of genotypes. For basal fluid secretion rates 6 measurements were taken over a 60 min period.

2.6 RNA extraction

Tubules were dissected from 40-50 male flies in *Drosophila* Schneider's medium, transferred in 200 µl of RLT buffer (Qiagen)containing 10 µl/ml ß-mercaptoethanol and homogenized using sonicator(Misonix, Inc., USA). The homogenate was centrifuged at 3000g for 3 min at 4˚C and the supernatant was transferred to a fresh tube. An equal volume of 70 % ethanol was added and mixed, transferred to a RNAeasy QIAGEN column. RNA extraction was done using Qiagen RNeasy Mini kit following manufacturer's protocol. To reduce genomic DNA contamination, a column DNA digestion step (using Qiagen DNAase kit) was carried out. RNA was eluted using 35 µl nuclease-free water and stored at -80˚C.

The quantity and purity of RNA was determined by Nanodrop spectrophotometer (ND-1000 V3.7.1). For measuring concentration of RNA, optical density (OD) at 260 nm was measured where OD of 1 at 260 nm corresponds to 40 µg/ml of RNA. The ratio of OD 260 nm and 280nm was checked for estimating purity of RNA and values between 1.8 and 2.0 were considered to be good quality.

The Glasgow Polyomics facility determined the RNA integrity using Agilent 2100 Bioanalyzer that uses RNA integrity number (RIN) software tool to assign values from 1-10 as an indicator for integrity of RNA. Higher RIN values indicated greater integrity. RNA with RIN value ≥7 was used for microarray experiment.

2.7 Oligonucleotide (primer) synthesis

2.7.1 Standard PCR primer design

Oligonucleotide primers were designed using web source Primer3, NCBI; FlyPrimerBank, DRSC and using software MacVector 11.1.1 (MacVector, Inc. UK). The design of the primer pair wherever possible was either (1) extending across an intron, or (2) one of the primer in the pair was designed across two exon boundaries. The lyophilised primers(Integrated DNA Technologies) were re-suspended in nuclease-free water and working concentration of 6.6µM was prepared from the stock of 100 µM. Primers were stored at -20 ˚C until further use. A list of primers used is in Table 2-5.

2.7.2 Taqman Gene Expression Assay design

Integrated DNA Technologies (IDT) PrimerQuest primer and probe design tool was used to design Taqman Gene Expression Assays and sent to IDT customer primer service for synthesis. The Taqman Gene Expression Assay consists of a pair of unlabelled PCR primers and TaqMan probe with a fluorophore 6-carboxyfluorescein FAM label on the 5' end, and minor groove binder (MGB) nonfluorescent quencher (NFQ) on the 3' end. The probe binds to a region of cDNA between which the primers are designed. During the PCR reaction when the *Taq* polymerase enzyme extends the primers and starts synthesising nascent strand, the enzyme's 5' to 3' exonuclease activity degrades the probe; fluorophore from the probe is released, breaking away from the quenching effect of the quencher. The fluorescence from the released fluorophore is detected in the quantitative PCR thermal cycler. The fluorescence detected is directly proportional to the fluorophore released and the amount of cDNA of interest present in the PCR. The contents of expression assay were briefly centrifuged at 750g, re-suspended in 500 µl TE buffer (10mM Tris, 0.1mM EDTA, pH8.0) resulting in 20X primer probe stock. The final concentration of 500 nM primers, 250 nM probe was used to run qRT-PCR. A list of primer and probe used in the experiments are listed in Table 2-5.

2.8 Polymerase Chain Reaction

2.8.1 cDNA synthesis

First strand cDNA (complementary DNA) synthesis from messenger RNA was carried out using the following reverse transcription reaction protocol. The reverse transcription reaction contained: 275 -500 ng of total RNA, 0.2 mM of each dNTP(Promega), 40 U RNaseOUT (Invitrogen), 10 mM dithiothreitol (DTT), 1X first strand buffer(Invitrogen), 1X oligo-dT (IDT,U.K.), final volume made up with nuclease free water to 18 µl. The reaction was mixed briefly and incubated at 42˚C for 10 min, cooled on ice for 5 min and 2 µl of Superscript II RNAse H^- Reverse Transcriptase (Invitrogen) was added to each reaction tube. The

reaction was incubated at 42˚C for 50 min and then incubated at 70˚C for 15 min to stop the reaction. The cDNA was stored at -20˚C.

2.8.2 Standard PCR

This protocol was used for optimizing annealing temperature of primers used for quantitative real-time PCR. In brief, each 25 µl reaction contained 1 µl of cDNA mixed with left and right primers at a concentration of 0.5 µM and pre-aliquoted ready-to-use DreamTaq Green PCR Master Mix (Thermo Scientific) that contained 2X DreamTaq Green buffer , dNTPS (0.4 mM each), and 4 mM $MgCl_2$ and enhanced DNA polymerase. The cycling was carried out in 0.2 ml PCR tubes using the Hybaid Gradient PCR Express-Gradient thermocycler for running a gradient of annealing temperatures across the block at the same time. A typical cycling procedure is described in Table 2-6.

Table 2-6 Taq DNA polymerase PCR cycling parameters

Step	Temperature, ˚C	Time	Number of cycles
Initial Denaturation	94˚C	2-5 min	1
Denaturation	94˚C	30 s	35 -40 cycles
Annealing	55-65˚C	30 s	
Extension	72˚C	30 s	
Final Extension	72˚C	5 min	1

2.8.3 *Pfu*-based Herculase II Fusion polymerase PCR

A high fidelity DNA polymerase *Pfu* (Agilent, U.K.) was used for high fidelity synthesis of long PCR product. The reaction mix was set up according to the manufactures' protocol as follows: 5X *Pfu* DNA polymerase buffer, dNTPs each at 25 mM, primers at 10 µM, 1 µl cDNA, 1.25 U *Pfu* DNA polymerase, final volume of 50 µl with sterile dH_2O. The reaction cycling parameter followed is described in Table 2-7.

Table 2-7 *Pfu*-based Herculase II fusion polymerase PCR cycling parameters

Step	Temperature, ˚C	Time	Number of cycles
Initial Denaturation	95˚C	2min	1
Denaturation	95˚C	30 s	35 -40 cycles
Annealing	55-65˚C	30 s	
Extension	72˚C	3 min	
Final Extension	72˚C	10 min	1

2.8.4 Quantitative Reverse Transcriptase PCR (qRT-PCR)

The sample cDNA used for quantitative PCRs was prepared as described above. In order to check for (1) genomic contamination, cDNA preparation from identical RNA samples were prepared without adding the reverse transcriptase enzyme and (2) background fluorescence, qRT-PCR reactions without cDNA were run alongside the sample cDNA qRT-PCR reactions. Reactions were set up in MicroAmp Optical grade PCR strips (Applied Biosystems). qRT-PCR reactions were set up in triplicates with primers of gene of interest (target gene). A standard gene (house-keeping gene), *Rpl32* that encodes for a ribosomal protein was used to quantify the relative amount of each gene.

Following amplification, each qRT-PCR reaction from a specific sample cDNA was analysed for its C_t (threshold cycle) value specific to target gene and *rp49* gene. Relative quantification for target gene in each sample cDNA was determined by normalizing target gene C_t value to its *Rpl32* C_t value. By calculating the ratio of the two compared samples' C_t values and using the $2^{-\Delta\Delta\ Ct}$ method (van Iterson et al., 2009), the relative fold change data of target gene between two samples was obtained. The assumption made for this type of comparative analysis was that the PCR efficiency of the amplicon of interest was steady over all the cycles. The results were plotted as mean ±S.E.M (where control = 1) using Graph Pad Prism 6.0 software. Student t-test method was used to determine statistical significance of data where three biological replicates were used. The qRT-PCR in which only two biological replicates were used, the mean values were plotted and no statistical test was carried out on it. qRT-PCR used to validate microarray experiment results had only 2 biological replicates hence no statistical test was applied for it. Depending on experimental design, two different qRT-PCR

methods to detect PCR products were used to quantify levels of expression of target gene.

2.8.4.1 SYBR Green-based qRT-PCR

For this method, the gene-specific primers were designed according to above section that yielded a PCR product of 150-500 bp and wherever possible spanned intron/exon boundaries of each gene of interest in order to control for possible genomic contamination. Each reaction setup contained: 10 µl of 2X SYBR Green real-time PCR master mix (Agilent Technologies) , 1 µl of each primer (0.3µM final concentration) and 1.5 µl cDNA, made up to a final volume of 20 µl with sterile dH_2O. The SYBR Green real-time PCR master mix contains the mutant Taq DNA polymerase, dNTPs, Mg^{2+}, a buffer specially formulated for fast cycling, and the double-stranded DNA-binding dye SYBR Green I to detect PCR product as it accumulates during PCR. The cycling was performed used an Opticon 3 thermal cycler according to the protocol described in Table 2-8. The CT value from the amplification curve was analysed using Opticon 3 software.

Table 2-8 SYBR Green based qRT-PCR cycling parameters

Step	Temperature , ˚C	Time	Number of cycles	Comments
Initial Denaturation	95˚C	10 min	1	To ensure template denature
Denaturation	95˚C	30 s	40 cycles	
Annealing	56-58˚C	30 s		Temperature varied depending on the gene-specific primer pair
Extension	72˚C	30 s		5 s per 100 bp product
Data Acquisition	-	-		Fluorescence data collection is performed after each cycle
Final Extension	72˚C	5 min	1	-
Melting curve	60-90 ˚C	1 s hold per 0.3 ˚C		Used to check the specificity of the amplified product

2.8.4.2 TaqMan-based qRT-PCR

The primers probe design for this qRT-PCR method is described in section above. Each 10 µl qRT-PCR reaction consisted of 5 µl Taqman Gene Expression Master Mix, 0.5 µl 20X Taqman probe, 3 µl nuclease-free water and 1.5 µl cDNA. Using the cycling parameter described in Table 2-9, cycling was carried out in Applied Biosystems StepOne/StepOnePlus Real-Time PCR amplification data was analysed using DataAssist Software.

Table 2-9 TaqMan-based qRT-PCR cycling parameters

Step	Temperature, ˚C	Time	Number of cycles
Initial Hold	50˚C	2min	1
Denaturation	95˚C	10 min	
Denaturation	95˚C	15 s	40 cycles
Annealing and Extension	60˚C	1 min	

2.9 Agarose Gel Electrophoresis

The quality and specificity of PCR products or DNA was assessed by running it on 1% TBE agarose gel made in 0.5% TBE, containing 0.1 µg/ml EtBr using 0.5% TBE as the electrophoresis buffer. Prior to loading, 6x loading dye was added to samples. The sizes of the samples were compared with 1 kb ladder (Invitrogen). Typically electrophoresis was carried out at 100 V and the DNA was visualised using high performance ultraviolet transilluminator (UVP, UK).

2.10 PCR/Gel purification

PCR products or DNA bands excised from the gel were purified using PCR/Gel purification kit (Invitrogen) according to manufacturer's protocol. DNA was eluted in 20-30 µl of nuclease-free water or EB buffer (Qiagen, U.K.).

2.11 Molecular Cloning

2.11.1 Plasmid vector

The plasmid vectors used for expression of genes of interest in *Drosophila* S2 cells were pAc5.1/V5-His and pMT/V5-His (Invitrogen). Both these vectors contain the C-terminal V5 epitope and 6xHis tags to aid the detection of recombinant protein. The Figure 2-2 summarizes pAc5.1/V5-His plasmid vector.

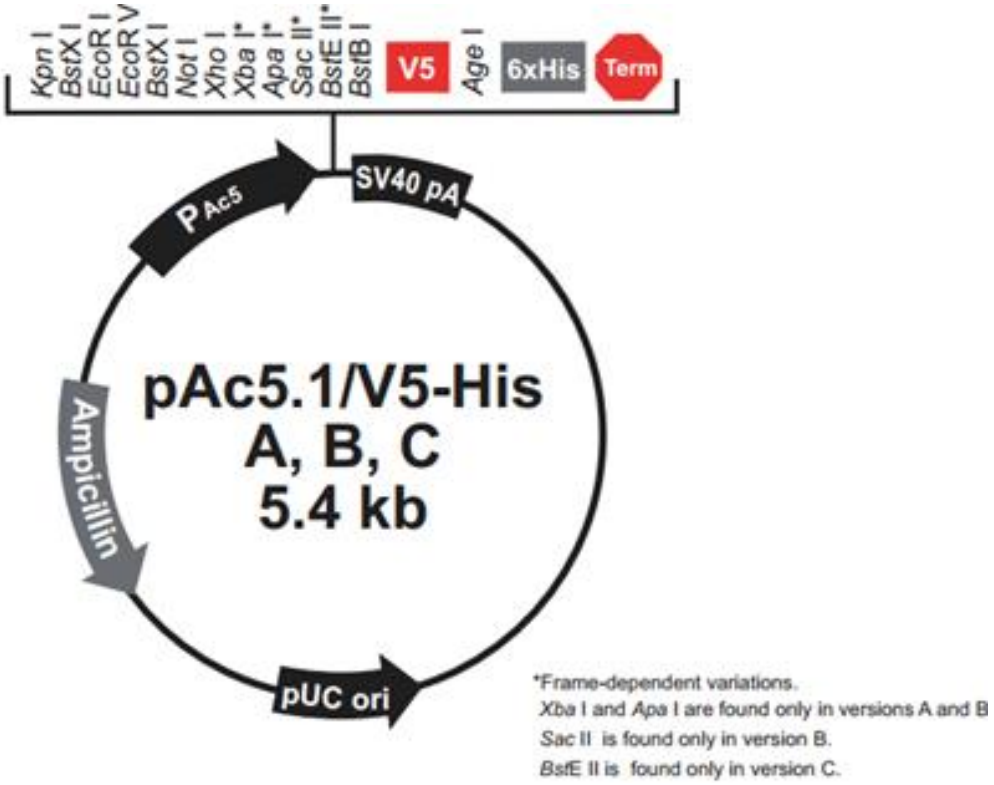


Figure 2-2: Map of pAc5.1/V5-His vector: The figure summarizes the features of the pAc5.1/V5-His A, B, and C vectors. The difference between the subtype vectors is frame dependent variation at certain sites as indicated in the diagram (www.invitrogen.com).

The pAc5.1/V5-His vector contained constitutively expressed the *Drosophila* actin 5C (Ac5) promoter and pMT/V5-His vector contained the inducible *Drosophila* metallothionein (MT) promoter for high-level expression of the gene of interest in S2 cells (Chung and Keller, 1990) and ampicillin resistance gene (*amp*^{R}) encoding ß-lactamase for selection of transformants in *E. coli*. The description of the plasmids used in this PhD work is stated below.

Table 2-10 A list of S2 cell expression constructs used for this study

Plasmid name	Vector backbone	Description	Source
pAc5.1- Relish-FLAG	pAc5.1/V5-His	The plasmid was constitutively expressed. Relish was tagged with FLAG at the N-terminal.	The plasmid was constructed in this study.
pAc5.1-Relish-cmyc	pAc5.1/V5-His	The plasmid was constitutively expressed. Relish was tagged with cMyc at the N-terminal	The plasmid was constructed by Dr. Jon Day (Dow/Davies lab)
pMT-DG1.V5	pMT/V5-His	The protein made was V5 tagged DG1 kinase at C-terminal and was expressed on induction with $CuSO_4$.	The plasmids were constructed by Dr. Mathew MacPherson (MacPherson et al., 2004b)

2.11.2 Restriction Digestion

The plasmid vector pAc5.1/V5-His was single or double restricted digested in a 50 µl reaction containing KpnI-HF and/or AgeI enzymes (at a ratio of 1 Unit of enzyme/µg of DNA), with appropriate enzyme specific buffer, bovine serum albumin (100 µg/ml), nuclease-free water and 1 µg of plasmid DNA. The reaction was incubated for 2 h at 37˚C followed by 15 min incubation with 1 U of calf intestinal alkaline phosphatase (Promega). The digested vector was ran on 1% TBE agarose gel and gel purified using PCR/Gel purification kit (Qiagen). The purified digested vector DNA was stored at -20˚C.

2.11.3 DNA ligation.

To generate the insert, sequence for a Kpn site and a FLAG (DYKDDDDK) epitope were included in the forward primer and Age1 site in the reverse primer. The *Relish* gene was amplified from cDNA and the PCR reaction was run in 1.5% TBE agarose gel. It was then isolated and purified using PCR/Gel purification (Qiagen).

For the ligation reaction, 150 ng of PCR product was ligated with 50 ng vector (molar ratio of vector: PCR product was 1:5). Ligation reactions were carried out using the Roche Rapid DNA Ligation Kit following manufacturer's protocol, and the reactions incubated at room temperature for 30 min before transformation into competent *E.coli* cells.

2.11.4 Transformation into *E. coli*

A 1:50 dilution of the ligation mixture was added to competent *E.coli* DH5α cells incubated on ice for 1 h, heat shocked at 42 ˚C for 30 s. The cells were allowed to recover for 2 h in 200 µl LB-broth and then 100 µl of transformed cells were spread onto LB-agar plates containing 100 µg/ml ampicillin and incubated overnight at 37˚C.

2.11.5 Identification of positive clones

The transformed *E. coli* were grown in the presence of 100 µg/ml ampicillin on LB plates. To identify the transformed *E. coli* expressing the plasmid with the insert of gene of interest, the transformed colony of *E.coli* from agar plate was picked using a sterile pipette tip and grown overnight at 37˚C in LB-broth containing ampicillin and the plasmid DNA was isolated using Qiagen mini kit (Qiagen). The isolated plasmid was then diagnostically checked by restriction digestion and PCR and sequenced as described below.

2.11.5.1 Diagnostic Restrict digest

Single or double restriction digestion of plasmid DNA was carried out according to the protocol described in above with enzymes *KpnI-HF*, *AgeI-HF* ,*EcorV* and *Cal-I* and their respective buffers. The digested products were ran in 1% TBE agarose gel and the DNA bands were assessed for the correct size if the plasmid contained the insert in right direction. All the enzymes were ordered from New England Biolabs, U.K.

2.11.5.2 Diagnostic PCR

To identify the presence and orientation of the insert in the vector, the isolated plasmid DNA was tested by PCR. Reactions were set up according to the protocol described above, using the forward primer that bound to the insert and the reverse primer that bound within the boundary of the insert and vector. PCR products were ran in 1% TBE agarose gel and positive bacterial colony plasmid DNA that contained the insert produced a band in the gel.

2.11.5.3 DNA sequencing

The DNA plasmid containing the insert identified using the methods mentioned above was sent for Sanger sequencing to the GATC Biotech Company, U.K. to check for any possible errors in the proof reading of the polymerase while making of the insert or any unpredicted errors that may have been incorporated during cloning procedures. The list of primers used for sequencing is listed in Table 5. The sequencing results received from GATC were assessed using software MacVector 11.1.1 (MacVector, Inc. UK).

2.11.6 Plasmid isolation

Bacterial cultures grown overnight were centrifuged down at 3000 g for 15 min at 4˚C. The pelleted cells were lysed using lysis buffer (Buffer P1 containing RNAse A and lyse reagent (Qiagen)) and transferred on the column for plasmid isolation. The isolation and purification of plasmid DNA was performed using Qiagen mini kit or maxi kit (Qiagen) following manufacturer's protocol and

plasmid DNA was eluted in 30 µl of water (for minipreps) or resuspended in 500 µl of water (for maxipreps) respectively.

2.12 *Drosophila* S2 cell culture

2.12.1 Passaging

Drosophila S2 cells (Invitrogen) were maintained in complete Schneider's medium (CSM) [Schneider's medium supplemented with 10% fetal calf serum (FCS)] at 28˚C. 10 ml of cells were kept in T75 flasks. S2 cells are round and loosely- attached when cultured in tissue culture flasks (Buster 2010). For general maintenance, cells were passaged at a density of 10^7 cells/ml. The high density cells were resuspended by gentle pipetting and then diluted 1:2 to a new flask by adding 5 ml of cells into 5 ml of fresh CSM. After passaging for two months, the cells were discarded.

2.12.2 Transient transfection of S2 cells

Exponentially growing S2 cells at 1 X 10^6 cell/ml density were seeded in a 12-well tissue culture plate and grown for 24 h at 28˚C. Transfections were carried out using Calcium Phosphate kit (Invitrogen) as per manufacture's protocol. In brief, 200 µl transfection reaction contained 7 µg of plasmid DNA, 12 µl 2M $CaCl_2$, 100 µl 2x HEPES buffered saline and sterile dH_2O. In case of co-transfection experiment, 3.5 µg of each plasmid DNA was taken with the total plamid DNA used for transfection being 7 µg. The transfection reaction was left to precipitate for one hour before adding to the seeded S2 cells. The cells transfected with single plasmid were grown for further 36 h and were then collected by centrifugation at 100 g for 4 min. The cells were resuspended in fresh CSM containing either NaCl (75 mM and 150 mM) or Sorbitol (0.125mM and 0.25mM) and treated for 30 min. As control of the experiment, S2 cells were treated for 30 min with either 5 µg/ml *E. coli* gram-negative peptidoglycan (PGN, Invivogen, U.K.) diluted in CSM or CSM. After treatment, S2 cells were centrifuged at 1000 g for 3 min. The supernatant was removed completely and the pellets were either frozen at -80˚C for protein analysis.

The cells that were co-transfected with the plasmids that had the vector backbone as pMT/V5-His were washed after 16 h. The cells were re-suspended in fresh CSM and re plated in the plate. To induce the expression of the protein, the gene was induced with 5 µl of 100 mM $CuSO_4$. The cells were further grown for 24 h and were then harvested by centrifugation at 100g for 4 min and used for immunocytochemistry study.

2.13 Protein analysis

2.13.1 Extraction

The pelleted S2 cells as described above were lysed in RIPA lysis buffer containing a protease and phosphatase inhibitor cocktail (Thermo, U.K.) and homogenized using sonicator (Misonix, Inc., USA). The homogeneous protein lysate was kept on ice for 30 min and then centrifuged at 5000 g for 5 min at 4°C. The supernatant was transferred into a fresh eppendorf tube and stored at -80°C until further use.

2.13.2 Protein quantification

The amount of protein was quantified using Bradford assay kit (BIO-RAD). A standard curve was generated using BSA standards in range of 0-5 µg in sterile dH_2O. Assay was carried out in a 96-well microtiter plate. 5 µl of BSA standard and protein supernatant were set up in triplicate and 200 µl of diluted Bradford dye reagent with ratio of 1:5 in dH_2O was added to each well and mixed by pipetting ensuring no generation of bubbles. The plate was incubated on a shaker at room temperature for 5 min. Using a plate reader, each well was recorded for its absorbance at 590 nm and standard curve was generated by plotting absorbance of BSA standards against the known concentration. The unknown protein sample absorbance was interpolated on the standard curve to calculate its protein concentration using Graph Pad Prism 6.0 software.

2.13.3 SDS-PAGE separation

Protein electrophoresis was performed using Novex NuPAGE electrophoresis system. 25 µg protein sample was prepared by adding 4X SDS-PAGE loading buffer and 1 µl of 0.5M DTT to final volume of 20 µl. Samples were denatured at 70° C for 10 min, vortexed and pulse-spun, before loading onto a 4-12 % Bis-Tris NuPAGE Gel (Invitrogen). The gel was then run in NuPAGE MOPS SDS running buffer (Invitrogen) at 160 V for first 5 min and then 130 V for next 50 min.

2.13.4 Western blotting

Proteins separated on NuPAGE Gel were transferred onto Hyband transfer membrane (Amersham) using Xcell II blot module at constant 70 V for 1 h. To check for the efficiency of the transfer, Hyband membrane was briefly stained with Ponceau S staining solution (recipe Table 2-4.e) and washed with distilled water for visualisation of the protein bands on the membrane. The membrane was blocked in blocking solution (PBS with 0.1% Tween 20, 5% and 5% non-fat dry milk powder) at room temperature for 2 h. The blocked membrane was washed on shaker 3 x 15 min with PBS with 0.1% Tween 20. The membrane was incubated overnight at 4°C with primary antibody diluted in blocking solution as indicated in Table 2-3. Membrane was thoroughly washed 4 x 15 min h in PBS with 0.1% Tween 20 while shaking at room temperature. It was blocked for 1 h in blocking solution. The membrane was incubated in HRP-conjugated secondary antibody diluted in blocking solution (description in Table 2-3) at room temperature for one hour.

The membrane was washed 4 x 15 min with PBS with 0.1% Tween 20. The secondary antibody was detected using ECL Western Blotting detection system (Amersham Biosciences) as manufacturer's instruction. The membrane was then exposed to X-ray film (Kodak) in the dark. The film was developed using XOMAT film processor with varying exposure times (15 s to 2 min).

2.14 Immunostaining of *Drosophila* tissues and cells

2.14.1 Immunohistochemistry (IHC) of intact *Drosophila* tissues

Intact tissues were dissected carefully in Schneider's medium. The tissues were incubated for 2 h either in Schneider's medium or Schneider's medium containing 5 µg/ml PGN or 300 mM NaCl. They were washed with 1X PBS (pH 7.4) and transferred into a 1.5 ml tube containing PBS (pH 7.4). Tissues were then fixed in 4 % PAF in 1xX PBS at room temperature for 20 min. The tissues were washed 3x with 1x PBS, then permeabilised with PBS with 0.5 % Triton X-100 (PBT) for 45 min. The tissues were then blocked with PBT + 10 % goat serum (Sigma) for 3 h at room temperature. Primary antibody was diluted in blocking solutions as described in Table 2-3 and then added to tissues. They were incubated overnight while shaking at 4˚C.The tissues were washed 4 x 15 min with PBT and incubated in blocking solution for 4 h. The tissues were then incubated with secondary antibody (diluted in blocking solution) (see Table 3 for description) overnight in the dark at 4˚C, while shaking. The tissues were then washed 3 x 20 min with PBT. The tissue cells' nuclei were stained using 500 ng/ml DAPI (4',6-diamidino-2-phenylindole)for 5 min. Tissues were washed 3 x 20 min with PBS, transferred on pre-treated Poly-lysine-coated microscope slides, mounted in Vectashield (Vector labs, U.K.), and sealed with glycerol/gelatin (Sigma) . The samples were viewed using a confocal microscopy system.

2.14.2 Immunocytochemistry (ICC) of S2 cells

After the S2 cells were harvested as described above, they were resuspended in 100 µl PBS and were transferred to pre-treated Poly-lysine-coated plates and were allowed to settle for 30 min. The excess PBS solution was removed. The cells were then fixed with 4 % PAF in PBS at room temperature for 20 min, washed 3X with PBS and permeabilised with PBT for 30 min. Blocking was done using PBT + 10 % goat serum (Sigma) for 1 h at room temperature, followed by a 2 h incubation with the primary antibody (Table 2-3) at room temperature. The cells were washed 4 x 15 min with PBT for 1 h and incubated in blocking solution for 1 h. The cells were then incubated with the secondary antibody (diluted in

blocking solution) (see Table 2-3 for description) for 2h. The cells were then washed 3 x 20 min with PBT for 1 h. In the S2 cells that were co-transfected, similar washing and antibody incubations were done for 2nd primary and secondary antibody. After the final washing step after the secondary antibody incubation, the cells' nuclei were stained using 500 ng/ml DAPI for 5 min. The cells were washed 3 x 15 min with PBS and mounted in Vectashield (Vector labs,U.K.).

2.15 Confocal Microscopy

Fluorescent imaging of the tissues and S2 cells was carried out using the LSM510 Meta confocal microscope system (Zeiss Technologies UK). A HeNe1 543nm laser and a 561-625 band pass filter were used for imaging the Alexafluor 568 secondary antibody. An Argon 488 laser and a 505-530 band pass filter were used for imaging the FITC antibody. The nucleus was stained with DAPI for its visualisation. The DAPI was excited using the standard UV source (mercury lamp) and the image captured using the confocal photomultipliers. The DAPI image was then merged with the other channels retrospectively using the proprietary LSM Meta 520 Meta Browser software. Either A 40x (oil immersion) or 63x objectives was used.

2.16 Microarray

2.16.1 Affymetrix Drosophila Genome 2.0 Array

Tubule dissections were performed on wild type and Relish mutant flies fed with and without 4% NaCl. The flies were dissected in the Schneider's medium and RNA extracted from tubule tissue and its quantity; quality and integrity was checked as described above. Four biological replicates for each genotype and treatment condition were used to create a transcript profile for each dataset. The biological replicates were biologically distinct samples to help capture any random biological variation (Blainey et al., 2014). Thus 16 microarrays were run to produce four dataset for each genotype and treatment condition. The RNA from each sample were prepared and supplied to The Glasgow Polyomics Centre, University of Glasgow (http://www.polyomics.gla.ac.uk/) who ran the

microarray experiment and carried out the normalization analysis. Target preparation from 500 ng RNA of each sample was done using the One-Cycle Eukaryotic Target labelling Assay protocol and kit (Affymetrix PN 900431) according to the manufacture's protocol. Figure 2-3 outlines 3' In vitro Transcription assay protocol assay and briefly describes the steps of preparation of cRNA (complementary RNA) and its hybridization to probes on the chip. The chip is then scanned to obtain "snapshot" of the transcriptome.

Briefly, double stranded DNA (dsDNA) was synthesised from each RNA sample in three-step process: (1) RNA structure was denatured in presence of PolyA control and T7 oligo (dT) primer mixture, (2) first strand synthesis was performed using superscript II, and (3) second strand synthesis was performed using DNA ligase and DNA polymerase. The ends of dsDNA were polished using T4 DNA polymerase. The dsDNA was then purified using a DNA clean up kit. Biotin-labelled cRNA was generated by 'In vitro Transcription' from the generated dsDNA library. The quality of cRNA was assessed by Agilent 2100 Bioanalyzer.

The cRNA was then fragmented into 35-200 base fragments using 5X Fragmentation Buffer and prepared for hybridization to the Drosophila Genome 2.0 Array. For target hybridization, 200 µl array target solution was prepared that contained 10 µg of fragmented cRNA, control oligo B2 and 20X hybridization control. 130 µl of this array target solution was hybridized with array at 60 rpm, 45˚C for 17 h.

The probe array was washed and stained using Streptavidin Phycoerythrin (SAPE) Antibody and SAPE stain. This process was performed on Fluidic station 450 using GeneChip Operating software (GCOS) to run the Midi_euk2v3 protocol in line with Affymetrix's instruction.

The array was scanned by Affymetrix Scanner 3000 7G generating intensity files (with extensions .CEL) that represented the raw data that were further analysed by Partek Genomic Suite 6.6 (version 6.13.0412) software.

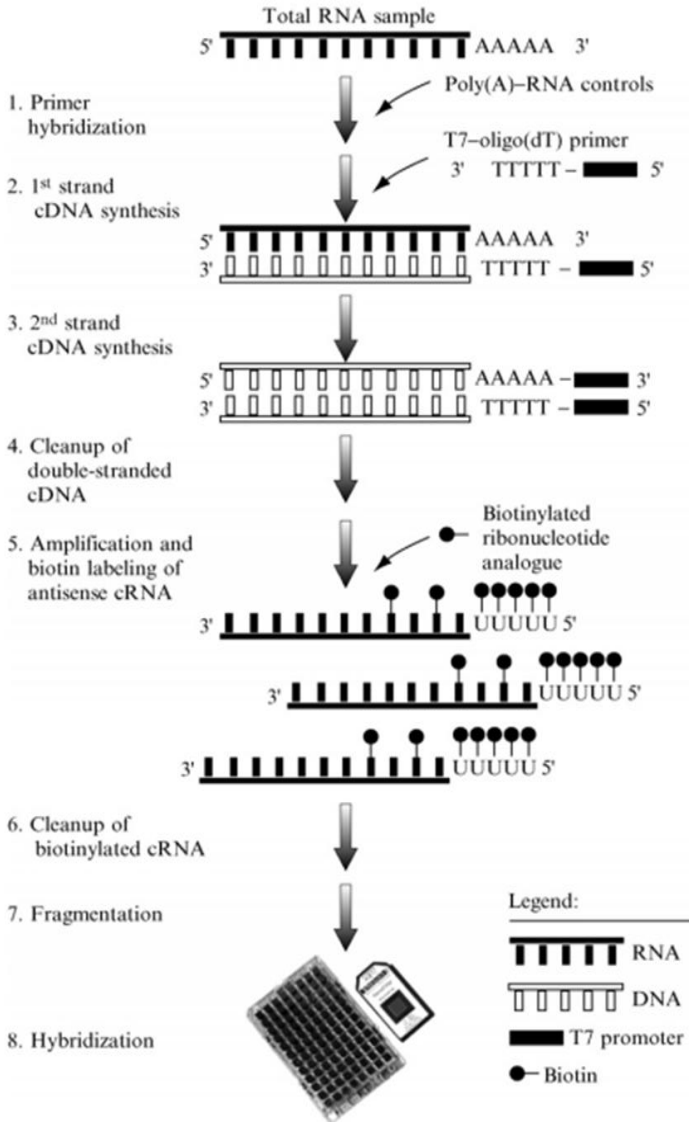


Figure 2-3: Outline of 3' In vitro Transcription assay protocol. The mRNA of each sample is prepared according to the above protocol for hybridizing it to the Drosophila Genome 2.0 Array (adapted from (Dalma-Weiszhausz et al., 2006))

2.16.2 Microarray data processing and analysis

The hybridization of biotin labelled cRNA to the probes of an array produces fluorescence; the fluorescence intensity of each probe in the array is captured and stored in a .CEL file. Thus scanning of an array chip for each sample produced a distinct .CEL file that contained the raw data. The bioinformatician at Glasgow Polyomics Centre used the Partek Genomic Suite 6.6 (version 6.13.0412) software to analyse these .CEL files to convert the probe intensities to biological information.

The probe that completely complements the target gene sequence is known as a perfect match probe (PM) and a probe with a single mismatch is known as mismatch probe (MM). Each gene on an array is represented in about 16-20 probes known as a probe set (Bolstad et al., 2003; Irizarry et al., 2003) . A probe set consists of a mixture of PM and MM. Raw probe intensity is the sum of a true hybridization signal, specific cross-hybridization signal, nonspecific binding signal, and small amounts of signal generated by system noise. A variety of algorithms such as MAS5, Robust Multi-Array Analysis can be applied to summarize either the intensities of PM and MM by subtraction of the raw MM probe intensity from the raw PM intensity or the intensities of PM only by subtraction of background intensity from the raw PM probe intensity, thus resulting into a single signal which correlate to the relative abundance of the gene transcript in a sample (Dalma-Weiszhausz et al., 2006).

Herein, Robust Multi-Array Analysis (RMA) algorithm was used to produce a single probe set intensity value. In this algorithm, the measurement of PM intensity is done by subtracting the background from the raw PM intensity. A pre-background adjustment for GC content was carried out which was then followed by application of RMA algorithm that used the quantile normalization to normalize the arrays, transform the probe intensity to log (base 2) values and apply median polish to control for the outlier probes (Bolstad et al., 2003; Irizarry et al., 2003).

The summarised probe set intensity values for each sample array was then grouped into four datasets (each dataset containing the average value of the four biological replicate sample array described in section 2.16.1): wild type salt stress, Relish mutant salt stress, wild type control fed and Relish mutant control fed. The relative fold change values generated between two datasets were statistically tested for its significance and output generated in form of a spreadsheet.

Statistical test applied on the compared datasets was two-way ANOVA (Genotype and Treatment as factors). To control for the false discovery rate (FDR),

Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) was applied that lead in generation of a step up *p*-value.

A “Salt Fly” database was made using MySQL queries. This database was made by me in collaboration with Dr. David Leader. The database contained the processed spreadsheet file which contained the *p*-value and step-up *p*-value result of the comparison done between the four datasets for each probe set. It also contained files downloaded from Flybase (an online database for genetic data for the insect family *Drosophilidae*) which contained information about each probe set with its matching unique gene identifiers (FBgn Number and CG number), gene name and symbols.

On applying specific MySQL queries on the database, statistically significant probe sets in one or more compared datasets were quickly and accurately extracted out from the database and the output generated as an excel spreadsheet along with information about those probe sets’ unique identifiers, name/symbol, the values of fold change between the two datasets along with the p-value and step up p-value for it.

The Principal Component Analysis is a visualisation method that reduces high dimensional data into two or three dimension representation. The bioinformatician at Glasgow Polyomics Centre generated the PCA plot using Partek Genomic Suite 6.6 (version 6.13.0412) software. In this PCA plot, the high dimensional variations detected for all the probe set across all the samples was reduced to two dimension representation. This is further explained in section 3.2.9.1

2.17 Bioinformatics

Flybase and Flymine are online databases (http://flybase.org/)(http://www.flymine.org/) containing detailed information about *Drosophila melanogaster* genes and integrated genomics data of *Drosophila*, *Anopheles* and other organisms. These were extensively used to study genes that were differentially expressed in tubules under effects of genotype or treatment or

both. Flymine v41.0 version of software was used to annotate the differentially expressed genes in tubules with Gene Ontology (GO) term based on their biological processes and find GO enrichment term based on *p*-value ≤0.05 (Lyne et al., 2007). The *p*-value for enrichment was calculated using the Hypergeometric distribution that calculated the statistical significance of the count for the number of times a GO term appeared for genes in a differentially expressed gene list of interest in respect to a reference population of genes and GO term. The below formula was applied:

$$p = \frac{(M \text{ choose } k)\,(N-M \text{ choose } n-k)}{N \text{ choose } n}$$

p the probability value
n the number of genes in the gene list of interest
N the number of genes in the reference gene population
K the number of genes annotated with a particular GO term in the gene list of interest
M the number of genes annotated with a particular GO term in the reference population

2.18 Percentage water loss after desiccation

To measure body weight, 9- to 12-day-old adult male flies of each genotype were put in group of 10-15 in empty vials and weighed in groups on an AND GR-202 precision balance (analytical weighing to within 0.0001 g). The weight of empty vials was noted prior to placing the flies. After weighing the flies, they were desiccated for 15 h in vials not containing food or water and weighed again. Water loss over 15 h was calculated for each genotype by subtracting weight at 15 h from that at 0 h. Experiments were run in duplicate with at least 20 flies of each genotype and sum of n = 110-105 flies for each genotype was plotted. Two-way ANOVA test was used to measure the statistical significance.

2.19 Measurement of hydrogen peroxide

40 pair of tubules were rapidly dissected from flies of specific genotypes and were added to 60 µl of 5 mM sodium phosphate (pH 7.4, Invitrogen). The tubules

were sonicated to release cellular contents, and briefly spun down at 5000 g at 4 °C. 50 µl of the supernatant was used immediately to measure H_2O_2 levels using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) as described by (Terhzaz et al., 2010) Total hydrogen peroxide levels were normalized to protein amounts in each homogenate using a Bradford assay (Bio-Rad). The assay was carried out as three technical replicates for each biological replicate sample. As only two biological replicates were used for each genotype, the values plotted were the mean values and no statistical test was done on it.

Chapter 3
Role of Relish in salt tolerance

Chapter 3

3.1 Introduction

3.1.1 Relish and Immune Deficiency (IMD) signalling pathway

The *Drosophila* transcription factor, Relish, is an orthologue of mammalian Nuclear Factor-kappa B (NF-κB) precursors p100 and p105 and is composed of a DNA binding Rel-homology domain and an inhibitory ankyrin-repeat domain (Dushay et al., 1996).The deletion mutant of the Relish gene, Rel^{E20} ascertained its role in *Drosophila's* humoral immune response and induction of battery of antimicrobial genes (Hedengren et al., 1999). The Relish mutants are highly susceptible to gram negative bacterial infection and also show susceptibility to certain fungal and viral infections (Hedengren-Olcott et al., 2004; Hedengren et al., 1999; Lemaitre et al., 1997; Tsai et al., 2008).The immune stress related-activation of Relish that primarily leads to induction of antimicrobial peptides (AMPs) is conserved across *Dipteran* and *Lepidopteran* insect species e.g. *Aedes ageypti*, *Manduca sexta* (Casanova-Torres and Goodrich-Blair, 2013; Gunaratna and Jiang, 2013; Schluns and Crozier, 2007; Shin et al., 2003).

Relish is a component of Immune Deficiency (IMD) signalling pathway that is predominantly activated in response to gram-negative bacterial infection and induces production of AMPs (Hedengren et al., 1999; Lemaitre et al., 1995). The signature AMP gene that is expressed on activation of the IMD signalling pathway is *Diptericin*. The Figure 3-1 summarizes the IMD signalling pathway. The membrane bound Peptidoglycan recognition protein (PGRP) -LC or cytoplasmic residing PGRP-LE receptor (not shown in the Figure 3-1) is activated by gram negative bacteria (Choe et al., 2002; Kaneko et al., 2006). This leads to interaction between proteins Imd (death-domain adaptor protein) and dFadd, along with activation of caspase DREDD (Death-related ced-3/Nedd2-like protein)(Leulier et al., 2002). Imd-dependent activation of dTAK1 (a MAPK kinase kinase) activates the DmIKK (*Drosophila* inhibitor of NF-κB (IκB)-kinase) complex which comprises of IRD5 (Immune response deficient 5) and KEY (Kenny) (Lu et al., 2001; Rutschmann et al., 2000; Vidal et al., 2001). The cytoplasm residing inactive Relish protein is phosphorylated by DmIKK complex and is cleaved by DREDD protein (Stoven et al., 2003). The N-terminal of endoproteolytically-cleaved Relish protein translocates from the cytoplasm to

the nucleus and controls the transcriptional expression of immune genes that help the organism to survive immune stress (Stoven et al., 2000). The IMD signalling pathway bifurcates at dTAK1 protein to initiate two signalling pathways: (1) Relish signalling pathway, and (2) c-Jun kinase (JNK) pathway (Silverman et al., 2003). Relish can form either homodimers or heterodimers with Dif (another NF-kappa B like protein) to modulate differential immune associated gene expression example gene expression of antimicrobial peptides such as *Cecropin, Drosomycin*. (Han and Ip, 1999; Tanji et al., 2010). The studies by Dijkers and O'Farrell (2007) demonstrated activation of Relish in the fat body by nitric oxide (NO) which is mediated by Calcineurin A1. This pathway of Relish activation is independent of IMD pathway and serves as an additional pathway to boost the immune response (Dijkers and O'Farrell, 2007).

