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**Using FlyAtlas to detect novel functions for well-known
genes in *Drosophila melanogaster***

A thesis submitted for the degree of
Doctor of Philosophy at the University of Glasgow

By

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The research reported within this thesis is my own work
except where otherwise stated, and has not been
submitted for any other degree

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Abstract

Drosophila melanogaster has been an important model organism for over a century, cumulating in a vast array of mutant and transgenic stocks, the publication of the genome, its subsequent annotation and more recently the production of the online gene expression database, FlyAtlas. Much of what we know about developmental biology was pioneered in *Drosophila* and it is possibly the most well studied and understood model organism, in terms of development, genetics and physiology. The so-called ‘omics’ era of biology has resulted in a relatively data poor discipline quickly becoming a data rich one. Therefore the need for a good model organism, which offers the balance between genetic power and relevance has never been more important, as scientists begin to evaluate and analysis this data. We will argue that *Drosophila melanogaster* offers the best opportunity to study the relevance of omics data.

FlyAtlas is an online resource, which allows scientists to look at tissue specific gene expression in the fruit fly *Drosophila melanogaster*. Unexpected expression patterns of previously characterised genes may hint at novel functions, thus helping to close the phenotype gap. To test this hypothesis we looked at the neuronal gene *Fasciclin 2 (fas2)*, which has been exhaustively characterised (over 500 papers), with neural functions ranging from axonal growth in development to synapse stabilization in the adult. Surprisingly FlyAtlas showed *fas2* is predominately expressed in the Malpighian tubule (a renal, rather than neural, tissue), hinting at a previously unreported function in this tissue. Results suggest *fas2* may play an important role in apical microvilli development and stability in the principal cells of the tubules. We have also shown that Fas2 may be involved in actin localisation. Fas2 shows dynamic localisation in response to cAMP and over expression of the protein results in a significant increase in secretion when tubules are stimulated with cAMP. We also present evidence that Fas2 co-localises with F-Actin bundles in response to cAMP, hinting at a role for the actin cytoskeleton in secretion.

Proteomics experiments carried out in order to determine Fas2’s, interacting partners proved problematic. For this reason 2D Blue Native PAGE and sucrose gradient techniques were optimised in order to facilitate this problem.

Unfortunately we were unable to isolate Fas2, however we have shown that BN-PAGE offers a robust protocol for the isolation of protein/protein complexes. We can also conclude from these experiments that 2D BN-PAGE offers an ideal comparative data source for transcriptomics data such as FlyAtlas.

The second gene tested in this study is the sex determination transcription factor *Doublesex (dsx)*. *Dsx* has been extensively studied in its role in differentiation of both the soma and to some extent the nervous system in males and females. FlyAtlas results indicate that it is also expressed in the Malpighian tubules, again hinting at previously unknown function in this tissue. Further to this the male and female transcripts of *dsx* are expressed in a sex specific manner. Our results confirm these observations and *dsx* was localised to the principal cells of the main and lower segments of the tubules. Male tubules however do not express *dsx* in the transitional segment whereas females do, suggesting that perhaps this segment of the tubule constitutes a previously unknown sex specific function. We have determined that Tra RNAi is effective at knocking down the female transcript in female tubules, allowing for the study of masculinised tubules in an otherwise female fly. Experiments concluded that although males and females show differential survival in response to bacterial infection, this is not controlled by *dsx* expression in the tubules. Preliminary results also suggest that two genes CG8719 and YP3 are differentially expressed in male and female tubules and offer ideal candidates to study *dsx* role in sexually dimorphic gene expression in the tubules.

In conclusion this study verifies the use of FlyAtlas to determine novel functions for well-known genes in *D.melanogaster*. In turn this indicates the importance of omics data, as a starting point for further functional analysis of both genes and proteins.

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Abbreviations

β -Gal	β -galactosidase
μ M	micromolar
AMP	anti-microbial peptide
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	adenosine 3',5' cyclic monophosphate
cDNA	complementary DNA
cGK	cGMP-dependent protein kinase
cGMP	guanosine 3',5' cyclic monophosphate
CREB	cAMP response element binding
CSM	complete Schneider's medium
CuSO ₄	copper sulphate
CyO	curly
DAPI	4, 6-diamidino-2-phenylindole
dbcAMP	dibutyryl adenosine 3', 5'-cyclic monophosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
Fas2	Fasciclin 2 protein
<i>fas2</i>	Fasciclin 2 gene
FITC	Fluorescein isothiocyanate
GFP	Green Fluorescent Protein
ICC	immunocytochemistry
IP	immunoprecipitation
KCl	potassium chloride
KH ₂ PO ₄	di-potassium hydrogen orthophosphate
LiCl	lithium chloride
M	molar
MgCl ₂	magnesium chloride
mM	millimolar
Na ₂ HPO ₄	di-sodium hydrogen orthophosphate
NaCl	sodium chloride
PBS	phosphate buffered saline
PCR	polymerase chain reaction
QRT-PCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
S2	Schneider line 2
SEM	standard error mean
UAS	upstream activating sequence
QRT-PCR	quantitative polymerase chain reaction

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Chapter 1

1 . Introduction

1.1 *Drosophila melanogaster*

The fruit fly *Drosophila melanogaster* has been an important model organism for over a century, resulting in a completed genome sequence and a vast array of transgenic and mutant lines (Adams and et.al, 2000). *Drosophila melanogaster* offer scientists the ideal balance between organismal complexity and genetic power, a combination often missing in other model organisms, such as the mouse (Dow, 2003a). This section discusses the power of the fruit fly as a model organism, with particular reference to the Malpighian tubules as a model system for epithelial biology. We will then go on to discuss its recent applications, including the development and uses of the online database FlyAtlas.

1.1.1 *History of Drosophila melanogaster*

From the earliest experiments *D. melanogaster* proved to be a powerful system in which to study genetics. The first mutation discovered in *D. melanogaster* was *white*: a simple change in eye colour from red to white, led to Morgan's discovery of sex-linked inheritance and his description of chromosomes and genes (Morgan, 1910). Most of what we know about the control of early embryonic development was pioneered in *Drosophila* studies, resulting in Edward B. Lewis, Christiane Nüsslein-Volhard and Eric F. Wieschaus being awarded the 1995 Nobel prize in medicine (Lewis, 1978; Nüsslein-Volhard and Wieschaus, 1980). There are several reasons why *D.melanogaster* is an attractive model organism and they are listed below:

- Short life cycle
- Cheap and easy to rear in large numbers
- Large stock centres
- Extremely well studied and characterised
- Genetic tools
- Sequenced and well-annotated genome
- 75% of known human disease genes have homologues in the fruit fly

The following sections will go into some detail about each of these aspects.

1.1.1.1 Life cycle of the fly and rearing

The fruit fly displays a holometabolous method of development: development stages are fourfold, embryonic, larval, pupal and adult (summarised in Figure 1.1). The process from embryo to adult takes roughly 10 days, dependent on temperature, meaning several generations can be studied in just a few weeks (Bate, 1993). The high fecundity of female flies, roughly 100 eggs per day, also means that large numbers of offspring can be collected in a very short timeframe (Reeve, 2001). After eclosion, females do not become receptive to males for 8-10 hr, virgins can also be distinguished from mated flies by size, pigmentation and the presence of a dark spot on their abdomen (Ashburner, 1989), thus facilitating genetic crosses. These aspects of the fly development, not only make them ideal models to study developmental biology but also to carry out genetic interaction studies. As a result of this *D. melanogaster* is one of the most well studied organisms with regards to development and genetics (Beckingham et al., 2005).

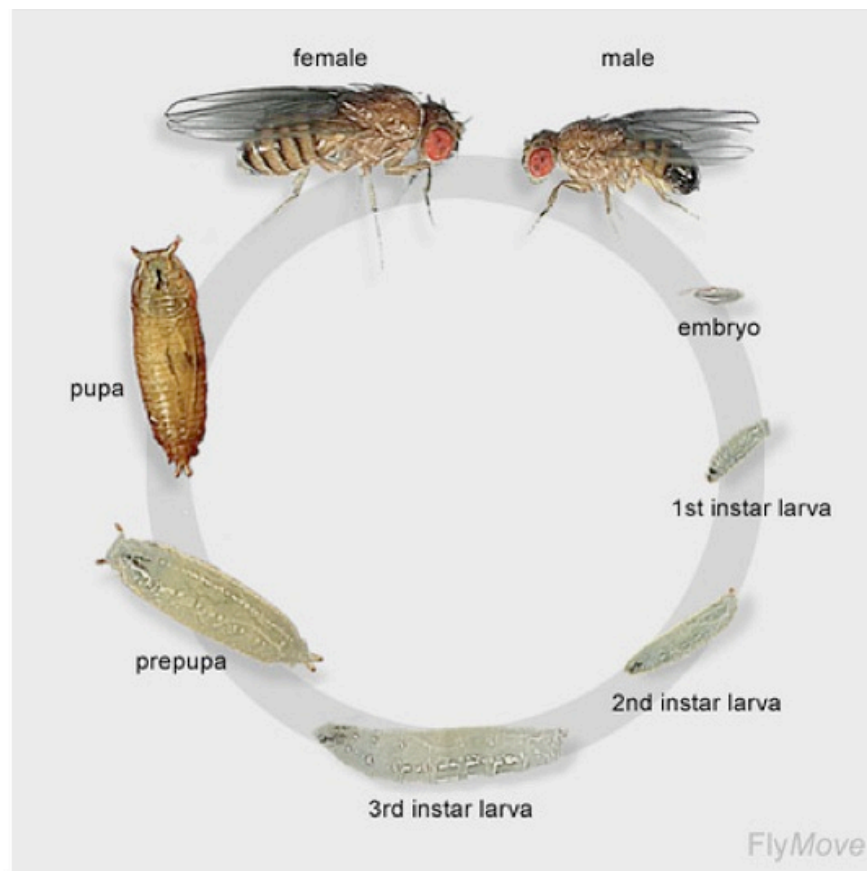


Figure 1-1 Life cycle of *Drosophila melanogaster*

Summary of all developmental stages of the fruit fly *D. melanogaster* <http://flymove.uni-muenster.de/>

1.1.2 *Drosophila*: useful genetic tools

Perhaps one of the most important reasons for choosing *D.melanogaster* as your model organism, is the ability to easily manipulate the fly's genome. Essential to this, is the presence of natural transposable elements in *D.melanogaster*, known as P elements (Charlesworth, 1989; Engels, 1992). Original P element experiments led to the discovery of many genes, which were involved in basic biological processes. Flies, which carry P elements inserted into these genes, often exhibit clear phenotypes and with the advent of plasmid rescue experiments it became possible to map these P elements to the gene in which they are inserted (Ballinger and Benzer, 1989; Bingham et al., 1982; Searles et al., 1982). Since this time however P elements, have been adapted in order to determine the expression profiles of thousands of genes, to produce thousands of genetically modified fly lines and to produce detailed genetic maps (Arias, 2008).

1.1.2.1 P elements and enhancer trapping experiments

P elements represent a classical transposable system, whereby transposons are able to 'jump' around the genome, facilitated by the transposase enzyme (Robertson and Engels, 1989). Researchers hijack this system in order to map the expression of genes, via enhancer trapping: trapping a reporter construct within the genome (Rubin and Spradling 1983; Bellen, O'Kane et al. 1989). In this technique the transposase gene in the P element is replaced with a reporter gene, consisting of various genetic markers, such as GFP, downstream of a weak promoter. Transgenic flies carrying these P elements are then crossed to a line which carries a $\Delta 2,3$ P-element, a defective transposon that can only express transposase in the germ line, and which itself is unable to move. The progeny from these crosses produce transposase, which then allows the P element/genetic marker to 'jump' to a new position within the genome. Subsequent progeny which have not inherited the $\Delta 2,3$ P-element are then selected, thus producing a fly line, which has a genetic marker 'stuck' in a new and potentially interesting area of the genome (Bellen et al. 1989). These studies were of great interest to the *Drosophila* community as more often than not, the genetic marker would be trapped close to a gene, and thus facilitated

the mapping of expression profiles for that gene. The ability to carry out plasmid rescue of the P elements also allowed scientists to detect where in the genome the P element had been inserted, and this along with vast amounts of cloning and complementation experiments, made the detailed annotation of the genome sequence all the more simple.

1.1.2.2 GAL4/UAS system

In the 1990s enhancer trapping technology was taken a step further with development of the GAL4/UAS system (Brand and Perrimon, 1993). Figure 1-2 summarises this system.

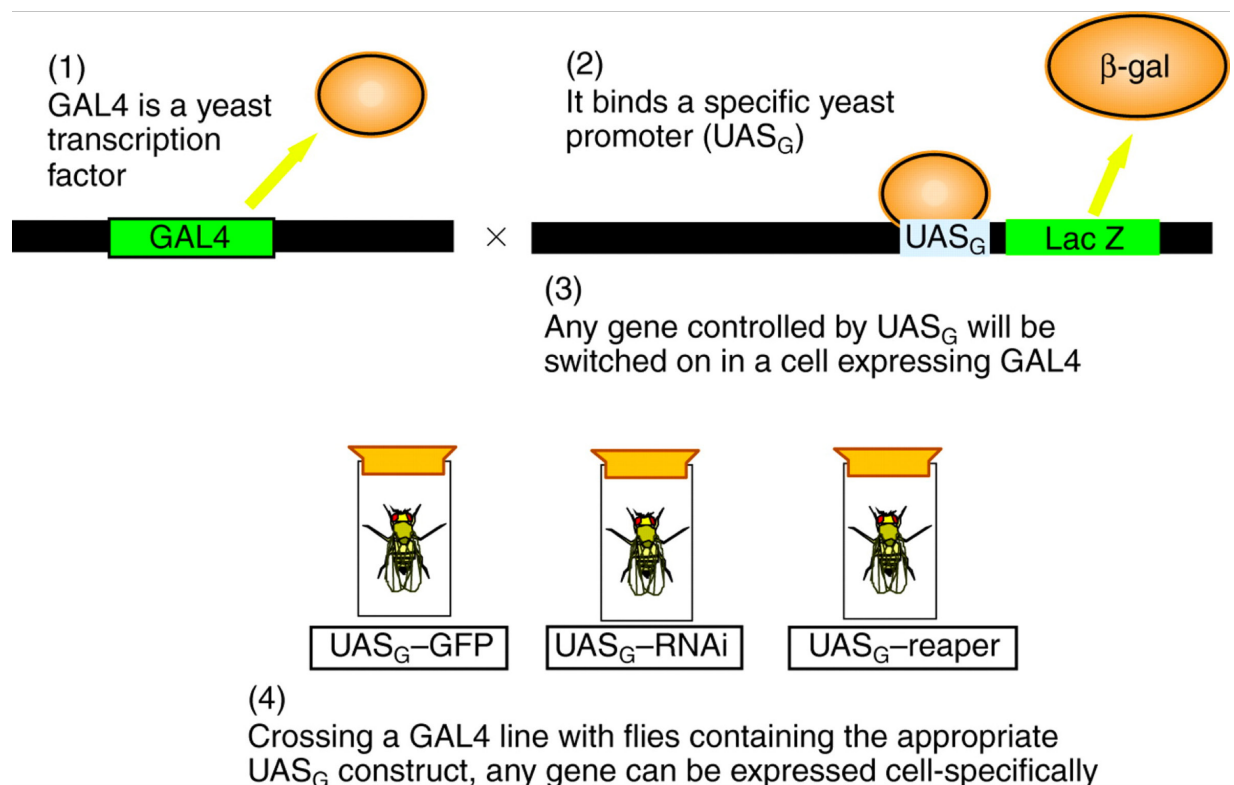


Figure 1-2 GAL4/UAS system in *Drosophila melanogaster*

In this system an enhancer-trap line expressing GAL4 in a tissue of interest is crossed to flies carrying a UAS-transgene. Expression of the transgene is subsequently driven in the GAL4 tissue in the resulting progeny (Dow, 2007).

The reporter gene in this system is the yeast transcription factor GAL4, which importantly has been shown to have little or no activity in *D.melanogaster* (Duffy, 2002). The GAL4 transcription factor is placed downstream of a tissue specific promoter (often discovered and evaluated through previous enhancer

trap experiments), thus GAL4 is only expressed in tissues where this promoter is activated. This line is then crossed to a line carrying the GAL4 recognition sequence, UAS, which is placed upstream of a reporter construct. Resulting progeny, express GAL4 in the tissue of interest, GAL4 then binds to UAS and initiates transcription of the downstream reporter. Adaptations of the technique have included the production of RNAi constructs, which target the degradation of gene transcripts for a particular gene of interest, in a tissue specific manner (Fortier and Belote, 2000; Lam and Thummel, 2000). Similarly we can now actively over-express or mis- express a particular gene.

1.1.2.3 Development of genetrap and EP lines

More recently P element technology has allowed the more subtle modification of native genes and proteins. Most notable is the production of so-called protein trap lines and EP lines. A protein trap line consists of the insertion of a GFP (or EGFP) within the open reading frame of a given gene, via an exon acceptor (Buszczak et al., 2007). These lines can be extremely useful as not only do they mark the expression profile and localisation of the protein, they do so without affecting the natural expression levels of the gene/protein, as would be the case if one were to over-express a tagged construct via GAL4/UAS. EP lines also involve the modification of the native gene sequences, in this case with the addition of a UAS promoter upstream of gene start site, allowing for targeted over or mis-expression of the native gene (Rorth, 1996; Rorth et al., 1998).

1.1.2.4 Sophisticated variants of the GAL4/UAS system

More recent developments in the GAL4/UAS system have resulted in highly sophisticated variants of the technique, which allow for better spatial and temporal control of gene expression. These include the development of the GAL80 system, whereby GAL80 inhibits GAL4 expression by actively binding to the protein (Ma and Ptashne, 1987). Expression of GAL80 therefore can be used to deplete GAL4 expression in a specific tissue (Lee and Luo, 1999). Temperature sensitive variants of GAL80 can be used to give a more detailed temporal control over GAL4 expression and therefore gene expression manipulation (McGuire et al., 2004). The production of the split GAL4 system has also made both temporal and spatial expression more accurate. In this case two separate lines are

generated, where one line carries half of the GAL4 sequence downstream of a tissue/cell specific promoter and the second line carries the second half downstream of a different promoter sequence. Once crossed GAL4 will only be expressed in tissue/cells, which express both halves of the GAL4, allowing for a more refined expression profile (Luan et al., 2006).

1.1.3 *Mosaics and Clonal analysis*

The development of genetic mosaic techniques, have been crucial to our current understanding of developmental biology in *D.melanogaster*. Genetic mosaics consist of flies, which contain clones of genetically distinct somatic cells. Over the past decades several approaches have been used to generate mosaic flies including chromosomal loss, mitotic recombination and cell transplantation (Kankel and Hall, 1976; Lawrence and Johnston, 1986). Traditional techniques relied on random recombination events, induced by irradiation, between homologous chromosomes. Figure gives an overview of irradiation-induced mitotic recombination between two chromosomes, one wt and one mutant. The resulting fly contains a group of cells within the wing blade, which are now mutant for a gene called *shaggy* surrounded by wt cells thus allowing a comparison of mutant and wt cells to be carried out (Blair, 2003). Unlike many mammalian genetic mosaics, which form a salt and pepper like formation of cells, daughter cells in *Drosophila* tend to form a close coherent clone of cells contained within one area of a tissue. The size of this area is dependant on the developmental stage at which the recombination takes place, the earlier the bigger the area. This allows for the subtle changes in a few cells by carrying out recombination at a later stage or change to a large number of cells by carrying out recombination at an earlier stage (Ashburner, 1989). Such experiments have been crucial in understanding the development of the wings, oocytes, and eye development (Ashburner, 1989; Brower et al., 1981).

Like the Gal4/UAS system a more refined technique for producing mosaic flies has been developed. The most commonly used technique involves the targeted recombination of DNA at FLPase recombination targets by FLP recombinase, a summary can be seen in Figure 1-3 (b). In the 1990s stocks were developed containing FLPase under the control of the heat shock promoter, allowing for the

induction of FLPase by heat-shock. Along with this came the development of stocks containing FRTs inserted in proximal locations on each of the chromosome arms. Thus it was possible to recombine specific areas of chromosomes, through the control of heat-shock induced FLPase activity. The benefit of this technique is two fold. Firstly, FRT-mediated recombination is slightly more efficient than irradiated recombination but still occurs at a low enough level to produce clones. Secondly, it offers a much less random technique whereby you know which areas within the chromosome have recombined (Chou, 1993; Chou, 1996).

Clonal or mosaic analysis have been and continue to be extremely important techniques for studying genetic changes in a specific tissue, that would otherwise be lethal to the fly. They therefore offer a vital role in understanding development at the genetic level at differential developmental time points. However in some tissues such as the Malpighian tubules mosaic analyses is more problematic. It would be possible to induce mosaics up until larval development but the use of clones after this point is limited by the arresting of cell division. Therefore if you wished to study a gene beyond embryonic development using clonal analysis the Gal4/UAS system offers a better system.

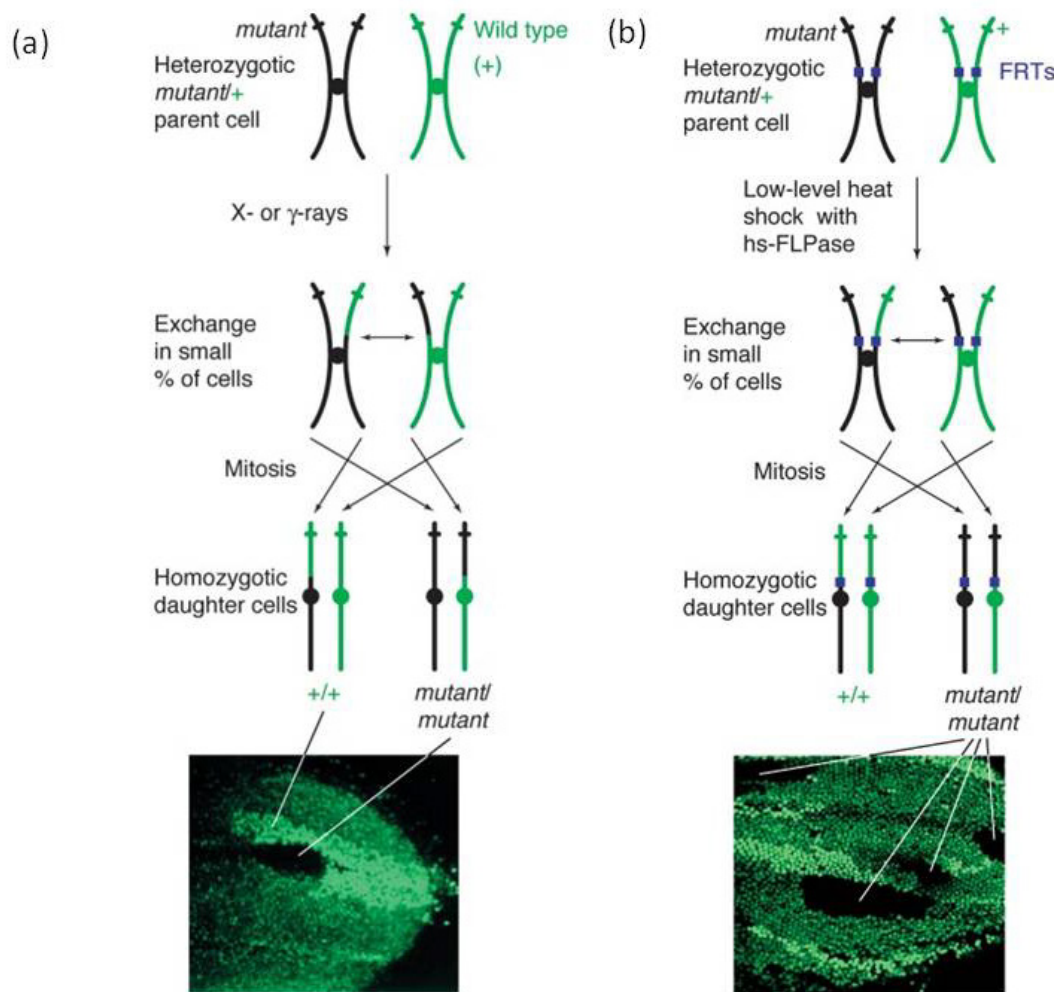


Figure 1-3 Summary of Mosaic analysis in Wing Disc Development

(a). Chromosome behavior during and after irradiation-induced mitotic recombination. The clones produced by the two homozygous daughter cells of a mitotic recombination event are shown below. The photograph shows a *shaggy* mutant clone, which lacks anti-Myc staining (green), and its sister 'twin spot' (+/+), which has a double dose of the Myc epitope, in a pupal wing blade. (b) FRT-induced mitotic recombination, catalyzed at FRTs by hs-FLPase. The photograph shows several *engrailed* clones, lacking anti-Myc staining (green), in a pupal wing blade. FRTs, FLPase recombination targets; hs-FLPase, heat-shock-induced FLP recombinase. (Blair, 2003)

1.1.4 Physiological studies in *D. melanogaster*

The development of these technologies has been instrumental in the rapid increase in physiological studies in *D. melanogaster* in recent years. As our understanding of physiology (how organisms work) has improved, it has become ever more apparent that traditional techniques, such as cell line experiments are limited in their scope. In order to understand a physiological process and the genes involved, it is important to study the process within its natural

physiological context i.e. the whole organism. By removing the process or the genes from their natural context you narrow the ability to truly understand their function within the whole organism. Dow and Davies have argued extensively that transgenic organisms, in which you can alter gene expression in a cell or tissue specific manner, will link this gap between gene, tissue and organism (Dow and Davies, 2003a). Choosing the right transgenic organism is ultimately a pay-off between biological relevance and genetic power and it can be argued that *Drosophila* offers the best trade-off between the two (Dow and Davies, 2003a). It is for this reason that *D.melanogaster* has become an increasingly obvious choice for physiological studies, as we can now effectively manipulate gene expression in a tissue specific manner, thus allowing the study of many biological processes *in vivo*. One such area of study is that of the epithelial physiology of the Malpighian tubules (Dow, 2003b; Dow and Davies, 2003b).

1.2 Malpighian tubules: a model system for epithelial biology

1.2.1 Introduction

Insect tubules have analogous functions to that of mammalian kidneys and to some extent livers (Dow et al., 1994b; Maddrell, 2004; Maddrell and Casida, 1971; Maddrell, 1981; Yang et al., 2007). They form the main excretory and osmoregulating organs of the insect, and classically provide an excellent system for the study of epithelial physiology. *D.melanogaster* tubules are among the smallest studied, measuring ~2mm in length and 35µm in diameter (Dow and Davies, 2003a). There are two pairs of tubules in *D.melanogaster*, one anterior and one posterior. Each pair, is joined by a common ureter to the alimentary canal and consist of two major cell types, principal and stellate (Wessing and Elchelberg, 1978). Recent studies have shown how complex and versatile insect tubules are. In particular the knowledge and genetic power available in *D.melanogaster* has lead to the discovery that Malpighian tubules are also involved in immunity (Davies et al., 2008; McGettigan et al., 2005). They have also been implicated in detoxification and metabolism (Chahine and O'Donnell, 2009, 2011; Dow and Davies, 2006; Evans et al., 2005; O'Donnell, 2009). Moreover, microarray studies have shown that many human disease genes have

D.melanogaster homologues that are most highly enriched in the tubules (Chintapalli et al., 2007). Therefore the tubules constitute a versatile phenotype for an array of studies. This section discusses how the Malpighian tubules are formed, their key role in osmoregulation and ion transport and looks at what we can learn about the processes in tubules from other tissues.

1.2.2 ***Embryonic development of the Malpighian tubules***

During embryogenesis the Malpighian tubules (MT) form from the evagination of cells from the junction between the hindgut and the midgut. The four tubules are derived from the shared hindgut primordium, through a sequence of cellular activities. The first step involves specification of the tubule specific cells within the hindgut. This involves the activation of genes such as transcription factors, *krüppel* (*Kr*) and *cut* (Ainsworth et al., 2000; Gloor, 1950; Hatton-Ellis et al., 2007; Weigel et al., 1990). Ectopic expression of both these genes at the anterior midgut/foregut boundary is enough to result in cells being specified as tubule cells. The second stage of development, involves the eversion of the four tubules from the hindgut primordium. Once cell fate is determined, expression of *Kr* is limited to four cluster of cells, which start to re-arrange to form four small buds (Ainsworth et al., 2000). These buds mark the beginning of the formation of the four tubules. Two of the buds project in a ventral direction, eventually becoming the posterior tubules and the second pair project in a dorsal direction, becoming the anterior pair. The distinction between the anterior pair and the posterior is determined by differing levels of signalling between the two (Hatton-Ellis et al., 2007). As the tubule buds develop, the cells undergo several regulated cell divisions. Cell division during this stage is controlled by the EGFR signalling pathway (Baumann and Skaer, 1993). By the end of cell division four short tubules, each containing around 8-12 cells around their circumference are formed. These then elongate over several hours, with cells reorganising to form four long tubules each with a lumen surrounded by only two cells. Elongation at this stage is controlled by several key genes and includes the recruitment of the actin cytoskeleton (Bates et al., 2008; Bradley and Andrew, 2001; Denholm et al., 2003b; Hatton-Ellis et al., 2007; Jack and Myette, 1999; Kerman et al., 2008; Lekven et al., 1998; Shim et al., 2001; Simoes et al., 2006) During this phase, cells from the caudal mesoderm begin to

invade the tubules. These cells will eventually become the stellate cells of the tubules (Denholm et al., 2003b). By the end of embryogenesis the four tubules are fully formed and contain two main cell types located in the secretory region: the principal cells (derived from the primordium cells) and the stellate cells (derived from the caudal mesoderm cells). At this stage the tubules appear to be functionally active in the transport of organic solutes such as urates, but the high levels of fluid secretion activity are not seen until after hatching (Beyenbach et al., 2010).

1.2.3 Larval and adult Malpighian tubules

As described in the previous section, by the end of embryogenesis the four tubules are fully formed, and have the ability to secrete organic compounds. Several changes, however, still occur during larval, pupal and adult development. Firstly and possibly most importantly, the tubules develop the ability to carry out high levels of fluid secretion during early larval development, as shown in *Rhodnius* (Skaer et al., 1990). Secondly, during pupal development the tubules are no longer required to carry out secretion (Bradley and Snyder, 1989). Studies also suggest that during this period cells undergo several changes, including the retraction of microvilli within the apical brush border of the principal cells (Bradley and Snyder, 1989; Ryerse, 1979). Thirdly, although stellate cells are formed during embryogenesis, they do not become ‘stellate’ shaped until several days after the adult fly has eclosed (Sözen et al., 1997). Little is understood about the processes behind these areas of tubule development, but perhaps the development of microarray studies and proteomics may help in elucidating the genes and proteins involved (Beyenbach et al., 2010).

Once thought of as a rather simple epithelial tissue, the tubules have recently been shown to be much more complicated. Gene expression profiling has shown that although there are predominantly two types of cells within the adult tubules, these cells can be further subdivided by their expression profile (Sözen et al., 1997; Wang et al., 2004). Figure 1.4 summarises the functional domains of the adult tubules. Previous studies had indicated the tubules consisted of clear physiological domains and these gene array studies served to prove these

findings are controlled at a gene expression level. These findings suggest a complex tissue, which carries out multiple processes by taking advantage of differential gene expression.

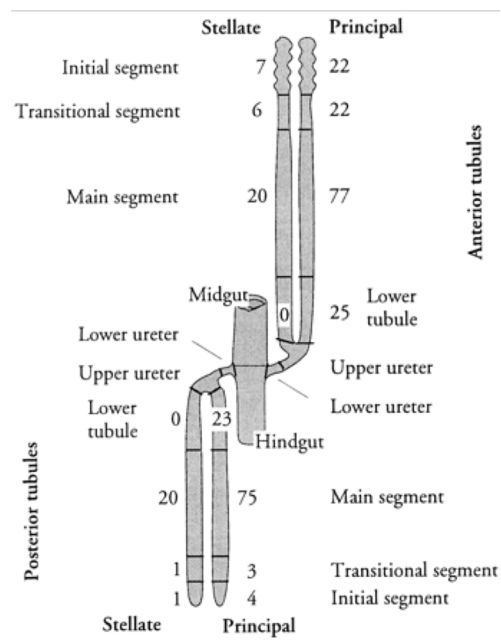


Figure 1-4 Summary of Functionally Distinct Regions of the Malpighian tubules (Sozen et al., 1997)

1.2.4 *Ion transport and Osmoregulation*

As previously stated the tubules are the main excretory and osmoregulating organs of the insect. Fluid secretion in the tubules is primarily under the control of the second messengers, cAMP, cGMP and Ca^{2+} , which act specifically on either cation or anion transport (A Riegel, 1998; Aston, 1975; Davies et al., 1995; Dow et al., 1994a; Dow et al., 1994b; Hegarty et al., 1991; Maddrell and Casida, 1971; Morgan and Mordue, 1984; O'Donnell et al., 1996; Sawyer and Beyenbach, 1985). The principal cells of the *Drosophila* tubules are responsible for the transepithelial secretion of Na^+ and K^+ while the stellate cells are responsible for the transport of Cl^- (Terhzaz et al., 1999; Torrie et al., 2004). A summary of the proteins, ions and second messengers involved in tubule transport can be seen in Figure 1.4. Key to the fluid secretion phenotype of the tubules is the vacuolar-type H^+ -ATPase (V-ATPase).

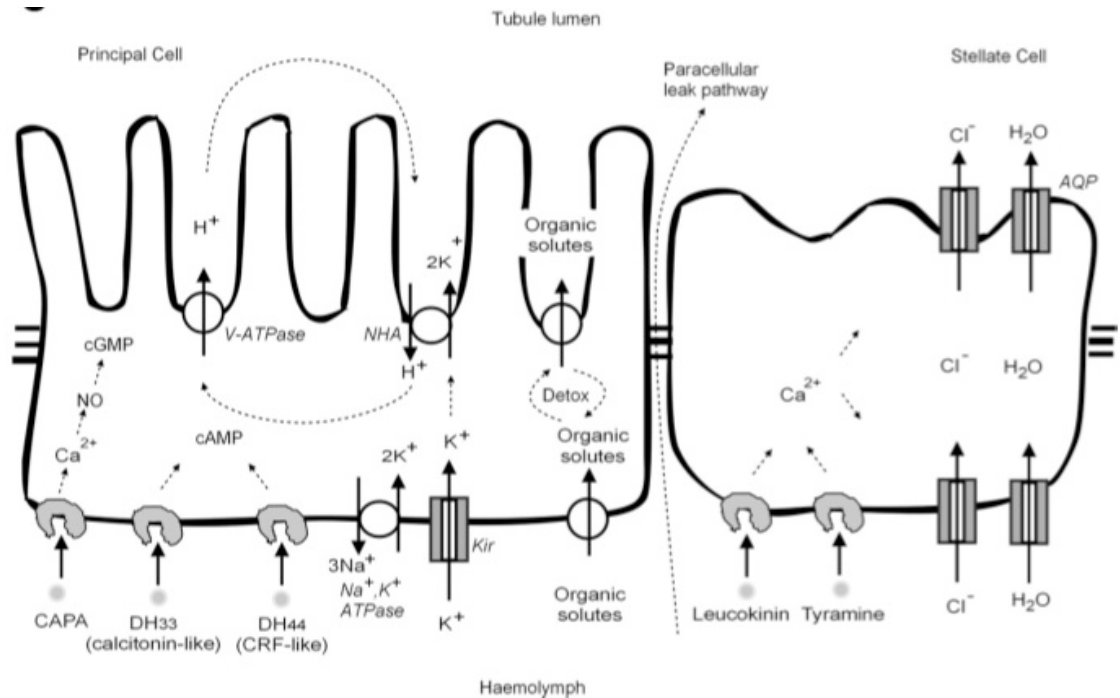


Figure 1-5 Sensitivity map of tubule physiology in *D.melanogaster* (Dow and Romero, 2010)

1.2.4.1 V-ATPase the primary ion pump

As summarised in Figure 1.5 the V-ATPase is the primary active ion pump, controlling fluid secretion in the Malpighian tubules. They do this by pumping H⁺ into the lumen, setting up a proton gradient across the apical membrane. This then drives the movement of alkali cations from the cell to the lumen through apical Na⁺ /H⁺ and/or K⁺/H⁺ exchangers (Dow et al., 1994b; Klein, 1992; Wieczorek, 1992). The V-ATPase of *D.melanogaster* is located to the apical brush border of the principal cells (Davies et al., 1996) and is made up of two complexes V₀ and V₁, each of which is made up of several subunits. V₀ is a stator, which is anchored to the cell membrane and V₁, a rotor, which translates H⁺ ions from one side of the membrane to the other (Beyenbach and Wieczorek, 2006; Meier et al., 2005; Murata et al., 2008). The V₀ subunit is permanently anchored to the apical membrane, the V₁ unit, however can dissociate and re-associate with the V₀ unit, thus controlling H⁺ ion transport (Beyenbach and Wieczorek, 2006; Sumner et al., 1995). Summarised in Figure 1-6

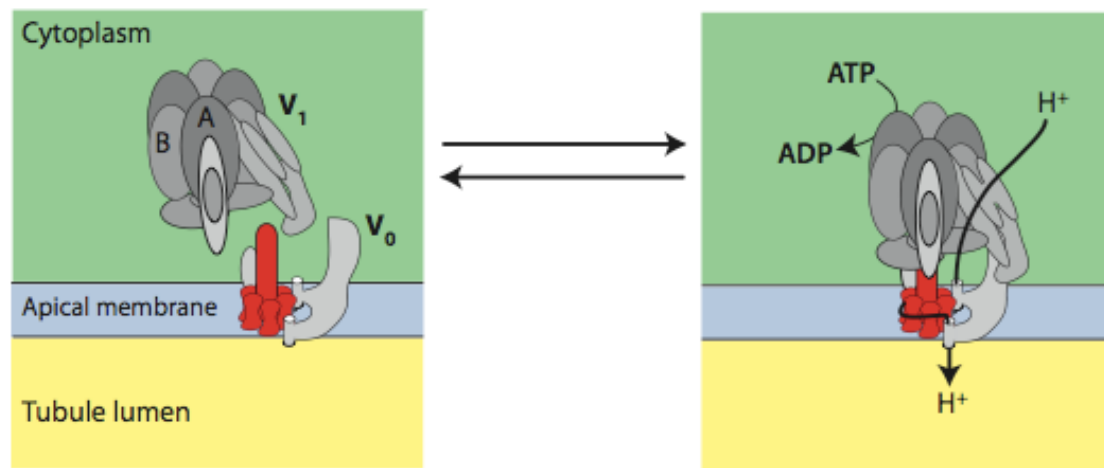


Figure 1-6 Summary of disassociation and re-association of V-ATPase subunits V_0 and V_1 (Beyenbach et al., 2010)

The activation of V-ATPase is highly energy consuming as is evident from the hydrolysis of ATP to ADP (Maddrell and O'Donnell, 1992; Wieczorek et al., 2000). It is not surprising therefore that cells require the ability to switch these enzymes on and off. The reversible association of the two complexes is just one level of control. There are several other levels. Firstly there is evidence in some organisms that V-ATPase subunit levels are transcriptionally controlled during certain stages of development. For example the cAMP-dependent signal transduction pathway is known to induce an up-regulation of subunit B during monocyte to macrophage differentiation in the mammalian haematopoietic system (Lee et al., 1997; Lee et al., 1995). There is also evidence in the tobacco hornworm, *Manduca sexta*, which shows that the promoter regions of the V_0 and V_1 complexes are differentially regulated, indicating another level of control (Gräf, 1996; Sumner and Wieczorek, 1995). In the Malpighian tubules of *D. melanogaster* it has been shown that V-ATPase responds to increased levels of cAMP and cGMP (Dow, 1998). The next section will discuss the role of cAMP in more detail.

1.2.4.2 Cyclic AMP induces fluid secretion

Although it has been known for several years that increasing intracellular levels of cAMP lead to a V-ATPase controlled increase in fluid secretion by the MT in *D. melanogaster*, as of yet the exact mechanisms have not been determined. Recent studies however suggest several areas of interest.

1.2.4.3 Involvement of cAMP in V-ATPase accumulation and activation

Several studies have indicated that increases in intracellular cAMP directly lead to the accumulation and activation of the V-ATPase complex within the microvilli (Beyenbach et al., 2009; Bradley, 1989; Bradley and Snyder, 1989; Dames et al., 2006; Karas et al., 2005; Wieczorek et al., 2000). As stated in section 1.2.4.1, one such mechanism is the direct transcriptional up regulation of V_1 subunits via cAMP signalling (Lee et al., 1995). More recently Beyenbach et al., produced evidence that protein kinase A (PKA) may induce assembly of the V-ATPase subunits via cAMP signalling, in Mosquito tubules (Beyenbach et al., 2009). Recent studies in the salivary glands of the Blowfly, have increased our understanding of how cAMP activates secretion through the V-ATPase (Dames et al., 2006; Rein et al., 2008a). Dames et al showed that accumulation, assembly and activation of the V-ATPase complex at the apical microvilli occurred through cAMP signalling, independently of an increase in intracellular Ca^{+} . This was of particular interest because previous studies had indicated that V-ATPase assembly and activation occurred through intracellular Ca^{+} increasing which in turn led to the phosphorylation of V-ATPase subunits allowing for their assembly and activation (Dames et al., 2006). The group also provided evidence that Subunit C of the V-ATPase in *Manduca sexta* is the only known subunit to be actively phosphorylated by PKA, This subunit is known to bind to both the V_1 and V_o subunits suggesting that it may play a crucial role in V-ATPase assembly and activation (Rein et al., 2008b). Therefore the exact mechanism, by which cAMP induces V-ATPase assembly and activation, is still unknown.

1.2.4.4 cAMP controls microvilli length and mitochondrial accumulation

V-ATPase activation is not the only mechanism governing fluid secretion in response to cAMP. Several studies, in both mammals and Diptera, indicate the importance of microvilli re-arrangement and the accumulation of mitochondria are also important (Bradley and Snyder, 1989; Paunescu et al., 2010). This is not surprising as microvilli provide the surface area required for V-ATPase accumulation and mitochondria are required to energise the V-ATPases. Interestingly studies suggest that during pupa development, mosquito tubules have a lowered response to cAMP, not due to desensitisation to cAMP, but more likely to a decrease in the length of microvilli and density (Bradley and Snyder,

1989). However recent studies have also indicated that cAMP plays an important role in the rearrangement of the actin cytoskeleton, which is key to the secretion phenotype (Karas et al., 2005). Bradley et al also showed that upon stimulation with 5HT (increases intracellular cAMP), microvilli in the tubules of *Rhodnius prolixus* extended by ~2.5 in length and 3 times in surface volume, in conjunction with the movement of mitochondria into the microvilli (Bradley and Satir, 1981). Interestingly mitochondrial movement was blocked by the actin inhibitor Cytochlasin B; suggesting that the rearrangement of actin cytoskeleton is involved. Along with this studies in Mosquito tubules indicated the role of actin in secretion phenotypes (Karas et al., 2005).

1.2.4.5 Conclusions

Much is known about the dynamics of fluid secretion in the Malpighian tubules in response to cAMP but the exact mechanisms underlying the response have yet to be fully understood. Studies in other Diptera indicate that cAMP may actively cause the phosphorylation of V-ATPase subunits, allowing for assembly of the active complex, via PKA. Others have suggested that other down stream effectors may be phosphorylated. The re-arrangement of mitochondria may also play an important role in activating fluid secretion and this in turn may be aided by the re-arrangement of the actin cytoskeleton. Further analysis of cAMP, actin remodelling and microvilli rearrangement may help understand the underlying mechanisms of cAMP induced fluid secretion. More recent applications in *Drosophila*, such as genomic and transcriptomic analysis may hold the key to understanding these mechanisms.

1.3 Recent applications in *Drosophila*

1.3.1 Sequencing and subsequent annotation of the *D.melanogaster* genome

The full genome of *D.melanogaster* was published in 2000, making it one of the first eukaryotic genomes to be fully sequenced (Adams and et.al, 2000). The raw genomic data acquired from a fully sequenced genome, is only useful to scientists if it can be accurately annotated. This inevitably requires previous

gene sequence data or an ability to accurately determine or predict where genes are in the genome (Stein, 2001). With this in mind the annotation of the *Drosophila* genome was aided by several factors. For example the *Drosophila* community have mapped new loci by recombination relative to known flanking data for many years, therefore already, in effect mapping the genome (Adams and et.al, 2000). Several tissues, notably the salivary gland, contain giant polytene chromosomes. These chromosomes have been studied in *Drosophila* since the 1930s, and consist of multiple identical sister chromatids, which have undergone many rounds of endoduplication. The resulting chromosome is easily seen under microscope conditions and forms distinct banding patterns, which are unique to each chromosome (Bridges, 1935). With the discovery of these giant chromosomes it became possible to map cloned genes to specific areas using *in situ* hybridisation (Langer-Safer et al., 1982). Many years of cloning genes also resulted in the production of large cDNA libraries. This made it possible for the *Drosophila* genome project to sequence the 5' ends of over 80,000 random clones, generating expressed sequence tags (ESTs). Once studied, these sequences fell into clusters of clearly similar sequences. Representative genes for each of these clusters were selected for full sequencing and aligned to the genome sequence, thus marking the most abundantly transcribed areas of the genome (Adams and et.al, 2000). Plasmid rescue of P-element insertions as discussed in section 1.1.2.1 also aided the cloning and sequencing of many genes, allowing for their subsequent genome annotation.

These reasons and the vast amount of cloned and previous sequenced data available for *Drosophila*, made the annotation of the genome sequence a much easier process than in other genome projects.

1.3.2 *New insights from the genome project*

With the production of the genome sequence came the realisation that although extremely well studied, much about *D.melanogaster's* genes are unknown. Dow estimated that around a third of a million research years spent on *Drosophila* studies, predominantly developmental, only identified around 20% of genes before the production of the genome project (Dow, 2003a). Given the wealth of information gathered about development in the fly and the insights this has gave

us into mammalian development, the prospects of determining what the other 80% of genes do is enticing. There is also the realisation that if a gene functions in development it may also have functions in the adult fly. Indeed the detailed microarray study carried out by (Chintapalli et al., 2007) showed that 90% of genes shown to have embryonic expression by *in situ* analysis, were expressed in the same tissue in the adult. Determining the function of genes in the adult fly, which have previously been identified as having a role in development, is difficult. Many such genes have severe if not lethal phenotypes when mutated, therefore the ability to study and manipulate these genes in a tissue specific manner becomes all the more important.

1.4 *Drosophila* and the post genomics era

The completion of the genome project in 2000, gave the *Drosophila* community a huge leap forward in fully understanding the genomics of the fly. Scientist can now easily clone any gene in *Drosophila* in a matter of weeks, can predict gene function through sequence analogy and have a final calculation in the number of protein coding genes in the fly (Ashburner and Bergman, 2005). However the sequencing of an organism's genome is only the advantages if we can fully understand the downstream mechanisms of genes i.e. transcripts, proteins, metabolism etc. For this reason the science community has seen an explosion in the number of so called 'omics' studies in recent years. These include the study of transcripts (transcriptomics), proteins (proteomics) and metabolites (metabolomics) to name a few (summarised in Figure 1.7)

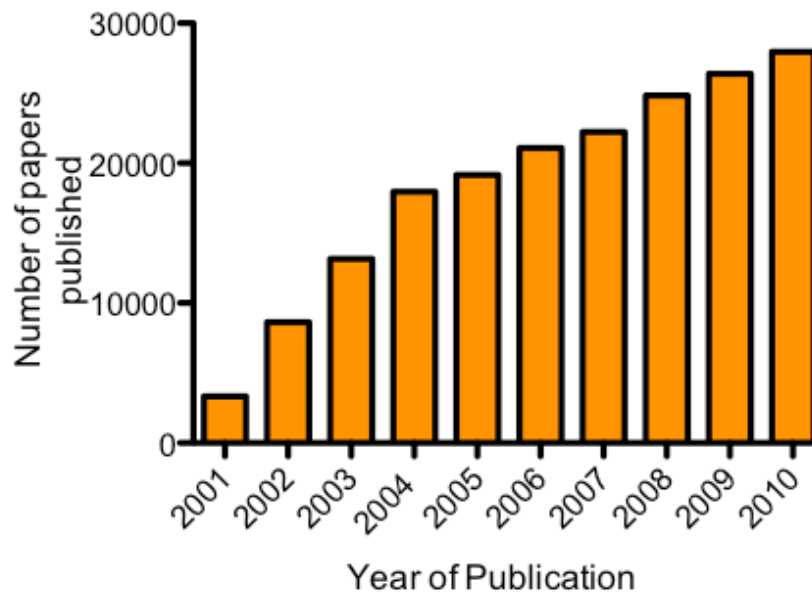


Figure 1-7 Comparison of Year on Year Publications Containing the Word 'Omics' in their title

Compiled using the online search engine ISI Web of Knowledge. Searches were carried out using the terms genomics, transcriptomics, proteomics and metabolomics and the year, in order to give an overview of the increase in 'omics' papers being published.

Like their predecessor, genomics, these studies aim to analyse the full complement of a tissue or cell in a high throughput manner. For example many proteomic studies have focused on understanding the differential expression or modifications of all proteins in cancerous and non-cancerous cells (Stevens et al., 2004). The large scale of data produced from any 'omics' study, must be rigorously analysed and confirmed, through more small-scale traditional methods and this often requires the ability to manipulate the genetics of an organism in a precise manner. For reasons discussed in Section 1.1 *Drosophila* studies have flourished in the post-genomic era. The ease with which the genome was annotated quickly led to the production of microarray genome chips allowing for whole transcriptomic analysis (discussed in more detail in section 1.4.1). Mutations in known metabolomic genes such as *rosy* also meant that tissue specific and whole fly metabolomic data could be gathered, and a comparison of mutant and non mutant flies led to significant changes to previous published metabolic maps (Kamleh et al., 2009).

1.4.1 *Transcriptomics of the fly*

The ultimate goal of any genome project, is to understand how an organism works on a genetic level, which genes are protein coding/expressed and how mutations and polymorphisms affect individual organisms. However simply knowing the sequence is not enough. It has been known for many decades that nearly all cells in organisms carry identical genomes, therefore what makes an eye cell different, from say, a kidney cell is the genes which are expressed in the cells and thus the proteins produced. Understanding which genes control development of different tissues or are disease causing, therefore became the ultimate goal of science. With this in mind the study of whole cell or tissue transcript, or transcriptomics, became increasingly popular.

1.4.1.1 Development of microarray technology

Microarray techniques stem from the development of Southern blotting technology, where the hybridisation properties of DNA are utilised in order to determine if a gene is expressed in a given sample. Unlike Southern blot analysis, where a single probe is used in order to detect a cloned gene for example, microarrays use many probes that are immobilised on an array or a chip. Once a genome has been fully sequenced and at least partially annotated it becomes possible to produce a whole genome chip, corresponding to all the genes that can be expressed in a given cell. Figure 1.8 gives an overview of how a microarray is carried out. If a genome is well annotated it is possible to not only look at individual genes but design probes against specific transcripts. One of the first whole genome arrays to be made commercially available was the Affymetrix *Drosophila* Genome Chip and later second version Affymetrix *Drosophila* Genome Chip 2 (<http://www.affymetrix.com>). The reasons that this chip was made available so soon after the publication of the genome are two fold. Firstly, many of the genes found in *D.melanogaster* were already well characterised making the annotation of the genome relatively easy. Secondly, the availability of large amounts of mutant stocks makes the fruit fly an ideal candidate in order to study transcriptomics. The Affymetrix *Drosophila* Genome 2 chip, consist of 18,880 probe sets, corresponding to 18,500 transcripts (<http://www.affymetrix.com>). Since its release 160 experiments and 3097

assays have been carried out using the chip (data from <http://www.ebi.ac.uk/arrayexpress>).

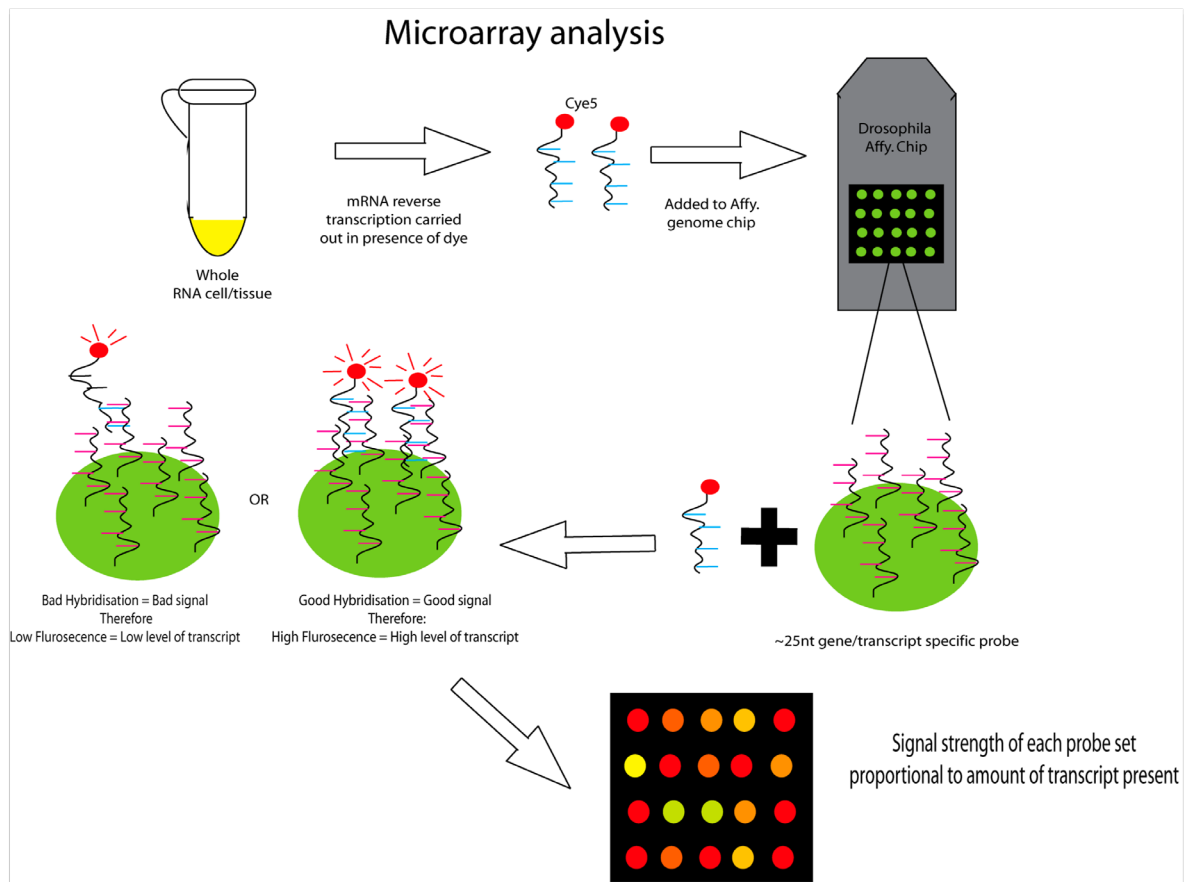


Figure 1-8 Summary of microarray technique

Whole tissue or cell RNA is extracted, mRNA is then synthesised to produce cDNA tagged with a fluorescence dye such as Cye5. Single stranded cDNA is then washed over genome chip, which contains an array of probes, representing the whole transcriptome of the fly. Several washes are then carried out before fluorescence is measured. The higher the amount of hybridisation, the higher the fluorescent signal. Therefore the signal directly proportional to mRNA levels of that given transcript.

1.4.1.2 FlyAtlas

FlyAtlas consists of 18 adult tissues, 8 larval and one S2 cell line (Chintapalli et al., 2007). As of August 2011, the full dataset consists of 44 Affymetrix chips, 18770 transcripts and 8,228,000 individual data points (www.flyatlas.org). The immense size of this data set is a perfect example of the mountains of data that microarray analysis can yield. One of the most valid outcomes of FlyAtlas was the realisation that when carrying out whole fly microarrays, many genes, which are actually extremely highly expressed in a specific tissue, are missed due to

high levels of background noise (Chintapalli et al., 2007). This once again highlights the importance of tissue/cell specific analysis when studying a gene or genes. Chintapalli et al showed that 90% of genes shown to have embryonic expression by *in situ* analysis, were expressed in the same tissue in the adult. Determining the function of genes in the adult fly, which have previously been identified as having a role in development, is difficult. Many such genes have severe if not lethal phenotypes when mutated. Again the GAL4/UAS system will be a valuable tool in overcoming this problem. However in order to do this we first need to know where the genes are expressed: FlyAtlas gives us the perfect starting point.

1.4.1.3 Recent advances in FlyAtlas and Microarray studies

Microarray technology not only allows scientists to analyse whole gene expression in a given tissue, but also allows us to study gene expression under different circumstances. Since the publication of FlyAtlas, Dow et al have continued to advance the use of microarray experiments, with particular attention to the Malpighian tubules. For example more recent studies have involved the assessment of differential gene expression between male and female tubules (Chintapalli et al manuscript submitted). They have also analysed gene expression in the response to different stimuli, such as cAMP and cGMP. Experiments such as these indicate the power of microarray analysis. If we take the example of cAMP stimulation of the Malpighian tubules, previous studies would have involved the identification of mutants, which lowered or increased the response of tubules to cAMP, looking for interactions with other genes and then mutating these and carrying out further studies. Using microarray analysis however, we are able to determine all genes that are up or down regulated in response to cAMP in the tubules. Both views are equally valid, but together offer a robust and complementary approach to determining downstream cAMP targets.

More recently, new advances in microarray technology have allowed FlyAtlas to be extended to include RNAseq data. Unlike traditional microarrays, RNAseq does not rely on the hybridisation of cDNA to probes, but rather high throughput sequencing of total mRNA fragments from a given sample (Wang et al., 2009). This has several advantages over traditional microarray analysis. Firstly it does not require the need for a prior knowledge of gene sequences, as there is no

need to produce probes for hybridisation. This allows for previously unidentified genes or gene variants to be detected. Secondly the noise level from RNAseq is significantly lower than microarray analysis, simply because you remove the hybridisation step. RNAseq is also a much more sensitive technique, in that it can detect genetic variation such as single nucleotide polymorphisms (SNPs) and has the ability to determine exon boundaries. Figure 1-9 gives a brief overview of RNAseq technology. With regards to FlyAtlas data from RNAseq analysis has been extremely useful in confirming previous expression profiles (as discussed in section).

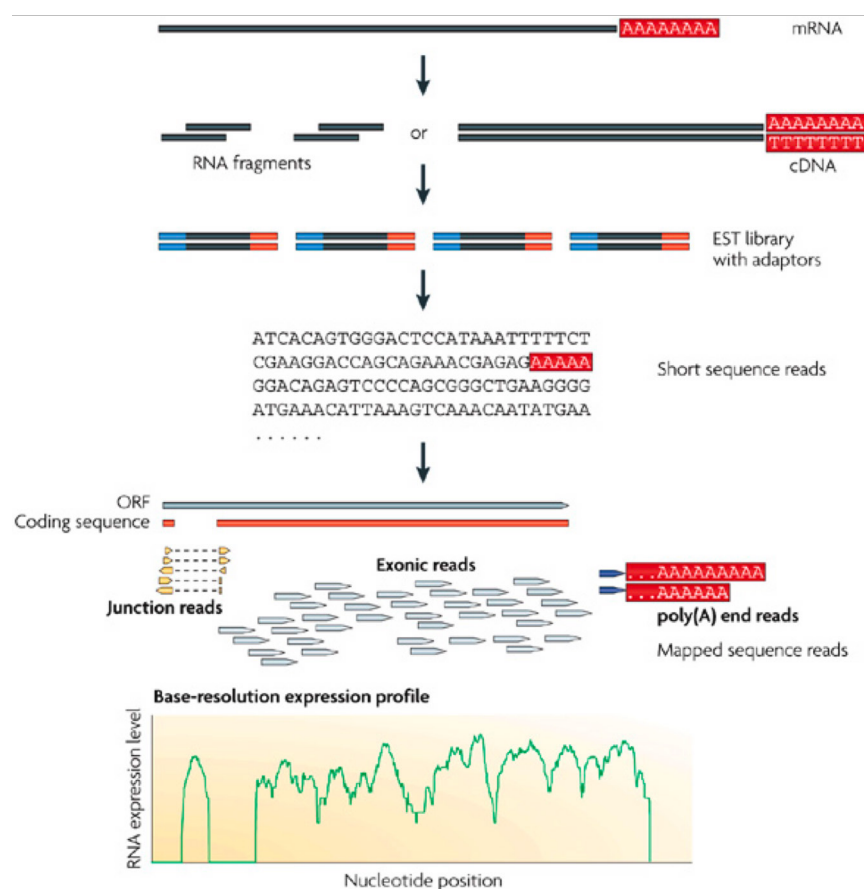


Figure 1-9 Overview of RNAseq

Briefly, long RNAs are first converted into a library of cDNA fragments through either RNA fragmentation or DNA fragmentation (see main text). Sequencing adaptors (blue) are subsequently added to each cDNA fragment and a short sequence is obtained from each cDNA using high-throughput sequencing technology. The resulting sequence reads are aligned with the reference genome or transcriptome, and classified as three types: exonic reads, junction reads and poly(A) end-reads. These three types are used to generate a base-resolution expression profile for each gene, as illustrated at the bottom; a yeast ORF with one intron is shown (Wang et al., 2009).

1.4.1.4 Unexpected expression patterns

Full analysis of FlyAtlas data, showed that some very well known and studied genes showed unexpected expression patterns. Many of these genes were involved in developmental processes and although showed expression in tissues previously indicated, they also showed extremely high expression in other tissues. Table 1-1 list some of these genes, their known function and their mRNA signal levels.

Table 1-1 Some genes that are predominantly expressed in unexpected places

Summary of genes which show unexpected expression patterns in FlyAtlas. Note boldface indicates maximum mRNA signal for each gene. (Adapted from Chintapalli et al 2007). Note table does not show all listed tissues on FlyAtlas, therefore if highest signal is not bold gene is expressed at a higher level in another tissue

Gene	Described in	Mean mRNA signal level							
		Brain	Head	Midgut	Tubule	Hindgut	Ovary	Testis	Accessory gland
<i>cry</i>	Circadian behavior	279	575	267	1,972	868	7	25	205
<i>fas2</i>	Neuronal fasciculation	129	66	49	1,676	78	5	9	53
<i>opd56d</i>	Olfaction	71	4,045	1	1	5,663	1	106	5
<i>kelch</i>	Nurse cell	181	185	22	16	22	5	6	6
<i>rpk</i>	Sensory neurons	1	0	1	7	0	904	47	0
<i>toe</i>	Eye, thorax	10	68	7	8	14	8	13	3,725
<i>vnd</i>	Embryonic CNS	6	4	289	5	6	3	2	8
<i>dsx</i>	Sex determination	21	119	89	140	106	9	1	8

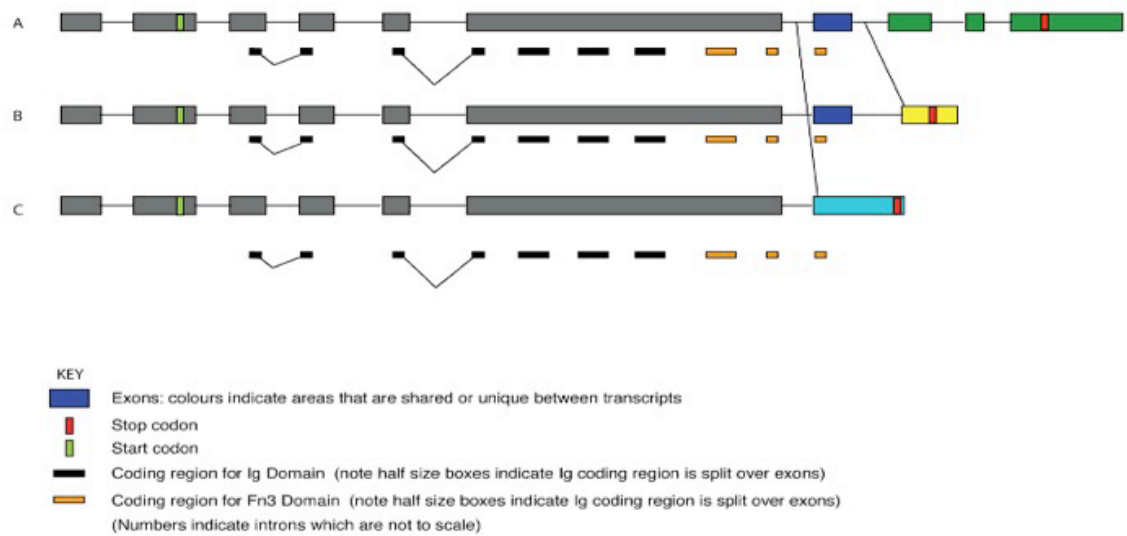
As we can see from this table, several genes show unexpected expression patterns in the MT. In particular *fasciclin 2* and *dsx* genes, which have been studied extensively in development, show high expression levels in the adult MT. It is tempting therefore to believe that these genes may play an important role in the MT and if so determining these roles may give an insight into physiological mechanisms.

1.5 Fasciclin 2

1.5.1 *Introduction*

Studies in grasshopper embryos had identified *fas2* as being a possible pathway recognition molecule, which allows growth cones to distinguish between different axon pathways (Harrelson and Goodman, 1988; Snow et al., 1988). In order to determine its function in the developing organism, *fas2* was cloned and characterised in *D.melanogaster* (Goodman et al., 1991). In situ hybridization mapped the gene to position 4B1-2 on the X chromosome. From this and subsequent studies it was determined that *fas2* is alternatively spliced to give three transcripts, resulting in 3 distinct protein isoforms, Fas2-PA, Fas2-PB and Fas2-C (Fig 1-10). The proteins are identical up to amino acid 737 and contain 5 Ig-like domains and 2 fibronectin type III domains. Fas2-PA and Fas2 -PB are transmembrane forms and the latter contains a PEST sequence in its cytoplasmic domain (Lin and Goodman, 1994), the function of which is unknown but other studies show the sequence acts as a target for rapid proteolytic degradation (Rogers et al., 1986). The third isoform, Fas2-PC is a glycosyl-phosphatidylinositol (GPI) membrane anchor protein and contains no transmembrane domain.

(a)



(b)

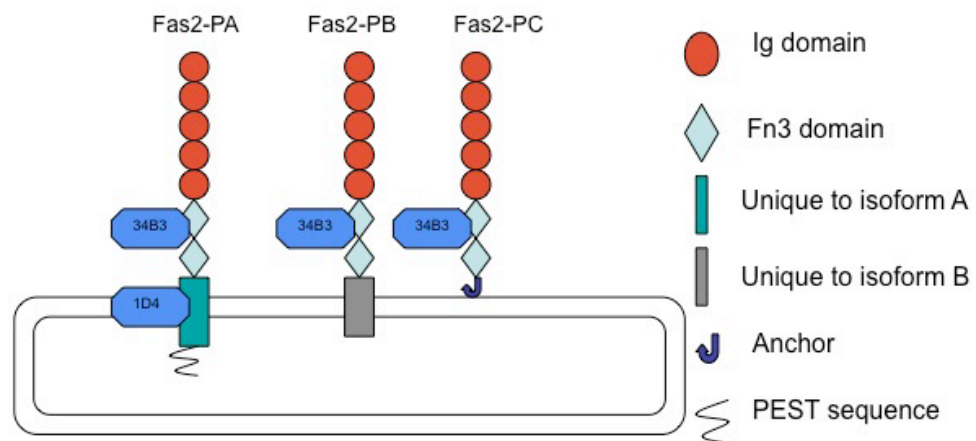


Figure 1-10 Gene and protein structure of Fas2

(a) Structure of *fas2* transcript including functional domains. The gene is alternatively spliced to produce 3 distinct transcripts. (b) Structure and domains of three Fas2 isoforms, including likely position of antibody epitope.

The protein is considered to be a homologue of the vertebrate protein NCAM (Goodman et al., 1991). However the proteins only share an amino acid identity of 26% across all seven extracellular domains, and they differ considerably in

their intracellular domains (Goodman et al., 1991). Interestingly the website database Homophila (www.superfly.ucsd.edu/homophila/) reports that *fas2* is also closely related to the gene Nephrin: a gene associated with the kidney disease, Finnish congenital nephrosis (Blast search indicates a 26% overall identity between Nephrin and Fas2 www.flybase.org). Patients exhibit high levels of proteinuria, ultimately leading to kidney failure (Patrakka et al., 2000). Nephrin acts as a structural adhesion molecule compulsory for the formation of the slit diaphragm in glomerular podocytes in human kidneys. Slit diaphragms are partly responsible for the selective filtration of molecules from kidneys, in a size dependent manner (Rodewald and Karnovsky, 1974; Wartiovaara et al., 2004). Indeed the classical *Drosophila* homologues of Nephrin *sticks* and *stones* (*sns*) and *hibris* (*hbs*), are found in nephrocytes, offering an attractive insight into the evolution of the glomerular kidneys (Weavers et al., 2009). Interestingly these nephrocytes are completely separate from the tubules. Therefore it is interesting that one possible homologue in flies, *fas2*, has not been investigated further, particularly now that we know that the gene is most highly expressed in the flies' tubules. However *fas2* does show functional similarities to NCAM and these may also give insights into its function in the tubules.

1.5.2 Oogenesis

Fas2 is first expressed during oogenesis. During mid oogenesis a cluster of six to eight border cells (BC) and two polar cells (PC), differentiate within the anterior follicular epithelium, causing the delamination of the BC (Niewiadomska, 1999; Szafranski and Goode, 2004). The polarity and movement of the cells is regulated by interactions between the proteins Fas2, Disc-large (Dlg) and Lethal-giant-larvae (Lgl). *Fas2* expression is selectively lost in BCs but not PCs, this in turn leads to the reorganisation of the three proteins in the BCs to a motile polarity and maintains Dlg and Lgl in the BCs, which in turn inhibits the rate of migration (Szafranski and Goode, 2004). *Fas2* COOH-terminus binds the PDZ domain of Dlg and this interaction is important in several other areas of development (see below). The protein also functions as a suppressor of epithelial invasion: along with several other proteins, such as Dlg, it actively suppresses invasion by organising the cooperative activity of distinct polarity and motility pathways (Szafranski and Goode, 2007). Interestingly it is believed that

this process may be partially governed through interactions with the actin cytoskeleton.

1.5.3 Axon growth and guidance

As discussed in Section 1.5.1, *fas2* was first cloned in order to understand its role in axon guidance during development. During development groups of axons known as growth cones extend out towards their intended synaptic target, aided by the adherence of growing axons to one another (Raper and Mason, 2010). The role of *fas2* in motor neuron growth cone guidance is well documented (Goodman et al., 1991; Grenningloh et al., 1991; Lin and Goodman, 1994). Mutations in the gene cause several phenotypes, which are dependant on both the dosage of the gene and in which area the gene is affected (Lin and Goodman, 1994). These include: the ‘bypass’ phenotype, where axons fail to enter their target and go past it; ‘detour’, where the axons enter their target at a different location; ‘stall’ where the once the axons enter their target they fail to then defasciculate; and ‘misroute’ where the axons meet a *fas2* positive cell and then move off target (Lin and Goodman, 1994). Essential to this processes is Fas2 ability to not only form homophilic cell adhesion complexes but also heterophilic complexes, with proteins such as Dlg (Lin and Goodman, 1994), this is summarised in Figure 1-10 . Fas2 functions in growth cone guidance do not solely rely on its properties as a cell adhesion molecule but also its ability to stabilize signalling of other molecules (Lin and Goodman, 1994).

1.5.4 Synapse Stability and Plasticity

The role of *fas2* in the stabilization and plasticity of neuromuscular junctions (NMJ) is extremely well studied. In order for a larval NMJ to form, pre- and postsynaptic interactions are extremely important (Kohsaka et al., 2007a). Expression of Fas2 at the presynaptic junction leads to the accumulation of Fas2 and Dlg at the postsynaptic junction, due to homophilic and heterophilic binding of Fas2, this in turn leads to the formation of the NMJ, as described in Figure 1-11 (Kohsaka et al., 2007a). Fas2 and Dlg complexes are also essential for synaptic stabilization and growth at the mature larval NMJ (Thomas and C.C. Garner, 1997). Decreasing *fas2* levels will also lead to an increase in synaptic

growth, showing that it also plays an important role here (Schuster et al., 1996). Structural plasticity is also controlled by *fas2* (Schuster et al., 1996). Interestingly long-term synapse plasticity is governed by both activity and cAMP-dependent process: cAMP-dependent plasticity requires a down-regulation of synaptic *fas2*. Indeed *fas2* has been shown to act in parallel with cAMP response element binding protein (CREB) in order to control synapse plasticity (Schuster et al., 1996). It is thought that increases in cAMP, cause the active removal of *fas2* from the synapse thus initiating synapse remodelling (Davis et al., 1996; Schuster et al., 1996).

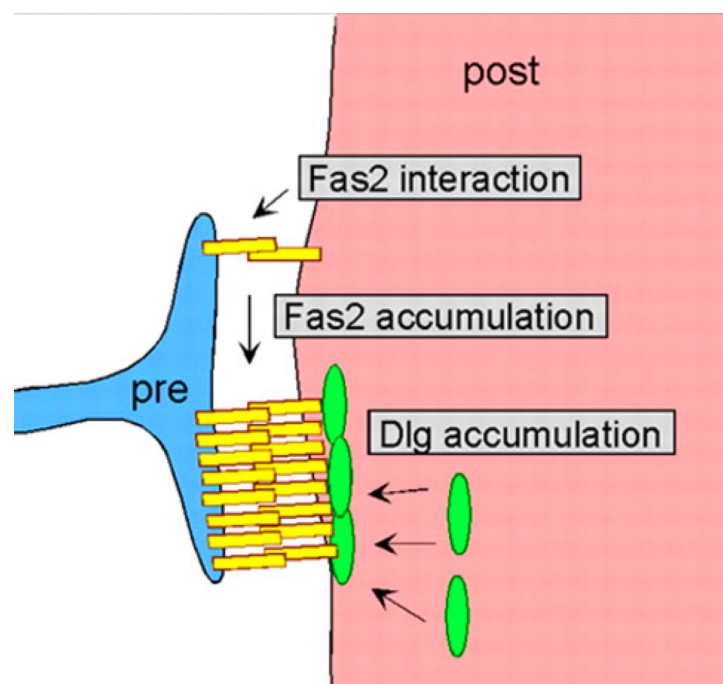


Figure 1-11 Schematic diagram describing a model of the postsynaptic assembly of Fas2 and Dlg (Kohsaka et al., 2007a)

1.5.5 Role in epidermal growth factor receptor (EGFR) and other signalling pathways

More recently Fas2 has been implicated in roles other than cell adhesion. For example Fas2 has been shown to be an important inhibitor of EGFR signalling during eye development (Mao and Freeman, 2009). This study is significant in that it indicates the importance of scaffolding proteins in signalling cascades. Previous studies have also showed that Fas2 interacts with several other signalling pathways such as FGRF (Forni et al., 2004). These and other studies

suggest that Fas2 is not only an important scaffolding cell adhesion molecule but also may be important for signalling processes.

1.5.6 Expression in the Malpighian tubules

As we have discussed *fas2* is extremely well characterised in terms of development and the nervous system and indeed there are over 500 references for *fas2* listed on FlyBase. We would therefore expected FlyAtlas to show a high expression level in the CNS of the fly. Table 1.2 shows results obtained.

Surprisingly it is predominantly expressed in the Malpighian tubules, a renal rather than neuronal tissue. Interestingly there is no known function for *fas2* in the Malpighian tubules.

Table 1-2 Summary of FlyAtlas results for all three transcripts of Fas2

Numbers indicate mean mRNA signal level in given tissue, boldface represents highest transcript level

TISSUE	Transcript A	Transcript B	Transcript C	Transcripts A, B and C
Brain	166	129	298	193
Head	58	67	234	114
Thoracoabdominal ganglion	261	198	417	340
Salivary gland	54	121	63	73
Crop	38	57	175	81
Midgut	51	49	63	35
Tubule	153	1676	393	813
Hindgut	46	78	147	79
Ovary	23	5	47	19
Testis	24	9	50	20
Male accessory glands	32	53	83	38
Adult carcass	38	82	135	92
Larval tubule	20	305	46	102
Larval fat body	31	26	181	52
Whole fly	28	79	78	51

The results indicate that transcript B of *fas2* appears to be tubule specific, with a ~10 fold increase in expression compared to other tissues. Transcripts A & C also show high levels of expression in the tubules. Previous studies have only hinted at *fas2* expression in the tubules (Campbell et al., 2009; Grenningloh et al., 1991). Interestingly transcript B and indeed the resulting protein are not as well studied as transcript A, which dominates studies carried out in

development. This suggests that *fas2-B* may have a previously unidentified function in the Malpighian tubules.

1.5.7 Possible role in the tubule

Understanding Fas2's interactions with other complexes and molecules can aid in determining its function in the tubules. For example it is interesting that Fas2 has multiple interactions with Dlg (see above), as this gene is expressed in the tubules. Strikingly Dlg sits at the lateral border during embryogenesis (Campbell et al., 2009) and the junctions between principal/stellate and principal/principal cells during larval development through to adulthood, where it acts to provide structural integrity. Secondly increases in cAMP levels in synapses lead to a decrease in Fas2 levels (Schuster et al., 1996). As cAMP is an important nucleotide involved with fluid secretion in the tubules then it may hint at a role for Fas2 in fluid secretion. EGRF signalling is also known to be essential for tubule formation and as *fas2* is known to interact with EGFR signalling it may hint at a role for *fas2* in tubule development (Baumann and Skaer, 1993; Kerber et al., 1998).

1.6 Doublesex

1.6.1 Introduction

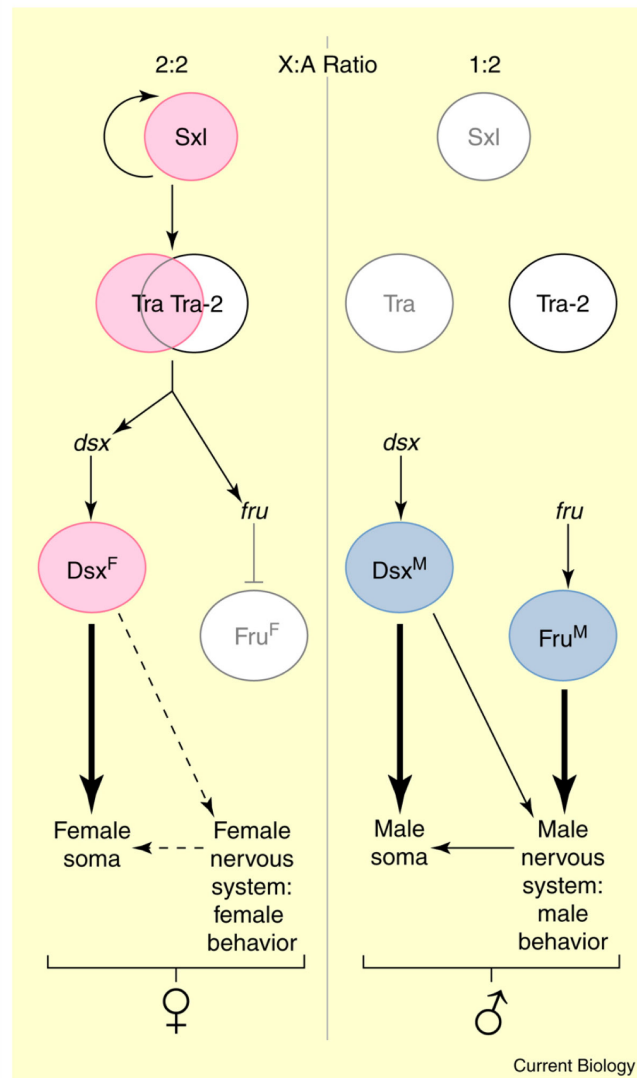
A second well-known gene, which is surprisingly abundant in the MT, is *doublesex* (*dsx*). Sex determination in the fruit fly is under the control of a sex determination hierarchy, which consists of several key genes. One such gene is the transcription factor *doublesex* (*dsx*). This gene is a member of the Dmrt protein family of transcription factors, which is an ancient conserved family found throughout the animal kingdom (Raymond et al., 2000; Raymond et al., 1998; Zarkower, 2002). During early embryonic development *dsx* is alternatively spliced to produce 3 isoforms, one male specific (*dsx^m*), one female specific (*dsx^f*) and a third as of yet undefined transcript, which appears to be functionally similar to *dsx^f*. The alternative splicing of *dsx* is controlled by the presence of other genes in the sex hierarchy as summarised in Figure 1-12. *Dsx* controls nearly all somatic sexual differences outside the nervous system, as

well as several nervous system characteristics (Lee et al., 2002; Mellert et al., 2010; Ridout et al.; Robinett et al., 2010; Sanders and Arbeitman, 2008).

1.6.2 *Targets of dsx*

The transcription of *dsx* is highly regulated throughout development, showing strict spatial and temporal expression (Rideout et al., 2010; Robinett et al.). As *dsx* is known to be a transcription factor, it is thought to control sexual dimorphisms through the direct binding of promoter regions of downstream genes (Baker and Ridge, 1980; Cho and Wensink, 1997). Both isoforms share identical DNA binding domains but differ in their C-terminal sequence, suggesting both proteins have the ability to bind the same sequences, but may differ in their function there after (Burtis et al., 1991; Erdman and Burtis, 1993). The variant C-terminal domains contain sex-specific regulatory elements as well as a homotypic domain that may again mediate protein:protein interactions (Burtis et al., 1991). Thus each isoform mediates the promotion or inhibition of sex specific gene expression through, as of yet undetermined co-factors, which bind the C-terminal domain of Dsx. Discovering true targets of the Dsx proteins, however has proved difficult, mainly due to being unable to distinguish direct binding from indirect binding (Luo et al., 2011). Many studies have focused on microarray analyses, where transcript levels are measured with and without *dsx* this does not however give an indication of direct targeting. At present there is only one known direct target of *dsx* and that is the fat body enhancer (FBE), which sits between the two Yolk proteins, *yp1* and *yp2* (Burtis et al., 1991). Dsx^M actively represses *yp1* and Dsx^F actively enhances expression. The Yolk proteins are important in female flies: they are involved in vitellogenesis, oogenesis and sex determination (Barnett et al., 1980; Soller et al., 1997; Yan and Postlethwait, 1990). There is however extensive evidence that *dsx* controls many other genes involved in sex determination (Lee et al., 2002; Mellert et al., 2010; Ridout et al.; Robinett et al., 2010; Sanders and Arbeitman, 2008).

(a)



(b)

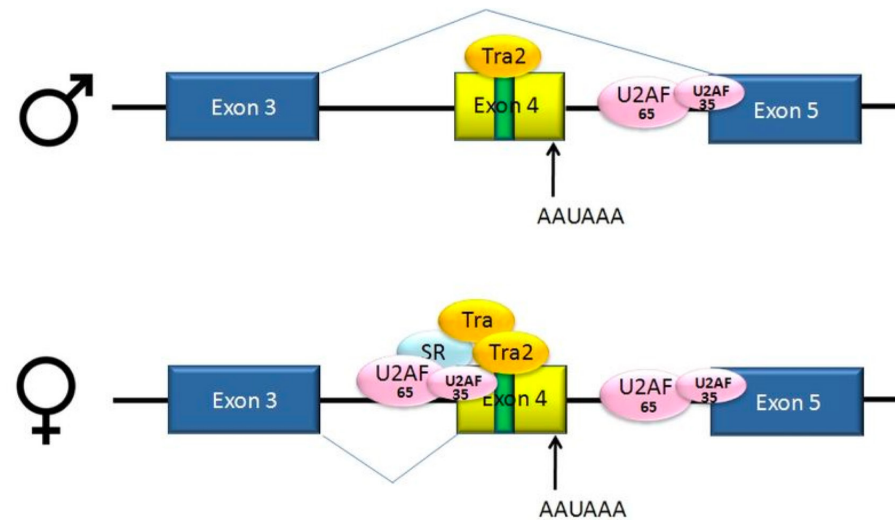


Figure 1-12 *Drosophila* sex hierarchy and the splicing of *dsx* to produce male and female variants

(a) Females: An equal ratio of X:A (X to autosome number) results in the expression of the gene *sex lethal* (*sxl*) this in turn leads to the active splicing of the gene *transformer* (*tra*) this splicing events results in an active form of *tra* which is then able to form a complex with transformer 2. This complex binds to the *dsx* gene and allows the splicing event, which leads to *dsx^F* being produced. The production of *Tra* also results in splicing of the gene *fru*, resulting in an inactive form of the protein. **Males:** In males *Sxl* is not produced and therefore no active form of *tra* is spliced and no *tra/tra2* complex forms, thus resulting in the default *dsx* transcript *dsx^M* being produced. Without the presence of *Tra*, *fru* is not spliced and an active form of *Fru* is produced, leading to the development of a male nervous system (Dornan 2011).

(b) Alternative splicing of *dsx* via the binding of *tra/tra2* complex, results in *dsx^F* non-binding of the complex results in *dsx^M* (Lynch and Maniatis, 1996)

It is now understood that *dsx* controls sexual characteristics and behaviour by the active suppression of genes: *Dsx^M* actively suppresses female genes and *Dsx^F* suppresses male specific genes. A further level of complexity in determining targets, is that most studies indicate differential tissue specific targets and usually only determine indirect targets (Camara et al., 2008; Christiansen et al., 2002).

1.6.3 *Expression in tubules and possible functions*

FlyAtlas reports that *dsx* is expressed at relatively high levels within the MT, again suggesting a previously unreported function for this gene summarised in Table 1.4.

Table 1-3 Summary of *doublesex* expression in *D.mealhogaster*
Numbers indicate mean mRNA signal level in given tissue.

TISSUE	Male	Female
Brain	18	21
Head	44	119
Thoracicoabdominal ganglion	59	55
Salivary gland	180	440
Crop	102	325
Midgut	51	89
Tubule	147	140
Hindgut	70	106
Ovary	8	9
Testis	21	1
Male accessory glands	62	8
Adult carcass	81	277
Larval tubule	29	41
Larval fat body	46	143
Whole fly	32	67

A recent, tubule specific microarray study, gave further insight into *dsx* expression in the tubules and also indicated that male and female tubules have a distinct set of sex specific genes, summarised in (Chintapalli et al. manuscript submitted). The tubules of the fly are important for many different physiological processes and have been shown to be involved in osmoregulation, immune response, detoxification and metabolism (Dow et al., 1994b; Maddrell, 2004; Maddrell and Casida, 1971; Maddrell, 1981; Yang et al., 2007).

Table 1-4 Summary of some of the genes differentially expressed in males and females

Dsx transcripts are highlighted in yellow. Immune genes are highlighted in green. Adapted from Chintapalli et al in press

Probeset ID	Gene Symbol	Gene Title	p-value(F vs. M)	Fold-Change(F vs. M)
Up in female				
1629545_at	Yp1	yolk protein 1	5.44661E-07	515.718
1631419_at	Yp3	yolk protein	4.41882E-07	514.01
1623655_at	Yp2	yolk protein 2	2.28907E-07	387.74
1633540_at	CG8147	CG8147	4.96236E-07	39.8852
1630600_at	Fst /// Scm	Frost /// Sex combs on midleg	2.12495E-05	31.4196
1637702_at	CG34427	---	1.1743E-07	28.8454
1641419_at	AttC	attacin	0.000672278	25.1226
1633820_at	Fad2	Fad2	0.000354706	22.46
1623776_s_at	Dsx	doublesex	1.42375E-10	19.3021
1627613_at	Mtk	Metchnikowin	7.05154E-05	12.0313
1627088_at	Fit	female-specific independent of transformer	0.000312061	9.92567
Up in males				
1623478_at	SPR	CG16752	3.0994E-07	-6.61793
1627653_at	l(1)G0469	lethal (1) G0469	3.61357E-05	-6.66035
1635189_at	Drs	drosomycin	0.00192381	-6.7587
1638816_at	CG3884	CG3884	0.00112492	-6.77181
1628982_at	NPFR1	neuropeptide F receptor	6.98814E-07	-6.90513
1632636_at	CG2145	CG2145	3.14302E-09	-8.61692
1638484_at	Hsp67Bc	Gene 3	0.000271373	-10.6377
1640799_at	Dsx	doublesex	6.94307E-13	-10.837
1628611_at	CG11241	CG11241	3.50671E-09	-11.1295
1637145_at	CG14787	CG14787	6.38554E-10	-18.3459
1633432_at	CG9657	CG9657	4.86936E-12	-41.6033
1633275_at	CG31562	CG31562	7.75546E-10	-56.7831

It is not surprising that sex specific genes are expressed in the tubules, as there are markedly different pressures on males and females with regards to physiology. For example, once mated, females are required to stage a higher level of immune response, as not only does the seminal package delivered by the male in itself pose an immune challenge, the increased time spent awake and eating needed to produce and lay eggs, also poses a higher chance of an immune challenge (Lazzaro et al., 2004; McKean and Nunney, 2005; Peng et al., 2005). Several recent studies have shown the importance of MT in immune response (Davies and Dow, 2009; McGettigan et al., 2005; Overend et al., 2011)

High levels of egg production in females, often leads to an increase in metabolism, due to the increased uptake of food. This in turn leads to higher

levels of osmoregulation and thus putting female tubules under a higher level of pressure than males (McGraw et al., 2004). Interestingly several of the genes highlighted as being differentially expressed in males and females are genes involved in metabolic and immune processes Table 1-4. This pose an interesting hypothesis that the tubules not only need to know what sex they are but may also play an important part in the physiological differences seen between males and females. The presence of differentially expressed *dsx* also hints at a role for this gene in determining the differences in male vs female gene expression in the tubules.

1.7 Aim of this study

We have discussed the advantages of *Drosophila melanogaster* as a model organism and recent advances in transcriptomics within the field. The question remains though of how one goes about using and assessing the data generated from such large-scale data sets such as FlyAtlas. As discussed in 1.4.1.4, FlyAtlas hinted at unexpected expression patterns of several very well known genes. This study aims to look at the expression of two of these genes, *fasciclin 2* and *doublesex*. Both of these genes show unexpectedly high expression patterns in the Malpighian tubules of the fruit fly hinting at previously unreported functions in this tissue. Therefore the aim of this study is to determine the function of these genes in the tubules. This study also aims to validate the use of FlyAtlas as a starting point in determining new functions for genes, and therefore the value of transcriptomics.

Chapter 2

2 Materials and methods

2.1 *Drosophila melanogaster*

2.1.1 *Drosophila* stocks

Strain	Genotype	Description	Origin	Reference
Canton S	Wildtype		Dow/Davies lab stock	
Gal4 Urate oxidase	w; Gal4 ^{UO} /Cyo	3rd instar larvae and adult GAL4 expression in main segment principal cells.	Dow/Davies lab stock	(Terhzaz et al., 2010)
Actin Gal4	w; Gal4 ^{actin} /Cyo	Ubiquitous driver	Bloomington Stock centre	FlyBase
c42-Gal4	w; +/+; c42/c42	Gal4 driver specific to the tubule principal cells	Dow/Davies lab stock	(McGettigan et al., 2005; Sözen et al., 1997)
c724-Gal4	w; +/+; c274/c724	Gal4 driver specific to the tubule stellate cells	Dow/Davies lab stock	(McGettigan et al., 2005; Sözen et al., 1997)
UAS- <i>fas2</i> -YFP	w; +/+; <i>fas2</i> / <i>fas2</i>	UAS construct <i>Fas2</i> -PB fused to YFP	Nose lab	(Kohsaka et al., 2007a)
UAS- <i>fas2</i> -YFP-Intra	w; +/+; <i>fas2</i> ^{intra} / <i>fas2</i> ^{intra}	UAS construct Intra-cellular domain of <i>fas2</i> fused to YFP	Nose lab	(Kohsaka et al., 2007a)
UAS- <i>fas2</i> -YFP-Extra	w; +/+; <i>fas2</i> ^{extra} / <i>fas2</i> ^{extra}	UAS construct Extra-cellular domain of <i>fas2</i> fused to YFP	Nose lab	(Kohsaka et al., 2007a)
UAS- <i>fas2</i> RNAi-KK	w; +/+; <i>fas2</i> ^{RNAiKK} / <i>fas2</i> ^{RNAiKK}	UAS construct Produces target down regulation of <i>fas2</i> under GAL4/UAS control	Vienna Drosophila Research Centre (VDRC) KK library	VDRC
UAS- <i>fas2</i> RNAi-V36351	w; <i>fas2</i> ^{V36351} / <i>fas2</i> ^{V36351}	UAS construct Produces target down regulation of <i>fas2</i> under GAL4/UAS control	Vienna Drosophila Research Centre (VDRC)	VDRC
UAS- <i>fas2</i> RNAi-V36350	w; <i>fas2</i> ^{V36350} / <i>fas2</i> ^{V36350}	UAS construct Produces target down regulation of <i>fas2</i> under GAL4/UAS control	Vienna Drosophila Research Centre (VDRC)	FlyBase
UAS- <i>fas2</i> RNAi-V8393	w; +/+; <i>fas2</i> ^{V38393} / <i>fas2</i> ^{V8393}	UAS construct Produces target down regulation of <i>fas2</i> under GAL4/UAS control	Vienna Drosophila Research Centre (VDRC)	
UAS- <i>fas2</i> RNAi-V8392	w; +/+; <i>fas2</i> ^{V38392} / <i>fas2</i> ^{V8392}	UAS construct Produces target down regulation of <i>fas2</i> under GAL4/UAS control	Vienna Drosophila Research Centre (VDRC)	
UAS- <i>fas2</i> -A-V5	w; +/+; <i>fas2A</i> ^{V5} /Cyo	<i>Fas2</i> -PA tagged with V5 construct under Gal4/UAS control	Generated for this study	

UAS- <i>fas2</i> -B-V5	w ⁻ ; +/+; <i>fas2</i> B ^{V5} /Cyo	<i>Fas2</i> -PB tagged with V5 construct under Gal4/UAS control	Generated for this study	
<i>fas2</i> EB112	w ⁻ ; +/+; +/+; <i>fas2</i> ^{EB112} /y	Imprecise P-Element excision resulting in <i>fas2</i> null. Homozygous lethal	Klāmbt lab	(Grenningloh et al., 1991)
<i>Fas2</i> -EP	w ⁻ ; +/+; +/+; <i>fas2</i> ^{EP} /y	Targeted up-regulation of native <i>fas2</i> via insertion of UAS upstream of <i>fas2</i>	Bloomington Stock centre	FlyBase
<i>fas2</i> proteintrap 788	w ⁻ ; +/+; +/+; <i>fas2</i> ^{genetrap} /y	<i>Fas2</i> genetrap line. Resulting from insertion of GFP ORF in 3' end of <i>fas2</i> gene	Klāmbt lab	
<i>fas2</i> proteintrap 377	w ⁻ ; +/+; +/+; <i>fas2</i> ^{genetrap} /y	<i>Fas2</i> genetrap line. Resulting from insertion of GFP ORF in 3' end of <i>fas2</i> gene. Resulting in <i>fas2</i> -PA containing a GFP	Klāmbt lab	
UAS- <i>tra</i> RNAi	w ⁻ ; +/+; <i>fas2</i> ^{RNAi} / <i>fas2</i> ^{RNAi}	UAS construct Produces target down regulation of <i>tra</i> under GAL4/UAS control	VDRC	VDRC
<i>dsx</i> ^{Gal4}	+; +; <i>dsx</i> ^{GAL4} /TM3, Sb, Ser, e	GAL4 element inserted into <i>doublesex</i> (<i>dsx</i>) locus via ends-in HR.	Goodwin lab	(Rideout et al., 2010)
UAS- <i>stingerII</i>	W ¹¹¹⁸ ; +; P(<i>w⁺mc</i> =UAS- <i>StingerII</i>)	Stable insulated nuclear enhanced (GFP).	Bloomington	

Table 2-1 List of All Lines Used in This Study

Table 2-1 lists all *Drosophila melanogaster* stocks used in this study. A description of each line is given along with the origin and reference for the line where applicable.

2.1.2 *Drosophila* rearing

Flies were reared in either vials or bottles containing standard *Drosophila* medium (Appendix 1) using a 12h/12h light/dark cycle. Flies were kept at either 22°C or 25°C as stated in text.

2.1.3 Dissection of *Drosophila* tissue

Adult flies were briefly anesthetized on ice, prior to acute dissection of tubules, gut or CNS in sterile Schneider's medium (Invitrogen, UK). For larval samples, larvae were dissected live. Whole flies were collected after briefly being anesthetized on either ice or CO₂.

For RNA extraction at least 30 flies were dissected. Protein samples required larger volumes of tissue dependant on application. Briefly, >50 flies worth of tubules were dissected for Western blot analysis, >150 for IP experiments and 3000 flies (3000 guts and 6000 pairs of tubules) were dissected for BN-PAGE analysis. Equal numbers of males and females were dissected unless otherwise stated. During all dissection protocols, 10 flies were placed on ice at a time in order to avoid prolonged exposure to cold temperatures. Tissue samples were also removed from Schneider's and placed in appropriate buffers every 30 min in order to maintain protein and RNA stability. For all protein work Schneider's medium was supplemented with Protease inhibitor as described in 2.11

Where appropriate, samples were incubated in 3 ml of sterile Schneider's medium contain the appropriate concentration of either cAMP, 8-Bromo-cAMP, CAPA, cGMP, forskolin or IBMX (all Sigma, UK).

2.1.4 *Embryo collection and preparation*

In order to collect *Drosophila* embryos, adult females were allowed to lay eggs on grape juice agar plates (Appendix 1) for approximately 16-24 hrs at 26°C. Embryos were then detached from the egg laying plate using a paintbrush and a stream of distilled water, and collected in a fine-mesh sieve. In order to prepare embryos for visualization they were first dechorionated, by placing the sieve into a 50% solution of bleach in distilled water for exactly 3 min, prior to several more washes with water. The embryos were then transferred to 5 ml heptane in a glass container using a paintbrush, and 5 ml of 4% paraformaldehyde (in PBS) was added, and the solution was shaken vigorously. Embryos were then fixed for 10-30 min at room temperature. After fixation, the aqueous bottom layer was completely removed, and a 5 ml solution of 95% methanol/5% EGTA (ethylene glycol tetra-acetic acid, pH 8.0) was added to the glass container, and swirled gently. De-vitellinized embryos sank to the bottom of the container. Embryos were then transferred to a clean 1.5 ml Eppendorf tube containing 1.0 ml of PBS using a Pipette with a cut tip. After the embryos had fallen to the bottom of the tube, most of the PBS was removed and several drops of PAT (PBS, 1% Triton-X, 0.1% bovine serum albumin (BSA)) were added. The embryos were then washed an additional three times with PAT, followed by two washes in PBS. To mount,

the PBS was removed and VectaShield (Vector Lab) was added, and the embryos were transferred to a polylysine slide for viewing as described in Section 2.12

2.2 RNA extraction

RNA extraction was carried out using the QIAGEN® RNeasy® Mini kit and the QIAGEN® RNase-free DNase set as described in the manual (Qiagen, UK). Typically 60 tubule pairs (30 flies), 10 heads and 5 whole fly samples were dissected as described in section 1.1.3. Whole fly samples were homogenized using a sterile blue probe and then sonicated tubule and gut samples were briefly sonicated. RNA was eluted in 25µl of RNase-free water or TE buffer. RNA quality and quantity was assessed as described in Section 2.8. All RNA samples were stored at -80°C and aliquoted to avoid repeated freeze/thawing.

2.3 First strand cDNA synthesis

Superscript™ II Reverse Transcriptase (Invitrogen, UK) was used in order to syntheses first strand cDNA from RNA. Reactions were carried out as manufacturer's instructions. All samples were stored at -20°C. Two samples were obtained from each RNA sample in order to facilitate in downstream applications and all samples were quantified as described in Section 2.8, thus allowing for equal concentrations of each sample to be used.

2.4 Oligonucleotide Synthesis

Primers for this study were designed using the application MacVector 11.1.1 or the online resource NCBI. Oligonucleotides were then synthesised by MWG Biotech custom primer service on a 0.01 µmol scale. All primers ordered were purified by High Purity Salt Free (HPSF®) technology, and their quality assessed by Matrix Assisted Laser Desorption Ionisation - Time of Flight (MALDI-TOF) analysis. A stock concentration of 100µM was obtained by re-suspending the lyophilised pellet in ddH₂O and stored at -20°C. A final working concentration of

6.6 μM was used in all experiments unless otherwise stated. All primers used in this study can be found in Appendix 2.

2.5 Polymerase chain reaction (PCR)

For all PCR procedures, cycling was performed using either, a Hybaid OmnE, Hybaid PCR Sprint or Hybaid PCR Express-Gradient thermocycler and DNA was subsequently separated by Agose gel electrophoresis as described in Section 2.6

2.5.1 *Standard PCR using Taq DNA polymerase*

For standard PCR amplifications a pre-aliquoted Thermoprime with Readymix™ PCR Buffer (ABgene, UK) was used. A standard cycle is summarised in Table 2-2. For each reaction 1 μl of each primer was added along with 1 μl of template (up to 1 ng of plasmid DNA, 100 ng of genomic DNA or 500 ng of cDNA) or ddH₂O for no-template controls, to 22 μl of mastermix.

Table 2-2 Typical PCR Cycle

Step	Temperature	Time		Comments
Initial Denaturation	94 °C	3 min		To ensure template denaturation
Denaturation	94 °C	30 sec	25 – 30 cycles	-
Annealing	50 – 60 °C	30 sec		Temperature is set depending on the melting temperature of the primers used; typically ~5 °C lower than T _m
Extension	72 °C	30 sec – 5 min		30 sec extension for each 500 bp of DNA to be amplified
Final Extension	72 °C	5 min		-

2.5.2 *PCR using proof reading Herculase II Fusion DNA Polymerase*

For all cloning protocols it was important PCR products contained no mistakes in sequence therefore the proof polymerase kit Herculase® II Fusion DNA Polymerase (Agilent Technologies, UK) was used, according to the manufactures

guidelines. An additional, 5 min incubation at 72 °C, with Taq polymerase was carried out, in order to obtain poly-A overhangs at the 5' and 3' ends of the PCR product. This facilitated downstream cloning reactions, as most plasmids used in this study require the poly-A overhangs in order to incorporate DNA into them.

2.5.3 *Reverse-Transcriptase (RT)- PCR*

RT-PCR was required for cloning, primer testing and gene analysis and was carried out in two stages. The first stage, cDNA synthesis, is described in 2.3. The second stage was carried out via standard PCR protocols as described in 2.5. Prior to carrying out PCR, all cDNA was quantified as described in 2.8 and equal concentrations of each sample were used. In order to control for possible genomic contamination primers used in RT-PCR were designed to span exon/intron boundaries. Amplified sequences could range in size from 20bp to 3.5kb.

2.5.4 *Quantitative reverse transcriptase (QRT)-PCR*

Gene expression levels were quantified using QRT-PCR. Initially, two-step QRT-PCR was carried out using the fluorescent double-stranded DNA dye DyNAmo™ SYBR® Green (Finnzymes, Finland). Prior to carrying out these experiments, cDNA was synthesised from the tissue of interest. Typically for each experiment 4 biological replicates were generated and 3 technical replicates were loaded for each. Primers were designed to produce products <500bp and were possible spanned exon/intron boundaries of the gene of interest. Primers were also designed against the gene alpha-tubulin in order that samples could be normalized against this gene. The cycle protocol can be seen in Table 2-3. The protocol was as manufacture recommends. Briefly, 25 µl 2x SYBR Green Master Mix, 2 µl each of primers (0.3 µM final concentration) and 1 µl of template cDNA (up to 500 ng) was made up to 50 µl using ddH₂O for each sample.

Table 2-3 Typical Cycling Condition for Two-Step QRT-PCR

Step	Temperature	Time		Comments
Initial Denaturation	95 °C	10 min		To ensure template denaturation
Denaturation	95 °C	20 s	35 - 45 cycles	-
Annealing	55 °C	20 s		Temperature is set depending on the melting temperature of the primers used; typically ~5 °C lower than T _m
Extension	72 °C	5 - 20 s		5 s per 100 bp of product
Data Acquisition	-	-		Fluorescence data collection is performed after each cycle
Final Extension	72 °C	5 min		-
Melting Curve	60 - 90 °C	1 s hold per 0.3 °C		Used to check the specificity of the amplified product

Samples were prepared on ice and loaded into optical grade PCR tube strips (MJ Research (StarLab,UK). Two blanks of mastermix only and no template control were loaded in triplicate. Prior to the experiment template amplicons were generated for each primer pair and standards ranging from 10^{-1} - 10^{-7} ng were created by serial dilution, allowing for absolute quantification of gene expression. Cycling was performed in Opticon™ 3 thermal cycler (BioRad, UK)

Following amplification Opticon™ 3 software was used in order to generate a standard curve. Absolute concentration was determined by placing the Cycle Threshold (Ct) value and the values from gene standards (RP49, Tubulin) onto the standard curve. Each sample of gene target was then normalized against alpha tubulin sample, resulting in a ratio of gene/alpha-tubulin. Results were then plotted as means \pm SEM (where control = 1) using GraphPad Prism 5.0 software. Statistical significance of data was determined by 2-way ANOVA and/or Student's *t* tests where appropriate.

Subsequent reactions were carried out using *Power SYBR® Green RNA-to-C_T™ 1-Step Kit*. Essentially the protocol is similar to that of 2-step QRT-PCR but omits the need to produce cDNA and instead converts RNA during the run. A typical cycle can be seen in Table 2-4

Table 2-4 Typical Cycling Conditions for One-Step QRT-PCR

Step	Temperature	Time		Comments
cDNA synthesis	48 °C	30 mins		Synthesis of cDNA
Initial Denaturation	95 °C	10 min		To ensure template denaturation
Denaturation	95 °C	20 s	35 - 45 cycles	-
Annealing	55 °C	20 s		Temperature is set depending on the melting temperature of the primers used; typically ~5 °C lower than T _m
Extension	72 °C	5 - 20 s		5 s per 100 bp of product
Data Acquisition	-	-		Fluorescence data collection is performed after each cycle
Final Extension	72 °C	5 min		-
Melting Curve	60 - 90 °C	1 s hold per 0.3 °C		Used to check the specificity of the amplified product

2.6 Agarose gel electrophoresis

After PCR was performed, samples were assessed via Agarose gel electrophoresis. Typically a 1% agarose gel was obtained using 0.5x TBE [90 mM Tris, 90 mM boric acid (pH 8.3), 2 mM EDTA], containing 0.1 µg/ml EtBr as described in Sambrook and Russell, 2001. Once set, gels were loaded into a Mini-Sub cell GT electrophoresis chambers (Bio-Rad, UK), containing 0.5% TBE. Samples were diluted in 6X loading dye [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol in water] and a 1KB ladder was loaded in order to compare band size (Invitrogen, UK). Gels were typically run at 150V until desired distance and visualised using a UV light transilluminator. If required, gel extraction was carried out as described in 2.7.

2.7 PCR Purification

Gel extraction was carried out using the QIAGEN® QIAquick Gel Extraction Kit according to the manufacturers' instructions. Alternatively PCR products were directly purified using QIAGEN® QIAquick PCR purification kit according to instructions. DNA was typically eluted in ddH₂O and stored at -20°C.

2.8 Quantification of Nucleic Acid

Nucleic acid quantity and quality was assessed using the NanoDrop 1000TM spectrophotometer (Thermo Scientific, UK) according to manufacturers' instructions. All samples were zeroed against a sample of the buffer in which they were diluted. Nucleic acid concentrations were measured at 260 nm (A_{260}) and 280 nm (A_{280}), quantity given as ng/ μ l. Purity was measured as a ratio of A_{260}/A_{280} , for RNA a value of 2.0 and for DNA a value of 1.8 indicated pure samples.

2.9 DNA Cloning

2.9.1 *E.coli* strains and plasmid

Listed below are the *E.coli* and plasmid strains used in this study.

Table 2-5 List of all *E.coli* strains and Plasmids used in this study

<i>E.coli</i>	
Strain	Genotype
DH5 α TM subcloning efficiency competent cells (Invitrogen)	(F ⁻ ϕ 80dlacZ Δ M15, Δ (lacZYA-argF), U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (r_K^- , m_K^+), <i>phoA</i> , <i>supE44</i> , λ^- , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>).
OneShot [®] TOP10 Competent cells (invitrogen)	F ⁻ <i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) Φ 80lacZ Δ M15 Δ lacX74 <i>recA1</i> <i>araD139</i> Δ (<i>ara leu</i>) 7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (StrR) <i>endA1</i> <i>nupG</i>
Plasmids	
Name	Purpose
pCR2.1 [®] -TOPO [®] TA vector	Standard cloning and sub-cloning reactions
pENTR TM /D-TOPO [®]	Gateway cloning
pRISE	Construction of RNAi constructs
pUAST	Cloning of <i>fas2</i> -V5

2.9.2 Cloning of *fas2*-V5 tagged constructs

To facilitate downstream experiments such as immunoprecipitation and localisation of individual isoforms, cloning was carried out to generate fly lines carrying *uas-fas2*-PA and *uas-fas2*-PB tagged with the 25 amino acid epitope V5. This short tag allows for easy detection with commercially available antibodies, whilst not interfering with protein function.

2.9.2.1 Primer Design and PCR amplification

Primers were designed to amplify the open reading frame (ORF) of *fas2*-PA and *fas2*-PB and include a 5' V5 tag. Restriction enzyme sites were also included in the 5' and 3' region in order to facilitate directional cloning into the pUAST vector upstream of 5 UAS open reading frames. PCR products were generated and purified as described in Sections 2.5 & 2.7, respectively.

A summary of the cloning procedure is given in Figure 2-1

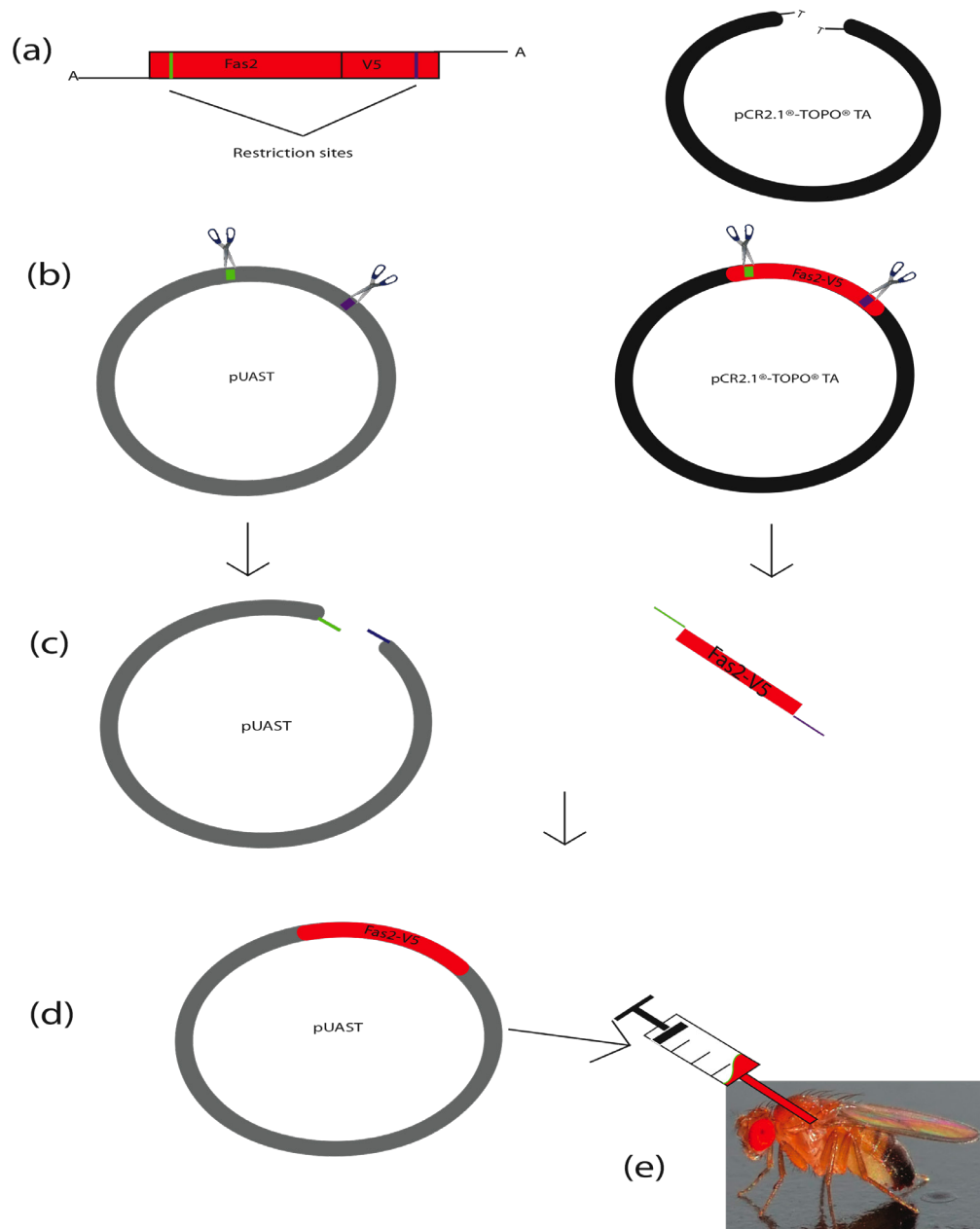


Figure 2-1 Cloning procedure for production of UAS-*fas2*-PA-V5 & UAS-*fas2*-PB flies

Summary of TA and direct cloning of *fas2*-PA-V5 and *fas2*-PB-V5. (a) PCR product containing Poly-A overhangs is introduced into pCR2.1®-TOPO® TA vector via Poly-T overhangs. Once amplified the *fas2*-PA-V5/*fas2*-PB-V5 is cut out with 2 distinct restriction enzymes (NotI & XbaI). (b & c) Sequentially the vector pUAST is also cut with these two enzymes thus allowing directional insertion of the *fas2* construct into the vector (d). Once insertion and direction were verified plasmid DNA was isolated as described in 2.7 and sent to BestGene inc, for injection into embryos (e).

2.9.2.2 DNA Ligation

Initial cloning was carried out as manufactures guidelines

(<http://products.invitrogen.com/ivgn/product/K450002#manuals>). Vector/insert ratio was worked out prior to the cloning reaction in order to obtain the

optimum molar ratio of 3/1 using the following equation:

$$\frac{\text{length of insert (in kb)}}{\text{length of vector (in kb)}} \times \text{ng of vector} = \text{ng of insert needed for a 1:1 ratio}$$

DNA ligation reactions were carried using the Roche Rapid DNA Ligation Kit according to the manufacturers' instructions. Reactions were incubated at room temperature for between 30 min and overnight were appropriate.

2.9.2.3 Transformation into *E.coli*

Plasmids were transfected into either DH5 α TM subcloning efficiency chemically competent cells (Invitrogen) or TOP10 cells by the addition of 50-100 ng of plasmid to 50 μ l of cells on ice. Following this samples were incubated on ice for 15 min followed by a heat-shock at 37°C for 30 s. Samples were then transferred back to ice for a further 2 min in order to quench the reaction. 950 μ l of L-broth (Appendix 1) was then added to each sample and incubated at 37°C for 30 min to allow expression of the *amp*^R gene. 50-100 μ l of each transformation was then spread onto L-Agar plates (Appendix 1) containing 100 μ g/ml ampicillin/kanamycin and incubated overnight at 37°C. When required, plates were also made to contain 50 mg/ml of X-Gal.

2.9.2.4 Identification of positive clones

All plasmids used in this study carry an antibiotic resistance gene, either ampicillin or kanamycin. Therefore, all *E.coli* successfully transfected with plasmid will grow on plates or liquid culture containing 100 μ g/ml of Ampicillin or 50 μ g/ml of Kanamycin, thus acting as the first level of identification. The plasmid pCR2.1®-TOPO® TA also contains the LacZ- α gene ORF in the multiple cloning region of the plasmid. This gene encodes the enzyme β -Galactosidase, which cleaves X-GAL to produce galactose and 5-bromo-4-chloro-3-hydroxyindole. This is then oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, and a blue colour is produced. Therefore *E.coli* containing the plasmid will appear blue on agar plates containing 50 μ g/ml of X-Gal. However if the fragment of interest

has been cloned into the vector this results in the disruption of the LacZ gene and thus a white colony is produced.

2.9.3 *Purification and Isolation of Plasmid DNA*

Small scale plasmid isolation was carried out using the QIAGEN® Qiaprep Spin Miniprep kit. For large scale isolation, as required for germline transformation, the QIAGEN® Qiagen Plasmid Maxi, Endofree Maxi kit was used, as manufacturer's guidelines. DNA was eluted in ddH₂O and stored at -20°C.

2.9.4 *Validation of Cloning by PCR and Restriction Digest*

As mentioned in the previous text all cloning reactions were validated by both PCR and restriction digest.

2.9.4.1 Validation via PCR

Primers were designed to anneal to the vector and insert respectively, thus confirming both the presence of the insert and the direction. Small amounts of individual clones were picked from plates using a sterile toothpick. This was then added to PCR reactions as stated in Section 2.5. PCR products were assessed via Agarose gel electrophoresis as described in Section 2.6

2.9.4.2 Validation via Restriction Digest

Restriction digest experiments were designed using the online resource NEBcutter (<http://tools.neb.com/NEBcutter2/index.php>). Either two enzymes were picked, where one cut within the insert and one within the vector. Alternatively, one enzyme, which cut both areas, was used. Before restriction digests were carried out, selected colonies were grown overnight at 37°C in 5 ml of L-broth supplemented with 100 µg/ml ampicillin or 50 µg /ml of kanamycin. Protocols were as manufacturer's instructions and reactions were typically incubated for several hours at 32°C prior to running on a 1% agarose gel for assessment. Restriction digest was also carried out, when required for sub-cloning.

2.9.5 Sequencing

Once cloning was complete plasmid DNA was sent to The DNA Sequencing and Services™ at the University of Dundee. Results were analysed using the software MacVector. Once confirmation of positive clones was obtained plasmid DNA was isolated and samples sent to Bestgene Inc. for the generation of fly lines.

2.10 *Drosophila* S2 cell culture

Once cloning was complete plasmids were transfected into *Drosophila* S2 cells in order to determine the presence of functioning protein.

2.10.1 *Passaging of S2 Cells*

Drosophila S2 cells (Invitrogen, UK) were maintained in complete Schneider's Medium (Invitrogen, UK) supplemented with 10% heat-inactivated Foetal Bovine serum) (CSM) at a temperature of 28 °C. Cells were typically kept in a total volume of 15 ml in 75cm³ flasks. For general maintenance, cells were passaged at a density of 10⁷ cells/ml. To do this, cells were re-suspended by gentle pipetting and then diluted 1:2.5 by adding 6 ml of cells into 9 ml of fresh CSM.

2.10.2 *Transient Transfection of S2 Cells*

Transient transfection was carried out in tissue culture six-well plates. 24 hours before transfection 6 x 10⁶ cells in a volume of 3 ml were placed into individual wells. Each 600 µl transfection, 19 µg of each plasmid DNA and 36 µl CaCl₂ (2 M - Invitrogen) were added to a sterile 1.5 ml eppendorf tube and made up to a total volume of 300 µl with dH₂O. This was mixed well and then added slowly over 1-2 min to 300 µl of 2 x Hepes buffered saline (HBS - 50 mM Hepes, 1.5 mM Na₂HPO₄, 280 mM NaCl, pH 7.1; Invitrogen, UK) with continuous mixing. Each 600 µl reaction was then left to precipitate for 30 min at room temperature before being added drop-wise to the seeded S2 cells whilst swirling continually to mix. Cells were then incubated for 16 - 24 hr at 28 °C. Following incubation, cells were re-suspended by gentle pipetting and transferred to a 15 ml falcon tube. Each sample was then pelleted by centrifugation at 1500g for 1 min at room

temperature and re-suspended in 3 ml fresh CSM to wash. This step was repeated twice more before cells were re-suspended in 3 ml of CSM and returned to the same six-well plate. If a plasmid encoding a metal inducible promoter was used, protein expression was induced in each 3 ml culture by the addition of 15 μ l of 100 mM CuSO_4 and expression was allowed to proceed for 40-42 hours.

2.11 Protein Analyses

2.11.1 *Protein Extraction*

Several protocols were used in order to extract protein. For Western Blot analysis, typically 50 flies (100 pairs of tubule) or 10 heads were dissected. Once dissected tubules were removed from the Schneider's medium every 30mins and transferred into SMART Buffer (Appendix 1). Once all tubules were collected samples were snap-frozen with liquid nitrogen and stored at -80°C . Whole flies were briefly anaesthetised on CO_2 or ice and placed straight into SMART buffer before being snap frozen. Once required, samples were defrosted on ice and homogenised firstly with a blue probe and secondly with a Microson™ Ultrasonic Cell Disruptor. Samples were then centrifuged at $10,000 \times g$ for 10 mins at 4°C using an Accuspin™ Micro R from Fisher Scientific in order to remove cell debris. Supernatant was removed and placed in a sterile Eppendorf and stored at -20°C or used directly for Western blot analysis.

For co-immunoprecipitation experiments, a larger quantity of protein was required (500-1,000 μg), so typically at least 150 fly tubules (300 pairs) were dissected and transferred into ~300-700 μl of 3T3 buffer (appendix). Initially 7 different detergents were assessed for their ability to solubilise membrane proteins, with the hope of finding one that was harsh enough to solubilise the protein but not disrupt protein/protein interactions. n-Dodecyl- β -maltoside (DDM) was determined to be the best and was used for all further applications.