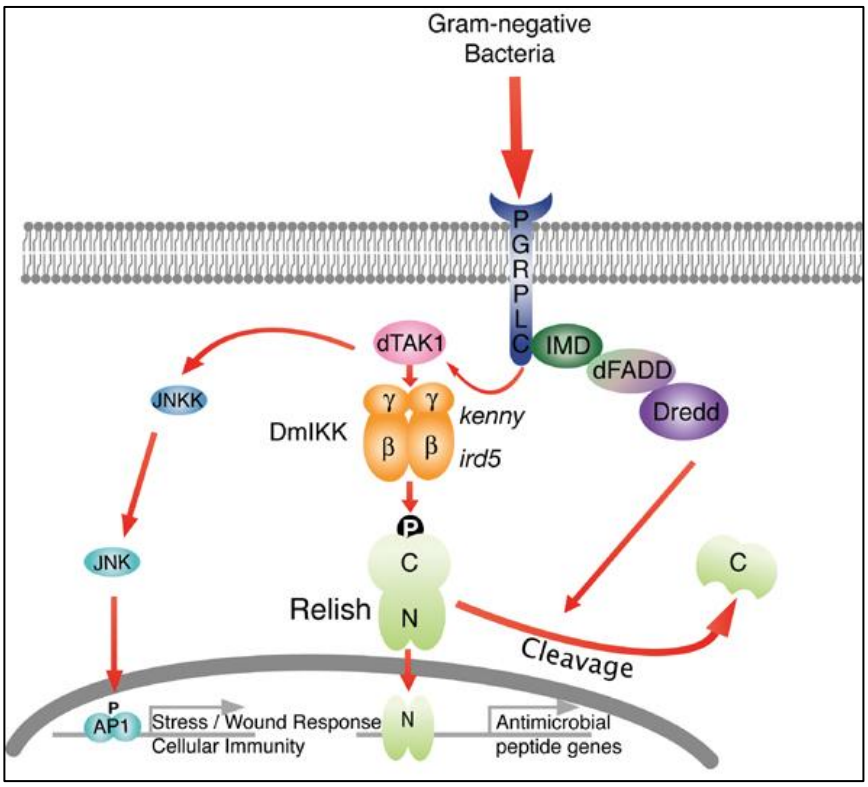


Figure 3-1: The Immune deficiency (IMD) signalling pathway. The membrane bound PGRP-LC receptor is activated by gram-negative bacteria that leads to interaction between proteins Imd (death-domain adaptor protein), dFadd and activation of caspase DREDD (Death-related ced-3/Nedd2-like protein). IMD-dependent activation of dTAK1 (*Drosophila* Transforming-growth-factor- -activated kinase 1) activates the Drosophila IKK (inhibitor of NF- B (I B)-kinase) complex which comprises of IRD5 (Immune response deficient 5) and Kenny. The inactive Relish is phosphorylated and cleaved by the DmIKK (*Drosophila* IKK complex) and DREDD respectively. The N-terminus translocates into the nucleus, inducing expression of AMP genes while the C-terminus resides in the cytoplasm. dTAK1 can also activate the c-Jun kinase (JNK) pathway that leads to expression of stress responsive genes (Figure reproduced by permission from Prof. Neal Silverman).

Relish activation during immune stress leads to upregulation of immune genes such as antimicrobial peptides, few genes are repressed by Relish (De Gregorio et al., 2002). Interestingly, Relish in the neuro-epithelium of the imaginal disc promotes rapid turnover of mRNA to regulate expression of genes required for the neuronal fate (Ayyar et al., 2007). Hence, Relish can regulate expression of genes either through binding to their promoter region to induce transcription or post-transcriptionally by controlling its mRNA degradation rate.

3.1.2 Relish activation in tubules

The production of AMP, Diptericin observed only in the tubule principal cells when flies or tubules are immune stressed with bacterial protein (LPS) confirms the role of *Drosophila* tubules as an independent immune-sensing tissue (McGettigan et al., 2005). It has been shown that similar to *Relish* expression, AMPs associated with IMD pathway- *Diptericin*, *AttacinA*, *CecropinA* and *Drosocin* are constitutively expressed and developmentally regulated in the tubules. Their expression starts from the late 3rd instar larval stage and continues to adulthood (Verma and Tapadia, 2012). In addition, overexpression of AMP (C*ecropin, Drosocin and AttacinA*) in the tubules of the adults increases their immune tolerance while down- regulation of *Diptericin* caused an immune sensitive phenotype in the larvae and adults. It is not yet known whether the AMPs produced in the tubules are secreted in the tubule lumen or the haemolymph.

In addition, immune stressor- peptidoglycan stimulates an increase in intracellular calcium in tubule principal cells (Overend et al., unpublished). The modulation in expression of immune associated genes (Nitric oxide synthase and forkhead (FOXO)) in the tubule principal cells affects the survival of the whole organism during gram-negative bacterial infection (Davies and Dow, 2009; Davies et al., 2012). Moreover, Relish in tubule principal cells is activated by peptidoglycan, NO through activation of soluble guanylate cyclase, ligand NPLP1-VQQ, and cGMP in dose dependent manner to induce production of the AMPs, specifically the antimicrobial peptide *Diptericin* (Davies et al., 2014; Overend et al., 2012)(Overend et al. unpublished). Taken together, this suggests that Relish can integrate signals from various external stimuli leading to a defined induction of genes to promote organismal survival.

In plants, salt stress caused due to high soil salinity manifests into osmotic stress followed by ionic stress (reviewed in (Gupta and Huang, 2014). In organism feeding on high salt diet or living in a saline condition, is challenged by salt stress caused due to increase in sodium ions in its extracellular fluid which causes efflux of water from the cells. Thus to survive, the organism needs to remove excess ions from the body to achieve ion-water homeostasis, and needs to repair and protect the cellular macromolecules affected due to ion toxicity. It was shown that Relish was activated and localised in the nucleus in response to salt diet and the expression of AMPs especially *Diptericin* was upregulated (Overend et al., 2012). In desiccation stress (a form of osmotic stress where the organism is deprived of food and water), flies with decreased expression of Relish in the principal cells produced a sensitive phenotype compared to its parentals (Terhzaz et al., 2012). In addition, desiccation stress related neuropeptide capa-1 leads to activation of Relish in tubules but does not induce expression of *Diptericin* but does induces transcription of other stress responsive genes (Terhzaz et al. 2014). Thus though desiccation stress and feeding on high salt diet produce osmotic stress, as the signalling during these stressors may involve different gustatory signals and subsequent behavioural responses, the study in this chapter has been focused on salt stress study only. In this chapter the role of Relish in salt stress tolerance is studied. A tubule specific transcriptome study was carried out using the Affymetrix Genome Chip 2.0, to (1) understand if there exists a functional overlap of immune and salt stress pathways working through the transcription factor -Relish, (2) find genes that are expressed under salt stress condition, and (3) find putative genes whose expression is controlled by Relish.

3.2 Results

3.2.1 High salt diet regime

Sterigoplous et al showed flies fed on diet containing 0.85 mol l^{-1} (4%) NaCl, survived up to 6 days, with median survival of flies being 4 days (Stergiopoulos et al., 2009). To ensure that all the flies fed on diet containing high salt, the flies were starved for five hours in empty vials and then placed on diet with 4% NaCl

in the evening which was the peak time for feeding behaviour (Wong et al., 2009). A similar feeding regime has been used for immune stress studies through natural infection (Kuraishi et al., 2011) and feeding assays for xenobiotic stress studies (Terhzaz et al., 2015a). After 5.5 days, 50% of the flies not starved (before feeding on high salt diet) were still alive. The starved flies had the same 50% survival rate. The flies fed on control diet not containing NaCl were all viable (data not shown). Thus, this feeding regime did not impact on stress survival phenotype (Figure 3-2).

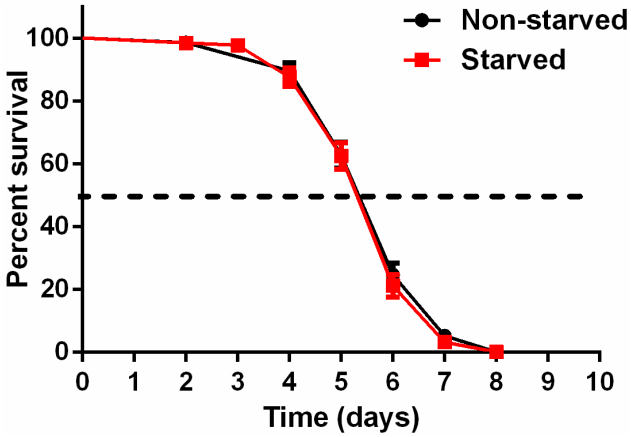


Figure 3-2: The high salt diet regime. Canton S males were either starved in empty vials for five hours (in red) or not (in black) prior to placing them onto diet containing 4% NaCl. Data are expressed as percent survival ± SEM, n>110 flies for each treatment.

3.2.2 Gender difference on survival on high salt diet

A recent study in *Drosophila* has shown that some genes are dimorphically expressed for example antimicrobial peptide *drosomycin* is expressed higher in females than males while the cytochrome *cyp6g1* is expressed stronger in males than females. (Chintapalli et al., 2012). There are also gender differences in response to dietary restriction, in which the extension of lifespan on dietary restriction regime was increased in females than males (Magwere et al., 2004). One of the factors contributing to this phenotype was suggested to be gender differences in energy requirement for reproduction processes (Magwere et al., 2004). To determine if there was a gender difference in wild type flies in response to a high salt diet, male and female flies were stressed separately on diet containing 4% NaCl. The number of viable flies was counted every 24 h until

100% mortality was reached. The median survival of the female flies was 4 days, whereas for male flies it was 5 days (Figure 3-2). Both male and female flies fed on diet without 4% NaCl were viable throughout the survival stress assay. This indicates that female flies are more susceptible to salt stress than male wild type flies. One of the factors may be the difference in rate of feeding between the sexes (Wong et al., 2009). As the male wild type flies were observed to be more robust than female flies during stress and to avoid the complexity of reproduction related metabolic requirements in females that may interfere in delineating the stress mechanisms, the further studies have been only carried out in males.

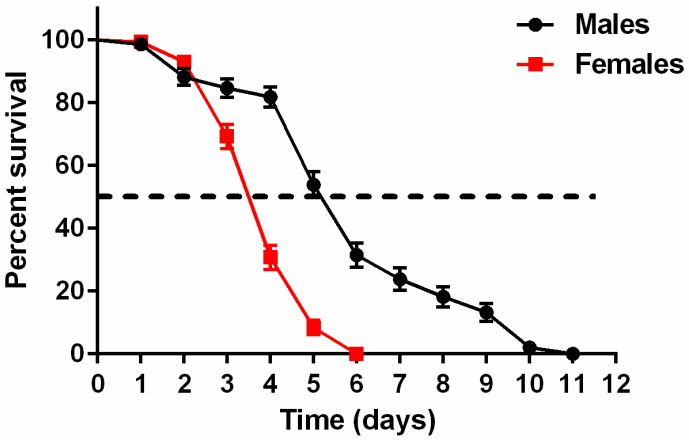


Figure 3-3: Difference in survival response on a high salt diet between male and female flies. Canton S male flies (in red) and female flies (in black) were fed with diet containing 4% NaCl. Data are expressed as percent survival ± SEM, ($p<0.001$ against female flies; Log rank test, Mantel-Cox).n>110 flies for each type.

3.2.3 Genetic background has an effect on survival on a high salt diet

Genetic background was seen to impact on dietary restriction based extension of life span (Bhandari et al., 2007). To determine whether genetic background played an effect in survival phenotype on sigh salt diet, Canton S wild type flies and Oregon R wild type flies were fed with 4% NaCl and their survival was assessed. 50% of the Canton S flies died at 6 days (Figure 3-4).The median survival of Oregon R flies was 5 days, indicating that there exists a difference in survivability between the two fly line background (Figure 3-4).

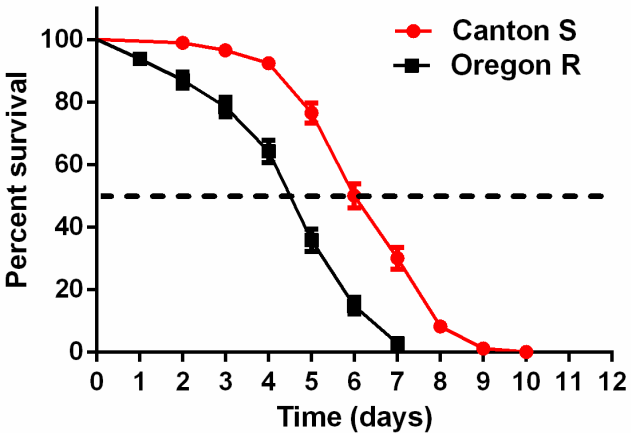


Figure 3-4: Difference in survival between wild type lines on a high salt diet. Male wild type Canton S flies (in red) and male wild type Oregon R flies (in black) were starved for five hours before placing them onto diet containing 4% NaCl. Data are expressed as percent survival ± SEM, ($p<0.001$ against Oregon R flies; Log rank test, Mantel-Cox). n>110 flies for each type.

For further studies Oregon R flies were used as wild type flies. This was due to (1) the original whole fly microarray data was carried out in Oregon R flies and (Stergiopoulos et al., 2009), and (2) the Relish mutant (Rel^{E20}) used for subsequent studies was backcrossed in Oregon R background.

3.2.4 Relish is important for salt stress tolerance

Relish (normally located in the cytoplasm) is localized to the nucleus under salt stress in *Drosophila* tubule's principal cells (Overend et al., 2012), indicating its activation during salt stress. To further investigate the role of Relish in salt stress tolerance, a salt stress survival assay on Oregon R and Relish mutant (Rel^{E20}) male flies was performed. As the Relish mutants were in wild type Oregon R fly line background (backcrossed by Prof. B. Lemaitre, EPFL), the wild type flies used for the further studies was Oregon R fly line. Wild type and Rel^{E20} male flies were starved for five hours prior to placing them onto diet with 4% NaCl. 50% of wild type male flies survived for 5 days (Figure 3-5), while the median survival for Rel^{E20} flies was 2 days.

This supports a role for Relish in tolerance to osmotic stress caused by a salt diet (salt stress). Further on the flies fed with high salt diet will be referred to as salt stressed.

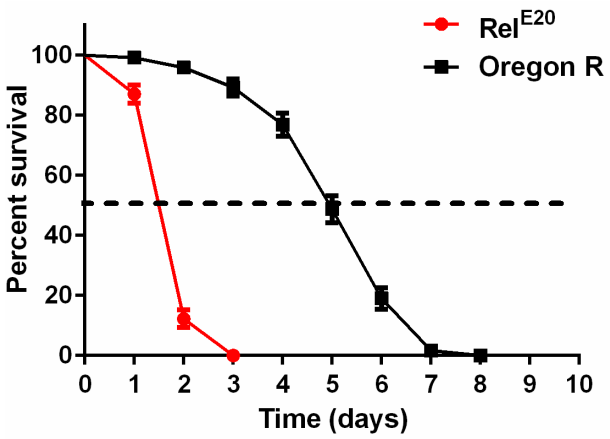


Figure 3-5: Survival of Relish mutants on high salt diet. Survival of Relish null mutants (Rel^{E20}) flies in Oregon R background compared against Oregon R wild type flies on high salt diet (4% NaCl). The flies were starved for five hours before placing them onto diet containing 4% NaCl. Data are expressed as percent survival ± SEM, ($p<0.001$ against Oregon R flies; Log rank test, Mantel-Cox).

3.2.5 Relish in tubule is important for salt stress tolerance

Next, the effect of repressing the expression of Relish in tissues in which it is highly expressed was investigated. The database, Flyatlas (flyatlas.org) shows the level of gene expression in various tissues in the larvae and adult fly. In adult fly, Relish is highly expressed in the epithelial tissues (tubules and gut) and the fat body- a tissue involved in immune sensing (Hedengren et al., 1999)(Figure 3-6).

To establish the potential tissue-specific roles of Relish during salt stress, the binary UAS/GAL4 system was used to regulate the expression of an RNAi that represses the expression of Relish gene in the tissue of interest. Relish was repressed by using UAS-Relish RNAi and the results were compared against UAS-Control RNAi. The fly lines expressing UAS-Relish RNAi (from Vienna Drosophila Stock Centre) and UAS-Control RNAi (gift from Dr. Edward Wilhelm Green, German Cancer Research Center, Heidelberg) used for the studies were in the similar genetic background and expressed the phiC31 vector inserted in a defined insertion site on chromosome 2. The UAS-Control RNAi expressed a hairpin nucleotide sequence that did not suppress the expression of any gene. As seen in section 3.2.3 that difference in the genotype background lead to difference in survivability on high salt diet, hence to negate for any background

effects that may occur, UAS-Control RNAi was used as control to compare survivability effect of UAS-Relish RNAi during salt stress.

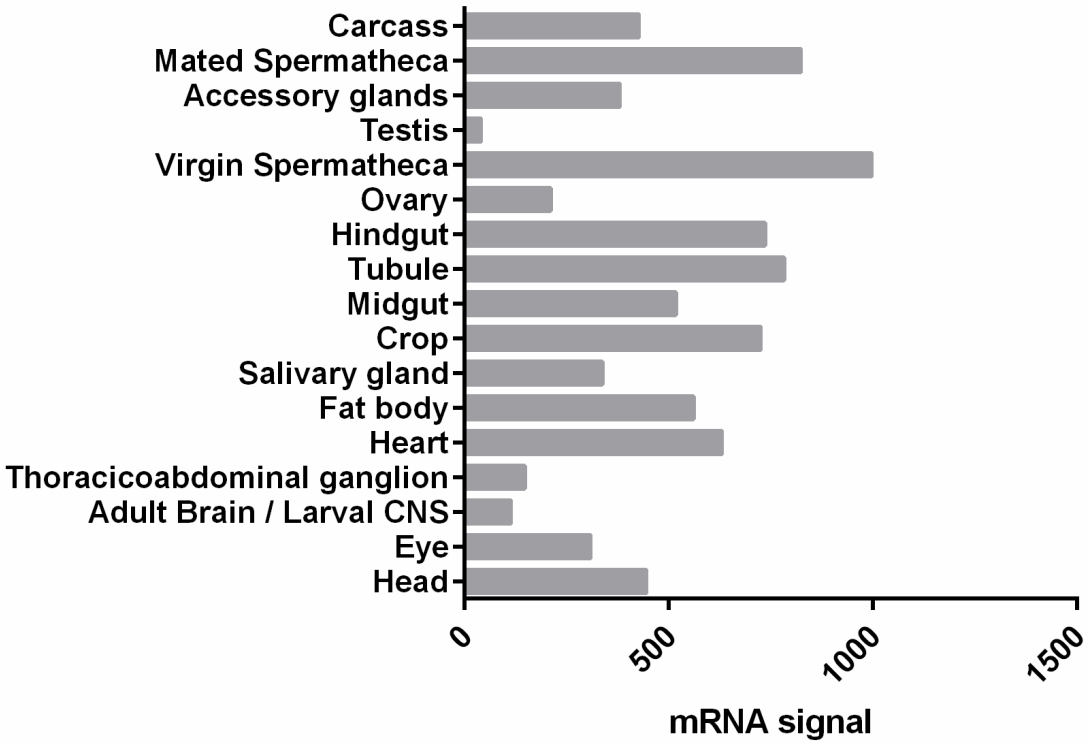


Figure 3-6: Flyatlas expression of Relish across all adult fly tissues. Data taken from Flyatlas (http://flyatlas.org/)

The UAS-Relish RNAi has been previously characterised in tubule principal cells for desiccation tolerance phenotype (Terhzaz et al., 2014). To confirm that the UAS-Relish RNAi was still suppressing the expression of Relish gene, the mRNA levels of Relish in tubule principal cells in progeny of flies that expressed UAS-Relish RNAi under the control of tubule principal cells specific GAL4 driver (CapaR-GAL4 - generated by Dr. S. Terhzaz) was assessed. A 50% decrease in mRNA transcripts was seen in the progeny compared to control flies that expressed an UAS-Control RNAi (Figure 3-7).

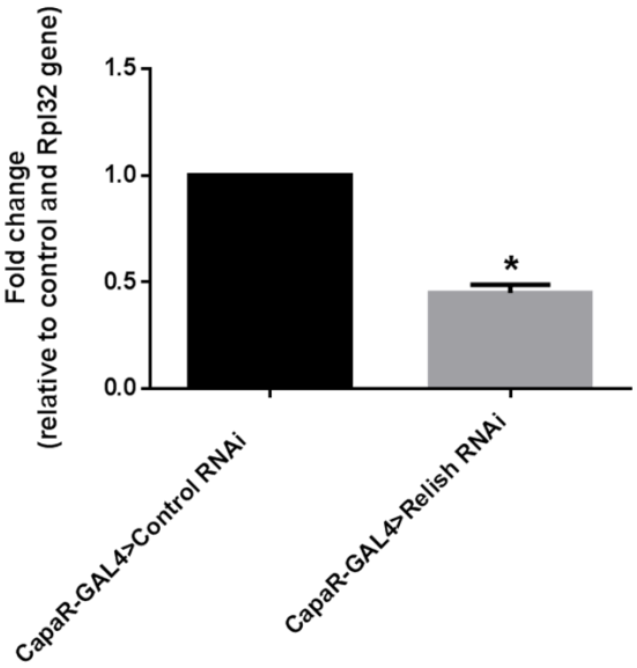


Figure 3-7: Tubule principal cell specific knock down of Relish. In tubules of male flies fed normally, Relish expression in tubules expressing UAS-Relish RNAi/UAS-Control RNAi under tubule principal specific CapaR-GAL4 was quantified by Q-RT-PCR. Data are expressed as Relish gene expression ratio compared to tubules from Control RNAi flies ± SEM (N = 3), where * indicates significance, $p < 0.05$.

The other tissue specific GAL4 drivers used were c564-GAL4 for fat body, Tsp42-GAL4 (generated by Dr. S. Terhzaz) for anterior region of midgut, and Alka-GAL4 (generated by G.Overend) for posterior region of midgut. The flies repressing Relish expression in specific tissues as compared to the control flies did not show any compromised survival phenotype when fed on normal diet (data not shown).

When Relish expression was repressed in the fat body, no difference in salt stress phenotype was observed between the experimental flies expressing Relish RNAi and control flies expressing the control RNAi (Figure 3-8). These data indicate that Relish expressed in fat body does not play a key role for coping with salt stress.

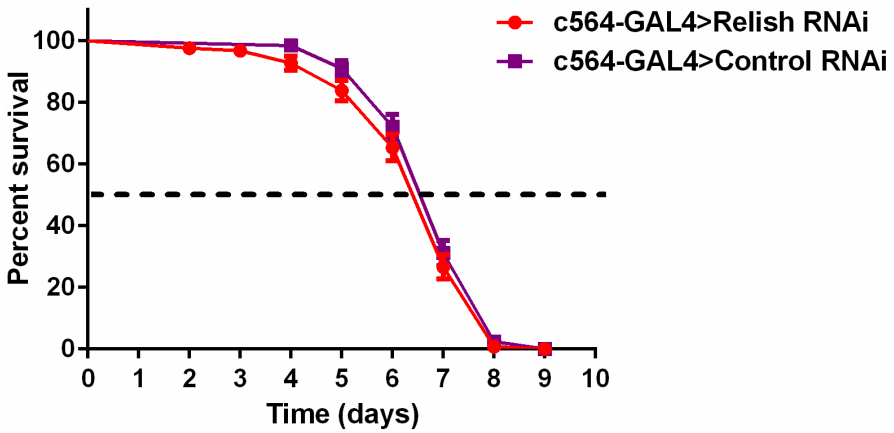


Figure 3-8: Survival on salt diet when Relish expression is suppressed in the fat body. Male flies of genotype c564-GAL4>UAS-Relish RNAi (in red) and c564-GAL4>UAS-Control RNAi (in purple) were starved for five hours before placing them onto diet containing 4% NaCl. Data are expressed as percent survival ± SEM, n>110 flies for each genotype.

The midgut is a tissue that expresses Relish in high abundance. It has six distinct morphological and molecular regions (Buchon et al., 2013). Relish expression was repressed in the anterior region using Tsp42-GAL4 and the posterior region using Alka-GAL4. These lines were crossed to Relish RNAi line and control RNAi fly lines, and the progenies were assessed for salt stress survival phenotype.

When salt stressed, the Relish RNAi in presence of Tsp42-Gal4 flies had a 50% survival rate at 5 days (Figure 3-9 A). A similar survival rate was observed in flies expressing control RNAi and Tsp42-GAL4 (Figure 3-9 A), indicating that repressing the expression of Relish in the anterior region of the midgut does not affect the survivability of flies during salt stress.

In flies with Relish RNAi and Alka-GAL4, the median survival rate on salt diet was 6.5 days (Figure 3-9 B). The control RNAi flies had a similar survival rate (Figure 3-9 B). Thus, there was no compromise in survival of flies with Relish expression being repressed in the posterior region of the midgut on high salt diet compared to control flies.

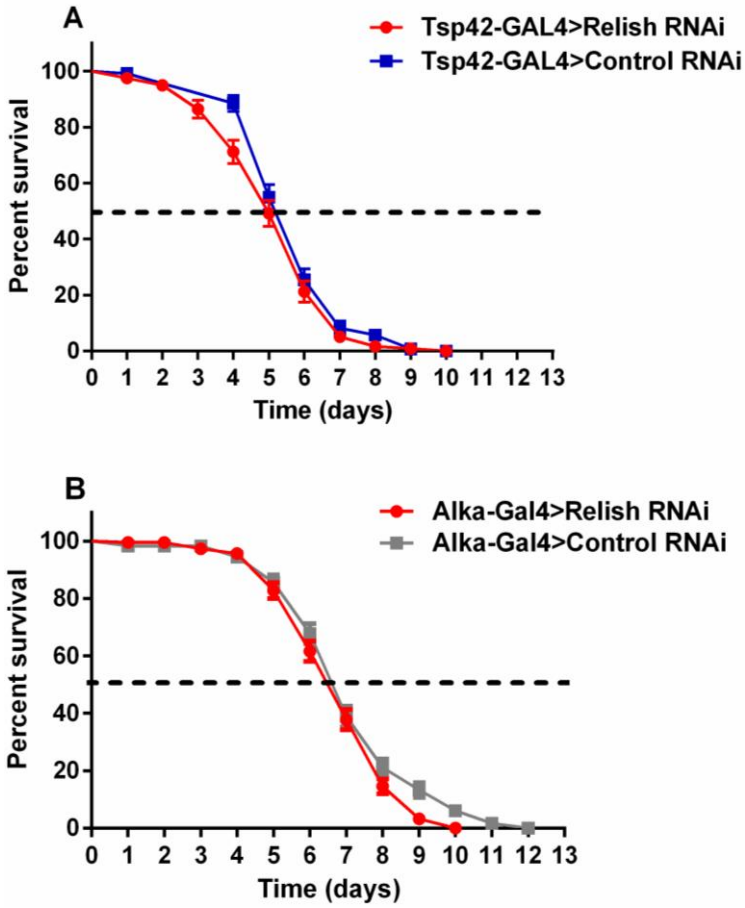


Figure 3-9: Survival on salt diet when Relish expression is suppressed in the gut. (A) The anterior midgut specific tissue GAL4 driver, Tsp42-GAL4, was used to supress the expression of Relish in the anterior midgut and in**(B)** the posterior midgut specific tissue GAL4 driver, Alka-GAL4, was used to supress the expression of Relish in the posterior midgut. Male flies of each genotype were starved for five hours before placing them onto diet containing 4% NaCl. Data are expressed as percent survival ± SEM, n>110 flies for each genotype.

The pluoripotent intestinal stem cells in midgut renew the epithelium every 1-2 weeks (Buchon et al., 2013). Hence it may be that the renewal of the midgut epithelium either (1) made the knockdown of Relish in efficient to produce a survival phenotype, or (2) other regions of gut may be increasing expression of Relish to compensate or (3) Relish in gut is not required in response to salt stress.

Relish is highly expressed in the Malpighian tubules (Figure 3-6). CapaR-GAL4 driver was used to repress the expression of Relish in the principal cells within the tubule. This GAL4 driver has a strong expression in the main segment region of the tubule (Terhzaz et al., 2012).

50% of flies expressing control RNAi and CapaR-GAL4 had a survival rate of 8 days, while the flies expressing Relish RNAi survived for 7 days (Figure 3-10 A) when salt stressed. This observation was confirmed using another tubule principal cell GAL4 driver, c42 GAL4 driver. The c42-GAL4-Relish RNAi and – control RNAi flies were tested for salt stress tolerance and the median survival of control RNAi was 4.5 days. When Relish was repressed, the survival rate was 3 days (Figure 3-10 B). This suggests that Relish in tubule principal cells plays a role in salt stress tolerance.

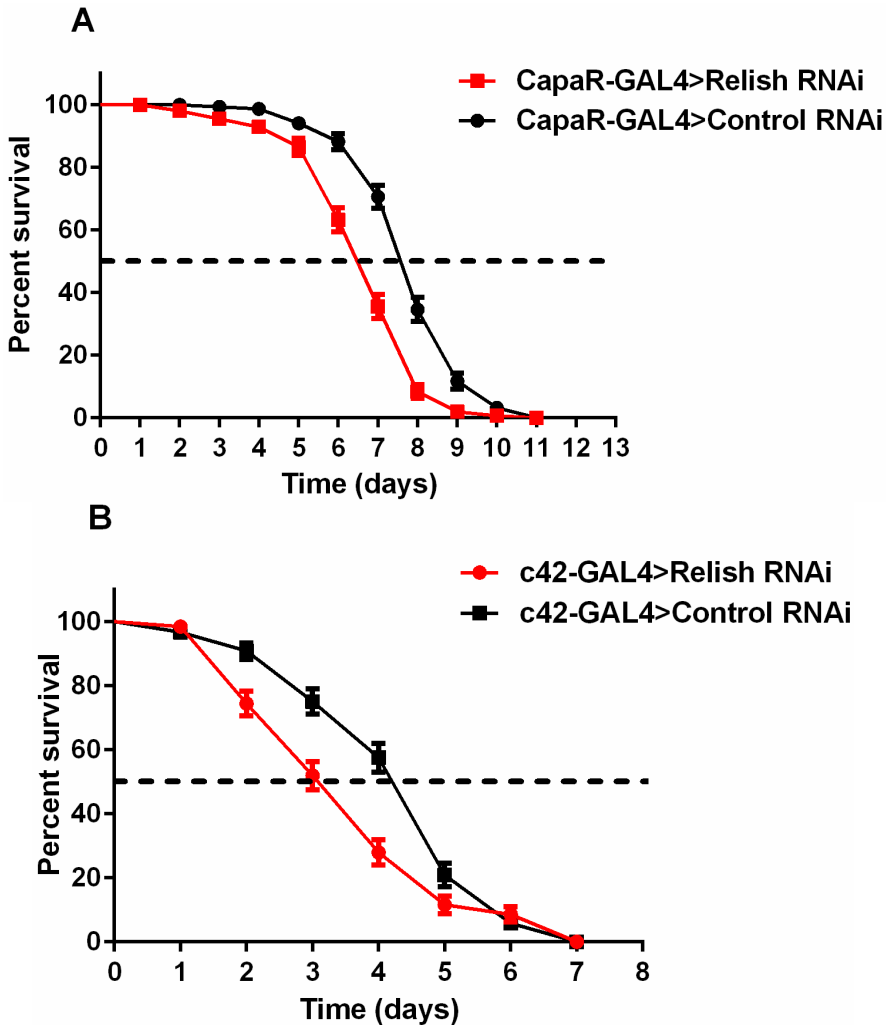


Figure 3-10: Survival on salt diet when Relish expression is suppressed in the tubules. Relish in tubule principal cells was down- regulated using A) CapaR-GAL4, and B) c42-GAL4 driver. Male flies of each genotype were starved for five hours before placing them onto diet containing 4% NaCl. Data are expressed as percent survival ± SEM, n>110 flies for each genotype. ($p<0.001$ against Control RNAi flies; Log rank test, Mantel-Cox).

3.2.6 In response to salt stress Relish is not activated in excised tubules and S2 cells

Previously, it was shown that Relish was activated in tubule principal cells of flies fed on 2% to 4% salt diet (Overend et al., 2012). Excised tubules treated with cyclic guanosine nucleotide (cGMP) showed Relish activation at 1nM while a higher concentration of 100 µM inhibited Relish activation (Davies et al., 2014). Therefore to study the *in vitro* effect of salt stress on Relish activation, the excised tubules and S2 cells were incubated in saline media and checked for Relish translocation to the nucleus.

Relish protein tagged at N-terminus with 6x histidine, was used to observe Relish localisation in tubule principal cells. In unstressed tubules, Relish remains in the cytoplasm (Figure 3-11C). When excised tubules were immune stressed by PGN, Relish localised into the nucleus (Figure 3-11 A). However, in excised tubules incubated with 340 mM NaCl solution for 2 h, there was no mobilisation of Relish into the nuclei of the tubule principal cells (Figure 3-11 B). No activation of Relish was observed in tubules treated with lower concentration of salt solutions (personal communication with Dr. G. Overend).

Drosophila S2 cells are derived from larval hemolymph and have been extensively used to study *Drosophila* Relish for their function in immune stress pathways triggered by lipopolysaccharide (LPS)(Hedengren et al., 1999; Stoven et al., 2000). Relish in S2 cells was shown to be activated by cGMP as well as under hypoxia conditions (Dijkers and O'Farrell, 2009).

On receiving an immune stress stimuli, Relish is endoproteolytically-cleaved, with the C-terminal domain fragment retained in cytoplasm and N-terminal domain fragment translocating into the nucleus to control expression of an array of immune related genes (Stoven et al., 2000).

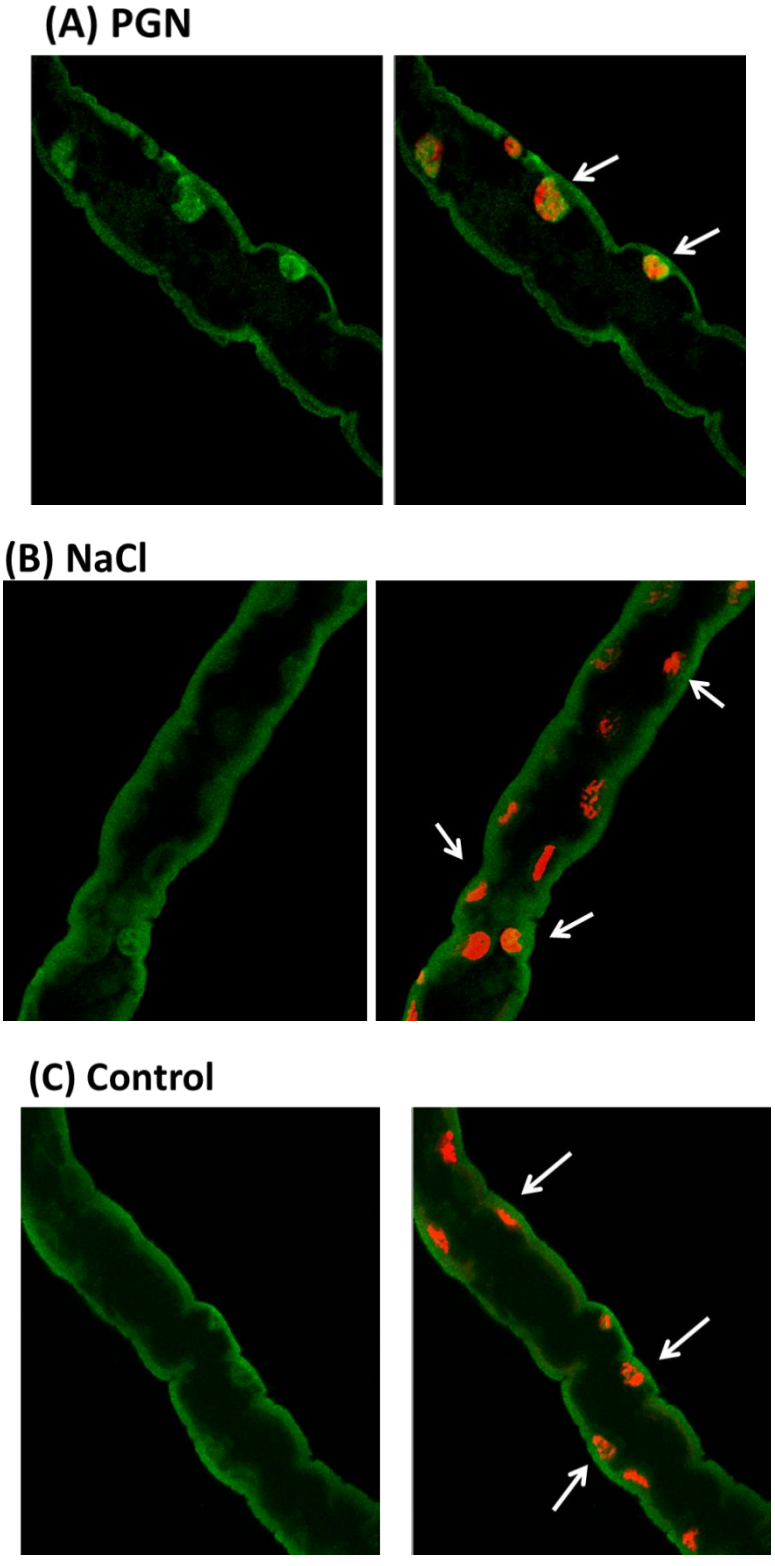


Figure 3-11: Relish activation in excised tubules. Tubules were isolated from 7-9 day old c42/UAS-Rel-His6 flies. Intact tubules were either treated with peptidoglycan (PGN) **(A)** or 340 nM NaCl. **(B**), or untreated **(C) in** Schenider's medium. Nuclei are labelled with DAPI (red). An anti-histidine antibody and a secondary antibody conjugated with FITC were used to detect Relish (green). Co-localization of Relish in nuclei is detectable as yellow or yellow/orange, and observed only in PGN stimulated tubules (A). The arrows point to the principal cell nuclei.

S2 cells treated with 400 mM NaCl activated c-Jun kinase (JNK) pathway but did not activate Relish (Chen et al., 2002b; Park et al., 2004). Since Relish is activated by cGMP at low concentrations but inhibited at higher concentration (Davies et al., 2014), could this be the case for osmotic stress too?

The haemolymph Na^+ concentration of adult flies fed on diet without salt was measured 80 mM and on diet containing 2% NaCl for 24 h was measured 100 mM, with an increase to 120 mM on chronic feeding for 7 days (Naikkhwah and O'Donnell, 2011). Hence S2 cells were treated with physiological tolerant concentrations of NaCl (75 mM or 150 mM NaCl). S2 cells were also treated with 0.125mM and 0.25mM sorbitol to generate osmotic stress, and 5 µg/ml of Gram negative bacterial protein peptidoglycan (PGN) as a positive control for 30 minutes.

FLAG-tagged Relish protein was overexpressed in S2 cells to determine if Relish activation was affected under osmotic stress caused by either NaCl or sorbitol. Relish activation was studied by immunoblotting cell lysates with antibody specific to C-terminal region of Relish protein that recognised full length protein and active fragment of the protein (Stoven et al., 2000; Stoven et al., 2003).

Untreated S2 cells only expressed the full length 110 kDa Relish protein; whereas S2 cells treated with PGN had both forms of the Relish protein, the full length 110 kDa protein as well as the 49 kDa fragment of active protein. All the cells treated by osmotic stress triggering solutions (either NaCl or sorbitol) display only the full length inactive Relish protein indicating that Relish is not active under these conditions (Figure 3-12).

Relish does not localise to nuclei under salt stress in the S2 cells and in excised tubules, this suggests that mechanisms of Relish activation in response to salt stress is different from the immune stress caused by PGN. It further indicates that the input signal for activating Relish in tubules during salt stress may be generated by another tissue.