For Blue-Native (BN)-PAGE experiments a far greater quantity of protein was required, therefore the tubules and guts of 3000 flies were dissected, resulting in 9.49 mg of protein. Samples were collected every 30 min and placed in a

small amount of Schneider's medium. They were then spun at 10,000 X g for 1 min and the Schneider's medium was removed before the samples were snap frozen in liquid nitrogen. Samples were then re-suspended in NATIVE-PAGE sample buffer (Invitrogen, UK), which contained 1% DM detergent and 1 in 100 dilution of protease inhibitor. All aliquots were then combined before sonication was carried out using the Microson™ Ultrasonic Cell Disruptor. Samples were then centrifuged at 20,000 X g for 30mins at 4°C. Supernatant was removed and placed in a sterile Eppendorf. Samples were then treated with Benzonase in order to remove any DNA contaminants. Briefly $MgCl_2$ was added to each sample to a final concentration of 2 mM along with 1-2 units of benzonase (Sigma, UK). Samples were then incubated at room temperature for 60mins before a further centrifugation at 20,000 X g for 30 min at 4°C. Supernatant was removed and transferred into a sterile Eppendorf and stored at -80°C.

All protein samples were quantified as outlined in 2.11.2 prior to further work.

2.11.2 Quantification Via Bradford Protein Assay

Protein levels were quantified using the Bradford protein assay. Typically, 1-5 μ l of sample was loaded on a 96 well plate and made up to 50 μ l with ddH₂O. 200 μ l of Bradford reagent (BioRad, UK) was then added. Standards ranging from 0.5 μ g - 5 μ g of protein standard (BSA, Sigma) were also loaded as above. Plates were then loaded into (plate reader) and absorption rate 590 nm was measured. A standard curve was generated from the standard and the protein samples plotted onto this curve.

2.11.3 Isolation of Membrane Protein Fraction by Sucrose Gradient

For BN-PAGE analysis membrane proteins were isolated by sucrose gradient centrifugation. Figure 2-2 summarizes this procedure. Briefly, 2.5 M, 2 M and 0.5 M sucrose dilutions were prepared by diluting 5 M sucrose in TKMD buffer (Appendix 1). 15.2 ml of 2.5 M sucrose was gently poured into a 37.5ml

UltraClear centrifuge tubes (Beckman Coulter). 2 ml of protein sample was then gently laid over this and carefully mixed with the sucrose layer. 12.5 ml of 2.0 M sucrose was carefully layered on top of this and a final layer of 0.5 M sucrose was added. Ultracentrifugation was carried out at 100,000 X g for 5h, at 4°C. Once complete, the membrane fraction can be found between 0.5 M sucrose and 2.0 M sucrose. This fraction was carefully removed by inserting a syringe into the side of the tube. The membrane fraction was washed with 2x lysis buffer before a further centrifugation at x 30,000 X g. The supernatant was carefully removed and the pellet re-suspended in 100 µl of lysis buffer.

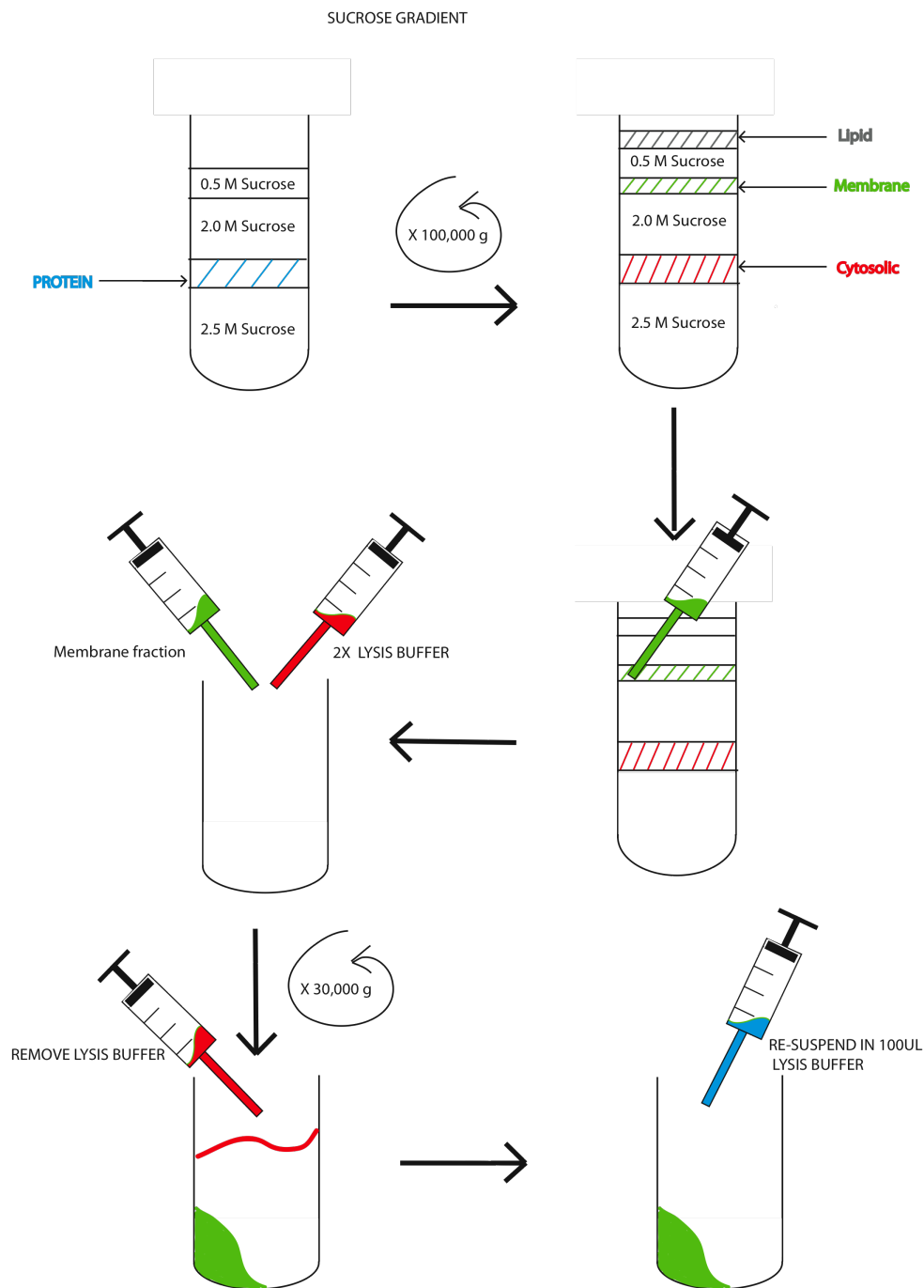


Figure 2-2 Summary of Isolation of Membrane Proteins by Sucrose Gradient

2.11.4 Protein Separation Via SDS-PAGE Electrophoreses

Once samples were prepared and quantified, they were diluted in 6X loading dye to a final concentration of 1x and β -mercaptoethanol was added (1:20). Samples were then boiled for ~5 min and placed back on ice. Electrophoresis was carried out using the Bio-Rad Ready Gel Mini-PROTEAN II Cell Module. Gels were either freshly prepared as described in Appendix 1, with either 10 or 16 wells or pre-

cast Bio-Rad Ready Gels 10-20% Tris-HCl buffered polyacrylamide gels. A Tris-Glycine running buffer was prepared as described in Appendix 1. Gels were run at 50V for ~30 min, or until the dye front had passed through the stacking gel at which point the voltage was increased to 150V and ran for a further 1h-1.5h. 2-5 μ l of ECL Plex Fluorescent Rainbow Marker (Amersham, UK) or BenchMark™ Pre-Stained Protein Ladder (Invitrogen, UK) was used for sizing proteins on the gel.

2.11.5 Western Blotting

Western blotting was carried out in order to quantify a particular protein via binding of an antibody. Gels were blotted onto Hybond ECL membrane (GE Healthcare, UK), using a Novex Xcell II™ blot module. Briefly, the gel was placed on 3mm Whatmen paper, the membrane was then placed over the gel and topped with further Whatmen paper and placed in the blot module. Transfer buffer was freshly prepared as described in Appendix 1. Transfer was carried out at 50V for 1h and the module was packed with ice in order to keep the temperature low throughout transfer.

2.11.6 Western Hybridisation

After transfer was complete blots were removed and incubated at 4°C overnight, in blocking solution (PBS 0.1% (v/v) Tween 20, 5% (w/v) Marvel milk). 3x 10 min washes with PBS-T (PBS & 0.1% Tween 20) followed. The blot was then incubated in primary antibody diluted in blocking solution (at various concentrations) for 3h at room temperature or overnight at 4°C. Washes were repeated as before. Secondary antibody, diluted 1:5000 in blocking buffer, was then added and incubated for 1h at room temperature, followed with 3 x 10min washes with PBS-T and a final wash with PBS.

2.11.7 Western Signal Detection

Two forms of detection were used in this study. The first utilized horseradish peroxidase (HRP)-Conjugated antibodies. The signal from these antibodies can be detected using chemiluminescence using the ECL™ Western Blotting analysis system (GE Healthcare, UK). The blot was incubated at RT for approximately 1min in equal volume of Reagent 1 and Reagent 2 before being wrapped in Saran

Wrap, and exposed to ECL film (GE Healthcare, UK). The blot was exposed for several time points in order to obtain optimal exposure and developed using the X-Omat film processor.

The second system utilized ECL Plex conjugated antibodies (GE Healthcare, UK). These antibodies are conjugated to a fluorescent probe and blots were directly visualized using a Typhoon Trio Variable Mode Imager (GE Healthcare, UK).

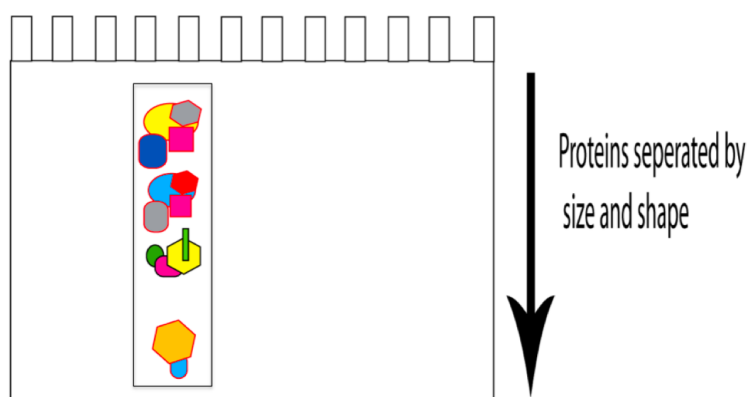
2.11.8 *Co-Immunoprecipitation (Co-IP)*

For Co-IP experiments, tissues were dissected as described in Section 2.1.3. The Pierce® Crosslink IP Kit (Thermo Scientific, UK) was used to carry out IPs. This protocol includes the cross-linking of antibody to a membrane, allowing the elution of protein without cross-contamination from antibodies. All steps were carried out as manufactures guidelines. Once eluted protein were loaded onto a standard SDS-PAGE gel and ran as described in Section 2.11.4. Gels were stained with SYPRO Orange (Sigma) as manufactures guidelines and visualized using the Typhoon.

2.11.9 *Protein separation by Blue Native (BN)-PAGE*

BN-PAGE involves the separation of proteins in 2 dimensions. It is particularly useful for the separation of membrane proteins as the proteins are coated with Commassie blue, thus hiding their negative charge, allowing proteins to separate more efficiently during electrophoresis. The first dimension in BN-PAGE separates proteins in their native state, allowing complexes to be kept together. The second dimension is carried out under denaturing SDS conditions as described in 2.11.4, therefore separating complexes into individual proteins, which are then separated by size and charge. Proteins, which may be forming complexes with each other, are therefore seen as a line of spots in the 2D gel (as seen in Figure 2-3).

1st Dimension Native gel



2nd Dimension SDS denaturing gel

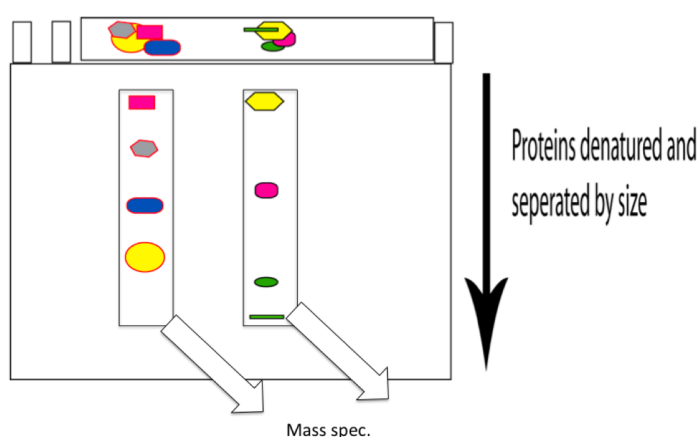


Figure 2-3 Summary of BN-PAGE procedure

The protocol used in this study was adapted from (Schagger and von Jagow, 1991) and used the Invitrogen NativePAGE™ Novex® Bis-Tris Gel System. All reagents used were from Invitrogen as described in Appendix 1. NativePAGE™ Novex® 3-12% Bis-Tris Gels 1.0mm, 10 well gels were placed in the Novex Xcell II™ Cell Module and each well was filled with dark blue cathode buffer, samples were then loaded prior to filling the cathode chamber to allow easy visualization of the wells. NativeMark™ Un-stained ladder was loaded as a standard. Gels were

ran in the cold room at 150V for 60 min and then 250V for 30-90 min, with an expected 12-16 mA at the start, reduced to 2-4 mA by then end of the run. When the dye front had reached 1/3rd of the way from the top of the gel, the cathode buffer was changed to light blue cathode buffer. Once the run was complete the lanes were carefully marked on the gel cassette prior to opening. Each lane of interest was then carefully cut out and placed in a sterile weigh boat. The gel strip was subsequently equilibrated for SDS-PAGE as follows:

1. Gel strip was incubated for 15-30mins in 5ml Reducing Agent
2. Reducing agent was removed and gel strip was incubated in 5ml Alkylating Solution for 15-30mins
3. After decanting Alkylating Solution, 5ml of Quenching Solution was added and incubated for 15mins

All steps were carried out at RT.

Gel strips were then immediately used for 2D SDS-PAGE. This was done using Novex® 4-20% Tris-Glycine pre-cast Gels with a 2D well. Each strip was gently inserted into the 2D well prior to loading the gel into the Novex Xcell II™ Cell Module. ECL Plex Fluorescent Rainbow Marker (Amersham, UK), was loaded into the first lane as a marker for protein size. 1X NU-PAGE MOPS SDS buffer was loaded and the gel was run as standard. Once complete the gel was stained with SYPRO-RUBY (Invitrogen, UK) as manufacturer's guidelines and visualized with the Typhoon.

2.11.10 *Identification of Proteins by Mass-Spectrometry*

Mass-spectrometry was carried out by proteomics department at the University of Glasgow. All peptide data was analysed using the online resource FlyBase and FlyMine.

2.12 Fluorescence Imaging of Tubules

2.12.1 *Live imaging of GFP fluorescence*

For live imaging of GFP expression in *Drosophila*, tissues were carefully dissected as described in Section 2.1.3 and mounted on pre-treated Poly-L-lysine-coated dishes in 100 μ l PBS for immediate viewing using the Zeiss 510 Meta confocal system. Where several N numbers were required all images were captured at exactly the same excitation settings. Where appropriated, cAMP was added to dishes as stated in the text. Images were analysed using LSM Image Browser software.

2.12.2 *Fixed imaging of fas2-GFP when stimulated with cAMP*

Tubules were carefully dissected as described in Section 2.1.3. They were transferred into dishes containing 3 ml Schneider's medium, with or without 10^{-4} cAMP. Samples were incubated for 10 min intervals up to and including 60mins. Tubules were then carefully stuck to Poly-L-Lysine pre-treated dishes in 100 μ l of PBS. PBS was then removed and replaced with ~200 μ l of fixation buffer (appendix) and incubated for 12 min. This time was determined after several experiments to give adequate fixation without disruption to the delicate microvilli of the tubules. After 12 min the tubules were washed 3 x 10min in PBS solution (appendix). At this point samples were either directly viewed, stained with Phalloidin or an immunocytochemistry (ICC) was carried out.

2.12.2.1 **Phalloidin Staining**

Phalloidin is a fungal toxin, which binds to and inhibits F-actin. When conjugated to a fluorescence tag, Phalloidin can be used to visualise F-Actin within a tissue. In order to do this, tissues must be first permeabilised by incubating samples in 0.2% Triton-100 diluted in PBS for 20-30 min. 100 nM of rhodamine tagged Phalloidin (supplier) was added and incubated for 30 min in the dark. Samples were then washed 3 x 10min with PBS. Finally samples were covered with VectoShield and visualized as described in 2.12.

2.12.3 Immunocytochemistry (ICC)

ICC was carried out in order to visualise native protein localization via antibody staining. Once fixed and permabilised as described above, samples were incubated in PAT solution (Appendix 2) for 2-3hrs in order to block. Primary antibody was diluted in PAT solution (varies concentrations as stated in text) and incubated with samples ON at 4°C. The following day samples were washed for 2hrs in PAT solution, changing every 10mins. PAT supplemented with 2% Goat serum (Sigma) was then added and the samples were blocked for 4hrs. Secondary antibody was again diluted in PAT solution supplemented with 2% goat serum. Sample and secondary antibody were incubated ON at 4°C. Subsequently samples were washed as before, with the addition of a final wash in just PBS. If required, the nuclear stain DAPI was added to samples for 30sec and washed with PBS 3 times. Samples were final covered with VectoShield and visualized as stated in Section 2.12.

A list of all antibodies used in this study, are listed in Appendix 2.

2.13 Fluid Secretion Assay

Tubules from 7 day old males and females were dissected as described in Section 2.1.3, taking extra care to avoid nicking the tubules. Fluid secretion assays were then carried out as described in (Dow *et al.* 1994 a; (Ramsay, 1954). Briefly, a Petri dish was filled with paraffin wax and depressions were made. A tiny metal pole was placed at equal distances from each depression. Petri dishes were then covered with Mineral Oil (Sigma). Each depression was filled with 9µl of bathing solution (1:1 *Drosophila* saline: Schneider's medium). Pairs of tubules, still attached by the ureter, were placed in the bathing solution. One tubule was then pulled out of the bathing solution with a fine glass rod and wrapped around the metal pole. As the tubules secrete, a bubble forms at the ureter. These bubbles are removed every 10mins and measured using an ocular micrometer and thus the rate of secretion in nl/min was calculated. Tubules were stimulated with 10^{-4} cAMP, 10^{-7} Drosokinin and 10^{-7} cGMP diluted in Saline/Schneider's solution.

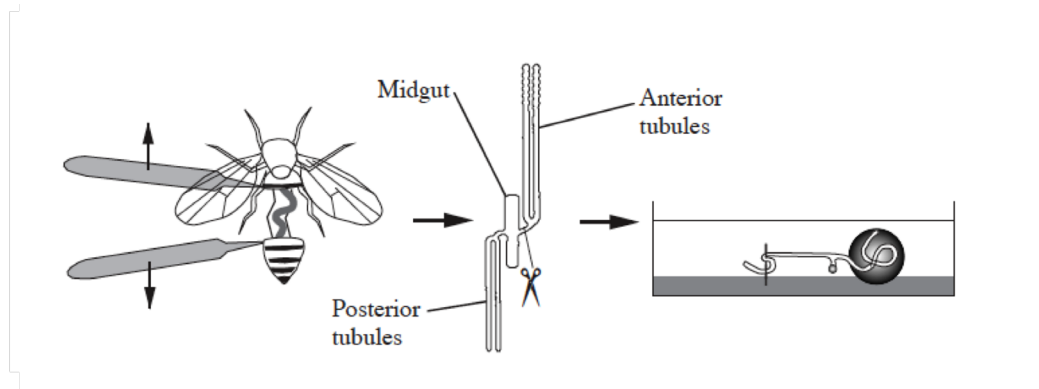


Figure 2-4 Schematic representation of fluid secretion assay

Data was analysed using Excel 12.9. and plotted using GraphPrism. Values were plotted as rate of secretion \pm SEM over time in mins, or as a percentage increase in secretion after stimulation. As summary of this technique is seen in Figure 2.4

2.14 Infection of adult flies with bacteria

Cultures of *E. coli* or *B. subtilis* (Selectrol freeze-dried pellets, TCS Biosciences) were grown overnight in 5 ml LB-broth to stationary phase at 37 °C. Bacterial challenge of adult flies was carried out using centrifugation, and resuspended in an equal volume of PBS. Flies were injected with 69 nl bacteria using a Nanoject II (Drummond Scientific) mounted to a micromanipulator. Microinjection needles (N-51-A glass capillaries) were pulled using a moving coil microelectrode puller (Campden Instruments limited). The tip of the needle was broken by touching to the flat plane of a pair of forceps, and the needle was backfilled with mineral oil prior to the uptake of bacteria. Where possible, the same needle was used for every fly. Flies were injected at the junction just below the first abdominal tergite.

Chapter 3

3 Results

3.1 Summary

This chapter discusses the validation of microarray results for *fas2* and the expression and localisation of the protein within the Malpighian tubules.

Quantative Reverse Transcriptase Polymerase Chain Reaction (QRT-PCR), confirmed the microarray results, concluding that all three *fas2* transcripts are highly expressed in the Malpighian tubules, with transcript B being the most highly abundant. RT-PCR also confirmed that only the three known transcripts are expressed. Protein expression was confirmed by Western blot analysis. We have also shown that Fas2 localisation is dynamic during development.

Both ICC and proteintrap analysis were used to determine the localisation of Fas2 protein. Localisation of the two isoforms Fas2-PA and Fas2PB were determined using the UAS-GAL4 system.

Fas2 is localised to the lateral between principal cells and between principal cells and invading stellate cells during late stages of embryonic development. During early larval development, Fas2 re-localises to the apical brush border in the principal cells of the tubule, with expression predominantly seen at the lower segment of the tubules. As development progresses, Fas2 expression moves up the tubule until all principal cells express Fas2 at their apical border. This expression stabilises and is seen in the adult tubules.

3.2 Introduction

As discussed in Section 1.4.1.4., FlyAtlas results suggested that *fas2* is most abundantly expressed in the Malpighian tubules (Table 1.2). As there is currently no known function for the protein in the tubules, *fas2* is an ideal candidate to test the ability of FlyAtlas to detect novel functions of genes and their products. FlyAtlas has been shown to be statistically robust and reproducible by QRT-PCR

(Chintapalli et al., 2007). However it is possible that probe sets many pick up non-canonical expression patterns. Typically there are several probe sets corresponding to known transcript sequences. Probe sets for *fas2* can be seen in Table 3-1.

Table 3-1 Affymetrix probe sets for *fas2*

Probe Identification	Transcript	Sequence Verified by FlyBase
1630160_at	B	Yes
1640163_at	A	Yes
1638956_at	C	Yes
1624774_at	A, B & C	Yes

Each of the probe sets for *fas2* correspond to areas which have been either cloned or sequenced previously (Goodman et al., 1991). However it is important to validate this data.

FlyAtlas utilised the Affymetrix *Drosophila* Genome 2 chips, consisting of 18,880 probe sets for 18,500 transcripts (Chintapalli et al., 2007). With the completion of genome projects, has come the realisation that the complexity of an organism does not correlate to an increase in protein-coding sequence, that is the total number of genes found in a given genome (Taft et al., 2007). Indeed some have argued that it is naïve to believe that proteins control all developmental and regulatory events in an organism. For example *C.elegans*, consist of only 1000 somatic cells but have essentially the same number of protein coding genes as vertebrates (Manak et al., 2006; Mattick, 2007; Mattick and Makunin, 2006; Taft et al., 2007). Therefore what makes a human more complex than a worm? One possibility is that more complex organisms have evolved to re-use proteins in many different processes and that the introduction of alternative splicing aids this (Taft et al., 2007). Tiling array studies such as that carried out by Manak et al have shown untranscribed regions of the genome may also play an important role. Their study detected newly predicted distal 5' exons for annotated genes; un-annotated transfrags that don't correlate with previously annotated areas and have little coding capacity and therefore may constitute new RNA classes; P

elements that insert into previously unidentified 5' exons that may explain deleterious mutations and 5' distal start sites (Manak et al., 2006). This along with the discovery of micro RNAs and long non-coding RNAs, has led many to believe that so-called non-coding regions of the genome are actually extremely important (Manak et al., 2006; Mattick, 2007; Mattick and Makunin, 2006; Taft et al., 2007).

In the context of this study, this may be of some relevance, as microarray studies such as that carried out for FlyAtlas, by nature only tell you about the expression of particular parts of a gene. Therefore it may be that this part actually constitutes an RNA regulatory molecule or a previously unidentified transcript. It is important therefore to determine if indeed the enrichment of the three *fas2* transcripts leads to similar levels of protein and if not are we perhaps seeing a form of non-canonical transcription.

3.3 Validation of *fas2* gene expression in the Malpighian tubules

3.3.1 *Validation of canonical transcription in the tubules*

As discussed in Section 1.4.1.3, RNA-seq is becoming the platform of choice for microarray analysis. Not only does the technique not require prior sequence information it also has the ability to detect previously unknown transcript variants. With the completion of FlyAtlas, a subsequent analysis was carried out using the Solexa Genome Analyzer II RNA-Seq system. The data generated can be found on the FlyAtlas server www.flyatlas.org. The results for *fas2* can be seen in Figure 3-1

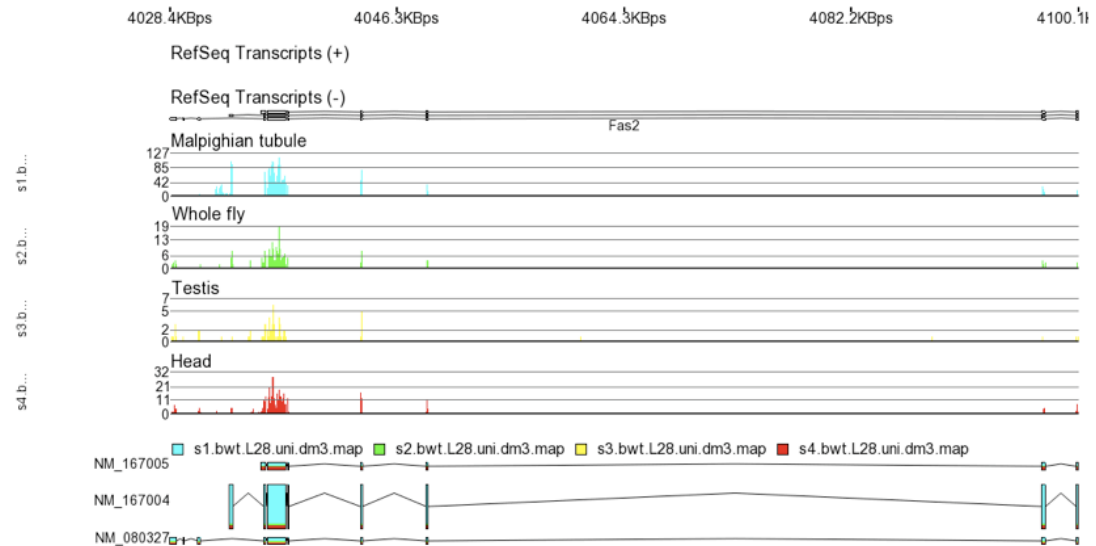


Figure 3-1 RNA-seq data generated by FlyAtlas for *fas2*

Known transcript data are compared to those generated from the Solexa RNA-seq run. RefSeq indicates all currently known and predicted exons within the *fas2* open reading frame. Data shown for Malpighian tubules, Whole fly, Testis and Head indicates actual RNAseq data as peaks in multiple runs. Transcripts seen in bottom of figure indicate those found via RNAseq sequencing and confirm the presence of all three known transcripts within the tubule, as indicated by blue colour in all three transcripts

The importance of this data is threefold. Firstly, the confirmation of the FlyAtlas data by an independent technology is very significant. Secondly, the Solexa data confirm that *fas2* RNA is most highly abundant in the tubules. Thirdly, the data confirm the presence of all three transcripts and no non-canonical transcription. It is important to note here that the peaks for small exons, such as those seen the 3' region of the gene, are smaller than those for larger exons. This confirms one of the caveats of RNA-Seq data, in that the ideal sequence length is ~50bp, anything below this size proves difficult to detect.

To further validate these results, primers were designed to span all exon boundaries within the three transcripts. RT-PCR was carried out as described in Section 2.5.3. The results can be seen in Figure 3-2.

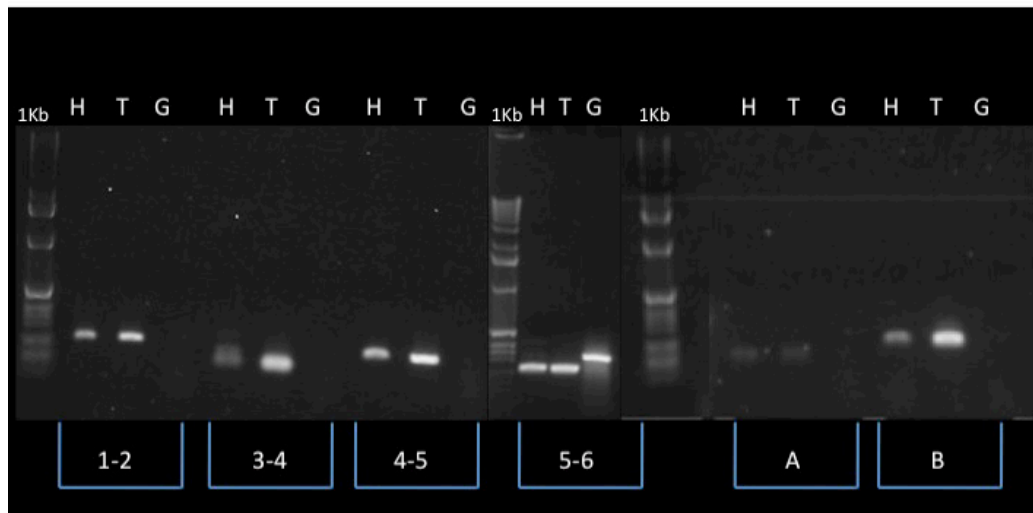


Figure 3-2 Exon boundaries of *fas2*

KEY: H=Head; T=Tubule; G=Genomic; 1Kb= 1Kb ladder. Numbers below bands indicate which exon boundary is represented. A & B indicate primers designed against unique sequences in transcripts A & B. Genomic samples were taken as a control. Note presence of band in genomic sample for exon 5-6, this is due to a short intron of 83 nucleotides. The presence of only one band in each sample confirms that all expression is canonical. NB RT-PCR was also carried out for transcript C and exons 2-3 data not shown.

These results indicate that only the three known transcripts of *fas2* are found in the tubules. Further proof was obtained during the cloning of the three transcripts, as sequencing data perfectly aligned with the known transcript data (Appendix).

We can conclude, with high confidence that tubules express cardinal transcripts of *fas2* at high levels.

3.3.2 Validation of expression levels by QRT-PCR

The results obtained from FlyAtlas were further validated by QRT-PCR. Primer sets were designed to detect each of the transcripts individually and together. Whole fly, head and tubule samples were dissected from 7 day old Canton S flies (wild-type) and QRT-PCR carried out. Figure 3-3(a)-(c) shows the results obtained for transcripts A, B and C, transcript A, transcript B and transcript C, respectively and compares them with the FlyAtlas results. All biological replicates were dissected within a short period of time where possible in order to minimise variation. Figure 3-3 (b) compares the enrichment values for transcript A. Both the head and the tubule data show excellent correlation between the QRT-PCR results and the microarray results, indicating that

transcript A is indeed up-regulated in the tubule when compared to whole fly (see below for p-values significance). Figure 3-3 (a) and (c-d), show an enrichment of *fas2* consistent or higher than that shown in the FlyAtlas data. The QRT-PCR data not only validates the array data but it is more robust, in that it shows more accurately the level of enrichment. Therefore we can conclude that *fas2-B* appears to be a tubule enriched transcript, with low levels seen in the whole fly and the head. Transcript A is enriched in the tubule when compared to whole fly but at a much lower level than B. Finally the results for *fas2-C* suggest that it is not significantly up-regulated in the tubule when compared to whole fly, but is present in the tubule. It is also important to note at this stage that these results do not rule out the possibility of high enrichment of *fas2* in other tissues or cells within the fly, which is not detectable by these methods. For example there is the strong possibility that if it were possible to isolate given neurons within the CNS and carry out a microarray analysis *fas2* would be significantly enriched. However such analysis requires precise dissecting and a significant amount of starting material, which at present is not achievable. Therefore a more detailed analysis of *fas2* expression by *in situ* hybridisation would be preferable in order to detect all tissue/cells in which *fas2* is highly enriched. However for the purpose of this study we can conclude that *fas2* is highly enriched within the tubule.

Interestingly recent studies suggest that *fas2-C* may be more highly expressed in the initial segment of the anterior tubules (Chintepalli et al unpublished). A bias towards either pair of tubules in one sample may explain this variation. Ideally a QRT-PCR should be carried out looking at each set of tubules individually to assess these findings.

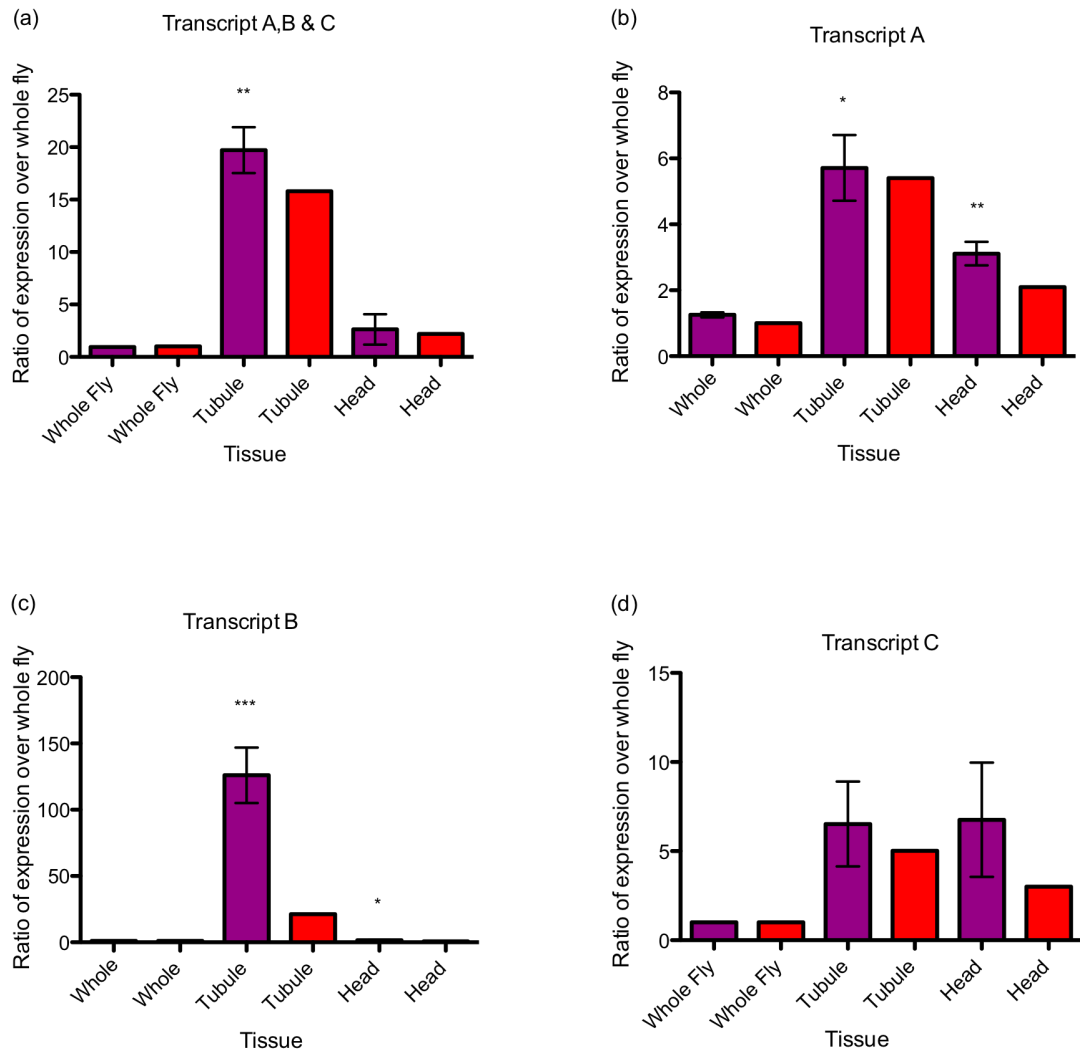


Figure 3-3 (a-d) Comparison of Q-PCR data and FlyAtlas data.

Shows a comparison of enrichment values, obtained from Q-PCR analysis and FlyAtlas, for *fas2* expression in the head and tubule when compared to whole fly (all samples taken from 7 day old Canton S wt). Bars shown in purple indicate Q-PCR results and those in red show FlyAtlas. Figure 1(a) shows results for all three transcripts (b) shows results for transcript A, (c) transcript B and (d) transcript C. Transcripts A and B and 3 transcripts together show significant differences in tubule and head enrichment compared to whole fly (*indicates P-value <0.05). C shows no significance difference in expression between the three tissues.

3.3.3 Confirmation of Fas2 Protein Expression in the Malpighian Tubules

In order to determine if high levels of *fas2* transcript correlated to protein expression, Western blot analysis was carried out. There are two commercially available monoclonal antibodies against Fas2, 1D4 and 3B34 both of which were obtained from the Developmental Studies Hybridoma Bank (first described in (Goodman et al., 1991)). The epitope for these antibodies is not published but

information provided by the supplier suggests that 1D4 attaches at the intracellular/cytoplasmic domains and 3B43 at the extracellular domain (see Figure 1-10 (b) for summary). Therefore we would expect that 1D4 detects isoform A and 3B43 to detect all three isoforms. The results from the western analysis can be seen in Figure 3-4

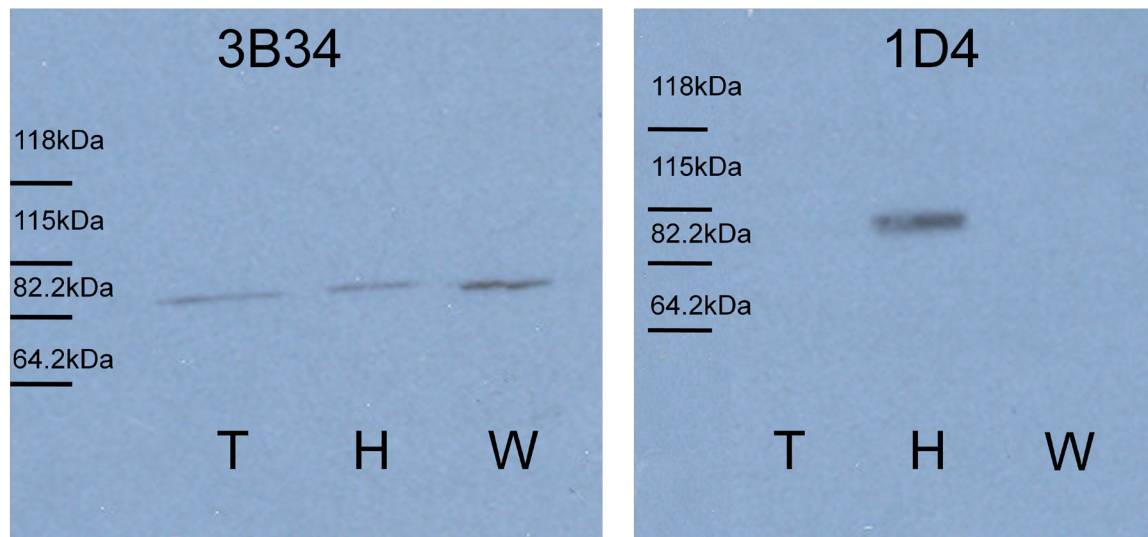


Figure 3-4 Western Blot Analysis of Fas2 expression

Western blot analyses were carried out using two commercially available antibodies. Results indicate that 3B34 detects isoform B and 1D4 isoform A. H= Head T=Tubule & W=Whole fly

Whole fly, head and tubule samples were dissected from 7day old Canton S and aliquoted into SMART buffer, a buffer used to extract membrane proteins. Figure 3-4 indicates that 3B34 shows a single clear band in each of the three samples. This is somewhat unexpected, as we would expect to see three bands in each of the samples corresponding to each of the three isoforms. The band that we do see however corresponds to isoform B, which has a predicted weight of 86.3kDa (weight cited by www.flybase.org). There are several reasons why we might see these results. Firstly this antibody may be specific to isoform B; this seems unlikely from the information given about the position of the epitope, as we know that the extracellular domain is identical in all three isoforms. Secondly it may be that isoforms A and C may have partners that attach to the extracellular domain making the epitope unavailable to the antibody. The fact that there is a differential expression pattern for the three isoforms suggests different functions so that they may well have different binding partners. Figure 3-4 for 1D4, shows a clear band ~90kDa in size in the head sample. Both the tubule and whole fly samples have no bands, suggesting no protein is present or there is too

little to be detected. FlyAtlas reports that *fas2-A* expression is most abundant in the brain and the QRT-PCR results show expression in the head is also high which may explain the resulting protein seen in the head sample. However these results show no protein in the whole fly or tubule sample. Interestingly there is a PEST motif, a motif that targets proteins for rapid degradation, in the C-terminal domain of isoform A (Grenningloh et al., 1991). This is consistent with a protein that is involved in signalling, therefore if Fas2-A is involved in signalling in the tubules it may explain why we see no results with 1D4: the protein is degraded at a fast rate making detection by Western blot analysis difficult. It is also interesting to note here that there is only one example of Western blots being carried out with these antibodies, suggesting that perhaps they are not ideal for this technique (Pascual et al., 2005). The results for both antibodies are somewhat surprising and further controls should ideally be carried out. For example it would be beneficial to carry out a Western blot including samples from Fas2 null flies and flies over-expressing Fas2 as controls. However time did not permit the inclusion of these experiments in this study and the expression of the *fas2* protein trap lines discussed in Section 3.4.2 confirmed the expression of Fas2 protein within the tubules.

So we can certainly conclude that the B isoform of the Fas2 protein is present in the tubules, but must also conclude that Ab. 1D4 is not ideal for our purposes.

3.4 Protein localisation

Several techniques were used in order to determine the localisation of Fas2 protein within the tubules.

3.4.1 *Immunocytochemistry (ICC)*

ICCs were carried out using the two monoclonal antibodies previously used for Western blot analysis. Figure 3-5 shows the results obtained. ICCs were also carried out on larval brain, as a control, staining seen here was consistent with previous studies (Goodman et al., 1991). Staining was confined to the principal cells of all four tubules and was completely absent from stellate cells. Staining was concentrated at the apical surface of the tubules.

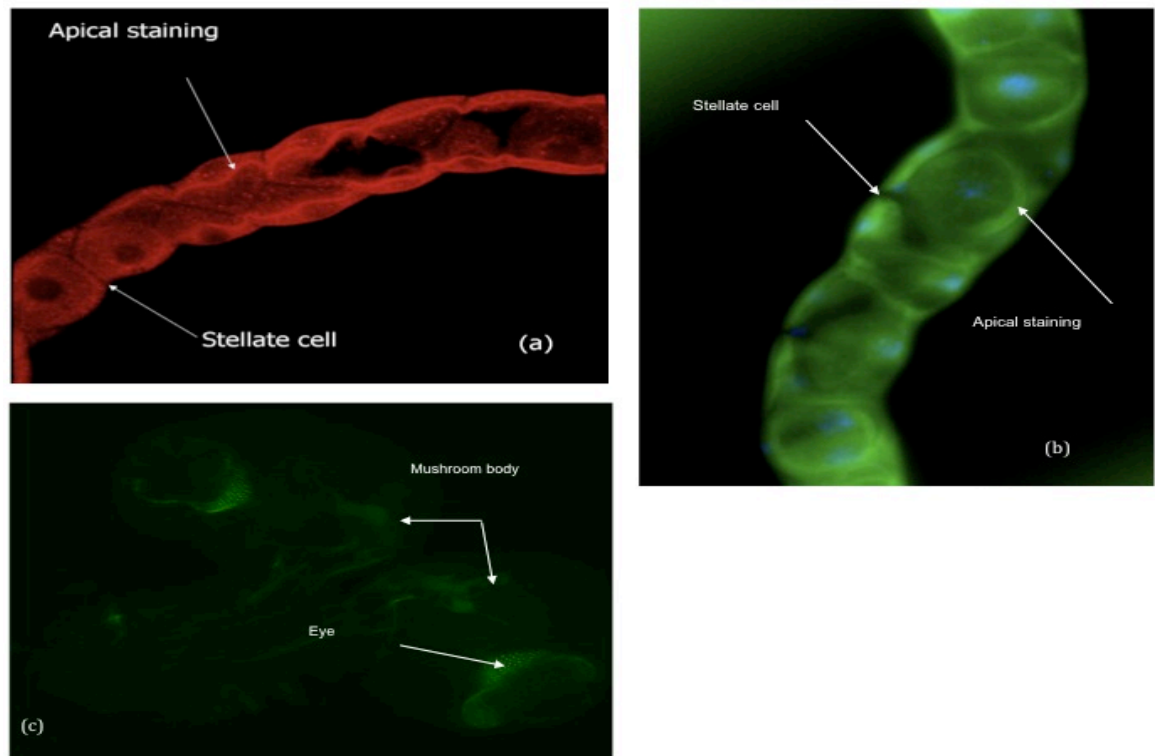


Figure 3-5 ICC showing localisation of Fas2 protein in the Malpighian tubules of adult flies and larval brain

Malpighian tubules were dissected from 7-day-old Canton s flies and ICCs were carried out using either antibody 1D4 (a) or 3B34 (b) Control experiments were also done on brain samples for 1D4 (not shown) and 3B43 (c) both of which confirmed that antibodies were working as staining was consistent with previous studies. Both antibodies show Fas2 is present in the tubules and appears to be concentrated at the apical membrane of the tubules. Staining was seen in the principal cells the length of both anterior and posterior tubules and was absent in all stellate cells.

From these results we can determine that Fas2 is expressed only in the tubule principal cells and appears to be localised to the apical brush border. This result was not consistent with those published previously, showing Fas2 localising to the lateral membranes during embryonic development (Campbell et al., 2009). Indeed when this study was started the hypothesis was that Fas2 was involved in septate junction stability in adult cells, as many proteins which localise to the lateral membrane during embryogenesis are involved in septate junction stability in the adult tubules (e.g. Dlg). It is therefore possible that subcellular localisation of Fas2 changes throughout the course of development. In order to determine if Fas2 localisation was dynamic during different stages of development, further studies were carried out as described in Section 3.4.2

3.4.2 Validation of protein expression & localisation throughout development using *fas2* protein trap line

To further validate the expression of Fas2 protein and to determine the localisation of the protein throughout development, two protein trap lines for *fas2* were obtained (kind donation from Klemmt laboratory). Protein trap lines are generated in order to 'trap' a tag or sequence of choice within a native gene within the fly (see Section 1.1.3.3). Previous studies have confirmed that this protein trap line conforms to wild type *fas2* as evident by its ability to rescue mutant phenotypes and co-localisation with wild type Fas2 (Silies and Klemmt, 2010). In this case an open reading frame for the GFP protein has been inserted into the native *fas2* gene, allowing visualisation of the protein *in vivo* by confocal imaging.

3.4.2.1 Localisation during embryonic development

As mentioned previously, studies had indicated that during late embryonic development Fas2 localises to the lateral membrane of MT cells. To validate these results late stage embryos were collected as described in Section 2.1.4, from the *fas2*-protein trap line. The results obtained can be seen in Figure 3-6.

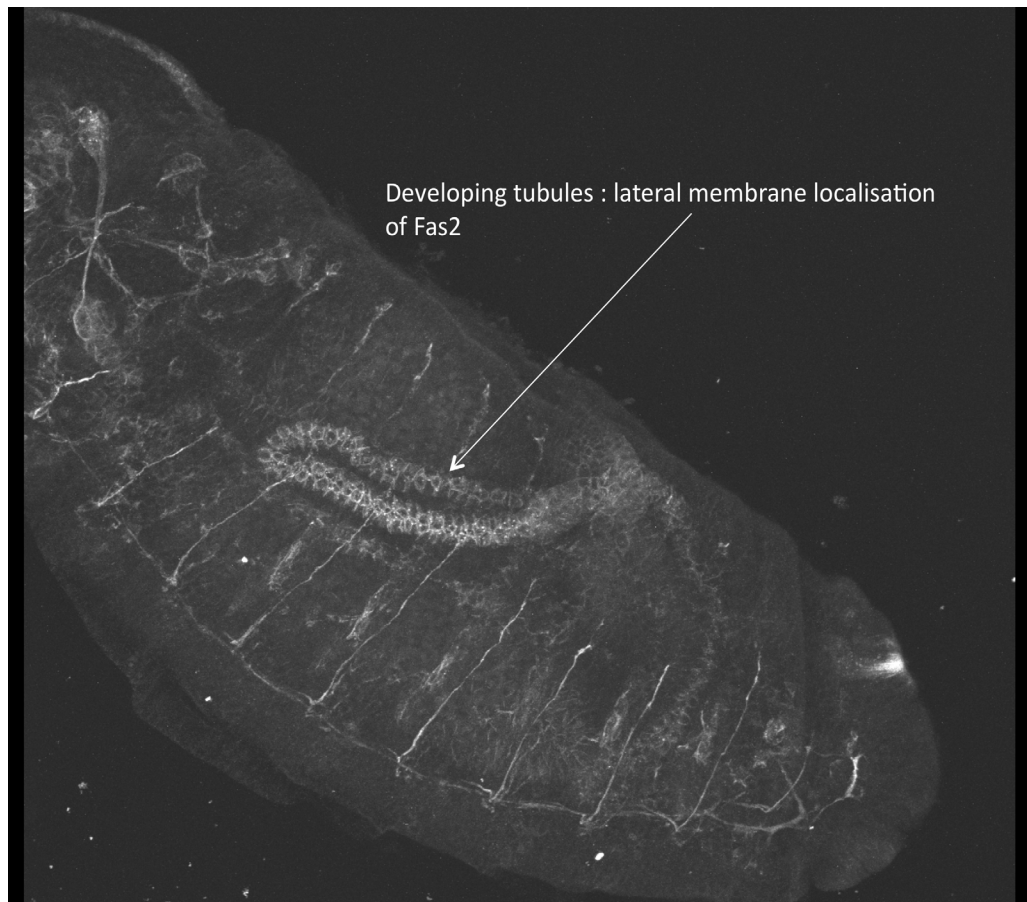


Figure 3-6 Localisation of Fas2 during late stage embryonic development

Embryos were collected as described in (section 2.1.4). Once fixed and mounted *fas2*^{proteintrap 788} was visualised using a confocal microscope and a full Z projection was obtained. Image shows a 16 stage embryo.

The results clearly show that *Fas2*^{proteintrap788} is localised to the lateral membrane in the developing tubule. This is consistent with previous studies such as those from the Skaer laboratory (Campbell et al., 2009). However as shown Section 3.4.1 in adult tubules, Fas2 is actually localised to the apical brush border, therefore at some stage Fas2 protein must relocate to this area.

3.4.2.2 Fas localisation during 1st instar to 3rd instar development

In order to determine if Fas2 relocates to the apical brush border during larval development, larvae were collected as described in Section 2.1.3. Larvae were aged by size and by development of mouth pieces as described in (Miller, 1950). Four stages of larval development were chosen, First Instar, Second Instar, Third Instar and Third Instar wandering larvae. Tubules were dissected, fixed and mounted as described in Section 2.12. The results obtained can be seen in Figure 3-7

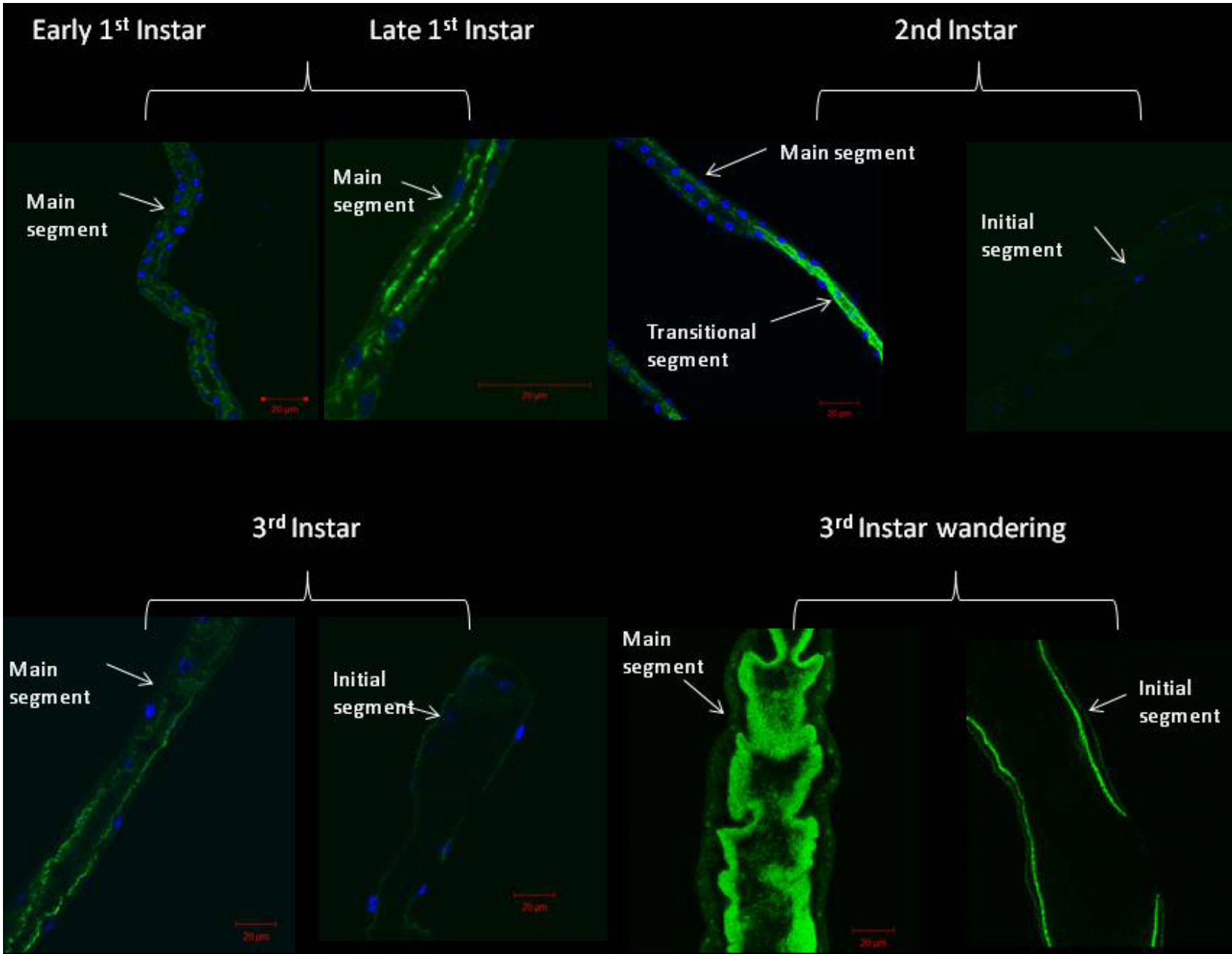


Figure 3-7 (a-h) Localisation of Fas2^{Protein trap 788} during larval development

Shows localisation of Fas2 protein trap line 788 (Green) nuclei stained with DAPI (blue) During larval development. Initial expression is seen mainly in the principal cells of the main segment (a-b). By 2nd instar development, expression has moved to the transitional segment where expression is intensified (c-d). By 3rd instar, expression of fas2 is seen in all principal cells, the full length of the tubule, with a peak in expression at the end of this stage (e-h). The pattern for adult tubules can be seen in Figure 3-8.

By early 1st Instar larval development, Fas2^{Protein trap 788} has already relocated from the lateral membrane to the apical brush border of the principal cells. Initially, expression is concentrated to the lower tubule and main segment Figure 3-7(a). As the larvae reach late 1st instar, Fas2^{Protein trap 788} expression increases in the main segment Figure 3-7(b), however little staining is seen in the initial segment. By 2nd Instar localisation is concentrated in the transitional segment of the tubule and has stabilised in the main segment Figure 3-7(c). No staining is seen in the initial segment Figure 3-7(d). By 3rd Instar staining is consistent throughout the full length of the lower tubule, main segment and transitional segment, Figure 3-7 (e). At this stage we also start to see staining in the initial segment of the tubule, Figure 3-7(f). By 3rd Instar wandering larvae Fas2^{Protein trap 788} is seen the full length of the tubule and is localised to the apical brush border of the principal cells. These results are intriguing as they suggest that Fas2^{Protein trap 788} expression moves from the proximal segment, through the main segment and transitional segment and finally to the initial segment, in conjunction with larval development.

3.4.2.3 Fas2 localisation in the tubules in adult flies

We have already shown by ICC experiments that in the adult fly, Fas2 localises to the apical brush border of the principal cells in the Malpighian tubules, here we show, using the protein trap line Fas2^{Protein trap 788}, that Fas2^{Protein trap 788} is localised to the apical brush border. As discussed in Section 1.2.5.2 the brush border of principal cells is made up of long dense microvilli, which provide a large surface area in which V-ATPase complexes and mitochondria are densely packed, allowing for maximum secretion levels (Bradley and Snyder, 1989; Terhzaz et al., 2009). Microvilli are stabilised by F-Actin and can be easily visualised by the binding of Rhodamine labelled Phalloidin. Fas2^{Protein trap 788} lines were dissected, fixed,

stained with phalloidin and mounted as described in the Section 2.12.2.1. The results can be seen in Figure 3-8.

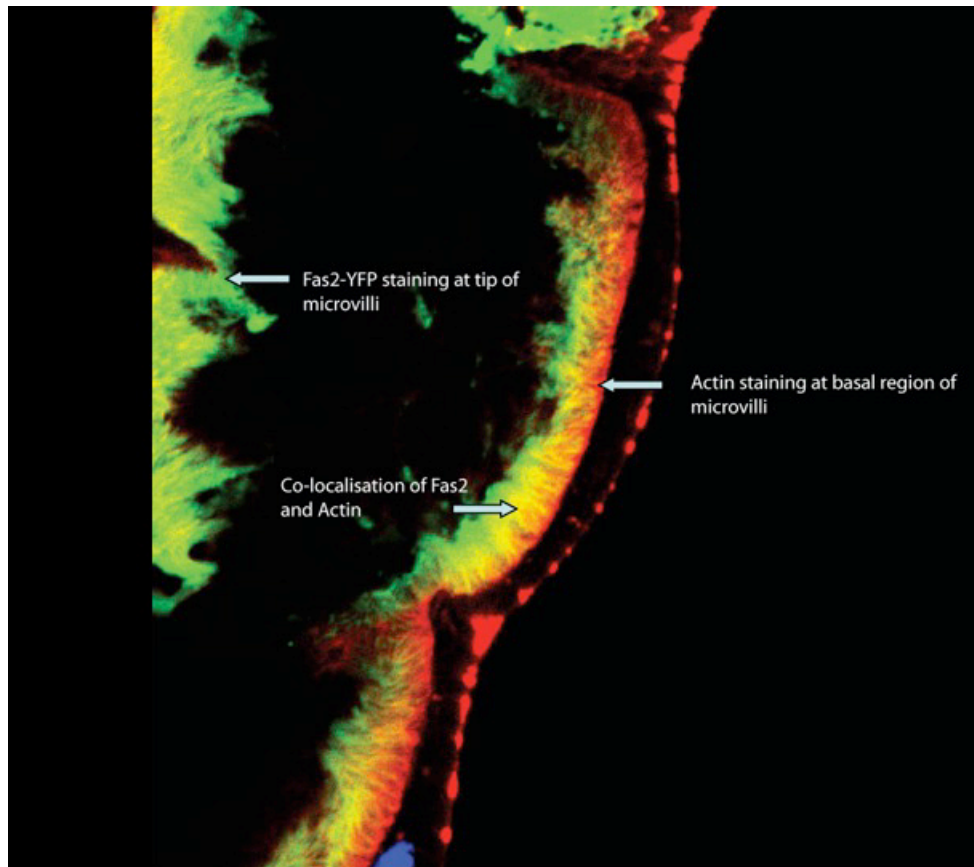


Figure 3-8 Adult tubule showing Co-localisation of Fas2^{Protein trap 788} and F-Actin

Red = Phalloidin; Green= Fas2; Blue= DAP I. Phalloidin staining indicates F-Actin projects from the base of the microvilli towards the tip. Fas2^{Protein trap 788} is localised at the tip and mid point of the microvilli, indicated by partial localisation with F-Actin.

Phalloidin stains the base of the microvilli and staining extends partially towards the tip. We can also see F-Actin bundles at the basal side of the tubules. Fas2 shows a partial co-localisation with F-Actin from the basal end of the microvilli, however Fas2 staining extends to the tip of the microvilli, where F-Actin is not found. These data confirm that Fas2 is specifically localised to the microvilli of the brush border.

3.4.3 *Cloning and localisation of Fas2-AV5 & Fas2-BV5*

In order to establish if the three isoforms of Fas2 localise to different areas within the tubules, each transcript was cloned upstream of UAS and a small V5 epitope was incorporated in order to aid visualisation. It is important to note here that such experiments are not completely conclusive in determining subcellular localisation, as often the over expression of a protein can lead to the miss localisation, or results which are not consistent with the behaviour of the native protein. The addition of a tag can also cause proteins to not fold correctly or to miss localise within the cell due to loss of binding partners for example. However the V5 tag used in this study is small at 25 amino acids and is unlikely to affect the protein. These experiments however can be used as a guide for further downstream experiments in order to determine isoform localisation. For example if there is a clear difference between tagged isoforms, expression of these proteins in a Fas2 null background would determine if the tagged proteins act as the Wildtype protein, via rescue experiments. Such experiments would also remove the issue of over expression. The development of flies over expressing individual isoforms would also aid downstream experiments such as those described in Chapter 4, by allowing the dissection of which isoform constituted which phenotype.

As isoform C carries a signal peptide at the N terminus and a GPI linked anchor at the C terminus, cloning of this isoform, with a tag proved difficult. Several attempts to clone a V5 tag between the GPI signalling motif and the last binding domain, proved difficult and time did not permit the conclusion of this cloning. However, Fas2-A^{V5} and Fas2-B^{V5} were successfully cloned as described in Section 2.9.2. The cloning protocol for these transcripts proved easier, however it was not without problems. Both transcripts, were extremely unstable within plasmids and often sequence re-arrangements and deletions occurred. Final cloning of these isoforms took several months and unfortunately time only permitted the evaluation of expression and localisation. Figure 3-9 shows the results obtained when each transcript is over expressed using the principal cell specific driver urate oxidase Gal4 (UO). Interestingly transcript A appears to localise to both the basal and apical membrane. This is unexpected as little or no basal staining is seen in previous

results. This may simply be due to artefact of over expressing the isoform, as it is not as highly expressed as Fas2-B: there is not sufficient space so Fas2-A localises to the basal membrane. Isoform B however localises to the apical brush border as expected.

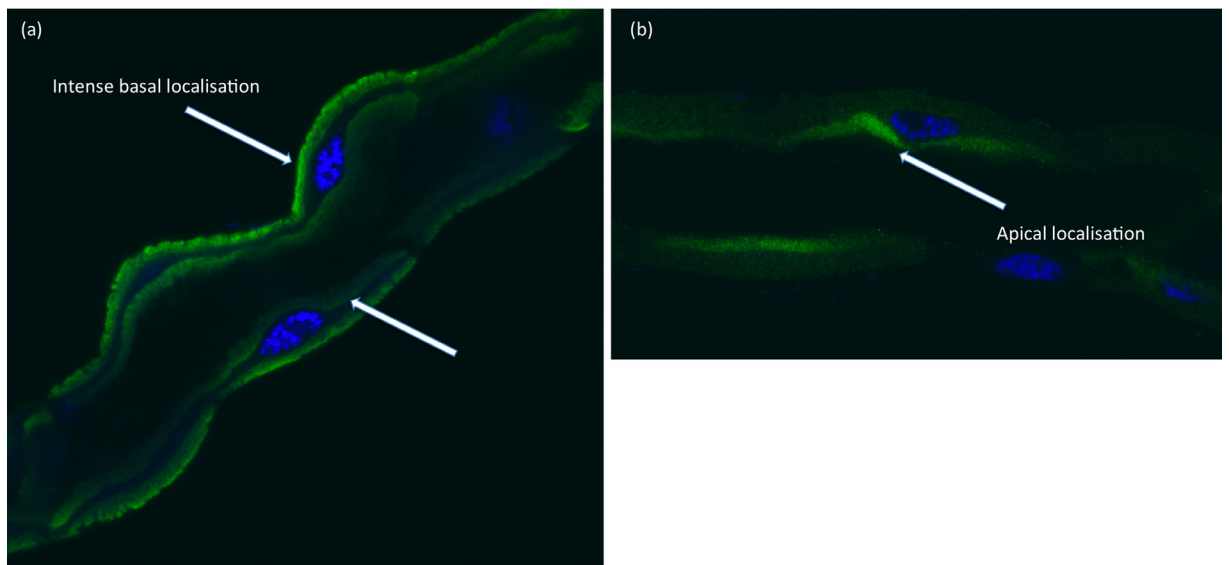


Figure 3-9 Localisation of Fas2-A^{V5} & Fas2-B^{V5}

The principal cell driver UO was used in order to drive expression of Fas2-A^{V5} & Fas2-B^{V5}. Tubules were then dissected and ICCs were carried out using an anti-V5 antibody. (a) Fas2-A^{V5} shows localisation to both the apical and basal membrane. The basal staining is somewhat unexpected but may be an artefact of over expression. (b) Clear apical staining of Fas2-B^{V5} is seen as expected. Flies were collected and dissected at 7 days old.

3.5 Discussion

In this Chapter we have validated the FlyAtlas results and shown that *fas2* mRNA is present at high levels (indeed higher than in the brain). We have also confirmed that the Fas2 protein is expressed in the tubules and shows dynamic localisation throughout development, shifting from the expected lateral membrane location in the embryo to a novel apical microvilli localisation in the larval and adult tubules. The multiple lines of evidence employed are important, as an extra non-neuronal role for *fas2* goes against the body of published work on this gene, which heavily emphasises a developmental role in the CNS.

During late embryonic development Fas2 is localised to the lateral membrane between cells. There are several hypotheses as to the role, which Fas2 may be playing here. Firstly Fas2 is known to stabilise NMJ in the larval brain, in conjunction with Dlg, which it directly binds through a PDZ domain found in Dlg (Kohsaka et al., 2007a). Previous studies have shown that throughout development Dlg is localised to the septate junctions of the tubules therefore it may be possible that Fas2 is involved in directing Dlg here during embryogenesis, where it remains throughout all stages of development (Campbell et al., 2009). As the antibody 1D4 works more efficiently in embryonic ICCs than larval or adult, we would argue that the isoform we see during embryogenesis is Fas2-A. If this is the case it may explain why Dlg does not re-localise with Fas2 during larval development: Fas2-A directs and tethers Dlg to the junctions, where it then attaches to other junctional proteins in order to remain attached to the membrane. Fas2-A is then degraded, but Dlg remains attached to other proteins. Dlg protein is highly abundant in adult tubules (own personal observations), however FlyAtlas data for adult tubules shows a very low level of expression, suggesting that protein levels are determined at embryogenesis and this protein remains throughout development and adulthood. Fas2 however shows both high levels of protein and transcript levels during larval development and adulthood. This further supports the argument that Fas2 may play a different role in embryogenesis than other stages. The small size of embryonic and indeed early larval tubules, make their inclusion in FlyAtlas impossible at present, however we would argue that if we could look at transcript levels and indeed protein levels at these early stages we would see a difference: *fas2-A* would be enriched in the embryonic tubules but *fas2-B* in larval tubules. We would therefore hypothesise that the expression of different isoforms of Fas2 during different stages of development, leads to different functions of Fas2. In order to verify this hypothesis, it would be crucial to carry out transcript specific *in situ* hybridisation throughout the different stages of development. Time however, did not permit the conclusion of such experiments in this study.

We have shown that between embryonic and larval development Fas2 shifts from the junctions to the apical brush border. As discussed in Section 1.2.3 although the tubules are fully formed by the end of embryogenesis and are functionally active in

the transport of organic solutes, they don't perform high levels of fluid secretion until after hatching (Skaer et al, 2005). There are several key reasons as to why this is the case. Firstly during embryogenesis the tubules are not only enclosed within the body cavity but also within the strong vitelline membrane of the embryo, making the excretion of fluids almost impossible. Therefore if the tubules were to carry out high levels of fluid secretion the fluid would become trapped within the membrane, eventually leading to death. Secondly evidence suggests that there is little or no expression of diuretic hormones/agonists, during embryogenesis. Thirdly and possibly most importantly the tubules are not fully mature at the end of embryonic development and are therefore likely to undergo further changes. We know that there are several key attributes required for the high level of secretion seen in larval and adult tubules, which are associated with the apical brush border. Firstly, the long dense microvilli allow for the large surface area required for fluid secretion to take place, along with this they also help control cell structure during secretion, when the cells expand and contract (Bradley and Snyder, 1989). One could argue that as there is no requirement for these microvilli during embryogenesis, they develop during early larval development. Indeed it has been shown in the in mosquito tubules, that changes in secretion rate throughout development are directly proportional to both the area and length of microvilli (Bradley and Snyder, 1989). Previous studies have also suggested that microvilli length and density are not static during different stages of the life cycle of insects and indeed during hormonal stimulation of the tubules. For example during the pupal stage of development in the lepidopteran *Calpodes ethhus*, there is a marked reduction in the need for fluid secretion, therefore the microvilli contract and shorten (Bradley and Snyder, 1989).

The microvilli also play an important role in localisation of mitochondria, which are required to energise the V-ATPase activity essential to fluid secretion. Studies in both Mosquito and the *Lepidopteran*, *Calpodes ethhus* have indicated that during stimulation or particular stages of development the contraction or expansion of the microvilli is directly responsible for the recruitment of mitochondria to the apical brush border (Bradley and Snyder, 1989). During stimulation with cAMP in mosquito tubules the microvilli extend and increase in density, along with this more

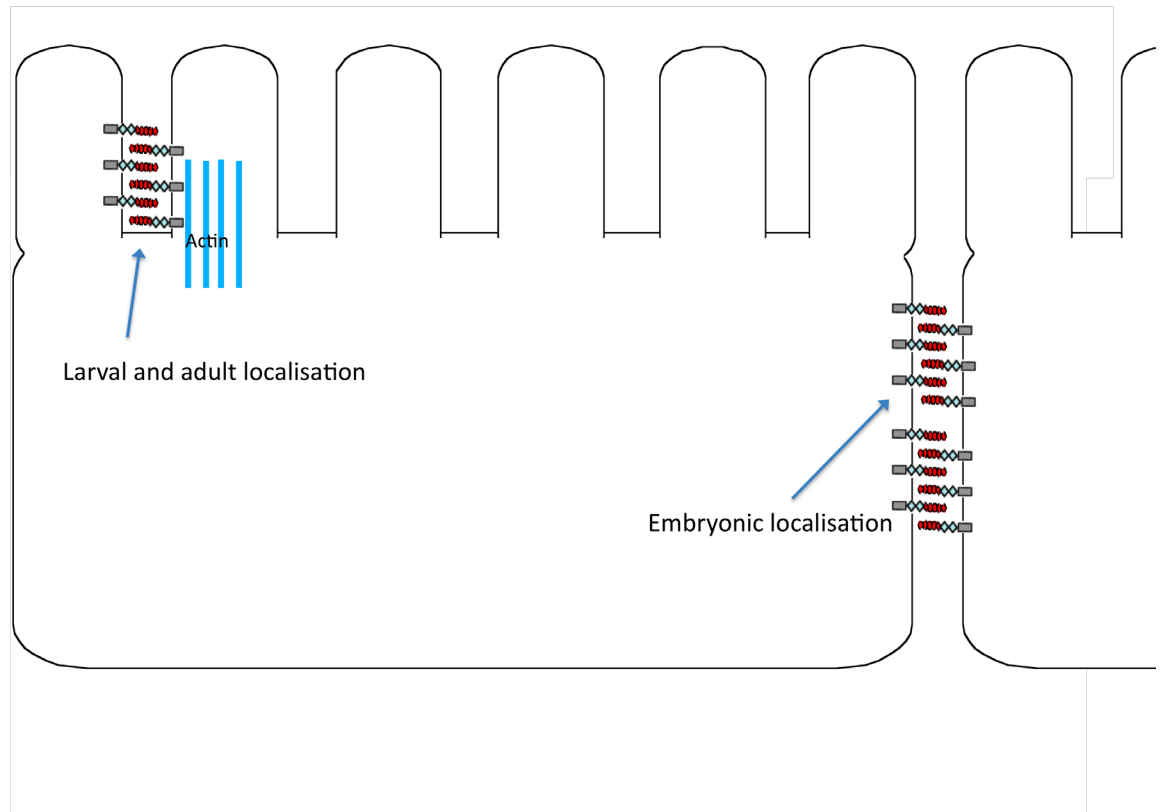
mitochondria are recruited to the brush border via the actin cytoskeleton (Bradley and Snyder, 1989). The actin cytoskeleton is essential for microvilli structure and without it they would collapse under the strain imposed on the cell during high rates of fluid secretion.

Perhaps the most important requirement for fluid secretion is the recruitment of V-ATPase complexes to the apical brush border. Previous studies have shown that during certain stages of development, the amount of V-ATPase present in the brush border is markedly reduced when there is a reduced need for fluid secretion (*Gräf, 1996; Sumner and and Wieczorek, 1995*). This is not surprising as the ion pump requires a vast amount of energy in order to work and it would be non-economical for the cells to constitutively activated.

To summarise, in order for high levels of fluid secretion to take place, the principal cells require:

1. Long dense microvilli, stabilised by the actin cytoskeleton
2. Mitochondria recruitment in order to power the V-ATPase
3. The presence of V-ATPase complexes at the apical brush border.

With this in mind could Fas2 be involved in one or more of these processes? Perhaps the movement of Fas2 along the length of the tubules during larval development indicates the development of microvilli; indeed we will present evidence that this is indeed the case in Chapter 4.

**Figure 3-10 Model of Fas2 localisation and function 1**

During embryogenesis Fas2 is localised to the lateral membrane between cells. In larval and adult flies Fas2, is localised to the apical microvilli showing some localisation with F-Actin

Chapter 4

4 Effect of *fas2* expression on the development of the Malpighian tubules

4.1 Summary

In this Chapter we discuss in more detail the use of the GAL4/UAS system and describe its use in manipulating the expression of a gene in both a systemic and tissue specific manner. Traditional mutational analyses rely on naturally occurring mutations and P element insertions in a gene of interest, or the creation of mutant libraries by random mutagenesis (e.g. x-ray, *ems*). Often these mutants pose problems when wishing to determine their function in a given developmental stage or tissue. In particular genes, which are important in developmental processes, are often lethal at an early age, making studies in the adult problematic. For reasons discussed in Section clonal analysis would not be possible with regards to studying tubules, therefore the GAL4/UAS system, is particularly important with regards to this study.

Here we show that systemic down regulation of *fas2*, utilising *fas2*^{RNAiKK} driven with *actin*^{GAL4}, results in a lethal phenotype shortly after eclosion. A comparison of tubule specific GAL4 drivers indicated that UO was more suited to this study due to the expression of C42 in the developing brain, however this was not without limitations. Urate oxidase is only expressed in the principal cells of the main segment from 3rd Instar development, making embryonic knock down impossible. Secondly as *fas2* is expressed in all principal cells the full length of the tubule, quantifying the level of knockdown was not accurate. Therefore in order to assess the true level of knockdown analysis of protein levels via Western blot analysis would have been beneficial. Secondly QRT-PCR analysis of larvae expressing *Actin*^{GAL4}, driving *Fas2*^{RNAiKK} could also have been assessed for levels of knockdown.

We have however found that knocking down *fas2* in the principal cells results in the depletion and stabilisation of apical microvilli. These results were confirmed using

fas2^{EB112} a heterozygous null line. Further, targeted over expression of *fas2* resulted in the opposite phenotype. Misexpression of *fas2* in the stellate cells appears to confirm the role of Fas2 in microvilli development and indeed possibly in Actin localisation. The misexpression resulted in increased microvillar length and density and an increased actin levels. This in turn led to the apparent distortion of stellate cells.

We also confirmed our previous observations that Fas2 does not alter Dlg localisation in larval or adult tubules, but were unable to demonstrate whether if there is an interaction between these molecules during embryonic development.

4.2 Down regulation of *fas2*

4.2.1 *Systematic down regulation of *fas2* via RNAi, results in death shortly after or during eclosion*

In order to determine the effect of down regulating *fas2* in the principal cells of the Malpighian tubules, several *fas2* RNAi lines were obtained from the Vienna *Drosophila* RNAi Centre (VDRC). All lines are homozygotic and all experiments were carried out at 26° C unless otherwise stated. To assess the quality of these lines, they were first crossed to the ubiquitous Gal4 driver, Actin^{Gal4}. Table 4-1 lists the RNAi lines assessed and their resulting phenotypes. As Actin^{Gal4} is balanced over Cyo, all crosses resulted in 50 % Cyo vs 50% Fas2^{RNAi}. Therefore results indicated in Table 4-1 were compared to control offspring expressing CYO.

VDRC Line	Resulting lethality
V8392	Lethality during late pupal development
V8393	Lethality during late pupal development, with some escapers
V36350	Death 3-4 days after eclosion
V36351	Death 1-2 days after eclosion
Fas2 KK	Lethality during late pupal development

Table 4-1 RNAi lines crossed to Actin Gal4 and resulting lethality

This table summarises the VDRC lines used in this study and their resulting phenotypes when driven with the ubiquitous driver Actin Gal4.

Previous studies have indicated the importance of *fas2* in embryonic development, larval development and adult neuronal stability (Adam et al., 2003; Beumer et al., 2002; Davis et al., 1996; Forni et al., 2004; Goodman et al., 1991; Grenningloh et al., 1991; Kohsaka et al., 2007a; Lin and Goodman, 1994; Rivlin et al., 2004; Szafranski and Goode, 2004). Indeed null mutations in *fas2* result in homozygote lethality early in embryonic development (Goodman et al., 1991; Grenningloh et al., 1991). This is not surprising considering its role in embryonic polarity. Mutations, which affect the amount of gene expression, also result in several

different phenotypes, which vary dramatically (as discussed in Chapter 1). We would therefore expect a good RNAi, driven by a ubiquitous driver, to result in early lethality in the developing fly. As we can see from Table 4.1 none of the RNAi lines resulted in embryonic lethality when driven with the ubiquitous driver Actin^{Gal4}. This suggested that the knockdown level was not sufficient to hinder the roles of *fas2* in embryonic development. However this may also be a result of the driver itself; although Actin^{Gal4} is a ubiquitous driver, levels of transcription are not equal in all cells/tissue (our unpublished observations). The RNAi knockdown however was enough to cause lethality before eclosion in several lines and therefore the line *fas2*^{RNAiKK} was selected for further analysis. The KK line represents a line with a known insertion site, allowing for maximum expression and theoretically less leakage (Dietzl et al., 2007).

Figure 4-1 shows the results obtained from crossing the VDRC line, *fas2*^{RNAiKK} to Actin^{Gal4}. The flies are clearly mutated suffering from several abnormal phenotypes, which result in death, before or during eclosion. These results suggest, that although the RNAi may not result in a substantial knockdown of *fas2*, the gene level is affected enough to elicit a phenotype, consistent with *fas2* disruption.

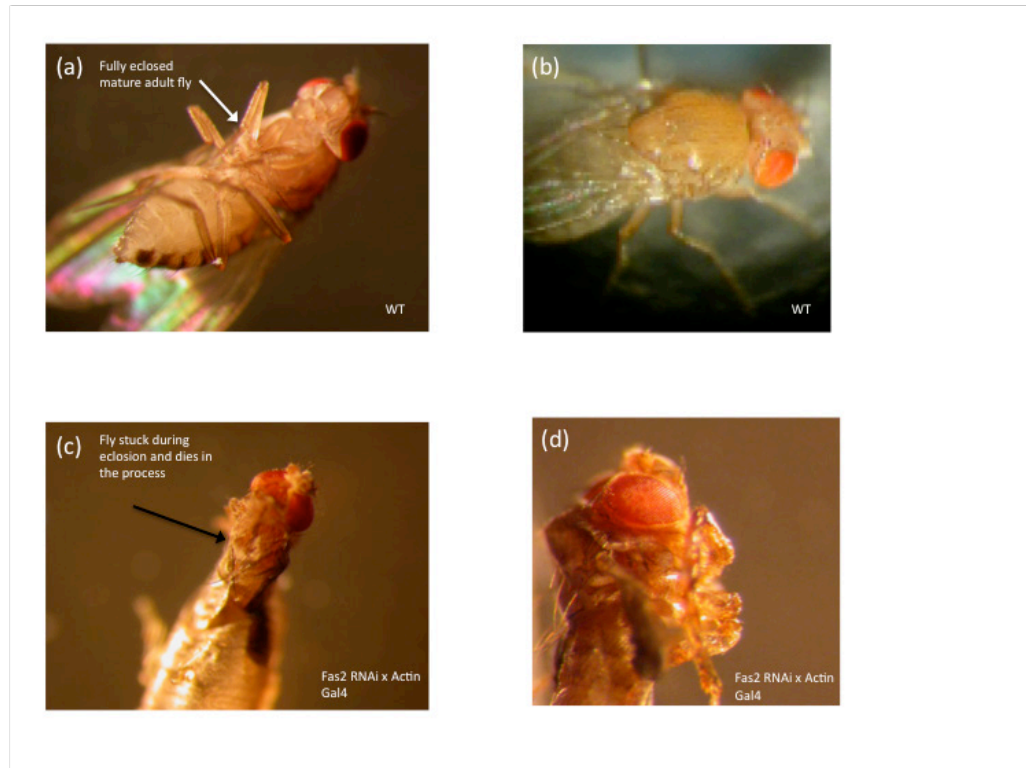


Figure 4-1 Effect of down regulation of Fas2 via RNAi on pupal development and eclosion

(a) shows typical WT development of eclosed fly; (b) Close up image of WT eye shape and development; (c) Actin Gal4 driving *fas2*RNAiKK results in non-eclosion from pupae and also; (d) as (c)

4.2.1.1 Conclusion

This section looks at the effect of down regulating *fas2* using the Gal4/UAS system. By systemically knocking down *fas2* with the ubiquitous driver Actin Gal 4 we have shown that the VDRC lines do affect *fas2* expression, however our results suggest that early development is not affected. This may be the result of a low level of knockdown, resulting in several phenotypes but not the expected lethality, as evident by the importance of *fas2* during early development. As discussed in Section 1.5.3 the affect of *fas2* mutations is both cell and dose dependant, suggesting that the affect of knocking down *fas2* in different cells and by differing amounts would lead to different phenotypes.

The driver line may also have an effect on the efficiency of the knockdown, however in our experience Actin^{GAL4} is efficient for the systemic knock down of genes, suggesting that the results we see here indicate that the RNAi is not at a sufficient level to cause similar effects to mutant lines. Indeed as heterozygote nulls of *fas2* survive adulthood but these RNAi crosses do not, it suggests that some cells/tissues where *fas2* is vital are expressing Actin^{GAL4} at a very high level, thus leading to death shortly after eclosion. These results suggest that *fas2*^{RNAiKK} is indeed effective at knocking down *fas2*, but this in turn is dependant on the efficiency of the driver line. As shown in Figure 4-3 we went on to further analyse the affect of *fas2*^{RNAiKK} expression in the MT and showed a low but significant level of knockdown of *fas2* in the tubules. However further analysis of transcript levels via *in situ* hybridisation or the quantification of protein levels by Western blot would be beneficial to this study. Time however did not permit the conclusion of such experiments.

The most important observation in this section is the importance of cell specific knockdown of genes, in order to determine their function. If we wished to determine the function of *fas2* in the adult Malpighian tubules for example, we could not use these crosses, as they die before they reach adulthood. Flies which, carry mutations in *fas2*, also may not be effective in studying its role in the tubules. For example we know the line *fas2*^{EB112} is homozygous lethal, therefore 50% is enough to maintain the fly but several phenotypes are observed (Goodman et al., 1991). We cannot be certain that this would be the case in the tubules. Therefore in an ideal world we would wish to knock down *fas2* in the tubules ONLY, as much as possible, thus avoiding early lethality.

4.2.2 Knockdown of *fas2*, specifically in the Malpighian tubule principal cells

4.2.2.1 Determination of GAL4 driver line to be used in this study

In order drive expression of *fas2*^{RNAiKK} in the principal cells of the tubules, two driver lines are available; C42, which drives expression in the principal cells, the

full length of the tubule (Sözen et al., 1997) and UO which drives expression only in the main segment principal cells from larval stage 3 (Friedman and Johnson, 1977; Terhzaz et al., 2010). As *fas2* is expressed in all principal cells in 3rd instar larvae, initial experiments concentrated on utilising C42. However it became apparent that these flies were eliciting phenotypes not consistent with tubule specific abnormalities, such as locomotor defects and unusual eye pigmentation. This suggested that C42 was driving RNAi expression in cells/tissues other than the tubules. In order to assess this C42 was used to drive expression of nuclear GFP, the full CNS of 2 day old pupal flies were then dissected and counter stained with the neuropil stain, nC82. The results are seen in Figure 4-2

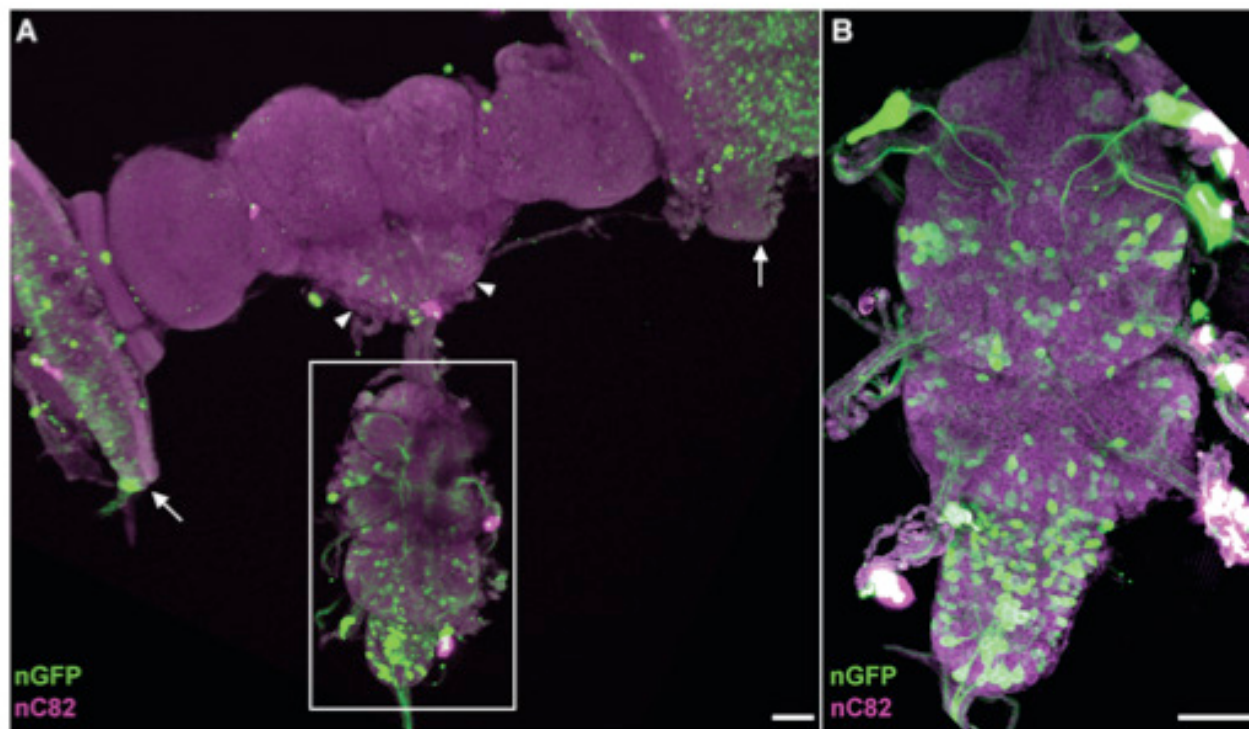


Figure 4-2 c42GAL4 driven nuclear GFP expression in the CNS of 2 day old pupa

(A) Maximal Z projection of a 2 day old pupal CNS exhibiting nGFP (green) expression in the developing photoreceptor lamina (arrows) and suboesophageal ganglion (arrowheads) within the brain and expression in the ventral nerve cord (VNC; white box). Dorsal view; anterior top. (B) Maximal Z projection of a 2 day old pupal VNC exhibiting extensive nGFP (green) expression in all ganglia. Ventral view; anterior top. neuropil counterstained with anti-nC82 (magenta). Scale bars = 50µm.

Results clearly show expression in regions where *fas2* is important, such as the developing photoreceptor lamina and several projecting neurons. For the purpose of this study it was decided that C42 would be excluded from further experiments

in order to eliminate any phenotype occurring from non-tubule expression. As crosses utilising UO showed no obvious phenotypes and previous observations suggested little expression of UO outside the tubules (data not shown), similar experiments were not carried out with UO in order to assess expression outside the tubule. In hindsight these experiments would prove useful in confirming UO as a tubule specific driver.

The following experiments, therefore used the more tubule specific driver UO.

4.2.2.2 QRT-PCR analysis confirms knockdown efficiency of *fas2* is dependent on driver line

As discussed in the previous section, experiments suggested that the knockdown efficiency of the VDRC lines was effective, but driver dependent, therefore *fas2*^{RNAiKK} was crossed with UO, tubules were dissected and knockdown efficiency was measured via QRT-PCR. These experiments were carried out at two different temperatures, as the Gal4/UAS system is known to be more effective at higher temperatures (Duffy, 2002). Therefore flies were reared at 22°C and 26°C. The results obtained can be seen in Figure 4-3. These results confirm the knockdown of *fas2*, but only at the higher temperature of 26°C. Although there appears to be only a 30% knockdown in tubules, these results are likely to underestimate the actual knockdown efficiency, as *fas2* is still expressed in the transitional, initial and lower segments of the tubules. In retrospect the knockdown efficiency would have been better detected by analysis of *fas2* expression in the larvae of flies expressing *fas2*^{RNAiKK} driven with *actin*^{GAL4}. Again assessment of protein levels via Western blot would also be beneficial.

We can, however conclude that *fas2*^{RNAiKK} is effective at knocking down *fas2* in the principal cells of the main segment when driven with UO, as is evident by microvilli defects detailed in Section 4.3

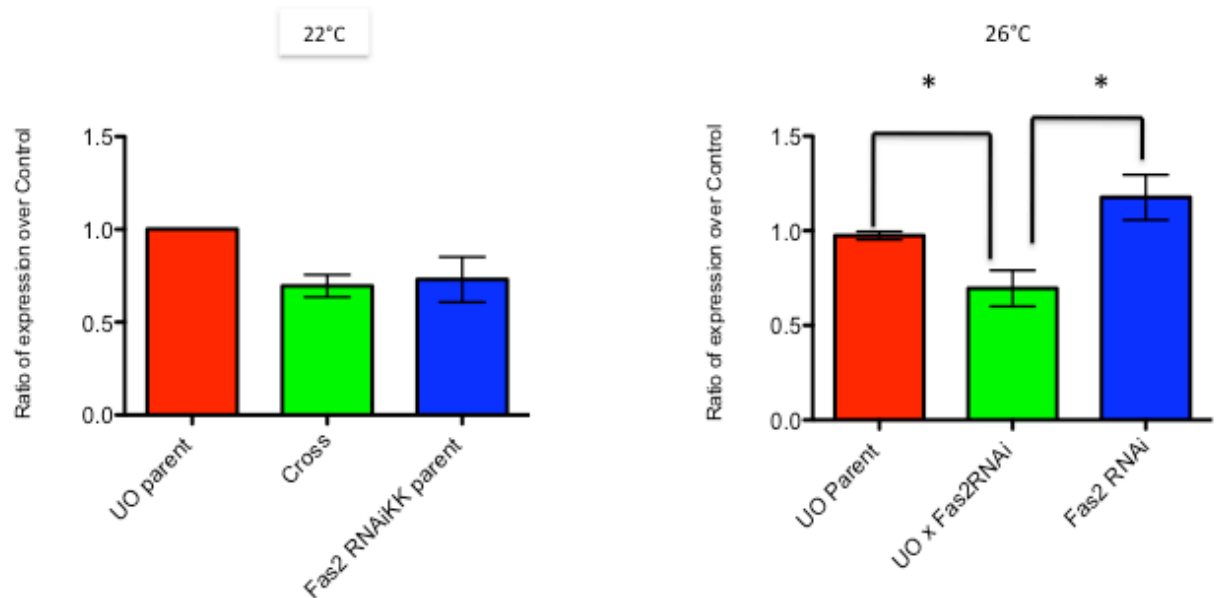


Figure 4-3 Analysis of RNAi efficiency by QRT-PCR

Comparison of the knockdown efficiency of *fas2*RNAiKK when driven with UO in the tubules at 22°C (left) and 26°C (right). Experiment compares the levels of *fas2* transcript in both parental lines and the cross. Results indicate that at 26° there is a small but significant decrease in *fas2* expression when compared to parental lines. P values are as follows UO parent compared to cross: $P = 0.0457$, Fas2 parent compared to cross: $P = 0.0345$ (t test analysis)

4.2.3 Down regulation of *fas2* in the principal cells does not affect the localisation of Dlg

As discussed in Chapter 3, it was initially thought that as Fas2 interacts with Dlg extensively throughout development, this would be the case in the Malpighian tubules. Indeed we have shown that during embryogenesis Fas2 localises to the lateral membrane of cells, consistent with Dlg staining (Campbell et al., 2009). However as is shown in Figure 4-4, Dlg remains localised to the junctional region in adult tubules unlike Fas2, which re-localises to the apical brush border. However as we can see in Figure 4-4 (b) Fas2 is always found directly apical to Dlg. We have

also discussed the possibility that Fas2 may be involved in the localisation of Dlg to the junctions during embryonic development. In order to test these hypotheses, we assessed the localisation of Dlg in adult tubules expressing *fas2RNAiKK* driven with UO Gal4. As can be seen in Figure 4-4 (b) the localisation of Dlg is unaffected and shows WT staining. From this we can conclude that it is probable that Fas2 does not interact with Dlg in the Malpighian tubules. These results do not however rule out the possibility that *fas2* interacts with Dlg during embryogenesis. Perhaps it would be better to assess this using an embryo specific, tubule Gal4 line, as UO is only expressed from 3rd instar (Friedman and Johnson, 1977; Terhzaz et al., 2010).

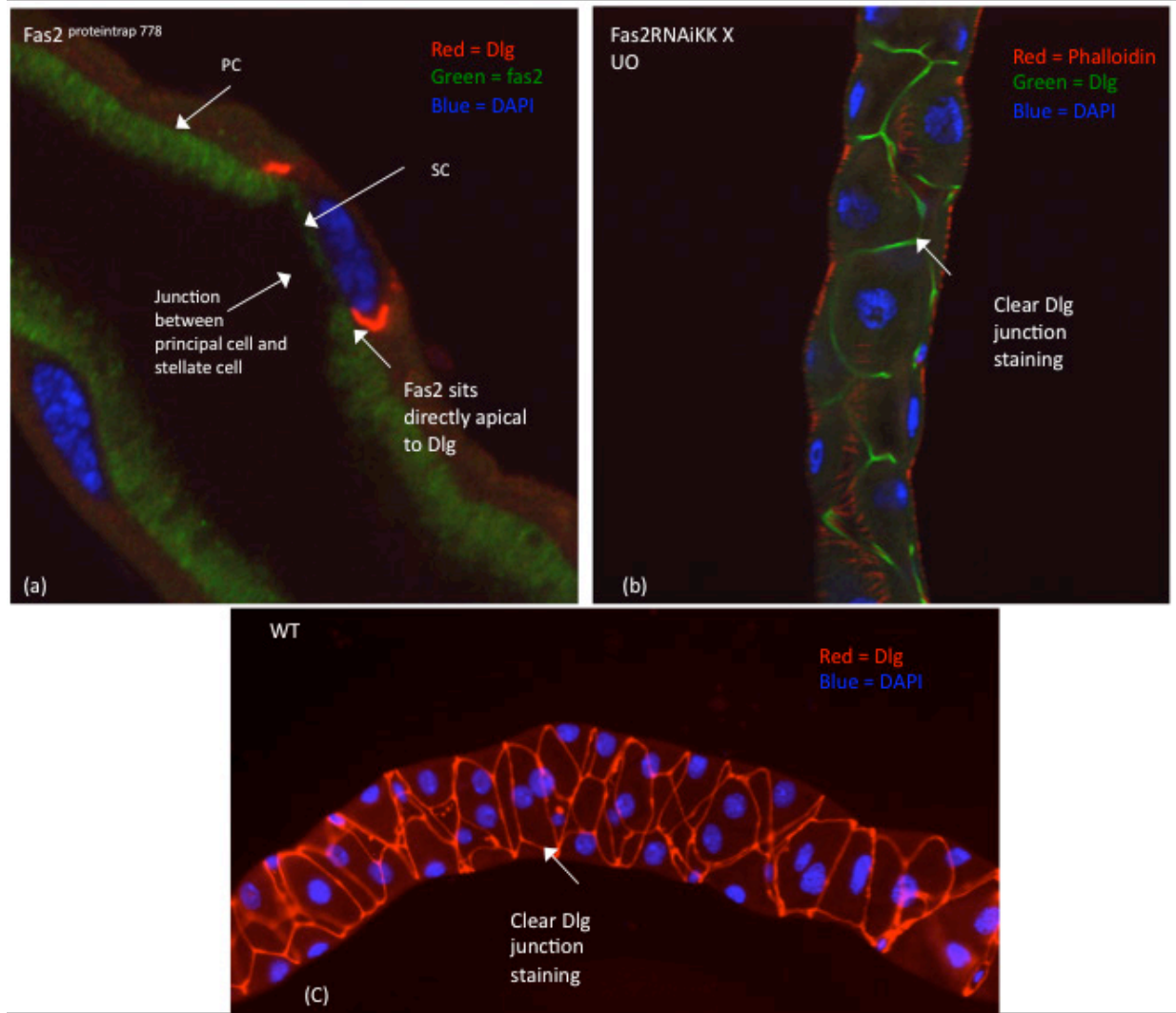


Figure 4-4 Localisation of Dlg in WT and *fas2RNAiKK* tubules

(a) Mid sectional scan of tubule. Localisation of *fas2* (*fas2*^{proteintrap778}) is shown to be directly apical to Dlg, Dlg stained with Anti-Dlg (1 in 500). (b) Tubules expressing *fas2RNAiKK* driven with UO Gal4 show no mis-localisation of Dlg as is evident by its WT staining (see (c) for WT staining). SC = Stellate cell PC= Principal cell.

4.3 Fas2 plays an important role in microvilli development and possibly Actin localisation

As Fas2 localises to the apical brush border of the principal cells, we analysed the effect of down regulation on the structure of both the tubules and the microvilli. As Fas2 plays an important role in fasciculation in neuronal development, we can speculate that it may be involved in the fasciculation of the microvilli during

development. In order to determine if this was indeed the case, tubules from the following fly lines were dissected and their tubules stained with Rhodamine labelled Phalloidin (a fungal toxin which binds F-actin):

- Canton S (WT)
- *Fas2*^{EB112} (Heterozygote null)
- *Fas2*^{RNAiKK} X UO
- *Fas2*^{EP} (Endogenous *fas2* tagged with UAS) X UO

All experiments were carried out at 26°C. These lines were chosen in order to establish the affect of both over and under expression of *fas2*. The results can be seen in Figure 4-5. The line *fas2*^{EB112}, is a 50% loss of function mutant, this line was included in order to determine if increasing the level of *fas2* down regulation to 50%, in all principal cells, would result in a more severe phenotype. As is evident in Figure 4-5, *fas2* appears to be extremely important in both the development and stabilisation of the microvilli. Knocking *fas2* expression down with *fas2*^{RNAiKK}, results in less dense shorter microvilli, Figure 4-5 (c). Evidence suggests that *fas2*^{EB112}, elicits a stronger phenotype than *fas2*^{RNAiKK}, suggesting that perhaps whether or not all cells are knocked down for *fas2* may be important. Over-expression of *fas2*, using the *fas2*^{EP} line crossed with *actin*^{GAL4} results in the opposite effect, in that microvilli are much longer and very dense. This line has been previously characterised and been shown to effectively over express endogenous *fas2* (Kraut et al., 2001).

We can also determine that in *fas2* mutant and RNAi expressing tubules F-Actin staining appears to be reduced and the opposite is again true in the over-expressor. What cannot be determined from this analysis, is if Fas2 is involved directly in F-Actin localisation, or if simply less, shorter microvilli result in less F-Actin. These results are intriguing as they suggest a very important role for *fas2* within the principle cells of the tubules. Time did not permit a full analysis of these results and in order to fully determine the role of Fas2 in microvilli stabilisation a full quantitative analysis of microvilli length is required. For each experiment at least 9 tubules were dissected and assessed. However, breakdown of the confocal microscope and software analysis resulted in incomplete scanning and generation of images for all tubules assessed. We can however conclude from these initial

experiments that the microvilli development and/or stabilisation was affected in all tubules assessed if *fas2* was over expressed or knocked down.

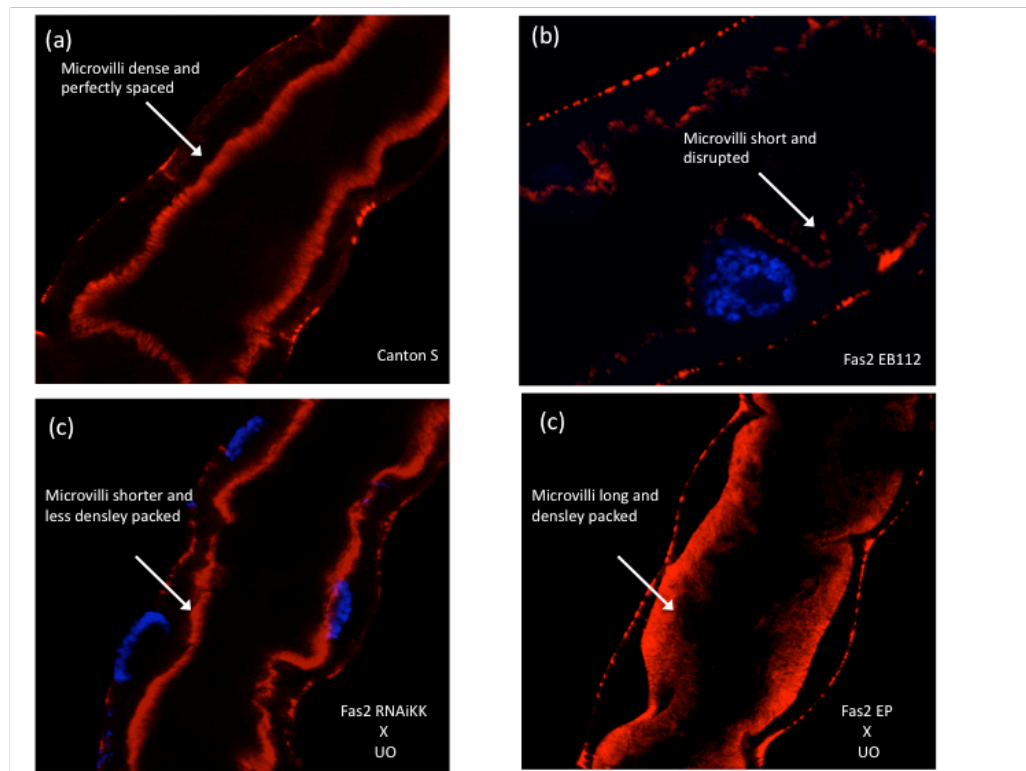


Figure 4-5 Effect of over-expression and under-expression of *fas2* on microvilli development
Adult tubules were dissected, fixed and stained and visualised on the same day. F-Actin staining with Rhodamine labelled Phalloidin. (a) Canton S tubule shows dense evenly spaced microvilli. However down regulating *fas2* using a mutant (50% loss of function) or by RNAi results in less dense, shorter microvilli (b) & (c). Over-expressing *fas2* results in the opposite effect, where microvilli are more densely packed and longer (d). Interestingly *fas2*^{EB112} shows a much more striking phenotype than *fas2*RNAiKK, suggesting that increasing the knockdown by ~20% significantly increase the mutant phenotype. Each experiment consisted of ≥ 9 tubules and this figure represents a typical picture.

4.3.1 Miss expression of *Fas2* in the stellate cells results in oversized microvilli and misshapen cells

In order to determine the effect of mis-expressing *fas2* in the stellate cells, the stellate cell Gal4 driver C724 was used in order to drive isoform B of *fas2*^{YFP} or *fas2*^{EP}. As was the case with C42, C724 driven lines also showed evidence of non-tubule expression (for example expression is seen in several axonal projections in the nervous system data not shown), however at present there is no stellate cell driver, which does not show some neuronal expression. Although the stellate cells

contain apical microvilli they are much shorter in both length and width than the principal cells (Wessing and Eichelberg, 1972). If indeed *fas2* is involved in microvilli development, it may be possible to induce longer denser microvilli in the stellate cells by mis-expressing *fas2*. Indeed this is the case as can be seen in Figure 4-6. The microvilli are not only slightly longer but they also span a much wider area when compared to normal stellate cells. Interestingly however this phenotype is not consistent in every stellate cell as can be seen in Figure 4-6. This suggests that some stellate cells have the ability to utilise *fas2* in order to develop longer microvilli and others within the same tubule do not. Further analysis of the stellate cells, which exhibit abnormal apical microvilli, also indicated that they were misshapen, appearing more principal shaped than stellate. There are several hypotheses for why this might be the case. Firstly simply the growth in width of the microvilli results in a distortion of the shape of the cell. Secondly *fas2* may be involved in determining the polarity of the principal cell, therefore mis-expressing it in the stellate cell, results in the cell losing polarity, disrupting the growth of the stellate shape. Further analysis of *fas2* mis-expression in the stellate cells is required. However time did not permit this to be undertaken in this study. Again quantification of microvilli length is also required.

We can therefore conclude that mis-expressing *fas2* in the stellate cells results in some cells developing longer wider microvilli, resulting in distortion of the cell shape.

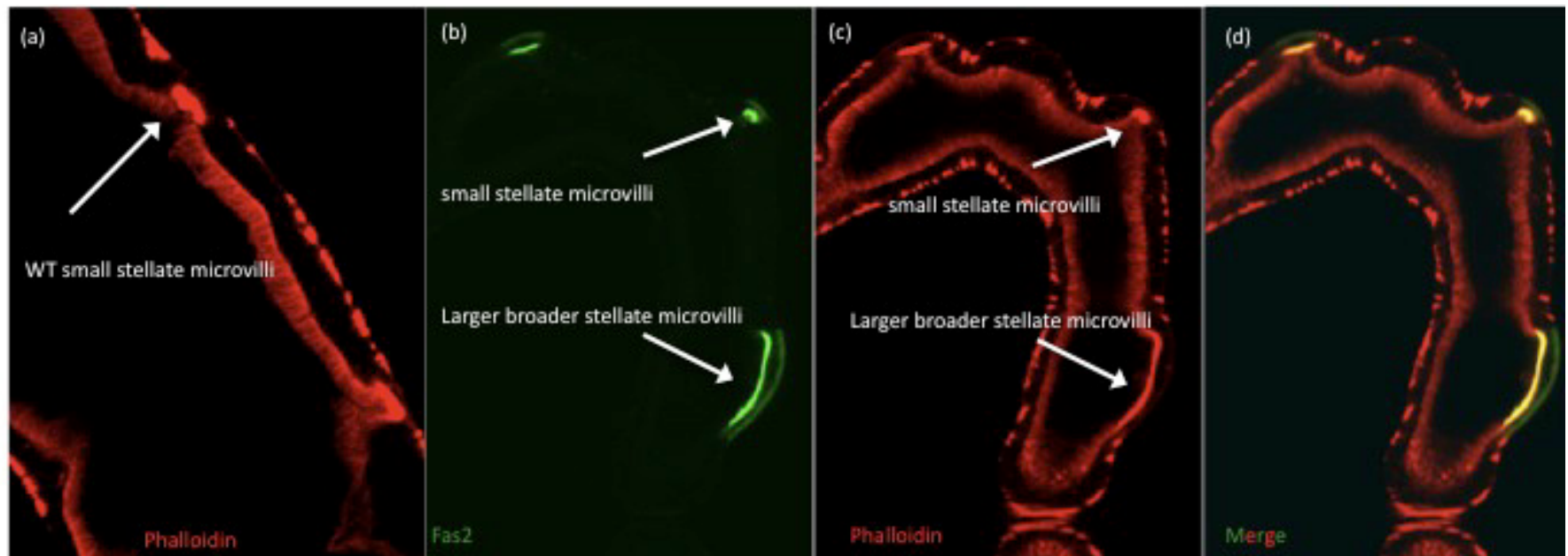


Figure 4-6 Miss expression of *fas2* in the stellate cells results in a border apical surface, with longer microvilli

(a) Canton S tubule stained with Phalloidin, indicates the short microvilli present in the stellate cells (b) Shows localisation of miss expressed *fas2* within the stellate cells, localised to the microvilli. One stellate cell shows abnormally long and broad microvilli (b),(c) &(d). This experiment was carried out multiple times and similar results obtained each time.

4.4 Conclusions

In this Chapter we discuss the effect of down regulating, over-expressing and mis-expressing *fas2* on the structure and development of the tubules. Adopting the Gal4/UAS system, we were able to affect the expression of *fas2* in the tubules. Our results indicate the importance of this technique, as global mutants of *fas2* often die early in development or shortly after eclosion, making it impossible to fully study the effect of *fas2* depletion in the tubules of adult flies.

We have shown that over expressing *fas2*^{RNAiKK} with the ubiquitous Gal4 driver *Actin*^{Gal4}, results in death shortly after eclosion. These results indicate that *fas2*^{RNAiKK} line is effective at decreasing *fas2* expression levels. However as the flies do develop to pupal stages, suggesting that knockdown is not sufficient to cause early lethality. This section indicates the importance of tissue specific regulation of gene expression in order to determine the function of a gene in an adult tissue.

RNAi knockdown of *fas2* in the tubule principal cells of the tubules results in significant knockdown of the gene transcript in comparison to control parental flies. We therefore assessed the tubules of knock down flies for abnormal development, with a particular focus on the microvilli, as this is where I have shown Fas2 to localise. Results indicated that *fas2* is important for both the development and/or stabilisation of the microvilli. Where *fas2* is down regulated, microvilli are shorter and less dense compared to control lines. We also show that the heterozygote null line *fas2*^{EB112} also elicits a similar, although more pronounced phenotype. Over-expression of *fas2* in the principal cells results in the opposite phenotype: with longer and denser microvilli. Actin localisation also appears to be affected in these lines: however it is unclear if this is an indirect result of less or more microvilli, or if the localisation of Actin is directly affected by the loss or gain of *fas2* expression. Further experiments are required in order to determine if Fas2 is required for the development of the microvilli in the tubules or simply for the stabilisation of the microvilli in the adult tubules. In order to distinguish between the two, experiments such as those shown in Figure 3-7 should be carried out in order to determine at which stage during development the microvilli are affected. If Fas2 is important for the

developing microvilli the we would expect to see a phenotype early in development. However if Fas2 is required for microvilli stabilisation then we may only see a phenotype in adult tubules.

Mis-expression of *fas2* in the stellate cells resulted in the development of slightly longer, border microvilli when compared to control lines. Interestingly, this effect was not evident in all stellate cells expressing *fas2*, suggesting that *fas2* mis-expression phenotypes are dependent on the cell they are expressed in. There are several hypotheses as to why this may be the case. Firstly studies suggest that although tubules predominantly consist of principal and stellate cells, these cells in them self show different gene expression profiles. Suggesting that although cells are named stellate cells there are actually atomically identical cells with different gene expression patterns. Therefore the affect, which Fas2 over expression has on a given stellate cell may be dependent on the expression of other genes in the cell. Secondly the level of drive expression often differs in cells with in the tubule. So it is likely that cells, which are not misshapen may be expressing lower levels of *fas2*. Cells, which exhibit the microvilli phenotype, also appear to be misshapen, exhibiting a more principal cell shape than stellate.

In conclusion this chapter indicates an important role for *fas2* in the development and stabilisation of apical microvilli in the principal cells of the Malpighian tubules. An updated model of Fas2 localisation can be found in Figure 4-7

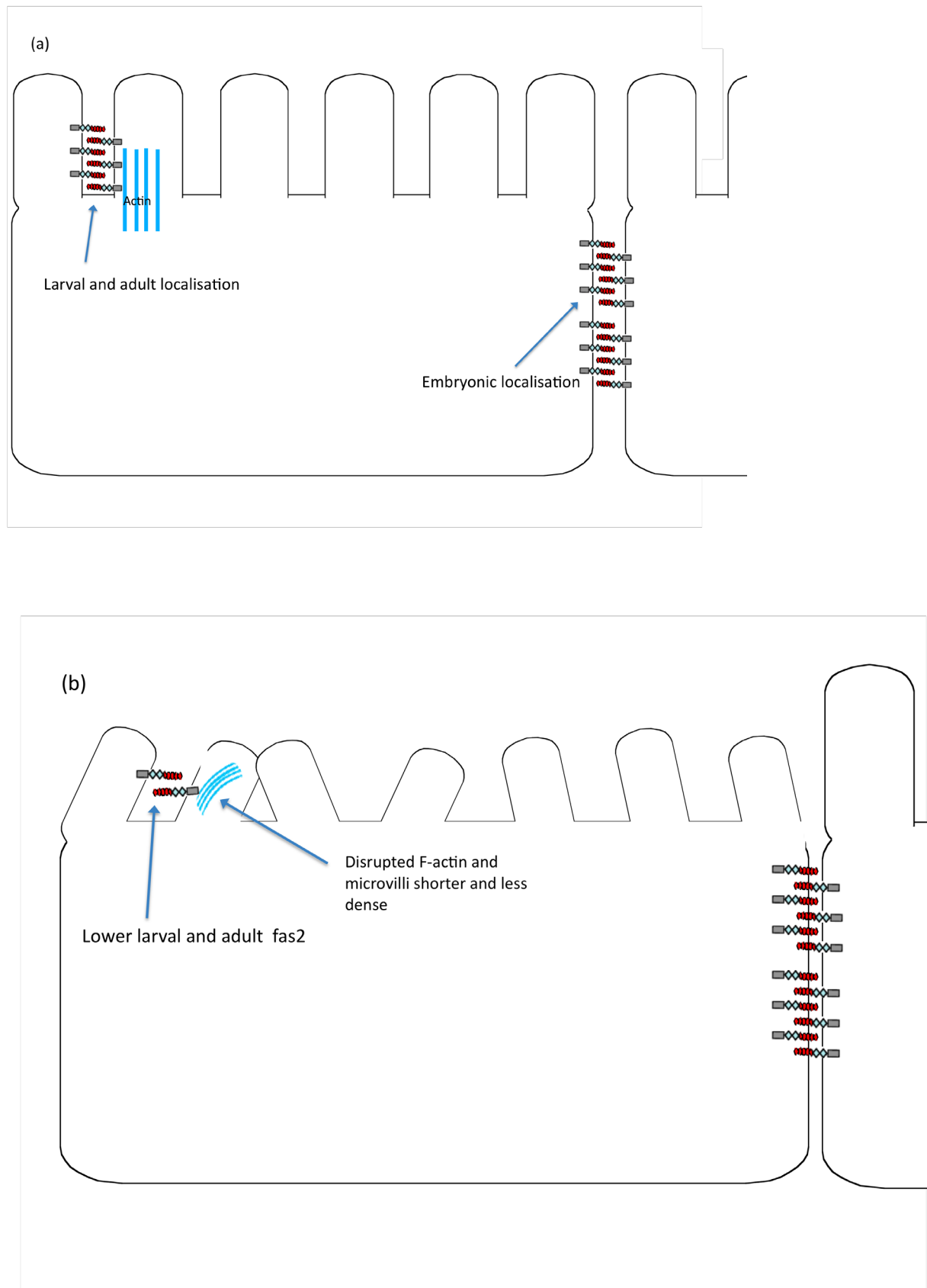


Figure 4-7 Model of Fas2 localisation and function 2

During embryogenesis Fas2 is localised to the lateral membrane between cells. (a) In larval and adult flies Fas2, is found to be localised to the apical microvilli showing some localisation with F-Actin.(b) When Fas2 is depleted this leads to disrupted, shorter less dense microvilli in the adult flies and F-actin.staining is also depleted. The opposite is true when Fas2 is over expressed.

Chapter 5

5 Interactions of *fas2* with cAMP & Actin and their relationship to fluid secretion

5.1 Summary

We have shown in the previous chapter that *fas2* is important for microvilli development, stabilisation and possibly for Actin localisation. As discussed in Section 1.2.5.2, microvilli are extremely important for the secretion phenotype of the tubules (Bradley and Snyder, 1989). Microvilli provide the surface area and stability required for secretion to take place. Studies suggest that when tubules are stimulated with cAMP, microvilli elongate, actin reorganisation occurs and finally, V-ATPase and mitochondria are recruited and activated (Bradley and Satir, 1981; Bradley and Snyder, 1989; Brown et al., 1993; Paunescu et al., 2010). Without microvilli tubules would lack not only the surface area required for mitochondria and V-ATPase accumulation but also the flexibility required to contract at such a high level. Therefore we can hypothesise that as *fas2* affects microvilli length, density and stabilisation we should see an effect on fluid secretion when *fas2* expression is up or down regulated.

We have discussed the possibility that Fas2 acts as a cell adhesion molecule in order to stabilise the microvilli, however this may not be the only function of Fas2 in the tubules. Previous studies have indicated that Fas2 plays an important signalling role in the nervous system (Mao and Freeman, 2009). Fas2 is also responsible for the recruitment of proteins both to and from the NMJ, in response to cAMP (Beumer et al., 2002; Kohsaka et al., 2007a). As cAMP is known to stimulate the recruitment of mitochondria and V-ATPase to the microvilli, there is the possibility that Fas2 may also be involved in this process. Secondly actin remodelling is an important process during secretion and recent studies have indicated the importance of cAMP in this process (Beyenbach et al., 2009; Karas et al., 2005). Indeed recent studies have

indicated that Fas2 may interact with several actin proteins, hinting at the possibility that Fas2 may be involved in actin organisation in the tubules.

This Chapter will discuss the experiments carried out in order to test these hypotheses and the results obtained. This study has concentrated on the role of cAMP in fluid secretion and has not looked at other stimuli such as cGMP or CAPA.

5.2 Wild type secretion response to cAMP

It has been known for several years that cAMP stimulates fluid secretion in the Malpighian tubules of *D.melanogaster* (Dow et al., 1994b; Riegel et al., 1998). Studies carried out using the adapted Ramsay assay, as described in Section 2.13, determined that low molar concentrations of cAMP elicit a marked increase in fluid secretion (Riegel et al., 1998). The direct mechanisms by which cAMP illicit this response is as yet unclear, however several recent studies have suggested the involvement of actin rearrangement, V-ATPase and mitochondria recruitment and activation and microvilli growth/rearrangement. Tubules are thought to actively take up cAMP through, an as of yet unidentified membrane transporter (Riegel et al., 1998). Many studies involving cAMP stimulation utilise the cell permeable analogue Dibutyryl-cAMP (db-cAMP), allowing for full uptake of the nucleotide through the cell membrane. cAMP is actively taken up by the principal cells, but dbcAMP is thought to enter both the principal and stellate cells equally. As stellate cells are known to respond to intracellular cAMP (Kerr et al., 2004), cAMP is preferred, for use as an agonist, because of its cell-specific action of the *fas2* expressing principal cells. We therefore carried out Ramsay secretion assays (Section 2.13), to compare the effect of cAMP and db-cAMP on secretion in wild type Canton S tubules. The results can be seen in Figure 5-1. As there was no significant difference between cAMP and dbcAMP, cAMP was used for all further experiments.

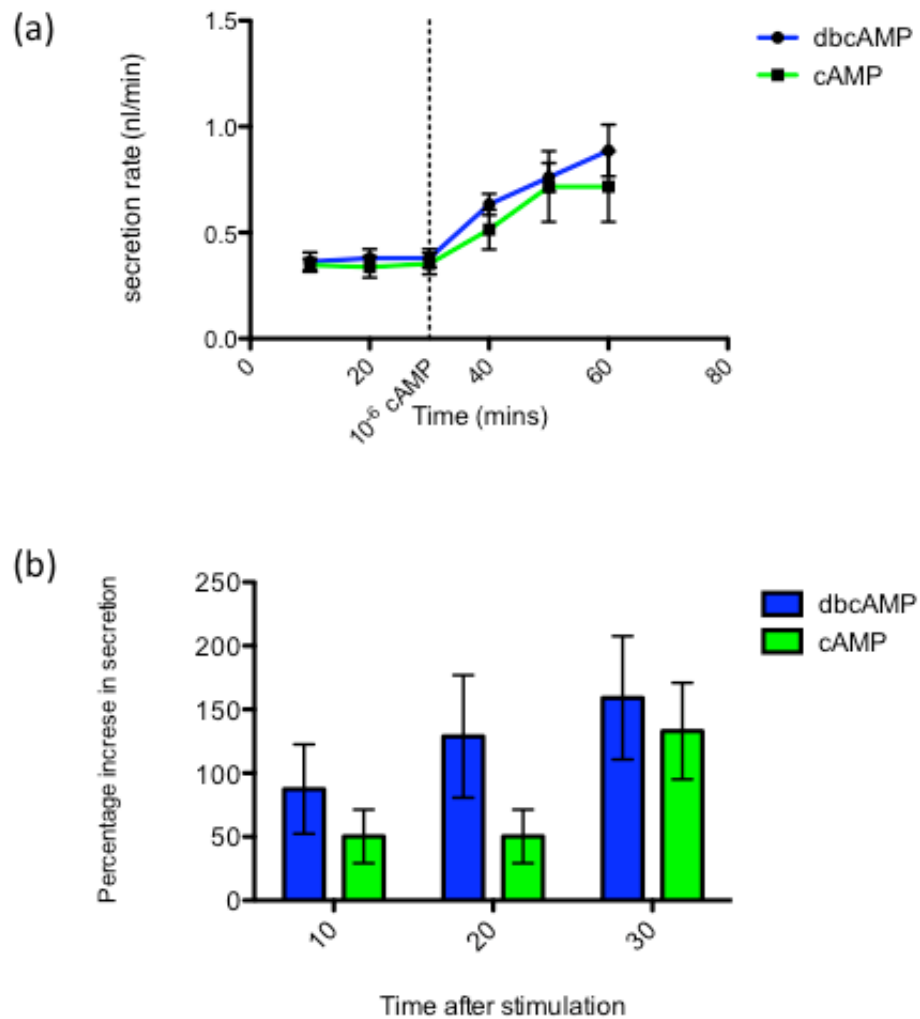


Figure 5-1 Comparison of the effect of 10^{-6} M cAMP & db-cAMP on the secretion of Canton S tubules

Tubules were dissected from 7-day-old Canton S flies and basal secretion was measured for 30 min before the addition of 10^{-6} M cAMP or 10^{-6} M db-cAMP. Secretion was then measured for a further 30 min. (a) Represents secretion curve of both sets of tubules (b) The percentage increase in secretion at each time point after stimulation, compared to mean basal secretion rate. There is no significant difference in secretion response to cAMP & db-cAMP ($P = >0.05$). Figure represents typical secretion assay, assay repeated 3 times with ~9 tubules per assay.