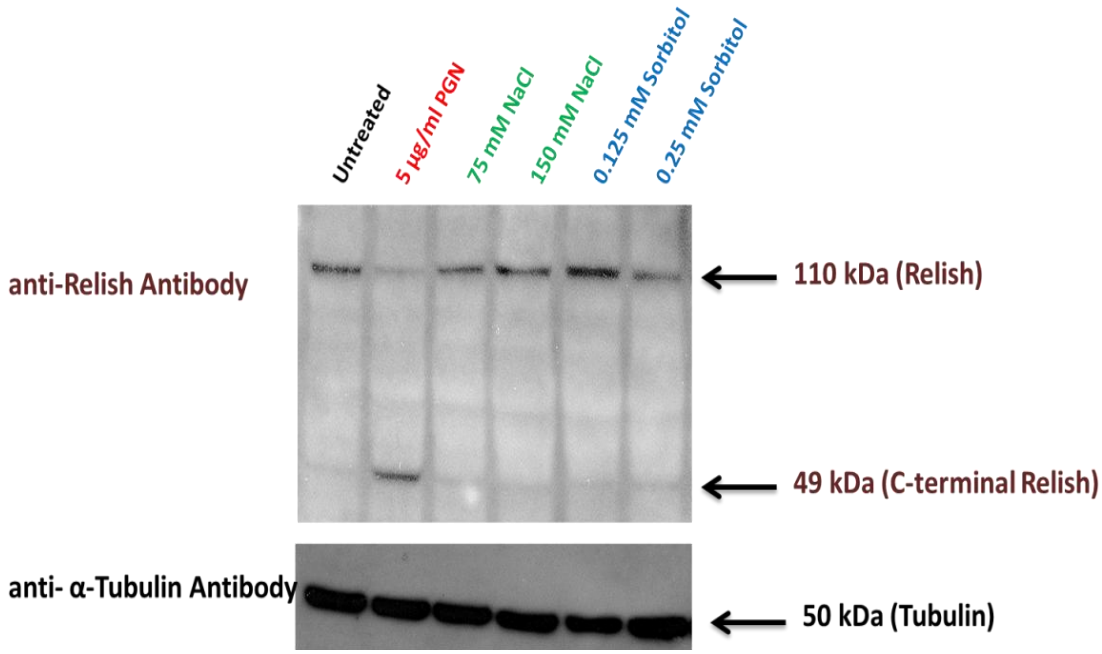


Figure 3-12: Immunoblot analysis of Relish activation in S2 cells. S2 cells were transiently transfected with Relish and treated for 30 min with NaCl concentrations 75 mM and 150 mM and an equivalent osmotic pressure generating concentration of Sorbitol. A positive control for Relish activation was peptidoglycan (immune stress). The western blot was incubated with antibody anti-Relish C21F3 that recognized the inactive full length Relish protein (110 kDa) and activated C-terminal Relish protein (49kDa). For loading control, the blot was re-probed with alpha-Tubulin antibody.

3.2.7 Antimicrobial peptide gene expression in salt stressed tubules of wild type flies

The whole fly microarray study of salt stressed wild type Oregon R flies showed that expression of immune genes such as *Metchnikowin*, *Drosomycin*, *Diptericin* were upregulated as an early response to high salt feeding (Stergiopoulos et al., 2009). Moreover, tubules of wild type Canton S flies stressed with 3% NaCl diet after 24 h showed an upregulation of *Diptericin* and *Cecropin* gene expression (Overend et al., 2012).

In order to determine which immune stress pathways are activated in wild type Oregon R fly tubules, the fold change gene expression of two AMPs *Drosomycin* and *Diptericin A* was quantified after 4 h and 24 h of feeding on diet with or without 4% NaCl. *Drosomycin* and *Diptericin A* are 'read- out' genes of immune stress pathways -Toll pathway and Immune Deficiency (IMD) pathway respectively.

After 4 h of salt stress, there was a 1.8-fold increase in the expression of *Drosomycin* (Figure 3-13 A) in the tubules of the salt stressed flies compared to control flies. The expression of *Diptericin A* in the tubules from salt stressed flies showed no significant change (Figure 3-13 A) compared to the tubules of normally fed flies.

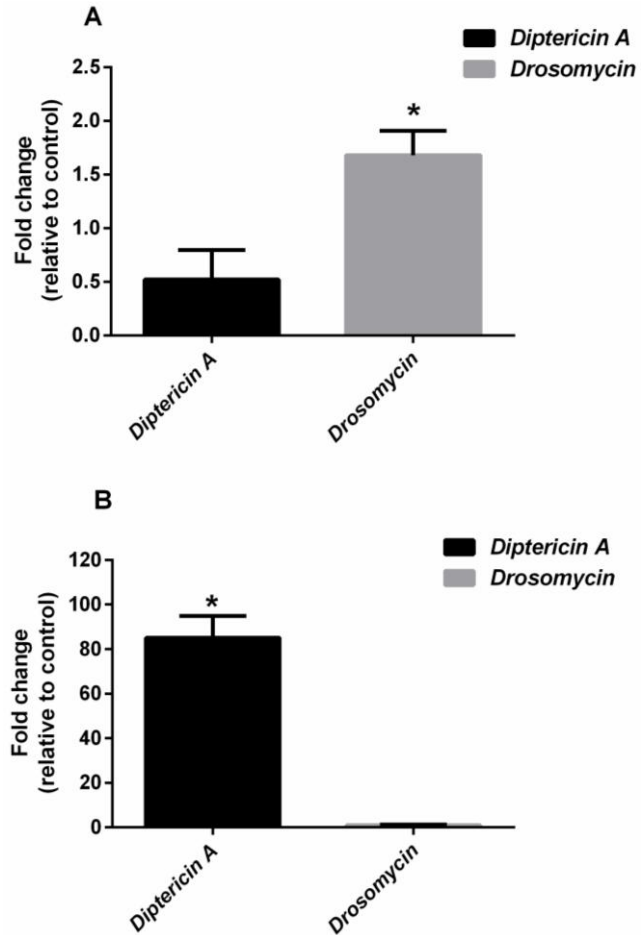


Figure 3-13: Antimicrobial peptide (AMP) gene expression in tubules of salt stressed flies. AMP expression was measured by Q-RT-PCR in tubules from 7 to 8 day-old Oregon R male adults fed on 4% NaCl containing diet for **(A)** 4 h and **(B)** 24 h. For each AMP gene, data are expressed as AMP gene expression ratio compared to tubules from normally fed flies ± SEM (N = 3), where * indicates significance, $p < 0.05$ relative to the normally fed.

A 100-fold increase in expression of *Diptericin A* was observed in tubules from salt stressed flies compared to tubules of normally fed control flies after 24 h. There was no change in expression for *Drosomycin* in tubules of salt stressed flies compared to control flies (Figure 3-13 B).Thus, while *Drosomycin* expression is increased early on during salt stress; it is down- regulated later on. In

contrast, *Diptercin A* is not induced (or required) early on to adapt to the salty diet, but it is required later on to adapt to salt stress.

To determine if the increase in expression of *Diptericin A* after 24 h salt stress at the mRNA level is reflected with an increase in the amount of Diptericin protein, the amount of Diptericin protein was assayed using immunohistochemistry.

The fly line *yw; Diptericin-lacZ; Drosomycin-GFP* was fed on diet with or without 4% NaCl for 24 h. In this fly line, the promoter region of *Diptericin* gene and *Drosomycin* gene is fused to the bacterial gene, LacZ and gene of green fluorescent protein (GFP), respectively (Tzou et al., 2000).The tubules were then excised from salt stressed and control flies and immunohistochemistry was carried out to detect the expression of beta- galactosidase (the product of *LacZ* gene) (Figure 3-14).

In tubules of control fed (unstressed) flies, a low beta- galactosidase staining was observed in the cytoplasm of the tubule cells which indicates to basal *Diptericin* production. The flies stressed with 4% NaCl diet had an increased amount of beta-galactosidase staining in the cytoplasm of the tubule cells compared to unstressed flies, implying the induction at the mRNA of *Diptericin A* is reflected in an increase in the amount of the total Diptericin protein in the tubules of stressed flies. No signal for GFP was detected in tubules of either the salt stressed or control flies.

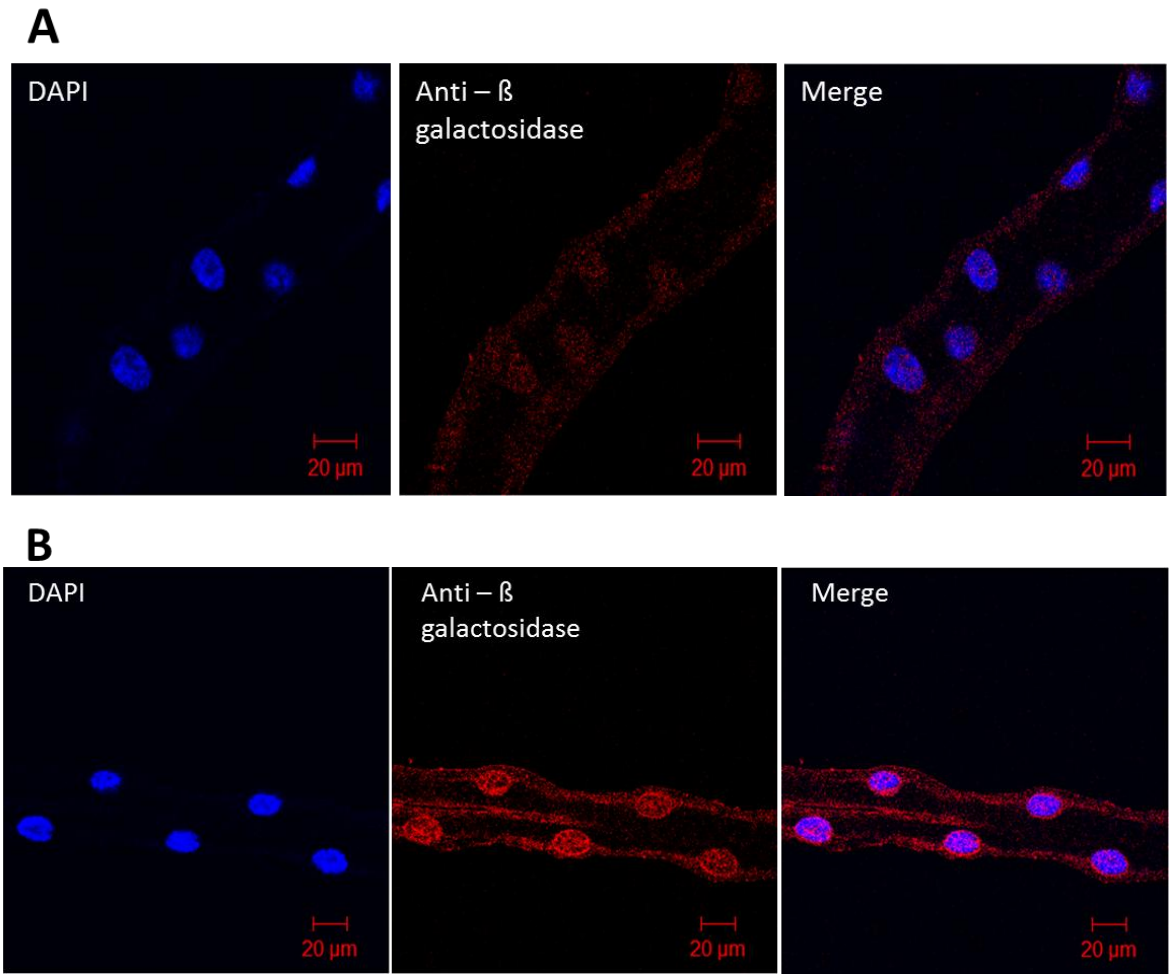


Figure 3-14: Expression of Diptericin in the tubules of salt stressed flies. Tubules were isolated from 7-9 day old *yw; Diptericin-LacZ; Drosomycin-GFP* flies fed on control diet **(A)** and diet containing 4% NaCl **(B).** Nuclei are labelled with DAPI (blue). Diptericin-LacZ protein was detected using antibody beta-galactosidase-specific antibody with a secondary antibody conjugated with Alexa Fluor (red). The protein was detected in cytoplasm.

3.2.8 Secretion assay of salt stressed wild type flies

To assess if the tubules were able to efficiently function to secrete fluid after 24 h of salt stress, an *in vitro* secretion assay was performed where the rate of fluid transport in intact excised tubules is assessed (Dow et al., 1994b). The fluid secretion rate of wild type flies salt stressed for 24 h did not show any difference when compared to tubules of control flies, both showing a fluid secretion rate of 0.5 nl/min. These results were similar to Naikkhwah and O'Donnell (2011), who observed no significant change in tubule fluid secretion rate of larvae reared on 2% NaCl diet for 24 h up to 7 days (Naikkhwah and O'Donnell, 2011). Thus this adaptability in adjusting fluid secretion rate may

help wild type flies to survive on high salt diet for long period of time (Figure 3-15).

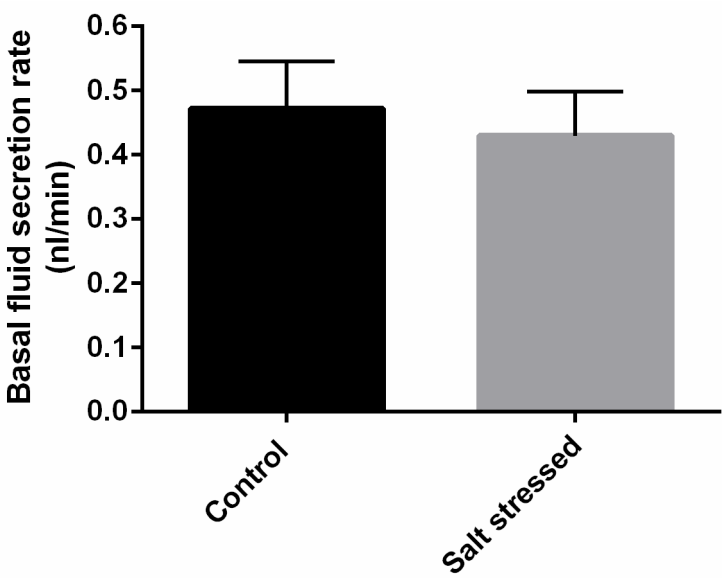


Figure 3-15: The basal fluid transport rates in tubules of salt stressed wild type flies. The basal fluid transport rates in tubules from 10 day old male adults was measured for 60 min in tubules of wild type fed for 24 h on control diet (in black) or diet with 4% NaCl (in grey).Data are expressed as fluid secretion rates (nanoliters/min) ± S.E.M.,N = 7 tubules.

3.2.9 Genes affected during salt stress and regulated by Relish

The expression of Relish in tubules of principal cells is important for salt stress tolerance as shown above. To identify genes in tubules which are critical for salt stress tolerance and are under control of Relish, a tubule-specific microarray study using the Affymetrix Genome 2.0 chip was performed.

It was observed that after 24 h of salt stress, compared to controls, there is an 100-fold upregulation of *Diptericin A* gene, a 'signature' Relish target gene .To ensure that 24 h time point did not drastically affect viability of Rel^{E20} (Relish mutant) flies fed with high salt, a survival assay of Rel^{E20} and wild type flies fed on 4% NaCl diet was carried out and the viable flies counted over a time course of 80 h (Figure 3-16). While the survival of wild type flies was not affected within 80 hours, 9% of the Rel^{E20} flies were dead after 24 h of salt stress (Figure 3-16). Hence the tubule specific microarray study was carried out on Rel^{E20} and wild type flies fed for 24 h with or without 4% NaCl added to its diet.

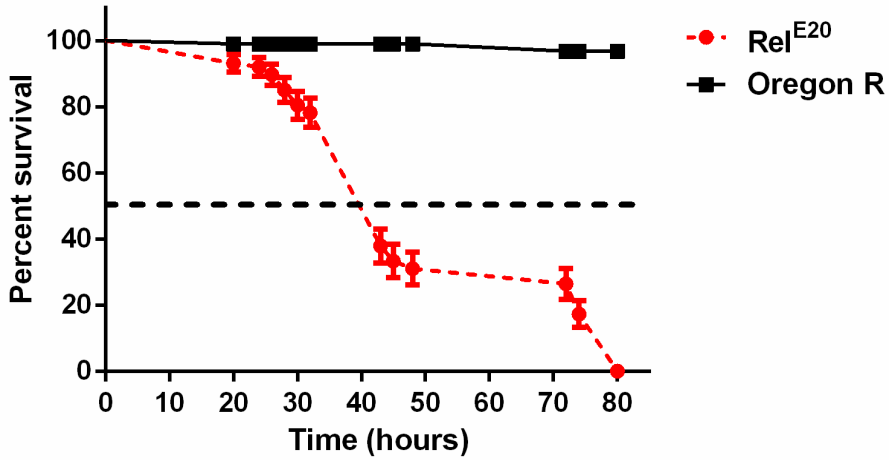


Figure 3-16: Survival of Relish mutants on high salt diet over 80 h. Survival of Relish null mutants (Rel^{E20}) flies in Oregon R background compared against Oregon R wild type flies on high salt diet (4% NaCl). The flies were starved for five hours before placing them onto diet containing 4% NaCl. Data are expressed as percent survival ± SEM, ($p<0.001$ against Oregon R flies; Log rank test, Mantel-Cox).

Tubules have their own circadian rhythm (Giebultowicz, 2001) with the secretion rate being most stable in the afternoon (unpublished data Kean L et al.). In neuronal cells, glia, and in the mushroom body of the brain, Relish mediated transcription was shown to be under the control of the circadian rhythm ((Kuo et al., 2010; Tanenhaus et al., 2012)). Therefore, it was imperative that tubule dissections were carried out when the tubule secretion rate was relatively stable as to negate any gene expression mediated by Relish due to circadian rhythm rather than salt feeding. Hence feeding regime was set such that the 24 h time point dissections were done in the afternoon, when the tubules' secretion rates were relatively stable and approximately 0.5 nanoliters/min over this period (unpublished data Kean L et al.). The RNA was isolated from the dissected tubules and performed in quadruplets for each genotype and condition and used for the microarray experiments. Before the RNA samples were sent to the Glasgow Polyomics Centre for the microarray experiment, some RNA samples were checked for the expression of *Diptericin A*. The results obtained for the wild type samples were similar to observed in section 3.2.7 that is 100-fold increase in *Diptericin A* was observed in salt stressed tubules compared to control tubules (data not shown). While for the salt stressed Rel^{E20} no transcripts for *Diptericin A* were detected (data not shown). In addition, the batch of food containing salt that was used to feed flies to salt stress for 24 h for the microarray experiment was checked for its saltiness by carrying out a survival assay of Rel^{E20} vs. wild type and the results obtained were similar to seen in

figure 3-16.Hence, this ensured that the RNA samples sent for the microarray experiment would produce a tubule transcriptome of salt stressed wild type and Rel^{E20} (discussed in the sections below) as well as for control fed wild type and Rel^{E20} (discussed in Chapter 4).

The Partek Genomics Suite software was used to generate box plots, array-array intensity correlation plots and principal component analyses (PCA). Quality control (QC) metrics were used to assess the quality of the microarray data. The PCA plot further discussed in section 3.2.9.1 is a key test that shows in the gene expression profiles of four datasets, the variation is due to factors such as genotype and stress treatment on transcriptional responses.

The Affymetrix Drosophila Genome 2 microarray used in this study has 14438 probe sets which covers 13250 *Drosophila* genes (Robinson et al., 2013). One of the limitation of this method for detecting differentially expressed genes under experimental conditions is that some genes (1367 of 13250 genes) were detected by more than one probe sets or some probe sets detected more than one gene (1188 of the 14438 probe sets) (Robinson et al., 2013). Therefore, in the results discussed below, the total number of probe sets and genes will not match.

Genes with a fold change of ≥ 1.5 were designated as upregulated, while genes with a fold change ≤ -1.5 were designated as down- regulated. A False Discovery Rate (FDR) of 5% was applied to the list of differentially expressed genes. It was observed that genes with a statistically significant fold change of ≥ 1.5 or ≤ -1.5 in the microarray result could not be validated with qRT-PCR (data not shown). This may be due to the difference in qRT-PCR and microarray experiments' sensitivities in detection for low-level gene expression changes or there may be a false detection of these genes in the microarray experiment. As the genes with the statistically significant fold change of ≥ 2 or ≤ -2 in the microarray array experiment produced similar and robust result when validated with qRT-PCR (Figure 3-22), this cut-off was used to perform Gene Ontology (GO) analysis for the differentially expressed genes.

To find the putative genes that were controlled by Relish under salt stress, an algorithm (Figure 3-20) was applied to the list of 164 differentially expressed genes in the wild type. The 1866 differentially expressed genes in RelE20 were genes altered as a result of salt stress.

3.2.9.1 Principal Component Analysis

Principal Component Analysis (PCA) is a statistical technique used to reduce and simplify dimensionality of large data sets into a linear format while preserving the variance in the data sets (Ringner, 2008). In a PCA plot, each dot represents a sample, and samples whose transcriptomes are similar group together to form a cluster. The farther away the two dots are on the plot, the larger is the variation in gene expression between the dots. Thus a PCA plot helps identify patterns in the global gene expression profiles between the samples and the variables that drive the variation in gene expression between the samples. PCA was carried out on four data sets: wild type, wild type salt stressed, Rel^{E20} and Rel^{E20} salt stressed. The dimensionality of the data sets was reduced to first two principal components. A total of 42.2% variance was captured in the first and the second principal components and was plotted two-dimensionally as shown in Figure 3-16.

The PCA plot showed that each genotype and diet condition formed distinct clusters (four clusters), each with four biological replicates being tightly clustered together (Figure 3-17). Though the replicates in the wild type control fed group are slightly scattered on PC2, which captured 13.1% of the total variance, they are tightly clustered on PC1 that captured 29.1% variance. The replicates in the wild type control fed and wild type salt stressed groups (demarcated by a pink ellipse) are clustered closely suggesting adaptability of the insect's osmoregulatory system to defend osmotic stress enabling it to survive for many days on high salt diet.

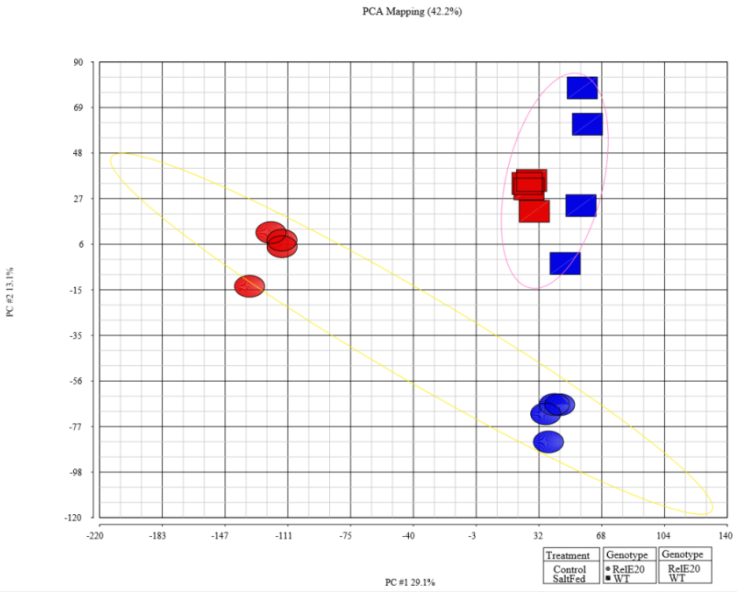


Figure 3-17: PCA of global gene expression profiles of Rel^{E20} and wild type data sets. Two dimensional plot of the PCA of Rel^{E20} and wild type data sets using the first two principal components of global gene expressions. The PCA plot shows four distinct clusters of the following datasets: wild type control fed (▪), wild type salt stressed (▪), Rel^{E20} control fed (●) and Rel^{E20} salt stressed (●), each with four biological replicates. The first principal component (PC1) captured 29.1% variance in the four combined datasets, while the second principal component captured 13.1% variance.

The wild type control fed and Rel^{E20} control fed are clustered similarly on PC1, which captured 29.1% of variance, but differently on PC2, which captured 13.1% of variance (Figure 3-17). This suggests that Relish in tubules is required for regulation of genes under normal control conditions. The cluster of Rel^{E20} salt stressed is markedly separated from Rel^{E20} control fed cluster as well as wild type salt stressed cluster on both the principal components. Altogether this plot highlights the importance of Relish protein in regulating expression of genes in tubules especially under osmotic stress caused by high salt feeding.

In Figure 3-18, the probe set counts based on fold change in tubules of salt stressed of wild type and Relish mutants (Rel^{E20}) are shown.

For further analysis only genes whose fold change was ≥2 or ≤-2 were considered. Under salt stress, in tubules of wild type 84 genes (64 probe sets) were upregulated and 80 genes (76 probe sets) were down regulated. While in Rel^{E20} tubules fed with salt for 24 h, 844 genes (859 probe sets) were upregulated and 1022 genes (1008 probe sets) were down- regulated compared to expression of these genes in tubules from control fed flies.

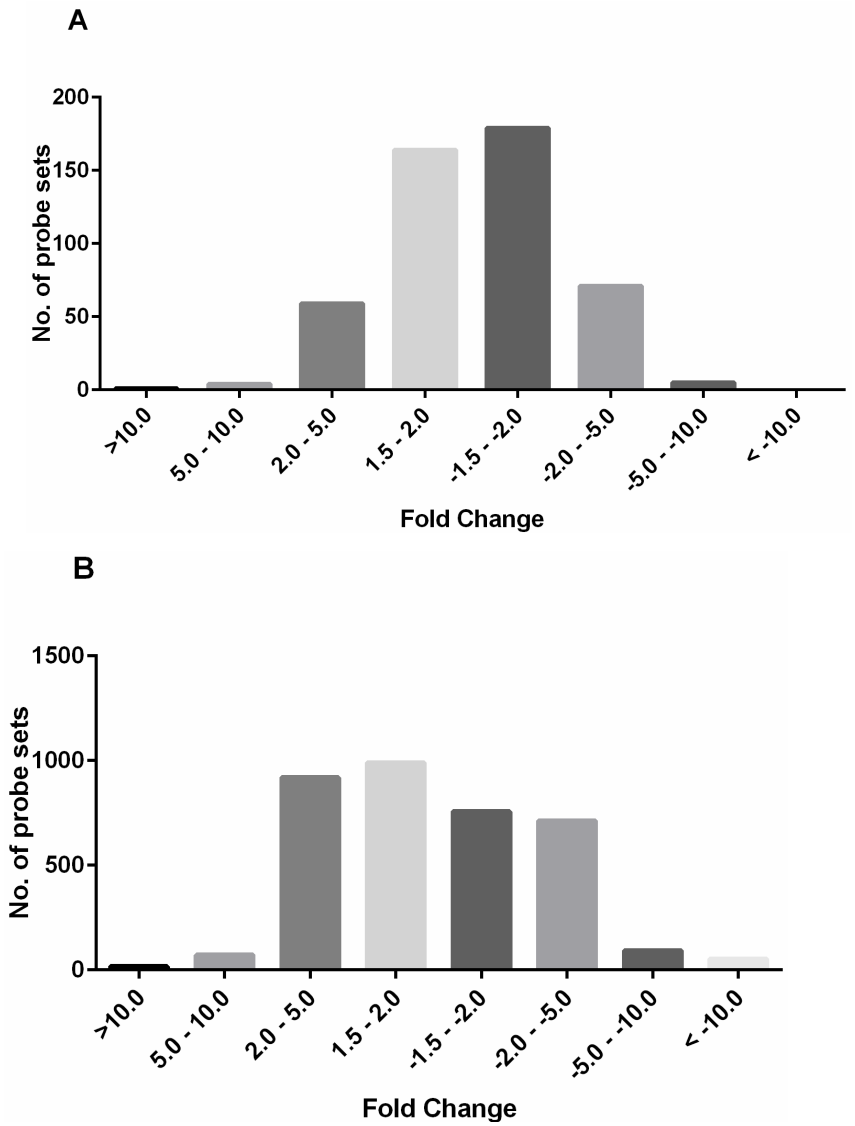


Figure 3-18: Number of probe sets significantly altered in salt stressed tubules of wild type and Rel^{E20}. The probe set counts were based on fold change between **(A)** tubules of salt stressed and control wild type, and **(B)** tubules of salt stressed and control Rel^{E20}. Data were statistically validated using two-way ANOVA test with Benjamini-Hochberg multiple test correction. The genes that had the corrected *p-value* ≤0.05 were considered significant.

3.2.9.2 GO classification of differentially expressed genes in wild type

The differentially expressed genes were analysed using Flymine tool for gene ontology (GO) enrichment in biological processes with *p-value* ≤ *0.05* (Lyne et al., 2007). The *p*- value was generated using the Hypergeometric test and was not corrected for multiple testing. In Table 3–1, top 10 GO enrichment terms for both the upregulated and down- regulated genes in wild type is listed. Many genes were annotated by more than two GO terms and many GO terms had similar genes being represented hence five broad biological process categories in

which the genes could be represented were metabolic process, transport, other functions, unclassified, and unknown (Figure 3-19).

Table 3-1: Top 10 GO terms significantly enriched in up and down- regulated gene list of wild type (salt stress vs. control).

Gene Ontology(GO) term		p-value
Up regulated		
GO:0006334	nucleosome assembly	2.16E-27
GO:0031647	regulation of protein stability	1.03E-03
GO:1901575	organic substance catabolic process	1.13E-03
GO:0006030	chitin metabolic process	1.95E-03
GO:0044255	cellular lipid metabolic process	3.89E-03
GO:1901564	organonitrogen compound metabolic process	9.29E-03
GO:0048878	chemical homeostasis	1.16E-02
GO:0055114	oxidation-reduction process	3.08E-02
GO:0055085	transmembrane transport	4.28E-02
GO:1901135	carbohydrate derivative metabolic process	4.47E-02
Down regulated		
GO:0055114	oxidation-reduction process	2.38E-04
GO:0044710	single-organism metabolic process	1.59E-03
GO:0006811	ion transport	2.01E-03
GO:0044255	cellular lipid metabolic process	2.51E-03
GO:0010033	hormone metabolic process	4.01E-03
GO:0042445	response to chemical	4.21E-03
GO:0007015	actin filament organization	6.56E-03
GO:0044281	small molecule metabolic process	6.76E-03
GO:0012501	programmed cell death	2.40E-02
GO:0009888	tissue development	4.81E-02

Table 3-1: Top 10 GO term significantly enriched in up and down- regulated gene list of wild type (salt stressed vs. control fed). *p-value* ≤0.05 was considered. Note that the substantial number of genes were found in more than one GO term.

In the upregulated gene list, the two probe sets *1631321_s_at* and *1629740_at* recognises 23 histone genes causing low *p-value* for GO term "nucleosome assembly" process. Genes involved in chitin and lipid metabolic processes are upregulated. The genes contributing in chitin metabolic processes are *CG14957, Mucin 18B (Muc18b), CG9307 (Cht5)* and *Imaginal disc growth factor 1 (Idgf1)* (Table 3-2). Genes that are involved in lipid metabolism are *CG10383, CG15534, CG4586, brummer (bmm)* and *Sterol regulatory element binding protein (SREBP formerly known as HLH106*) (Table 3-2). The genes in these processes are catabolic in nature. Thus, it may be speculated that these genes were being upregulated to produce glucose by breakdown of chitin and lipids for energy to cope with salt stress or form compatible osmolytes to maintain cell integrity.

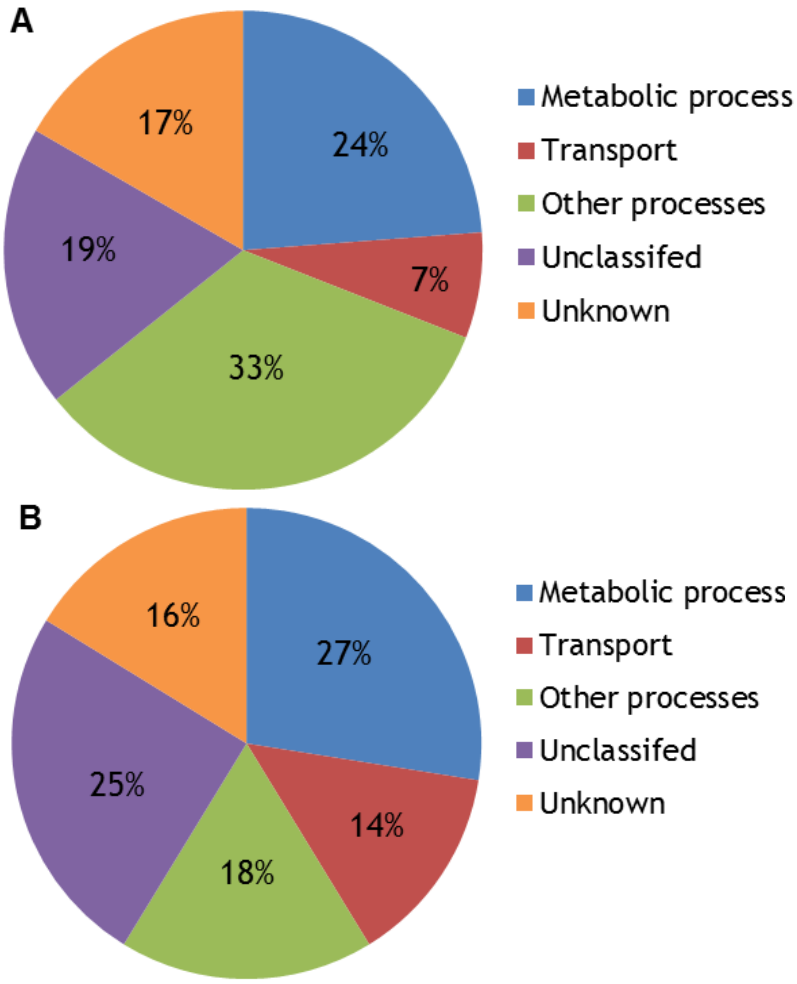


Figure 3-19: Biological process classification of genes differentially expressed in tubules of salt stress wild type. Categorising genes into three broad biological process categories, unclassified and unknown for **(A)** upregulated genes and **(B)** down-regulated genes in tubules of salt stress wild type flies.

In the down- regulated set of genes, there are three genes related to hormone metabolic process and that have high enrichment in tubules. These are *Juvenile hormone epoxide hydrolase 1* (*Jheh1)* and *Juvenile hormone epoxide hydrolase 3 (Jheh3)*, involved with juvenile hormone catabolic process, and *Ecdysone oxidase* (*Eo)* involved with ecdysone metabolic process which are all down- regulated (Table 3-3). Juvenile hormone (JH) and ecdysone hormones are primarily been associated with growth, moulting and metamorphosis (Hartfelder, 2000; Truman and Riddiford, 2002). A recent study has shown *Jheh1* to be involved in oxidative stress (Guio et al., 2014) while the physiological role of *Jheh3* other than in moulting is less understood.

Table 3-2: Genes upregulated in tubules of salt stress wild type segregated according to GO term.

			Salt fed		
Flybase ID	**Gene Symbol**	**Description**	**WT**	**Rel**	**Dependence**
Metabolic processes					
FBgn0039788	Rpt6R	ATPase activity	2.6	-1.2	D
FBgn0039769	CG15534	sphingomyelin phosphodiesterase activity	2.4	1.5	D
FBgn0037236	Skp2	unknown	2.1	1.9	D
FBgn0026371	SAK	protein tyrosine kinase activity / ATP binding	2.0	1.1	D
FBgn0027571	CG3523	fatty acid synthase activity	2.1	-1.1	D
FBgn0004907	14-3-3zeta	protein binding	5.7	8.1	I
FBgn0038020	GstD9	glutathione transferase activity	4.3	51.5	I
FBgn0010222	Nmdmc	methylenetetrahydrofolate	4.0	7.6	I
		dehydrogenase (NAD+) activity			I
FBgn0029924	CG4586	acyl-CoA oxidase activity	3.6	5.2	I
FBgn0035412	CG14957	chitin binding	2.7	1.1	I
FBgn0003887	betaTub56D	GTPase activity	2.5	3.2	I
FBgn0020416	Idgf1	chitinase activity	2.4	19.7	I
FBgn0261283	HLH106	sequence-specific DNA binding transcription factor activity	2.3	3.8	I
FBgn0032699	CG10383	hydrolase activity, acting on ester bonds	2.3	20.0	I
FBgn0036449	bmm	triglyceride lipase activity	2.2	10.3	I
FBgn0031000	Muc18B	chitin binding	2.0	19.0	I
FBgn0038180	Cht5	chitinase activity	2.0	4.1	I
FBgn0036290	CG10638	alditol:NADP+ 1-oxidoreductase activity	2.2	6.4	I
FBgn0031432	Cyp309a1	oxidoreductase activity, iron ion binding	2.1	25.1	I
FBgn0036806	Cyp12c1	iron ion binding, electron carrier activity	2.1	6.5	I
Transmembrane transport					
FBgn0022355	Tsf1*	iron ion transmembrane transporter activity	3.0	1.6	D
			3.2	3.4	
FBgn0034882	CG5398	water channel activity	2.5	-1.3	D
FBgn0038717	CG17751	secondary active organic cation transmembrane transporter	2.2	-8.2	D
FBgn0037387	CG1213	glucose transmembrane transporter activity	2.1	-1.3	D
FBgn0037807	CG6293	L-ascorbate:sodium symporter activity	2.4	5.2	I
FBgn0038799	CG4288	high affinity inorganic phosphate:sodium symporter	2.0	5.2	I
Response to lithium ion					
FBgn0034381	List	potassium:amino acid symporter activity,	2.0	1.8	D
		neurotransmitter transporter activity			
Regulation of DNA repair					
FBgn0019686	lok	ATP binding , protein kinase activity	2.0	2.4	I
Regulation of transcription from RNA polymerase III promoter					
FBgn0061362	CG33785	DNA-directed RNA polymerase activity	2.3	2.7	I
Behavioral response to starvation					
FBgn0033926	Arc1	nucleic acid binding	8.2	14.5	I
Medium-term memory					
FBgn0010015	CanA1	Ca2+-dependent protein serine/threonine phosphatase	2.7	2.2	I

Table 3-2: Genes upregulated in tubules of salt stress wild type segregated according to GO term. The first column denotes Flybase Id, second column - Gene Symbol, third column-gene description, the next two columns denote fold change (with multiple correction p-value≤0.05) of WT -Wild type salt stress vs. Wild type control, Rel- Rel^{E20} salt stress vs and Rel^{E20} control respectively. Dependence symbols denote "I" Independent of Relish and "D" Relish dependent during stress.* at gene symbol denotes detected by more than two probe sets.

cont. Table 3-2

			Salt fed		
Flybase ID	**Gene Symbol**	**Description**	**WT**	**Rel**	**Dependence**
Nucleosome assembly					
FBgn0051617	His1:CG31617	DNA binding	3.0	-1.8	D
FBgn0053801	His1:CG33801	DNA binding	3.0	-1.8	D
FBgn0053804	His1:CG33804	DNA binding	3.0	-1.8	D
FBgn0053807	His1:CG33807	DNA binding	3.0	-1.8	D
FBgn0053810	His1:CG33810	DNA binding	3.0	-1.8	D
FBgn0053813	His1:CG33813	DNA binding	3.0	-1.8	D
FBgn0053816	His1:CG33816	DNA binding	3.0	-1.8	D
FBgn0053819	His1:CG33819	DNA binding	3.0	-1.8	D
FBgn0053822	His1:CG33822	DNA binding	3.0	-1.8	D
FBgn0053825	His1:CG33825	DNA binding	3.0	-1.8	D
FBgn0053828	His1:CG33828	DNA binding	3.0	-1.8	D
FBgn0053831	His1:CG33831	DNA binding	3.0	-1.8	D
FBgn0053834	His1:CG33834	DNA binding	3.0	-1.8	D
FBgn0053837	His1:CG33837	DNA binding	3.0	-1.8	D
FBgn0053840	His1:CG33840	DNA binding	3.0	-1.8	D
FBgn0053843	His1:CG33843	DNA binding	3.0	-1.8	D
FBgn0053846	His1:CG33846	DNA binding	3.0	-1.8	D
FBgn0053849	His1:CG33849	DNA binding	3.0	-1.8	D
FBgn0053852	His1:CG33852	DNA binding	3.0	-1.8	D
FBgn0053855	His1:CG33855	DNA binding	3.0	-1.8	D
FBgn0053858	His1:CG33858	DNA binding	3.0	-1.8	D
FBgn0053861	His1:CG33861	DNA binding	3.0	-1.8	D
FBgn0053864	His1:CG33864	DNA binding	3.0	-1.8	D
Unclassified					
FBgn0028949	CG15254	metalloendopeptidase activity	5.0	1.3	D
FBgn0037038	CG11037	serine-type endopeptidase activity	3.3	1.1	D
FBgn0034407	DptB	antibacterial humoral response	2.8	-1.2	D
FBgn0035617	l(3)psg2	regulation of programmed cell death	2.6	1.2	D
FBgn0000046	Act87E	structural constituent of cytoskeleton	2.4	-1.4	D
FBgn0037468	CG1943	wing disc dorsal/ventral pattern formation	2.4	1.6	D
FBgn0034657	LBR	receptor activity	2.0	-1.5	D
FBgn0037151	CG7130	response to heat	3.9	5.4	I
FBgn0039319	CG13659	transferase activity	3.3	3.3	I
FBgn0026582	CG9418	DNA binding	2.9	4.8	I
FBgn0061361	CG33786	translation regulator activity	2.3	2.7	I
FBgn0033699	RpS11	structural constituent of ribosome	2.3	4.4	I
FBgn0037391	CG2017	GTPase activity	2.3	8.6	I
FBgn0031114	cactin	defense response	2.1	3.2	I
FBgn0038966	pinta	vitamin E and retinoid binding	2.1	4.7	I
FBgn0036875	CG9449	acid phosphatase activity/ involved in phagocytosis	2.1	1.8	I
			2.4	1.7	I
Unknown					
FBgn0003022	Ote	unknown	2.2	1.2	D
FBgn0037850	CG14695	unknown	11.0	24.6	I
FBgn0053468	CG33468	unknown	5.3	66.9	I
FBgn0033928	Arc2	unknown	4.4	17.1	I
FBgn0035868	CG7194	unknown	3.4	22.5	I
FBgn0033945	CG12868	unknown	2.7	5.1	I
FBgn0036467	CG12310	unknown	2.6	3.0	I
FBgn0033519	CG11825	unknown	2.5	5.8	I
FBgn0040723	CG5011	unknown	2.5	8.8	I
FBgn0259711	CG42365	unknown	2.5	19.8	I
FBgn0015010	Ag5r	unknown	2.3	13.3	I
FBgn0038353	CG5399	unknown	2.2	7.1	I
FBgn0036419	CG13482	unknown	2.1	7.0	I
FBgn0036735	Edc3	unknown	2.0	7.9	I

Also genes involved in response to chemical stimulus (Table 3-3) are down-regulated indicating that the first few hours of feeding on high salt diet produces a response similar to any chemical stimulus but as the feeding on salt diet continues, the response to salt stress becomes more specific.