5.2.1 Determination of optimal cAMP concentration

Previous studies suggest a cAMP concentration of as little as 200nmol L^{-1} elicit an effect on fluid secretion (Riegel et al., 1998). In order to assess the dose response to cAMP in tubules, secretion assays were carried out using three different concentrations of cAMP. The results can be seen in Figure 5-2

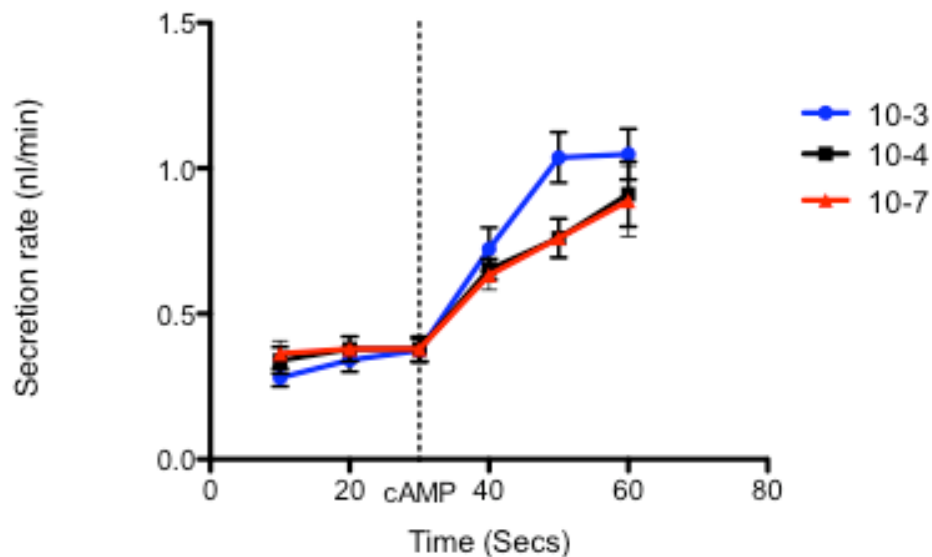


Figure 5-2 Effect of different concentrations of cAMP on the fluid secretion rate of Canton S tubules

Tubules were dissected from 7-day-old Canton S flies and basal secretion was measured for 30 min before the addition of 10^{-3}M , 10^{-4}M & 10^{-7}M cAMP (as a final concentration in bathing solution). Secretion rate was then measured for a further 30 min. Represents secretion curve of all sets of tubules. 10^{-3} produced the highest response in secretion. Figure represents typical secretion assay, assays were repeated 3 times with ~9 tubules per assay.

Results indicate that 10^{-3}M cAMP, induces the highest response, however as this is somewhat higher than the level of cAMP found *in vivo*, a lower level of cAMP would be more beneficial to experiments. As 10^{-4}M and 10^{-7}M gave a similar response a concentration of 10^{-6}M , was used for all subsequent experiments. This concentration is in line with that used in previous experiments (Dow et al., 1994b).

5.2.2 *Conclusions*

We have confirmed previous observations that cAMP induces an increase in fluid secretion rate in the Malpighian tubules and that cell preamble analogues are not required, which may elicit a phenotype not consistent with *in vivo* mechanisms.

5.3 Down regulation of Fas2 results in a decrease in fluid secretion response to cAMP

5.3.1 *Introduction*

We have shown that the down regulation of *fas2* expression by RNAi or by mutation, leads to a decrease in microvilli length, density and stability. In order to assess if this phenotype affects fluid secretion, Ramsay assays were carried out as described in Section 2.13. As the microvilli are disrupted, we would expect to see not only a decrease in basal secretion but also in stimulated secretion. We therefore analysed both basal and cAMP stimulated fluid secretion in both RNAi and *fas2*^{EB112} mutant lines.

5.3.2 *Down regulation of Fas2 via fas2RNAiKK driven with UO*

The results obtained when *fas2*^{RNAiKK} is driven with the principal cell driver UO, can be seen in Figure 5-3. There is no significant difference in basal secretion rates in the tubules of progeny of the cross, when compared to both parental lines. This is of interest as it suggests that the decrease in microvilli length and density does not seem to have an effect on basal fluid secretion.

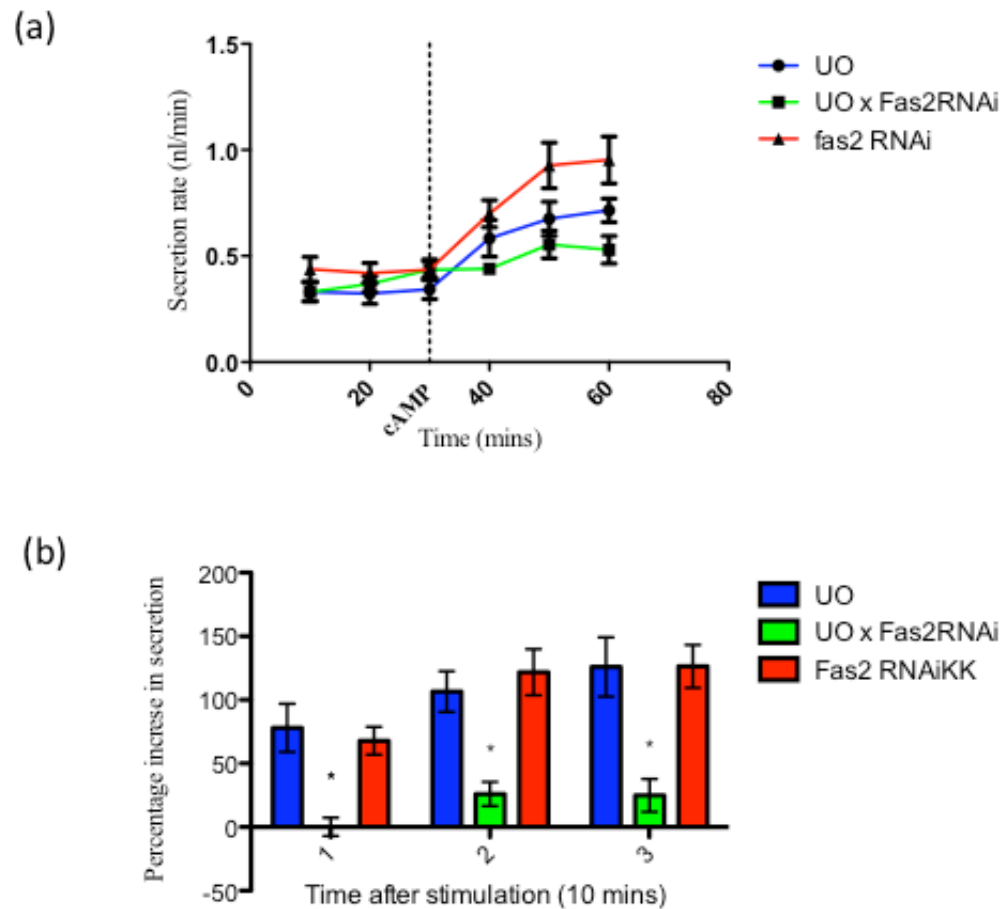


Figure 5-3 Effect of down regulating Fas2, via RNAi, on fluid secretion rate of the tubules in response to cAMP

Tubules were dissected from 7-day-old UO, Fas2RNAiKK and UO x Fas2RNAiKK progeny and basal secretion was measured for 30 min before the addition of 10^{-6} M cAMP. Secretion rate was then measured for a further 30 min. (a) Indicates secretion curve of three sets of tubules. (b) Indicates percentage increase in fluid secretion at each time point after addition of cAMP. For each time point the knock down tubules show a significantly smaller percentage increase when compared to parental lines. Figure represents typical secretion assay, assay repeated 3 times with ~9 tubules per assay. * in (b) indicates P value > 0.05 P values are indicated in table below statistical analysis student t test unpaired

	P Value		
	10 min	20 min	30 min
UO parent vs cross	0.029	0.001	0.0016
Fas2RNAiKK parent vs cross	0.002	0.005	0.0002
Parental vs parental	0.6459	0.5378	0.9885

This result however may again be skewed, due to the driver line UO. UO is only expressed in the main segment of the tubules and indeed is not equally expressed in all cells (See Figure 5-8). Therefore not all cells will have a decreased microvilli

structure and therefore may compensate for the knockdown cells. Although we do not see a decrease in basal secretion levels, there is a significant decrease in secretion response to cAMP. These results are intriguing as they suggest that perhaps the stability and surface area of the microvilli is more important after stimulation with cAMP. Indeed several studies have suggested that microvilli extension is crucial to cAMP induced secretion, if however the microvilli are shorter and less dense then perhaps they cannot extend. A reduction in microvilli length and density would also decrease the amount of space available for incoming mitochondria and V-ATPase subunits, again resulting in a decrease in fluid secretion. There is also the possibility that Fas2 may play another role in fluid secretion downstream of cAMP. We will present evidence that this is indeed the case.

Therefore we can hypothesize that Fas2 knock down leads to an instability in microvilli, which does not affect basal secretion levels but is sufficient to disrupt accelerated fluid secretion induced by cAMP.

5.3.3 Down regulation of *fas2*: *EB112* heterozygous null.

We have shown in the previous Chapter that the heterozygous null line *fas2*^{EB112}, showed a more pronounced microvilli phenotype than the RNAi knock down of Fas2 using UO. We therefore carried out secretion assays in order to determine if these lines also showed a defect in cAMP induced secretion response. The more pronounced phenotype may also elicit a decrease in basal secretion, which is not seen in the RNAi cross. The results obtained can be seen in Figure 5-4. The EB112 line consists of a P-element insertion in the endogenous *fas2* locus. This insertion leads to the loss of Fas2 production and therefore a Fas2 null fly. As the P-element insertion is homozygous lethal, the P-element insertion is balanced over the x chromosome balancer FM7w. With this in mind only female flies could be used for this experiment as males carrying the P element insertion die before eclosion. Interestingly *fas2* mutant tubules, elicit a decreased response to cAMP but also have a highly increased basal secretion level. This was an unexpected result, as it suggests that these tubules are able to secrete at a higher basal level. It may

suggest that highly deformed microvilli lead to a ‘leaky’ tubule, which lacks the ability to control fluid secretion, without well-structured apical constraints.

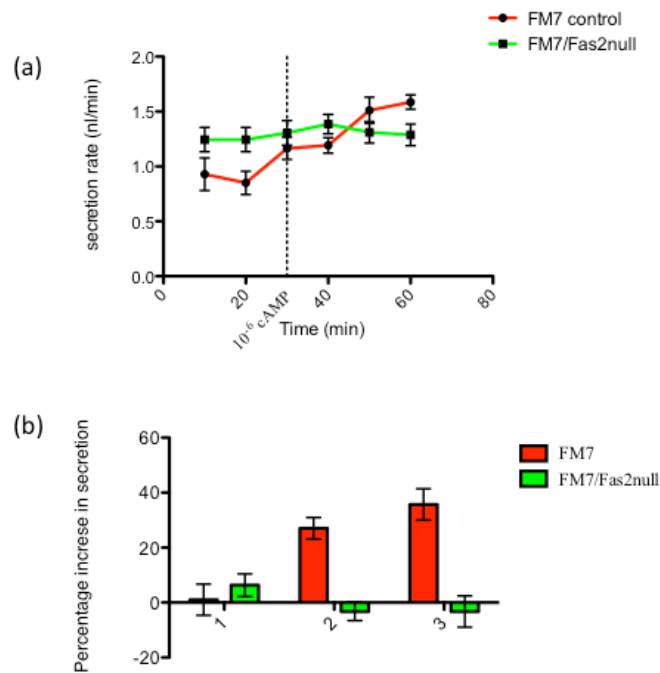


Figure 5-4 Effect of 50% reduction of Fas2 on fluid secretion response to cAMP

Tubules were dissected from 7-day-old females, *fas2*^{EB112}/Fm7 & Fm7 lines and basal secretion was measured for 30 min before the addition of 10^{-6} M cAMP. Secretion rate was then measured for a further 30 min. (a) Indicates secretion curve of three sets of tubules. (b) Indicates percentage increase in fluid secretion at each time point after addition of cAMP. There appears to be a slight increase in basal fluid secretion For, each time point the cross tubules show a significant decrease in percentage decrease when compared to parental lines. Figure represents typical secretion assay, assay repeated 3 times with ~9 tubules per assay. P values are indicated in table below statistical analysis (student t test unpaired)

	P Value		
	10 min	20 min	30 min
<i>Fas2</i> ^{EB112} vs FM7	0.4551	<0.0001	0.0006

5.3.4 Conclusions

These results confirm that *fas2* plays an important function in the Malpighian tubules, as halving the gene results in a significant decrease in fluid secretion response to cAMP. The results however indicate that *fas2* may play a role other than microvilli stabilisation.

5.4 Over expression of *fas2* in the principal cells of the tubule results in a significant increase in fluid secretion in response to cAMP

5.4.1 *Introduction*

In the previous section we showed that down regulation of *fas2* results in a significant decrease in response to cAMP induced fluid secretion. We therefore hypothesised that the opposite would be true if we over expressed *fas2* in the principal cells. In order to test this hypothesis we conducted the following crosses: UO x *fas2*^{YFP}, UO x *fas2*^{EP} and finally UO x *fas2*^{extraYFP}. A description of each of these lines is given in Table 2-1. Briefly *fas2*^{YFP} is a UAS line, consisting of several GAL4 binding domains (UAS) upstream of the open reading frame of *fas2-B* fused to YFP. Therefore driving expression of this construct results in over expression of only isoform B of Fas2, where localisation and expression can be assessed by YFP visualisation. *fas2*^{EP} consists of a insertion of UAS upstream of the endogenous *fas2* locus, resulting in expression of both endogenous Fas2 and over expression of endogenous Fas2 via a Gal4 driver line. *fas2*^{extraYFP} is similar to *fas2*^{YFP} but in this case only the extra cellular domain of isoform B is over expressed.

5.4.2 *Over expression of *fas2*-YFP with the principal cell driver UO results in a slightly increased response to cAMP*

In Chapter 3 we showed that over expression of isoform B of Fas2 tagged with YFP, resulted in increased microvilli length and density. In order to assess if this in turn leads to an increased fluid secretion rate we carried out equivalent experiments to those carried out in Section 5.3. The results obtained can be seen in Figure 5-6. The results indicate that there is no significant difference in basal secretion. There is however a small, but significant increase in stimulated response to cAMP. We can therefore conclude that we see the opposite effect as that seen when we decrease *fas2* expression.

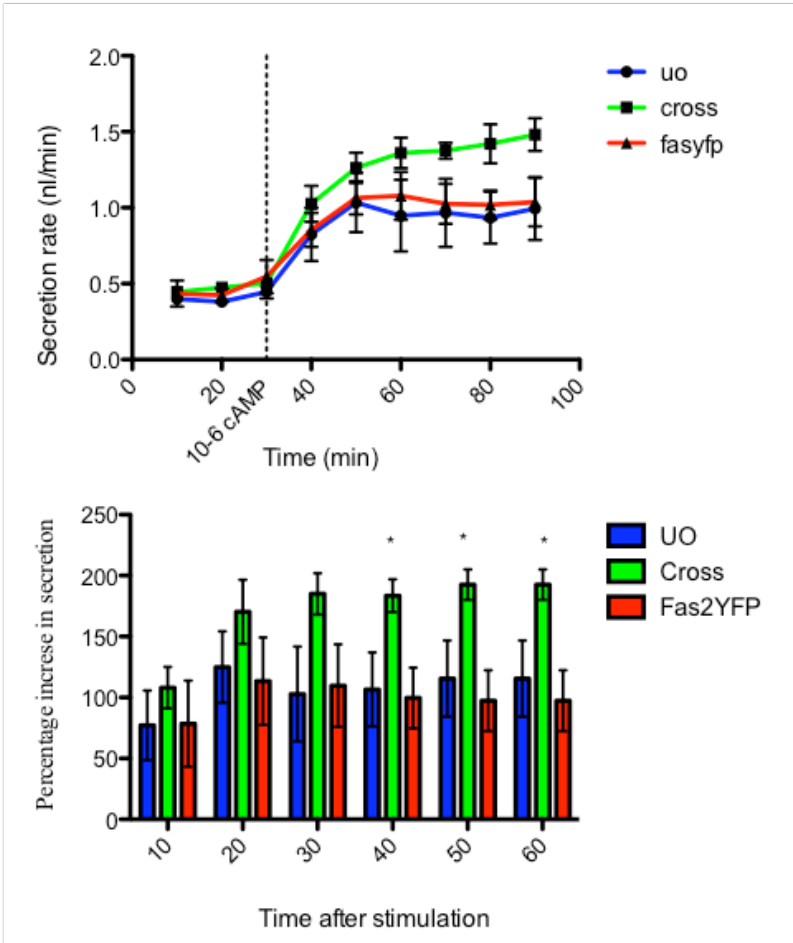


Figure 5-5 Effect of $fas2^{YFP}$ over expression in principal cells on fluid secretion in response to cAMP

Tubules were dissected from 7-day-old UO, $fas2^{YFP}$ & UO X $fas2^{YFP}$ lines and basal secretion was measured for 30 min before the addition of 10^{-6} M cAMP. Secretion rate was then measured for a further 30 min. Secretion curve of three sets of tubules are shown. For time points 40, 50 & 60 min there is a significant increase in percentage increase in secretion in the tubules from the cross when compared to BOTH parent (as indicated by * = $P < 0.05$)

	P Value					
	10	20	30	40	50	60
UO parent vs cross	0.3734	0.2758	0.0815	0.0429	0.0451	0.0451
FasYFP vs cross	0.4444	0.2228	0.0650	0.0124	0.0058	0.0058
Parental vs parental	0.9764	0.8051	0.8998	0.8652	0.6711	0.6711

5.4.3 ***Over expression of endogenous *fas2* increases fluid secretion response to cAMP***

We have shown that over expressing endogenous *fas2* with the principal cell driver UO leads to a marked increase in microvilli length and density. The line *fas2*^{EP}, leads to an increase in expression of endogenous *fas2* when driven with UO. Therefore all three transcripts will be over expressed in comparison to *fas2*^{YFP} (transcript B). We may expect therefore to see a more pronounced secretion phenotype when driving the expression of *fas2*^{EP} compared to *fas2*^{YFP}. We once again carried out secretion assays, stimulating with cAMP.

Figure 5-6 shows the results obtained when *fas2*^{EP} is driven with UO. As with previous results we see no significant difference in basal secretion levels. However there is a highly significant increase in stimulated response to cAMP. This increase is extremely remarkable as it represents a ~400% increase when compared to parental response to cAMP. Interestingly this response is not evident until 20 min after stimulation, this timeframe allows for the recruitment of V-ATPase and mitochondria to the microvilli. However it is tempting to believe that *fas2* may be playing a role other than microvilli development, as the secretion response is so markedly increased. A large surface area would allow increased space for mitochondria and V-ATPase recruitment, but without an increased signalling mechanism to recruit and activate the mitochondria and V-ATPase, this space would not be utilised. Therefore we would argue that *fas2* is playing a further role down stream of cAMP. It is also interesting to note that marked difference between over expression of one transcript and all three. This result suggests that although transcripts A & C are expressed at lower levels in the tubules they too may play an important role.

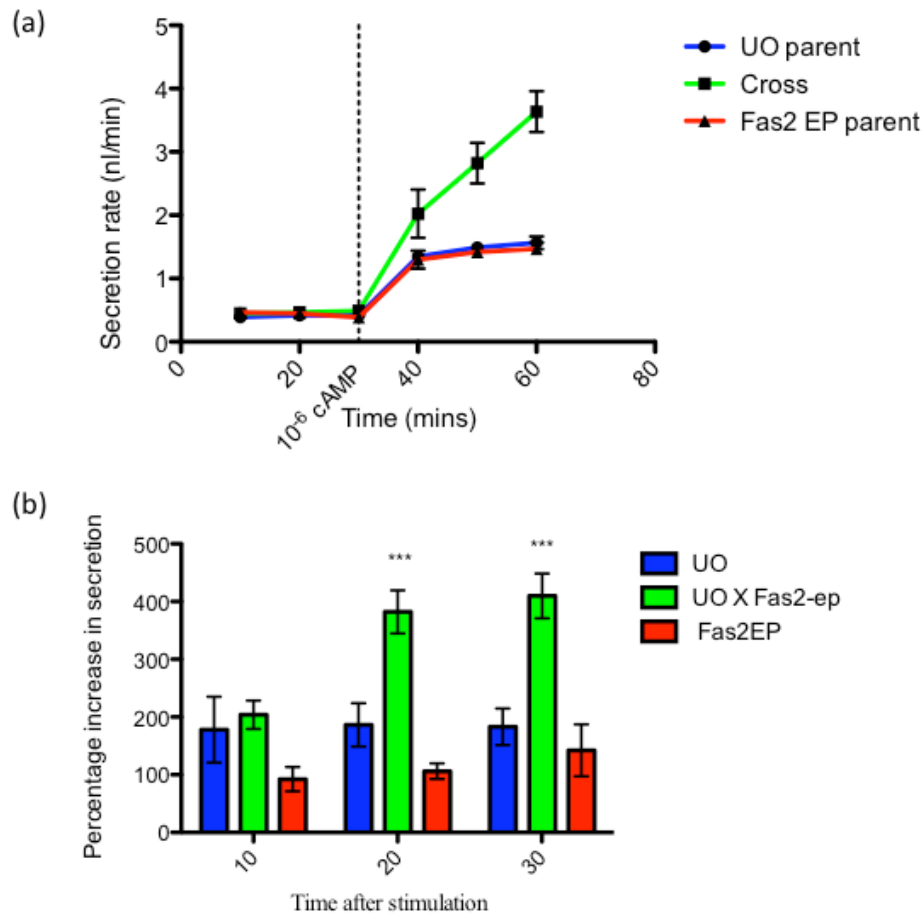


Figure 5-6 Effect of over expression of fas2 via fas2^{EP} x UO on cAMP induced fluid secretion in the Malpighian tubules

Tubules were dissected from 7-day-old UO, Fas2^{EP} and UO x Fas2^{EP} lines and basal secretion was measured for 30 min before the addition of 10⁻⁶ M cAMP. Secretion rate was then measured for a further 30 min. (a) Indicates secretion curve of three sets of tubules. (b) Indicates percentage increase in fluid secretion at each time point after addition of cAMP. For each time point the tubules from the cross show a significant increase in percentage increase when compared to parental lines. Figure represents typical secretion assay, assay repeated 3 times with ~9 tubules per assay. P values are indicated in table below statistical analysis = unpaired t Test * indicates where value is significant (P < 0.05) when compared to BOTH parental lines.

	P Value		
	10 min	20 min	30 min
UO parent vs cross	0.6860	0.0041	0.0011
Fas2EP parent vs cross	0.0085	0.0001	0.0014
Parental vs parental	0.2252	0.096	0.4644

5.4.4 ***Stimulation with Drosokinin increases response to cAMP***

In order to determine if the increase in cAMP involved activation of the V-ATPase, tubules were further stimulated with drosokinin after incubation with cAMP. Drosokinin activates the chloride shunt conductance of the stellate cells, when activated, this is rapidly followed by a collapse in the transepithelial potential (Beyenbach et al., 2010; Bradley, 1989; Maddrell and O'Donnell, 1992; Maddrell, 1981; O'Donnell et al., 1996). The movement of Cl^- across the membrane allows for the formation of a circuit, thus increasing secretion. Chloride movement is activated when V-ATPases are activated, thus giving an overall control of fluid secretion. When the tubules are stimulated with cAMP, H^+ ions are pumped across the membrane via V-ATPase, this then forces the movement of Cl^- out of the stellate cells. When drosokinin is added to the bathing solution, it effectively removes the brake from the Cl^- shunt and Cl^- then 'floods' across the membrane (Maddrell and O'Donnell, 1992). This in turn leads to an increase in V-ATPase activity and thus fluid secretion increases further. As we have only over expressed *fas2* in the principal cells of the tubules any effect drosokinin has on fluid secretion would be through a secondary effect on V-ATPase activity. We therefore repeated the secretion assays carried out in Section 5.4.3, with the addition of 10^{-7} drosokinin at 60 min. If over expression of *fas2* increases the activity of V-ATPase, either indirectly or directly then addition of drosokinin should further increase fluid secretion. The results obtained can be seen in Figure 5-7. Again we see a significant increase in fluid secretion response to cAMP in tubules over expressing *fas2*. With the addition of drosokinin at 60 min (30 min after cAMP stimulation), we see a further increase in secretion response when compared to parental lines. We can therefore speculate that *fas2* acts upstream of V-ATPase activation, thus increasing either the amount of V-ATPase in the microvilli or indeed the amount of activated V-ATPase.

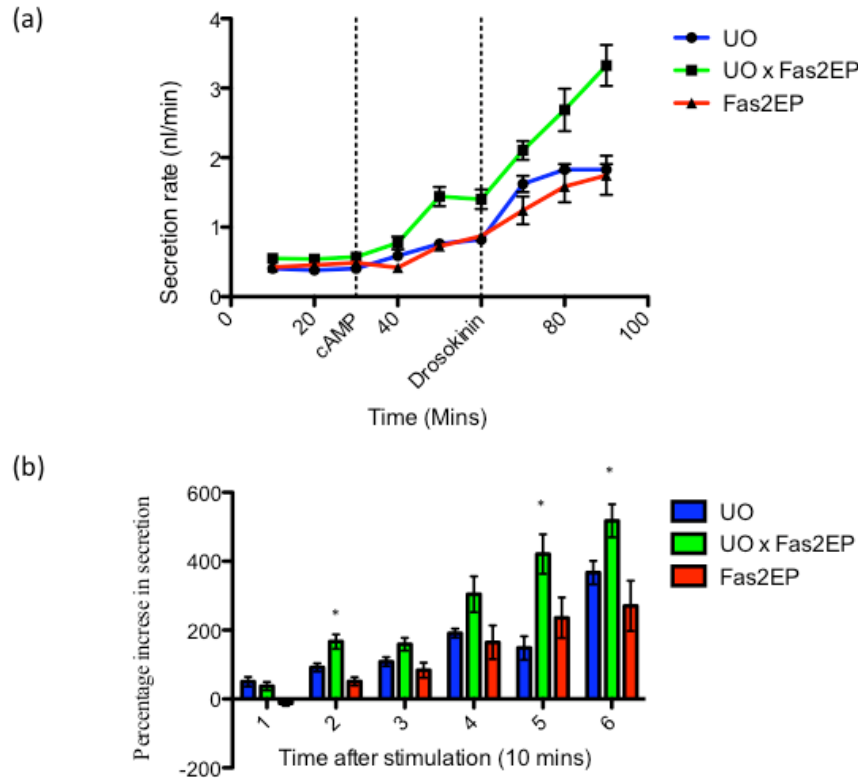


Figure 5-7 Effect of over expression of *fas2* in the principal cells on fluid secretion response to cAMP and Drosokinin

Tubules were dissected from 7-day-old UO, *Fas2^{EP}* and UO x *Fas2^{EP}* lines and basal secretion was measured for 30 min before the addition of 10^{-6} M cAMP. Secretion rate was then measured for a further 30 min. After 60 min 10^{-7} M drosokinin was added and secretion measured for a further 30 min. (a) Indicates secretion curve of three sets of tubules. (b) Indicates percentage increase in fluid secretion at each time point after addition of cAMP & drosokinin. For each time point the tubules from the cross show a significant increase in percentage increase when compared to parental lines. Figure represents typical secretion assay, assay repeated 3 times with ~9 tubules per assay. P values are indicated in table below. Student unpaired t Test * indicates where value is significant when compared to BOTH parental lines. (P < 0.05)

	P Value					
	10	20	30	40	50	60
UO parent vs cross	0.5042	0.0153	0.0592	0.929	0.0028	0.0342
Fas2EP vs cross	0.0225	0.0044	0.0332	0.1215	0.0342	0.0156
Parental vs parental	0.0092	0.0549	0.3247	0.5447	0.0719	0.2143

It is important to note here that the response of tubules from the progeny of UO x *fas2^{EP}*, over expressing *fas2*, is slightly decreased when compared to previous experiments. When compared to the parental line *fas2^{EP}*, the cross exhibits a

significant increase in response to both cAMP and drosokinin at all time points except 40 min. However when compared to the UO parent, the cross is only significantly different at time points 20, 50 and 60. These experiments were carried out several months after the initial secretion assay experiments and it may be possible that the stock of cAMP was slightly degraded, resulting in an overall decrease in cAMP response. It is also possible that in these experiments UO was expressed at a lower level resulting in a decreased level of *fas2* over expression. As can be seen in Figure 5-8 the expression of UO in cells is not consistent throughout the tubule and indeed both temperature and feeding patterns of the flies will affect its expression. These experiments highlight the importance of day-to-day and fly batch variance in secretion assay experiments. Due to the decrease in response seen at this stage, new cAMP was ordered and previous experiments repeated in order to confirm the heightened response to cAMP in tubules over expressing *fas2*. Results indicated that secretion phenotypes seen in 5.4.3 were indeed a true phenotype. Unfortunately, time did not permit the repeat of the above experiment. Although slightly variable these results indicate that drosokinin does indeed have an additive effect on increased secretion response to cAMP, in tubules over expressing *fas2*. We would therefore argue that *fas2* acts upstream of cAMP in order to directly or indirectly increase the recruitment and activation of V-ATPase.

5.4.5 *Expression of only the extracellular domain of fas2 results in increased response to cAMP when compared to controls*

Previous studies have suggested that the extracellular domain of *fas2* is sufficient to cause accumulation of *fas2* at the NMJ (Kohsaka et al., 2007b). These results were of great interest as they suggest that interactions with intracellular proteins, such as Dlg, are not required for the accumulation of Fas2 at the NMJ. These results also indicated the importance of homophilic cell adhesion, as the expression of Fas2 at the postsynaptic region directly effected the accumulation of Fas2 at the presynaptic region (Kohsaka et al., 2007b). As the extracellular domain contains the PDZ binding domain of Fas2 it is not surprising that it is responsible for cell adhesion and targeting. In order to determine if the extracellular domain of Fas2 is responsible for the increased secretion response of tubules over expressing Fas2,

secretion assays were carried out as before. We utilised the line $Fas2^{extraYFP}$ (kindly provided by Prof. Nose lab) in order to over express the extracellular and the transmembrane domain of Fas2. These lines express fas2 extracellular domain tagged to YFP. As can be seen in Figure 5-8, $Fas2^{extraYFP}$ localises to the apical brush border as expected, suggesting that the lack of the full intracellular domain does not affect localisation.

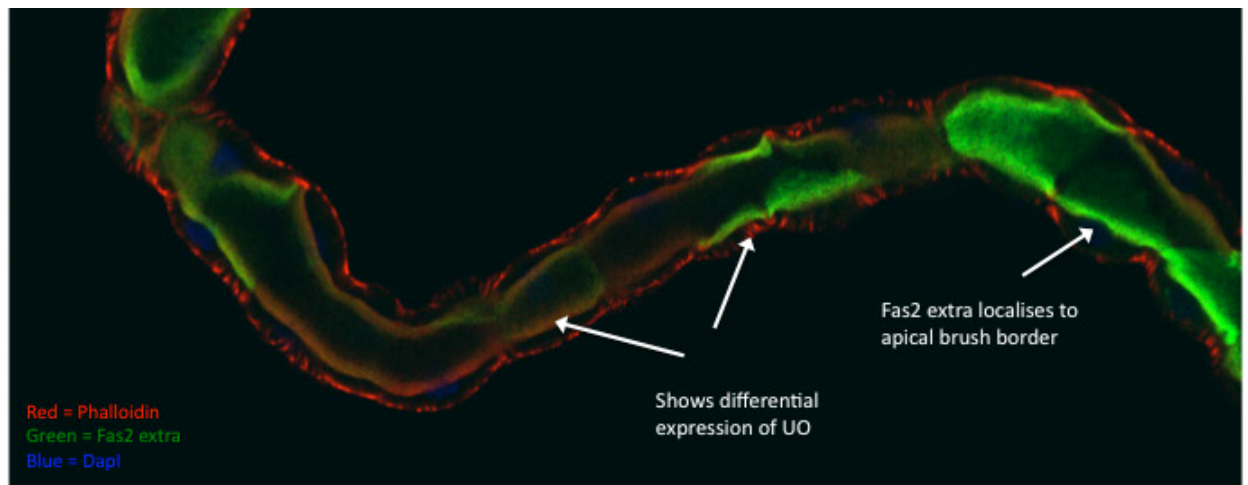


Figure 5-8 Localisation of $Fas2^{extraYFP}$ when driven with UO

$fas2^{extraYFP}$ was driven with the UO driver. The lack of a full intracellular domain does not affect the localisation of $Fas2^{extraYFP}$. This figure also indicates the differential expression levels of UO as evident by differing levels of YFP. Flies reared at 26°C

This figure also indicates the differential levels of UO expression in different cells of the main segment, as indicated by differing levels of $fas2^{extraYFP}$ over expression. We can also see that areas where *fas2* is more highly expressed appear to have denser microvilli.

5.4.6 Secretion response to *FasEXTRA* over expression

As $fas2^{extraYFP}$ localises to the apical brush border as expected, secretion assays were then carried out. As previously, $fas2^{extraYFP}$ was driven with the principal cell driver UO. Results obtained can be seen in Figure 5-9. These results indicate that only the extracellular domain of Fas2 is required to elicit a significant increase in fluid secretion rate in response to cAMP, when compared to parental controls. Although predicted due to evidence from previous studies, these results are somewhat

surprising. Figure 3-10 indicates the predicted position of Fas2 within the apical microvilli. As the extracellular domain is likely to be positioned within the lumen, it is surprising that this domain has an effect on fluid secretion. As cAMP is taken up by the tubules it acts to increase fluid secretion through several mechanisms within the cell. We would therefore expect proteins within the cell to affect fluid secretion rate. We will, however present evidence that Fas2 acts through a feedback mechanism in order to increase fluid secretion. It also interesting to note that the percentage increase in fluid secretion is again substantially lower than that seen when over expressing Fas2^{EP}. This again highlights that over expression of more than one isoform of Fas2, as in Fas2^{EP}, results in a more pronounced phenotype than the over expression of only one isoform, as seen in Fas2^{extraYFP} and Fas2^{YFP}.

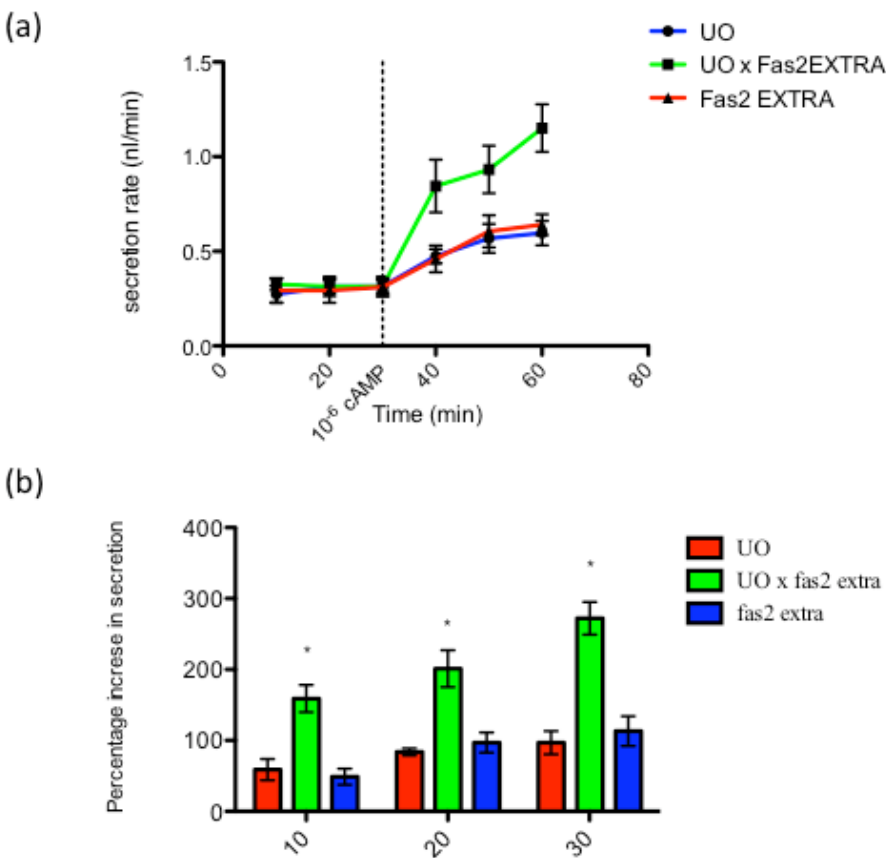


Figure 5-9 Effect of over expression of fas2^{EXTRA} on the secretion rate of Malpighian tubules in response to cAMP stimulation

Tubules were dissected from 7-day-old UO, Fas2^{EP} and UO x Fas2^{EP} lines and basal secretion was measured for 30 min before the addition of 10⁻⁶ M cAMP. Secretion rate was then measured for a further 30 min. Af (a) Indicates secretion curve of three sets of tubules. (b) Indicates percentage increase in fluid secretion at each time point after addition of cAMP. For each time point the tubules from the cross show a significant increase in percentage increase when compared to parental lines. Figure represents typical secretion assay, assay repeated 3 times with ~9 tubules per assay. P values are indicated in table below statistical analysis = unpaired t Test * indicates where value is significant (P<0.05) when compared to BOTH parental lines.

	P Value		
	10 min	20 min	30 min
UO parent vs cross	0.0039	0.0064	0.0002
Fas2 Extra parent vs cross	0.0038	0.0270	0.0014
Parental vs parental	0.6271	0.3675	0.5510

5.5 Possible signalling mechanism for fas2 induced fluid secretion

5.5.1 Introduction

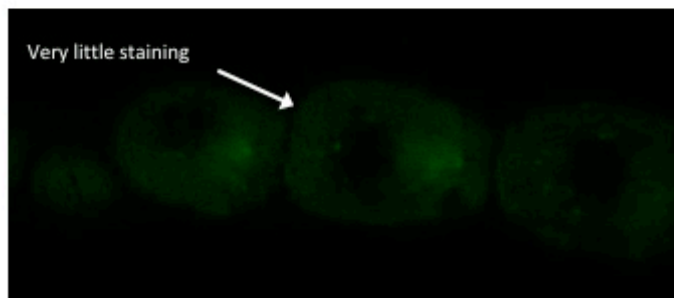
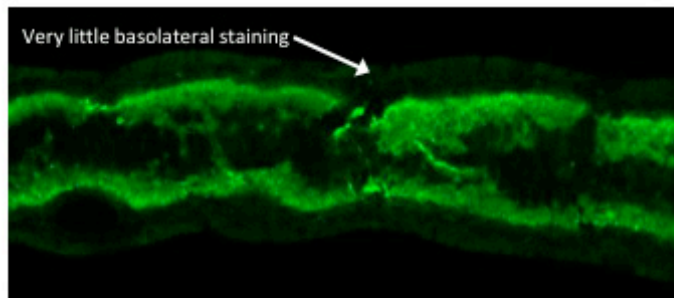
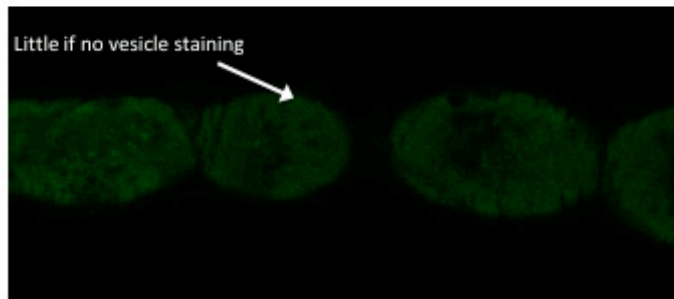
We have presented evidence here that *fas2* is necessary to induce a stimulated fluid secretion response to cAMP. We have however not determined if this is simply due to microvilli integrity, or if other factors downstream of cAMP involve *fas2*. As we have discussed in previous sections, disruption of *fas2* expression does not lead to significant changes in basal fluid secretion rates, hinting that it may also play another role, other than microvilli stabilisation. Indeed Fas2 has been shown to play an important role as a signalling molecule in the CNS (Mao and Freeman, 2009). It therefore may be possible that Fas2 plays a role in signalling the movement of other proteins to and from the microvilli in response to cAMP. As discussed in previous sections there are, several proteins, which are known to move in response to cAMP, including V-ATPase subunits and Actin (Beyenbach et al., 2009; Bradley and Satir, 1981; Karas et al., 2005). In the CNS, Fas2 is known to actively move away from the NMJ, inducing the movement of other proteins both to and from the NMJ (Beumer et al., 2002). During embryogenesis Fas2 is also known to be crucial in determining polarity, also through its associations with other proteins (Szafranski and Goode, 2004). It therefore may be possible that Fas2 moves away from the microvilli, in response to cAMP and thus allows the movement of actin and/or V-ATPase. In order to test this hypothesis, we utilised the *fas2*^{proteintrap788} line in order to determine if Fas2 protein moves in response to cAMP.

5.5.2 *Fas2 re-localises to the basolateral membrane and back in response to cAMP stimulation*

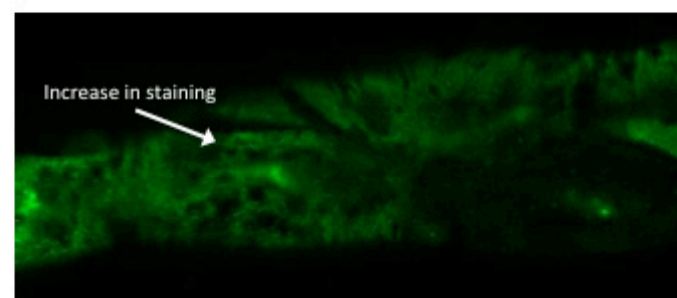
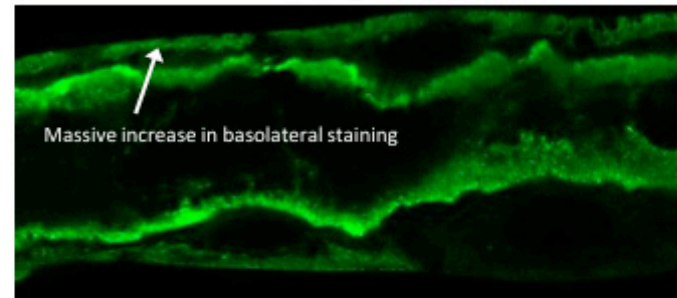
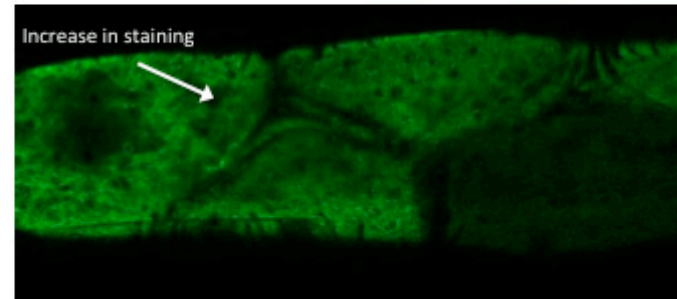
We utilised the *fas2*^{proteintrap788} line in order to visualise Fas2 protein localisation in response to cAMP. Initial experiments were carried out under live conditions, however this did not allow enough time to gather information from every focal plane, meaning that movement on the surface of the tubule may be missed. For

these reasons tubules were incubated for 10, 20, 30, 40, 50 and 60 min intervals in 10^{-6} McAMP or as a control Schneider's medium, fixed and visualised using a Confocal microscope . All samples were prepared on the same day and each image was acquired using the same Confocal settings. For each sample set, a Z-stack was taken in order to visualise the tubules at all focal planes. Each sample consisted of at least 9 tubules. Time however did not permit the imaging of each tubule per set, however the same phenotype was observed in each case. The results can be seen in Figure 5-10.

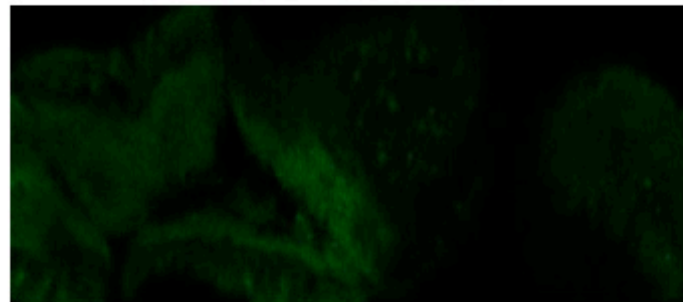
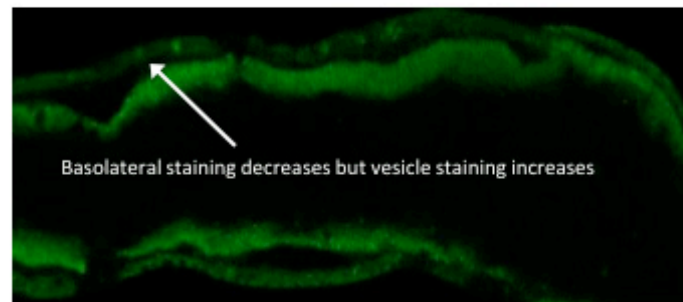
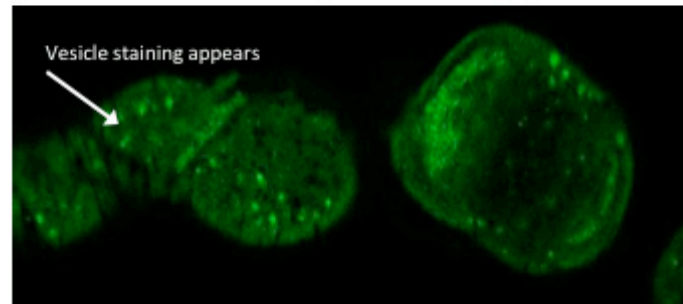
Control



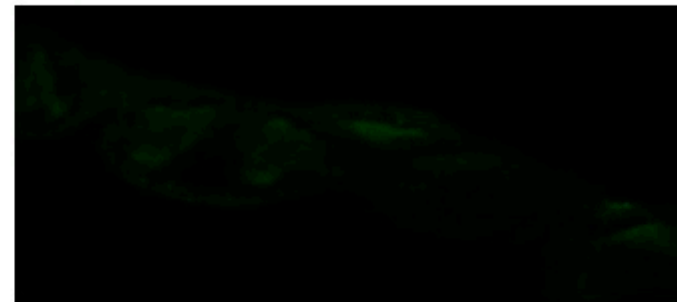
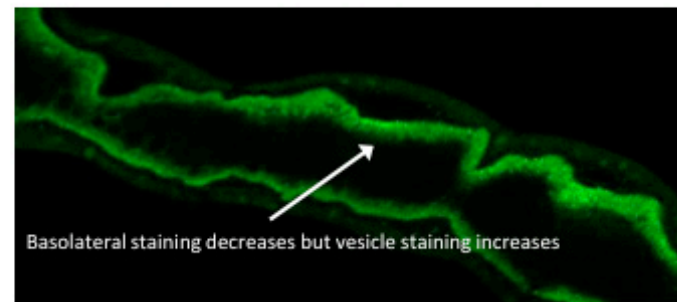
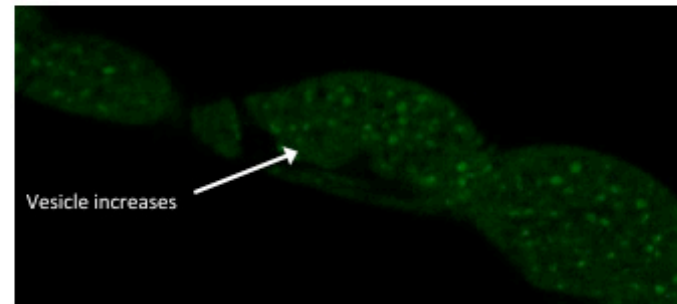
10 minute cAMP stimulation



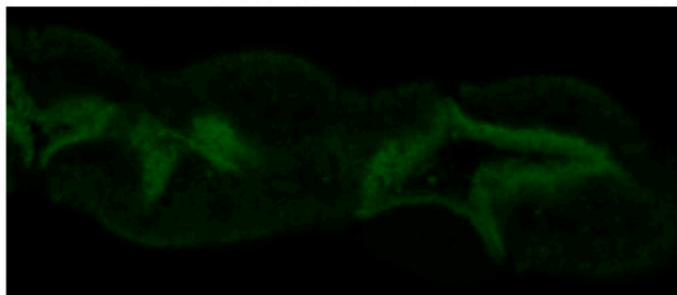
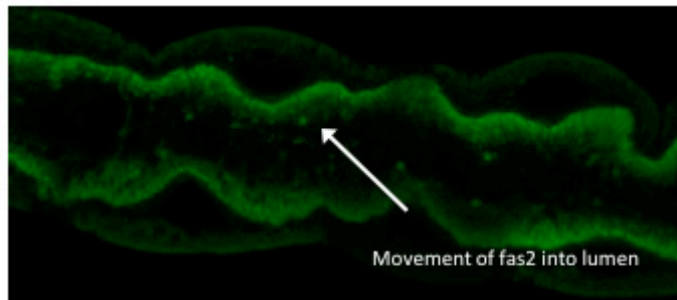
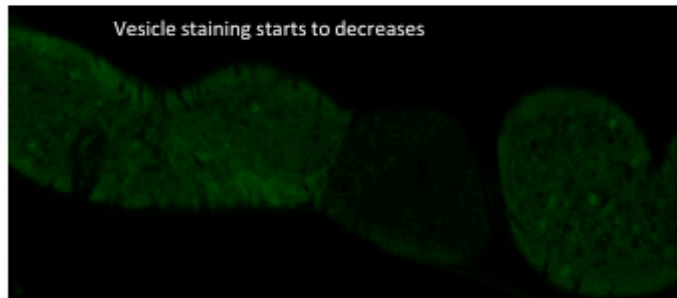
20 minute cAMP stimulation



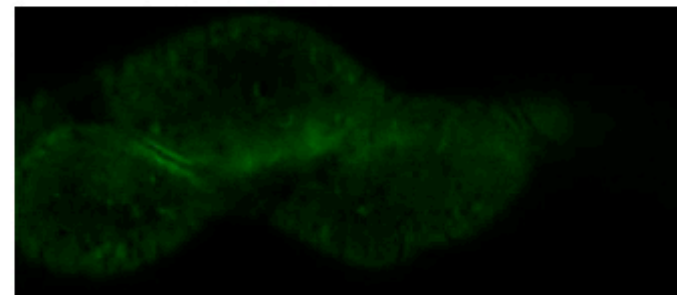
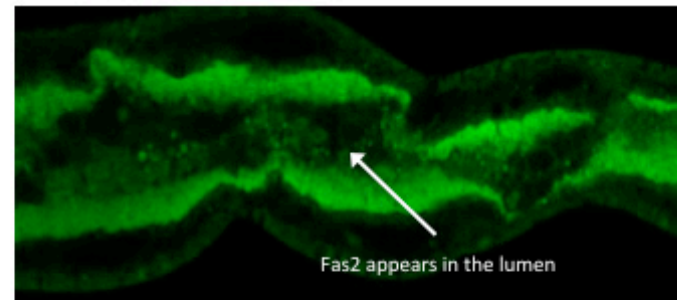
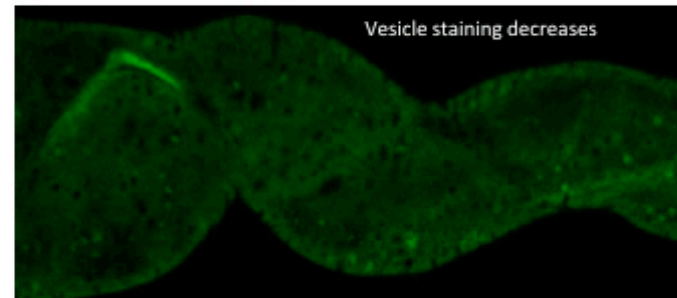
30 minute cAMP stimulation



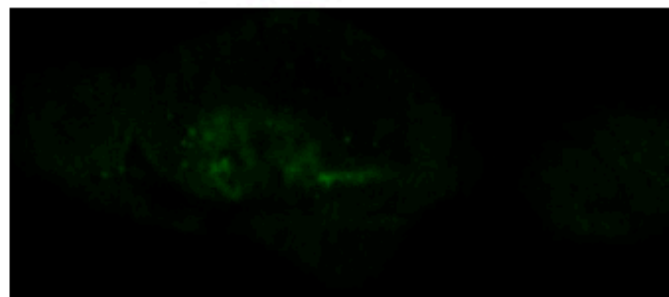
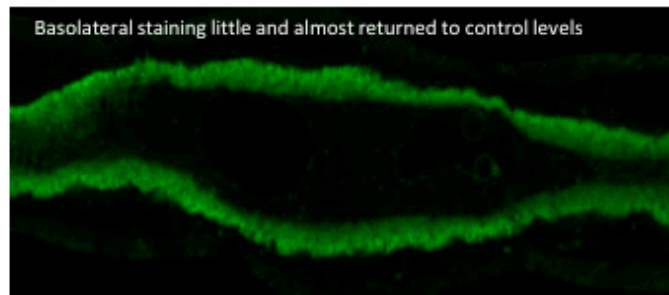
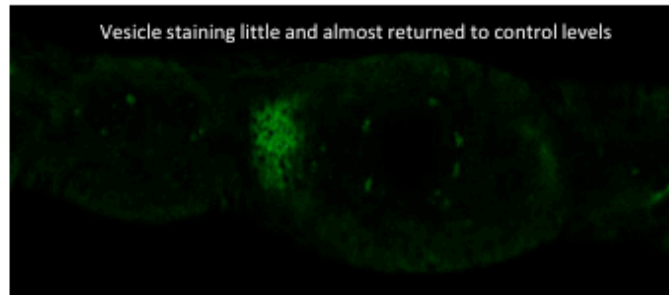
40 minute cAMP stimulation



50 minute cAMP stimulation



60 minute cAMP stimulation



Control

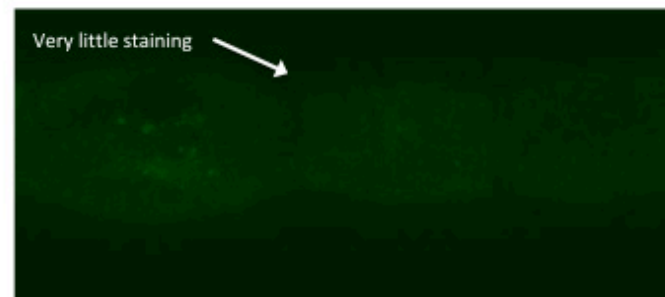
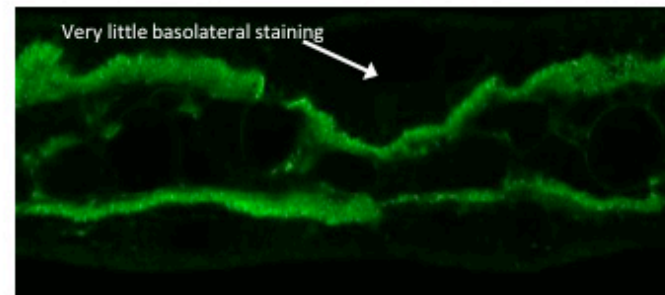
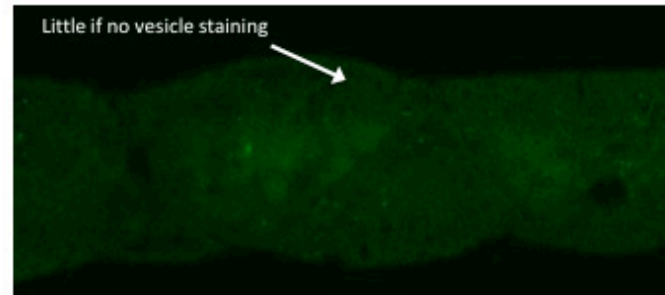


Figure 5-10 Localisation of Fas2 in the tubules in response to cAMP stimulation

Tubules were dissected as described in Section 2.12.2. They were subjected to incubation with 10^{-6} cAMP in Schneider's medium or Schneider's medium as a control, for the following time points 10, 20, 30, 40, 50, & 60 min. Tubules were then fixed and mounted before being visualised using a confocal microscope. All images were acquired using the same settings and experiments were carried out on the same day. Controls for each time point showed no difference when compared to each other and first time point tubules. Each panel indicates a top (basal cortex), middle (lumen and apical brush border) and bottom (basal cortex) slice of the tubule.

The results indicate that Fas2 movement is indeed induced by cAMP stimulation. After 10 min, Fas2^{Proteintrap 788} becomes much higher at the basal membrane than in control tubules. Fas2^{Proteintrap 788} is also increased at both surfaces of the tubule when compared to control. There also appears to be less Fas2^{Proteintrap 788} at the apical microvilli, suggesting that Fas2^{Proteintrap 788} has been internalised and moved to the basal membrane. After 20 min of incubation Fas2^{Proteintrap 788} starts to appear in vesicles along the surface of the tubule and the basal membrane. By 30 min vesicle number has increased (as assessed by manual counting of vesicles) and Fas2^{Proteintrap 788} appears to move back towards the apical brush border. After 40 min of incubation, little Fas2^{Proteintrap 788} is seen at the basal membrane and vesicles appear to fuse with the apical brush border. Fas2^{Proteintrap 788} in Vesicle also starts to appear in the lumen of the tubules suggesting that Fas2^{Proteintrap 788} is being secreted into the lumen or the degradation of Fas2^{Proteintrap 788} leads to YFP appearing in the lumen. By 50 min, luminal Fas2^{Proteintrap 788} has increased and very little is seen at surface of the tubules. By 60 min, Fas2^{Proteintrap 788} is only present at the apical brush border and staining resembles that of control tubules. These results may indicate a 'signalling' role for Fas2 in response to cAMP movement. Intriguingly, a comparison of these results with secretion assay results, also indicate a signalling role. After a 10 min cAMP incubation tubules appear to show no significant increase in fluid secretion in tubules where fas2 is over expressed (Figure 5-6). If we compare this to the results seen in Figure 5-12, where after 10 min Fas2^{Proteintrap 788} has moved to the basal membrane, it suggests that the movement back to the apical brush border is more important for an increased fluid secretion rate. It is therefore possible that Fas2 may play an important role in trafficking a protein or proteins back to the apical brush border. Several studies indicate the importance of Fas2 as a 'signalling' protein in the nervous system, whereby the movement of Fas2 to and from the synapse or neural muscular junction leads to the signalling of other proteins to move towards or

away from the junction (Beumer et al., 2002; Kohsaka et al., 2007a; Szafranski and Goode, 2004). Therefore Fas2 acts as a signal for the accumulation or disassociation of other proteins either via cell adhesion or protein/protein interactions.

As we can see from Figure 5-10 Fas2^{Proteintrap 788} appears to be secreted into the lumen of the tubules after 50 min incubation with cAMP. As it would appear that Fas2 is important for the stabilisation of microvilli and it is present at all times, we would expect Fas2 which has been secreted, to be replaced. Indeed most proteins that are involved in trafficking are often recycled once used. We therefore determined the mRNA levels of *fas2* after a 3hr stimulation with cAMP, at three different concentrations. Results can be seen in Figure 5-11

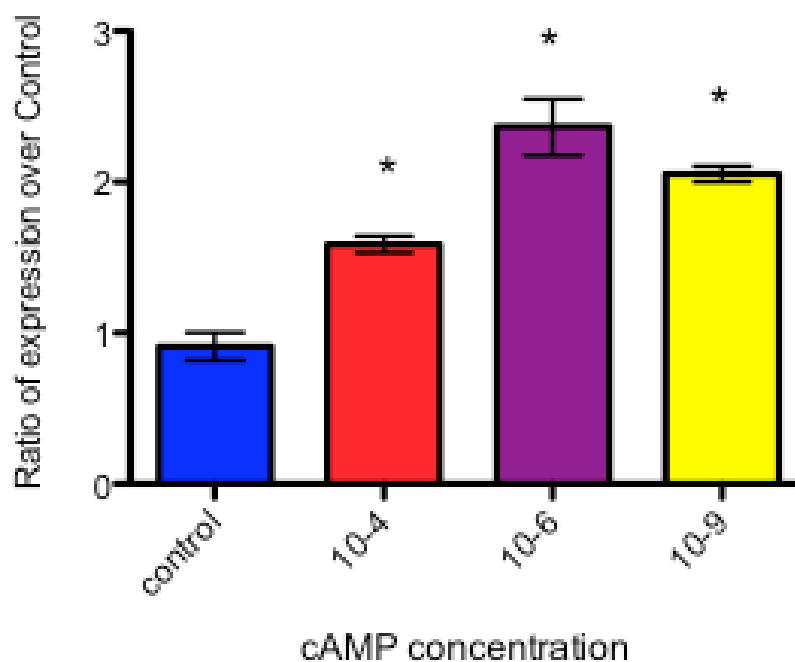


Figure 5-11 Comparison of *fas2* expression in response to different concentrations of cAMP
Tubules from 7-Day-old Canton S were incubated for 3hr in varying concentrations of cAMP. Results are expressed as ratio compared to control, where control = 1. *Fas2* expression significantly increases in a dose dependent manner. P values are as follows: 10⁻⁴M p = 0.0245; 10⁻⁶M = 0.0196; 10⁻⁹M = 0.0085. N=4 mRNA levels were measured by QRT-PCR as described in Section 2.5.4

These results indicate that *fas2* expression in the tubules is increased in response to cAMP, in a dose dependent manner. We can therefore conclude that *fas2* may play a role in trafficking, as it appears to be up regulated in response to cAMP, suggesting the need to replenish protein concentrations. Although these results differ from those of the secretion assay seen in Figure 5-2, where there is no significant secretion response between 10^{-6} M 10^{-4} M cAMP, this does not undervalue these results. The main reason for this is that during secretion assay stimulation the tubules are exposed to cAMP for 30-40min and measured. In the case of the above experiment the tubules are exposed for 3 hr. It therefore may be possible that tubules exposed to higher concentrations of cAMP for a longer time require more Fas2 production in order to stabilise the microvilli and continue high levels of secretion. It would therefore be important to repeat these experiments in order to compare the results, by using similar time points. However time did not permit these experiments.

5.5.3 **Conclusions**

The results presented in this section suggest that Fas2 may play an important signalling or trafficking role in response to cAMP stimulation in the tubules. These results are intriguing, as we know that V-ATPase subunits are recruited to the apical brush border in response to cAMP stimulation (Beyenbach et al., 2009; Dames et al., 2006; Rein et al., 2008b). The vesicle staining seen in Figure 5-10, therefore may represent the movement of V-ATPase subunits back to the apical brush border along with Fas2. Interestingly a recent proteomics study suggested that Fas2, may interact with several Vo subunits of V-ATPase (Rees et al., 2011). Although we were able to show that Fas2 co-localises with V-ATPase in un-stimulated tubules, time constraints meant that this could not be studied further with regards to cAMP stimulation (Figure 5-12).

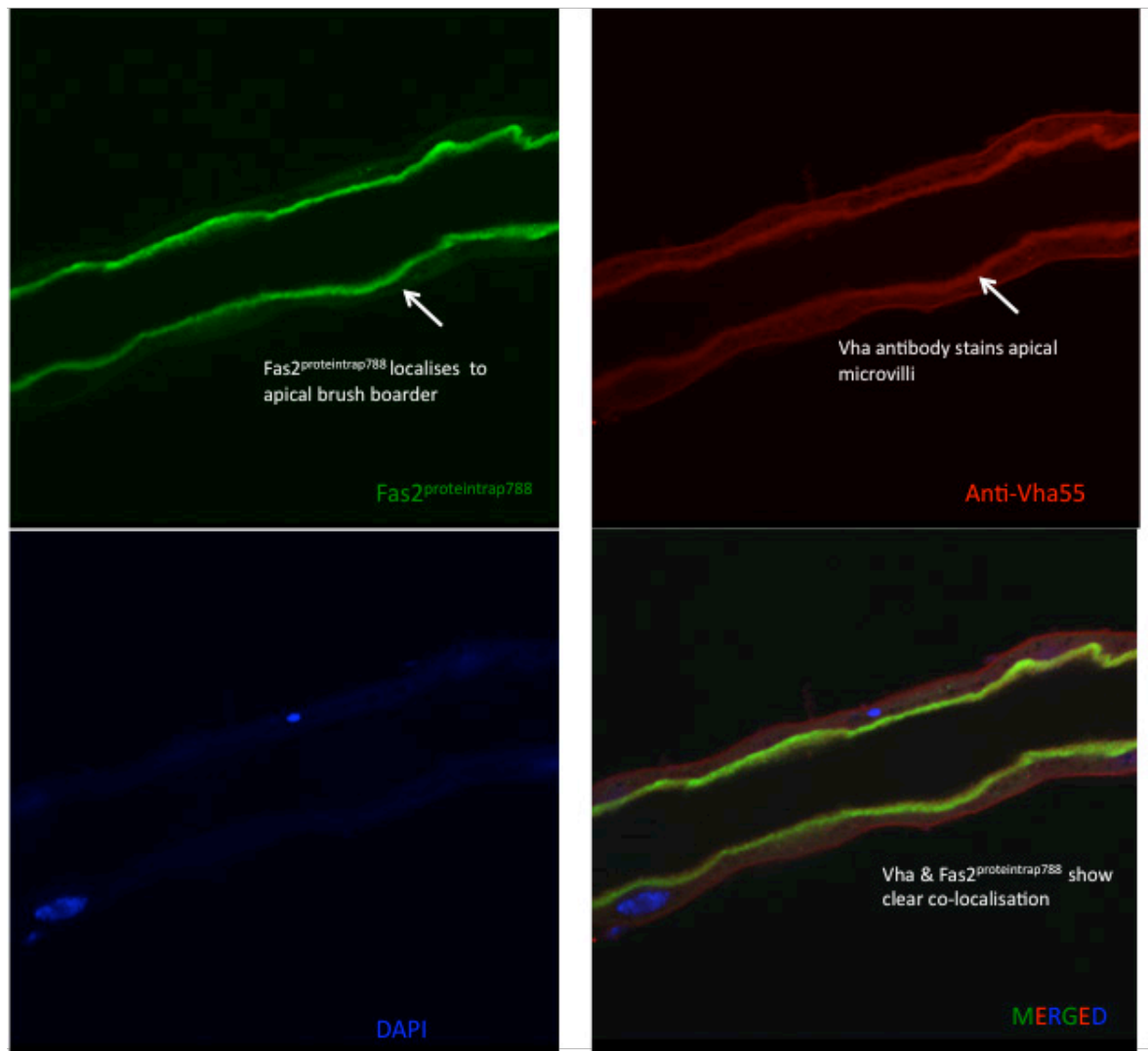


Figure 5-12 Co-localisation of Fas2 & V-ATPase

Tubules were dissected from 7-day-old Fas2^{proteintrap788} flies and ICC was carried out as described in Section 2.12.3 using an antibody against the Vha55 subunit of V-ATPase. Image shows each channel separately and merged. Clear co-localisation of Fas2 and Vha55 is seen at the apical brush border

At present these results only hint at a role for Fas2 in ‘signalling’ or trafficking and several key experiments are required in order to establish if this is the case. For example experiments such as those carried out in Chapter 6 would be beneficial to determining Fas2 interacting partner, both during stimulated and un-stimulated conditions. At present we can only conclude that cAMP cause dynamic changes in Fas2^{Proteintrap 788} localisation, resulting in an increase in secretion rate.

5.6 Association of the Actin cytoskeleton and fluid secretion

5.6.1 Introduction

Another possible mechanisms, by which Fas2 induces fluid secretion is an interaction with the actin cytoskeleton. We have shown that actin appears to increase at the apical brush border when *fas2* is over expressed in the principal cells of the tubule. Other studies have indicated the re-arrangement of the actin cytoskeleton is important for normal fluid secretion response to cAMP (Karas et al., 2005). We therefore studied the association of F-Actin with fluid secretion and its interactions with Fas2. Several studies in mammalian systems have hinted at the importance of the actin cytoskeleton, in recruitment of mitochondria and V-ATPase to the microvilli (Paunescu et al., 2010).

5.6.2 *Fas2 co-localises with F-actin after 10 min stimulation with cAMP*

In order to determine if Fas2 movement in response to cAMP stimulation may involve the movement of F-Actin as well, the experiment carried out in Section 5.5.2 was repeated with the addition of Phalloidin staining, in order to visualise F-Actin localisation. Unfortunately due to the breakdown of the confocal microscope these experiments could not be completed. However preliminary results indicate that after a 10 min incubation with cAMP Fas2^{proteintrap788} re-localises and co-localises with F-Actin filaments at the basal cortex of the tubule. These results can be seen in Figure 5-13.

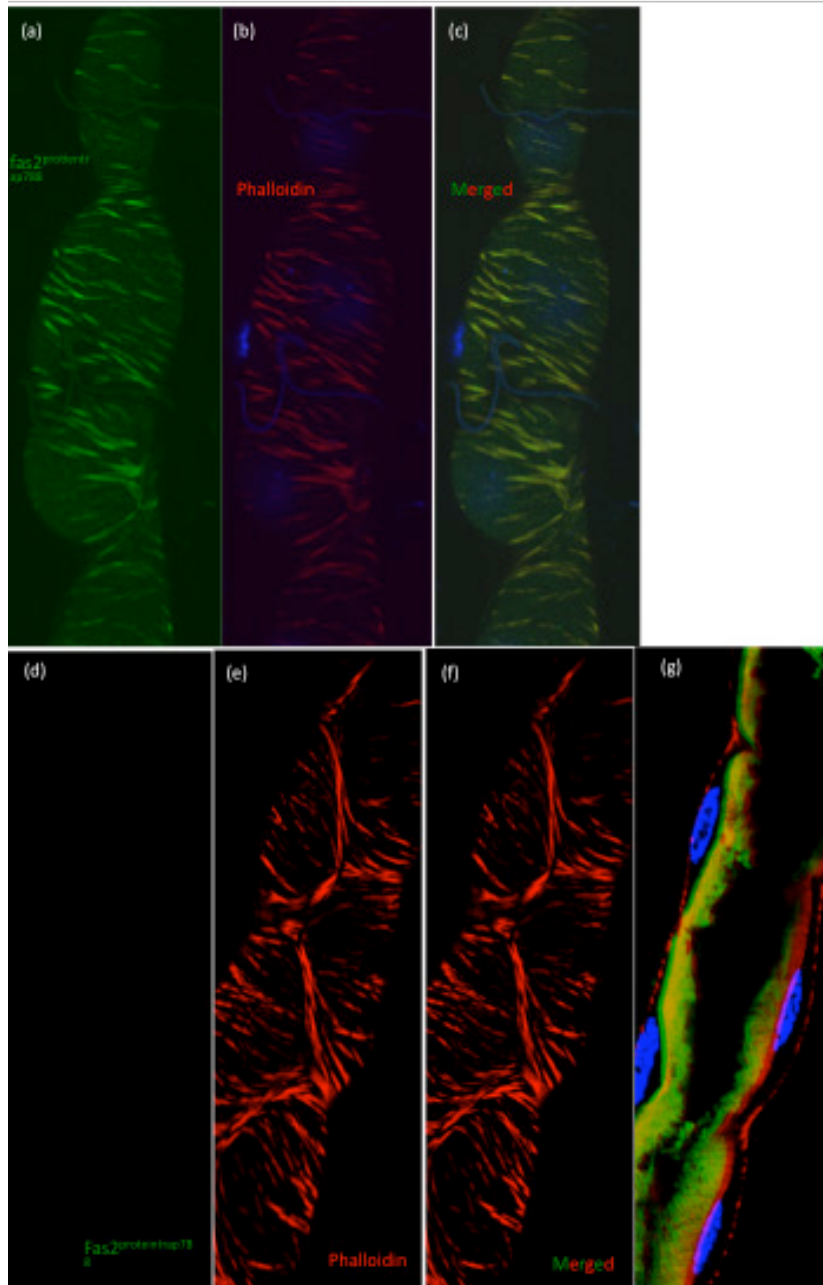


Figure 5-13 Localisation of Fas2^{proteintrap788} and F-Actin in response to cAMP stimulation for 10 min

Tubules were dissected and incubated for 10 min in 10^{-6} M cAMP., fixed and mounted. (a-c) Represent incubated tubules where green = fas2^{proteintrap788} red= Phalloidin and yellow = Co-localisation. (d-f) are un-stimulated tubule expressing fas2^{proteintrap788} and stained with phalloidin. No Co-localisation is seen in the control tubule, but fas2 and F-actin clearly co-localise when stimulated with cAMP. (g) mid section of tubule shown in (d-f) in order to show Fas2^{proteintrap788} is present in the tubule Each image shows the basal cortex of the tubules.

5.6.3 *Actin de-polymerization and polymerization are necessary for normal secretion phenotype.*

Previous studies have indicated the importance of actin polymerisation and de-polymerisation in order to elicit a normal secretion phenotype. In response to cAMP or a blood meal, mosquito tubules re-organise their actin cytoskeleton. It has been suggested that this allows the movement of V-ATPase and mitochondria into the microvilli (Bradley and Snyder, 1989; Karas et al., 2005; Paunescu et al., 2010). Incubation with the F-Actin inhibitor Phalloidin led to no marked decrease in basal secretion in mosquito tubules. However there was a decrease in secretion response to cAMP. Cytochalasin D, which initiates de-polymerisation of F-actin, in contrast has both a reduced basal and cAMP stimulated secretion rate when compared to controls. In order to establish if this is the case in *D.melanogaster* tubules, secretion assays were carried out as before with the addition of Cytochalasin D and Phalloidin. The result can be seen in Figure 5-14

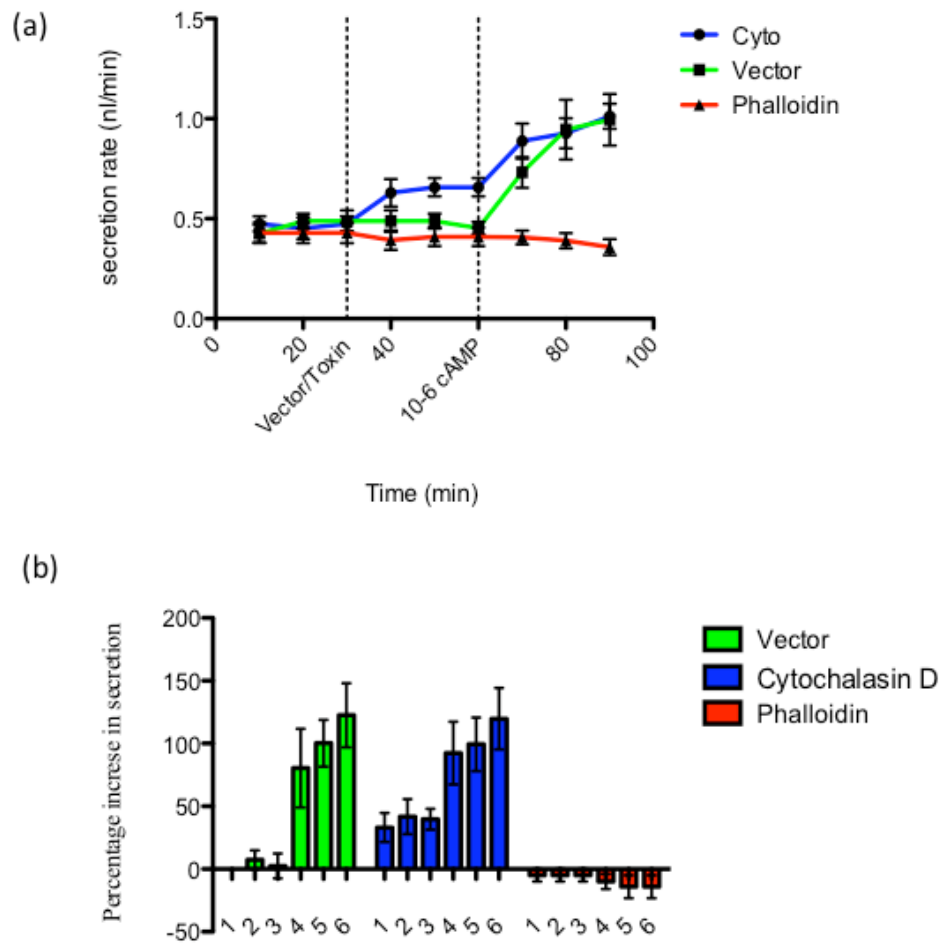


Figure 5-14 Effect of Cytochalasin D and Phalloidin on basal and cAMP induced fluid secretion

Tubules were dissected from 7-day-old Canton S and basal secretion was measured for 30 min before the addition of Cytochalasin D, phalloidin or PBS+DMSO. Secretion rate was then measured for a further 30 min. After 60 min 10^{-6} M cAMP was added and secretion measured for a further 30 min. (a) Indicates secretion curve of three sets of tubules. (b) Indicates percentage increase in fluid secretion at each time point after addition of Cytochalasin D, phalloidin or Vector. Cytochalasin D appears to increase basal secretion and has no significant effect on cAMP induced secretion. Phalloidin however, significantly reduces both basal and stimulated response. The addition of the vector solution (PBS + 0.001% DMSO) had no effect on fluid secretion. Figure represents typical secretion assay, assay repeated 3 times with ~9 tubules per assay. P values are indicated in table below statistical analysis = unpaired t Test. * indicates where value is significant when compared to BOTH parental lines ($p < 0.05$)

	P-value				
	10	20	30	40	50
Cyto vs control	0.0462	0.0616	0.0205	0.7740	0.9768
Phalloidin vs control	0.3739	0.2028	0.5219	0.0223	0.0006

Our results indicate that Phalloidin has both a significant effect on basal secretion and cAMP stimulated secretion. This result is consistent with the hypothesis that F-Actin filaments, de-polymerises in response to cAMP, in order for the actin cytoskeleton to re-organise and aid the recruitment of mitochondria and V-ATPase subunits to the microvilli. As Phalloidin inhibits this process it is not surprising that both basal and stimulated secretion rates are lowered. These results are somewhat similar to those found in mosquito tubules, but differ in that basal secretion is increased in response to Cytochalasin D and there is no apparent effect on stimulated response. This however may be due to differences in experimental procedure and must be repeated. It is possible that the initial treatment with Cytochalasin D results in a toxic response by the tubules, thus forcing an increase in fluid secretion to clear the tubules of the toxin. Further experiments would also included the staining of the tubules with Phalloidin after incubation in each of the above conditions, in order to assess the localisation and form of the F-actin i.e. polymerised or de-polymerised. It would also be prudent to repeat these experiments in tubules both over expressing and down regulating Fas2 in order to determine if Fas2 plays a role in actin re-organisation.

5.7 Discussion

In this chapter we discuss the effect *fas2* expression has on the secretion response of the tubules to cAMP. Initial experiments determined the wild type response to cAMP and dbcAMP. These experiments indicated that there was little difference in secretion response between cAMP and dbcAMP. We also established that a concentration of 10^{-6} M cAMP was adequate to elicit a response in wild type tubules.

Utilising the lines discussed in Section 5.3.1, secretion assays were carried out in order to determine if *fas2* expression affects the tubules secretion response to cAMP. The marked changes in microvilli structure and actin localisation, suggest that it may. Knocking down expression of *fas2* resulted in a significant decrease in stimulated fluid secretion rate but not basal secretion. The line *fas2*^{EB112} showed a slightly decreased basal rate and a decreased stimulated response.

Conversely, over expression of *fas2* dramatically increased the stimulated secretion rate, but had no effect on basal secretion.

Further analyses indicated that Fas2 is actively removed from the apical brush border in response to cAMP, and rapidly accumulates at the basal membrane, before moving back to the apical membrane in vesicle structures. In addition it would appear that this movement coincides with co-localisation with F-Actin bundles as shown in Figure 5-12.

These findings suggest that Fas2 is not only involved in microvilli development and stabilisation, but also may play a 'signalling' and/or trafficking role in response to cAMP. This is evident by the dynamic localisation of Fas2 in response to cAMP. Evidence from the neural muscular junction suggests that the movement of Fas2 away from the membrane, in response to cAMP increases, 'signals' the movement of other proteins either to or from the NMJ (Beumer et al., 2002; Kohsaka et al., 2007a). Results presented here suggest that there maybe a similar process occurring in the tubules. In particular we have presented evidence that this involves the co-localisation of Fas2 with F-Actin. It therefore is possible that the microvilli phenotype is in response to an increase or decrease in actin localisation to the microvilli. F actin and indeed the actin cytoskeleton in general has been indicated to play a role in both mitochondria and V-ATPase, recruitment and activation, and is therefore likely to be vital to stimulated fluid secretion (Bradley and Snyder, 1989; Dames et al., 2006; Karas et al., 2005; Ryerse, 1979). Further analysis of proteins involved in actin de-polymerisation and polymerisation would be beneficial in studying this phenomenon. Analysis of the response of Fas2-B and Fas2-A would also have been significant, as it is unclear if both isoforms move in response to cAMP or just one. Time however did not permit these experiments. Most importantly, determining if other proteins are moving with Fas2 or not. We have shown that there is a movement of Fas2 to localise with F-Actin bundles at the basal cortex of the tubule, but there is a possibility that other proteins, such as V-ATPase subunits are also present.

Figure 5-14 represents the final model for Fas2 function. In brief, Fas2 is localised to the apical brush border and co-localises with F-Actin. In response to cAMP Fas2 is internalised and moves to the basolateral membrane. During this

time Fas2 may also initiated the depolymerisation of F-Actin allowing for the movement of V-ATPase subunits and/or mitochondria into the microvilli. Fas2 then moves back to the apical brush border, either alone or in complex with other proteins (e.g. actin or V-ATPase subunits), where it is secreted into the lumen or reinserted into the microvilli. When fas2 is depleted this results in shorter less dense microvilli and less F-Actin localisation. There is in turn less Fas2 to be internalised, possibly resulting in less actin depolymerisation and thus less movement of V-ATPase/mitochondria, resulting in a decreased secretion response. Alternatively less fas2 results in less protein complexes returning to the microvilli again resulting in a lowered secretion response. The opposite is seen when Fas2 is over expressed. The following lists the outcomes of this study for which we have presented good evidence of the hypothesis present in Figure 5-14

- (a) We have shown that Fas2 has differential localisation in embryos compared to larval and adult tubules
- (b) Fas2 is involved in microvilli development/stabilisation (although further analysis and quantification required)
- (c) Fas2 depletion or over expression results in significant changes to the cAMP induced secretion response, most probably due to the affect on microvilli

The following hypotheses are based on limited data and preliminary data:

- (a) Fas2 is internalised in response to cAMP stimulation. It then re-localises to the basal cortex where it co-localises with F-Actin bundles. Fas2 then moves back to the apical brush border. We hypothesises that this may suggest a further role for Fas2 in which it actively moves other proteins to or from the apical brush border through protein protein interactions or it triggers the movement by reducing the tight binding of microvilli through Fas2-Fas2 interactions. This in turn may trigger the re-arrangement of the Actin cytoskeleton, required for increase fluid secretion. However the evidence presented in this study, is not substantial enough to prove or disprove this hypothesis and further experiments are required.

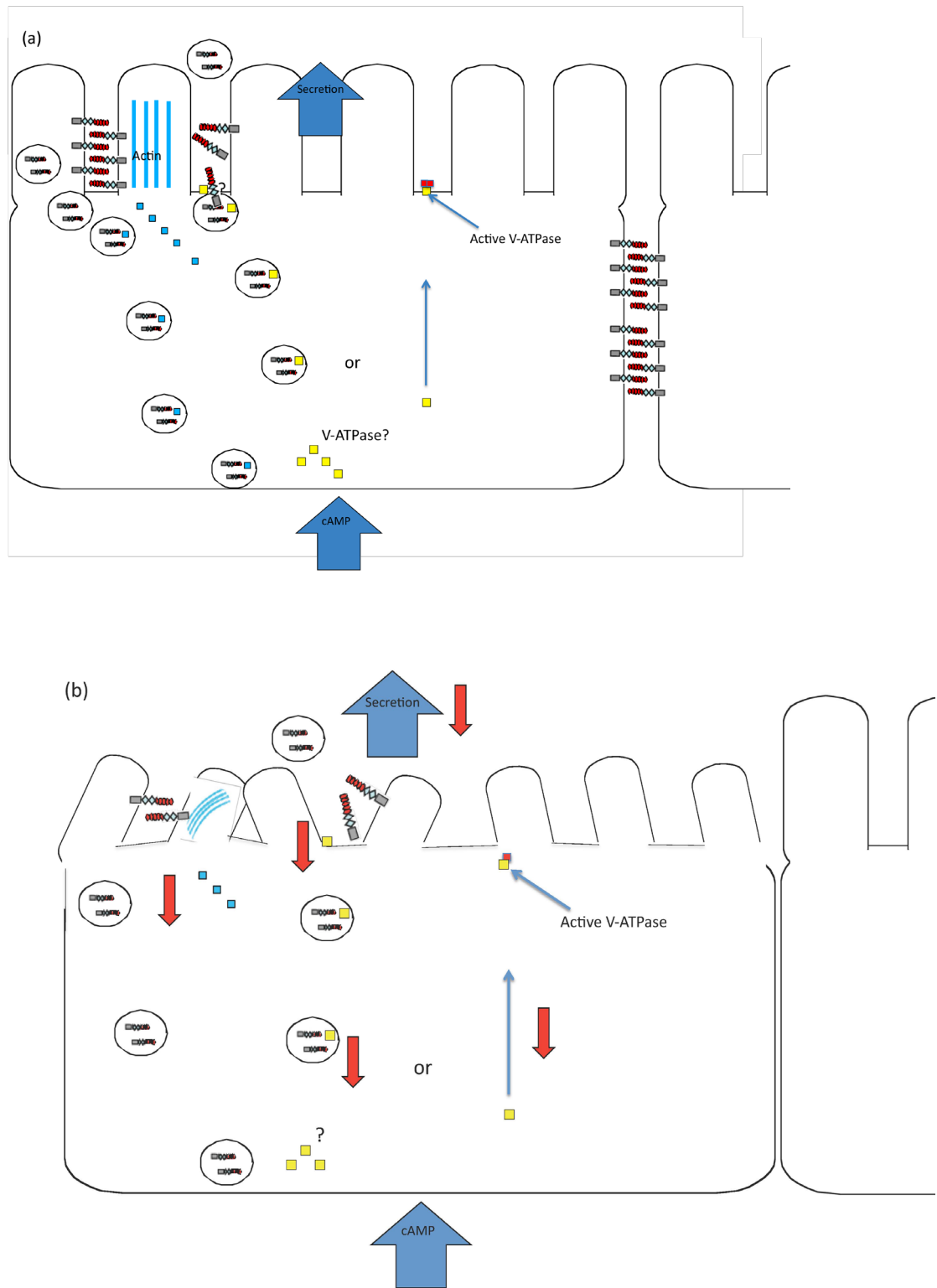


Figure 5-15 Model of Fas2 localisation and function 3

During embryogenesis Fas2 is localised to the lateral membrane between cells (a) In response to cAMP Fas2 is internalised and moves to the basolateral membrane. During this time Fas2 may also initiate the depolymerisation of F-Actin allowing for the movement of V-ATPase subunits and/or mitochondria into the microvilli. Fas2 then moves back to the apical brush border, either alone or in complex with other proteins (e.g. actin or V-ATPase subunits), where it is secreted into the lumen or reinserted into the microvilli. (b) When Fas2 is depleted this results in shorter less dense microvilli

Chapter 6

6 BN-PAGE analysis

6.1 Summary

The last decade has seen a marked increase in the number of proteomic studies, where high-throughput techniques are employed in order to analysis global protein expression. Before the advent of high-throughput techniques, scientists relied on antibody experiments, such as Western blot analysis, immunocytochemistry (ICC), immunoprecipitation (IP) and co-immunoprecipitation (coIP). These techniques are extremely robust and can give valuable insights into protein/protein interactions (O'Farrell, 2008). However in conjunction with large-scale proteomics work we can gain a clearer picture of the global proteomics of a cell or tissue. This Chapter looks at the application of sucrose gradients for membrane separation and the optimisation of 2D Blue-native PAGE in order to detect protein/protein complexes.

6.2 Co-Immunoprecipitation and membrane proteins

In order to understand the function of a protein it is often beneficial to determine the proteins it interacts with. Indeed all mechanisms in biology are essentially controlled by which proteins interact with each other. Traditional techniques involve the use of antibodies in order to ‘pull-down’ protein complexes from a given sample. Immunoprecipitation (IP) is a technique whereby antibodies are bound to beads (covalently or not), these beads are then added to a given protein sample, where the antibody then attaches to the desired protein. Through a series of centrifugations, all other proteins are eluted and your desired protein is left bound to the beads. Under particular lysis conditions protein complexes can be left intact, allowing for the pull down of proteins along with your bait. The use of coIPs however is not without its problems. Membrane protein complexes can be particularly difficult to coIP, as harsh lysis conditions are required to remove proteins from the membrane, often leading to the disruption of complexes (Sambrook and Russell, 2006). A second drawback to coIP experiments is the need for a good antibody against at least one of the proteins in the complex. This can be problematic if for example you wish to determine novel interacting proteins, if an antibody is not available for your protein of interest (Santoni et al., 2000).

6.3 Two dimensional (2D) gel electrophoresis

2D gel electrophoresis was first developed in the 1970s (Klose, 1975; MacGillivray and Rickwood, 1974; O’Farrell, 1975). However the use of 2D gels did not reach its peak until the development of microanalytical techniques, to analyse the low levels of protein found in 2D gels. The first such technique was Edman sequencing (Aebbersold et al., 1987; Matsudaira, 1987; Rosenfeld et al., 1992), however this was quickly surpassed by the development of mass spectrometry (MS) (Cottrell, 1994; James et al., 1994; Yates et al., 1993). MS has since become an extremely sensitive technique with the ability to detect single post-translational modifications within a given protein (Gorg et al., 2000; Gorg et al., 2004; Wilkins et al., 1999). Traditional 2D gel electrophoresis consists of two separate 1D electrophoresis steps, separating proteins by their isoelectric point (pI) and then by their masses. This involves firstly the

application of isoelectric focusing (IEF) in the first dimension and the application of SDS-PAGE in the second dimension (Gorg et al., 2000). This method is extremely effective, in that it allows for the separation of over 2000 proteins (Gorg et al., 2004). This therefore allows the separation and identification, through MS, of thousands of proteins from a given sample.

6.3.1 *Membrane proteins: 2D gel electrophoresis*

Although now the method of choice, for large-scale proteomic work, 2D gel electrophoresis is not without its problems. In particular, traditional 2D gels have a bias against hydrophobic membrane proteins (Santoni et al., 2000). Indeed the higher the hydrophobicity of a protein, the lower the efficiency of separation by conventional 2D methods. The main reason for this is the mechanisms involved in IEF separation in the first dimension. IEF separates proteins along a pH gradient until they reach a position where their overall net charge is equal to zero (Righetti et al., 1999). By nature proteins are polyampholytes, consisting of both positively and negatively charged molecules. Therefore each protein has a unique pH value, which it will migrate to, defined by its pI. Traditional 2D gels consist of a polyacrylamide matrix which contains an immobilised pH gradient (IPG) (Gorg et al., 2000; Gorg et al., 2004; Righetti et al., 1999). In order for efficient separation to take place in the IPG gel, low levels of ions must be present, because they migrate in the electric field and interfere with the focusing of the polyampholytic proteins in the pH gradient. For this reason lysis buffers used for 2D analysis must not contain highly ionic detergents such as SDS (Braun et al., 2007). There are therefore three main reasons that traditional 2D gels hinder the efficient separation of membrane proteins: (i) membrane proteins require robust lysis buffers, containing ionic detergents, in order to extract them from their lipid bilayer and keep them solubilised in an aqueous solution. The lysis buffer required for 2D IPG gels is therefore less efficient at extracting membrane proteins; (ii) proteins become less soluble at their pI resulting in the aggregation of highly hydrophobic proteins; (iii) once the first dimension is complete, transfer of hydrophobic proteins from the IPG strip to the SDS gel is extremely difficult. It is therefore evident that in order to study membrane proteins another system is required.

6.4 Blue Native PAGE as an alternative to colPs and traditional 2D gel electrophoresis

Recent predictions suggest that up to one third of genes in many organisms encode membrane proteins (Stevens and Arkin, 2000; Wallin and von Heijne, 1998). It is therefore not surprising that the pitfalls of membrane proteomics are beginning to be addressed. The development of 2D blue native gel electrophoresis (BN-PAGE) is just one example of this. Unlike conventional 2D analysis proteins are first separated on a native gel. Protein complexes are therefore kept in their native state. 2D gel analysis, utilising a 1D native gel are not uncommon; however again the separation of hydrophobic membrane proteins is often inefficient, as protein complexes often form aggregates. BN-PAGE electrophoresis overcomes this problem by masking the charge of all proteins with the binding of the chemical Coomassie blue; thus making all protein complexes negatively charged. Importantly the addition of Coomassie blue at low levels does not cause the disassembly of protein complexes (Schagger and von Jagow, 1991). Protein complexes then migrate to the positive electrode according to size and shape (Schagger et al., 1994; Schagger and von Jagow, 1991; Schamel, 2008). Protein complexes therefore migrate intact along the native gel. This method allows for a much higher resolution of membrane protein complexes. After completion of the first dimension protein complexes are then separated under SDS denaturing conditions. Figure 2-3 gives an overview of 2D BN-PAGE electrophoresis. As we can see protein complexes will migrate in a vertical straight line once the 2D has been run, allowing for the identification of all proteins in a given complex. BN-PAGE is therefore the method of choice when wishing to determine membrane protein-protein interactions for the following reasons: (i) membrane complexes can be separated with high resolution in the 1D and (ii) with a good 2D resolution protein complexes can be identified as a vertical line of spots allowing for the identification of proteins via MS, without the need for a working antibody.

6.4.1 *BN-PAGE in the context of this study*

As we have shown in the previous chapters the membrane protein Fas2 is extremely important in microvillar development and stabilisation and for a

normal fluid secretion response to cAMP. We have speculated that Fas2 may interact with both actin and V-ATPase subunits in order to increase the fluid secretion rate of the tubules in response to cAMP. In order to determine if this was indeed the case I initially wished to carry out colP experiments in order to determine which proteins Fas2 interacts with under both stimulated and unstimulated conditions. However as discussed in Section 3.3.3, commercially available antibodies against Fas2 proved to be inefficient in both ICC and Western blot analysis of the tubules. It was therefore concluded that colP experiments in the tubules would also prove difficult with these antibodies. I therefore carried out colP experiments utilising the fas2^{proteintrap788} line and an antibody against GFP. Unfortunately these experiments failed to work and I was unable to purify Fas2 and any interacting partners. All control experiments were carried out and it would appear that antiGFP was unable to bind properly to Fas2^{proteintrap788}, as the protein was often seen in the unbound fraction. This suggests that perhaps the epitope for GFP is unavailable, perhaps shielded by an interacting partner. This may have been overcome with the addition of harsher detergents in order to disrupt protein/protein interactions, but as the purpose of this experiment was to establish the interacting partners of Fas2 this was not carried out. I therefore decided to carry out BN-PAGE analysis in order to determine if it was possible to isolate Fas2 using this technique. Along with this the development and adaptation of BN-PAGE for *D.melanogaster* tissue samples, also offered the chance to characterise other protein complexes within the tubules. In the future this method could therefore be used in order to determine the membrane proteome of the Malpighian tubules.

6.5 Results

6.5.1 *Determination of lysis buffer detergent*

Lysis conditions are extremely important, as we require a detergent that is harsh enough to extract proteins from the membrane but not disrupt protein/protein interactions. Often both the type and concentration of a detergent can prove vital to the success of a proteomic analysis. Effectively, a detergent, which extracts membrane proteins and solubilises them without affecting protein/protein interactions is required. Figure 6-1 summarises the process

involved in extracting and solubilising proteins in non-ionic detergents. Detergents remove proteins from the membrane by intercalating into the phospholipid bilayers and solubilising lipids and proteins (Seddon et al., 2004). Non-ionic detergents are preferred for membrane protein isolation, as they effectively extract the proteins without disrupting protein/protein interactions, whilst solubilising the proteins (Seddon et al., 2004). Importantly the detergent required is dependent on the protein or proteins and the membrane you are working with. Therefore ideally, a large-scale analysis of different detergents would be required in order to determine the best detergent for your experiment. This would include calculating the critical micelle concentration (CMC); that is the lowest concentration at which a detergent forms micelles, for all detergents to be tested. This is important as ideally one would wish to use a detergent at the lowest possible concentration (le Maire et al., 2000).

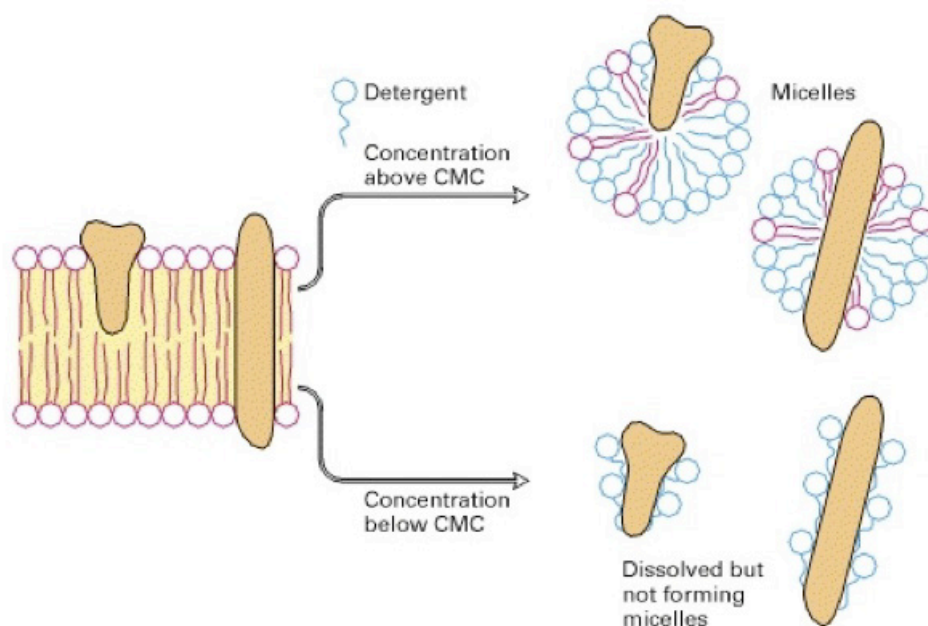


Figure 6-1 Solubilization of integral membrane proteins by non-ionic detergents

At a concentration higher than its critical micelle concentration (CMC), a detergent solubilizes lipids and integral membrane proteins, forming mixed micelles containing detergent, protein and lipid molecules. At concentrations below the CMC, many detergents (e.g., octylglucoside) can dissolve membrane proteins without forming micelles by coating the membrane-spanning regions. From <http://www.ncbi.nlm.nih.gov/books/NBK21589/>

Secondly, several detergents should be tested in order to assess their ability to both extract and solubilise the protein of interest. This would be done by carrying out multiple extraction protocols, including several detergents at differing concentrations. A Western blot analysis would then be carried out in

order to determine which lysis protocol produced the most abundant amount of your protein.

In the case of this study, in order to initially optimise full protein solubilisation and extraction from *D.melanogaster*, a more crude approach was taken. Around 100 whole flies were briefly anaesthetised on CO₂ before being homogenised into lysis buffer samples containing seven different detergents at an initial concentration of 1%. The detergents tested can be seen in Table 6-1

Table 6-1List of commonly used detergents for membrane protein extraction and solubilisation

Detergent	Properties
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	Zwitterionic
Triton X 100	Non-ionic
Cymal	Non-ionic
n-Dodecyl- β -maltoside (DDM)	Non-ionic
dodecyl maltoside (DM)	Non-ionic
fos-choline	Non-ionic
n-octyl p-D-glucopyranoside (OG)	Non-ionic

A basic lysis buffer of 150mM NaCl, 50mM HEPES plus 1% detergent was chosen in order to assess the solubilisation and extraction efficiency of whole protein preps. Crude analysis was carried out where whole fly protein preps were measured by Bradford assay (as described in Section 2.11.2). This was carried out in order to determine the efficiency of each detergent at total protein extraction. The results can be seen in Table 6-2

Table 6-2 Lysis buffer detergents and resulting total protein extraction efficiency

Detergent	Protein concentration mg/ml
OG	2.863
Fos-Choline	2.856
DDM	2.704
DM	2.697
Cymal	3.306
Chaps	3.195
Triton X 100	2.787

These results indicate that Cymal and Chaps are the most efficient detergents. However overall there was no substantial difference between the detergents. Samples were then diluted to a concentration of 2.7 mg/ml (the lowest concentration of protein in detergent test) and whole protein samples run out on a SDS gel. This was carried in order to determine if the proteins solubilised in the lysis buffer varied depending on the detergent used. The results can be seen in Figure 6-2

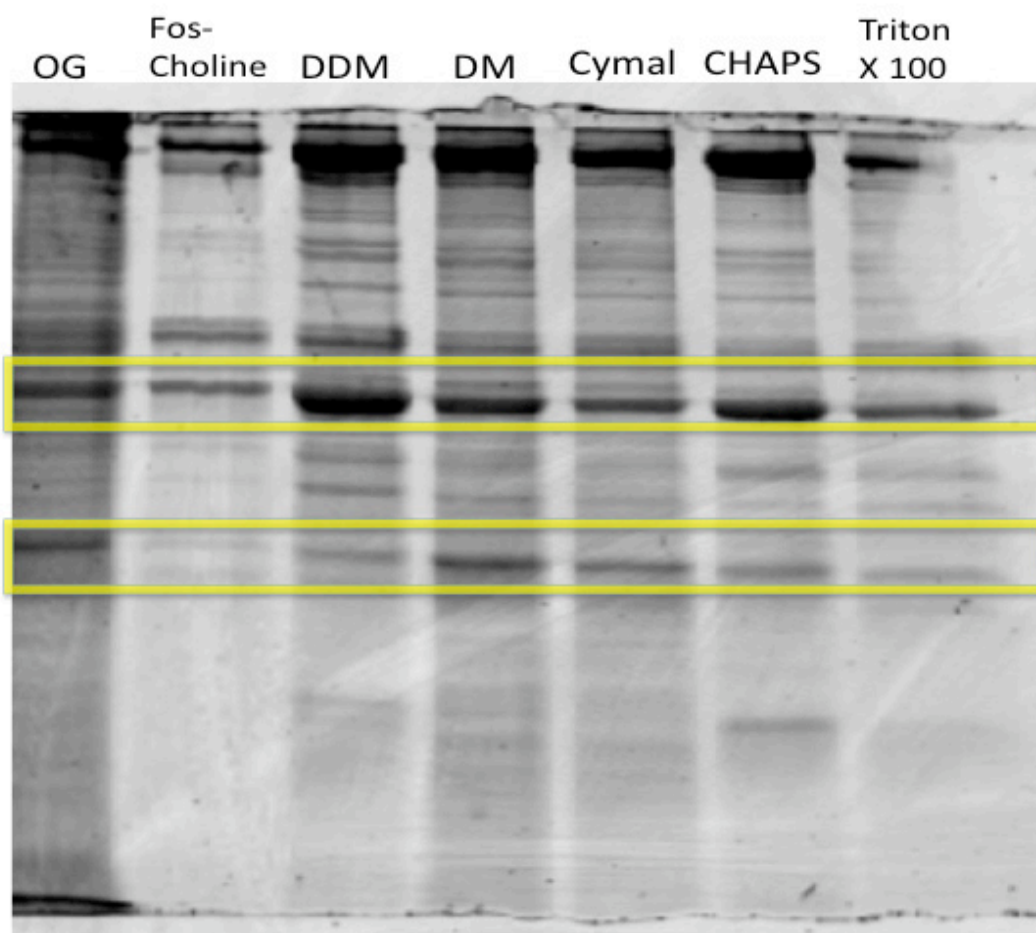


Figure 6-2 Comparison of total protein extraction with different detergent lysis buffers
Whole fly protein samples were prepared in lysis buffers containing 7 different detergents. Once diluted to a concentration of 2.7mg/ml each sample was ran on an SDS-PAGE gel in order to determine if detergents affect the abundance of in individual proteins.

The results indicate that there is a slight difference in the abundance of certain bands between detergents. For example in the area highlighted in the figure several bands appear to be stronger in the DDM sample; however, one band is significantly stronger in all other samples. These results again highlight the

importance of detergent selection, as your protein of choice may not be suitably solubilised with certain detergents.

6.5.1.1 Conclusions

This selection highlights the importance of detergent selection. We have shown, somewhat crudely, that with regards to whole fly samples each of the seven detergents was equally efficient at whole protein extraction. With more time these experiments would have been continued in order to determine the best detergent concentration for effective extraction and solubilisation of Fas2 and its interacting proteins. However for the purpose of BN-PAGE optimisation experiments DDM was chosen as the detergent, simply due to the presence of all highly abundant protein bands.

6.5.2 *Isolation of membrane proteins by sucrose gradient electrophoresis*

6.5.2.1 Introduction

The concentration and chemical diversity of individual proteins within a given sample is so diverse and that in itself, poses one of the intrinsic problems of 2D proteomics. In a typical human cell for example, the most abundant protein is actin; which, can be found at around 10^8 molecules per cell. Other proteins however, such as, transcription factors are found at much lower levels, with around 100-1000 molecules per cell (Rabilloud, 2002). This makes identification of both sets of proteins extremely troublesome on the same 2D gel. It is therefore essential to prepare samples in a way which excludes unwanted proteins, but enriches desired proteins. The most common application used is that of separation of proteins by sucrose gradient centrifugation. Typically this process involves the enrichment of a particular membrane or organelle within the cell. Most notably this technique has been used in order to isolate, mitochondria proteins (Taylor et al., 2003), chloroplast proteins (Singh et al., 2008) and membrane proteins.

In the context of this study we wished to isolate membrane proteins from tubules and guts of *D.melanogaster*. If we were to run whole protein preps through the 2D BN-PAGE protocol, this is likely to result in aggregation of highly abundant proteins, such as actin: the staining procedure would fail to identify low abundant proteins. Initial experiments concentrated on optimisation of the protocol, using whole fly preps. Sucrose gradients were carried out, as described in Section 2.11.3. Traditional experiments rely on large-scale isolation of proteins from cell cultures. In this study however, tissue samples were prepared. This resulted in a much-decreased starting concentration of protein. Therefore the final concentration of protein after sucrose gradient centrifugation was much lower than ideally suited to 2D BN-PAGE electrophoresis. Results for one such prep can be seen in Table 6-3

Table 6-3 Comparison of protein concentration before and after sucrose gradient centrifugation

Samples from ~300 whole flies were prepared as described in Sections 2.1.3 & 2.11.1. Sucrose gradient centrifugation was carried out as described in Section 2.11.3 Protein concentration was measured both before and after separation.

Starting concentration of samples (mg/ml)	Membrane fraction concentration (mg/ml)	Cytosol fraction concentration (mg/ml)
25.0	0.4747	0.144
28.55	0.5670	0.234
24.08	0.3450	0.210

These results indicate the significant loss of protein when carrying out sucrose gradient centrifugation. The most significant effect of this loss is the inability to detect low abundance proteins in downstream experiments. These results also indicated the importance of starting concentration and for this reason it was decided that both tubule and guts would be dissected for tissue specific experiments. With this in mind ~3000 flies were dissected and full alimentary canal and tubules were collected as described in Sections 2.1.3 & 2.11.1. This resulted in a final protein concentration of 9.493 mg/ml. 1.5 ml of this sample was then run through the sucrose gradient centrifugation protocol as described previously. Unfortunately however, all protein from this sample was lost during this protocol. It is likely that this may be due to a lack of separation during centrifugation and/or a low abundance of starting material. Time constraints meant that this experiment could not be repeated. Therefore all downstream

BN-PAGE experiments reported here used whole protein preparations (BN-PAGE was carried out using whole fly membrane preps but no detectable protein was found after running the second dimension).

6.5.2.2 Conclusions

Sucrose gradient centrifugation is the ideal protocol for separation of membrane fractions. However in this study we have highlighted the pitfalls of the procedure when starting material is low. With more time it would have been possible to optimise this protocol. It may also have been prudent to carry out a simple centrifugation step in order to pellet membrane proteins such as that carried out by Beynebach et al. This would perhaps of resulted in a significantly lower loss of starting material (Beynebach et al., 2009).

6.6 BN-PAGE results

BN-PAGE experiments were carried out using whole fly and tubule/gut protein preps in order to optimise the protocol. Initial experiments were carried out using a protocol from several different sources. However these experiments proved inefficient as can be seen in Figure 6-3

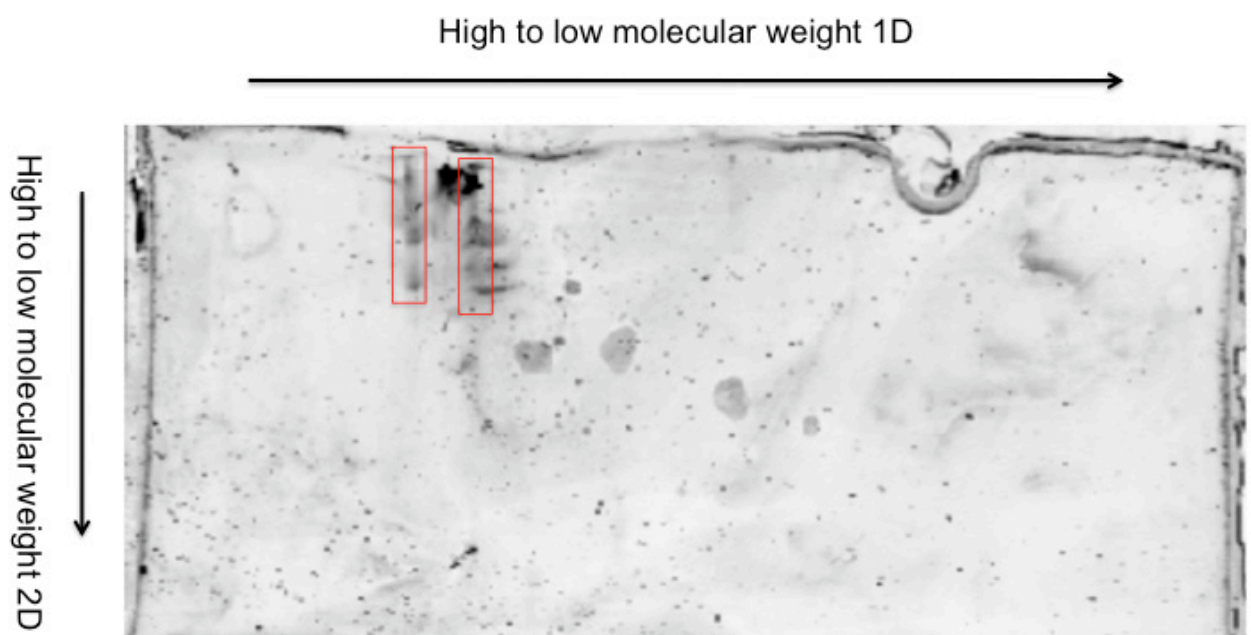


Figure 6-3 Initial 2D BN-PAGE results from whole fly protein samples

Whole fly protein samples were run on 2D BN-PAGE gels and protein visualised with Sypro Orange (Sigma, town). Red boxes indicate possible protein complexes. However overall levels of protein are significantly lower than expected.

Proteins were visualised in the 2D using the protein stain Sypro Orange. As we can see in Figure 1.3, there is an extremely low amount of overall protein on the gel. However there do appear to be several complexes shown. Further research indicated that Invitrogen (Invitrogen, Paisley), supplied a fully equipped kit for 2D BN-PAGE analysis, we therefore purchased this kit in the hope that ready-made solutions and gels would produce better results.

The above stated samples were re-run using the Invitrogen kit (as described in M&M). The resulting 2D gel was stained with Sypro Orange (Invitrogen, Paisley) and visualised. Results can be seen in Figure 6-4

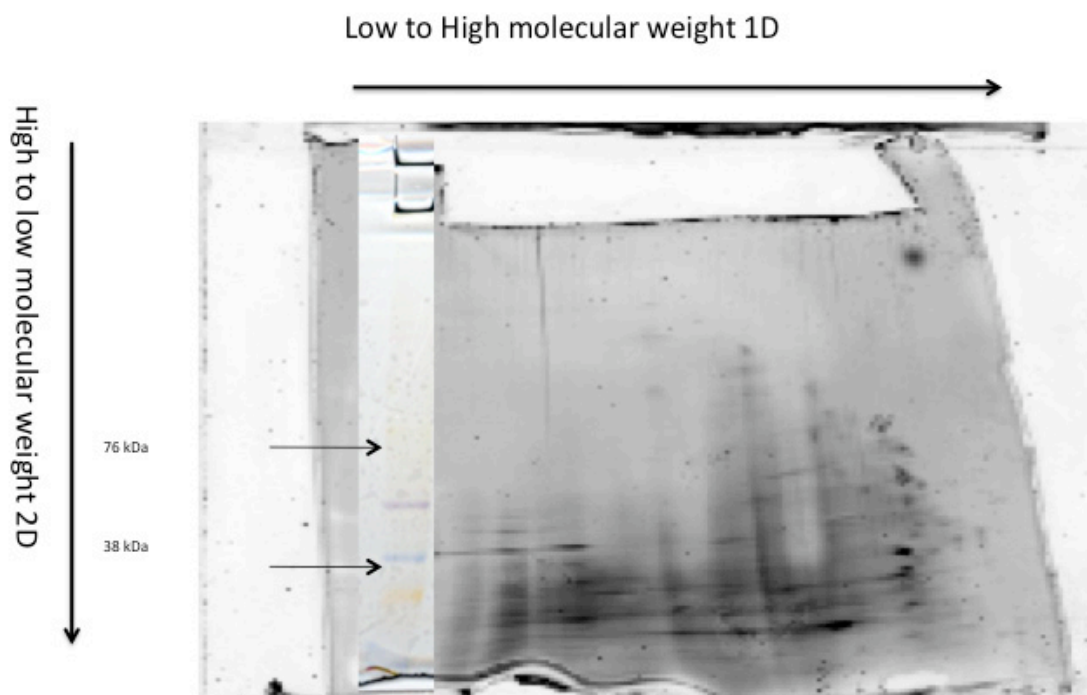


Figure 6-4 BN-PAGE 2D gel using Invitrogen BN-PAGE kit and whole fly protein samples

As we can see from this gel there is a significant increase in the number of proteins present in this gel. We can also clearly see the apparent separation of

protein complexes, as is evident by the presence of vertical lines of protein spots. In order to determine if this was indeed the case, samples were chosen from this gel and sent for MS. Figure 1.5 indicates the area of the gel samples were taken from. This process proved difficult, as Sypro Orange only visible under fluorescent conditions. Therefore images were required using the Typhoon Trio image detector and printed to the exact size of the gel. Conventional MS runs also rely on an automated robot, which can pick spots at precise areas on the gel. Unfortunately, a malfunction meant that for these experiments this was not possible. Therefore strips were manually cut, divided into equally sized pieces and sent for Trypsin digest and MS at the proteomics unit at Glasgow University.

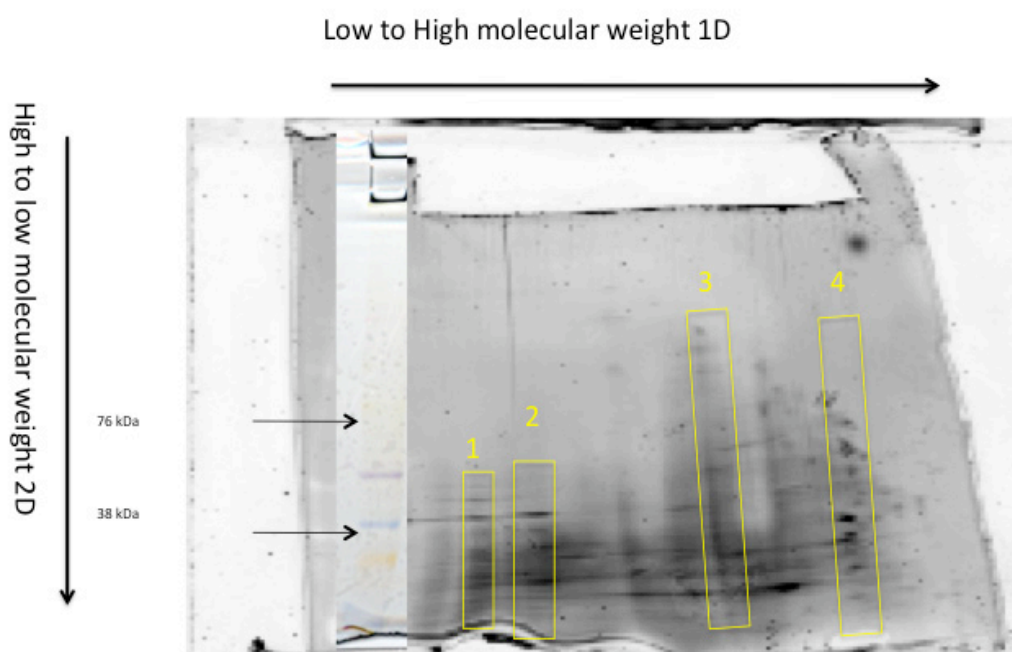


Figure 6-5 Areas of 2D BN-PAGE sent for MS analysis

Representation of areas sent for MS analysis, in order to determine whether vertical strips represent protein complexes.