Table 3-3: Genes down- regulated in tubules of salt stress wild type segregated according to GO term.

			Salt fed		
Flybase ID	Gene Symbol	Description	WT	Rel	Dependence
Metabolic process					
FBgn0050489	Cyp12d1-p	oxidoreductase activity, iron ion binding	-2.9	-1.0	D
FBgn0053503	Cyp12d1-d	oxidoreductase activity, iron ion binding	-2.9	-1.0	D
FBgn0063498	GstE2	glutathione transferase activity	-2.3	1.0	D
FBgn0042094	Adk3	nucleoside triphosphate adenylate kinase activity	-2.3	-1.5	D
FBgn0015546	spel1	DNA-dependent ATPase activity , damaged DNA binding	-2.1	-1.6	D
FBgn0033980	Cyp6a20	oxidoreductase activity, iron ion binding	-2.0	1.1	D
FBgn0040629	CG18673	carbonate dehydratase activity	-7.6	-5.2	I
FBgn0013772	Cyp6a8	alkane 1-monooxygenase activity, iron ion binding	-7.3	-3.3	I
FBgn0036821	CG3961	long-chain fatty acid-CoA ligase activity	-4.8	-59.6	I
FBgn0015034	Cyp4e1	oxidoreductase activity, iron ion binding	-3.8	-5.7	I
FBgn0034406	Jheh3	epoxide hydrolase activity	-3.4	-5.5	I
FBgn0010053	Jheh1	juvenile hormone epoxide hydrolase activity	-2.8	-2.1	I
FBgn0036975	CG5618	sulfinoalanine decarboxylase activity	-2.7	-2.3	I
FBgn0002939	ninaD	scavenger receptor activity	-2.6	-5.6	I
FBgn0033981	Cyp6a21	oxidoreductase activity, iron ion binding	-2.5	-3.2	I
FBgn0026718	fu12	1-acylglycerol-3-phosphate O-acyltransferase activity	-2.5	-3.7	I
FBgn0030597	Eo	ecdysone oxidase activity	-2.4	-3.2	I
FBgn0042137	CG18814	alcohol dehydrogenase (NAD) activity	-2.3	-6.0	I
FBgn0262559	Mdh2	L-malate dehydrogenase activity	-2.3	-6.0	I
FBgn0014032	Sptr	sepiapterin reductase activity	-2.2	-8.3	I
FBgn0027843	CAH2	carbonate dehydratase activity	-2.1	-2.6	I
FBgn0032603	CG17928	oxidoreductase activity, iron ion binding	-2.1	-2.1	I
Response to chemical					
FBgn0034085	Ptp52F	axon guidance	-2.1	-1.3	D
FBgn0035542	DOR	ecdysone receptor-mediated signaling pathway	-2.1	3.1	D
FBgn0013772	Cyp6a8	response to caffine	-7.3	-3.3	I
FBgn0020545	kraken	response to toxic substance / digenstion	-2.4	-3.1	I
FBgn0026718	fu12	behavioral response to ethanol	-2.5	-3.7	I
FBgn0027932	Akap200	behavioral response to ethanol	-2.1	-3.5	I
FBgn0031998	SLC5A11	behavioral response to nutrient	-2.1	-4.7	I
FBgn0033782	sug	response to starvation	-5.6	-5.8	I
FBgn0037890	CG17734	behavioral response to ethanol	-2.3	-3.3	I
FBgn0050489	Cyp12d1-p	response to DDT	-2.9	-1.0	I
FBgn0053503	Cyp12d1-d	response to DDT	-2.9	-1.0	I
FBgn0260005	wtrw	response to humidity	-2.0	-2.4	I
Transmembrane transport					
FBgn0038720	CG6231	secondary active organic cation transmembrane transporter	-2.6	-1.0	D
FBgn0033391	CG8026	folic acid transporter activity	-2.2	4.2	D
FBgn0026439	Eaat1	glutamate:sodium symporter activity	-4.2	-38.3	I
FBgn0038752	CG4462	secondary active organic cation transmembrane transporter	-3.8	-19.2	I
FBgn0053181	CG33181	cation transmembrane transporter activity	-2.5	-4.9	I
FBgn0039927	CG11155	kainate selective glutamate receptor activity	-2.4	-4.5	I
FBgn0034275	CG5002	high affinity sulfate transmembrane transporter activity	-2.2	-4.4	I
FBgn0031998	SLC5A11	Na+-dependent multivitamin transmembrane transporter	-2.1	-4.7	I
FBgn0259214	PMCA	calcium-transporting ATPase activity	-2.1	-2.2	I
FBgn0260005	wtrw	calcium channel activity, cation channel activity	-2.0	-2.4	I
FBgn0019952	Orct	organic cation transmembrane transporter activity	-2.0	-8.8	I

cont.Table 3-3

			Salt fed		
Flybase ID	**Gene Symbol**	**Description**	**WT**	**Rel**	**Dependence**
Tissue development and cell death					
FBgn0028360	l(1)G0148	protein serine/threonine kinase activity	-3.8	-1.0	D
FBgn0259730	tal-1A	unknown	-2.6	1.9	D
FBgn0259733	tal-AA	unknown	-2.6	1.9	D
FBgn0259731	tal-2A	unknown	-2.6	1.9	D
FBgn0259732	tal-3A	unknown	-2.6	1.9	D
FBgn0015546	spel1	DNA-dependent ATPase activity , damaged DNA binding	-2.1	-1.6	D
FBgn0027932	Akap200	protein kinase A binding / autophagic cell death	-2.1	-3.5	I
FBgn0019952	Orct	organic cation transmembrane transporter activity	-2.0	-8.8	I
Positive regulation of cell adhesion					
FBgn0034199	Gbp	cytokine activity	-2.4	-2.4	I
Positive regulation of mitosis					
FBgn0029879	APC7	mitotic anaphase-promoting complex activity	-2.5	-1.6	D
Amine biosynthetic process					
FBgn0037723	SpdS	spermidine synthase activity	-3.2	-6.7	I
Unclassified					
FBgn0025814	Mgstl	glutathione transferase activity	-2.6	-1.98	D
FBgn0032726	CG10621	selenocysteine methyltransferase activity	-2.5	-1.1	D
FBgn0015568	alpha est1	carboxylic ester hydrolase activity	-2.2	-1.96	D
FBgn0033204	CG2065	oxidoreductase activity	-2.2	3.0	D
FBgn0037046	CG10581	nucleoside-triphosphatase activity	-6.1	-11.8	I
FBgn0034736	CG6018	carboxylic ester hydrolase activity	-3.8	-6.0	I
FBgn0033917	CG8503	histone deacetylase binding	-2.8	-4.0	I
FBgn0030362	regucalcin	invovled in multicellular organism reproduction	-2.8	-2.7	I
			-2.3	-2.5	I
FBgn0032162	CG4592	dodecenoyl-CoA delta-isomerase activity	-2.6	-12.3	I
FBgn0033904	CG18327	transmembrane transporter activity	-2.5	-5.9	I
FBgn0051233	CG31233	aminopeptidase activity	-2.4	-8.3	I
FBgn0038733	CG11407	long-chain fatty acid transporter activity	-2.4	-12.4	I
FBgn0032666	CG5758	synaptic target recognition	-2.3	-2.0	I
FBgn0036759	CG5577	phosphatase activity	-2.3	-5.2	I
FBgn0033905	CG18324	transmembrane transporter activity	-2.3	-3.2	I
FBgn0052191	CG32191	N-acetylgalactosamine-4-sulfatase activity	-2.2	-2.9	I
FBgn0041234	Gr59f	taste receptor activity	-2.1	-2.6	I
FBgn0038135	CG8773	aminopeptidase activity	-2.1	-9.0	I
FBgn0037215	CG12582	beta-mannosidase activity	-2.0	-2.1	I
FBgn0037934	CG6830	transferase activity	-2.0	-2.4	I
Unknown					
FBgn0031910	CG15818	unknown	-4.1	1.7	D
FBgn0036576	CG5151	unknown	-3.0	-1.5	D
FBgn0035793	CG7546	unknown	-2.6	-1.98	D
FBgn0041160	comm2	unknown	-2.1	1.8	D
FBgn0030271	CG15202	unknown	-2.1	-1.9	D
FBgn0032957	CG2225	unknown	-2.0	-1.4	D
FBgn0028532	CG7968	unknown	-7.9	-2.4	I
FBgn0033607	CG9062	unknown	-3.4	-3.6	I
FBgn0037779	CG12811	unknown	-2.4	-4.6	I
FBgn0027793	CG14787	unknown	-2.4	-2.5	I
FBgn0040531	CG11741	unknown	-2.2	-2.9	I
FBgn0032322	CG16743	unknown	-2.0	-3.5	I
FBgn0039109	CG10365	unknown	-2.0	-2.7	I

Table 3-3: Genes down- regulated in tubules of salt stress wild type segregated according to GO term. The first column denotes Flybase Id, second column - Gene Symbol, third column-gene description, the next two columns denote fold change (with multiple correction p-value≤0.05) of WT -Wild type salt stress vs. Wild type control, Rel-Rel^{E20} salt stress vs and Rel^{E20} control respectively. Dependence symbols denote "I" Independent of Relish and "D" Relish dependent during salt stress

It is worth noting that the transcription factor *sugarbabe (sug)* is down-regulated in response to salt stress (Figure 3-22 and Table 3-3). It has been shown that *sug* expression is highly induced in response to a high sugar diet and it represses genes involved in fat breakdown (Zinke et al., 2002). Thus the down regulation of *sug*, may be increasing the breakdown of fat for energy requirement to cope with stress.

3.2.9.3 GO classification of differentially expressed genes in Rel^{E20}

Table 3-4 shows GO classification of 844 genes that are upregulated and 1022 genes that are down-regulated in tubules of salt stressed Rel^{E20} compared to its unstressed (control) counterpart.

Table 3-4: Top 20 GO term significantly enriched in up and down- regulated gene list of Rel^{E20} (salt stress vs. control).

Gene Ontology(GO) term		p-value	No. of genes
Up regulated			
GO:0048731	system development	2.00E-10	232
GO:0050896	response to stimulus	3.30E-10	267
GO:0044767	single-organism developmental process	3.33E-09	310
GO:0032502	developmental process	5.87E-09	312
GO:0009653	anatomical structure morphogenesis	1.11E-08	185
GO:0065007	biological regulation	8.79E-08	313
GO:0007275	multicellular organismal development	9.15E-08	265
GO:0051716	cellular response to stimulus	4.06E-07	185
GO:0050794	regulation of cellular process	9.15E-07	269
GO:0050789	regulation of biological process	1.11E-06	288
GO:0048856	anatomical structure development	1.30E-06	279
GO:0048869	cellular developmental process	1.00E-05	221
GO:0030154	cell differentiation	1.48E-05	213
GO:0016043	cellular component organization	3.93E-05	234
GO:0044707	single-multicellular organism process	8.33E-05	292
GO:0071840	cellular component organization	2.60E-04	235
GO:0044763	single-organism cellular process	4.36E-04	418
GO:0044699	single-organism process	6.35E-04	504
GO:0032501	multicellular organismal process	8.49E-04	319
Down regulated			
GO:0044281	small molecule metabolic process	8.30E-26	140
GO:0044710	single-organism metabolic process	1.45E-18	253
GO:0055114	oxidation-reduction process	4.03E-16	99
GO:0019637	organophosphate metabolic process	1.39E-15	80
GO:0055086	nucleobase-containing small molecule metabol	1.60E-13	63
GO:1901564	organonitrogen compound metabolic	2.80E-10	106
GO:0006793	phosphorus metabolic process	4.64E-10	126
GO:0006082	organic acid metabolic process	8.47E-10	61
GO:0006796	phosphate-containing compound metabolic	1.28E-08	120
GO:0055085	transmembrane transport	9.14E-08	69
GO:1901135	carbohydrate derivative metabolic process	2.01E-06	71
GO:0016310	phosphorylation	5.54E-06	70
GO:0008152	metabolic process	7.68E-06	500
GO:0044711	single-organism biosynthetic process	1.12E-05	70
GO:0044765	single-organism transport	3.26E-05	152
GO:1902578	single-organism localization	6.79E-05	161
GO:0006810	transport	6.73E-04	160
GO:0051234	establishment of localization	9.87E-04	164
GO:0044237	cellular metabolic process	0.001487	386
GO:0051179	localization	0.021932	181

Table 3-4: Top 20 GO term significantly enriched in up and down- regulated gene list of Rel^{E20} (salt stress vs. control fed). *p-value* ≤0.05 was considered. Note that, substantial number of genes were found in more than one GO term.

The number of genes denoted in Table 3-4 are the count of genes from the differentially expressed gene list of Rel^{E20} (salt stress vs. control) that grouped in that specific GO category.

25% of upregulated genes were not mapped under any GO term. Genes associated with developmental processes are highly enriched followed by genes involved in response to stimuli. 267 genes associated with response to stimuli (Table 3-4), 132 genes responded to stress which could be further grouped under four major groups: defense response, response to abiotic stress, response to nutrient levels, and regulation of stress response. The genes are listed in Table 3S-1.

In down- regulated genes, 29% genes are not categorised under any GO categories. Most of the down- regulated genes are involved with metabolic processes. Other than the GO term related to metabolic processes, genes were enriched in 'Transporter' category. 160 genes were associated with GO of transporter (Table 3-4) of which 69 are 'transmembrane transport' (Table 3S-2).

It is interesting to see that there are no overlapping GO categories for the up and down- regulated genes. This implicates that in absence of Relish and its target genes and in response to stress, sets of specific genes are triggered but not repressed, or excessively repressed as a rescue system, or are not induced.

3.2.9.4 Genes affected by Relish during salt stress

The above results demonstrate that Relish regulation during salt stress needs to be finely tuned for the organism's survival to stress. An *in silico* approach was utilized to find genes that are controlled by Relish (Figure 3-20). Genes that were identified as upregulated or down- regulated in wild type tubules of flies fed on high salt diet (salt stressed) compared to normal (control) diet were then examined to see how were they expressed in tubules of salt stressed Rel^{E20} compared to control. Using this approach, putative genes were identified in tubules that were controlled by Relish as "Dependent" or were "Independent" of Relish during salt stress. This approach does not directly determine how Relish

regulates the expression of gene but helps recognise the genes, whose expression is controlled by Relish during salt stress.

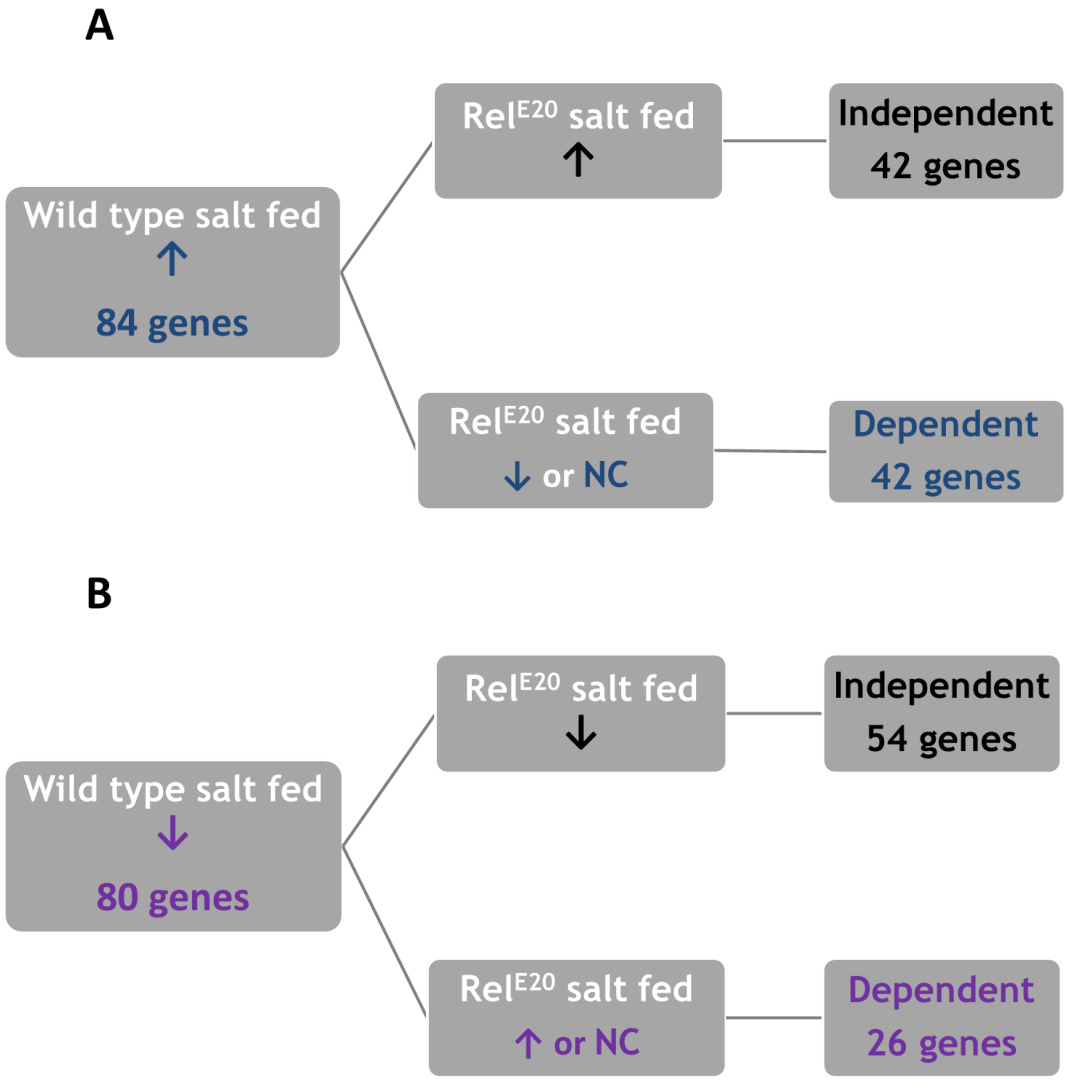


Figure 3-20: Flowchart describing the analysis done to identify Relish Independent and Dependent genes. The wild type data sets of upregulated genes **(A)** and down-regulated genes **(B)** were compared against the upregulated and down- regulated data sets of Rel^{E20} to identify dependence of the genes on Relish for their expression in response to salt stress. ↑ denotes upregulated, ↓ denotes down- regulated, and NC denotes no change.

Table 3-2 summarises a list of genes that are upregulated in wild type tubules and their dependence on Relish during salt stress.

Diptericin B (DptB) is a known Relish target gene having Relish binding sites in its promoter region (Lee et al., 2001). In the above described analysis it was classified amongst the Relish "Dependent" genes (Table 3-2). The microarray result for *DptB* was validated with qRT-PCR (Figure 3-21). RNA was isolated from Rel^{E20} and wild type flies fed a diet with or without salt for 24 h and was

quantified to determine the amount of *Diptericin*. Rel^{E20} flies are incapable of inducing *Diptericin* on immune stress (Hedengren et al., 1999), thus expression of *Diptericin* is a "read out" of Relish activation. As shown earlier, *Diptericin A* expression is induced in salt stressed tubules of Relish (Figure 3-13). Hence the read out of *Diptericin A* was used as a positive control for the samples while validating the expression pattern of genes from the microarray results (Figure 3-21).

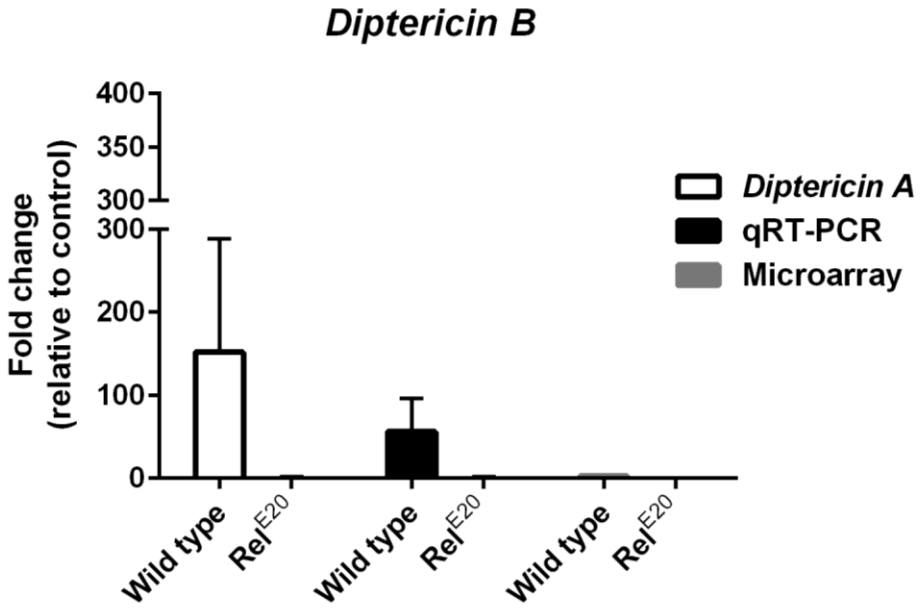


Figure 3-21: Validation of microarray result of *Diptericin B* by qRT-PCR. Tubules from 7-9 day old Rel^{E20} and wild type flies fed on diet with or without 4% NaCl were dissected. *Diptericin A* and *Diptericin B* primers were used in the qRT-PCR reaction for quantifying mRNA transcripts. The qRT-PCR result (in black) is of 2 independent biological replicates. The microarray data (in grey) is result from 4 biological replicates.

DptB expression was observed in wild type tubules but not detected in Rel^{E20} tubules (Figure 3-21). Thus, it is possible to conclude that genes grouped as "Dependent" require Relish for its induction. The other genes identified in this group are known to be involved in varied functions: nucleosomes assembly or transmembrane transporter genes. Thus, Relish can control the transcription of a diverse set of genes in response to a specific stress input.

The microarray result for selected genes were validated by qRT-PCR and shown in Figure 3-22. The genes selected for validation were highly expressed in tubules such as transcription factor *sug*; transporters- *CG6293, CG17751 and CG11407;* and enzymes – *CG10621* and *Cyp4e1*.

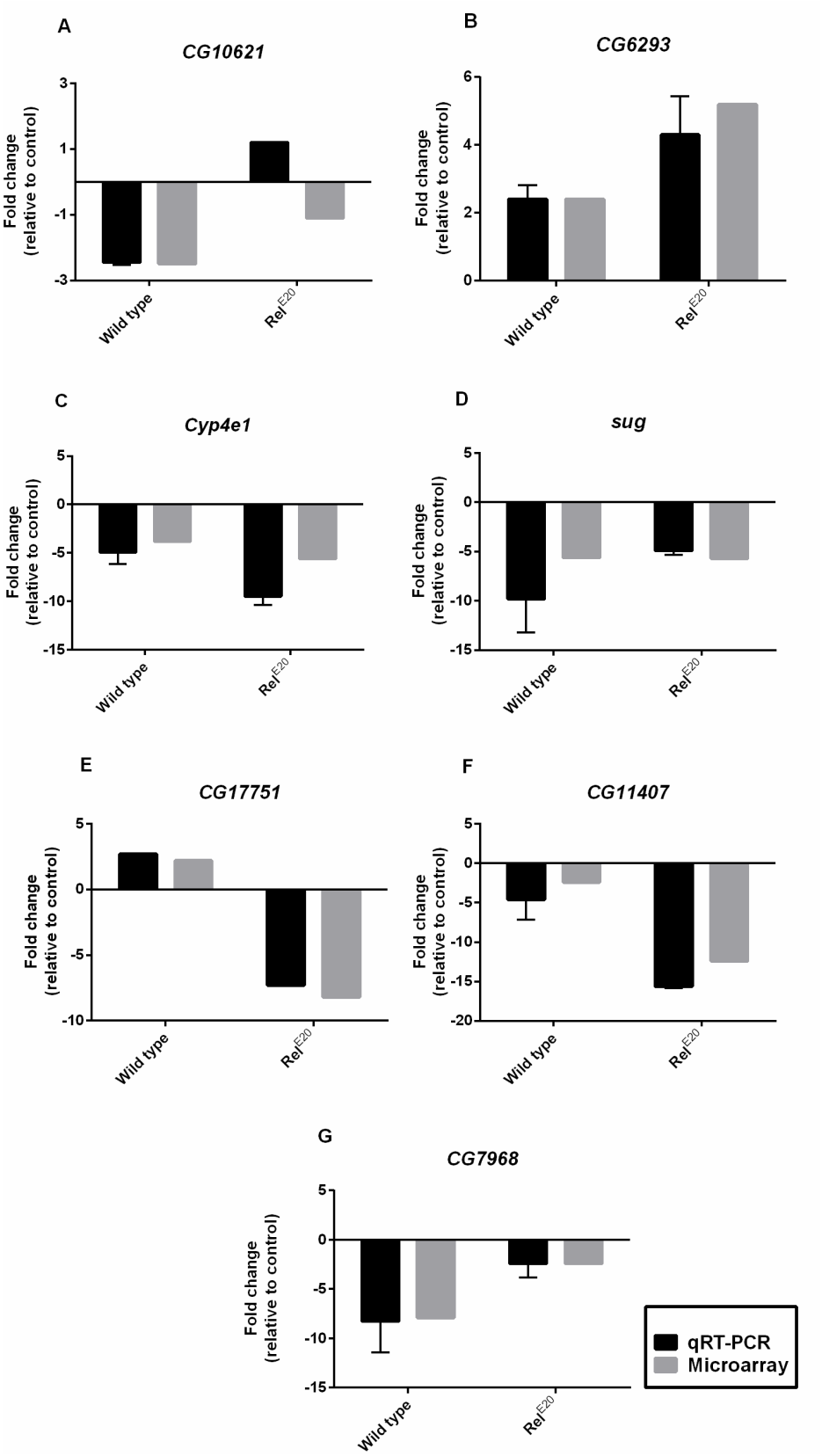


Figure 3-22: Validation of microarray result by qRT-PCR. Tubules from 7-9 day old Rel^{E20} and wild type flies fed on diet with or without 4% NaCl were dissected. Gene specific primers were used in the qRT-PCR reaction for quantifying mRNA transcripts. The qRT-PCR result (in black) is of 2 independent biological replicates. The microarray data (in grey) is result from 4 biological replicates.

Of the 844 genes that are upregulated in Rel^{E20} upon salt stress, 44 are also upregulated in wild type tubules upon salt stress and thus are regarded as independent of Relish. It is interesting to see that many genes that are independent of Relish are hyper-activated in Rel^{E20} (Table 3-2). For example *bmm, CG6293* and *CG13482* show a fold change of 2.2, 2.4, and 2.1 in tubules of wild type but their fold change in Rel^{E20} is 10.3, 5.2, and 7.0 respectively. This indicates that induction of these genes is independent of Relish but require Relish and its target genes for its appropriate regulation.

Interestingly, 14 genes specific to the c-Jun kinase (JNK) cascade (Table 3-5) and *hemipterous (hep)*(a substrate of MAPK kinase kinase*)* with a fold change of 1.95 were upregulated in Rel^{E20} tubules under salt stress. However in wild type tubules under salt stress no change was seen. The JNK pathway is one of the three Mitogen Activated protein kinase (MAPK) pathway. The hep> dJNK> Jra/Kay make the core JNK signalling pathway where MAPK kinase kinase activates hep , which in turn activates dJNK leading to activation of Jra and Kay for transcriptional activation of genes (Stronach, 2005). The Connector of kinase to AP-1 (Cka) organises and regulates the core JNK module (Chen et al., 2002a). Except for dJNK, all the components of JNK pathway along with *slpr* (another MAPK kinase kinase that is important for activation of dJNK (Stronach and Perrimon, 2002)) were seen to be upregulated (Table 3-5) indicating that the JNK pathway was being activated in response to salt stress in Rel^{E20} mutants.

In addition it was observed that target genes of JNK pathway activation: *puckered (puc), CG13482* and *Neural Lazarillo (Nlaz)* (Hull-Thompson et al., 2009; Park et al., 2004) were upregulated in tubules of salt stressed Rel^{E20} flies and showed a significant fold change of 1.9, 3.4 and 2.2 respectively. In wild type tubules of salt stress only *CG13482* showed a fold change of 2.0. Park et al., 2004 have shown that *puc and CG13482* genes peak rapidly in immune challenge flies causing JNK pathway activation and quickly come back to baseline in presence of Relish (Park et al., 2004). Further their activation is independent of Relish. In addition, Rel^{E20} flies challenged by immune stress either had increased duration or intensity of expression of these JNK target genes (Park et al., 2004). Taken together, it can be suggested that on high salt feeding, the JNK pathway

is activated as an early response in tubules independent of Relish; but that Relish and its target genes is required to attenuate the JNK pathway activation, similar to results seen by Park et al. for immune stress in flies.

Table 3-5: Genes associated with JNK cascade in tubules of salt stressed Rel^{E20}

Flybase ID	Gene Symbol	Ontology Term
FBgn0043364	cbt	JNK cascade
FBgn0010341	Cdc42	JNK cascade
FBgn0044323	Cka	positive regulation of JNK cascade
FBgn0033153	Gadd45	JNK cascade
FBgn0001291	Jra	JNK cascade
FBgn0001297	kay	JNK cascade
FBgn0031885	Mnn1	regulation of JNK cascade
FBgn0010909	msn	JNK cascade
FBgn0003053	peb	negative regulation of JNK cascade
FBgn0015286	Rala	negative regulation of JNK cascade
FBgn0003209	raw	regulation of JNK cascade
FBgn0014020	Rho1	JNK cascade
FBgn0003371	sgg	negative regulation of JUN kinase activity
FBgn0030018	slpr	positive regulation of JNK cascade

The *foraging (for or dg2)* gene was upregulated only in salt stressed tubules of Rel^{E20} by a fold change of 2.3 (Figure 3-23).

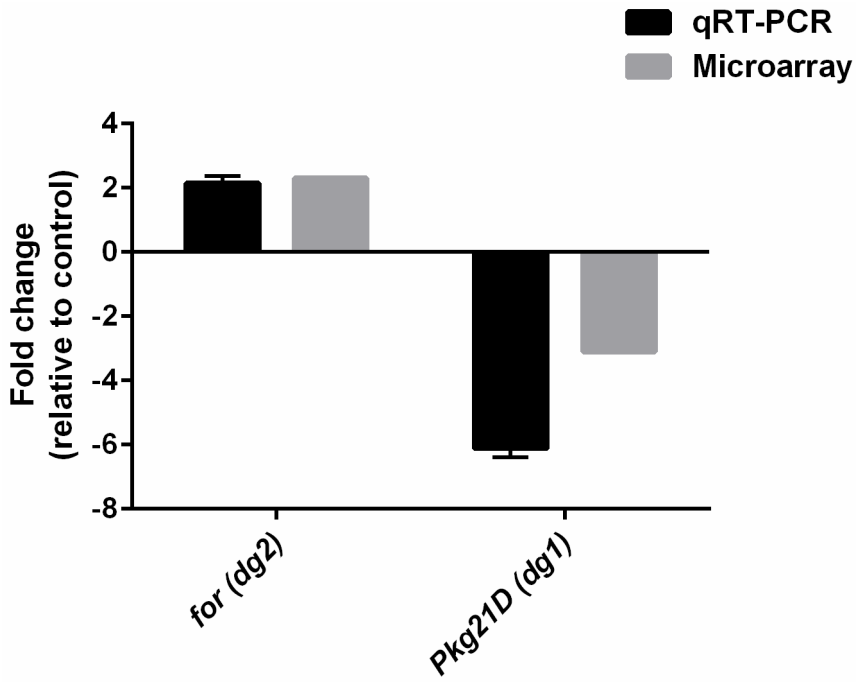


Figure 3-23: Validation of microarray result by qRT-PCR for genes *foraging (dg2)* and *Pkg21D (dg1)*. Tubules from 7-9 day old Rel^{E20} and wild type flies fed on diet with or without 4% NaCl for 24 h were dissected. Gene specific primers were used in the qRT-PCR reaction for quantifying mRNA transcripts. The qRT-PCR result (in black) is of 2 independent biological replicates. The microarray data (in grey) is result from 4 biological replicates.

It has been shown that tubule principal cells which overexpress *dg2* increase fluid secretion when stimulated with neuropeptide capa-1 (MacPherson et al., 2004b). This increase in fluid secretion has been attributed to the ability of capa-1 to inhibit cGMP-specific phosphodiesterase (cG-PDE) activity leading to increase in 3′,5′-cyclic guanosine monophosphate (cGMP) pools potentially activating apical V^+ATPases (MacPherson et al., 2004a; Terhzaz et al., 2006). The gene for the dual specific phosphodiesterase, *Pde 11,* was upregulated by 2.4 fold change in salt stressed Relish mutants. There was no upregulation of these two genes in salt stress tubules of wild type after 24 h feeding on high salt diet. In addition, another tubule enriched cGMP dependent kinase *Pkg21D* was observed to be down- regulated by a fold change of -7.0 in tubules of salt stressed Rel^{E20} (Figure 3-23). Hence it may be inferred that during salt stress cGMP signalling in tubules may play a role in fluid secretion to regain water-ion homeostasis and require Relish and its target genes to regulate this mechanism. A loss of this regulation seen in the Relish mutant may be leading to hyper-activation of this mechanism making Rel^{E20} sensitive to salt stress.

It is interesting to note that many genes that are independent of Relish are transmembrane transporters (Table 3S-2). 13 that were also down- regulated in wild type tubules were also down- regulated in salt stressed tubules of Rel^{E20} (Table 3S-2).146 genes annotated as transporter genes were specifically observed to be down- regulated only in salt stress tubules of Rel^{E20} (Table 3S-2). These include water channel gene (*Drip),* chloride channels (*Clc-a, Best1, Best2)*, the ABC and cGMP transporter gene white (*w)* (Table 3S-2). *Clc-a* and *Drip* have been shown to be stellate cell specific (Cabrero et al., 2014; Kaufmann et al., 2005). The abundantly expressed and highly enriched potassium channels- Inward-rectifying potassium channels (*Ir and Irk3)* were also observed to be down- regulated (Table 3S-2).

These results imply a role for Relish in the transcriptional maintenance of chloride channels to ensure appropriately diuresis occurs in the tubules in response to neuropeptides during salt stress. The results also indicate that cGMP signalling may play a role during salt stress to secrete excess Na^+ and Cl^- ions to gain water and ion balance and this signalling may be modulated through Relish.

Table 3-3 summarises a list of genes that are down- regulated in wild type tubules and their dependence on Relish during salt stress. Twenty three genes classified as “Dependent” varied in functions, further highlighting that wide range of genes that Relish can down regulate.

3.2.9.5 Comparing predicted Relish controlled genes to whole fly microarray data of flies overexpressing *Diptericin*

Zhao et al (2011) performed a microarray experiment on whole flies over expressing *Diptericin* in all the tissues and demonstrated that over expression of *Diptericin* in flies increased their tolerance to hyperoxia (Zhao et al., 2011). *Diptericin* is induced in tubules of wild type flies and not Relish mutants in response to salt stress (Figure 3-13, Figure 3-21). Thus we wondered if the increase expression of Diptericin in tubules during salt stress affected regulation of genes in tubules of wild type flies. Hence an analysis was carried out to search for common genes between the data set of Relish ‘Dependent’ and ‘Independent’ genes in this study, and the Diptericin microarray data set generated by Zhao et al. (2011). Only the genes enriched in the tubules were considered for the analysis and five genes were found common (Table 3-6).

Table 3-6: Comparing predicted Relish controlled genes to whole fly microarray data of flies overexpressing *Diptericin*

			Diptericin	Dependence	Tubule mRNA
	Flybase ID	Gene Symbol	overexpression	on Relish	Enrichment
1630528_at	FBgn0038717	CG17751	4.1	D	58.3
1624953_at	FBgn0036806	Cyp12c1	-1.5	I	4.1
1632021_at	FBgn0033980	Cyp6a20	-2.5	D	2.7
1639539_at	FBgn0015034	Cyp4e1	-1.5	I	4.2
1640755_at	FBgn0013772	Cyp6a8	-1.5	I	28.5

Table 3-6: Comparing predicted Relish controlled genes to whole fly microarray data of flies overexpressing *Diptericin*. The first column denotes Flybase Id, second column – Gene symbol, third column is the value from microarray data of whole fly overexpressing *Diptericin* from Zhao et al., 2011. Note the original fold change ratio values of the data converted into whole numbers. The fourth column is the “Dependence” on Relish protein predicted from the *in silico* analysis. Dependence symbols denote “D” as dependent and “I” as independent. The fifth column in the tubule mRNA enrichment values from Flyatlas (http://flyatlas.org/).

CG17751, a tubule specific gene according to Flyatlas data, was upregulated in the data set of salt stress tubules of wild type but down- regulated in Rel^{E20} (Figure 3-23F). This gene is also upregulated in flies overexpressing *Diptericin*. Also according to *in silico* analysis carried out in this study, it was categorised as

a Relish dependent gene during salt stress. This suggests that the induction of *Diptericin* during salt stress influences the expression of *CG17751*. *Cyp6a20* followed a similar pattern for being categorised as Relish dependent gene. In the *in silico* analysis, the genes *Cyp4e1, Cyp6a8* and *Cyp12c1* were categorised as independent of Relish activation during salt stress. These genes are highly enriched in other epithelial tissues eg- *Cyp6a8* in abundantly expressed and highly enriched in crop. Hence this may be the contributing factor in difference between the results seen in whole fly microarray data of Zhao et al. (2011) vs the tubule data of this study. Thus, it may be suggested that Relish target genes exert its effect directly or indirectly on expression of other genes required for salt stress tolerance.

3.3 Discussion

3.3.1 Relish activation in response to salt stress

Relish in tubules is important for tolerance to salt stress (a form of osmotic stress) caused due to high salt feeding. However, incubation of S2 cells and excised tubules in solution triggering osmotic stress does not activate Relish (Figure 3-10, Figure 3-11). These results are similar to those observed by Chen et al.2002. They showed in *Drosophila* S2 cells that *Drosophila* Jun kinase (dJNK) was activated on high salt stress but not Relish (Chen et al., 2002b). Also in mammalian human embryonic kidney cells, HEK293 cells, it was shown that osmotic stress lead to activation of TAK1-JNK pathway, although TAK1-mediated NF-kappa B activation was blocked by TAO2 (thousand-and-one amino acid kinase 2) (HuangFu et al., 2006). This implies that activation of Relish in tubules of flies fed on high salt diet is mediated by an *in vivo* mechanism triggered in response to stress. This highlights that tissue-tissue communication is occurring in response to salt stress. It is possible that neuropeptides play this role. The neuropeptide

NPLP1-VQQ can activate Relish and increase expression of *Diptericin* via receptor Gyc76C in the tubules of principal cells (Overend et al., 2012). A targeted knockdown of this receptor in principal cells causes sensitivity to salt stress in flies. Another neuropeptide, capa-1, triggers activation of Relish in tubule

principal cells but does not increase expression of *Diptericin* (Terhzaz et al., 2014). Capa-1 can also activate the NF-kB pathway in HEK293 cells (Terhzaz et al., 2014). Capa-1 levels increase by 1.8 fold in whole flies that were salt fed compared to control (personal communication Dr. Selim Terhzaz). Thus, capa-1 could also be a likely candidate in activation of Relish in response to salt stress either working in parallel or synergistically with neuropeptide NPLP1-VQQ to tolerate salt stress.

After 24 h of salt stress, no difference was observed in secretion rate of tubules of salt stressed wild type flies compared to control (Figure 3-14). Similarly, Naikkhwah and O'Donnell, (2012) observed no change in fluid secretion rates of larval tubules when fed a 2% NaCl diet for 24 h and suggested presence of haemolymph "factor" that contributed to an increase the intracellular Ca^{2+} levels in tubules cells.

Calcineurin Ca^{2+}-dependent phosphatase (Can A1) has been shown to be involved in promoting Nitric oxide (NO) induced Relish translocation to nucleus (Dijkers and O'Farrell, 2007). The microarray data shows *Can A1* expression to be upregulated by 2.7-fold and 2.2-fold in tubules of salt stressed wild type flies and Relish mutants compared to their respective controls (Table 3-2). This suggests that (1) increase in intracellular Ca^{2+} may be occurring in response to salt stress that leads to activation of Relish, (2) this activation of Relish may be mediated by Can A1, and (3) upregulation of CanA1 gene expression is independent of Relish. Also, NO and cGMP has been shown to mobilise Relish in nuclei of tubule principal cells ((Davies et al., 2014), unpublished data, Overend et al.).

NPLP1-VQQ stimulation in tubules produces a small but significant increase in intracellular Ca^{2+} in principal cells and cGMP levels, and is a partial agonist to fluid secretion phenotype of the tubule (Overend et al., 2012). Capa-1 signalling is through an increase in intracellular Ca^{2+} pools and NO/cGMP pathway (Kean et al., 2002). As both neuropeptides are able to signal through similar secondary messengers in the principal cells and can increase intracellular Ca^{2+} pools in principal cells of tubule, they both may be involved in salt stress response in

activation of Relish through Ca^{2+} /NO-cGMP signalling pathways. These neuropeptides may be either working additively or synergistically for steady regulation of water and ion balance to combat salt stress.

3.3.2 Antimicrobial peptide expression in tubules of salt stressed tubules

An upregulation of *Diptericin A* and *Diptericin B* was observed after 24 h of salt feeding in tubules of wild type but not in the Relish mutant flies. Diptericin A is a signature antimicrobial peptide of IMD pathway activation; hence its upregulation at mRNA level as well as protein level confirms nuclear translocation of Relish and activation of IMD pathway. After 4 h of salt feeding, upregulation in antimicrobial peptide *Drosomycin* was seen in tubules of wild type. The levels of Drosomycin were back to baseline even after feeding on salt diet continued for 24 h. The microarray data showed that salt stress tubules of wild type had a significant upregulation of antimicrobial peptide *Attacin A/B* with a FC of 1.8. While in Rel^{E20}, a very high upregulation of *Drosomycin* (FC=7.4) and *Attacin A/B* (FC=2.4) was observed after 24 h of salt feeding (Table 3S-2). The upregulation of *Drosomycin* and *Attacin* in salt stress tubules of Rel^{E20} and wild type flies may be attributed to activation of FOXO. It has been shown that FOXO can induce strong expression of Drosomycin and mild expression of *Attacin*, independent of immune stress pathways (Becker et al., 2010). An upregulation of FOXO and its target genes *dDOR* (Francis et al., 2010b), *InR* (Puig and Tjian, 2005), and *GATAd* (Alic et al., 2011) was observed in tubules of salt stressed Rel^{E20} but a downregulation of dDOR was seen in tubules of salt stressed wild type. Other evidence that FOXO activity is induced under salt stress response is the upregulation of 14-3-3 zeta gene in tubules of wild type and Rel^{E20} (Table 3-2). 14-3-3 proteins bind and regulate FOXO activity (Brunet et al., 1999; Nielsen et al., 2008; Tzivion et al., 2011). Interestingly only the zeta sub-type of 14-3-3 group of genes is being upregulated under salt stress response, implying the specificity of this isoform in regulation of FOXO under salt stress. Thus it can be inferred that FOXO activity is induced as an early response to salt stress for adaption to this stress.

As Relish and its target genes may influence the expression of other genes, a comparison of the tubule microarray dataset of salt stressed flies to tubule enriched genes of whole fly microarray dataset of flies overexpressing *Diptericin,* a Relish target gene was carried out. Two genes*CG17751* and *Cyp6a20* showed similar trend in expression in both data sets. These genes were affected in their expression levels in salt stress tubules of Rel^{E20} compared to wild type. This confirms our hypothesis that Relish and its target genes play a role in regulating the expression of other genes in tubules during salt stress.

3.3.3 Activation of stress pathways in response to salt stress

The JNK pathway is one of the evolutionarily conserved Mitogen-activated protein kinase (MAPK) signal transduction pathway which is a critical regulator in lifespan expansion and plays an important role in stress tolerance (Biteau et al., 2011; Stronach, 2005). In response to salt stress an upregulation of the JNK target gene *CG13482* was observed in tubules of salt stressed wild type and Rel^{E20} (Table 3-2) and genes of the JNK cascade were upregulated in tubules of Relish mutants only (Table 3-5). In tubules of salt stressed Rel^{E20}, a prolonged or hyper expression of JNK target genes *puckered* and *CG13482* was observed. The IMD pathway bifurcates at protein dTAK1 into the JNK and Relish pathways with two signalling cascades regulating two different groups of target gene that are different in function and induction kinetics (Park et al., 2004). It has been shown in S2 cells that Relish target genes target dTAK1 to attenuate JNK pathway activation during immune stress (Park et al., 2004). However, Kim et al. show that JNK activates AP1 complex which complexes with STAT92E to repress Relish activated genes (Kim et al., 2007; Kim et al., 2005). Thus a cross-talk occurs between the two pathways during immune stress to maintain homeostasis. A similar cross talk may be occurring in tubules of wild type flies upon salt stress that helps the wild type flies to survive for longer period. Because genes in JNK cascade are upregulated in Rel^{E20} tubules only and not in wild type tubules (Table 3-4) after 24 h of salt stress, it may be concluded that there is a prolonged activation of JNK pathway due to loss of Relish.

A study by Inoue et al., showed that in flies, D-MEKK1 that activates the p38 MAPK pathway was required for tolerance to salt stress (Inoue et al., 2001). Recently, it was shown that double mutants of p38a and p38c , components of the p38/MAPK pathway was responsible for salt stress tolerance (Chakrabarti et al., 2014). In the salt stress tubules of Rel^{E20} and wild type, p38c is upregulated by fold change of 4.1 and 1.7, respectively, compared to tubules from the control fed flies. This indicates that other MAPK kinase pathways are involved in tolerance to stress. A study on S2 cells shows that activation of dJNK in response to salt stress is reduced only on simultaneous suppression of 4 MAPK kinase kinases: *D-MEKK1, TAK1, ASK1 and MLK2* (Chen et al., 2002b). Taken together it can be inferred that the prolonged JNK pathway activation and high upregulation seen of p38c gene in salt stress tubules of Rel^{E20} was to compensate for the loss of activated Relish pathway during salt stress.

During oxidative stress, the JNK pathway plays a protective role by inducing autophagy genes, *Autophagy-related 1* (*ATG1)* and *Autophagy-related* 18 (*ATG18)* (Wu et al., 2009). The JNK pathway can also antagonize insulin/IGF signaling (IIS) pathway (Wang et al., 2005) and a fine tuning between JNK pathway and IIS pathway is required for achieving balanced metabolic homeostasis (Karpac and Jasper, 2009). FOXO can be activated by JNK pathway independent of IIS signalling but a chronic activation of this activation is deleterious (Karpac et al., 2013). In Table 3S-1 that lists the genes that are associated with response to stress, *FOXO*, *Insulin Receptor (InR)* and autophagomse related genes *ATG1*, *ATG18* were observed to be upregulated in salt stress Relish mutants only. These genes play an important role in response to starvation (Erdi et al., 2012; Scott et al., 2004). It is difficult to dissect out whether upregulation of genes of FOXO/IIS signalling pathway and autophagy in tubules of salt stress Rel^{E20} is a starvation response or hyper-activation of JNK pathway due to absence of Relish leading to inappropriate regulation of these mechanisms. A starvation survival assay shows more than 50% of Rel^{E20} flies die between 22 h and 38 h and 50% wild type flies die within 40 h (Figure 3S-1). This necessitates studying the expression of these genes in salt stress flies at time points other than 24 h to delineate if the hyper-activation of these genes in

response to salt stress is because of mis-regulation of JNK pathway due to absence of Relish or whether the fly is compensating for the absence of Relish.

The whole fly microarray data of salt stressed wild type flies showed *Tot M* (a target gene of JAK-STAT signalling pathway) is upregulated after 4 and 16 hours of salt feeding indicating the activation the pathway (Stergiopoulos et al., 2009). Signal-transducer and activator of transcription protein at 92E (Stat92E) transcriptionally activates *Suppressor of cytokine signaling at 36E* (*Socs36E)* and *Protein tyrosine phosphatase 61F* (*PTP61F*) genes which are known negative regulators of the pathway (Baeg et al., 2005; Stec et al., 2013). In mammalian cells the activation of JAK-STAT pathway has been attributed to cell shrinkage caused by hyperosmotic stress (Gatsios et al., 1998). Thus, the upregulation of genes of JAK-STAT pathway in response to salt stress in tubules is not surprising. As *Socs36E* (FC=6.7), *PTP61F* (FC=2.8) and *STAT92E* (FC=2.1) genes were seen to be upregulated in tubules of Rel^{E20} but not in wild type flies at time point chosen for performing microarray, suggests that the JAK-STAT pathway is activated on high salt feeding at earlier time point and that Relish or its targets may be required for possibly adjusting the cell volume leading to rapid attenuation of the pathway.

3.3.4 Adapting to salt stress

Cells use many redundant strategies to adapt to osmotic stress through accumulating osmolytes such as sugars and enhancing volume to regain intracellular homeostasis (Burg et al., 2007). Also osmotic stress has shown to cause DNA damage, a temporary cell cycle arrest, cytoskeletal rearrangement and chromatin condensation (Albiez et al., 2006; Dmitrieva and Burg, 2007). A modulation in lipid metabolism related genes and an upregulation of genes involved in chitin breakdown in salt stressed tubules of wild type and Rel^{E20}(Table 3-2) was observed. This alteration in metabolism may be a coping mechanism to adapt to salt stress by accumulating osmolytes and generating energy.

It was interesting to observe that certain class of genes associated with tissue development, mitosis and regulation of programme cell death were specifically

altered only in stressed wild type tubules not in Rel^{E20} (Table 3-5). Certain transporters like glucose membrane transporter such as *CG1213*, amino acid symporter *List* and putative aquaporin *CG5398* are specifically upregulated only in wild type tubules. In addition, a gene encoding for structural component of cytoskeleton (*Act87E*) and genes for histone proteins were upregulated in wild type tubules and not in Rel^{E20} (Table 3-2). This implies that these genes are being specifically controlled by Relish and may be important for adaption of tubule cells to osmotic stress caused by high salt diet. It will be useful to study the promoter region for these genes to see if there are Rel binding sites in them.

In salt stressed tubules of Rel^{E20}, genes encoding heat shock proteins are upregulated (Table 3S-1) and may be an osmoprotective response against salt stress. This osmoprotection may be through heat shock proteins' ability to inhibit apoptotic pathways (Takayama et al., 2003) and contribute to protein homeostasis (Takeuchi et al., 2015). A down regulation of the plasma membrane *Ca^{2+} ATPase* (*PMCA*), *Ca^{2+} /Na^{2+} exchanger* (*Calx*) and Inward rectifying potassium channels (*Ir* and *Irk3*) was observed. Chloride channels *Clc-a* and *Best2* and the volume sensitive chloride channel, *Best1,* were also down- regulated (Table 3S-2). It is intriguing that these ion transporter and water channel activity genes are being down regulated. It may be postulated that absence of Relish and its target genes in response to salt stress causes loss of transcriptional regulation of these genes. Another explanation may be that down regulation of these genes was a potential mechanism to combat osmotic stress but needs further investigation by assessing gene expression over a time course of salt stress.

All four of the seven AQP/MIP (*CG4019*, *CG17664*, *CG7777* and *DRIP*) genes known to be abundantly expressed in tubules are down- regulated (Table 3S-2). It has been observed that stellate cell specific knockdown of either *CG7777* or *Drip* showed increased basal rate of fluid secretion compared to their parental controls (Cabero et al. unpublished). It was observed that if *CG7777* expression was decreased in stellate cells, expression of *Drip* was increased and vice versa (Cabrero et al. unpublished). Hence it may be that simultaneous down regulation of *Drip* and *CG7777* may be occurring in salt stress Rel^{E20} as a mechanism to regulate ion balance within the tubules by increasing fluid secretion rate. The

preliminary data shows that Relish protein is expressed only in tubule principal cells (Figure 3S-2). Thus the effect on the stellate cell specific aquaporins may be an in direct effect exerted by Relish in response to salt stress.

Recent studies in the lab have revealed principal cell specific *CG17664* and *CG1409* water channels encode aquaglyceroporins. If these channels were facilitating the movement of glycerol outside the cells then it may suggest that the down regulation of these genes was to decrease the movement of glycerol outside the cells leading to accumulation of glycerol inside the cells as an osmoprotectant.

Another explanation to the down regulation of aquaporins in Rel^{E20} can be understood from a recent study in *Aedes aegpypti* that showed all the aquaporins in tubules being down- regulated 24 h post blood meal (Drake et al., 2015). As the female mosquito can lose up to 75% of blood meal water in the first three hours of feeding, this down regulation of aquaporins at 24 h time point was attributed to prevent excessive water loss (Drake et al., 2015). It may be speculated that a similar mechanism may be occurring in salt stress tubules of *Drosophila* to prevent excessive loss of fluid and this regulation of aquaporins may be controlled by Relish as in Rel^{E20} a hyper repression of the genes is seen. Thus to delineate if Relish controlled the regulation of aquaporins' expression under salt stress it is necessary to check their expression in salt stress Relish mutants and wild type within the first few hours on salt feeding.

Some neuropeptides are involved in fluid secretion. *Diuretic Hormone* (*Dh* also known as *Dh44)* (FC 7.4) and *Ion transport peptide* (*ITP)* (FC 3.9) are upregulated in salt stressed Rel^{E20}. *Dh* hormone is known to increase fluid secretion in tubule principal cells through cAMP signalling (Cabrero et al., 2002). In locust, *ITP* was shown to have an anti-diuretic effect by increasing fluid absorption of chloride, sodium and potassium ions transduced by cAMP messengers (Phillips et al., 1998). Tubules have a small subset of cells displaying neuronal markers which may be regarded as neuroendocrine cells (Sozen et al., 1997). Thus neuropeptides *Dh* and *ITP* may be produced in these cells in salt stress Rel^{E20} tubules suggesting a localised rescue compensatory mechanism to regain water-

ion homeostasis. As Naikkhwah and O'Donnell , predicted that other tissues may be playing a role in removing excess Na^+ and Cl^- ions (Naikkhwah and O'Donnell, 2011), we hypothesised that due to loss of Relish in Rel^{E20} , production of these neuropeptides take places in tubules to exert its affect such that *Dh* may be acting on tubules to increase fluid secretion while *ITP* may be acting on hindgut to reabsorb excess fluid.

The results of this chapter demonstrates that Relish in tubules plays an important role in salt tolerance by regulating a range of genes that play diverse role in adaptation and tolerance to high salt feeding caused osmotic stress. Additionally, it was observed that in absence of Relish and under salt stress, certain stress pathways in tubules were being hyper-activated and alteration of specific genes was taking place perhaps as a compensatory mechanism for loss of Relish and to help the organism to tolerate salt stress.

The Figure 3-24 summarizes a mode of activation of Relish and other stress pathways triggered in tubules of wild type in the first 24 h of salt feeding and is proposed based on the transcriptomic study performed in tubules of wild type and Rel^{E20} after 24 h of salt feeding and data mining.

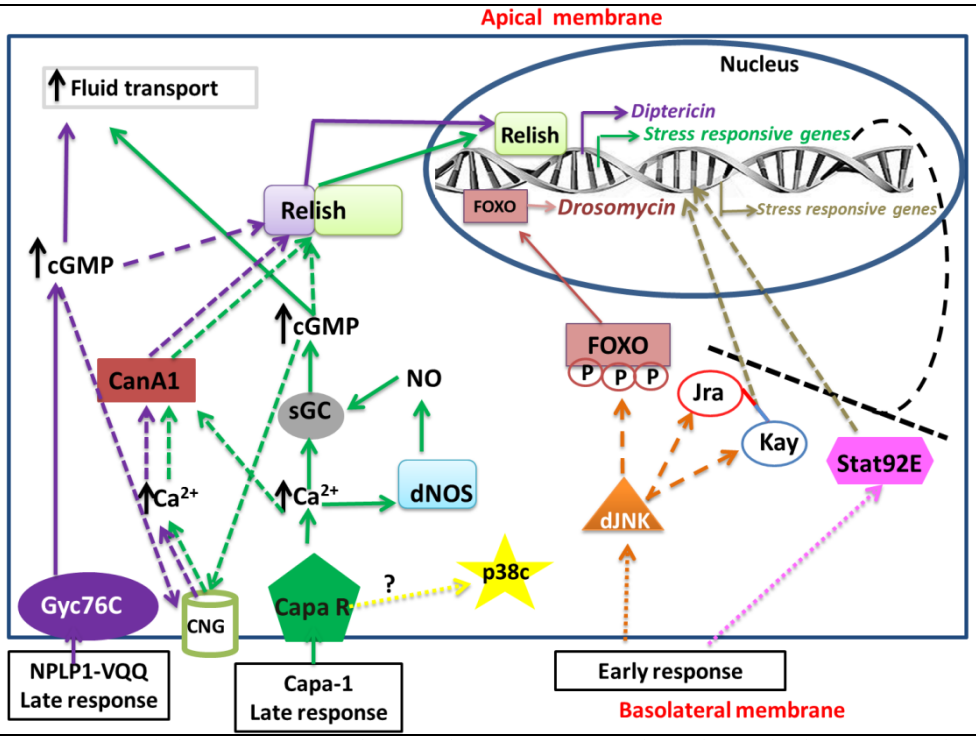


Figure 3-24: A model on stress pathways being triggered in the tubule principal cell of wild type flies in the first 24 h of salt feeding. The JNK pathway is triggered as an early response which activates FOXO. On activation, FOXO enters the nucleus, increasing expression of *Drosomycin*. The activation of JNK pathway activates Jra and Kay which make the dAP-1 complex. The JAK-STAT pathway is activated in response to osmotic stress, causing activation of Stat92E. The activation of dAP-1 and Stat92E lead to transcription of stress responsive genes. The trigger for activation of JNK and JAK-STAT pathways in response to salt stress is unknown but assumed to be from basolateral membrane. As the stress continues, neuropeptides released in haemolymph in response to salt stress trigger their respective receptors at the basolateral membrane. The role of Gyc76C and NPLP1-VQQ in response to salt stress, increase in *Diptericin* expression and Relish activation is established (Overend et al., 2012). Whether the activation of Relish by this receptor is through an increase in either Ca^{2+} from cyclic nucleotide gated channels (CNG) or cGMP (or both) is not known. The microarray data suggests that Ca^{2+}-dependent phosphatase CanA1 may play a role in activation of Relish leading to increase in *Diptericin*. In addition, activation of capa receptor (CapaR) by capa-1 increases intracellular Ca^{2+} from internal Ca^{2+} stores and CNG causing increases in cGMP through NO pathway (Kean et al., 2002). Capa-1 is shown to activate Relish but not induce the expression of *Diptericin* (Terhzaz et al., 2014). Under salt stress, activation of Relish by capa-1 may be via CanA1 or cGMP leading to expression of other stress responsive genes. Capa-1 may also trigger p38/MAPK pathway as suggested by Terhzaz et. al, 2014. Capa-1 and NPLP1-VQQ may be increasing the fluid secretion additively or synergistically to regain water-ion homeostasis during salt stress. As Relish target genes can inhibit JNK pathway (Park et al., 2004), and AP-1 complex along with STAT92E can inhibit Relish activated genes (Kim et al., 2007), there may be a cross talk occurring between Relish and JNK pathway to maintain homeostasis during salt stress. The dotted lines depict the predicted pathways (coloured denotes activated pathways and black denotes inhibited pathways) and solid lines depict the pathways established in literature for tubule principal cells.

3.4 Supplementary Data

Figure 3S-1: Survival of Relish mutants on starvation assay.

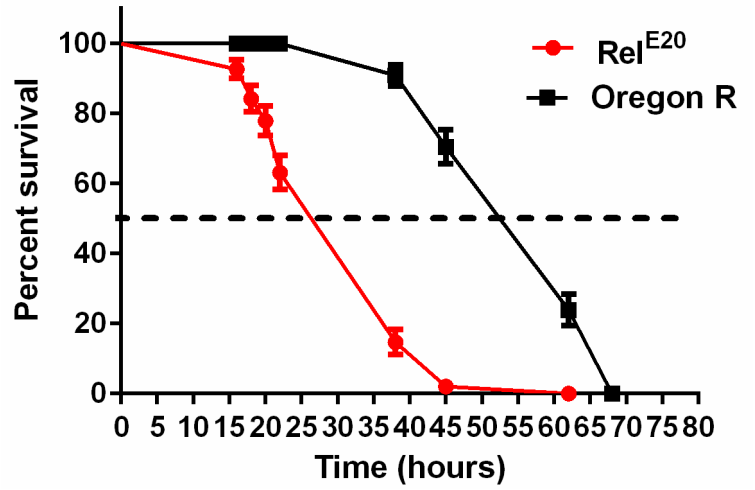


Figure 3-S1: Survival of Relish mutants on starvation assay. Survival of Relish null mutants (Rel^{E20}) flies in Oregon R background compared against Oregon R wild type flies on starvation. The flies were placed onto vial containing only agar. Data are expressed as percent survival ± SEM, ($p<0.001$ against Oregon R flies; Log rank test, Mantel-Cox).

Figure 3-S2: Expression of Relish in tubule cells.

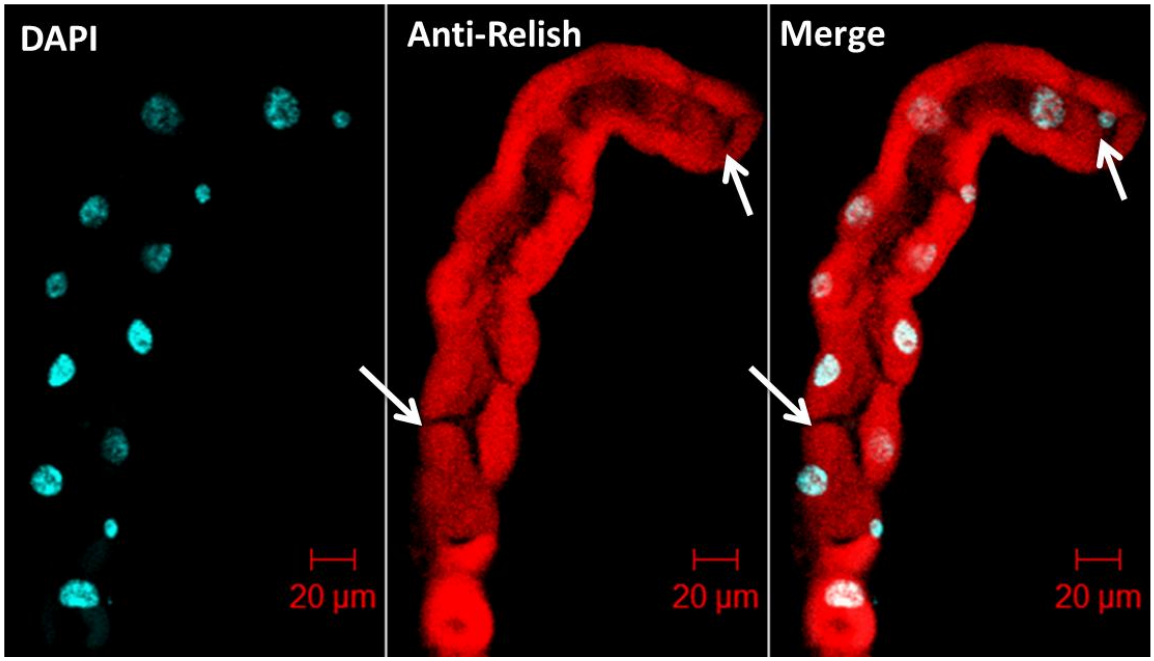


Figure 3-S2: Expression of Relish in tubule cells. Tubules were isolated from 7-9 day old wild type flies. Intact tubules were probed with Relish antibody that was specific to C-terminal region of Relish protein. The Anti-Relish antibody (Anti-Relish 21CF3) recognised full length inactive protein and the cytoplasmic C-terminus fragment of the active protein (Stoven et al., 2000). Nuclei are labelled with DAPI (blue). Relish protein was detected in cytoplasm using Relish antibody with a secondary antibody conjugated with Alexa Fluor (red). Nuclei stained with DAPI. The protein was detected only in the principal cells. The arrows point to the stellate cells that did not express any protein. The stellate cells were recognised based on their star shape and small nuclei.(Work done in association with Dr. S. Terhzaz).

Table 3-S1: The upregulated genes in salt stress Rel^{E20} tubules classified under GO term "Response to stress".

Response to nutrient levels		Response to abiotic stimulus		Defense response		Regulation of response to stress	
Flybase ID	**Gene Symbol**	**Flybase ID**	**Gene Symbol**	**Flybase ID**	**Gene Symbol**	**Flybase ID**	**Gene Symbol**
FBgn0004852	Ac76E	FBgn0031322	CG5001	FBgn0082598	akirin	FBgn0082598	akirin
FBgn0033926	Arc1	FBgn0037151	CG7130	FBgn0035850	Atg18a	FBgn0035850	Atg18a
FBgn0260945	Atg1	FBgn0043002	Chrac-14	FBgn0012042	AttA	FBgn0010015	CanA1
FBgn0037363	Atg17	FBgn0044323	Cka	FBgn0041581	AttB	FBgn0010341	Cdc42
FBgn0035850	Atg18a	FBgn0263864	Dark	FBgn0031114	cactin	FBgn0000308	chic
FBgn0032935	Atg18b	FBgn0033188	Drat	FBgn0010015	CanA1	FBgn0044323	Cka
FBgn0036746	Crtc	FBgn0264490	Eip93F	FBgn0010341	Cdc42	FBgn0260632	dl
FBgn0023094	cyc	FBgn0020497	emb	FBgn0033301	CG12780	FBgn0260866	dnr1
FBgn0263864	Dark	FBgn0011205	fbl	FBgn0030320	CG2247	FBgn0020497	emb
FBgn0034915	eIF6	FBgn0037724	Fst	FBgn0263864	Dark	FBgn0038197	foxo
FBgn0038197	foxo	FBgn0034335	GstE1	FBgn0260632	dl	FBgn0004507	GlyP
FBgn0001226	Hsp27	FBgn0001168	h	FBgn0260866	dnr1	FBgn0019686	lok
FBgn0001230	Hsp68	FBgn0026575	hang	FBgn0026404	Dronc	FBgn0031885	Mnn1
FBgn0013984	InR	FBgn0264562	Hr4	FBgn0283461	Drs	FBgn0037705	mura
FBgn0020278	loco	FBgn0001224	Hsp23	FBgn0005660	Ets21C	FBgn0028411	Nxt1
FBgn0263593	Lpin	FBgn0001225	Hsp26	FBgn0004507	GlyP	FBgn0039044	p53
FBgn0034219	mthl4	FBgn0001226	Hsp27	FBgn0001226	Hsp27	FBgn0003053	peb
FBgn0262656	Myc	FBgn0001230	Hsp68	FBgn0020278	loco	FBgn0030310	PGRP-SA
FBgn0028411	Nxt1	FBgn0013275	Hsp70Aa	FBgn0264975	Nrg	FBgn0043575	PGRP-SC2
FBgn0039044	p53	FBgn0013276	Hsp70Ab	FBgn0028411	Nxt1	FBgn0020621	Pkn
FBgn0015279	Pi3K92E	FBgn0013277	Hsp70Ba	FBgn0030310	PGRP-SA	FBgn0015286	Rala
FBgn0014018	Rel	FBgn0013278	Hsp70Bb	FBgn0043575	PGRP-SC2	FBgn0003209	raw
FBgn0036187	RIOK1	FBgn0051354	Hsp70Bbb	FBgn0015791	Rab14	FBgn0014018	Rel
FBgn0264357	SNF4Agamma	FBgn0013279	Hsp70Bc	FBgn0015286	Rala	FBgn0014020	Rho1
FBgn0086676	spin	FBgn0013984	InR	FBgn0014018	Rel	FBgn0263289	scrib
FBgn0027605	Vps4	FBgn0027338	Kap-alpha3	FBgn0014020	Rho1	FBgn0015541	sda
		FBgn0020278	loco	FBgn0263289	scrib	FBgn0003371	sgg
		FBgn0004512	Mdr49	FBgn0015296	Shc	FBgn0030018	slpr
		FBgn0031885	Mnn1	FBgn0031973	Spn28Dc	FBgn0264357	SNF4Agamma
		FBgn0052296	Mrtf	FBgn0028988	Spn42Dd	FBgn0031973	Spn28Dc
		FBgn0002887	mus201	FBgn0016917	Stat92E	FBgn0036389	ssp2
		FBgn0039044	p53	FBgn0086358	Tab2	FBgn0016917	Stat92E
		FBgn0003301	rut	FBgn0041180	Tep4	FBgn0086358	Tab2
		FBgn0015541	sda	FBgn0022355	Tsf1	FBgn0003862	trx
		FBgn0052423	shep	FBgn0010812	unc-45	FBgn0010812	unc-45
		FBgn0030018	slpr				
		FBgn0016917	Stat92E				
		FBgn0010812	unc-45				

Table 3-S2: The down- regulated genes in salt stress Rel^{E20} tubules classified under GO term "Transporters".

Transporters		Transmembrane transporters	
Flybase ID	Gene Symbol	Flybase ID	Gene Symbol
FBgn0052446	Atox1	FBgn0016119	ATPsynCF6
FBgn0002921	Atpalpha	FBgn0016120	ATPsynD
FBgn0037218	aux	FBgn0038387	blp
FBgn0040238	Best1	FBgn0011211	blw
FBgn0035696	Best2	FBgn0036199	Bmcp
FBgn0260857	Bet1	FBgn0013995	Calx
FBgn0260859	Bet3	FBgn0002022	Catsup
FBgn0260860	Bet5	FBgn0039296	CG10420
FBgn0086694	Bre1	FBgn0039644	CG11897
FBgn0000212	brm	FBgn0033196	CG1358
FBgn0032790	CG10194	FBgn0035173	CG13907
FBgn0039107	CG10300	FBgn0039641	CG14511
FBgn0034081	CG10731	FBgn0037485	CG14606
FBgn0034535	CG11110	FBgn0038262	CG14857
FBgn0039927	CG11155	FBgn0028886	CG15279
FBgn0039840	CG11340	FBgn0031517	CG15406
FBgn0033558	CG12344	FBgn0039844	CG1607
FBgn0037845	CG14694	FBgn0033443	CG1698
FBgn0033316	CG14749	FBgn0034883	CG17664
FBgn0032449	CG17036	FBgn0038717	CG17751
FBgn0033905	CG18324	FBgn0051229	CG31229
FBgn0033904	CG18327	FBgn0051792	CG31792
FBgn0053181	CG33181	FBgn0052669	CG32669
FBgn0259221	CG42321	FBgn0053281	CG33281
FBgn0038049	CG5844	FBgn0053282	CG33282
FBgn0263398	CG6364	FBgn0040350	CG3690
FBgn0037846	CG6574	FBgn0034885	CG4019
FBgn0039687	CG7593	FBgn0250757	CG42235
FBgn0039741	CG7943	FBgn0038752	CG4462
FBgn0036920	CG8004	FBgn0029932	CG4607
FBgn0038135	CG8773	FBgn0034275	CG5002
FBgn0030669	CG9240	FBgn0039207	CG5789
FBgn0037714	CG9396	FBgn0039223	CG5805
FBgn0031813	CG9527	FBgn0038407	CG6126
FBgn0028382	cyp33	FBgn0036843	CG6812
FBgn0029131	Debcl	FBgn0038938	CG7084
FBgn0026479	Drp1	FBgn0038716	CG7342
FBgn0000618	e(y)2	FBgn0037140	CG7442
FBgn0026439	Eaat1	FBgn0033635	CG7777
FBgn0033483	egr	FBgn0039696	CG7837
FBgn0037913	fabp	FBgn0033048	CG7881
FBgn0024689	fws	FBgn0034063	CG8389
FBgn0037881	GCC88	FBgn0034479	CG8654
FBgn0022160	Gpo-1	FBgn0031520	CG8837

cont. Table 3-S2: The down- regulated genes in salt stress Rel^{E20} tubules classified under GO term "Transporters".

Transporters		Transmembrane transporters	
Flybase ID	**Gene Symbol**	**Flybase ID**	**Gene Symbol**
FBgn0032250	holn1	FBgn0029950	CG9657
FBgn0265042	Irk1	FBgn0039787	CG9702
FBgn0032706	Irk3	FBgn0051116	ClC-a
FBgn0037374	jagn	FBgn0027610	Dic1
FBgn0002567	ltd	FBgn0015872	Drip
FBgn0026207	mbo	FBgn0263916	Ent2
FBgn0035473	mge	FBgn0039487	gb
FBgn0031640	Mon1	FBgn0038376	Hmt-1
FBgn0002939	ninaD	FBgn0001296	kar
FBgn0053554	Nipped-A	FBgn0004513	Mdr65
FBgn0031381	Npc2a	FBgn0058263	MFS17
FBgn0034118	Nup62	FBgn0025684	MFS18
FBgn0050277	Oatp58Da	FBgn0002778	mnd
FBgn0034715	Oatp58Db	FBgn0265351	nac
FBgn0036732	Oatp74D	FBgn0019952	Orct
FBgn0041585	olf186-F	FBgn0036007	path
FBgn0036005	pall	FBgn0259214	PMCA
FBgn0031282	Pex12	FBgn0036770	Prestin
FBgn0033380	Phax	FBgn0031049	Sec61gamma
FBgn0003118	pnt	FBgn0003360	sesB
FBgn0050181	ppk3	FBgn0031998	SLC5A11
FBgn0030057	Ppt1	FBgn0016041	Tom40
FBgn0038519	Prx3	FBgn0260005	wtrw
FBgn0016701	Rab4	FBgn0033665	Zip48C
FBgn0053180	Ranbp16	FBgn0038312	Zip88E
FBgn0087002	Rfabg	FBgn0028516	ZnT35C
FBgn0028699	Rh50		
FBgn0014877	Roe1		
FBgn0011232	scat		
FBgn0015808	ScpX		
FBgn0017482	T3dh		
FBgn0260744	Tango9		
FBgn0021795	Tapdelta		
FBgn0035110	thoc7		
FBgn0014396	tim		
FBgn0027359	Tim8		
FBgn0027358	Tim9b		
FBgn0032397	Tom70		
FBgn0266723	Trs31		
FBgn0266722	Trs33		
FBgn0036666	TSG101		
FBgn0030872	Ucp4A		
FBgn0041174	Vhl		
FBgn0003996	w		
FBgn0033692	wash		
FBgn0004643	Zw10		

Chapter 4

Relish regulated genes in unstressed tubules

Chapter 4

4.1 Introduction

NF-κB is expressed in all mammalian cell type and tissues. It is a transcription factor that responds to number of diverse external stimuli. It also plays an important role in multiple physiological and pathological processes (as reviewed in (Vallabhapurapu and Karin, 2009)). Besides its known role in immunity, NF-κB regulates a range of genes that are involved in cell survival, differentiation, proliferation and energy homeostasis (Guttridge et al., 1999; Mauro et al., 2011)(reviewed(Hayden and Ghosh, 2012). Genome wide microarray studies of cancer cells and cells derived from mice deficient in NF-kB proteins or expressing NF-kB repressors, have identified more than 500 genes that are regulated by NF-kB in a cell type- and stimulus specific manner (Cardozo et al., 2001; Li et al., 2001; Tian et al., 2005). In addition, chromatin immunoprecipitation (ChIP) studies determined a large number of genes with NF-κB binding sites in promoter/enhancer regions (Martone et al., 2003; Oeckinghaus and Ghosh, 2009).

In *Drosophila*, studies on Relish (a *Drosophila* NF-κB-like protein) have been focussed on its role during immune stress (Brown et al., 2009; De Gregorio et al., 2002; Delaney et al., 2006; Hedengren et al., 1999; Silverman et al., 2003), in maintenance of gut epithelium associated with commensal microbiota (Broderick et al., 2014; Guo et al., 2014), and hypoxia (Bandarra et al., 2014; Dijkers and O'Farrell, 2009). A recent study in Activating Transcription Factor 3 (ATF3) mutants has indicated a role for Relish in lipid metabolism (Rynes et al., 2012). In addition, localised DNA damage causes Relish in fat body to be activated by FOXO in response to suppressed insulin/IGF signalling to regain growth homeostasis (Karpac et al., 2011). In tubules, Relish is activated by neuropeptides capa-1 (through CapaR receptor) and NPLP1-VQQ (through Gyc76C receptor), cyclic guanosine monophosphate (cGMP) (Davies et al., 2014; Overend et al., 2012; Terhzaz et al., 2014). Additionally, Relish in tubule principal cell is required for tolerating salt stress and desiccation stress (Terhzaz et al., 2014). Overall this demonstrates that Relish responds to stimuli other than immune stress for regulating specific set of genes for optimal working of an organism especially during stress.

A study by Wagner et al. (2008) showed that the Drosophila airway epithelial cells that acted as first line of defence against airbone pathogens, highly expressed Relish as well as basal Diptericin levels. The tubules are autonomous immune-sensing tissue (McGettigan et al., 2005). A preliminary immunohistochemistry study showed that Relish protein in the unstressed tubules was localised in the principal cells of the tubules (Figure 3-S2). In the immunohistochemistry study, a weak signal of Diptericin protein was detected in the unstressed tubules compared to the stressed tubules (Figure 3-14). When levels of Gyc76C (a receptor for NPLP1-VQQ) is suppressed, basal levels of *Diptericin* decrease in the tubules (Overend et al., 2012). Taken together, this implies that in the unstressed tubules a basal Relish activity is occurring. This *in vivo* activity may be regulated by a signal generated in an autocrine fashion within the tubule cells or relayed by a signal generated in another tissue or may be under a circadian rhythm as seen for Relish- mediated transcriptional activity in the nervous system ((Kuo et al., 2010; Tanenhaus et al., 2012)).
In this chapter, using Relish mutant (Rel^{E20}) flies, a study was carried out to identify genes in the tubules that are regulated by basal Relish activity.

4.2 Results

4.2.1 Relish affects basal fluid secretion rate in tubules

The main segment of the tubules secretes fluid (primary urine) (O'Donnell and Maddrell, 1995). This segment is composed of ~77 principal cells per tubule (Figure 1-2). Moreover, according to the *in silico* analysis carried out on the microarray data of the salt stressed tubules (discussed in the previous chapter), 4 of 6 upregulated genes encoding for the transmembrane transporters in the tubules of the wild type flies were “Relish Dependent” (Table 3-2). In the preliminary immunohistochemistry study Relish was observed to be localised in the principal tubule cells (Figure 3-S2), this led to assessing the basal fluid secretion rate of Relish mutant tubules under unstressed conditions.

Figure 4-1, shows results of secretion assay using tubules isolated from wild type and Rel^{E20} flies. The basal fluid secretion rate for wild type tubules was 0.63±0.05 nl/min (Figure 4-1), whereas, the rate for tubules isolated from Rel^{E20}

was 0.48±0.03 nl/min. This small yet significant difference in basal fluid secretion rate, illustrates that Relish and its target genes play a key role in regulating fluid secretion functionality of tubules.

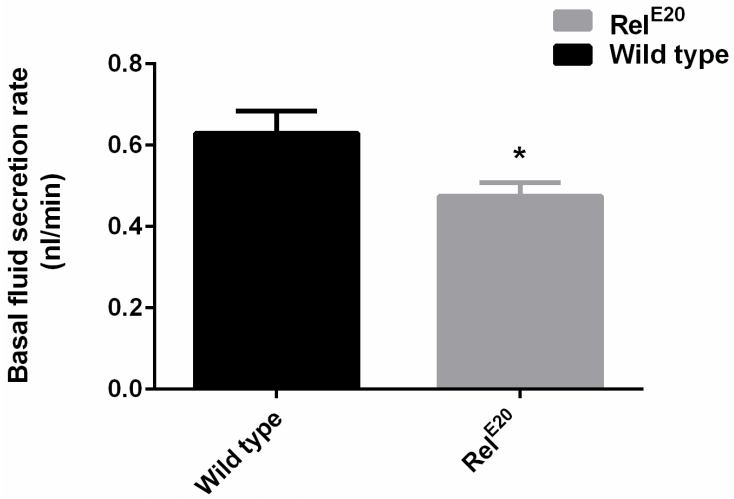


Figure 4-1: Impact of Relish on fluid secretion rate. The basal fluid secretion rate of Rel_{E20} and wild type. Tubules were dissected from 7-9 day old flies. Data are expressed as fluid secretion rate (nanolitres/min) ± S.E.M., N=26 tubules. The basal fluid secretion rate of tubules from wild type was 0.62 nl/min and Rel^{E20} was 0.47 nl/min. Data was assessed for statistical significance using Student's t test, * denotes p-value<0.001.

4.2.2 Differentially expressed genes in the tubules of Relish mutant

In chapter 3, it was observed that during salt stress a large number of genes were differentially altered in the tubules of Rel^{E20} flies. Some genes were hyper-activated or -repressed in salt stressed tubules of Rel^{E20} compared to its expression seen in the tubules of salt stressed wild type flies. Also, the PCA analysis of the gene clusters of control fed (unstressed) tubules of wild type and Rel^{E20} flies showed a 13.1% variance on PC2 on principal component axis (in Figure 3-17). This led to identifying the genes that are regulated by Relish in tubules under unstressed conditions. Figure 4-2 shows the probe set counts based on fold change with *p-value* ≤ *0.05* corrected for multiple test between Rel^{E20} and wild type tubules.

For further analysis only genes whose fold change was ≥2, or ≤-2 were considered. In Rel^{E20} tubules 254 genes (233 probe sets) were upregulated and 194 genes (186 probe sets) were down- regulated compared to expression of these genes in tubules from wild type.

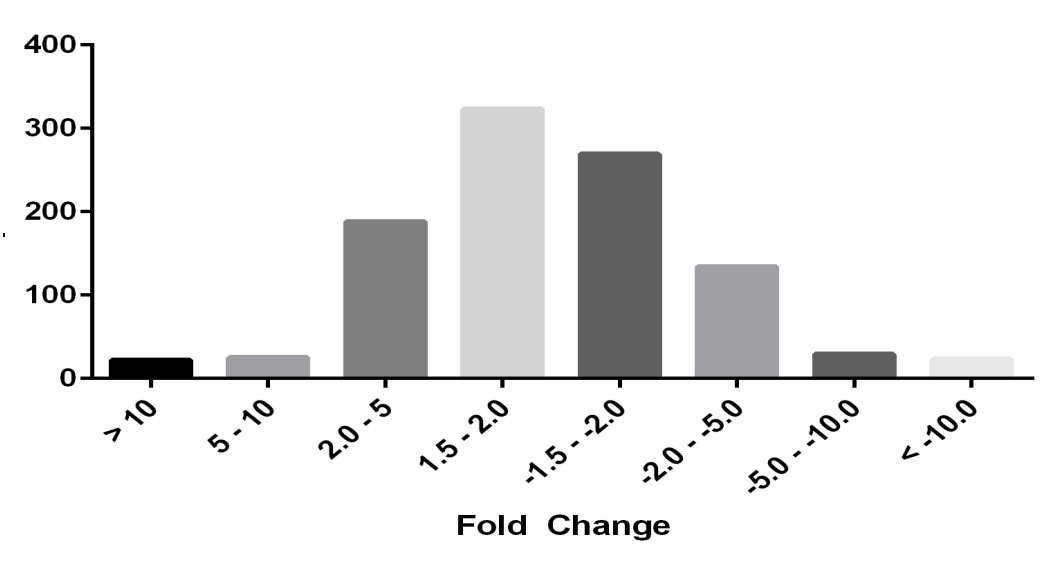


Figure 4-2: Number of probe sets significantly altered in Rel^{E20}. The probe set counts were based on fold change between Rel^{E20} and wild type tubules. Data were statistically validated using two-way ANOVA test with Benjamini-Hochberg multiple test correction. The genes that had the corrected *p-value* ≤0.05 were considered significant.