6.6.1.1 MS results suggest vertical lines represent protein complexes

Peptide results from MS analysis were analysed using the online database FlyBase. Each peptide was assigned to a gene and a gene list compiled. A full list of all proteins found in each lane can be found in Appendix 3. Each lane was

then analysed using the online database FlyMine (<http://www.flymine.org/>). This software allows for the analysis of gene/protein list, giving various details of their relationship to one another. Included in this analysis is a calculation of tissue expression profiles, embryonic developmental expression profiles, gene ontology enrichment (GO) and pathway enrichment values. Each of one of these analyses can help us to determine the likelihood that proteins may act together in a complex. For example proteins involved in complexes must be expressed in the same tissues, are often expressed at the same developmental stages, will be involved in the same pathway and will often share GO terms. GO terms are a list of characteristics associated with a given gene. If there is significant enrichment of GO terms for a list of genes or proteins it suggests they may interact or at least be involved in similar processes. For each lane the above analyses were analysed and results are summarised in Figure 6-6 - Figure 6-9

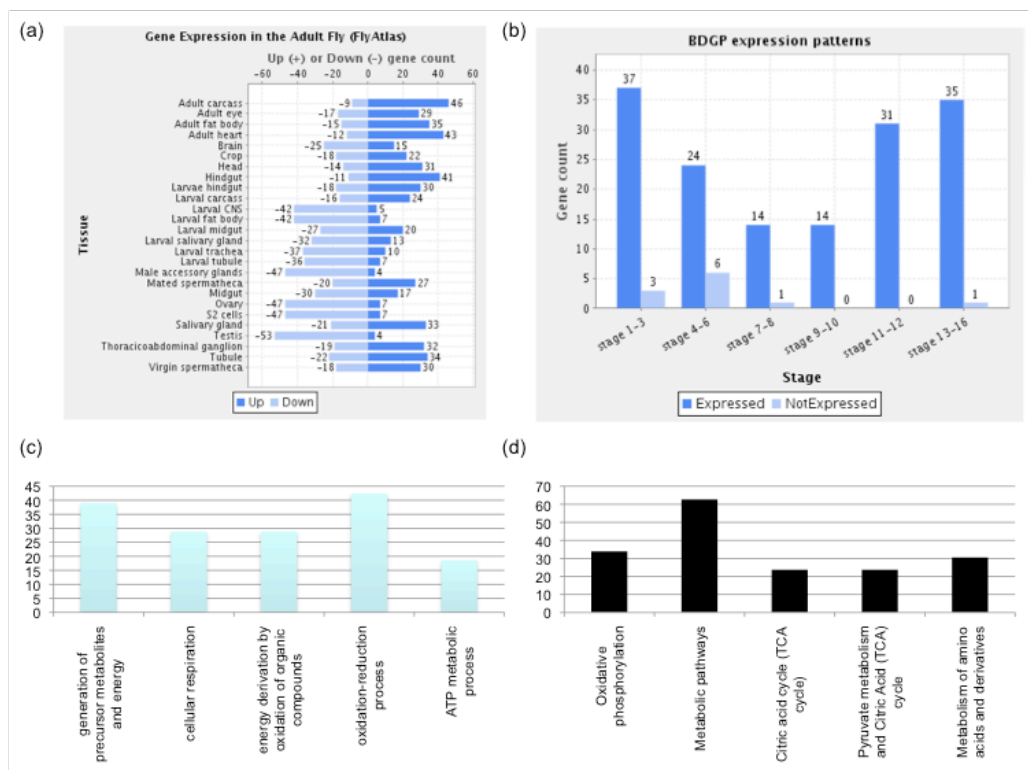


Figure 6-6 Comparison of expression profile, GO term enrichment and pathway enrichment of proteins isolated from Lane 1 of BN-PAGE gel

Summary of analysis carried out on proteins isolated from lane one of BN-PAGE gel. (a) Expression profile of genes (assumed from protein extraction) from FlyAtlas. Values indicated the number of genes (from protein list) expressed in each tissue. (b) Expression profile of gene (assumed from protein extraction), during different embryonic developmental stages. (c) summary of top five most enriched GO terms (d) summary of top five most enriched pathways

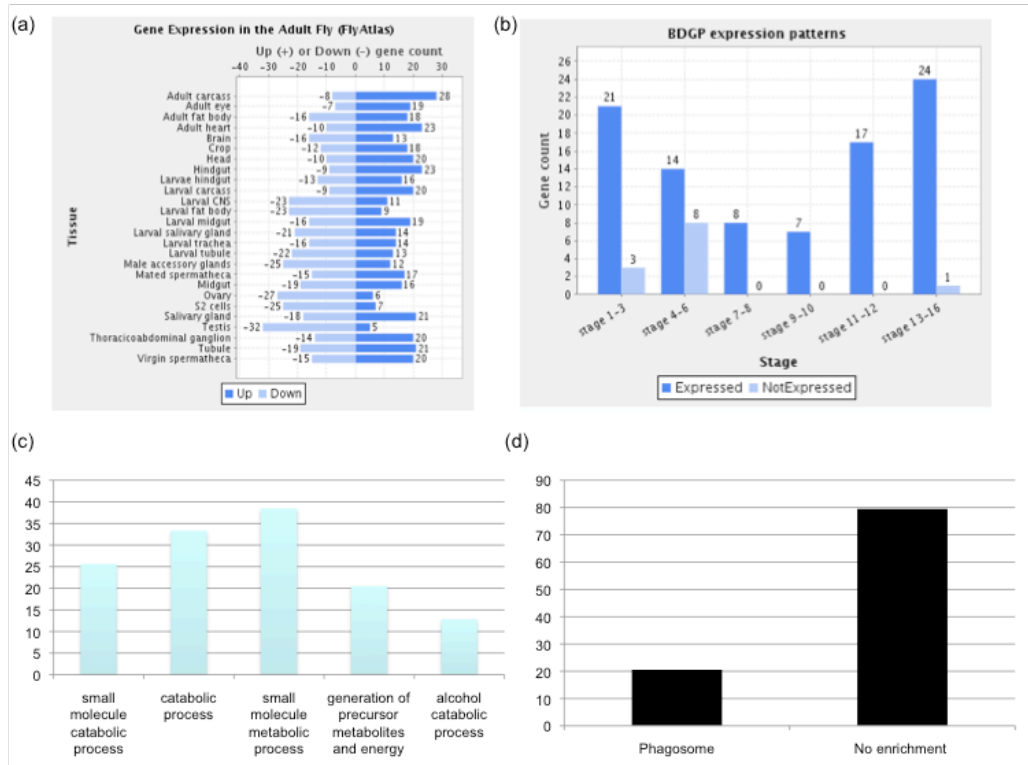


Figure 6-7 Comparison of expression profile, GO term enrichment and pathway enrichment of proteins isolated from Lane 2 of BN-PAGE gel

Summary of analysis carried out on proteins isolated from lane two of BN-PAGE gel. (a)

Expression profile of genes (assumed from protein extraction) from FlyAtlas. Values indicated the number of genes (from protein list) expressed in each tissue. (b) Expression profile of gene (assumed from protein extraction), during different embryonic developmental stages. (c) summary of top five most enriched GO terms (d) summary of top five most enriched pathways

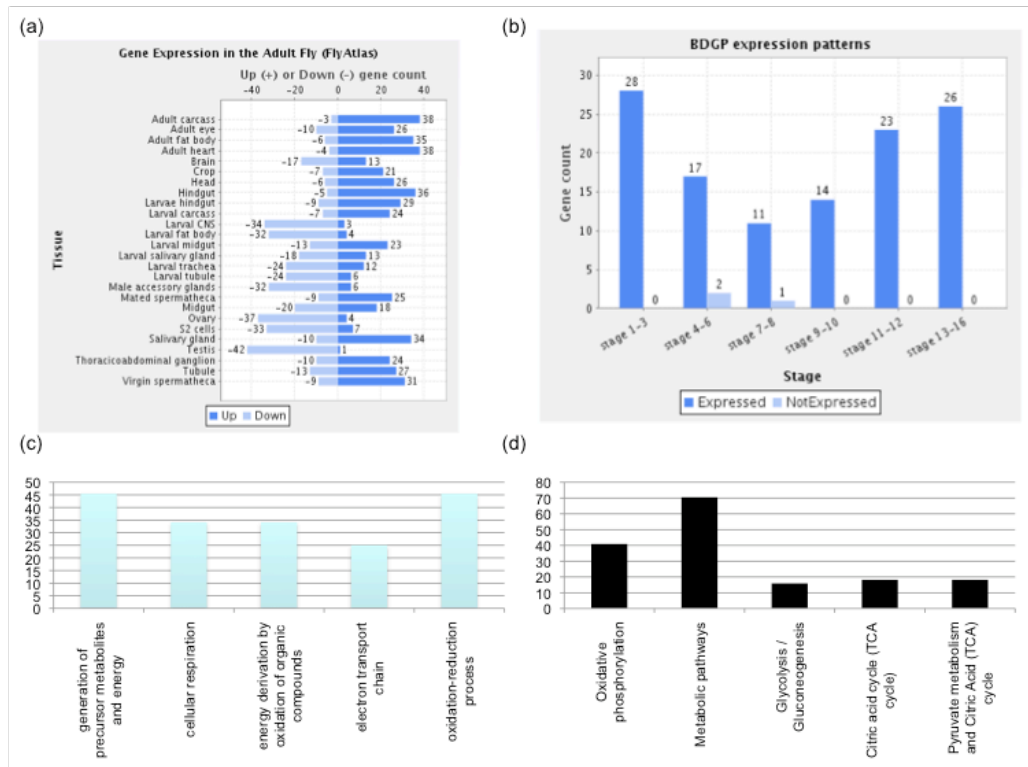


Figure 6-8 Comparison of expression profile, GO term enrichment and pathway enrichment of proteins isolated from Lane 3 of BN-PAGE gel

Summary of analysis carried out on proteins isolated from lane three of BN-PAGE gel. (a) Expression profile of genes (assumed from protein extraction) from FlyAtlas. Values indicated the number of genes (from protein list) expressed in each tissue. (b) Expression profile of gene (assumed from protein extraction), during different embryonic developmental stages. (c) summary of top five most enriched GO terms (d) summary of top five most enriched pathways

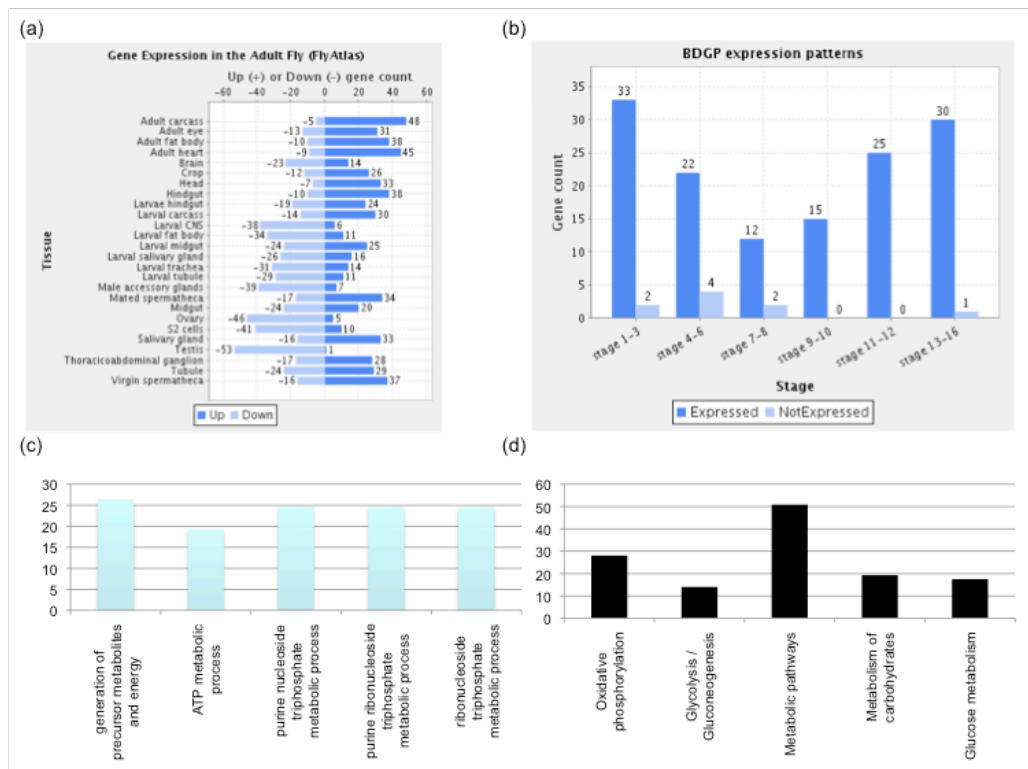
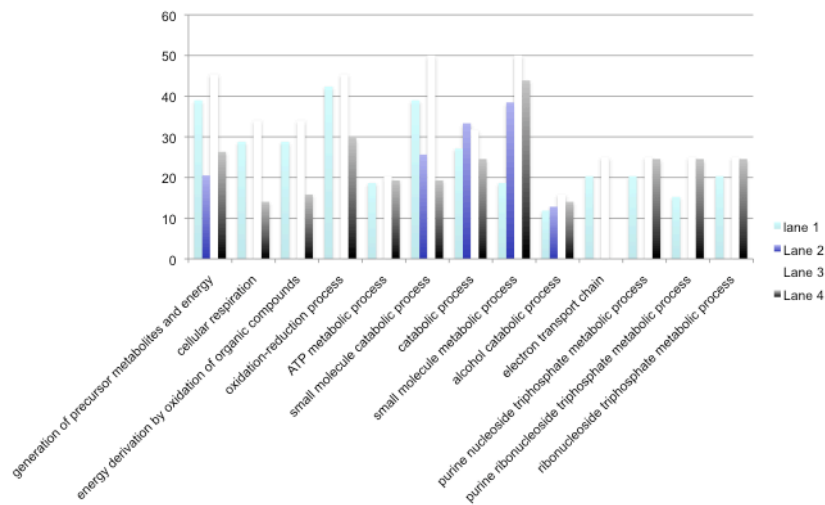


Figure 6-9 Comparison of expression profile, GO term enrichment and pathway enrichment of proteins isolated from Lane 4 of BN-PAGE gel

Summary of analysis carried out on proteins isolated from lane four of BN-PAGE gel. (a) Expression profile of genes (assumed from protein extraction) from FlyAtlas. Values indicated the number of genes (from protein list) expressed in each tissue. (b) Expression profile of gene (assumed from protein extraction), during different embryonic developmental stages. (c) summary of top five most enriched GO terms (d) summary of top five most enriched pathways

The results presented in Figure 6-6 - Figure 6-9 indicate the strong possibility that the proteins in each lane are indeed complex forming proteins. The sheer volume of proteins in some lanes was surprising, however it is likely that as the lanes were cut manually there is some cross over between adjacent lanes. What is more surprising is the observation that all proteins isolated from the four lanes, show significantly high enrichment in similar GO terms and pathway enrichment, most of which involve mitochondrial processes, summarised in Figure 6-10 There are several reasons why this may be the case. Firstly it is possible that mitochondrial and mitochondrial-associated proteins are simple highly enriched due their large abundance in the fly. Secondly it is possible that our lyses procedure did not fully lysis cells and vesicle compartments and/or mitochondria were left intact and thus enriched in downstream steps. Even so it appears that BN-PAGE has worked successfully to isolate and detect protein/protein interactions.

(a)



(b)

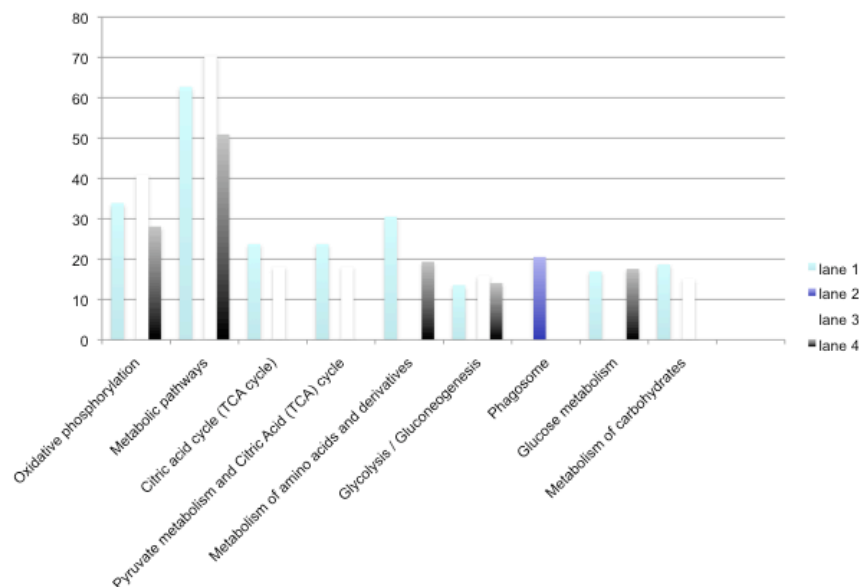


Figure 6-10 Summary of GO terms and protein pathways enriched in all four lanes

Analysis was carried out to determine highly enriched (a) GO terms and (b), pathways in all four lanes. Most significantly lane 2 was the only lane to contain proteins involved in phagosomes.

Figure -11 highlights the significance that, although proteins found in all four lanes function in similar pathways, there is still good separation of protein complexes across the gel. Of the 175 proteins isolated only 63 were found in more than one lane, whilst 112 were unique to an individual lane. It is likely that

the majority of those found in more than one lane are proteins, which function in more than one complex.

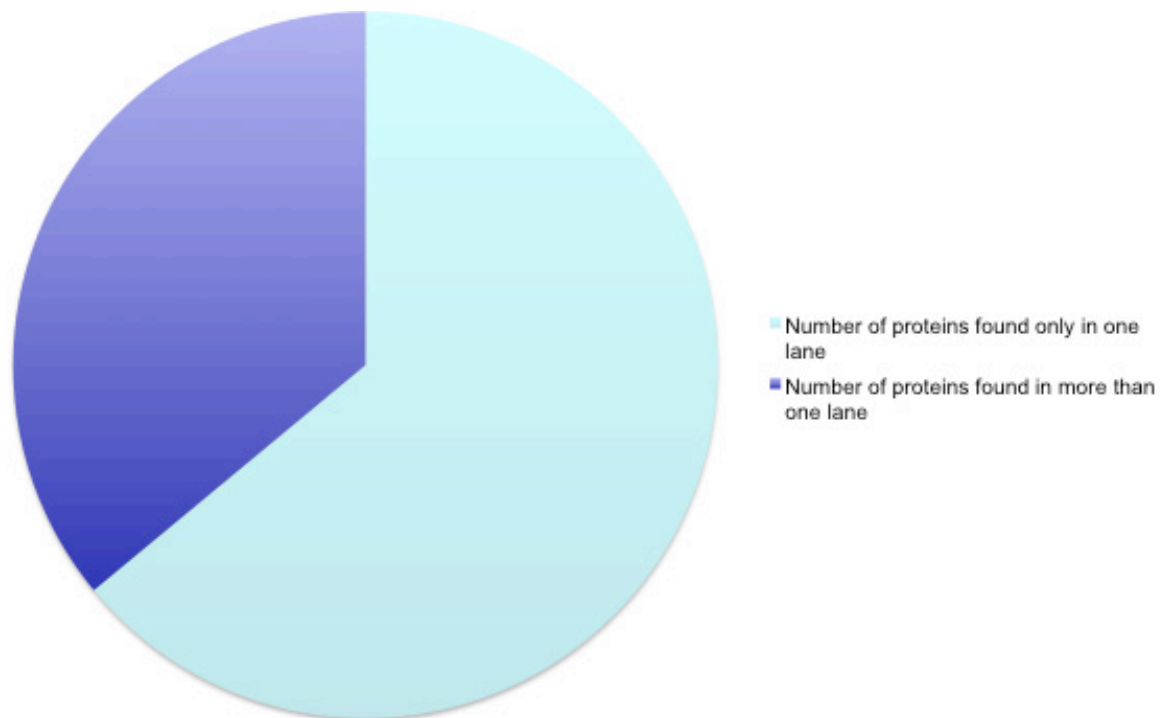


Figure 6-11 Analysis of proteins found within one lane or more lanes

6.6.2 String analysis of known and predicted protein/protein interactions

The final analysis carried out on the BN-PAGE data utilised the online software programme String V 9.0. (<http://string.embl.de/>). Once a list of genes or proteins is integrated into the programme, a search is carried out against databases of known and predicted protein/protein interactions. A schematic representation of the evidence base for protein/protein interactions, between your lists is then generated. The program also includes proteins it deems 'missing' from your list, in order to complete your interaction map. The results from each lane can be seen in Figure 6-12 - Figure 6-16 In each Figure (a) represents protein interactions inclusive of 'missing' proteins and (b) represents interactions with only the proteins extracted from each lane. The results from this analysis clearly confirm that each lane corresponds to likely protein complexes. Although diagrams appear more robust when 'missing' proteins are included, this does not diminish our results, as it is likely that these proteins

were simply missed in our analysis. Figure 6-16 represents 38 randomly chosen proteins, which have been run through the same analysis. The low numbers of interactions validate our results conclusively.

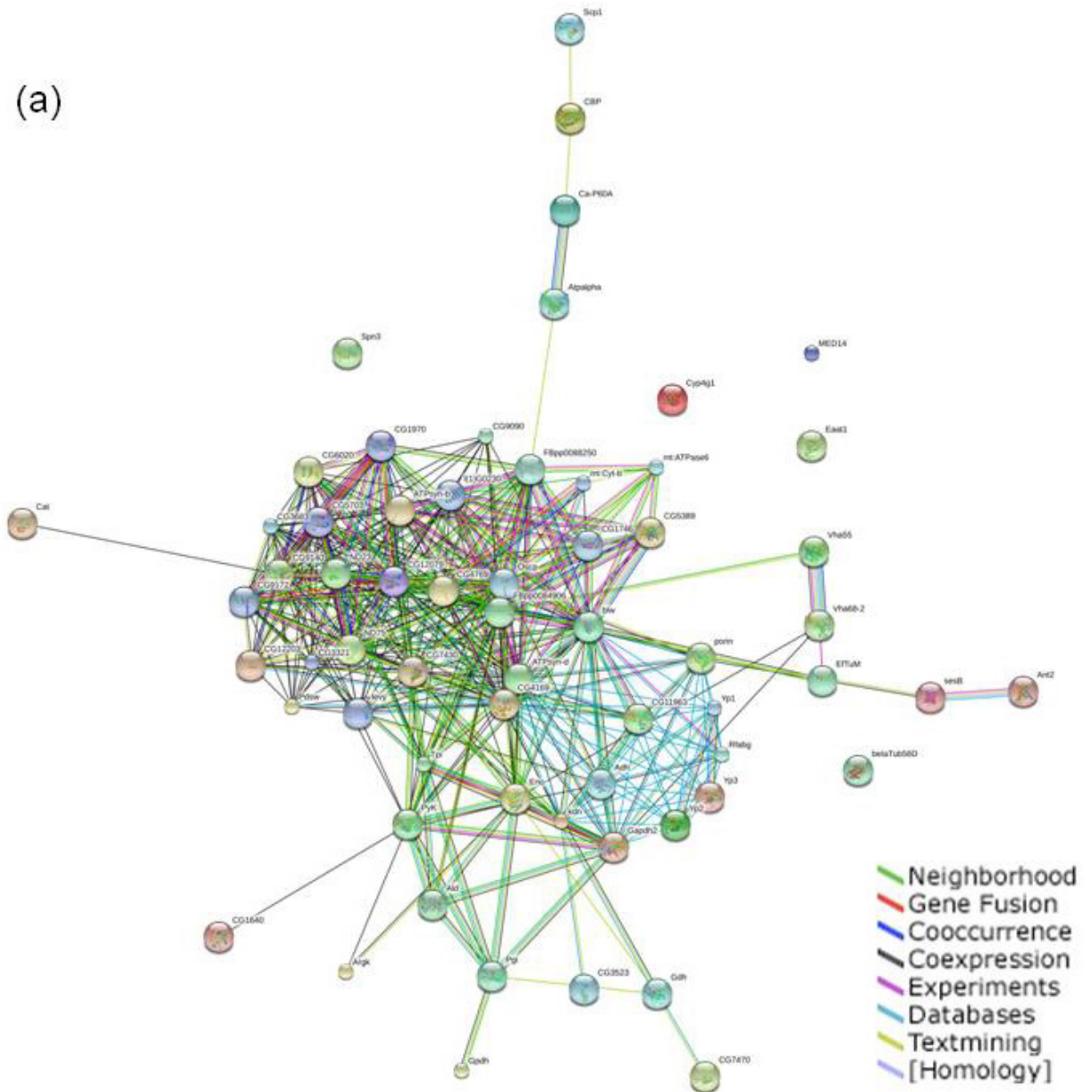


Figure 6-12 Analysis of known and predicted protein interactions using String of V. 9.0 between proteins extracted from Lane 1

Proteins extracted from Lane 1 were analysed using online software String V.9.0. Coloured lines correspond to evidence as indicated. (a) Protein interactions including those of 'missing interaction partners' (b) Protein interactions of only those proteins extracted from Lane 1

(b)

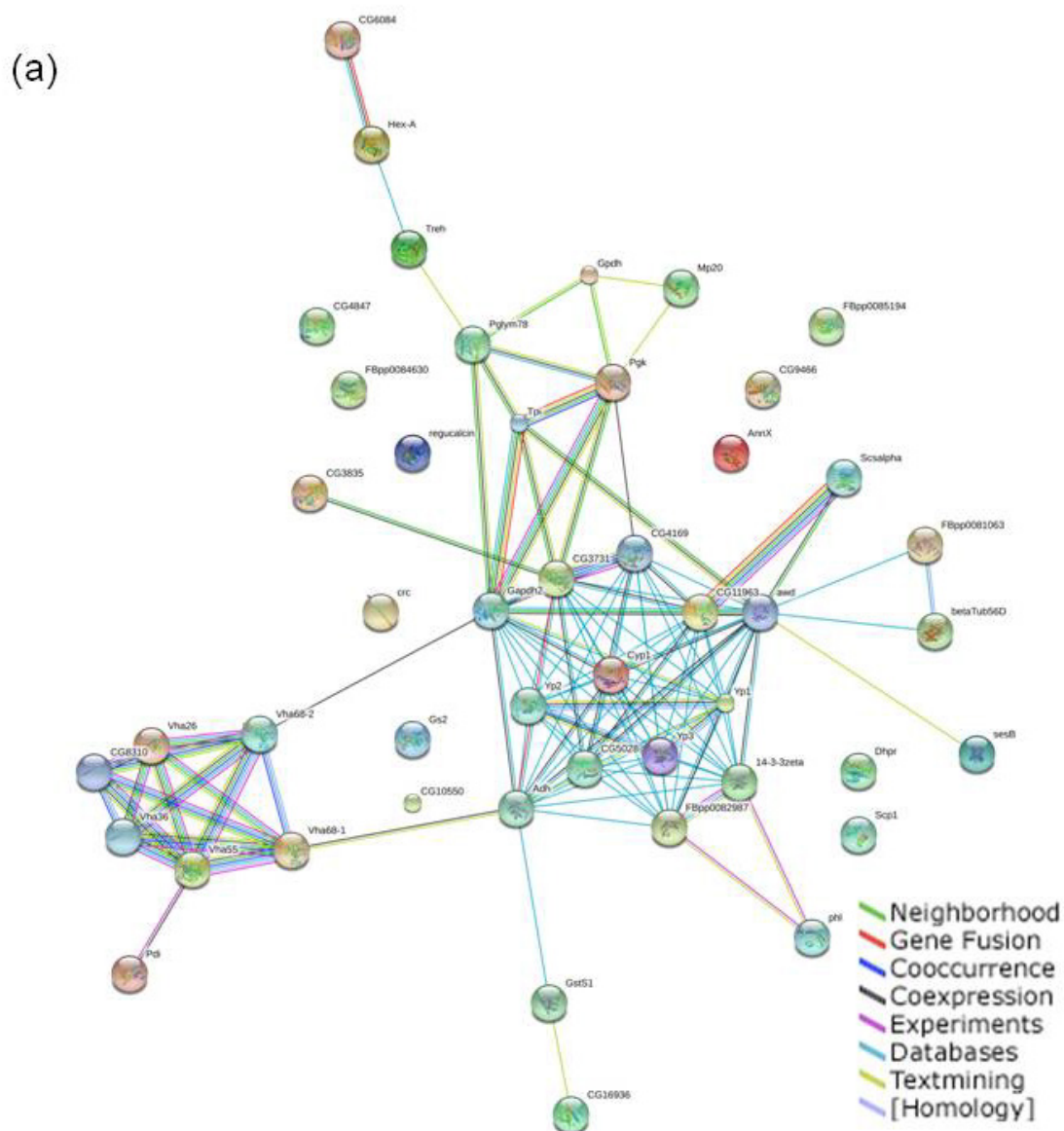


Figure 6-13 Analysis of known and predicted protein interactions using String of V. 9.0 between proteins extracted from Lane 2

Proteins extracted from Lane 2 were analysed using online software String V.9.0. Coloured lines correspond to evidence as indicated. (a) Protein interactions including those of 'missing interaction partners' (b) Protein interactions of only those proteins extracted from Lane 2

(b)

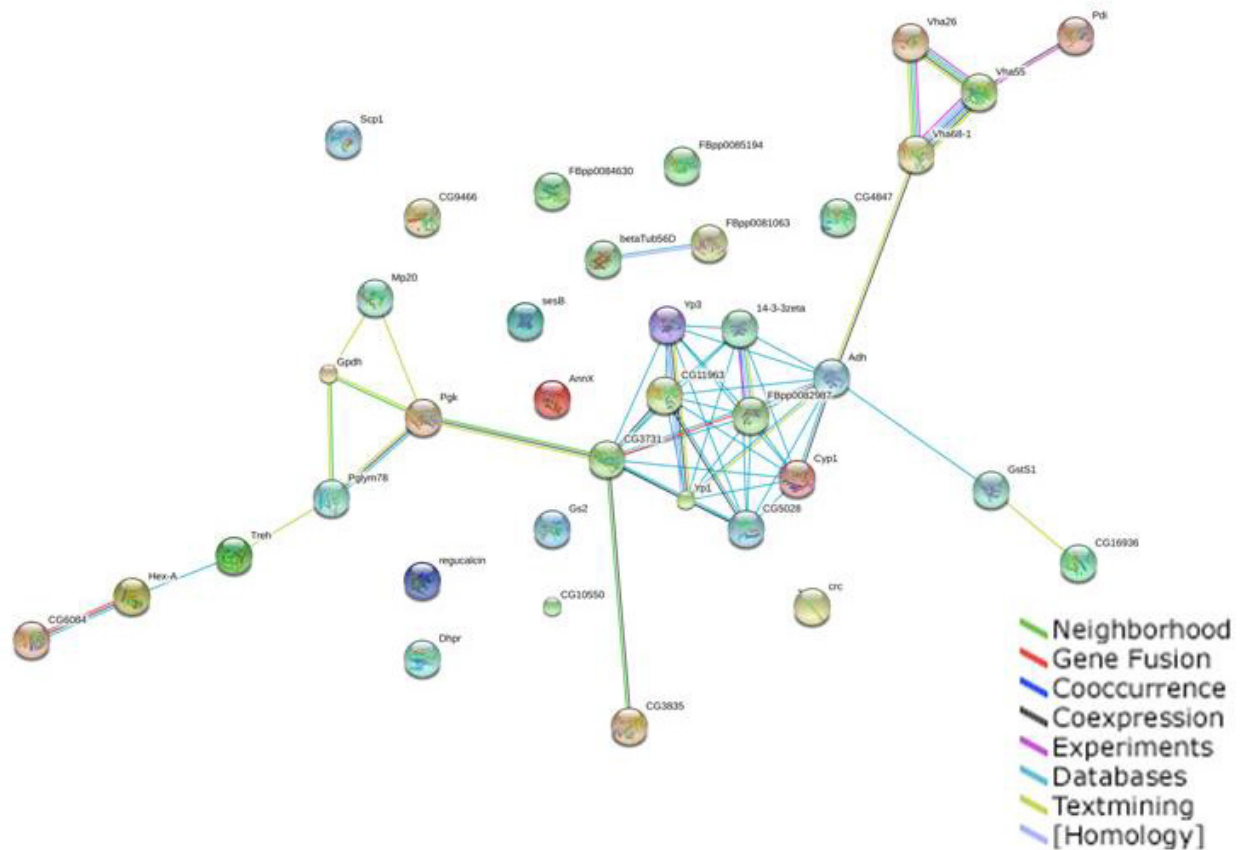


Figure 6-13 Analysis of known and predicted protein interactions using String of V. 9.0 between proteins extracted from Lane 2

Proteins extracted from Lane 2 were analysed using online software String V.9.0. Coloured lines correspond to evidence as indicated. (a) Protein interactions including those of 'missing interaction partners' (b) Protein interactions of only those proteins extracted from Lane 2

(a)

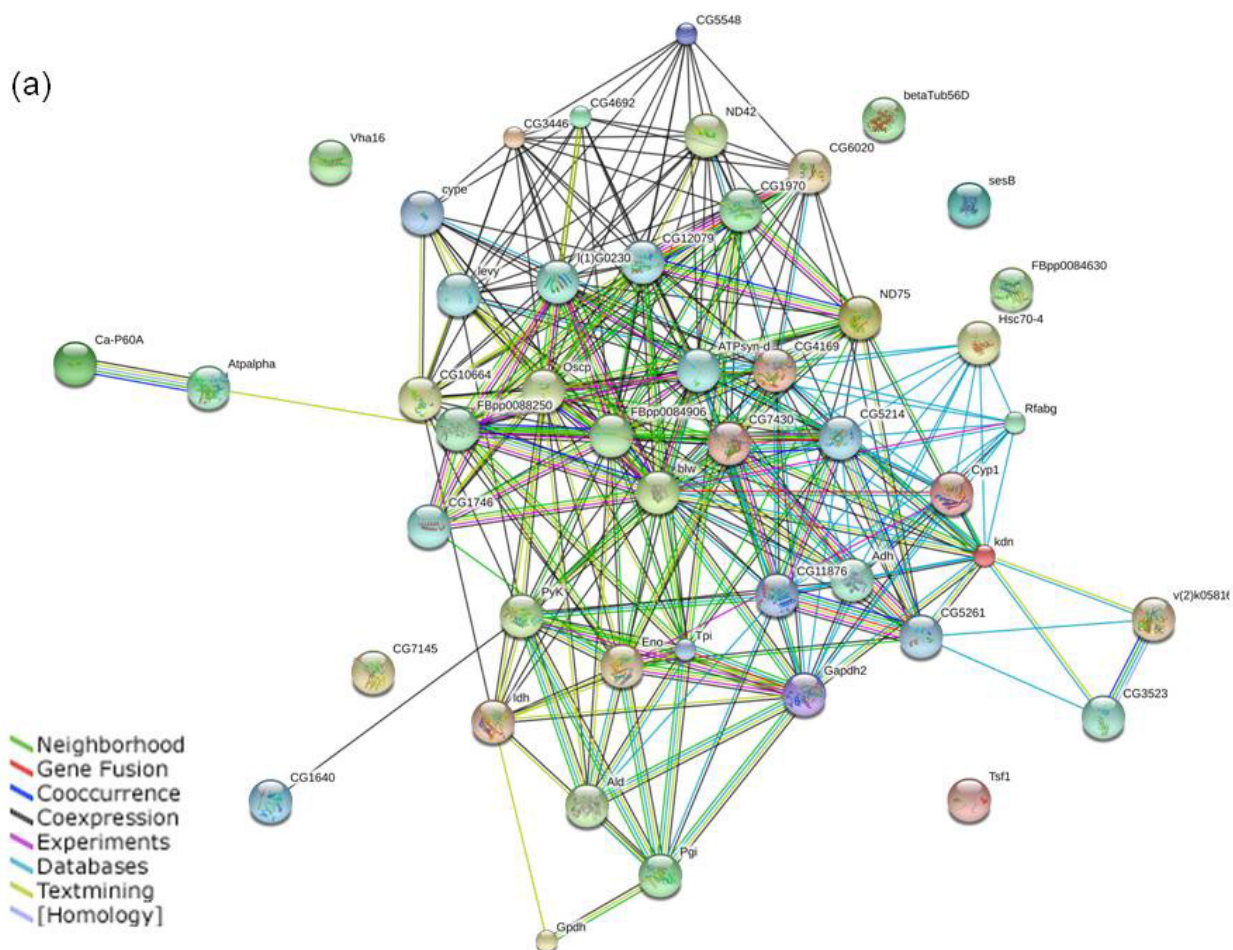


Figure 6-15 Analysis of known and predicted protein interactions using String of V. 9.0 between proteins extracted from Lane 4

Proteins extracted from Lane 4 were analysed using online software String V.9.0. Coloured lines correspond to evidence as indicated. (a) Protein interactions including those of 'missing interaction partners' (b) Protein interactions of only those proteins extracted from Lane 4

(b)

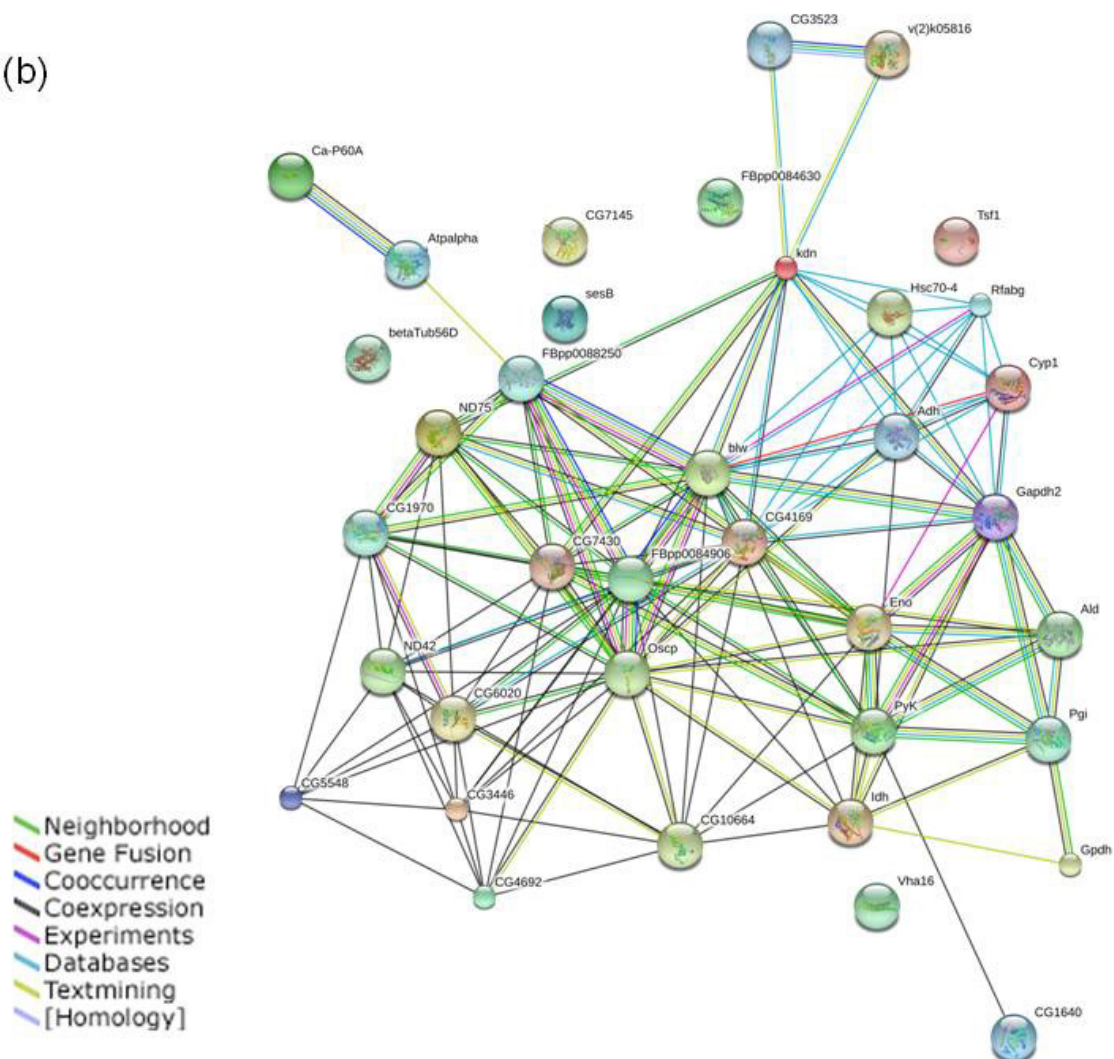


Figure 6-14 Analysis of known and predicted protein interactions using String of V. 9.0 between proteins extracted from Lane 3

Proteins extracted from Lane 3 were analysed using online software String V.9.0. Coloured lines correspond to evidence as indicated. (a) Protein interactions including those of 'missing interaction partners' (b) Protein interactions of only those proteins extracted from Lane 3

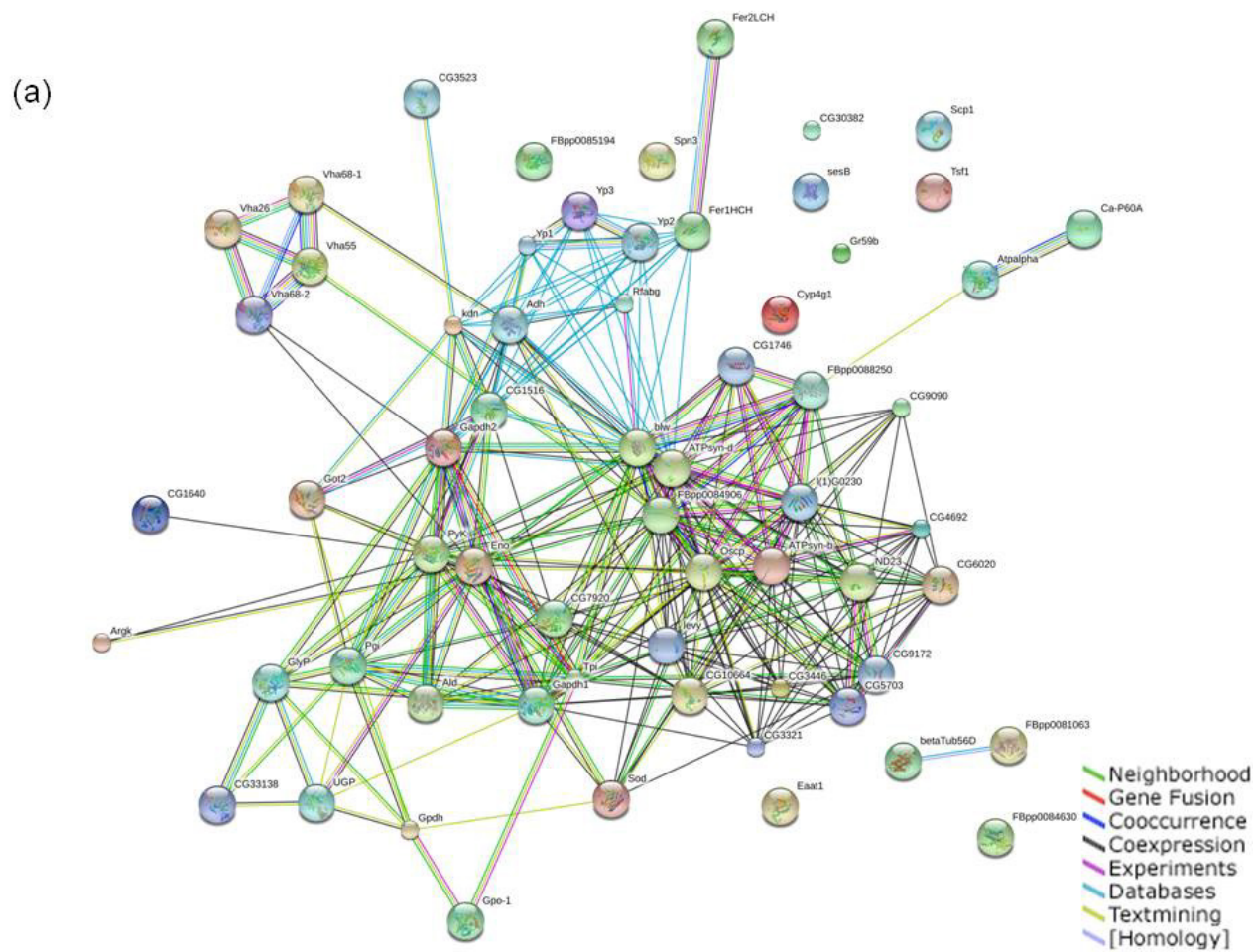


Figure 6-15 Analysis of known and predicted protein interactions using String of V. 9.0 between proteins extracted from Lane 4

Proteins extracted from Lane 4 were analysed using online software String V.9.0. Coloured lines correspond to evidence as indicated. (a) Protein interactions including those of 'missing interaction partners' (b) Protein interactions of only those proteins extracted from Lane 4

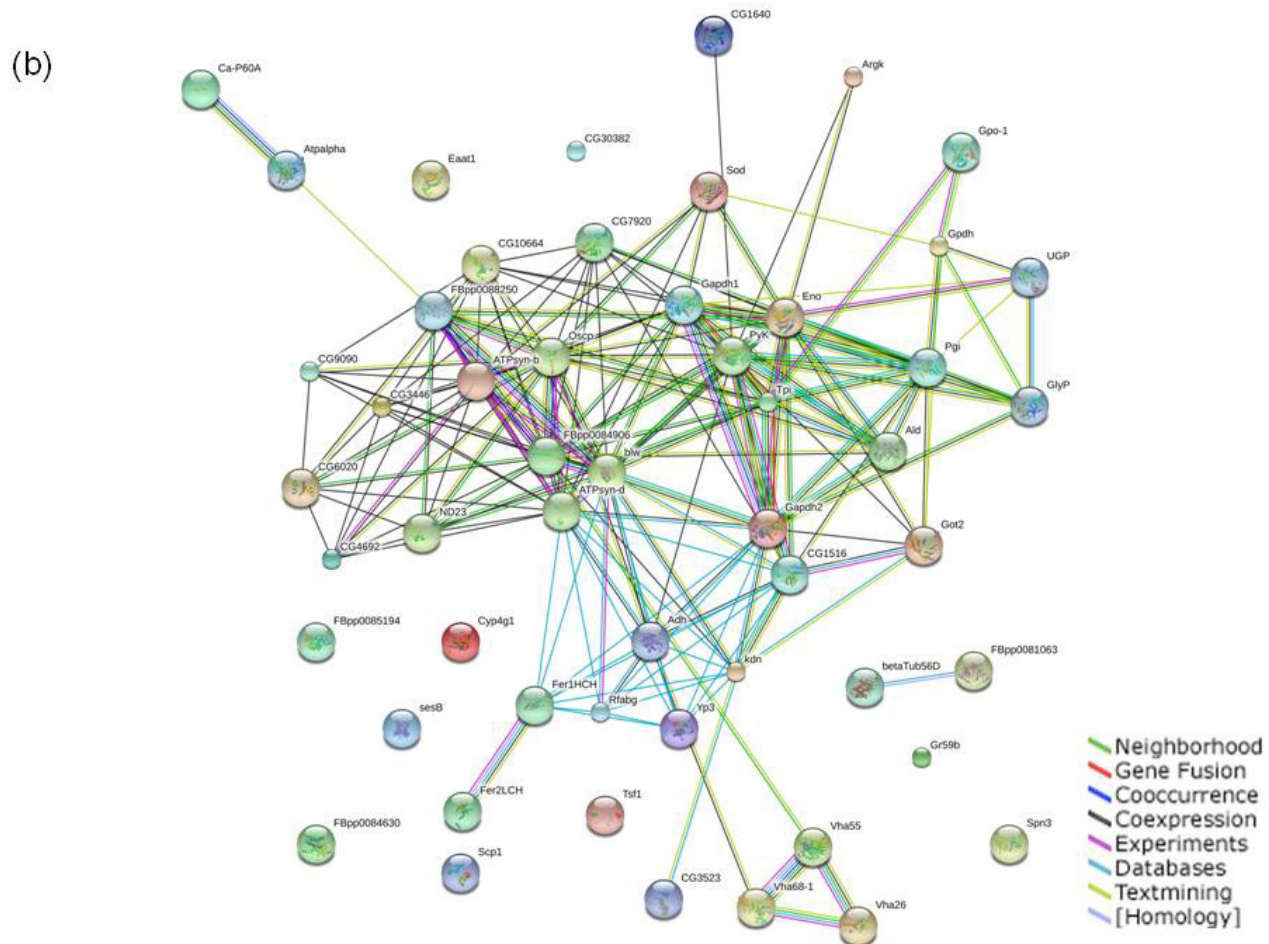


Figure 6-15 Analysis of known and predicted protein interactions using String of V. 9.0 between proteins extracted from Lane 4

Proteins extracted from Lane 4 were analysed using online software String V.9.0. Coloured lines correspond to evidence as indicated. (a) Protein interactions including those of 'missing interaction partners' (b) Protein interactions of only those proteins extracted from Lane 4

(b)

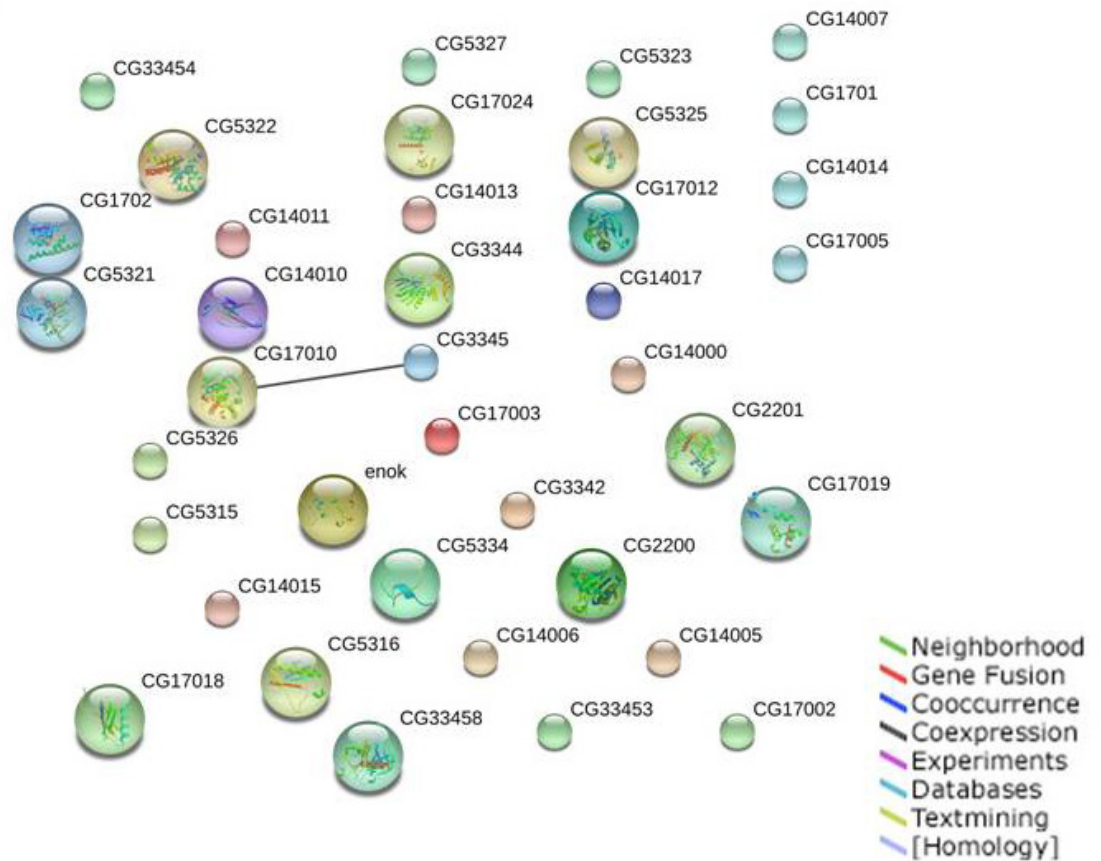


Figure 6-16 Analysis of known and predicted protein interactions using String of V. 9.0 between 38 randomly selected proteins extracted

38 random proteins from were analysed using online software String V.9.0. Coloured lines correspond to evidence as indicated. (a) Protein interactions including those of 'missing interaction partners (b) Protein interactions of only those proteins selected

6.7 Summation

In this Chapter we have demonstrated the pitfalls associated with sucrose gradient centrifugation, when trying to isolate membrane proteins from tissue samples. We have also shown that BN-PAGE is a robust technique for the separation of protein complexes. The BN-PAGE procedure laid out in this study, was ultimately designed to help determine the protein interactions of Fas2 under stimulated and unstimulated conditions, within the Malpighian tubules. However time constraints made this impossible. It took several months to optimise the BN-PAGE protocol, using whole fly total protein samples and indeed I was unable to successfully isolate tubule/gut proteins on BN-PAGE. However I have successfully demonstrated a proof of principle, in that BN-PAGE is a technique, which can be successfully used to isolate membrane protein complexes. With more time and the optimisation of sucrose separation, this technique could be used in order to determine the full proteome of the tubules and indeed protein complexes. Further to this we could extend the procedure further to look at protein complex formation in response to stimuli such as cAMP. These experiments, although time consuming and expensive could be instrumental in determining key aspects of tubule physiology.

Chapter 7

7 Doublesex

7.1 Summary

This chapter discusses experiments carried out in order to determine the function of *dsx* in the tubules. The results represented here are initial experiments and are not as extensive as those carried out on *fas2*. We do however confirm the presence of *dsx* in the tubules and show that they are expressed in a sex specific manner: *dsx^F* is expressed in female tubules and *dsx^M* is expressed in male tubules. Furthermore we show that expression is limited to the principal cells of main and lower segments of the tubule. In females expression is also seen in the transitional segment.

We also tested the ability of *tra^{RNAi}* in knocking down both *tra* and *dsx^F* in the tubules of female flies, with the aim of masculinising female tubules in an otherwise female fly. We then determined if this in turn had an effect on the immune response in the females.

Several genes were selected from a sex specific microarray, in order to determine if *dsx* is responsible for the sexual dimorphic expression of genes in the tubules. Further microarray analysis suggested that *dsx^M* and *dsx^F* show differential responses to the neuropeptide CAPA in the tubules, we therefore assessed *dsx* mRNA in response to CAPA.

7.2 Introduction

As discussed in Section 1.6.1 the gene *dsx* is instrumental in sex determination in the fruit fly *D.melanogaster*. It acts as the final ‘switch’ in the sex determination hierarchy and ultimately leads to both how a fly looks and acts, with regards to sex (Baker and Ridge, 1980; Billeter et al., 2006; Bownes et al., 1983; Hildreth, 1965; Raymond et al., 2000; Rideout et al., 2010; Sanders and Arbeitman, 2008; Villella and Hall, 1996; Waterbury et al., 1999; Zarkower, 2002). Alternative splicing of *dsx* results in two sex specific transcripts, the female transcript *dsx^F* and the male transcript *dsx^M*. A third transcript is also produced, *dsx^{Alt}*, which results in the same protein isoform of *dsx^F*, but shows expression in both males and females (Dornan et al. personal communication & our observations). The resulting transcription factors not only define whether a fly is male or female but also how they behave. FlyAtlas indicates that *dsx* is expressed in the MT, suggesting sex specific functions of the MT cells. Indeed a recent microarray analysis indicates several genes show sex specific expression in the MT (Chintapalli et al manuscript submitted). It is tempting therefore, to believe that *dsx* may at least, in part control sex specific gene expression. A recent microarray analysis has indicated that the tubules of the fly show differential gene expression between males and females. In order to determine if *dsx* transcripts play a role in this, it was necessary to specifically masculinise or feminise the tubules in the opposite background. RNAi lines against *dsx* target both transcripts, therefore producing an intersex phenotype (Rideout et al., 2010). This line therefore is not beneficial to study *dsx^F* and *dsx^M* specific targets. This study therefore utilised the line *tra^{RNAi}* in order to specifically knockdown *dsx^F*, and allow for *dsx^M* to be spliced in the tubules of female flies. The protein Tra is required in order to form a complex with Tra2, which then binds to the *dsx* locus and allows for splicing of *dsx^F*. Therefore knocking down *tra* should decrease *dsx^F* and increase *dsx^M*. The benefits of such a system are twofold. Firstly it allows for the study of direct and indirect targets of both *dsx* transcripts, by comparing expression in parental and RNAi driven lines. Secondly, in the context of the tubules it allows for the assessment of the physiological effect of masculinating tubules in an otherwise female fly. As the tubules form the main excretory and osmoregulating organs in the fly, there is a strong possibility that gene expression is controlled at a sex specific level, as males and

females are under different physiological stresses. Therefore if we masculinise female tubules, does this affect the response to pressures such as increased secretion due to increased food intake after mating? The tubules have also been indicated as an immune sensing system and have been shown to react in both independent and systemic infection (McGettigan et al., 2005). Females are known to be under a higher pressure with regards to immune response, as both mating and increased food intake expose them to higher levels of microbes (Lawniczak et al., 2007). Indeed females are known to respond better to bacterial infection than males (Lawniczak et al., 2007). If the tubules are involved in this response then they may require the need to communicate with other tissues, such as the gut. This Chapter will discuss *dsx* expression in the tubules, the specific knockdown of *dsx^F* in the tubules and a possible role for *dsx* in sexually differential gene expression and immunity.

7.3 Results

7.3.1 *Confirmation of differential dsx expression in male and female tubules*

As stated in Section 1.6.3, FlyAtlas results suggest that the male and female transcripts of *dsx* are expressed in a sex specific manner in the tubules. In order to confirm this report, primers were designed against *dsx^F* and *dsx^M* and QRT-PCR was carried out. Primers were also designed against the third transcript *dsx^{Alt}*, currently thought to be an alternative variant of *dsx^F* but also known to be expressed in males (Dornan et al, personal communication). The results can be seen in Figure 7-1

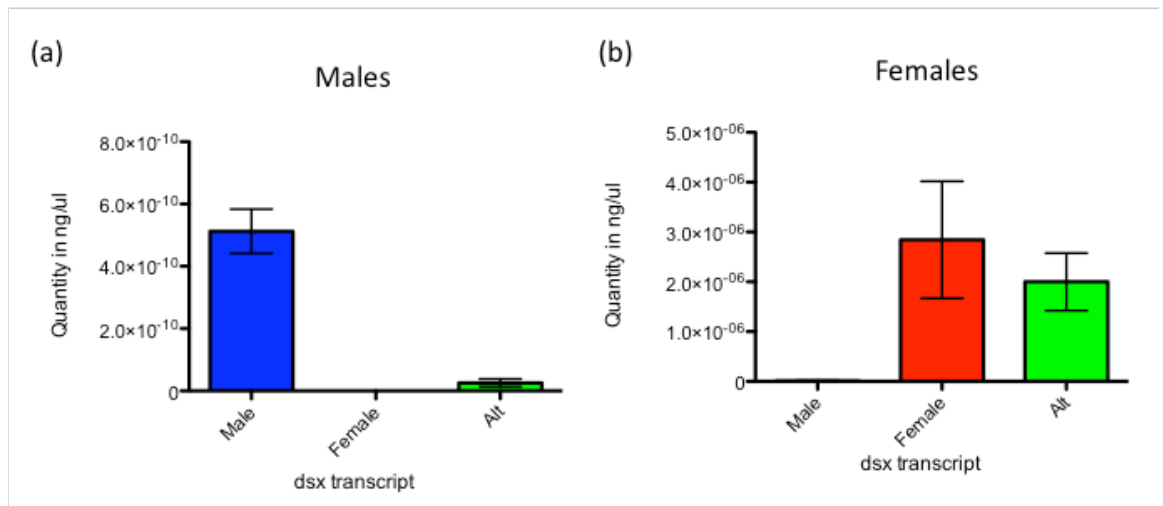


Figure 7-1 Expression of *dsx* transcripts in male and female tubules

Tubules were dissected from 7-day-old flies male and female flies. QRT-PCR was then carried out in order to determine the expression of each transcript in either males or females. (a) male tubules (b) female tubules. N = 4

The results confirm the presence of *dsx* transcripts in both males and females. They also confirm that *dsx^M* is only expressed in male tubules and *dsx^F* is only found in female tubules. Interestingly female tubules show much more variance in *dsx^F* expression than male tubules, suggesting that perhaps *dsx* expression is dynamic in females and may be under different pressures. We also noted the expression of *dsx^{Alt}*, in both male and female tubules, although in much lower amounts in the male. We can therefore confirm that *dsx* is indeed expressed in the tubules in a sex specific manner.

7.3.2 Expression profile of *dsx^M* and *dsx^F* in the tubules

In order to determine which cells express *dsx* in the tubules, a *dsx^{GAL4}* line was utilised to drive expression of *dsx^{RFP}*, a nuclear marker. The *dsx^{GAL4}* is an enhancer trap line consisting of a Gal4 insertion downstream of the *dsx* promoter, obtained by homologous recombination within the *dsx* open reading frame (Rideout et al., 2010). Several flies were dissected at 7-days-old and ≥ 9 tubules were assessed. Results can be seen in Figure 7-2, results indicate that *dsx* is only expressed in the principal cells of the tubules (as evident by nucleus size). Furthermore no expression is seen in the initial segment in either males or females. However females express *dsx* in the transitional segment and males do not. This is interesting as it suggests that perhaps the transitional segment in females may carry out a previously unidentified role, specific to females.

However as these results only represent one population of flies dissected on the same day it would be vital to repeat these results. Additionally pictures taken at a higher magnification would enable the determination of expression in the transitional segment more precisely. It would also be interesting to carry out experiments such as those described in Section 3.4.2, in order to determine *dsx* expression throughout development in the tubules.

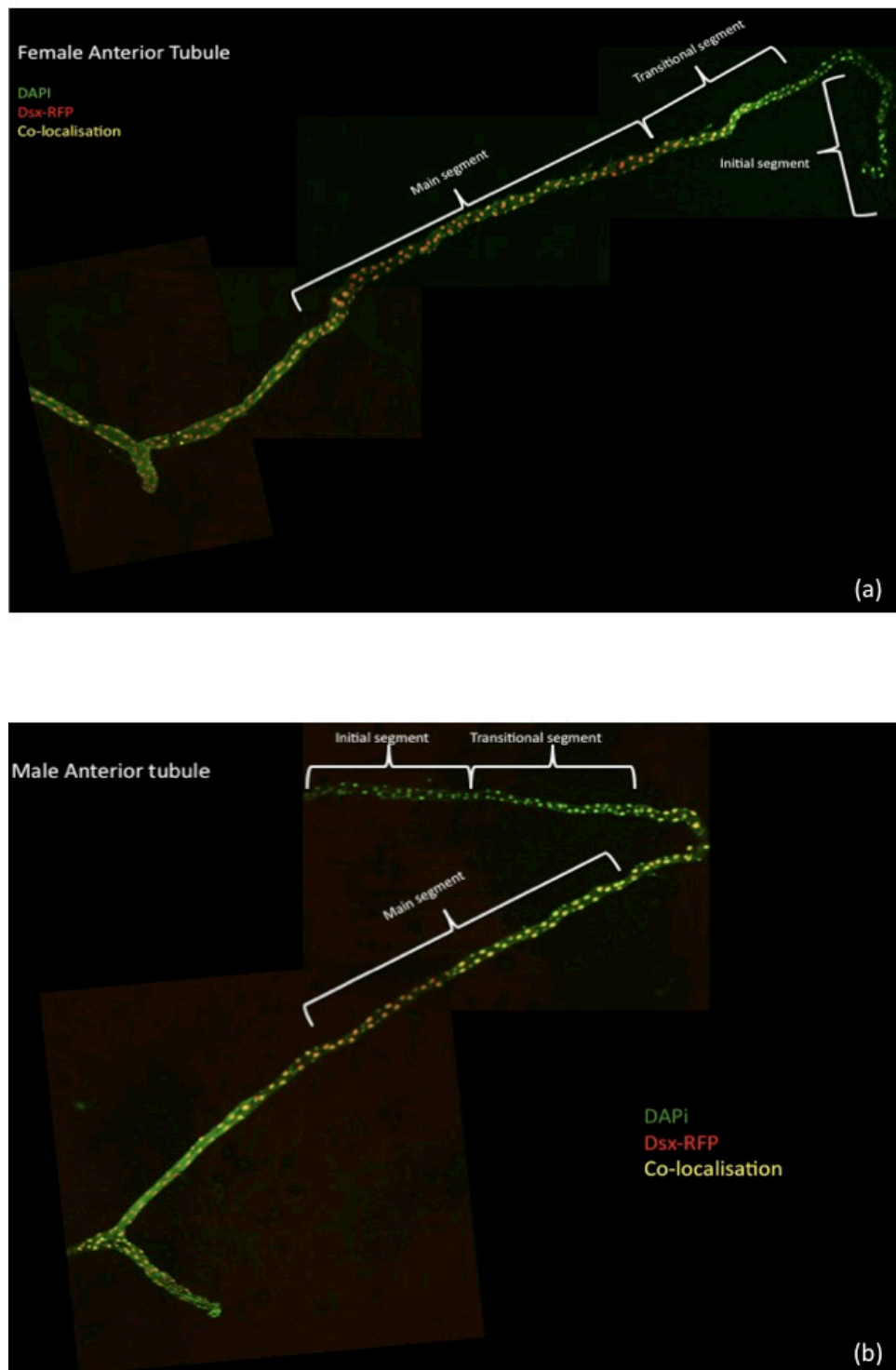


Figure 7-1 Expression profile of *dsx* in adult tubules

dsx^{GAL4} was utilised to drive expression of *dsx^{RFP}*. (a) Female tubules express *dsx* in the lower main and transitional segment, only in principal cells (b) Males show no expression in transitional segment, but otherwise are the same as females

7.3.3 Specific knock down of *dsxF* in the tubules of female flies

Utilising UO^{GAL4} , *tra*^{RNAi} was driven in the principal cells of the tubules main segments. Female flies were then dissected at 7-days-old and QRT-PCR carried out. The results can be seen Figure 7-2.