A set of genes that are abundantly expressed in tubules according to Flyatlas data and their expression significantly altered in the microarray data were selected for qRT-PCR to validate their fold change (FC) values observed in the microarray (Figure 4-3). The fold change trend observed in the microarray and qRT-PCR for all tested genes was similar.

In Figure 4-3, five genes (*sug*, *CG12290*, *Ndae1*, *NPFR1* and *Cyp4e1*) in Rel^{E20} tubules were upregulated in tubules from Rel^{E20}, indicating that in the absence of Relish, the expression of these five genes are induced. Thus, Relish may be involved in suppressing their expression under unstressed condition. *Cyp4e3*, *CG6293* and *CG7968* were down- regulated in Rel^{E20} microarray data set compared to wild type. As the qRT-PCR data confirms that these three genes are down- regulated in tubules of Rel^{E20} (Figure 4-3), it indicates that in the absence of Relish the expression of these genes is repressed. Thus, these genes need Relish for their appropriate basal expression.

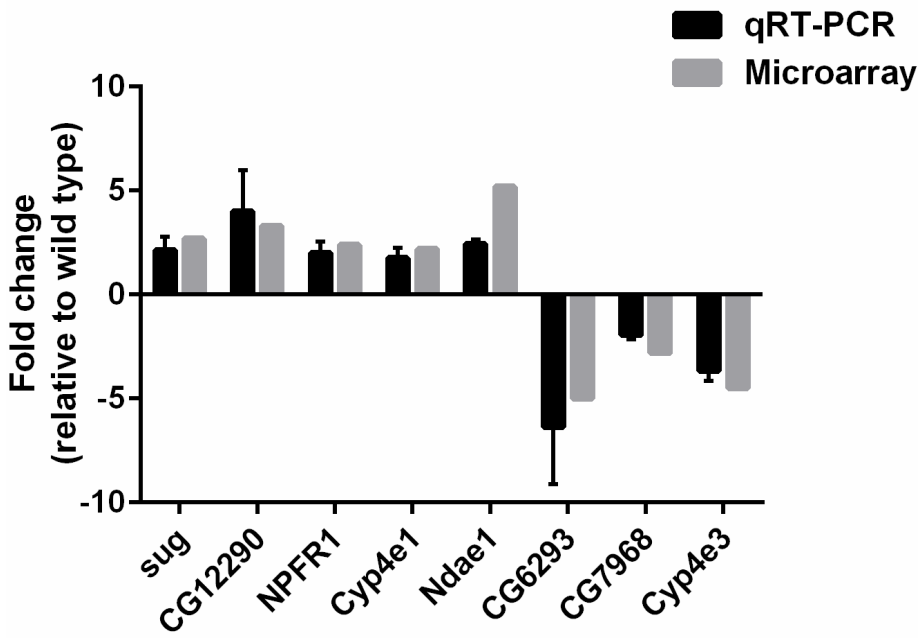


Figure 4-3: Validation of microarray result by qRT-PCR. Tubules from 7-9 day old Rel^{E20} and wild type flies fed on normal (control) food were dissected. Gene specific primers were used in the qRT-PCR reaction for quantifying mRNA transcripts. The qRT-PCR result (in black) is of 2 independent biological replicates. The microarray data (in grey) is result from 4 biological replicates.

4.2.3 GO analysis of Relish regulated genes

The differentially expressed genes were analysed for gene ontology (GO) enrichment in biological processes with *p-value* <0.05. The top 10 GO enrichment terms that covered the most number of differentially expressed genes and had a *p-value* ≤ 0.05 are enlisted in Table 4–1.

As shown in the Figure 4-4, 20% of upregulated and 27% of down- regulated genes were categorised under metabolic process. Most consisted of genes whose products are enzymes. The mRNA expression of detoxification enzymes belonging to certain families of glutathione-S-transferases (GSTs) and cytochrome P450 (CYPs) were altered in absence of Relish protein in tubules (Table 4-2). Tubules play a crucial role in detoxification and express large amount of detoxifying enzymes such as GSTs and CYPs (Wang et al., 2004; Yang et al., 2007). As many types of GSTs and CYPs have been associated with insecticide resistance in many insect species (Amichot et al., 1998; Gong et al., 2013; Kostaropoulos et al., 2001; Le Goff et al., 2003; Liang et al., 2015; Ortelli

et al., 2003), it is interesting that their mRNA expression in tubules is altered by loss of Relish.

Table 4-1 : Top 10 GO terms enriched for up- and down- regulated gene list of Rel^{E20} vs wild type.

Gene Ontology(GO) term		p-value
Up regulated		
GO:0044699	single-organism process	0.002
GO:0044710	single-organism metabolic process	0.004
GO:0050794	regulation of cellular process	0.008
GO:0050789	regulation of biological process	0.014
GO:0065007	biological regulation	0.022
GO:0023052	signaling	0.032
GO:0044700	single organism signaling	0.032
GO:0006810	transport	0.035
GO:0051234	establishment of localization	0.040
GO:0007154	cell communication	0.042
Down regulated		
GO:0055114	oxidation-reduction process	5.30E-08
GO:0044710	single-organism metabolic process	6.60E-08
GO:0044281	small molecule metabolic process	0.0002
GO:0009056	catabolic process	0.0014
GO:0044699	single-organism process	0.005
GO:0006810	transport	0.012
GO:0051234	establishment of localization	0.021
GO:0006950	response to stress	0.0230
GO:0044765	single-organism transport	0.024
GO:0044763	single-organism cellular process	0.031

Table 4-1: Top 10 GO terms enriched for up- and down- regulated gene list of Rel^{E20} vs wild type. The top 10 GO term covering most number of genes and significantly enriched in up- and down- regulated gene list of Rel^{E20} vs wild type. *p-value* ≤ 0.05 was considered. A substantial number of genes were found in more than one GO term.

In insects, CYPs are linked to insecticide resistance (Amichot et al., 1998; Daborn et al., 2001; Gong et al., 2013; Le Goff et al., 2003), and in humans, they play a role in drug breakdown and resistance (reviewed (Rodriguez-Antona and Ingelman-Sundberg, 2006)). In humans, NF-κB regulates expression and activity of various CYPs (reviewed (Zordoky and El-Kadi, 2009)). Thus, it is not surprising to observe that on loss of Relish in tubules, the mRNA expression of CYPs is altered. Interestingly genes of the same family of CYPS were observed to be differentially expressed in Rel^{E20}. *Cyp4e1* was upregulated but *Cyp4e3* was down- regulated (Figure 4-3). Similarly, *Cyp6a13* was induced whereas *Cyp6a20* and *Cyp6a8* were repressed in Rel^{E20} (Table 4-2).

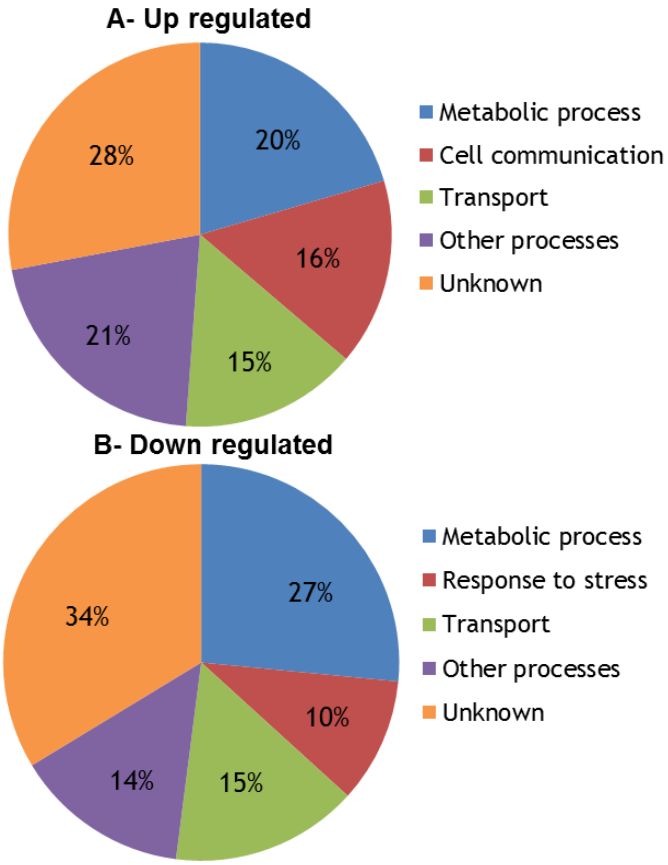


Figure 4-4: Biological process classification of genes differentially expressed in Rel^{E20} vs wild type. The percentage of differentially expressed genes classified into five broad biological process categories and unknown for **(A)** upregulated genes and **(B)** down-regulated genes in tubules of Rel^{E20} compared to wild type under unstressed conditions.

GSTs are a diverse family of enzymes found in all animals and are the most commonly and efficiently used detoxification system (Enayati et al., 2005). In insects, their expression is most commonly induced during xenobiotic stress (reviewed (Enayati et al., 2005)). In unstressed tubules of Rel^{E20}, five GSTs (*GstE5*, *GstD2*, *GstZ2*, *GstD9* and *GstE1*) were upregulated and only 1 GST (*GstD5*) was+ down- regulated (Table 4-2). A recent study by of Zhao et al (2011) demonstrated that flies overexpressing *Diptericin* (a Relish target gene) had an increased GST activity and were more tolerant to oxidative stress compared to the parental flies (Zhao et al., 2011).

Table 4-2: *GST*s and *CYP*s expression in tubules affected by loss of Relish

		Fold change		Flyatlas data	
		Unstressed Rel/WT	Diptericin overexpression	mRNA signal Tubule	Enrichment (Tubule vs WF)
Glutathione S transferase (GST)					
FBgn0063495	GstE5	9.6	-1.6	319.6	7.2
FBgn0010038	GstD2	5.1	-	39	1
FBgn0037697	GstZ2 (CG9363)	4.1	-	12	0.8
FBgn0038020	GstD9	3.1	-	99.7	0.8
FBgn0034335	GstE1	2.8	1.9	579.4	2.6
FBgn0010041	GstD5	-4.6	-	35.9	3.3
Cytochrome P450 (CYP)					
FBgn0015038	Cyp9b1	4.8	-	11.9	0.3
FBgn0033304	Cyp6a13	3.5	-	406.1	4.3
FBgn0023541	Cyp4d14	3.1	-	74.1	2
FBgn0033775	Cyp9h1	2.9	-	11.9	0.6
FBgn0053503	Cyp12d1-d	2.7	-	67.8	8.5
FBgn0050489	Cyp12d1-p	2.7	-	947.7	15.4
FBgn0036806	Cyp12c1	2.6	-1.5	293.7	4.1
FBgn0015034	Cyp4e1	2.2	-1.5	326.2	4.2
FBgn0033980	Cyp6a20	-9.5	-2.5	433.5	2.7
FBgn0030615	Cyp4s3	-6.5	-	100.1	3.5
FBgn0015035	Cyp4e3	-4.5	-2.6	825.4	10.2
FBgn0035790	Cyp316a1	-3.9	-	6	3.3
FBgn0038194	Cyp6d5	-3.3	-	3147.6	3.1
FBgn0013772	Cyp6a8	-2.2	-1.5	3140.8	28.5

Table 4-2: *GST*s and *CYP*s expression in tubules affected by loss of Relish. List of the genes encoding GST and CYP whose expression is changed in the Relish mutant. The first column denotes the fold change of the gene expression in tubules of unstressed Relish mutant (Rel) compared to wild type (WT). The second column denotes fold change value from microarray data of whole fly overexpressing *Diptericin* from Zhao et al., 2011. Note the original fold change ratio values of the data are converted into whole numbers. The third and fourth columns are the Flyatlas data of tubule mRNA signal in wild type tubules and enrichment in wild type tubules compared to the whole fly. Red- denotes upregulated, Blue- denotes down regulated, '-' denotes no change.

Similar to the *in silico* study carried out in Chapter 3 section 3.2.9, a comparison of the microarray data of Zhao et al (2011) was done with the tubule microarray data of unstressed Rel^{E20} vs wild type flies. An upregulation in expression of genes: *GstE5, Cyp12c1* and *Cyp4e1,* is observed in unstressed tubules of Rel^{E20} while their expression was repressed in the flies overexpressing *Diptericin* (Table 4-2). The suppression in expression of genes *Cyp6a20, Cyp4e3, Cyp6a8* and *GstE1* was seen in both the compared datasets (Table 4-2). The other genes detected in both the datasets are enlisted in Table 4S-1.

In comparing the whole fly microarray data of Zhao et al (2011) and the tubule microarray data here, it is necessary to consider that variability may arise due to difference in tissues used for generating both the data sets. However, this result

does suggest that there exists an association between Relish and expression of these genes.

28% and 34% of genes in up- and down- regulated gene lists were annotated as unknown genes that did not have any annotated GO for biological process (Figure 4-4).16% of genes in upregulated genes were cell communication related genes e.g. neuropeptide receptor *NPFR1,* but this GO category was not found in the down- regulated genes. In the down- regulated gene list, 10% were categorised as response to stress genes e.g. *Immune regulated catalase (IRC)*, and yet none of the upregulated genes were in this category. This specific categorisation of genes whose expression is affected in tubules on loss of Relish protein indicates importance of Relish for tight regulation of genes required for maintaining tissue homeostasis and detoxification process especially during stress.

For the upregulated genes (Table 4-3), 16% of genes that were assigned GO for cell communication process consisted of genes that encoded either G-protein receptors (e.g. *CG12290*, *NPFR1* and *mthl9*) or signal transduction proteins (e.g. *unc-13*, *Graf,* or transcription factor *sug*). Of 15% genes under the GO category of transport, 40% were transmembrane transport genes (such as Na(+)-driven bicarbonate exchanger, *Ndae1,* putative zinc transporter *CG2177,* and water channel *CG17664*) while 32% were known to be involved in vesicle-mediated transport (e.g. *crq* and *SNAP*).

Genes such as *car*, *TBPH, santa-maria and unc-13* show a large fold change in tubules of Rel^{E20} compared to wild type. According to Flyatlas, these genes are highly enriched in brain and thoracic ganglion tissues of *Drosophila* while their expression in tubules is low. This dysregulation in expression of genes in tubules highlights the importance of Relish in maintaining tight regulation of these genes in specific tissues.

Table 4-3: Top 30 upregulated genes in tubules of Rel^{E20} categorised according to biological processes.

Metabolic process			Cell communication			Transport		
FBgn	Gene Symbol	FC	FBgn	Gene Symbol	FC	FBgn	Gene Symbol	FC
FBgn0025697	santa-maria	19.3	FBgn0003360	sesB*	125.0	FBgn0003360	sesB*	125.0
FBgn0034356	CG10924	18.6	FBgn0025790	TBPH	25.0	FBgn0259221	CG42321	62.3
FBgn0000592	Est-6	18.3	FBgn0025697	santa-maria	19.3	FBgn0010549	l(2)03659	43.4
FBgn0001258	ImpL3	11.5	FBgn0000257	car	10.2	FBgn0036816	Indy	18.3
FBgn0000257	car	10.2	FBgn0003285	rst	7.3	FBgn0033558	CG12344	10.7
FBgn0050104	CG30104	9.7	FBgn0003984	vn	5.3	FBgn0000257	car	10.2
FBgn0063495	GstE5	9.6	FBgn0030685	Graf	4.8	FBgn0039902	CG2177	8.4
FBgn0027914	Gen	9.1	FBgn0035802	CG33275	3.7	FBgn0003475	spir	5.7
FBgn0000406	Cyt-b5-r	8.2	FBgn0025726	unc-13	3.7	FBgn0033260	Cul-4	5.3
FBgn0261261	plx	7.8	FBgn0250791	Snap	3.4	FBgn0261285	Ppcs	5.3
FBgn0036367	CG10116	5.8	FBgn0039419	CG12290	3.3	FBgn0259111	Ndae1	5.2
FBgn0033260	Cul-4	5.3	FBgn0001137	grk	2.9	FBgn0034883	CG17664	5.1
FBgn0261285	Ppcs	5.3	FBgn0000533	ea	2.8	FBgn0033196	CG1358	4.2
FBgn0010038	GstD2	5.1	FBgn0035131	mthl9	2.7	FBgn0015924	crq	3.7
FBgn0041712	yellow-d	4.9	FBgn0051720	CG31720	2.7	FBgn0037140	CG7442	3.7
FBgn0015038	Cyp9b1	4.8	FBgn0033782	sug	2.7	FBgn0025726	unc-13	3.7
FBgn0033846	mip120	4.6	FBgn0086604	CG12484	2.7	FBgn0037912	sea	3.6
FBgn0033539	Git	4.6	FBgn0003392	shi	2.6	FBgn0037913	fabp	3.6
FBgn0034364	CG5493	4.6	FBgn0037105	S1P	2.6	FBgn0250791	Snap	3.4
FBgn0039114	Lsd-1	4.2	FBgn0039212	Syx18	2.6	FBgn0027581	CG6191	3.2
FBgn0030761	CG9784	4.1	FBgn0016672	lpp	2.6	FBgn0051792	CG31792	3.1
FBgn0037697	CG9363	4.1	FBgn0011591	fng	2.5	FBgn0035173	CG13907	2.9
FBgn0033304	Cyp6a13	3.5	FBgn0031016	kek5	2.5	FBgn0035770	pst	2.9
FBgn0050481	mRpL53	3.3	FBgn0027932	Akap200	2.5	FBgn0030574	CG9413	2.8
FBgn0034588	CG9394	3.2	FBgn0051612	CG31612	2.5	FBgn0051547	CG31547	2.7
FBgn0023541	Cyp4d14	3.1	FBgn0037408	NPFR1	2.4	FBgn0003392	shi	2.6
FBgn0038020	GstD9	3.1	FBgn0029791	CG4096	2.4	FBgn0028699	Rh50	2.6
FBgn0014427	CG11899	3.0	FBgn0033273	CG2183	2.4	FBgn0039212	Syx18	2.6
FBgn0033775	Cyp9h1	2.9	FBgn0051140	CG31140	2.4	FBgn0027287	Gmap	2.6
FBgn0038704	CG5316	2.8	FBgn0051721	Trim9	2.3	FBgn0039840	CG11340	2.4

Table 4-3: Top 30 upregulated genes in tubules of Rel^{E20} categorised according to biological processes. The first column denotes Flybase gene number (FBgn), second column - Gene Symbol, third column- FC denotes fold change (with multiple correction p-value≤0.05).Note that, few genes are represented in more than one biological process.* at gene symbol denotes detected by more than two probe sets.

Amongst down- regulated genes (Figure 4-4), 10% were categorised as response to stress and consisted of genes (e.g. *Drosomycin (Drs)* and *Immune regulated catalase (IRC)*) that are known to be upregulated in response to immune stress (Ha et al., 2005; Lemaitre et al., 1996) , oxidative stress (*Sod3*) (Jung et al., 2011) and starvation stress (*Ac76e*) (Mattila et al., 2009) (Table 4–4).

A gene of interest is the transporter gene *CG6293* (a sodium ascorbate transporter) which was down- regulated in Rel^{E20} tubules (Table 4–4). Flyatlas data of *CG6293* shows high mRNA expression of this gene in epithelial tissues: tubules, hindgut and ovary. The FC of -5.0 was observed in the tubules of Rel^{E20} compared to wild type (Figure 4-3, Table 4-4). SVCT1/2 transporters encoded by

genes *SLC23a1/a2* are human orthologs of *CG6293*. The mammalian sodium ascorbate transporters are involved in absorption of Vitamin C (in form of ascorbic acid) from the diet (Tsukaguchi et al., 1999). These transporters play a vital role in maintaining ascorbate pools within the cell and contribute in redox homeostasis of the cell (reviewed (Savini et al., 2008)). Thus, the influence of Relish protein on mRNA expression of on the *Drosophila* sodium ascorbate transporter and possibly subsequent pools of ascorbate within the tubule cells shows the role Relish can play in regulation of metabolic homeostasis.

Glut1 encodes *a* glucose transporter is the *Drosophila* orthologue of human *Glut1* sharing 68% identity (Escher and Rasmuson-Lestander, 1999). In humans, *Glut1* is involved in maintaining energy homeostasis of cell (Takata, 1996). The *Drosophila Glut1* has 19 transcripts and the gene is recognised by two probe sets (1628757_at and 1634033_s_at). According to Flyatlas, *Glut1* shows high expression and enrichment in tubules compared to whole fly for both the probe sets. A significant fold change in Rel^{E20} tubules compared to wild type for 1628757_at (FC = -3.7) and 1634033_s_at (FC=-1.4) (Table 4–4) is observed. It is not known which transcripts are expressed in tubules but nonetheless it can be inferred that absence of Relish protein significantly affects the expression of *Glut1*. This down regulation of *Glut*1 which would result in less glucose being transported into the tubule cells may be contributing to the lower basal fluid secretion rate in Rel^{E20} tubules compared to wild type seen in Figure 4-1.

4.2.4 H_20_2 quantification in tubules

The above microarray data results of mis- regulation of GSTs and CYPs showed that absence of Relish in tubules altered expression of genes that play a role in regulation of redox balance (above and Discussion). Therefore, H_20_2 levels were examined in tubules of Rel^{E20} and wild type under unstressed conditions (Figure 4-5). In wild type tubules, H_20_2 levels were 0.22 µM H_20_2 /µg protein. In Rel^{E20} tubules, H_20_2 measured was 0.07 µM H_20_2 /µg protein which was lower than wild type. This suggests that loss of Relish protein causes mis-expression or reduced expression of genes such as *SOD3* that are required for generation or breakdown of reactive oxygen species like H_20_2. This alteration of gene expression may be

resulting in either more than the normal amount of H_20_2 being broken down or that Rel^{E20} tubules generate less H_20_2.

Table 4-4: Top 30 down- regulated genes in tubules of Rel^{E20} categorised according to biological processes.

Metabolic process			Transport			Response to stress		
FBgn	Gene Symbol	FC	FBgn	Gene Symbol	FC	FBgn	Gene Symbol	FC
FBgn0002939	ninaD	-43.6	FBgn0260858	Ykt6	-102.8	FBgn0002939	ninaD	-43.6
FBgn0033631	Sod3*	-28.1	FBgn0002939	ninaD	-43.6	FBgn0014018	Rel	-32.7
FBgn0040629	CG18673	-19.3	FBgn0262007	CG42825	-23.8	FBgn0033631	Sod3*	-28.1
FBgn0015286	Rala	-17.8	FBgn0001128	Gpdh	-7.7	FBgn0015286	Rala	-17.8
FBgn0003082	phr*	-14.9	FBgn0030746	CG9981	-7.1	FBgn0023511	Edem1	-15.4
FBgn0042173	CG18853	-14.9	FBgn0050345	CG30345	-5.8	FBgn0003082	phr*	-14.9
FBgn0033980	Cyp6a20	-9.5	FBgn0034715	Oatp58Db	-5.5	FBgn0042173	CG18853	-14.9
FBgn0001128	Gpdh	-7.7	FBgn0039487	gb	-5.5	FBgn0033980	Cyp6a20	-9.5
FBgn0030615	Cyp4s3	-6.5	FBgn0031515	CG9664	-5.3	FBgn0015575	Î±-Est7	-8.0
FBgn0025837	CG17636	-6.3	FBgn0037807	CG6293	-5.0	FBgn0033518	Prx2540-2	-5.6
FBgn0039756	CG9743	-6.3	FBgn0029950	CG9657	-4.7	FBgn0033520	Prx2540-1	-5.6
FBgn0033518	Prx2540-2	-5.6	FBgn0031010	CG8028	-4.6	FBgn0039487	gb	-5.5
FBgn0033520	Prx2540-1	-5.6	FBgn0035968	CG4484	-4.6	FBgn0031515	CG9664	-5.3
FBgn0033521	CG12896	-5.6	FBgn0264574	Glut1	-3.8	FBgn0024836	stan	-5.2
FBgn0022359	Sodh-2	-5.3	FBgn0038198	Npc2b	-3.6	FBgn0005636	nvy	-5.1
FBgn0010041	GstD5	-4.6	FBgn0041585	olf186-F	-2.9	FBgn0038465	Irc	-3.7
FBgn0015035	Cyp4e3	-4.5	FBgn0034794	Gmer	-2.9	FBgn0003435	sm	-3.2
FBgn0022774	Oat	-4.2	FBgn0051103	CG31103	-2.8	FBgn0004852	Ac76E	-2.7
FBgn0038922	CG6439	-4.0	FBgn0038718	CG17752	-2.6	FBgn0020270	mre11	-2.6
FBgn0035790	Cyp316a1	-3.9	FBgn0021953	Fatp	-2.6	FBgn0026751	XRCC1	-2.6
FBgn0040211	hgo	-3.7	FBgn0261285	Ppcs	-2.5	FBgn0003256	rl	-2.5
FBgn0038465	Irc	-3.7	FBgn0036141	wls	-2.4	FBgn0000055	Adh	-2.5
FBgn0038198	Npc2b	-3.6	FBgn0022942	Cbp80	-2.4	FBgn0022942	Cbp80	-2.4
FBgn0038194	Cyp6d5	-3.3	FBgn0032699	CG10383	-2.3	FBgn0010381	Drs	-2.3
FBgn0035203	CG9149	-3.0	FBgn0036843	CG6812	-2.2	FBgn0036381	CG8745	-2.2
FBgn0034794	Gmer	-2.9	FBgn0038747	RhoGAP92B	-2.1	FBgn0013772	Cyp6a8	-2.2
FBgn0036449	bmm	-2.9	FBgn0033443	CG1698	-2.1	FBgn0036101	NijA	-2.2
FBgn0031321	Tgt	-2.8	FBgn0005632	faf	-2.1	FBgn0036843	CG6812	-2.2
FBgn0004852	Ac76E	-2.7	FBgn0259173	corn	-2.0	FBgn0026718	fu12	-2.1
FBgn0001098	Gdh	-2.6	FBgn0027655	htt	-2.0	FBgn0005632	faf	-2.1

Table 4-4: Top 30 down- regulated genes in tubules of Rel^{E20} categorised according to biological processes. The first column denotes Flybase gene number (FBgn), second column - Gene Symbol, third column- FC denotes fold change (with multiple correction p-value≤0.05).* at gene symbol denotes detected by more than two probe sets. Note that, few genes are represented in more than one biological process.

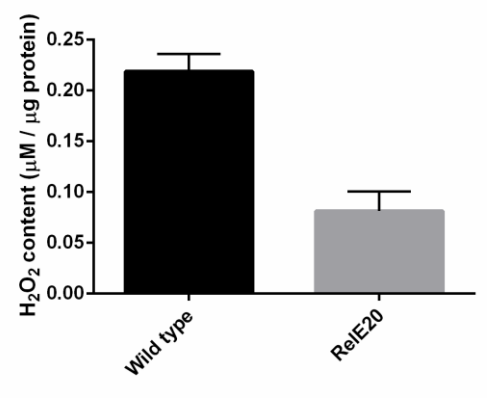


Figure 4-5: H_20_2 quantity in tubules of Rel^{E20}. Tubule homogenates of wild type and Rel^{E20} from 15-20 flies were assayed for H_20_2 levels (according to protocol of (Terhzaz et al., 2015a)). Total H_20_2 levels were normalised to protein amounts and data expressed in μM/μg. The result is from two independent biological replicates for each genotype and the mean of the values has been plotted with ±S.E.M.

4.3 Discussion

4.3.1 Role of Relish in detoxification process

Tubules are enriched with a wide variety of detoxifying genes encoding for cytochromeP-450 and GST (Wang et al., 2004; Yang et al., 2007). GSTs have a diverse range of functions from detoxification to cell signalling (Pajaud et al., 2012). The most investigated role of GSTs is its detoxification function through conjugation of glutathione to various electrophilic compounds thus protecting various cellular components (Habig et al., 1974). GSTs expression is induced on insecticide treatment and is associated with insecticide resistance (Kostaropoulos et al., 2001; Lumjuan et al., 2011; Ortelli et al., 2003). GSTs convert insecticide to produce water-soluble metabolites that can easily be excreted and neutralise pesticide produced toxic oxygen free radical species to less harmful form (Enayati et al., 2005) .The 9.6 fold upregulation in expression of *GstE5* in Rel^{E20} compared to wild type is particularly interesting. The flies over expressing *Diptericin* showed a decrease in *GstE5* (Zhao et al., 2011).This indicates that Diptericin is required to suppress the expression of *GstE5*,hence the absence of Diptericin production in tubules of Relish mutant leads to upregulation of *GstE5* in tubules of Rel^{E20}. According to Flyatlas data, *GstE5* is 7.2 fold enriched in tubules compared to whole fly (Table 4-2) with its high expression being limited to epithelial tissues (crop, midgut, tubules and hindgut)

and low expression in other tissues like the head. Although Zhao et al studied the whole flies and the study here is limited to the tubules, gene expression pattern for GstE5 and Relish in the epithelial tissue identical. Hence it can be suggested that the expression of *GstE5* is regulated by Relish through Diptericin.

Recent studies revealed mammalian GSTs to have a wider physiological role such as modulating c-Jun N-terminal kinase (JNK) signalling pathway (Thevenin et al., 2011) and acting as carrier of nitric oxide (Lok et al., 2012), and act as key regulators to stress kinase pathways (Pajaud et al., 2012). Taking into account studies of GSTs in human, it may be speculated that loss of Relish protein and its subsequent target genes lead to alteration of redox homeostasis in tubule cells. This may result in a disruption in the control of certain stress related pathways affecting expression levels of GSTs.

As mentioned earlier, CYPs play an important role in drug metabolism and drug breakdown and drug interaction. NF-kB regulates CYPs' gene expression by various mechanism such as directly binding to promoter of the CYP gene causing an increase or decrease in its mRNA expression (Abdel-Razzak et al., 2004; Bell and Strobel, 2012; Dulos et al., 2005) or indirectly by repressing nuclear receptors that regulate CYPs (Tian et al., 1999). In insects, CYPs are induced on treatment with insecticides (Amichot et al., 1998; Daborn et al., 2001; Le Goff et al., 2003) and plant toxins, (Danielson et al., 1998) and play a critical role in insecticide resistance. CYPs have other physiological roles such as synthesis of juvenile hormones (reviewed (Feyereisen, 1999)) and cuticular hydrocarbon biosynthesis (Qiu et al., 2012). In the Malpighian tubules, 28% of all CYP P450 genes are expressed over 2.8 fold (Wang et al., 2004). In the data set, studied here a modulation in tubule of two CYP families: *Cyp4e (Cyp4e1* and *Cyp4e3*) and *Cyp6a (Cyp6a13, Cyp6a20* and *Cyp6a8*) which are differentially up- and down-regulated in tubules of Rel^{E20} mutants compared to wild type suggest that Relish and its target genes can differentially regulate particular CYP family genes.

The gene encoding for Cyp4e3 is highly enriched and abundantly expressed in the tubules, fat body, head and brain. Recently, it was demonstrated that

Cyp4e3 overexpression or suppression in tubule principal cells caused the flies to survive better or worse respectively compared to parental flies when exposed to the insecticide, permerthin (Terhzaz et al., 2015a). Additionally, Terhzaz et al (2015) saw an increase in the expression of *Diptericin* (a Relish target gene) and increase in H_2O_2 levels in tubules when *Cyp4e3* expression in principal cells was suppressed. While Terhzaz et al. showed that under unstressed conditions, decrease in expression of *Cyp4e3* leads to activation of Relish subsequently inducing expression of *Diptericin,* the tubule microarray data here in shows that absence of Relish protein causes decrease in expression of *Cyp4e3*. In addition, *Diptericin* overexpression in whole flies showed a down regulation in the expression of *Cyp4e3* (Zhao et al., 2011). Therefore, it may be that mRNA expression of *Cyp4e3* is under a feedback mechanism regulated by Relish and modulated by redox state of tissue.

The two peroxiredoxins encoding genes (Prx2540-2, Prx2540-1) were highly down- regulated in Rel^{E20}. These peroxiredoxin are enriched in tubules and are involved in the breakdown of hydrogen peroxide (H_20_2). Superoxide Dismutase 3 (Sod3), an extracellular SOD, also involved in breakdown of oxygen free radicals to H_20_2 (Blackney et al., 2014) was also highly down- regulated in tubules of Rel^{E20}.

The promoter region of SOD1 (Cu/Zn-SOD -mammalian ortholog of *Sod3*) contains NF-kB binding sites (Rojo et al., 2004). The expression of *Sod1* is upregulated by phosphatidylinositol 3 kinase (PI3K) /Akt pathway through activation of NF-kB (Rojo et al., 2004). Based on this study, it can be postulated that in *Drosophila* a similar regulation of *Sod3* may be occurring. Therefore, it may be postulated that in Rel^{E20} tubules, the baseline mRNA expression of *Sod3* is not induced appropriately because of the lack of Relish protein.

Relish is required for the maintenance of appropriate H_2O_2 levels in tubules since Rel^{E20} has less amount of H_2O_2 than wild type levels (Figure 4-5). This and the genes discussed above indicate that Relish potentially regulates optimal redox status of tubules through balancing either directly or indirectly the basal expression of detoxifying enzymes and other contributing redox genes. This

regulation by Relish may be through various physiological inputs from stress kinase pathways, by H_2O_2 itself, or both of which are triggered in response to different stress agents like infection or xenobiotic substance.

4.3.2 Role of Relish in fluid transport and metabolism

A study by Hasler et al. (2008) demonstrated vasopressin independent, liposaccharide-mediated NF-kB activation led to decrease in mRNA expression of the human aquaporin, AQP2, in renal collecting duct principal cells (Hasler et al., 2008). The chromatin immunoprecipitation study showed the *AQP2* gene had two putative NF-kB binding sites at its promoter region (Hasler et al., 2008). The gene expression encoding protein CG17664, *Drosophila* homologue of human aquaporins, had a 5.1 fold increase in tubules of Rel^{E20}. Of the four aquaporins found in tubules, *CG17664* is highly abundant and very highly enriched in tubule and is regarded to have a tubule specific-role (Wang et al., 2004). Recent study has shown CG17664 as an aquaglyceroporin expressed only in the tubule principal cells (Cabrero et al. unpublished). Thus, Relish is required for the repression of *CG17664*, indicates that some Relish-target genes are involved in controlling water or glycerol homeostasis.

In Activating transcription factor 3 (ATF3) mutants, Relish has been suggested to be playing a role in lipid metabolism and Rel^{E20} larvae had lower amount of triglycerides than wild type (Rynes et al., 2012), in data set of tubules of adult flies studied here, five genes (*Npc2b*, *bmm*, *Gpdh*, *CG9149* and *CG9743*) that are associated with lipid metabolism were highly down- regulated (Table 4–4), although transcription factor *sug* that represses the breakdown of dietary fat was upregulated (Zinke et al., 2002). In humans, obesity, inflammation and activation of NF-κB have been linked (Cai et al., 2005; Carlsen et al., 2009; Shoelson et al., 2003; Tang et al., 2010). Recently, *Drosophila* has been recognised as a model to study obesity (Baker and Thummel, 2007; Trinh and Boulianne, 2013) and hence investigation of the above genes during stress (immune stress and high salt diet intake) can help gain further insights of obesity and NF-κB.

Trehalose is the main source of energy in insects and glucose is important for trehalose anabolism. It was observed that on a carbohydrate free diet, the tobacco hornworm *Manduca sexta*, maintained its trehalose levels through gluconeogenesis (Thompson et al., 2003). Tubules are highly enriched with sugar and glucose transporters hence suggesting sugar transport as a key role of tubules (Wang et al., 2004). It was earlier suggested that one of the probable reasons for the decreased fluid secretion rate seen in tubules of Rel^{E20} flies was the decrease in glucose being transported inside the tubule cells due to decrease in expression of *Glut1* transporter. As tubules are high energy demanding tissues, we wondered if decrease in transport of glucose in the cells, altered metabolic pathways of cell to generate glucose. The mRNA expression of *CG10924* gene, a predicted phosphoenolpyruvate carboxykinase (PEPCK) that has moderate level expression in tubules according to Flyatlas, increased 18.6 fold in tubules of Rel^{E20}. This increase in a gene associated with gluconeogenesis is interesting as it reflects a probable change in metabolic status that occurs in tubules in absence of Relish protein further highlighting the role of Relish and its target genes on metabolism.

The screening of mutations in flies and genome wide RNAi study in S2 cells have identified genes that regulate the IMD pathway and maintain the basal Relish signalling activity (Foley and O'Farrell, 2004; Khush et al., 2002) . The study undertaken in this chapter demonstrated that loss of basal Relish activity in tubules of Rel^{E20} can affect the expression of wide range of genes, majority of which play a role in detoxification, redox and metabolic processes. This implies that Relish responds to other physiological signals other than immune stress to regulate genes for optimal functioning of the organism. Under unstressed conditions and in absence of Relish, the dysregulation of these genes do not seem to drastically affect the survival of Rel^{E20} flies which are viable under unstressed conditions (Hedengren et al., 1999). Upon countering stress (for example gram negative infection) that require specific regulation of genes by Relish, Rel^{E20} become sensitive to stress. The genetic and functional studies on the genes identified in this study can potentially help understand how Relish controls the expression of these genes and how loss of this regulation is associated with pathology and insecticide resistance.

4.4 Supplementary Data

Table 4-S1: Comparing differentially regulated genes in unstressed Relish mutants to whole fly microarray data of flies overexpressing Diptericin.

Upregulated in Relish mutants			
		Unstressed	Diptericin
FBgn	CGNum	Rel/WT	overexpression
FBgn0033234	CG11893	49.0	-3.3
FBgn0063495	GstE5	9.6	-1.6
FBgn0003285	rst	7.3	-1.9
FBgn0051975	CG31975	3.2	-1.9
FBgn0034335	GstE1	2.8	1.9
FBgn0036806	Cyp12c1	2.6	-1.5
FBgn0022710	Ac13E	2.3	-1.9
FBgn0033234	CG8791	2.2	-1.5
FBgn0015034	Cyp4e1	2.2	-1.5
FBgn0039316	CG14149	2.2	-1.5
FBgn0033873	CG6337	2.1	-1.9
FBgn0004369	Ptp99A	2.1	-1.5
FBgn0051901	Mur29B	2.1	-1.6
Down regulated in Relish mutants			
		Unstressed	Diptericin
FBgn	CGNum	Rel/WT	overexpression
FBgn0033789	CG13324	-20.6	-1.9
FBgn0023511	Edem1	-15.4	2.2
FBgn0039085	CG10170	-13.3	-1.7
FBgn0003082	phr	-11.7	1.9
FBgn0033980	Cyp6a20	-9.5	-2.5
FBgn0053346	CG33346	-7.3	-3.8
FBgn0034480	CG16898	-6.9	-1.5
FBgn0035043	CG4781	-6.6	-1.6
FBgn0025837	CG17636	-6.3	2.3
FBgn0031515	CG9664	-5.3	-1.7
FBgn0015035	Cyp4e3	-4.5	-2.6
FBgn0020270	mre11	-2.6	-3.5
FBgn0038718	CG17752	-2.6	-1.7
FBgn0052368	CG32368	-2.3	-2.4
FBgn0013772	Cyp6a8	-2.2	-1.5

Table 4-S1: Comparing differentially regulated genes in unstressed Relish mutants to whole fly microarray data of flies overexpressing Diptericin. The first column denotes Flybase Id, second column – Gene symbol, third column is fold change of the gene expression in tubules of unstressed Relish mutant (Rel) compared to wild type (WT), fourth column is the value from microarray data of whole fly overexpressing Diptericin from Zhao et al., 2011. Note the original fold change ratio values of the Zhao et al., 2011 data converted into whole numbers. The FC values in red are denoted for a gene whose expression is suggested to be regulated by Diptericin in Zhao et. al study.

Chapter 5
In vivo stress associated role of cGMP-dependent kinases

Chapter 5

5.1 Introduction

cGMP is an intracellular second messenger that is involved in regulating diverse physiological processes such as behaviour, circardian rythmn, vascular relaxation/remodelling, platelet function and gastrointestinal and kidney functioning (Hofmann et al., 2006). In tissues, cGMP is synthesized by two classes of guanylate cyclases (GCs): membrane bound GC and cytosolic GC. The membrane bound GCs are linked to receptors and are activated by peptide ligands *eg*. natriuretic peptides. The cytosolic soluble GCs are primarily intracellular targets of the gaseous signalling molecule, nitric oxide (NO). Cyclic nucleotide gated channels (CNG), cGMP kinases (cGKs) and cGMP-regulated phosphodiesterases are important targets for cGMP action. cGMP is hydrolysed by phosphodiestereases (PDEs). Thus the activities of GCs and PDEs dictate the intracellular levels of cGMP (Davies, 2006; Francis et al., 2010a).

Components of the cGMP signalling pathway are conserved in Drosophila (Davies, 2006) and fluid secretion by *Drosophila* Malpighian tubules is finely regulated by cGMP signalling (Broderick et al., 2003; Davies et al., 1995; Dow et al., 1994a). The cGMP-generating receptor guanylate cyclases (Gyc76c, CG5719, and CG9783) are highly enriched in *Drosophila* Malpighian tubules (Davies, 2006). Manipulation of cGMP signalling components using transgenic methods has shown that ectopic expression of human GC-A receptors and its activation with Atrial natriuretic peptide leads to generation of cGMP and increased fluid secretion by *Drosophila* Malpighian tubules (Kerr et al., 2004). The NO producing enzyme NOS (NO synthase) increases cGMP content only in the tubule principal cells through NO-stimulated soluble GC (sGC) present in the cells (Broderick et al., 2003). The tubules express all six PDEs: *Drosophila* PDE1, *dunce*(PDE4), PDE6, PDE8, PDE9 and PDE11. The biochemical assays show that PDE6 is a cGMP-specific PDE, and PDE1 and PDE11 are dual-specific PDEs. Through structural analysis it is inferred that PDE4 and PDE8 are cAMP-specific PDE and PDE9 is a c-GMP specific PDE. (Day et al., 2005). The two functionally characterised cGKs of Drosophila - Dg1(Pkg21D) and Dg2 (foraging, for) are highly expressed in tubules (MacPherson et al., 2004b). Also, tubule principal cells express several CNG channels as cGMP-induced calcium influx has been shown to occur (MacPherson et al., 2001).

Thus the tubules contain signalling components required for generation and breakdown of cGMP, and transducing cGMP signals.

cGMP signalling is also regulated by neuropeptides. For example, the capa-1 neuropeptide, which is associated with desiccation and cold tolerance (Terhzaz et al., 2015b), stimulates tubule fluid secretion via the production of cGMP via NO production, activation of soluble guanylate cyclase and inhibition of cG-PDE activity in the tubule principal cells (Broderick et al., 2003; Davies et al., 1995). Thus, capa can regulate the cGMP content in the tubule principal cells. This diuretic function of capa-1 peptide is conserved among Dipterian insect species (Pollock et al., 2004) (Coast, 2009). . However, in *Rhodinus prolixus* , Cap2b belonging to capa peptide family produces an antidiuretic effect on the tubules and cGMP has shown to contribute to this effect (Quinlan et al., 1997). Also identification of an antidiuretic hormone acting on the tubules in beetles seems to exert its effect through cGMP (Eigenheer et al., 2002).

The vertebrate and *Drosophila* cGKs share functional and structural similarities and based on sequence predictions Dg1 and Dg2 can be assigned as a cGKII homologue, and cGKIα and cGKIß homologue respectively (MacPherson et al., 2004b). Unlike Dg1 localisation which is seen in cytosol, cGKII is membrane bound but similar to Dg1 tissue expression, cGKII is highly expressed in kidney, intestinal mucosa but also in brain, smooth muscles. While Dg2 is expressed as membrane bound in Drosophila cells, its vertebrate homologue cGKI is cytosolic in expression and is expressed in cerebellum and few neuronal cell types (reviewed in (Hofmann et al., 2006; MacPherson et al., 2004b)). The activity of Dg2 can be inhibited by vertebrate cGK inhibitor, Rp-8-CPT-cGMPs but Dg1 is insensitive to this inhibitor (MacPherson et al., 2004b).

The cognate serine/threonine cGKs - Dg1 and Dg2, are stimulated by cGMP at micromolar levels (Dg1 EC_{50} = 0.09 ±0.04 µM and Dg2 EC_{50}= 0.13±0.04 µM, 0.32±0.14 µM (MacPherson et al., 2004b)) and activated by cAMP at ~20µM concentration (Foster et al., 1996; MacPherson et al., 2004b).

Flyatlas data shows *Dg1* to be expressed in high abundance and highly enriched in the tubules and hindgut of adult fly and larvae. *Dg2* is highly expressed in all the tissues of adult fly and larvae (Chintapalli et al., 2007; Robinson et al., 2013).There are 12 putative transcripts detected for *Dg2* gene (information from Ensemble: http://www.ensembl.org/index.html) but only two transcripts (*Dg2P1* and *Dg2P2)* have been functionally characterised in *Drosophila* tubules (MacPherson et al., 2004b). Also two naturally occurring polymorphs: for^R and for^s, are found for *Dg2* gene that show difference in behaviour (Sokolowski, 2001) and tubule functionality (MacPherson et al., 2004a).

The targeted expression of cGKs in tubule principal cells showed that Dg1 is localised in the cytosol and may transduce a cytosolic cGMP signal. Dg2 is localised to the membrane where Dg2P1 is expressed in the apical membrane while Dg2P2 is found at the basolateral and apical membrane, and hence Dg2 may transduce the cGMP signal generated at the membrane (MacPherson et al., 2004b). The differential localisation of cGKs in the tubule cells, their response to different intracellular cGMP pools, and their differential expression in fly tissues indicate that they may be transducing different cGMP-generating signals, leading to different physiological effects at tissue and whole organism (Davies et al., 2014).

Overexpression of Dg1 or Dg2 in tubule principal cells increases fluid secretion in tubules in response to capa peptide and/or cGMP (MacPherson et al., 2004b); although an overexpression of these kinases does not increase cGMP content in the tubules (Davies, 2006). In *Drosophila*, capa-1 peptide is associated with cold and desiccation tolerance (Terhzaz et al., 2015b) and one of its signalling features is through nitric oxide/cGMP (Broderick et al., 2003). Hence do the cGKs transduce the cGMP signal of capa-1 related stress signalling for regulating water and ion homeostasis for organismal survival during stress?

In chapter 3, it was observed that the expression of *Dg1* and *Dg2* was significantly altered only in salt stressed tubules of Rel^{E20}. Also, it was postulated that Relish in tubule principal cells may be activated through Ca^{2+} /NO-cGMP signalling under salt stress mediated by neuropeptide NPLP1-VQQ and

a candidate neuropeptide capa-1. In addition, the previous studies have shown activation of Relish in tubules by (a) receptor guanylate cyclase Gyc76C in response to salt stress (Overend et al., 2012), (b) neuropeptide capa-1 (Terhzaz et al., 2014), and (c) cGMP in a dose dependent manner (Davies et al., 2014). In this chapter an attempt has been made to elucidate *in vivo* role of cGKs in tubule principal cells under different stress conditions and whether it can activate Relish.

5.2 Results

5.2.1 Characterization of cGMP dependent kinases RNAi lines

When this study was undertaken the UAS-cGK RNAi lines generated in the lab prior to start of this PhD work gave inconsistent results for stress experiments. This was attributed to the fly lines discontinuing to express the RNAi. Hence, cGK RNAi lines used for the current study were KK lines ordered from Vienna Drosophila Resource Centre (VDRC) (Table 2-1). The fly lines expressing UAS-Dg1 RNAi and UAS-Dg2 RNAi (VDRC), and UAS-Control RNAi (gift from Dr. Edward Green, German Cancer Research Center, Heidelberg) used for the studies were in the similar genetic background and expressed the phiC31 vector inserted in a defined insertion site on chromosome 2. The UAS-Control RNAi expressed a hairpin nucleotide sequence that did not suppress the expression of any gene. The hairpin nucleotide sequence expressed by UAS-Dg1 RNAi and UAS-Dg2 RNAi was against mRNA of Dg1 and all known Dg2 transcripts, respectively; thus inducing the suppression of respective gene in a particular tissue when the UAS was expressed under a tissue specific GAL4 driver such as tubule principal specific driver – CapaR-GAL4 or expressed in all the tissues of the fly using Actin-GAL4/Cyo. In CapaR-GAL4, the expression of GAL4 is under control of the promoter region of the gene CapaR, which is constitutively and highly expressed in the tubule principal cells. Similarly in Actin-GAL4/Cyo driver line, GAL4 expression is under the control of promoter region of Actin gene that is expressed globally in all cells and of the fly. Hence these GAL4 driver lines were used when the UAS expression of either of the cGKs was to be directed either in a specific tissue or expressed in all tissues.

The UAS RNAi lines were crossed to the ubiquitously expressing GAL4 driver line Actin-GAL4/Cyo to assess if knockdown of these genes in all the tissues was lethal for fly development and to validate the UAS-RNAi lines (Figure 5-S1 describes the crossing scheme). In the Actin-GAL4, Actin gene promoter sequence is fused with GAL4 sequence. The wing phenotype was used as an indicator of whether the eclosed fly expressed the GAL4 driven-*Dg1* or -*Dg2* UAS-RNAi line. The curly wings indicated that the fly did not express UAS RNAi. Straight wings were an indicator of flies expressing the gene specific UAS RNAi line in all the tissues. The Figure 5-1 shows the number of eclosed flies and their wing phenotype for each of the cGK kinase.

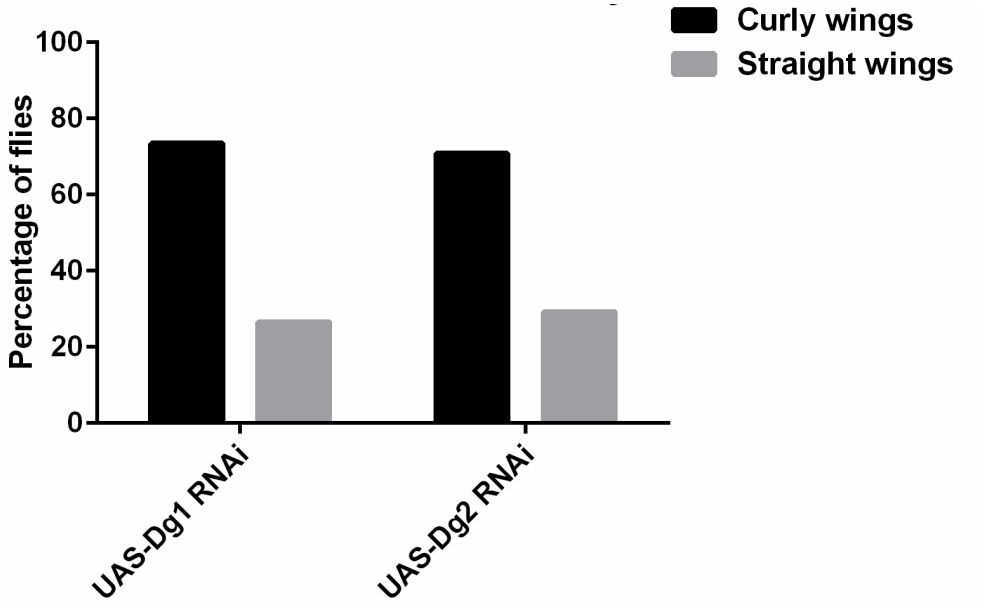


Figure 5-1: Characterization of cGK gene RNAi lines. The UAS-Dg1 RNAi and UAS-Dg2 RNAi male flies were mated with Actin-GAL4/Cyo female flies. The progeny flies were scored for curly and straight wings. The straight wing flies ubiquitously expressed the RNAi while the curly wing flies did not. The eclosion of 100 flies was assessed for each of the UAS-RNAi lines.

The eclosion of 100 flies was assessed for each of the UAS-RNAi lines. For Dg1 RNAi, 30 flies that eclosed had straight wings and 70 flies had curly wings. Similarly for Dg2 RNAi, 31 and 69 of flies had straight and curly wings respectively indicating the ubiquitous knockdown of either of these kinases affected eclosion. As eclosion hormone-triggered cGMP signalling is known to play a role in ecdysis (Truman, 1992), data in Figure5-1 suggests that the UAS-RNAi line produced a phenotypic effect.

5.2.2 Percentage knockdown of cGK genes in cGK RNAi

Flyatlas data show that *Dg1* and *Dg2* are highly expressed in tubules of adult fly and larvae (Chintapalli et al., 2007; Robinson et al., 2013). As such, this study was focused on the role of *Dg1* and *Dg2* in tubule principal cells only. The targeted knockdown of the specific cGKs in the principal cells was thus performed using the CapaR-GAL4 driver (Terhzaz et al., 2012) and the results compared to control flies that expressed UAS-control RNAi (Table 2-1) and CapaR-GAL4.

Quantification of *Dg1* gene knockdown in tubules indicated a 70% decrease compared to control flies (Figure 5-2 A). Furthermore, to assess if down regulating the expression of *Dg1* affected the expression of *Dg2*, the relative mRNA expression of *Dg2* in tubules of these flies compared to control flies was quantified and no significant difference was observed (Figure 5-2A).

Similarly, quantification of *Dg2* gene knockdown in tubules was assessed in flies expressing UAS-Dg2 RNAi under the control of CapaR-GAL4. The progeny showed a small yet significant 30% knockdown for expression of *Dg2* compared to control flies. A down regulation in expression of *Dg1* gene was observed in the tubules of progeny compared to control, but this down regulation was not significant (Figure 5-2B).

It can be concluded that under unstressed conditions expression of Dg1 and Dg2 was independent of each other.

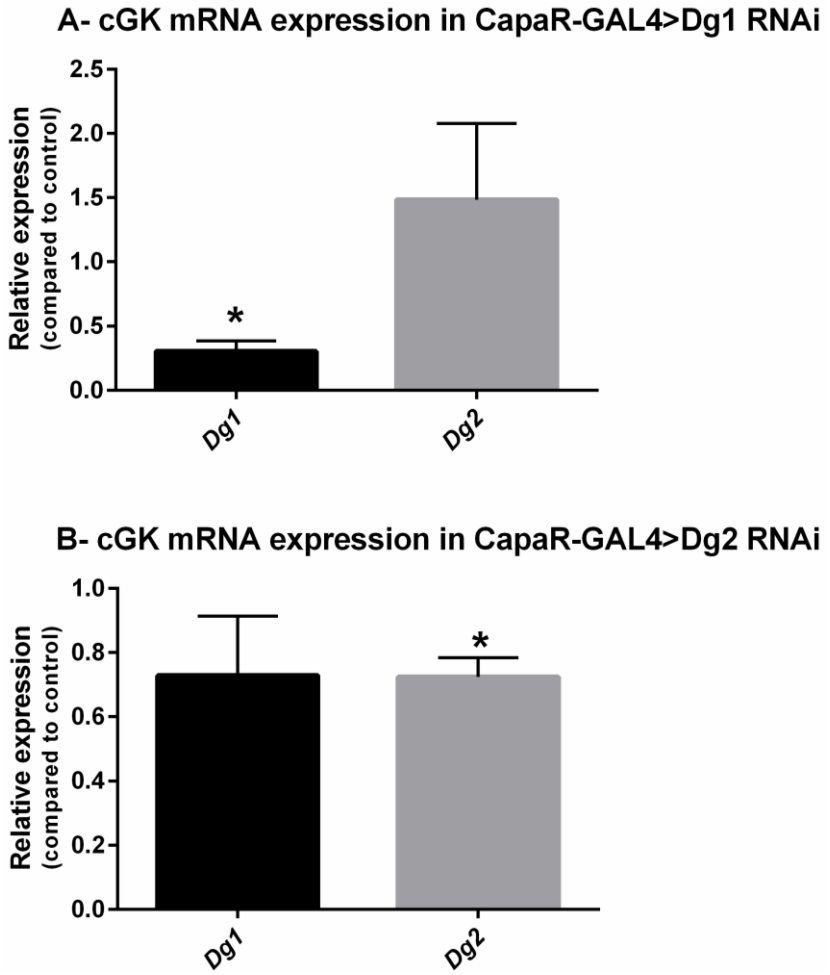


Figure 5-2: Tubule principal cell-specific knock down of cGK genes. In tubules of male flies fed normally, expression of *Dg1* (in black) and *Dg2* (in grey) in tubules expressing (A) UAS–*Dg1* RNAi or UAS-Control RNAi (B) UAS–*Dg2* RNAi or UAS-Control RNAi, under tubule principal cell specific CapaR-GAL4 was quantified by Q-RT-PCR. Data expressed as cGK gene expression ratio in tubules of cGK RNAi compared to that in Control RNAi tubules ± SEM (N = 3). Student t-test was used for testing statistical significance, where * indicates significance against Control RNAi, $p<0.05$.

5.2.3 Survival on different stressors

5.2.3.1 Osmotic stress

Salt stress or desiccation stress (loss of water) can potentially manifest into increase in ion concentration in the haemolymph, requiring optimal function of osmoregulatory tissues for survival. Down regulation of Relish in tubule principal cells causes loss of salt tolerance (chapter 3) and sensitivity to desiccation (Terhzaz et al., 2014). To investigate the role of cGKs in salt stress during these stressors, salt stress survival assay study of flies in which Dg1 or Dg2 expression was down- regulated down- regulated in tubule principal cells using CapaR-GAL4

was carried out. The flies expressing UAS-Control RNAi in presence of CapaR-GAL4 were used as control flies.

On a high salt diet, 50% of flies with down- regulated Dg1 in tubule principal cells survived for 5.5 days compared to the control flies that survived for 6.5 days (Figure 5-3A).

A similar result was observed in flies with down- regulated Dg2 in tubule principal cells, where the median survival of flies was 5.5 days while the medial survival of control flies was 6.5 days (Figure 5-3B). This suggests that Dg1 and Dg2 are important for salt tolerance. The survival of flies expressing UAS-Dg1 RNAi and UAS-Dg2 RNAi in presence of CapaR-GAL4 fed on normal diet was unaffected and showed similar viability to that of flies with UAS-Control RNAi and CapaR-GAL4 (data not shown).

To investigate if suppression of Dg1 and Dg2 expression in the tubule principal cells affected desiccation survival, flies expressing UAS-Dg1RNAi or -Dg2 RNAi and CapaR-GAL4 were placed in empty vials containing no food or water. After 12 h of stress the flies were counted every hour for death events.

The median survival of flies with down- regulated Dg1 was 19 h and they survived better than control flies whose median survival was 16 h (Figure 5-4A). A similar increase in survival phenotype was observed in flies with targeted knockdown of Dg2 in tubule principal cells whose median survival was 20 h compared to the control flies whose median survival was 17 h (Figure 5-4B).

Thus unlike Relish whose down regulation in tubule principal cells causes sensitivity to desiccation stress, down regulation of Dg1 or Dg2 in tubule principal cells helps improve desiccation tolerance.

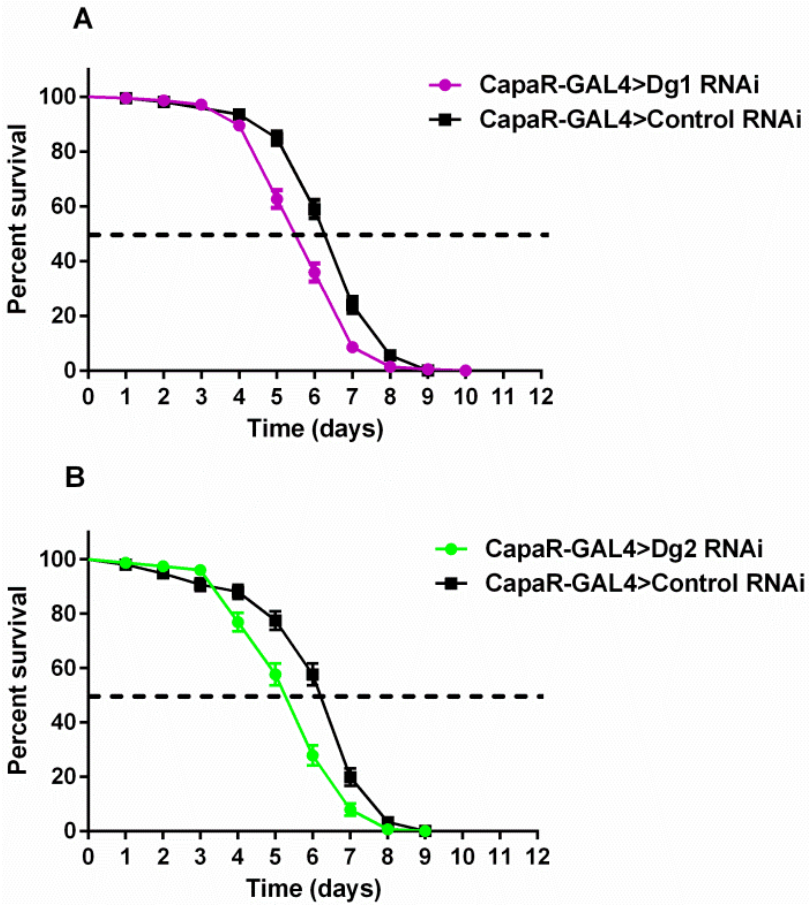


Figure 5-3: Survival during salt stress when Dg1 or Dg2 expression is suppressed in tubule principal cells. Male flies expressing Dg1 RNAi and CapaR-GAL4 (purple) (A) or Dg2 RNAi and CapaR-GAL4 (green) (B) and control flies expressing UAS-Control RNAi and CapaR-GAL4 (black) were starved for five hours before placing them onto food containing 4% NaCl. Data are expressed as percent survival ± SEM, n>110 flies for each genotype. ($p<0.001$ against Control RNAi flies; Log rank test, Mantel-Cox).

5.2.3.2 Immune stress

Insect tubules constitute an independent immune-sensing tissue (McGettigan et al., 2005) . Nitric oxide can modulate immune response (Dijkers and O'Farrell, 2007) as well as translocate Relish in tubule principal cells (Overend et al., unpublished data). Hence to deduce if the cGKs played a role in immune stress via transducing NO-generated cGMP, septic infection with non-lethal Gram-negative bacterial pathogen *Erwinia carotovora carotovora 15 (Ecc15)* was carried out. The flies were reared and subjected to septic infection with *Ecc15* or were mock-infected with saline at 25°C. *Ecc15* infection triggers the IMD pathway and induces antimicrobial peptide production through the NO signalling pathway (Basset et al., 2000; Dijkers and O'Farrell, 2007). Hence, Relish mutant

(Rel^{E20}) flies septically infected with *Ecc15* were used as positive control for this experiment.

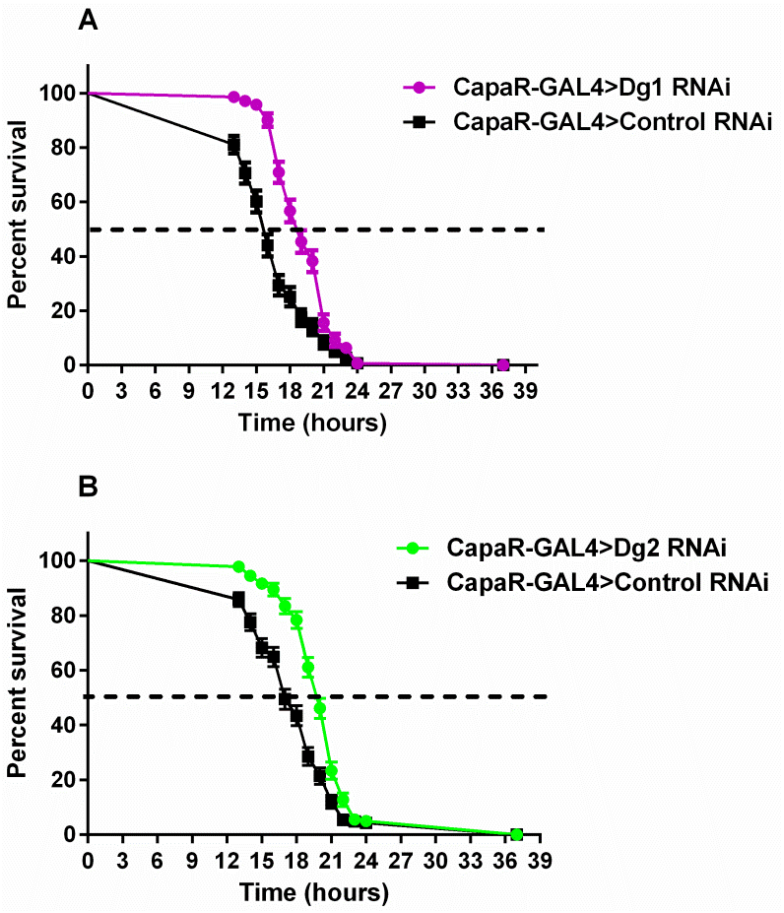


Figure 5-4: Survival during desiccation when Dg1 or Dg2 expression is suppressed in tubule principal cells. Male flies expressing Dg1 RNAi/CapaR-GAL4 (purple) (A) or Dg2 RNAi/CapaR-GAL4 (green) (B) and control flies expressing UAS-Control RNAi/CapaR-GAL4 (black) were placed in empty vials without food or water. Data are expressed as percent survival ± SEM, n>110 flies for each genotype. ($p<0.001$ against Control RNAi flies; Log rank test, Mantel-Cox).

The Rel^{E20} flies died within 24 h of septic infection. There was no significant difference in survival between flies with down- regulated Dg1 in tubule principal cells and control flies (Figure 5-5A). The flies with down- regulated Dg2 showed a reduced survival to infection but this sensitivity was observed only after 15 days of infection (Figure 5-5B). This difference may be due to age related impairments of signalling modules controlled by Dg2, as the mock infected flies too showed a decreased survival rate. Interestingly, this reduced survival due to age was exacerbated after *Ecc15* infection.

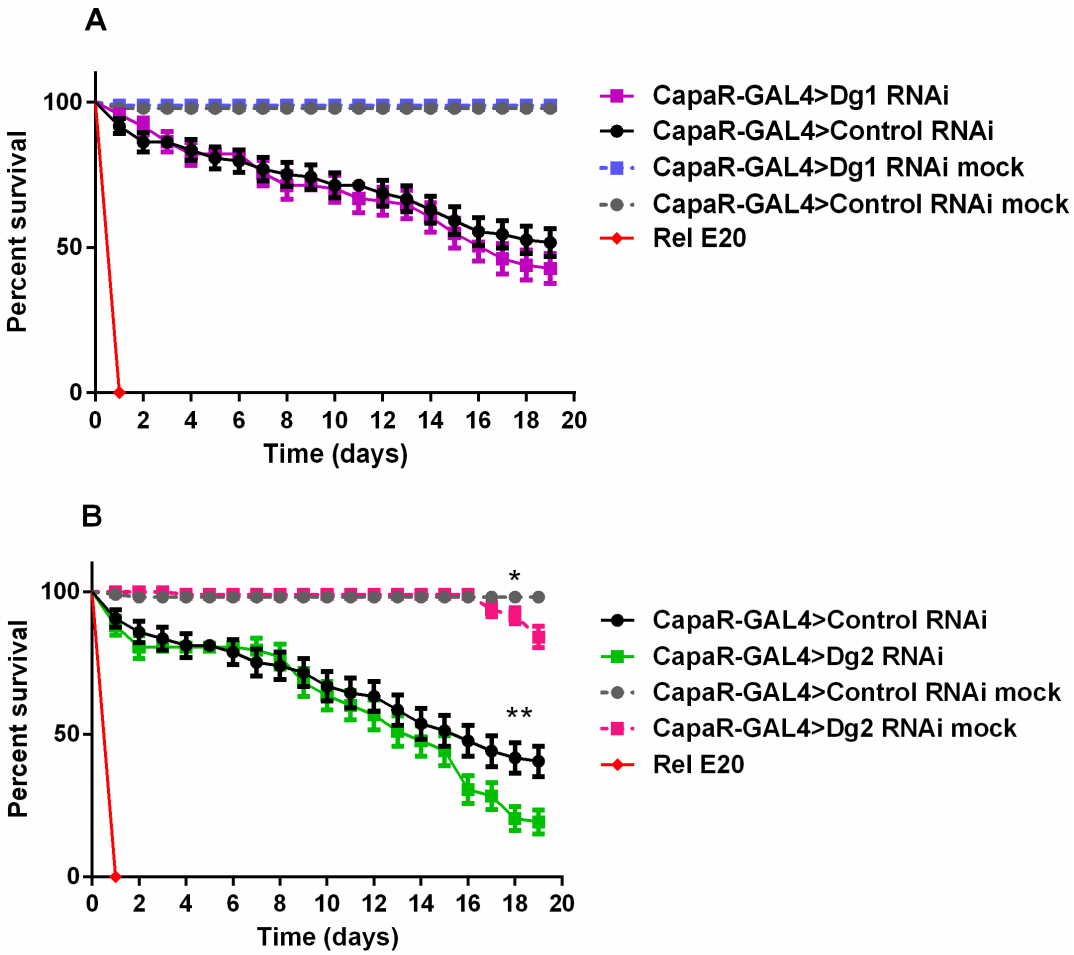


Figure 5-5: Survival after septic infection when Dg1 or Dg2 expression is suppressed in tubule principal cells. Male flies expressing Dg1 RNAi/CapaR-GAL4 (purple) (A) or Dg2 RNAi/CapaR-GAL4 (green) (B) and control flies expressing UAS-Control RNAi/CapaR-GAL4 (black) and Rel^{E20} (red) were septically infected with *Erwinia carotovora carotovora 15* and flies were reared at 25°C. Data are expressed as percent survival, n≥90 flies for each genotype. * denotes the $p<0.05$ against Control RNAi flies during mock treatment and ** denotes the $p<0.05$ against Control RNAi flies during septic infected with bacteria; Log rank test, Mantel-Cox)

5.2.4 Fluid secretion rate

Given that osmotic stress survival phenotypes were observed in flies with down-regulated Dg1 or Dg2 compared to control, and that tubules are key osmoregulatory tissues, the basal fluid secretion rate of these tubules under unstressed condition was assessed. No difference was observed in the basal fluid secretion rate between cGK RNAi and control RNAi (Figure 5-6). The basal fluid secretion rates for Dg1 RNAi, Dg2 RNAi and control RNAi were 0.31 nl/min, 0.34 nl/min and 0.35 nl/min respectively.

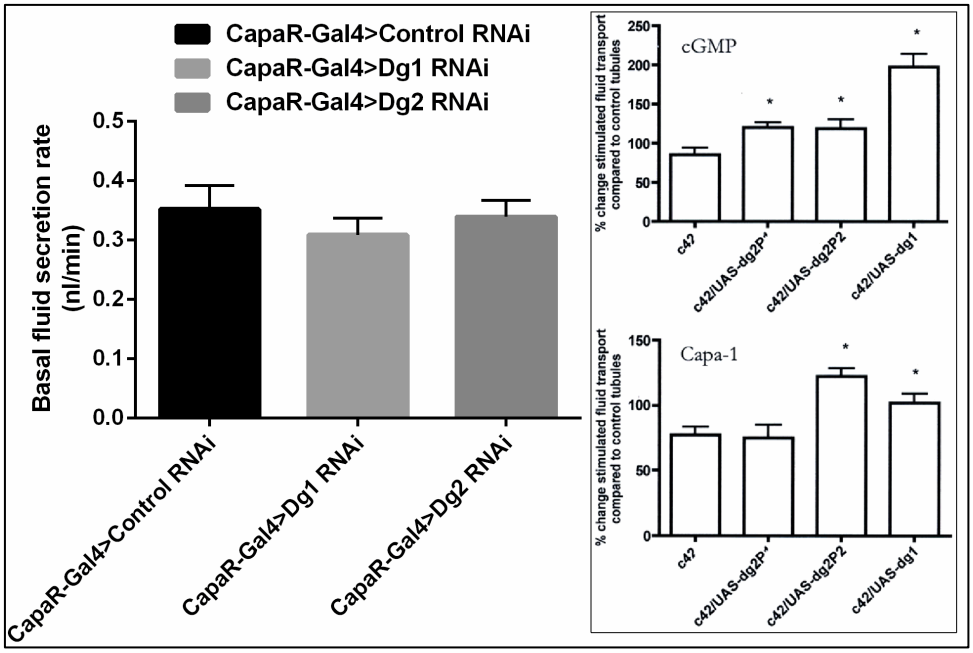


Figure 5-6: The basal fluid transport rates in tubules of flies when Dg1 or Dg2 expression is suppressed in tubule principal cells. The basal fluid transport rates in tubules of 10-12 day old male adult flies expressing UAS-Dg1 RNAi or UAS-Dg2 RNAi and CapaR-GAL4, control flies expressing UAS-Control RNAi and CapaR-GAL4 was measured for 60 mins.Data are expressed as fluid secretion rates (nanoliters/min) ± S.E,M., N=16 tubules. Inset: Percentage increase in fluid secretion rates of tubules overexpressing *Dg1*, *Dg2P1* and *Dg2P2* transcripts in tubule principal cells on stimulation with cGMP and capa-1 (MacPherson et al., 2004b).

Due to time constraints fluid secretion rate on stimulation with cGMP and capa-1 were not assessed. Previously, on stimulation with cGMP or capa-1, an increase in fluid secretion was observed for tubules overexpressing either Dg1 or Dg2 in principal cells (Inset Figure 5-6). Hence, it is likely that significant down regulation of these cGK genes in tubule principal cells will produce a reverse effect in fluid secretion when stimulated with cGMP or capa-1.

5.2.5 Percentage water loss during desiccation stress

As flies with down- regulated Dg1 in tubules survived longer during desiccation stress, the amount of water loss was determined in these flies when desiccated for 15 h. The percentage water loss in flies with down- regulated Dg1 was 23.7 % after 15 h of desiccation which was significantly lower compared to control flies that had a 28.0 % of water loss (Figure 5-7). As the UAS-Dg2 RNAi flies were not performing reliably by this stage of the project, this and following experiments were performed only on UAS-Dg1 RNAi flies.

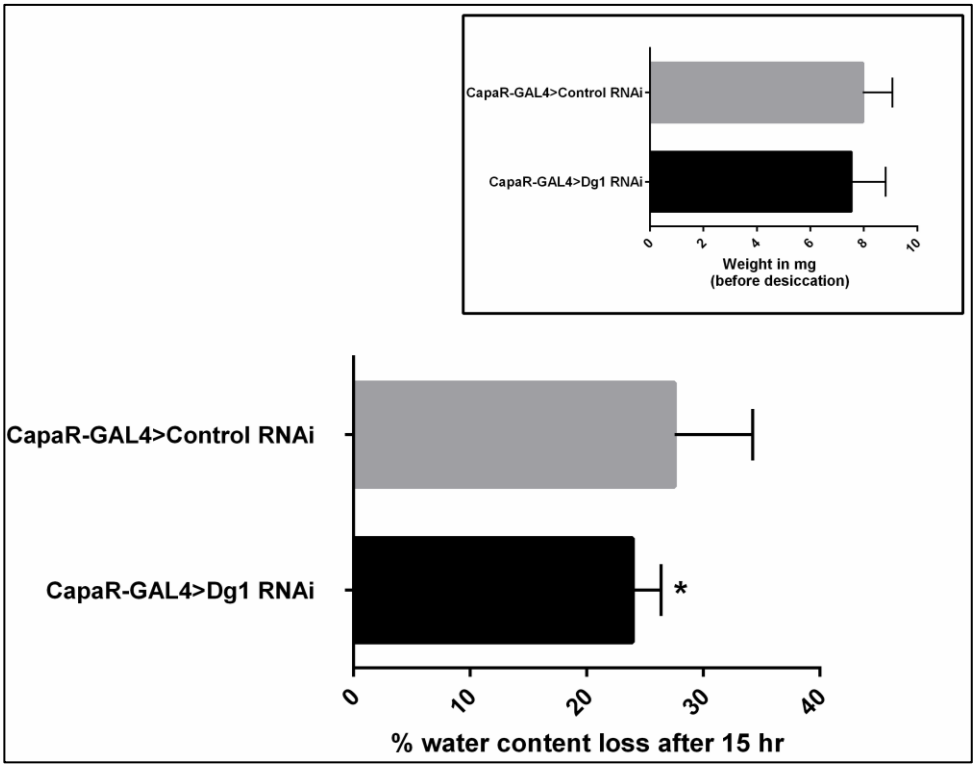


Figure 5-7: Percentage water loss after 15 hour of desiccation in flies with Dg1 supressed in tubule principal cells. 9-11 day old flies expressing UAS-Dg1 RNAi or UAS-Control RNAi and CapaR-GAL4 were weighed and kept in empty vials without food or water (inset). After 15 h, the flies were weighed and percentage of water loss was calculated. Data expressed as ± S.E.M. n≥ 110 flies ($p<0.05$ against Control RNAi flies; Two-Way ANOVA used for testing statistical significance).

The water loss in UAS-Dg1 RNAi is lower than control flies – may be due to tubules but perhaps also due to hindgut/absorption effects.

The percentage water loss correlates to shrunken abdomen phenotype when flies are desiccated (Terhzaz et al., 2015b).The shrunken abdomen was observed to be more pronounced in control flies compared to flies with down- regulated Dg1 (Figure 5-8).

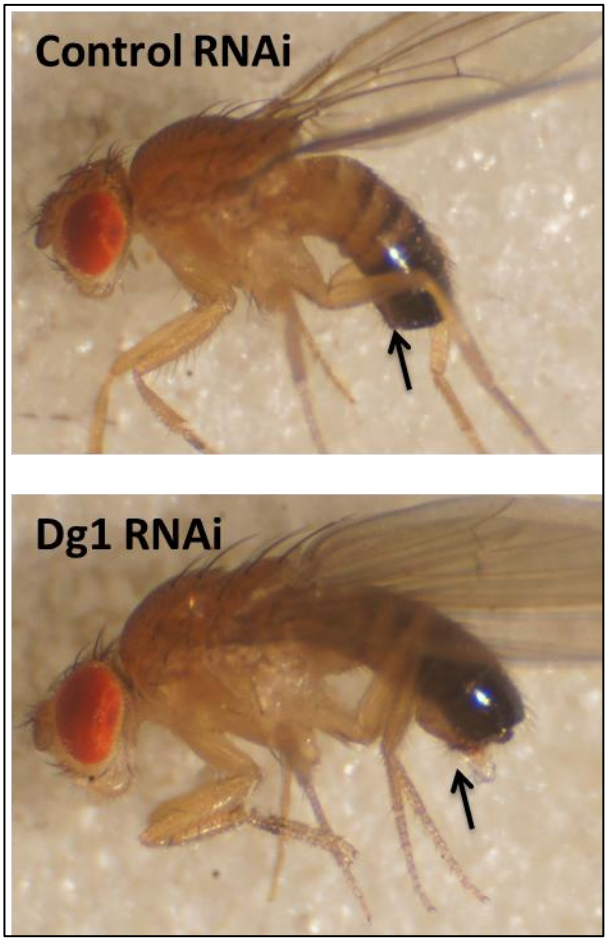


Figure 5-8: Effect of 15 hour of desiccation in flies expressing UAS-Dg1 RNAi or UAS Control RNAi and CapaR-GAL4. 9-11 day old flies expressing UAS-Dg1 RNAi or UAS-Control RNAi and CapaR-GAL4 were kept in empty vials without food or water. The photo was taken 15 h after desiccation. The arrows point to shrunken abdomen in each panel.

5.2.6 Expression of the immune gene, Diptericin, is reduced upon down regulation of Dg1 kinase

The down regulation of receptor GC-Gyc76C in tubule principal cells causes decreased basal *Diptericin* levels (Overend et al., 2012). Thus to investigate if Dg1 was downstream of Gyc76C, basal *Diptericin* levels were measured in the tubules of flies with down- regulated Dg1. The tubules expressing down-regulated down- regulated Dg1 had 37% decrease in basal levels of *Diptericin* compared to control flies (Figure 5-9).

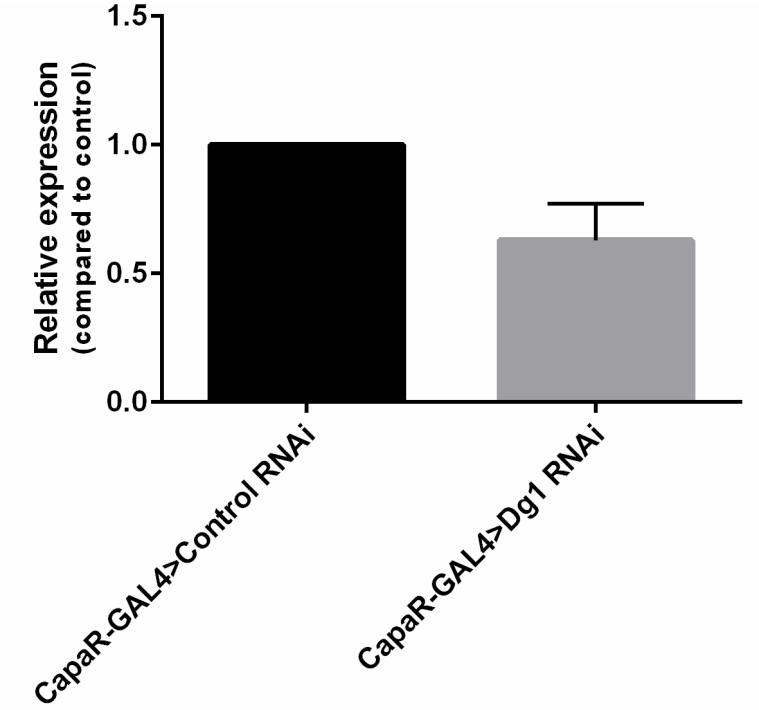


Figure 5-9: Basal Diptericin gene expression in tubules from flies expressing Dg1 RNAi in tubule principal cells. In tubules of male flies fed normally, *Diptericin* gene expression in tubules expressing UAS–Dg1 RNAi/UAS-Control RNAi under tubule principal specific CapaR-GAL4 was quantified by Q-RT-PCR. Data expressed as *Diptericin* expression ratio in tubules of Dg1 RNAi compared to Control RNAi. (N = 2). The values plotted were the mean ± S.E.M. of two independent biological replicates.

5.2.7 Co-transfection study of Dg1 and Relish S2 cells

As Diptericin expression indicates Relish activity (Hedengren et al., 1999; Stoven et al., 2000), the data in Figure 5-7 led us to investigate if Dg1 could potentially activate Relish. This was performed in Drosophila S2 cells. S2 cells were transiently co-transfected with Dg1 and Relish expression plasmids. The FLAG epitope of previously constructed FLAG-tagged Relish plasmid for S2 cells (in chapter 3) did not work efficiently for immunohistochemistry. Hence a c-myc-tagged Relish plasmid constructed by Dr. Jon Day (Dow/Davies labs, University of Glasgow) was used for this study. The activity of Relish protein expressed by this plasmid was validated by incubating S2 cells with the IMD pathway activator peptidoglycan (PGN) (concentration 30 µg/ml) and nuclear mobilisation of Relish was assessed by immunohistochemistry (Figure 5-S2). The previously characterised Dg1 plasmid expressing V5 tagged Dg1 in S2 cells (MacPherson et al., 2004b) was also used for this co-transfection study.

Dg1 protein and inactive Relish protein are localised in cytoplasm (MacPherson et al., 2004b; Stoven et al., 2000). On co-transfecting the S2 cells, localisation of the V5 tagged Dg1 and c-myc tagged Relish proteins were assessed by immunohistochemistry using mouse-anti-V5 and rabbit-anti-c-myc antibodies. The S2 cells transfected with only Relish plasmid and stimulated with PGN showed Relish localised in the nucleus (Figure 5-10A and Figure 5-S2). In S2 cells co-expressing Dg1 and Relish protein, no Relish translocation was observed (Figure 5-10B).

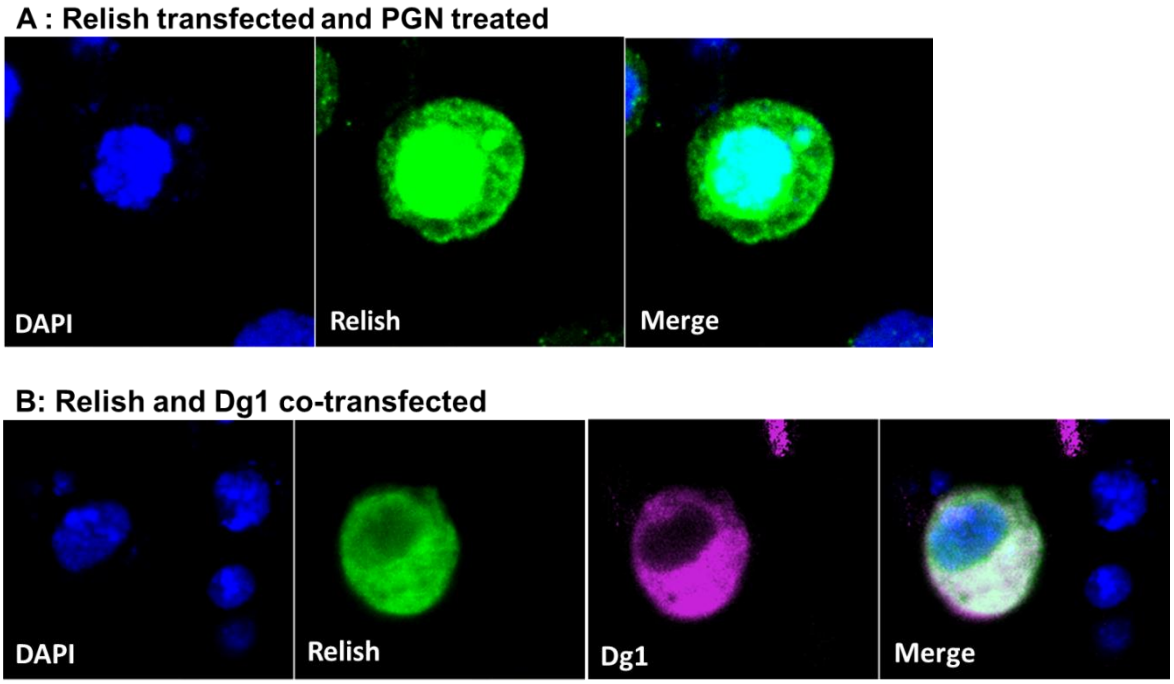


Figure 5-10: Relish translocation study in S2 cells. (A) S2 cell expressing c-myc-Relish protein was stimulated with 5 µg/ml PGN and nuclear mobilisation of activated Relish protein was studied by probing S2 cells with anti-cmyc antibody. **(B)** S2 cell co-expressing cmyc tagged Relish and V5 tagged Dg1. The cell was probed with rabbit-anti-c-myc and mouse-anti-V5 antibodies. No nuclear Relish translocation was observed in (B) as seen in (A). The nuclei stained DAPI is shown in blue, c-myc-Relish in green and Dg1-V5 in purple.

Thus it may be inferred that Dg1 does not activate Relish under unstressed conditions but one cannot discount the probability that in *in vivo* conditions, or under stressed or neuropeptide-stimulated conditions, Dg1 may be able to phosphorylate Relish, thus warranting further studies of Relish translocation under stressed conditions or neuropeptide stimulation in tubules down regulating Dg1 and overexpressing Relish.

5.3 Discussion

The *Drosophila* Malpighian tubules are blind-ended, hence the absorption of water, ions and solutes occur by transepithelial flux (Beyenbach et al., 2010; Dow and Davies, 2001). At low cGMP concentration, an increase in transepithelial potential of *Drosophila* Malpighian tubules is observed which correlates to increase in fluid secretion but at non-physiologically high cGMP concentration, a reduction in transepithelial potential is observed along with decreases in fluid secretion (Davies et al., 1995). cGMP increases transepithelial cation transport across the main segment of *Drosophila* tubules (Bijelic and O'Donnell, 2005) and can activate cGKs in tubules (MacPherson et al., 2004b). In addition, one of the aspects of cGMP mediated fluid transport phenotype is dependent on Ca^{2+} signalling as it was observed that exogenous cGMP directly or partially through cGKs (studied by pharmacological inhibition of cGK) activates CNG- Ca^{2+} channels to increase intracellular calcium levels leading to increase in fluid transport ((Broderick et al., 2003; MacPherson et al., 2001), (Broderick, 2002)). Also, it was observed that in tubules treated with cG-PDE inhibitors, fluid secretion was stimulated at cGMP concentration (10^{-7}M) unlike the untreated tubules which did not produce any change in fluid secretion when stimulated with 10^{-7}M cGMP (Broderick, 2002). In addition, capa neuropeptide inhibits the cG-PDE activity to increase fluid secretion (Broderick et al., 2003). This indicates that the intracellular levels of cGMP and its downstream effectors such as cGKs, cG-PDEs and CNGs channels play an important role in secretion and absorption functionality of the tubules.

As mentioned earlier that the two functionally characterised cGKs- Dg1 and Dg2; in tubule principal cells have different cellular localisation and hence may be transducing differentially localised cGMP signals. It was postulated in Chapter 3 that capa-1 and NPLP1-VQQ is released in response to salt stress, activate Relish in tubule principal cells through Ca^{2+}/NO-cGMP pathway. NPLP1-VQQ increases cGMP concentration in tubules and a small increase in Ca^{2+} concentration that may be attributed to influx of Ca^{2+} through CNG(Overend et al., 2012). Gyc76C receptor, localised at the basolateral membrane is activated by NPLP1-VQQ, and its knockdown in tubule principal cells produces a salt-sensitive phenotype

(Overend et al., 2012). It is likely that one of the downstream effectors of cGMP signalling generated by the activation of Gyc76C is Dg2 localised at the basolateral membrane.

The subcellular localization of cGMP is an important determinant of the tubule fluid transport phenotype (Broderick et al., 2003). The overexpression of cytosol localised Dg1 increases fluid secretion in response to cGMP and capa-1 (MacPherson et al., 2004b). Thus it may be inferred that Dg1 is transducing cGMP pools generated by NO-activated sGC in cytoplasm by capa-1. In addition, the overexpression of dNOS in tubule principal cells significantly increases cGMP (Broderick et al., 2003) and dNOS-associated increase in tubule principal cell Dg1 activity but not Dg2 activity has been observed (Overend et al, unpublished).

Taken together, it may be inferred that during salt stress one of the downstream effectors of capa-1 is Dg1 which transduces the cytoplasmic cGMP signal produced by capa-1 activated sGC. Hence down regulation of Dg1 in tubule principal cells causes the observed salt sensitive phenotype. Overexpression of *Dg2P2* in tubule principal cells shows an increase in tubule fluid secretion rate on stimulation with capa-1 hence Dg2 may also be a downstream effector of capa-1 in response to salt stress. Altogether it may be suggested that during salt stress, Dg1 and Dg2 in tubule principal cells are downstream targets of cGMP signals generated by capa-1 and NPLP1-VQQ neuropeptide; absence of which leads to sensitivity to salt stress.

The capa-1 neuropeptide has been associated with desiccation tolerance. The down regulation of capa-1 in the capa producing neurons or down regulating the capa-1 receptor in tubule principal cells, increases tolerance to desiccation compared to the parentals (Terhzaz et al., 2012; Terhzaz et al., 2015b). As down regulating Dg1 or Dg2 in tubule principal cells increases survival during desiccation, it further affirms that Dg1 and Dg2 are downstream effectors of capa-1. Terhzaz et al.,2015 observed that down regulating capa-1 production decreased water loss in flies during desiccation stress. Similarly during desiccation stress, percentage water loss in flies with down- regulated Dg1 in tubule principal cells is low compared to control flies. This implies that *in vivo,*

Dg1 has a role in fluid secretion during desiccation stress. As the difference in percentage water loss between the control and flies with down- regulated Dg1 is small, this suggests that more than one downstream effector of capa-1 in tubule principal cells may exist which contributes to fluid secretion phenotype.

Although Relish in tubule principal cells is activated and mobilised to the nucleus by NPLP1-VQQ/Gyc76C and Capa-1/CapaR, none of these peptides /receptors show an immune stress phenotype when infected with non-lethal pathogen (Overend et al., 2012; Terhzaz et al., 2014). No immune stress phenotype was observed when flies expressing either of the cGK genes were down- regulated in tubule principal cells and infected with non-lethal bacteria *Ecc15*. However, a decrease in basal *Diptericin* levels was observed in tubules with down- regulated Dg1 compared to control, a result similar to when Gyc76C is down- regulated in tubule principal cells (Overend et al., 2012).

Altogether the data suggest that cGK-regulated physiological effects in the tubule principal cells can be either beneficial or detrimental to survival, depending on the type of stress. cGMP targets may not always transduce fluid secretion phenotype but can be involved in modulating activity of other proteins, producing various physiological effects (Broderick et al., 2003). As seen in vertebrates, cGKs phosphorylate different substrates, which affect physiological outcomes (Hofmann et al., 2006). For example, cGKIα in the HEK293 kidney cell line phosphorylates transient receptor potential isoform 3 (TRPC3) causing inhibition of store operated calcium influx (Kwan et al., 2004). Also, cGKII in an intestinal cell line (IEC-CF7) phosphorylates CFTR channel leading to stimulation of chloride channel (Vaandrager et al., 1997). In Drosophila, cGK- Dg2 has been shown to phosphorylate the forkhead box transcription factor FoxO in dopaminergic neurons that causes neurodegeneration in a *Drosophila* model of Parkinson's disease (PD) (Kanao et al., 2012). Thus other than contributing to a fluid transport phenotype during osmotic stress, Dg1 and Dg2 may phosphorylate other substrates that modulate physiology of the tubule to tolerate stress.

The NO activated sGC pathway in tubule principal cell increases *Diptericin* levels (Davies and Dow, 2009) and activates Relish in tubules (Overend et al., unpublished). The down regulation of Gyc76C receptor in tubule principal cells decreases basal *Diptericin* levels and no Relish activation is observed on stimulation with NPLP1-VQQ (Overend et al., 2012). To investigate if Dg1 can activate Relish, Dg1 and Relish proteins were expressed in Drosophila S2 cells. The overexpression of Dg1 in tubule principal cells increases the cGK activity in tubules by 6.16± 0.7 fold over the wild type tubules under unstressed conditions (MacPherson et al., 2004b). A similar likelihood was considered for S2 cells overexpressing Dg1. Overexpressing Dg1 and Relish in S2 cells did not activate Relish. This may be due to Dg1 activity not being sufficient for activating Relish, requiring an optimum level of cGMP (possibly produced by stimulus) to potentiate its activity.

Application of exogenous cGMP to tubules can significantly elevate cA-PDE activity in the tubules (Broderick, 2002). A pilot peptide array study has shown Dg1 to interact with Pde11, a dual specific phosophodiesterase (Finlayson, 2010). This interaction of Dg1 and Pde11 may likely be contributing in maintenance of intracellular cGMP and cAMP levels during stress, in turn dictating the fluid secretion phenotype and activity of other cyclic nucleotide dependent proteins. Therefore, further studies to find the interacting partners of cGKs in tubule principal cells will increase the understanding of the activity of these kinases in the insect renal system especially during stress.

From the data in this chapter and from the understanding acquired from previous *in vitro* secretion assays and pharmacological studies performed on *Drosophila* tubules, it is plausible to assign an *in vivo* stress-associated physiological role to Dg1 and Dg2 expressed in the principal cells of the Malpighian tubules. However, further studies are warranted prior to assigning them this *bona-fide* role.

5.4 Supplementary Data

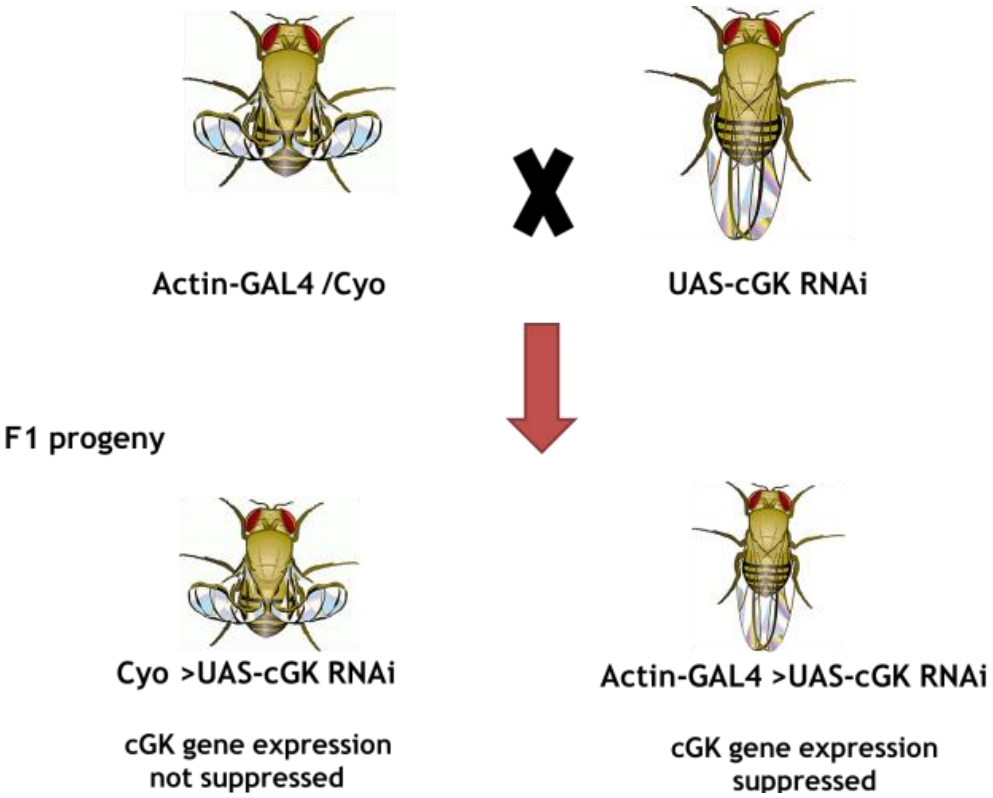


Figure 5-S1: Cross scheme for characterization of cGK gene RNAi lines.: The UAS cGK RNAi (UAS-Dg1 RNAi or UAS-Dg2 RNAi) male flies were mated with Actin-GAL4/Cyo female flies. The F1 progeny were assessed for their wing phenotype. The curly wings indicated that the fly did not express UAS RNAi. Straight wings were an indicator of flies expressing the gene specific UAS RNAi line in all the tissues.

Figure 5-S2: Relish translocation study in S2 cells.

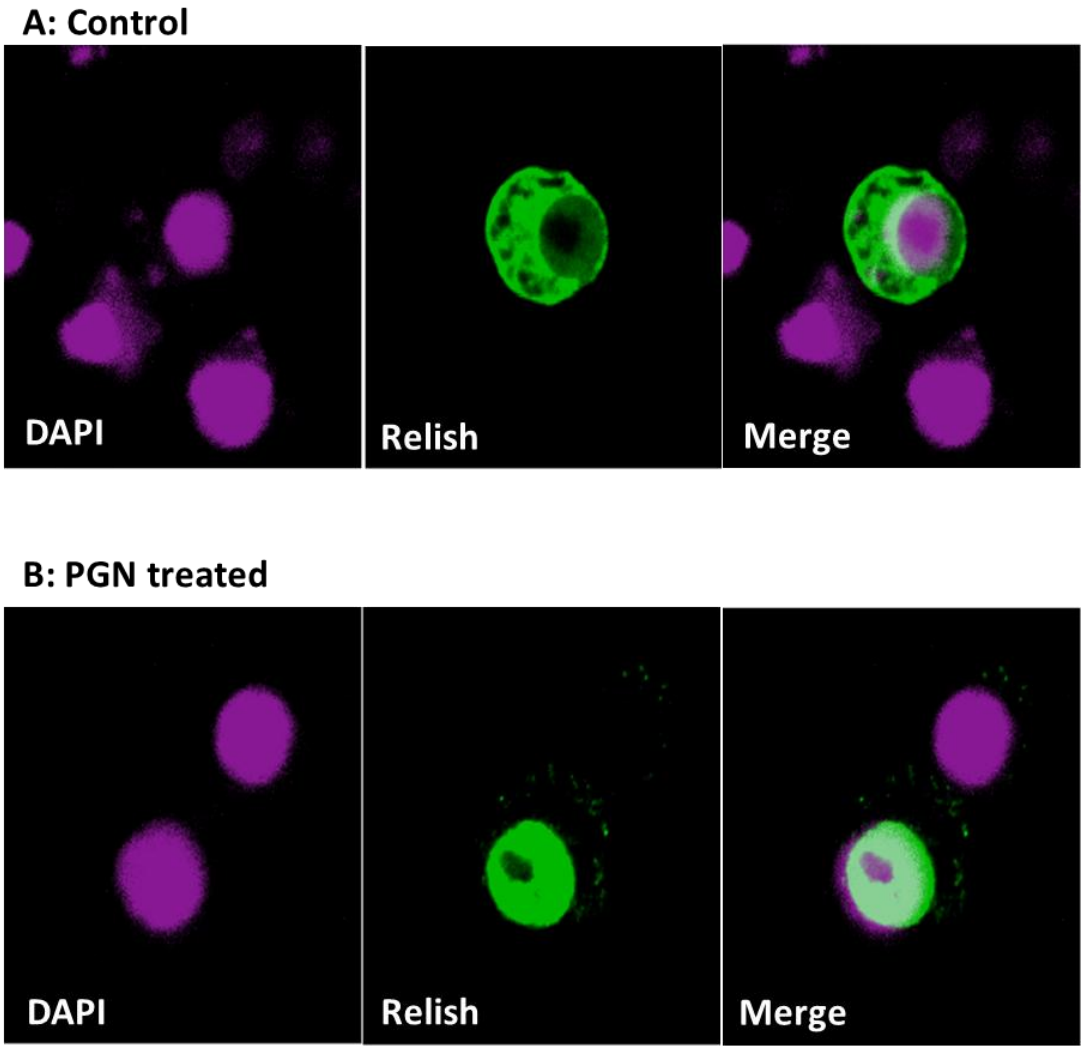


Figure 5-S2: Relish translocation study in S2 cells. S2 cell expressing c-myc-Relish protein was incubated in control medium (A), or 30 µg/ml PGN containing medium (B) for 30 mins. The nuclear mobilisation of activated Relish protein was studied by probing S2 cells with anti-c-myc antibody. The nuclei were stained with DAPI. The nuclear Relish translocation was observed as seen in (B). The nuclei stained DAPI is shown in purple and c-myc-Relish in green.

Chapter 6

Summary, Conclusion and Future Work

Chapter 6

6.1 Summary and Conclusion

The Malpighian tubules are blind-ended hence the fluid secretion and absorption takes place through transpethilal flux and osmosis (reviewed in (Dow, 2009; Dow and Davies, 2003)). As the tubules are not innervated, communication with other tissues occurs through factors floating in haemolymph that bind to the receptors on the tubule cells (Soderberg et al., 2011). The strategic location of these osmoregulatory epithelial tissues in the insect body helps them to communicate to the midgut and hindgut as well as the cuticle (Chintapalli et al., 2012). Hence the genetically tractable Malpighian tubules of the *Drosophila* make an excellent model for studying *in vivo* epithelial stress handling mechanisms.

6.1.1 Relish, cGMP-dependent kinases and neuropeptides

The studies carried out in this PhD work have demonstrated that Relish, an important transcription factor in humoral immune response of the insect(Hedengren et al., 1999) in required for salt stress tolerance and is activated by an *in vivo* mechanism in tubules. The likely mechanism of this activation may be through the neuropeptide receptor signalling. In accordance to results of Naikkhwah and O'Donnell, (2012), there was no change in basal fluid secretion rates between the stressed and unstressed tubules of wild type flies. Furthermore, Naikkhwah and O'Donnell, (2012) suggested that a haemolymph factor that increases intracellular calcium (Ca^{2+}) may be playing a role in response to salt feeding to increase fluid secretion. As neuropeptides/receptor : NPLP1-VQQ/Gyc76C and capa/capaR leads to increase in intracellular calcium and cGMP and have previously demonstrated to activate Relish (Broderick et al., 2003; Overend et al., 2012; Terhzaz et al., 2014), it may be that *in vivo,* these neuropeptides are activating Relish and working additively or synergistically in response to salt stress. In addition, the microarray data of salt stressed wild type and Relish mutants showed an upregulation of *CanA1* that encodes Calcineurin A1(a calcium and calmodulin dependent serine/threonine phosphatase) which is known to activate Relish in response to immune stress (Dijkers and O'Farrell, 2007). Altogether the data suggests that Ca^{2+}/ cGMP signalling may play a role in Relish activation in response to salt stress.

One cannot discount the fact that salt stress increases the chloride ion load in the haemolymph. Thus during salt stress, the secretion of chloride ions from the tubules may occur via the stellate cells. This secretion mechanism is likely triggered by either neuropeptide Drosokinin and/or biogenic amine tyramine which increase intracellular calcium ions accompanied by increase in fluid secretion and chloride conductance (Cabrero et al., 2013; O'Donnell et al., 1998; Terhzaz et al., 1999).

The flies with down- regulated cGMP-dependent kinases (cGKs) (Dg1 or Dg2) in the tubule principal cells were sensitive to salt stress but tolerant to desiccation stress compared to the control flies. Previously it was demonstrated that an overexpression of either of the cGMP-dependent kinase (cGKs) in the tubule principal cells increases the fluid secretion rate of tubules when stimulated with cGMP or neuropeptide capa-1(MacPherson et al., 2004b). The survival assay results here in and the previous *in vitro* secretion assay results of the cGKs overexpressors in the principal cells suggest these kinases as downstream effectors of the neuropeptides NPLP1-VQQ and capa-1 that are released during osmotic stress (salt stress and/or desiccation stress). The response of these kinases to the neuropeptide signalling is either beneficial or detrimental depending on the type of stressor.

The expression of *Dg1* and *Dg2* in the wild type tubules under unstressed conditions was observed to be independent of each other. In the microarray data, the expression of these kinases remained unaltered after 24 hour of salt stress in the tubules of the wild type. In contrast, in the salt stress tubules of Relish mutants, the expression of *Dg2* was upregulated while *Dg1* expression was down regulated. Was this modulation in expression of these genes in the salt stressed Relish mutants due to loss of transcriptional regulation by absence of Relish or a rescue mechanism for survival?

Relish activation in tubule principal cells via soluble guanylate cyclase (sGC) and NPLP1-VQQ via receptor Gyc76C has been previously demonstrated (Overend et al., 2012)(Overend, unpublished). To study if the cGMP signals produced by sGC and NPLP1-VQQ/Gyc76C were transduced via Dg1, Dg1 and Relish protein were

overexpressed in the *Drosophila* S2 cell model system. A protein-protein interaction with Dg1 and Relish under resting (unstressed) condition could not be established but an interaction between them may occur *in vivo* triggered by a neuropeptide signal. This in turn may be dictating the transcriptional regulation of Dg1.

In *Drosophila*, there are six diuretic neuropeptides and a biogenic amine identified that stimulate the principal and stellate cells in the main segment of the tubule to secrete fluid. It has been suggested that some of these diuretic neuropeptide may act as "clearance" factors, working by increasing secretion of the tubules and water ion reabsorption by the lower tubules and hindgut to passively clear the haemolymph of waste products (O'Donnell et al., 2003). In addition, it has been suggested by Naikkhwah and O'Donnell, (2012) that other tissues may be involved in removal of excess sodium ions from the haemolymph of the *Drosophila*. Hence it can be hypothesised that multiple tissues and multiple neuropeptides work in coordination to tolerate salt stress. The other neuropeptide that may play a role in salt tolerance is Diuretic hormone 44 (Dh44) that stimulates fluid secretion in the tubules through cAMP signalling(Cabrero et al., 2002). The whole fly knockdown of Dh44 receptor 2 (Dh44-R2) produced a salt sensitive phenotype (Hector et al., 2009). In addition, in the microarray data here in the expression of Dh44 was upregulated in salt stressed tubule of Relish mutants that we suggested as a localised rescue system. Moreover, suppression in expression of Dh44-R2 in the tubule principal cells made the flies more sensitive to salt stress compared to the control flies (Figure 6-1). cAMP and cGMP can increase transepithelial cation flux in the tubules(Bijelic and O'Donnell, 2005). Thus, it may be postulated that the synchronised effects of Ca^{2+}/cGMP signalling neuropeptides Gyc76C and capa-1, and cAMP signalling neuropeptide Dh44 may be acting on the tubules to increase secretion of Na^{+} ions as well as modulating one other's diuretic effects perhaps through activation of phosphodiesterases.

Simultaneously efflux of chloride ions may be occurring via the stellate cells through Ca^{2+} signalling by diuretic peptide Drosokinin and/or biogenic amine tyramine. It is plausible that *in vivo* the ligands (NPLP1-VQQ, Dh44, Drosokinin)

may be stimulating their respective receptors on the hindgut thus acting as "clearance factors" as mentioned earlier.

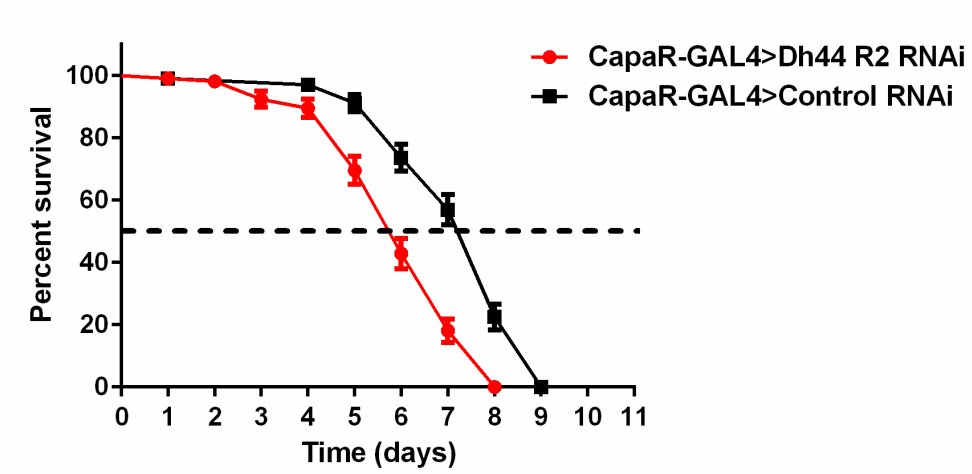


Figure 6-1: Survival on salt stress when Dh44-R2 expression is suppressed in the tubules. Dh44-R2 in tubule principal cells was down- regulated using CapaR-GAL4 driver. Male flies of each genotype were starved for five hours before placing them onto diet containing 4% NaCl. Data are expressed as percent survival ± SEM, n>110 flies for each genotype. ($p<0.001$ against Control RNAi flies; Log rank test, Mantel-Cox). The percentage knockdown of Dh44-R2 in tubule principal cells was ~60 % (Cannell E., unpublished). The median survival of CapaR-GAL4>Dh-44 R2 flies was 6 days and CapaR-GAL4>Control RNAi was 8 days.

An interesting finding in our microarray data was increase in expression of Ion transport peptide (ITP) for salt stressed tubules of Relish mutants. ITP stimulates chloride transport and fluid reabsorption in the hindgut of desert locust (Phillips et al., 1998). In *Drosophila* ITP localisation has been seen in the brain and hindgut-innervating abdominal ITP neurons (Dircksen, 2009; Dircksen et al., 2008). The recent study has assigned ITP as a peptide associated with clock-mediated behavioral control (Hermann-Luibl et al., 2014). The Flyatlas data shows *ITP* expression in tubules as low (mRNA signal 92 ± 19), thus the increase in expression of this peptide ~4 fold in salt stressed tubules of Relish mutants provides a new insight for this peptide and suggests a potential communication occurring between hindgut and tubules.

6.1.2 Relish regulated genes and pathways

An upregulation of stress response pathways, especially the c-Jun kinase (JNK) pathway was observed in salt stressed tubules of the Relish mutants. This suggested that Relish and its target genes are required to attenuate the response

of these stress response pathways. The JNK pathway is known to be stimulated as a response to salt stress (HuangFu et al., 2006; Ouwens et al., 2001) and an upregulation of one of the known target gene of the JNK pathway (*CG13482*) in salt stressed tubules of wild type and Relish mutants was observed. This and other microarray data suggests that similar to immune stress (Kim et al., 2007; Park et al., 2004) there occurs a cross talk between Relish and JNK pathway during salt stress in the tubule. However, in absence of Relish and its target genes, the JNK pathway becomes hyper activated and may be playing a redundant role as a survival mechanism.

In *Drosophila* larvae, different concentrations of dietary salt, alter feeding behaviour (Niewalda, Russell 2010). Whether the adult flies also have a change in feeding behaviour in respect to dietary salt is not known. It was seen that starvation response genes- *FOXO*, *Insulin Receptor (InR)* and autophagomse related genes *ATG1*, *ATG18* were being upregulated in salt stress Relish mutants only. This raises questions whether the mutant flies were starving or whether the hyper activation of JNK pathway was antagonising the insulin/IGF signalling (IIS) pathway (Karpac and Jasper, 2009) leading to starvation response. A localised DNA damage causes Relish in fat body to be activated by FOXO in response to suppressed IIS activity to regain growth homeostasis (Karpac et al., 2011). Hence it may be speculated that a similar mechanism of FOXO related activation of Relish may be required in the tubules to gain homeostasis and regulate IIS pathway during salt stress. The loss of this mechanism in Relish mutants may be leading to starvation like response. Alternatively, as Relish is expressed in the nervous system and mediates transcription which is controlled by the circadian rhythm (Tanenhaus et al., 2012) ; it may be that any feeding behavioural response related to feeding on salt diet and controlled by Relish activation may be absent in the Relish mutants. Thus, it will be useful to measure the rate and volume of food intake by using methods such as capillary-based feeding method, in Relish mutant and wild type adult flies. This measurement would help in differentiating whether the upregulation of starvation response genes in the tubules of salt stress Relish mutants was due to alteration in feeding behaviour or the hyper activation of JNK pathway leading de-regulation of IIS pathway.

A down regulation of transporter genes in the salt stressed Relish mutants may be accounted for loss of transcriptional regulation of these genes due to absence of Relish and its targets genes. Another explanation may be that down regulation of these genes was a potential mechanism to combat osmotic stress. Both these explanation need further investigation by assessing gene expression over a time course of salt stress as well as studying the expression of these genes under another osmotic stressor such as desiccation stress.

The preliminary immunohistochemistry study demonstrated Relish to be localised in the principal cells. Also a basal activity of Relish was detected in the resting (unstressed) tubules, as a weak expression of Diptericin protein (a Relish target gene) was observed in immunohistochemistry studies. The basal fluid secretion rate was significantly lower in the Relish mutants than the wild type illustrating that Relish plays a role in fluid secretion phenotype of the tubule. In Relish mutants, under unstressed conditions the mRNA expression of detoxification enzymes belonging to certain families of glutathione-S-transferases (GSTs) and cytochrome P450 (CYPs) in tubules were altered. In addition, the expression of genes encoding for transporters and water channels were also altered. For example: the expression of *Glut1* (encoding a glucose transporter), *CG6293* (encoding a sodium ascorbate transporter) was down-regulated while *CG17664* encoding for aquaglyceroporin was upregulated. The majority of genes whose expression was affected in the tubules of unstressed Relish mutants played a role in detoxification, redox and metabolic processes. This implies that *in vivo*, Relish may be responding to other physiological signals other than stress signals and controlling expression of genes for optimal working of the organism. While drawing conclusions from the results seen in the tubules of Relish mutants under unstressed conditions, there is a possibility that Relish regulation of certain genes in tubules is affected by a Relish-dependent signal generated in another tissue; thus, the effects seen in the tubule for that particular gene may not be a direct cause of loss of Relish in the tubule.

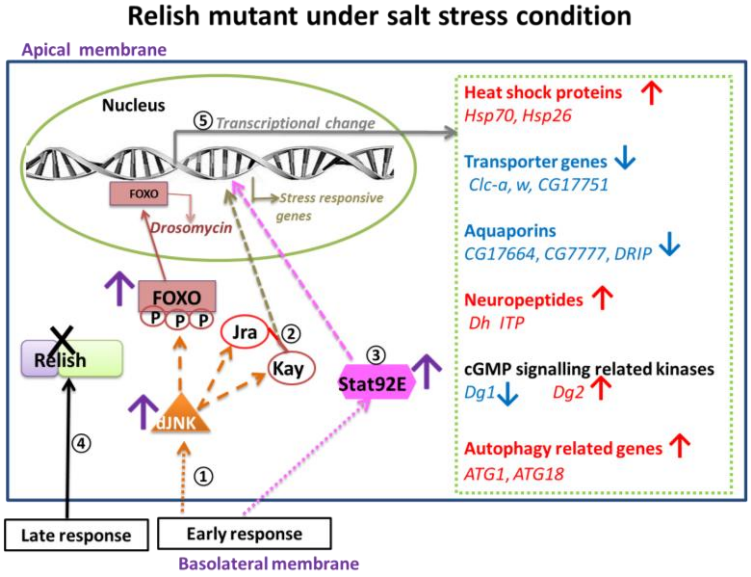
As suggested in this study that Relish and its target genes exert its effect on the expression of other genes under stressed and unstressed conditions, a comparison was performed between the tubule microarray data studied here and

Diptericin (a Relish target gene) overexpressing whole fly microarray data of Zhao et al. (2011). A variation factor in the comparison of the datasets is the different tissues used for generating the datasets. While the study here is limited to tubules, Zhao et al. studies are in whole fly.

The expression of *CG17751* (encoding for a tubule specific organic cation transmembrane transporter) and *Cyp6a20* (encoding for a type of cytochrome P450 expressed only in adult tissues), were recognized in the *in silico* analysis as Relish dependent genes under salt stress. The expression pattern of these genes under stress was a similar in the flies overexpressing Diptericin. Similarly, the fold change expression of *GstE5* and *Cyp4e1* in tubules of unstressed Relish mutants vs. wild type compared to *Diptericin* overexpressing flies vs its parental, suggested that the regulation in expression of these genes required the basal activity of Relish. These results affirmed the methodology of the *in silico* study carried out here in to identify Relish dependent genes during salt stress in tubules and also the hypothesis that at least one known Relish target gene was affecting the expression of other genes.

The Figure 6-2 summarizes the molecular changes that occur in the Relish mutants compared to the wild type under salt stress and control conditions. This summary is proposed based on the

A



B

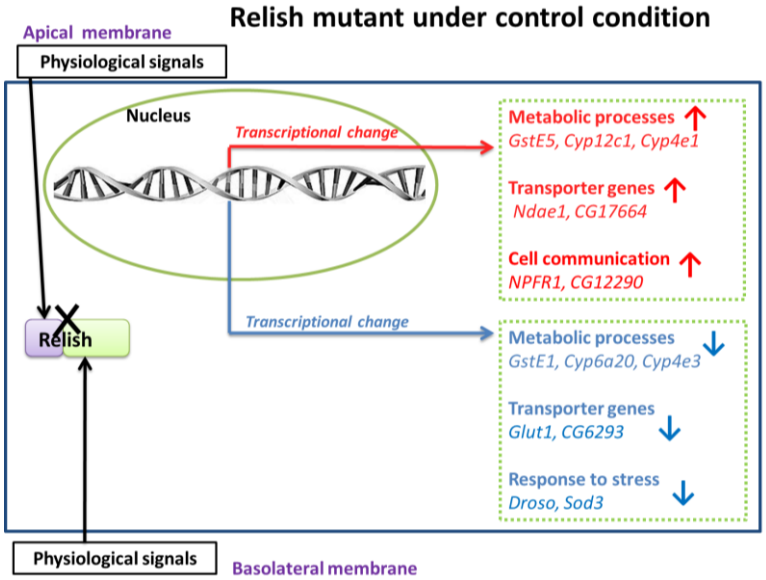


Figure 6-2: Summary of the molecular changes that occur in the tubule principal cell of Relish mutant (Rel^{E20}) under salt stress and control conditions. This is proposed based on the tubule microarray data. **(A)** The molecular mechanism changes in Rel^{E20} under salt stress. ①The early response to salt stress causes the JNK pathway to be activated, which activates FOXO. On activation, FOXO enters the nucleus, increasing expression of *Drosomycin*. ②The activation of JNK pathway activates Jra and Kay which make the dAP-1 complex. ③The JAK-STAT pathway is activated in response to salt stress, causing activation of Stat92E. The activation of dAP-1 and Stat92E leads to transcription of stress responsive genes. ④As the stress continues (late response), neuropeptides released in haemolymph in response to salt stress trigger their respective receptors at the basolateral membrane leading to Relish activation in wild type, which in turn leads to activation of genes that attenuate the JNK and the JAK-STAT pathway (not shown). In Rel^{E20}, absence of Relish protein (marked as X) causes hyper activation of the stress pathways discussed above (shown in purple arrows ↑). ⑤The absence of Relish protein also causes differential expression of the genes shown in the green box. **(B)** Under control conditions, in response to physiological signals and in absence of Relish protein in Rel^{E20} (marked as X), certain groups of genes are differentially expressed. The genes marked in red and arrows ↑ are upregulated. The genes marked in blue and arrows ↓ are down- regulated.

6.2 Future work

Based on the above findings, the PhD work can be extended to provide further insights into the epithelial stress handling mechanisms in the *Drosophila* Malpighian tubules. Future work could be carried out in the following areas:

1) *In vivo* hindgut studies

The hindgut is the final check point where water and ions are reabsorbed or excreted out in urine(Chintapalli et al., 2012). The Flyatlas data shows Relish, Gyc76C and Dh44-R2 are expressed in the hindgut. Also, Dg1 is very highly abundant and highly expressed in the hindgut. The functional studies of these genes in the hindgut will expand the understanding and provide useful insights of the effects of Relish regulated genes, Dg1, and neuropeptide-receptor (NPLP1-VQQ/Gyc76C and Dh44/Dh44-R2) in the absorptive/secretory mechanisms of epithelial tissues in *in vivo* context.

2) Studying the osmotic stress responses on down regulating Ion transport peptide in neurons

In *Drosophila*, Ion transport peptide (ITP) is localised in the hindgut-innervating abdominal ITP neurons. Hence the survival assay studies and functional studies on the hindgut in response to osmotic stress on knock down of ITP in the neurons will provide useful insights into stress associated water-ion homeostasis strategies of the insect.

3) Peptide array studies

Dg1 and Dg2 peptide array studies in the tubules in response to cGMP treatment will help identify the *in vivo* Dg1 and Dg2 targets.

4) Study of c-Jun kinase pathway in tubules

A very high expression of dJNK (Drosophila c-Jun N-terminal kinase) in the tubules indicates to its importance in the tissue. The salt stress survival studies and gene expression studies in dominant negative dJNK flies and in Relish

mutant flies expressing dominant negative dJNK will help understand (1) whether FOXO-activation in the tubules during salt stress response is due to activation of JNK pathway or IIS pathway, and (2) identify the degree of redundancy between the JNK and Relish pathways in response to salt stress.

5) Combinatorial gene studies of GstE5, CG17664 and CG17751

The study here identifies genes *GstE5*, *CG17751* and *CG17664*, whose regulation in tubules is indicated to be dependent on Relish. GSTs are detoxifying enzymes that are associated with insecticide resistance (Enayati et al., 2005). An increase in expression of GstE5 has been detected in DDT insecticide resistant *Aedes ageypti* (Lumjuan et al., 2011). In addition, CG17664 is an aquaglyceroporin (Cabrero et al, unpublished) which is one of the four known important aquaporin genes in *Aedes ageypti* that is down- regulated for appropriate diuretic function of female mosquitoes after a blood meal (Drake et al., 2015). The Flyatlas data shows the expression of *CG17751* to be very highly enriched and abundantly expressed only in the larval and adult tubules. In *Drosophila* the high expression of *GstE5* is limited to epithelial tissues (crop, midgut, tubules and hindgut) and *CG17664* is tubule principal cell specific gene. Hence, doing a combinatorial knockdown study of these genes when the flies are exposed to a combination of stressors can render useful stress response insights in insects which can be explored for target development of insecticides.

6) Co-expression genes studies of tubules treated with an immune stressor and cGMP

The tubule microarray study here in shows an overlap of immune associated genes with salt stress response genes. The studies also indicate cGMP signalling to play a role in salt stress response. The co-expression gene studies of tubules treated with immune stressor and cGMP will help identify common genes that can be functionally studied for understanding the fluid secretion functionality of the tubules during stress.

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