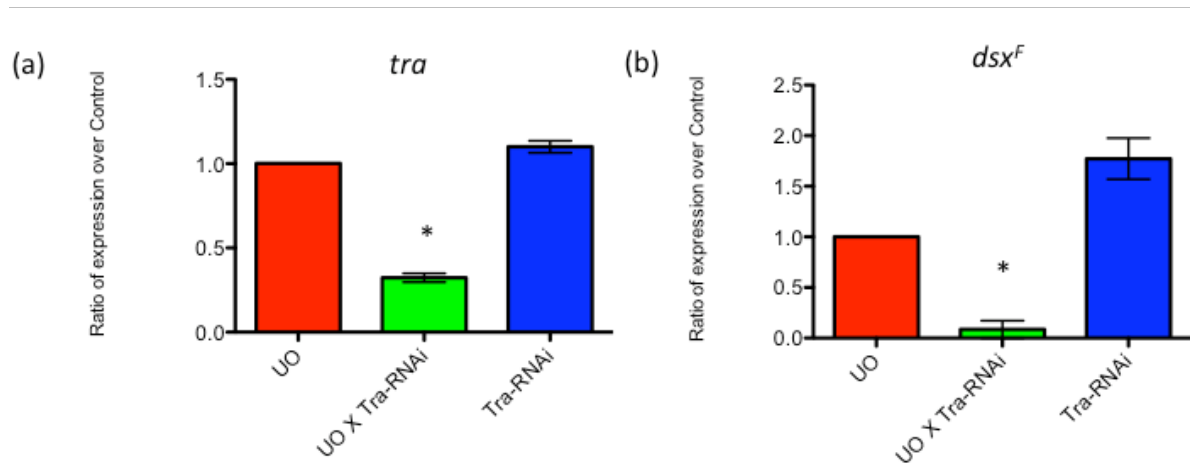


Figure 7-2 Efficiency of *tra*^{RNAi} at knocking down *tra* and *dsxF*

7-day-old female tubules expressing *tra*^{RNAi} & UO, were dissected and compared to parental lines. Expression of *tra* and *dsxF* was compared in all sets. (a) expression of *tra* (b) expression of *dsxF*. In both cases expression was significantly lower than both parental lines. Student unpaired t test analysis. N=4

	P values	
	<i>tra</i>	<i>dsxF</i>
UO vs cross	0.0086	<0.0001
Cross vs <i>tra</i> ^{RNAi}	0.0166	0.0033
Parental vs parental	0.0624	0.0330

Results indicate that *tra*RNAi is extremely effective at knocking down both *tra* and *dsxF*. Further analysis is required in order to test if *dsxF*^M is also upregulated in the female tubules. We can conclude however that this line is effective at disrupting both *dsxF* and *tra* expression.

7.3.4 Differential immune response in males and females

Several studies have indicated that the pressure of mating in females has led to an increased immune response, resulting in females responding better than

males to microbe infection (Lawniczak et al., 2007; McGraw et al., 2004; McKean and Nunney, 2001; Peng et al., 2005). However there is debate in the field as to whether or not this is simply a matter of size, males being smaller than females, or if increased immune systems after mating prime females for a response to further infection (Lawniczak et al., 2007; McGraw et al., 2004; McKean and Nunney, 2001; Peng et al., 2005). In this study we wished to analyse whether or not masculinising the tubules of females, by down regulating *tra* and *dsx^F*, affected immune response to *Bacillus* infection. Firstly we determined if indeed there was a difference between males, females and virgin females, as some studies suggest that virgin females respond in the same fashion as males (Lawniczak et al., 2007). 7-day-old females, virgin females and males were collected and injected with *Bacillus*, a Gram-positive bacterium known to kill *Drosophila* within 2-3 days of infection (Davies et al., 2009). The results obtained can be seen in Figure 7-4. Results confirm that males die significantly quicker than females. However in our findings, there was no significant difference between females and virgin females. If time permitted it would have been beneficial to determine if this was the case across a broad spectrum of bacteria and fungi. We can conclude therefore that males appear to be more sensitive to *Bacillus* infection than females.

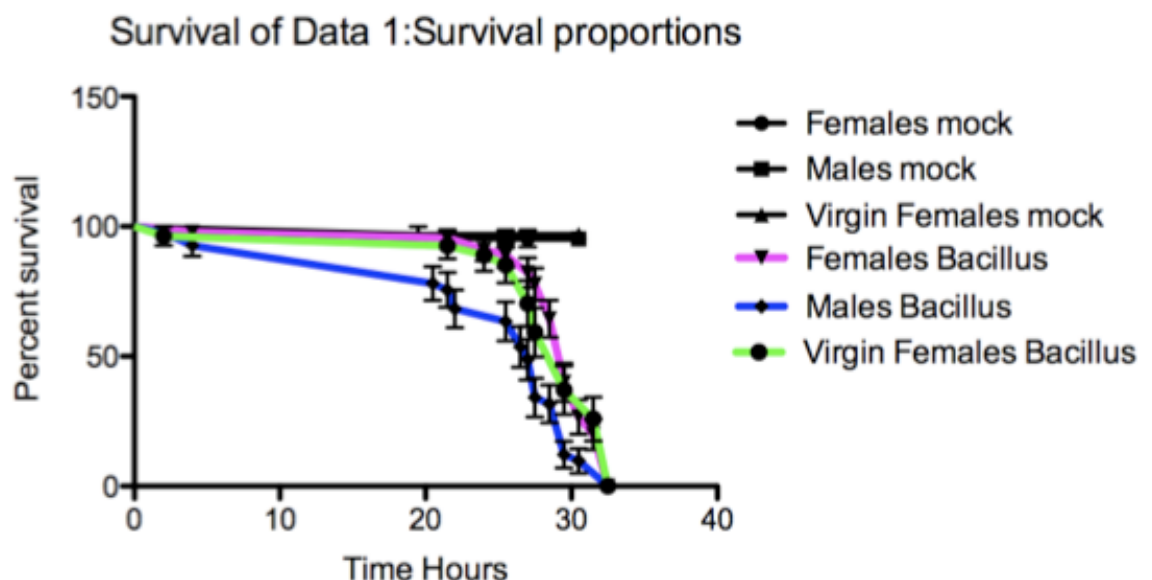


Figure 7-3 Analysis of male, female and virgin females response to *Bacillus* infection

7-day-old males, females and virgin females were infected with *Bacillus*, as described in section. Flies were also mock infected with PBS. All three *Bacillus* groups die significantly quicker than mock controls ($P < 0.0001$). In infected flies the males die significantly faster than both females and virgin females ($P = 0.0113$). $N = 30$ per set

7.3.5 Masculinisation of female tubules has no effect on survival of females infected with *Bacillus*

As we have established that males are more sensitive to *Bacillus* infection than females, we assessed whether loss of *dsx^F* expression in the tubules may play a role in this. Crosses carried out in Section 7.3.3 were repeated and lines subjected to infection with *Bacillus*. The results can be seen in Figure 7-5. Again there is a significant reduction in survival of mock versus infected flies. We also again see that males die significantly quicker than all infected females. There is however, no difference between female flies expressing *tra^{RNAi}* in the tubules and parental lines. These results suggest that *dsx^F* expression, at least in the tubules does not govern female's higher survival in response to *Bacillus* infection.

Survival of Males and females: Survival proportions

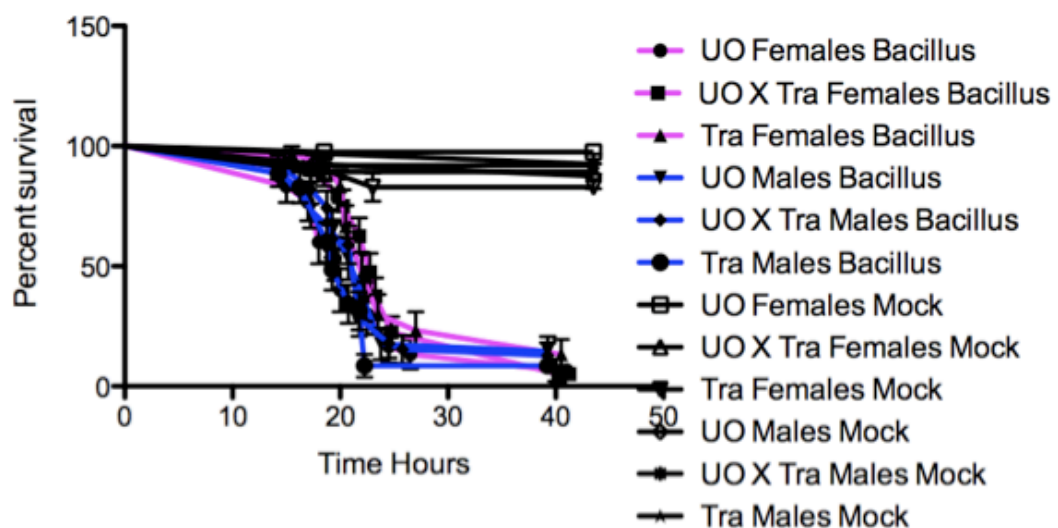


Figure 7-4 Effect on survival to *Bacillus* infection when female tubules do not express *dsx^F*
7-day-old males and females from indicated crosses were infected with *Bacillus*, as described in section 2.14. Flies were also mock infected with PBS. All six *Bacillus* groups die significantly quicker than mock controls ($P < 0.0001$). In infected flies the males die significantly faster than both females and virgin females ($P = < 0.0001$). $N = 30$ per set

7.3.5.1 Conclusions

We have confirmed previous evidence that males and females show different viability in response to *Bacillus* infection, but virgin females show no difference to

mated females. We have also concluded that this is unlikely to be affected by the expression of *dsx^F* transcripts in the tubules. The experiments conducted in this section are preliminary and by no means conclusive. Further analysis with more bacteria and fungi infections would be beneficial, before we can rule out a role for *dsx* in tubule immune response.

7.3.6 *Determination of sexually dimorphic gene expression in the tubules*

As discussed in Section 7.2, several genes have been shown to exhibit differential gene expression between male and female tubules. Table 1.4, summarises some of these genes. In order to test whether or not *dsx* plays a role in the expression of these genes, we planned to masculinise female tubules as described in Section 7.3.3 and assess the level of expression of several of these genes. However time did not permit this analysis. Genes were however selected, and RT-PCR was carried out in order to determine if these genes were differentially expressed in males and females. Unfortunately the genes selected for male up regulation failed to amplify in tubule samples at all and further analysis is required. Of the three genes selected for female up regulation, two showed a higher level of expression in female tubules, YP3 and CG8791. Therefore these genes would be ideal candidates for further analysis.

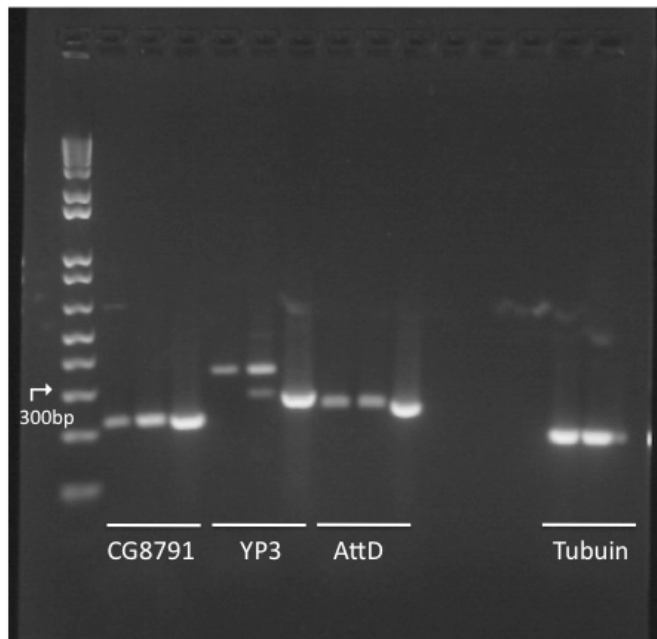


Figure 7-5 Expression of female specific genes in tubules of males and females

Gel represents expression of mRNA for each gene listed. Lane 1= male Lane 2 = female and lane 3 = whole fly for each gene.

7.4 Summation

In this chapter we have presented early preliminary results on the expression and function of *dsx* in the tubules of the fly. We can conclude that *dsx* is expressed in a sex-specific manner in male and female tubules and indeed the expression of many genes is also sexually dimorphic. We have not however determined if this is due to *dsx* expression or not. We have shown that *dsx^F* can be efficiently knocked down in female tubules and that this may in turn help to determine *dsx* function in downstream experiments. We have shown that immunity and survival in the fly is dependent on sex, but we cannot determine from our results if *dsx* expression in the tubules plays a role in this. In conclusion the results presented here tell us little about the function of *dsx* in the tubules, but prove that further experimentation is warranted.

Chapter 8

8 Discussion, Future Work & Overall Conclusions

8.1 Discussion and Future Work

8.1.1.1 Different roles for *fas2* in developmental stages

In this study we have shown that Fas2 localises to the lateral membrane in the developing embryonic tubules. This is consistent with previous studies (Denholm et al., 2003a). However during early larval development *fas2* re-localises to the apical brush border of the principal cells. Furthermore, it appears that *fas2* is expressed in a progressive manner, starting at the base of the tubules and progressing to the initial segment, throughout larval development, until expression is stabilised and Fas2 is expressed in all principal cells the full length of the tubule. These results are intriguing for two reasons. Firstly they suggest two separate roles for Fas2 in the embryo and later development stages. We have argued that this is due to differential expression of two separate isoforms of Fas2, however we have not proved this conclusively. Secondly the progressive expression of Fas2 along the tubules hints at a role in the development of the microvilli, as is further evident by data presented here. We have also demonstrated that Fas2 does not interact with Dlg in larval or adult tubules but have not ruled out an interaction in the embryo.

8.1.1.1.1 *Future work*

In order to fully understand the role of *fas2* in embryonic development further studies are required. One such study would be the analysis of Dlg localisation in *fas2*^{EP} lines. If Fas2 is responsible for localisation of Dlg, then it is likely to be disrupted in embryos mutant in *fas2*. It would also be of interest to look at the effect of EGFR signalling in these embryos as not only is this important for tubule development but Fas2 is known to inhibit EGFR signalling (Baumann and Skaer, 1993; Denholm et al., 2003a; Jung et al., 2005; Mao and Freeman, 2009). A closer look at microvilli development in the larvae would also be of interest. In particular electron microscope analysis of developing microvilli in, wild type tubules, tubules over-expressing Fas2 and tubules depleted Fas2 would be of interest. Finally it would be interesting to look at *fas2* expression and

localisation in the pupal tubules and indeed microvilli length, as it is known in some Diptera, that microvilli contract during this time. The hypothesis that Fas2 movement in response to cAMP is involved in the secretion phenotype is based on extremely preliminary data and is by no means conclusive. However we feel that the data presented here warrants a further analysis of Fas2 movement, as evidence from Fas2 role in the nervous system suggest this may be of some importance.

8.1.1.2 Fas2 is crucial to wild type microvilli development and stabilisation in adult flies

We have shown clearly in this study that *fas2* is key to normal microvilli length and that it may also play an important role in actin localisation. Over-expression of *fas2* results in longer, denser microvilli than wild type tubules and indeed the opposite is true in tubules with depleted *fas2* levels. Due to the continual breakdown of equipment, we were unable to fully quantify the length and density of microvilli, however the experiment was repeated in at least none tubules per genotype and the results were consistent in all tubules assessed. We are therefore confident that Fas2 is vital for microvilli stabilisation and/or development.

Fas2 depletion or over-expression also results in an increase or decrease in F actin localisation. We presented evidence that this is possibly due to a direct interaction between F-Actin and Fas2, as Fas2 is found to co-localise with F-Actin bundles on the surface of the tubule after stimulation with cAMP. However with more time and money this hypothesis would have been much more thoroughly examined as the evidence presented here is not conclusive enough to determine if this is the case.

We also can conclude from this study the importance of tissue specific manipulation of genes involved in development, when wishing to study their role in adult flies.

8.1.1.2.1 Future work

Here, in particular, electron microscopy experiments would be beneficial, in order to quantify the length and density of microvilli in tubules with differing

levels of *fas2* expression. Quantification of F-Actin fluorescence would also be advantageous, these experiments were in fact carried out but due to equipment failure final analysis could not be completed.

8.1.1.3 *Fas2* may play a signalling role in response to cAMP, which is crucial for Wildtype fluid secretion response

Depletion or over-expression of *fas2* in the tubule principal cells results in a decreased or increased cAMP induced fluid secretion rate. These results suggest that *fas2* is vital for normal secretion response to cAMP. Indeed, only the extracellular domain of *fas2* is required in order to illicit an increased response to cAMP. We have not however, determined if this is simply an affect of increased microvilli surface area or due to a signalling mechanism of *fas2*, such as the recruitment of proteins to or from the microvilli. We have however produced evidence that this increased response is likely to be due to increased V-ATPase activity as addition of Drosokinin to the bathing solution further increase secretion rate.

We present evidence here that in response to cAMP, Fas2 is internalised and moved to the basolateral membrane before moving back to the apical brush border, where it is re-inserted into the microvilli or secreted into the lumen. This result suggests that a signalling mechanism is involved in the increased and decreased response to cAMP induced secretion. If this is the case it is intriguing that only the extracellular domain of Fas2 is required for increased fluid secretion, as this suggest a signalling mechanism to Fas2 in the lumen. Intriguingly, we have also shown that Fas2 movement in response to cAMP, results in co-localisation with F-Actin bundles at the basolateral membrane. This result is of significance as previous studies in mosquito tubules have suggested that actin cytoskeleton re-arrangement is crucial to increased fluid secretion in response to cAMP (Bradley and Snyder, 1989; Karas et al., 2005). Preliminary results have further validated this, by showing that F-actin inhibitors, phalloidin and Cytochalsin D affect both basal and cAMP induce fluid secretion. These results are preliminary and act as an indication of the need for further analysis of Fas2 movement in response to cAMP. Perhaps simply the release of Fas2 from the microvilli allows movement of other proteins into the microvilli, or the

microvilli become less structured allowing for the flexibility required for fluid secretion to take place.

We have not however ruled out the possibility that Fas2 may be involved in the movement of other proteins such as V-ATPase subunits.

8.1.1.3.1 *Future work*

This study only looked at fluid secretion in response to cAMP, it would therefore also be beneficial to look at several other stimuli such as CAPA and cGMP. Further complete analysis of cAMP response would be strengthened by the inclusion of secretion assays, where CRF was used as a stimulus. CRF is a neuropeptide, which is known to increase intracellular cAMP levels.

Ultimately, determining if Fas2 movement in response to cAMP involves the recruitment of other proteins such as V-ATPase subunit, or actin, will give us further insight into Fas2 role in fluid secretion. This would involve co-localisation experiments and BN-PAGE analysis of both stimulated and un-stimulated tubules. Determining if Fas2 works upstream or downstream of actin cytoskeleton re-arrangement could also be determined by repeating secretion assays with F-Actin toxins.

8.2 BN-PAGE analysis: conclusions and future work

In this study we have optimised a BN-PAGE protocol for use in detecting protein/protein interactions in the fly. This procedure would be extremely beneficial in determining not only proteins, which form complexes with Fas2, after cAMP stimulation in the tubules, but could effectively be used to study the whole proteome of the tubule. In particular, stimulations with different second messengers and neuropeptides could be carried out and a full proteome protein complexome, comparison done. Although expensive and time consuming this could ultimately led to unparalleled insights into tubule physiology.

8.3 Doublesex: conclusions and future work

We have confirmed, in this study, that *dsx* transcripts are differentially expressed in a sex specific manner in the tubules. Further to this we have localised expression to the principal cells of the lower and main segment of the tubule. One difference is seen between male and female expression patterns: Dsx^M is not found in the transitional segment but Dsx^F is. This result is of some interest as it suggests a previously unidentified, sex specific function of this segment in female tubules. We have also shown that tubules can effectively be ‘masculinised’, in an otherwise female fly, by over expressing *tra*^{RNAi}. It would therefore be possible to carry out a microarray analysis on these tubules with the intention of discovering direct and indirect targets of Dsx^M and Dsx^F . We have also identified two genes, which could be used to validate these experiments.

As the tubules are known to be involved in immune response, it was hypothesised that male/female difference in immune response may be governed by *dsx* expression in the tubules. However our results indicate that this is not the case, at least with regards to one bacterium. Further analysis of immune response in males and females is required though.

8.4 Overall Conclusions and Importance of Study

The aim of this study was to determine the value and use of large-scale ‘omics’ data in determining novel functions for well-known genes. As discussed in Chapter 1, the growth of large-scale omics studies has significantly increased over the last decade and is like to continue in growth as technology improves and becomes cheaper. Studies such as FlyAtlas are extremely beneficial at producing an over-all picture of the transcriptome of different tissues. Transcriptomic data has also become more widely used with regards to disease vs healthy or stimulated vs un-stimulated studies, whereby the transcriptome of one state is compared to the other in order to determine which genes are disease causing or responsive to stimuli. With such studies come terabytes of data, which then requires full analysis, quantification and verification through standard small-scale experiments such as mutant studies. In this study we have

shown that *Drosophila melanogaster* offers an ideal opportunity to carry out such experiments. This study in particular showed the benefit of ‘omics’ data in determining novel functions for genes that have been previously well characterised in developmental or with regards to other tissues. The unexpectedly high expression of *fas2* and *dsx* in the Malpighian tubules hinted at a previously unknown function for these genes, in this tissue. This offered an ideal opportunity to determine the value of transcriptomic data with regards to expression vs function i.e. does high expression of a gene indicate a key function in that tissue. We opted to study genes, which had unusually high expression patterns in the MT, as this tissue offers a robust phenotype for epithelial physiology and as such there are several techniques, which one can use in order to help determine the function of a gene/protein within these tissues. Although the overall aim of this study was to determine novel functions for these two genes in the tubule, the importance of this study to the general science community is two-fold. Firstly, studies of gene function within the MT are extremely important. The MT offers a robust phenotype for the study of epithelial biology and can give biologist insights into the development and function of their human homologue, the kidneys. The MTs are also increasingly important tissues with regards to insecticide development as they are the key tissue involved in detoxification. As such several proteins expressed in the tubules are now targets for insecticides. Therefore discovering novel functions for genes within the MT is of interest to both the study of epithelial tissues and with regards to insecticide development. Secondly, this study offers an opportunity to determine the value and robustness of transcriptomic data with regards to determining novel functions for genes.

We have shown with regards to *fas2* that the high expression level do indeed indicate novel functions and as discussed in this Chapter we have evidence to suggest a key role for Fas2 in microvilli stabilisation and/or development in the Malpighian tubules. Depletion or over expression of Fas2 results in a decrease or increase in cAMP stimulated fluid secretion but not basal secretion levels. This suggests that the change to microvilli in mutant, over-expressors or RNAi lines against *fas2*, is not enough to disrupt basal secretion. This is not surprising in the case of lowering basal secretion, as it is likely that any mutation which affects basal secretion significantly would be lethal to the fly. A basal level of secretion

is required in order to maintain a balanced and functional metabolism and a basic level of osmoregulation, without which the fly would quickly die. We have also shown that Fas2 localisation is dynamic in response to cAMP stimulation and argue that this suggests Fas2 may be involved in a signalling mechanism, whereby it signals the movement of other proteins either to or from the apical brush border. The results presented here however are not conclusive enough to determine if this is the case and as discussed in the previous Sections several key experiments are required in order to determine if this is indeed the case. We have however shown that Fas2 co-localises with F-actin bundles on the basal cortex of the tubules ONLY after stimulation with cAMP. This is of some interest as F-Actin is known to undergo depolymerisation and re-polymerisation in response to cAMP in mosquito tubules. This re-arrangement of the Actin cytoskeleton is essential to the secretion phenotype. It would therefore be beneficial to determine the re-arrangement of F-Actin in Fas2 mutant or Fas2 over-expressor lines, in order to determine if Fas2 plays a role in this mechanism and that also leads to changes to in cAMP induced secretion response. Perhaps the most exciting outcome of this study is the potential role of Fas2 in microvilli stabilisation and/or development. As discussed throughout this thesis the tubules offer a robust phenotype for the study of epithelial biology and *D.melanogaster* is a key model organism. Understanding development and stabilisation of microvilli in human epithelial cells is of increasing importance as several diseases are known to involve disruption of microvilli. For example celiac disease leads to the atrophy of microvilli within the small intestines. This is due to an immune response to gluten, consumed through diet (Alaedini and Green, 2005). This induced immune response leads to the damage of microvilli within the intestines leading to several symptoms in patients including vitamin and mineral deficiencies, due to the inability to malabsorption of nutrients from food (Alaedini and Green, 2005; Dyduch et al., 1993; Shiner and Birbeck, 1961). Figure 8-1 shows an example of a biopsy from the small intestine of a celiac patient (Shiner and Birbeck, 1961). Each biopsy shows differing degrees of atrophy and interestingly several show similar images to those seen in our study. For example microvilli appear less dense, shorter and less well packed.

The only current treatment for Celiac disease involves the exclusion of gluten from the diet. Although effective, it can take up to 6 months for microvilli to

heal and in some case the damage is beyond repair. Therefore it would be beneficial to develop drugs in order to speed up or increase the development of microvilli in these patients in order to overcome the effects of years of malabsorption. This is just one example of the importance of microvilli in epithelial disease in humans, there are several other, including bacterial infections which can be devastating if not treated adequately. Studying how to treat microvilli damage and potentially speed up the process in human epithelial tissue would be extremely beneficial would therefore be extremely beneficial. With this in mind the further analysis of Fas2 involvement in microvilli development or stabilisation is extremely important as it may, in part lead to a fly model to study microvilli stabilisation and perhaps a model to study the effects of microvilli damage and repair.

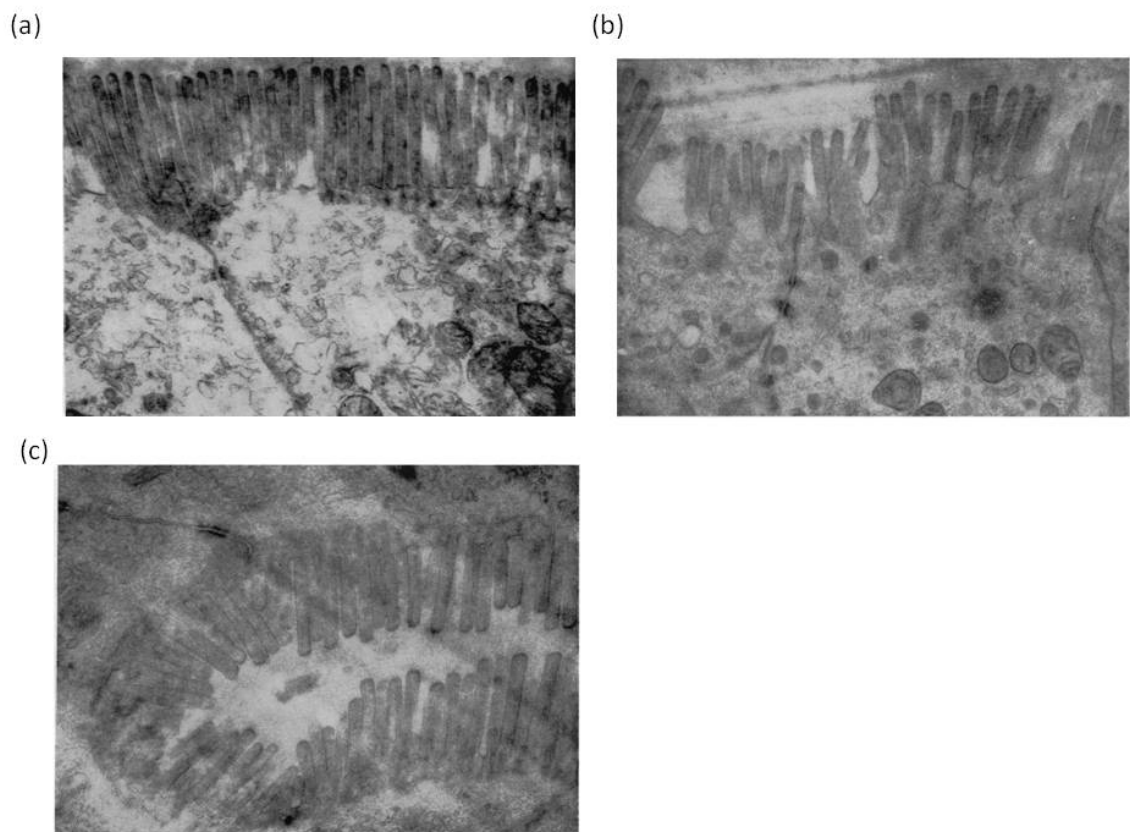


Figure 8-1 Comparison of Microvilli in the Small Intestine of Patients with Celiac Disease

- (a) Electron micrograph from control case C.B. showing normal microvilli and upper part of small intestinal surface cell. (b) Electron micrograph from coeliac patient J.N. showing short, irregular and loosely packed microvilli (c) Electron micrograph from control case F.T. showing short, though regular microvilli with variation in length. Adapted from (Shiner and Birbeck, 1961)

We have also shown that BN-PAGE analysis offers an opportunity to carry out 2D gel analysis on membrane protein complexes. This part of the study however was not fully completed due to time constraints. However we have shown that the procedure can be used in whole fly samples in order to detect protein-protein complexes and in theory with several more months of work could be used to study protein-protein interactions in the tubule. These experiments would also be beneficial in determining any interacting partners that Fas2 may have in the tubule, under both cAMP stimulated and un-stimulated conditions. As discussed in Chapter 1 transcriptomic data is only valid if used in conjunction with protein analysis, as the expression of a gene does not always lead to a functional protein. Therefore combining FlyAtlas data with a full proteome of the fly would be extremely beneficial to the science community. Traditional proteomic analysis is problematic with regards to membrane proteins, therefore BN-PAGE could offer a opportunity to look at membrane proteins. This again underlines the importance of a combination of large scale and small scale studies in order to obtain the most accurate and appropriate results. During the proteomic work in this study, it became apparent that obtaining large amounts of starting material for fly tissues can be problematic. With more time and money however this can be easily overcome, by increasing the number of dissections and the isolation procedure.

The results presented here for the role of *dsx* in the tubules are preliminary. Time did not permit the completion of many of the experiments planned at the beginning of the study. However we have shown that *dsx* is expressed in the tubules and we have determined several sex specific genes which are differentially expressed between males and females and therefore may be targets of *dsx*. Understanding differences between male and female tubules may be of importance with regards to immunity and metabolism and again underline the importance of sex biased in all experiments. Our experiments neither, indicated or ruled out a role for *dsx* expression in tubules effecting male/female differences in immunity and therefore with more time and money the role of *dsx* in the tubules would have been much more thoroughly examined. In particular the masculinisation of female tubules in an otherwise female fly or vice versa would have been of particular interest. Overall these experiments would have given an insight into the role of physiological dimorphism in the whole fly, i.e.

does every tissue in the fly require the ‘knowledge’ of which sex it is? For example if one tissue is masculinised in a female fly can it still interact with other tissues in a normal manner? These are basic questions in physiology which have still to be answered and may help to understand some of the difference between males and females, in particular in response to disease.

Overall this study has proved the ability of transcriptomic data to indicate the potential of novel functions for well-known genes. We have also shown the importance of follow up experiments in order to assess such data. Most importantly we have shown that even with an ideal model in which to test the robustness of transcriptomics data, there are many pitfalls. Determining the role of *fas2* and *dsx* in the tubules was not fully realised, within the time constraints of this study and several key experiments still are required. Showing, therefore that ‘omics’ data in some organisms is still in its infancy, as follow up experiments would prove even more difficult. However we believe we have shown the robustness and value of ‘omics’ with regards to *Drosophila melanogaster*, a organism which has been extensively studied and much is known about developmental, neurobiology, physiology and gene function, in that we have shown that with the aid of FlyAtlas we were able in part to determine a novel function for *fas2* within the tubules. The importance of which is validated by the study of *fas2* for over a decade, without the discovery of a function in the Malpighian tubules. To conclude with more money and more time perhaps FlyAtlas would have aided the discovery of more gene/protein function by highlighting the tissue in which to study the gene.

Appendices

Appendix 1: Media and solutions used in this study

Drosophila Media

Standard growth media per litre of water

10 g agar
15 g sucrose
30 g glucose
35 g dried yeast
15 g maize meal
10 g wheat germ
30 g treacle
10 g soya flour

Escherichia coli growth media

L-broth per litre of water

10 g Bacto-tryptone
5 g dried yeast
10 g NaCl

L-agar per litre of water

10 g Bacto-tryptone
5 g dried yeast
10 g NaCl
15 g Bacto-agar

12% SDS PAGE recipe

	5 ml	10 mL	15 mL	20 mL	25 mL	30 mL	40 mL	50mL
H ₂ O	1.7	3.4	5.1	6.8	8.5	10.2	13.6	17.0
4x Stacking	1.25	2.5	3.75	5.0	6.25	7.5	10.0	12.5
30% Acryl/0.8%bis-Acryl	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
10% APS	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020

Stacking Gels

	1 mL	2 mL	3 mL	4 mL	5 mL	6 mL	8 mL	10 mL
H ₂ O	0.573	1.147	1.72	2.29	2.87	3.44	4.59	5.73
4x Stacking	0.25	0.5	0.75	1.0	1.25	1.5	2.0	2.5
30% Acryl/0.8%bis- Acryl	0.167	0.333	0.5	0.667	0.833	1.0	1.33	1.67
10% APS	0.010	0.020	0.030	0.040	0.050	0.060	0.080	0.100
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.010

SDS PAGE Buffers

4x Resolving Buffer:

1.5 M Tris-Cl, pH 8.8

0.4% SDS

4x Stacking Buffer:

0.5 M Tris-Cl, pH 6.8

0.4% SDS

2x Sample Buffer:

125 mM Tris-Cl, pH 6.8

20% Glycerol

4% SDS

10% BME

0.04% Bromophenol Blue

0.4% SDS

Appendix 2: Primers used in this study

Primers:

	Forward	Reverse
Fas2 primer list		
QRT-PCR primers		
3		
transcripts	TCAGTCGGCGTCTTTTTGGC	CTTGGGCAGCCTGGTGTTGTTTC
transcript		
B	CGACAATGCTGCTGATTTTGC	AACGGAAACAGAAAGAAACCC
transcript		
A	CACGCCATTGGACGAAAAGG	ATTCAAGTGCTCTTCCCCCAGC
transcript		
C	ACCGTTGAGGTGATGGAGACC	TGTGAACAGTCGTTTGTGTAGCG
RT-PCR		
exon 2-3	GCAATGGAAGGACAATCGGAAC	ACAGCGAGGTAATCATCAGGGC
exon 3-4	CCCTGATGATTACCTCGCTGTC	GCCTTTACCTCGCACATTACCAC
exon 5-6	TCCAGCACGACAAAGAGGAACC	ATGCGATAGTATGTGTTGCCAC
Doublsex		
Alternative	GCAGCAGCAGAAGCAAAAAAGC	TGATTCCAGCACCGATAGCAACG
Female	AGGTGGTAGGTCATCGGGAACATC	TCGGGGCAAAGTAGTATTCGTTAC
Male	GTGGAATAAATCGCACTGTAGCC	GAGTCGGTGGACAAATCTGTGTG
Tra	CCCAGCATCGAGATTCCCGTGG	GGAGCGAGTGCGTCTGGTGG
CG8791	GGCCTGGAGGTCTTGCGGTG	AAGCAGTCCTGCCGGCACCT
YP3	GCCCGGATTCGGCGAGGATG	TGGTCAGGGTGGAGCCGAGG
ATD	GGAGCGGTCAACGCCAATGGT	CGGCGGTCATGCCGAAGTGA

V5 PRIMERS

Start site	ATAAGAATGCGGCCGCTAAACTATATGGGTGAATTGCCGCCAAATTCAGTCG GCTCTAGAGCTTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCT
Isoform A V5	TACCCACCGCCGAATTCTTCCC GCTCTAGAGCTTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCT
Isoform B V5	TACCAGCAGTGTGCGTCGTCGG GCTCTAGAGCTTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCT
Isoform C V5	TACCGGCTAACATTGAGGCTAG

Appendix 2

Sequencing results from fas2-V5 cloning aligned to Flybase sequence

```

10    20    30    40    50    60    70    80
Untitled
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ACTGACCCG
-----
Consensus:
ATGGGTGAATTGCCGCCAAATTCAGTCGGCGTCTTTTTGGCGCTGCTCCTCTGCAGCTGCTCTTTAATAGA
ACTGACCCG
-----
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aTGGGTGAATTGCCGCCAAATTCAGTCGGCGTCTTTTTGGCGCTGCTCCTCTGCAGCTGCTCTTTAATAGAA
CTGACCCG
03_006.ab1
aaaaatgaactttatTTTGGCGCTGCTCCTCTGCAGCTGCTCTTTAATAGAACTGACCCG
05_004.ab1
06_003.ab1
08_001.ab1
07_002.ab1

          90    100    110    120    130    140    150    160
Untitled
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Consensus:
TGCGCAGTCCCCCATCCTGGAGATTATCCCAAACAAGAAGTCCAGCGCAAGCCAGTGGGCAAGCCCCTGA
TCCTCACCT
-----
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TCCTCACCT
03_006.ab1
TGCGCAGTCCCCCATCCTGGAGATTATCCCAAACAAGAAGTCCAGCGCAAGCCAGTGGGCAAGCCCCTGA
TCCTCACCT
05_004.ab1
06_003.ab1
08_001.ab1
07_002.ab1

          170    180    190    200    210    220    230    240
Untitled
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CTGCCCAAG
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Consensus:
GCCGGCCACAGTTCCTGAGCCGTCCCTGGTCGCCGATCTGCAATGGAAGGACAATCGGAACAACACCATT
CTGCCCAAG
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GCCGGCCACAGTTCCTGAGCCGTCCCTGGTCGCCGATCTGCAATGGAAGGACAATCGGAACAACACCATT
CTGCCCAAG
03_006.ab1
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CTGCCCAAG
05_004.ab1

```

06_003.ab1
08_001.ab1
07_002.ab1

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Untitled

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Consensus:

CCGAATGGACGCAACCAGCCGCCGATGTACACGGAAACGCTGCCCCGGCGAAAGTTTGGCCCTGATGATTAC
CTCGCTGTC

04_005.ab1 CCGAATGGACGCAACCAGCCGCCG

03_006.ab1

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05_004.ab1

06_003.ab1

08_001.ab1

07_002.ab1

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Consensus:

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04_005.ab1

03_006.ab1

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05_004.ab1

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06_003.ab1

08_001.ab1

07_002.ab1

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Consensus:

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TGTGCGAG

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08_001.ab1

07_002.ab1

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Consensus:

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GTATGTGGT

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GTATGTGGT

05_004.ab1

GTAAAGGCCGATCCCAATCCAACAATCGACTGGCTGCGCAACGGAGATCCGATCCGCACGACCAACGACAA
GTATGTGGT

06_003.ab1

08_001.ab1

07_002.ab1

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Consensus:

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TTATCGAAA

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03_006.ab1

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TTATCGAAA

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TTATCGAAA

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08_001.ab1

07_002.ab1

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Consensus:

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TCGAGATGC

05_004.ab1

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TCGAGATGC

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Consensus:

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Consensus:

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Consensus:

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05_004.ab1

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07_002.ab1

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Consensus:

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TGAATTTGC

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03_006.ab1

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07_002.ab1

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Consensus:

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03_006.ab1

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06_003.ab1

TCCGGACTTTAGCCACATGAAGGAGCTGCCTCCGGTTTTCTCGTGGGAGCAGCGCAAGGCGAATCTCAGCT
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07_002.ab1

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Consensus:

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CTAAAGATT

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07_002.ab1

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Consensus:

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CATTGCGAC

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03_006.ab1

05_004.ab1

06_003.ab1

GTCGGCACTGGACCCCGCAGCGATTTGATTGTGCATCCCGTAACGAGGCAGTATTACTCTGGATACAAGTG
CATTGCGAC

08_001.ab1

07_002.ab1

1530 1540 1550 1560 1570 1580 1590 1600

Untitled

CAACATCCATGGAACCGCTGAACATGATATGCAACTGAAGGAGGCACGTGTCCCTGATTTTGTGTCCGAAG
CCAAACCCA

Consensus:

CAACATCCATGGAACCGCTGAACATGATATGCAACTGAAGGAGGCACGTGTCCCTGATTTTGTGTCCGAAG
CCAAACCCA

04_005.ab1

03_006.ab1

05_004.ab1

06_003.ab1

CAACATCCATGGAACCGCTGAACATGATATGCAACTGAAGGAGGCACGTGTCCCTGATTTTGTGTCCGAAG
CCAAACCCA

08_001.ab1

cCA

07_002.ab1

1610 1620 1630 1640 1650 1660 1670 1680

Untitled

GTCAACTGACCGCCACCACGATGACCTTCGACATTCGCGGCCCATCAACCGAACTAGGCCTGCCCATTCTGG
CGTACAGT

Consensus:

GTCAACTGACCGCCACCACGATGACCTTCGACATTCGCGGCCCATCAACCGAACTAGGCCTGCCCANTCTG
GCGTACAGT

04_005.ab1

03_006.ab1

05_004.ab1

06_003.ab1

GTCAACTGACCGCCACCACGATGACCTTCGACATTCGCGGCCCATCAACCGAACTAGGCCTGCCCATTCTGG
CGTACAGT

08_001.ab1

GTCAACTGACCGCCACCACGATGACCTTCGACATTCGCGGCCCATCAACCGAACTAGGCCTGCCCCA-
TCTGGCGTACAGT

07_002.ab1

1690 1700 1710 1720 1730 1740 1750 1760

Untitled

GTGCAGTATAAGGAGGCCCTAAATCCGGACTGGTCGACGGCCTATAACCGCAGTTGGTCACCAGATTGCGC
GTACATTGT

Consensus:

GTGCAGTATAAGGAGGCCCTAAATCCGGACTGGTCGACGGCCTATAACCGCAGTTGGTCACCAGATTGCGC
GTACATTGT

04_005.ab1

03_006.ab1

05_004.ab1

06_003.ab1

GTGCAGTATAAGGAGGCCCTAAATCCGGACTGGTCGACGGCCTATAACCGCAGTTGGTCACCAGATTGCGC
GTACATTGT

08_001.ab1

GTGCAGTATAAGGAGGCCCTAAATCCGGACTGGTCGACGGCCTATAACCGCAGTTGGTCACCAGATTGCGC
GTACATTGT

07_002.ab1

1770 1780 1790 1800 1810 1820 1830 1840

Untitled

GGAGGGACTGCGACCACAGACGGAGTACAGCTTCCGATTTGCCGCCCGCAACCAGGTGGGATTAGGAAATT
GGGGCGTCA

Consensus:

GGAGGGACTGCGACCACAGACGGAGTACAGCTTCCGATTTGCCGCCCCGAACCAGGTGGGATTAGGAAATT
GGGGCGTCA

04_005.ab1

03_006.ab1

05_004.ab1

06_003.ab1

GGAGGGACTGCGACCACAGACGGAGTACAGCTTCCGATTTGCCGCCCCGAACCAGGTGGGATTAGGAAATT
GGGGCGTCA

08_001.ab1

GGAGGGACTGCGACCACAGACGGAGTACAGCTTCCGATTTGCCGCCCCGAACCAGGTGGGATTAGGAAATT
GGGGCGTCA

07_002.ab1

1850 1860 1870 1880 1890 1900 1910 1920

Untitled

ATCAGCAACAGTCGACACCACGACGATCGGCTCCCGAGGAGCCCCAAGCCATTGCATAATCCCGTCCAGCAC
GACAAAGAG

Consensus:

ATCAGCAACAGTCGACACCACGACGATCGGCTCCCGAGGAGCCCCAAGCCATTGCATAATCCCGTCCAGCAC
GACAAAGAG

04_005.ab1

03_006.ab1

05_004.ab1

06_003.ab1

ATCAGCAACAGTCGACACCACGACGATCGGCTCCCGAGGAGCCCCAAGCCATTGCATAATCCCGTCCAGCAC
GACAAAGAG

08_001.ab1

ATCAGCAACAGTCGACACCACGACGATCGGCTCCCGAGGAGCCCCAAGCCATTGCATAATCCCGTCCAGCAC
GACAAAGAG

07_002.ab1

1930 1940 1950 1960 1970 1980 1990 2000

Untitled

GAACCGGTGGTCGTGTCGCCCTATTCCGATCATTTTCGAGCTGCGCTGGGGCGTGCCCCGCCGACAACGGAG
AGCCTATTGA

Consensus:

GAACCGGTGGTCGTGTCGCCCTATTCCGATCATTTTCGAGCTGCGTTGGGGCGTGCCCCGCCGACAACGGAG
AGCCTATTGA

04_005.ab1

03_006.ab1

05_004.ab1

06_003.ab1

GAACCGGTGGTCGTGTCGCCCTATTCCGATCATTTTCGAGCTGCGTTGGGGCGTGCCCCGCCGACAACGGAG
AGCCTATTGA

08_001.ab1

GAACCGGTGGTCGTGTCGCCCTATTCCGATCATTTTCGAGCTGCGTTGGGGCGTGCCCCGCCGACAACGGAG
AGCCTATTGA

07_002.ab1

2010 2020 2030 2040 2050 2060 2070 2080

Untitled

TAGGTACCAGATCAAATACTGTCCGGGCGTTAAGATCAGCGGCACCTGGACGGAAGTGGAGAACTCCTGCA
ACACCGTTG

Consensus:

TAGGTACCAGATCAAATACTGTCCGGGCGTTAAGATCAGCGGCACCTGGACGGAAGTGGAGAACTCCTGCA
ACACCGTTG

04_005.ab1
03_006.ab1
05_004.ab1
06_003.ab1
TAGGTACCAGATCAAATACTGTCCGGGCGTTAAGATCAGCGGCACCTGGACGGAAGTGGAGAACTCCTGCA
ACAC
08_001.ab1
TAGGTACCAGATCAAATACTGTCCGGGCGTTAAGATCAGCGGCACCTGGACGGAAGTGGAGAACTCCTGCA
ACACCGTTG
07_002.ab1

2090 2100 2110 2120 2130 2140 2150 2160

Untitled
AGGTGATGGAGACCACATCCTTCGAGATGACCCAGCTGGTGGGCAACACATACTATCGCATTGAACTGAAG
GCGCACAAC

Consensus:
AGGTGATGGAGACCACATCCTTCGAGATGACCCAGCTGGTGGGCAACACATACTATCGCATTGAACTGAAG
GCGCACAAC

04_005.ab1
03_006.ab1
05_004.ab1
06_003.ab1
08_001.ab1
AGGTGATGGAGACCACATCCTTCGAGATGACCCAGCTGGTGGGCAACACATACTATCGCATTGAACTGAAG
GCGCACAAC
07_002.ab1

2170 2180 2190 2200 2210 2220 2230 2240

Untitled
GCCATCGGCTATTCATCGCCTGCTTCCATTATCATGAAGACGACACGAGGAATTGACGTCATCCAAGTGGCT
GAGCGACA

Consensus:
GCCATCGGCTATTCATCGCCTGCTTCCATTATCATGAAGACGACACGAGGAATTGACGTCATCCAAGTGGCT
GAGCGACA

04_005.ab1
03_006.ab1
05_004.ab1
06_003.ab1
08_001.ab1
GCCATCGGCTATTCATCGCCTGCTTCCATTATCATGAAGACGACACGAGGAATTGACGTCATCCAAGTGGCT
GAGCGACA
07_002.ab1

2250 2260 2270 2280 2290 2300 2310 2320

Untitled
GGTCTTCTCCTCGGCGGCCATCGTGGGCATCGCAATCGGCGGTGTCCTCCTGCTTCTGTTTCGTGGTCGACC
TACTCTGCT

Consensus:
GGTCTTCTCCTCGGCGGCCATCGTGGGCATCGCAATCGGCGGTGTCCTCCTGCTTCTGTTTCGTGGTCGACC
TACTCTGCT

04_005.ab1
03_006.ab1
05_004.ab1
06_003.ab1

08_001.ab1

GGTCTTCTCCTCGGCGGCCATCGTGGGCATCGCAATCGGCGGTGTCTCCTGCTTCTGTTCTGTGGTCGACC
TACTCTGCT

07_002.ab1

2330 2340 2350 2360 2370 2380 2390 2400

Untitled

GCATCACCGTCCACATGGGCGTCATGGCCACGATGTGCCGCAAGGCCAAGCGATCGCCTTCCGAAATCGAC
GACGAGGCC

Consensus:

GCATCACCGTCCACATGGGCGTCATGGCCACGATGTGCCGCAAGGCCAAGCGATCGCCTTCCGAAATCGAC
GACGAGGCC

04_005.ab1

03_006.ab1

05_004.ab1

06_003.ab1

08_001.ab1

GCATCACCGTCCACATGGGCGTCATGGCCACGATGTGCCGCAAGGCCAAGCGATCGCCTTCCGAAATCGAC
GACGAGGCC

07_002.ab1

2410 2420 2430 2440 2450 2460 2470 2480

Untitled

AAGCTGGGCAGTGGCCAGCTGGTAAAGGAGCCACCGCCGTCACCGTTGCCACTGCCGCCGCCCGTCAAACCT
GGGCGGTTT

Consensus:

AAGCTGGGCAGTGGCCAGCTGGTAAAGGAGCCACCGCCGTCGCCGTTGCCACTGCCGCCGCCCGTCAAACCT
GGGCGGTTT

04_005.ab1

03_006.ab1

05_004.ab1

06_003.ab1

08_001.ab1

AAGCTGGGCAGTGGCCAGCTGGTAAAGGAGCCACCGCCGTCGCCGTTGCCACTGCCGCCGCCCGTCAAACCT
GGGCGGTTT

07_002.ab1

2490 2500 2510 2520 2530 2540 2550 2560

Untitled

GCCCATGAGCACGCCATTGGACGAAAAGGAGCCGCTCCGCACGCCAACAGGCAGCATCAAACAGAACTCGA
CCATCGAAT

Consensus:

GCCCATGAGCACGCCATTGGACGAAAAGGAGCCGCTCCGCACGCCAACAGGCAGCATCAAACAGAACTCGA
CCATCGAAT

04_005.ab1

03_006.ab1

05_004.ab1

06_003.ab1

08_001.ab1

GCCCATGAGCACGCCATTGGACGAAAAGGAGCCGCTCCGCACGCCAACAGGCAGCATCAAACAGAA

07_002.ab1 tggccccgccacatgCAGCATC-AACAGAACTCGACCATCGAAT

2570 2580 2590 2600 2610 2620 2630 2640

Untitled

TCGACGGGCGATTCTGTGCACTCGCGCAGTGGCGAGATAATCGGGAAGAATTCGGCGGTGGGTAAGCCTAT
CCCTAACCTT

Consensus:

TCGACGGGCGATTTCGTGCACTCGCGCAGTGGCGAGATAATCGGGAAGAATTCGGCGGTGGGTAAGCCTAT
CCCTAACCT

04_005.ab1

03_006.ab1

05_004.ab1

06_003.ab1

08_001.ab1

07_002.ab1

TCGACGGGCGATTTCGTGCACTCGCGCAGTGGCGAGATAATCGGGAAGAATTCGGCGGTGGGTAAGCCTAT
CCCTAACCT

2650 2660

Untitled CTCCTCGGTCTCGATTCTACG

Consensus: CTCCTCGGTCTCGATTCTAC

04_005.ab1

03_006.ab1

05_004.ab1

06_003.ab1

08_001.ab1

07_002.ab1 CTCCTCGGTCTCGATTCTACt

Sequencing results for fas2-B-V5 aligned to FlyBase sequence

10 20 30 40 50 60 70 80

Untitled_3

ATGGGTGAATTGCCGCCAAATTCAGTCGGCGTCTTTTTGGCGCTGCTCCTCTGCAGCTGCTCTTTAATAGA
ACTGACCCG

Consensus:

ATGGGTGAATTGCCGCCAAATTCAGTCGGCGTCTTTTTGGCGCTGCTCCTCTGCAGCTGCTCTTTAATAGA
ACTGACCCG

10_015.ab1

aTGGGTGAATTGCCGCCAAATTCAGTCGGCGTCTTTTTGGCGCTGCTCCTCTGCAGCTGCTCTTTAATAGAA
CTGACCCG

09_016.ab1

cacgtaacttatTTTGGCGCTGCTCCTCTGCAGCTGCTCTTTAATAGAACTGACCCG

11_014.ab1

12_013.ab1

14_011.ab1

13_012.ab1

90 100 110 120 130 140 150 160

Untitled_3

TGCGCAGTCCCCCATCCTGGAGATTTATCCCAAACAAGAAGTCCAACGCAAGCCAGTGGGCAAGCCCCTGA
TCCTCACCT

Consensus:

TGCGCAGTCCCCCATCCTGGAGATTTATCCCAAACAAGAAGTCCAACGCAAGCCAGTGGGCAAGCCCCTGA
TCCTCACCT

10_015.ab1

TGCGCAGTCCCCCATCCTGGAGATTTATCCCAAACAAGAAGTCCAACGCAAGCCAGTGGGCAAGCCCCTGA
TCCTCACCT

09_016.ab1

TGCGCAGTCCCCATCCTGGAGATTTATCCCAAACAAGAAGTCCAACGCAAGCCAGTGGGCAAGCCCCTGA
TCCTCACCT

11_014.ab1

12_013.ab1

14_011.ab1

13_012.ab1

170 180 190 200 210 220 230 240

Untitled_3

GCCGGCCACAGTTCCTGAGCCGTCCCTGGTCGCCGATCTGCAATGGAAGGACAATCGGAACAACACCATT
CTGCCCAAG

Consensus:

GCCGGCCACAGTTCCTGAGCCGTCCCTGGTCGCCGATCTGCAATGGAAGGACAATCGGAACAACACCATT
CTGCCCAAG

10_015.ab1

GCCGGCCACAGTTCCTGAGCCGTCCCTGGTCGCCGATCTGCAATGGAAGGACAATCGGAACAACACCATT
CTGCCCAAG

09_016.ab1

GCCGGCCACAGTTCCTGAGCCGTCCCTGGTCGCCGATCTGCAATGGAAGGACAATCGGAACAACACCATT
CTGCCCAAG

11_014.ab1

12_013.ab1

14_011.ab1

13_012.ab1

250 260 270 280 290 300 310 320

Untitled_3

CCGAATGGACGCAACCAGCCGCCGATGTACACGGAAACGCTGCCCGCGAAAGTTTGGCCCTGATGATTAC
CTCGCTGTC

Consensus:

CCGAATGGACGCAACCAGCCGCCGATGTACACGGAAACGCTGCCCGCGAAAGTTTGGCCCTGATGATTAC
CTCGCTGTC

10_015.ab1 CCGAATGGACGCAACCAGCCGC

09_016.ab1

CCGAATGGACGCAACCAGCCGCCGATGTACACGGAAACGCTGCCCGCGAAAGTTTGGCCCTGATGATTAC
CTCGCTGTC

11_014.ab1

12_013.ab1

14_011.ab1

13_012.ab1

330 340 350 360 370 380 390 400

Untitled_3

GGTGAAATGGGCGGCAAGTACTACTGCACCGCCTCCTATGCAAATACGGAGATCCTCGAGAAGGGCGTCA
CAATTA AAA

Consensus:

GGTGAAATGGGCGGCAAGTACTACTGCACCGCCTCCTATGCAAATACGGAGATCCTCGAGAAGGGCGTCA
CAATTA AAA

10_015.ab1

09_016.ab1

GGTGAAATGGGCGGCAAGTACTACTGCACCGCCTCCTATGCAAATACGGAGATCCTCGAGAAGGGCGTCA
CAATTA AAA

11_014.ab1

tACGGAGATCCTCGAGAAGGGCGTCACAATTA AAA

12_013.ab1

14_011.ab1

13_012.ab1

410 420 430 440 450 460 470 480
Untitled_3
CTTACGTGGCCATCACCTGGACAAATGCCCCTGAGAATCAGTACCCCACTCTTGGCCAAGACTATGTGGTAA
TGTGCGAG

Consensus:
CTTACGTGGCCATCACCTGGACAAATGCCCCTGAGAATCAGTACCCCACTCTTGGCCAAGACTATGTGGTAA
TGTGCGAG

10_015.ab1
09_016.ab1
CTTACGTGGCCATCACCTGGACAAATGCCCCTGAGAATCAGTACCCCACTCTTGGCCAAGACTATGTGGTAA
TGTGCGAG
11_014.ab1
CTTACGTGGCCATCACCTGGACAAATGCCCCTGAGAATCAGTACCCCACTCTTGGCCAAGACTATGTGGTAA
TGTGCGAG
12_013.ab1
14_011.ab1
13_012.ab1

490 500 510 520 530 540 550 560
Untitled_3
GTAAAGGCCGATCCCAATCCAACAATCGACTGGCTGCGCAACGGAGATCCGATCCGCACGACCAACGACAA
GTATGTGGT

Consensus:
GTAAAGGCCGATCCCAATCCAACAATCGACTGGCTGCGCAACGGAGATCCGATCCGCACGACCAACGACAA
GTATGTGGT

10_015.ab1
09_016.ab1
GTAAAGGCCGATCCCAATCCAACAATCGACTGGCTGCGCAACGGAGATCCGATCCGCACGACCAACGACAA
GTATGTGGT
11_014.ab1
GTAAAGGCCGATCCCAATCCAACAATCGACTGGCTGCGCAACGGAGATCCGATCCGCACGACCAACGACAA
GTATGTGGT
12_013.ab1
14_011.ab1
13_012.ab1

570 580 590 600 610 620 630 640
Untitled_3
GCAAACCAATGGCCTGCTAATCCGAAATGTCCAGGAGAGCGATGAAGGCATCTACACTTGCCGTGCAGCCG
TTATCGAAA

Consensus:
GCAAACCAATGGCCTGCTAATCCGAAATGTCCAGGAGAGCGATGAAGGCATCTACACTTGCCGTGCAGCCG
TTATCGAAA

10_015.ab1
09_016.ab1
GCAAACCAATGGCCTGCTAATCCGAAATGTCCAGGAGAGCGATGAAGGCATCTACACTTGCCGTGCAGCCG
TTATCGAAA
11_014.ab1
GCAAACCAATGGCCTGCTAATCCGAAATGTCCAGGAGAGCGATGAAGGCATCTACACTTGCCGTGCAGCCG
TTATCGAAA
12_013.ab1
14_011.ab1
13_012.ab1

650 660 670 680 690 700 710 720

Untitled_3

CTGGTGAGCTATTGGAACGCACCATTTCGCGTGGAGGTCTTCATTAGCCGGAGATCATATCGCTACCCACC
AATCTGGAG

Consensus:

CTGGTGAGCTATTGGAACGCACCATTTCGCGTGGAGGTCTTCATTAGCCGGAGATCATATCGCTACCCACC
AATCTGGAG

10_015.ab1

09_016.ab1

CTGGTGAGCTATTGGAACGCACCATTTCGCGTGGAGGTCTTCATTAGCCGGAGATCATATCGCTACCCACC
AATCTGGAG

11_014.ab1

CTGGTGAGCTATTGGAACGCACCATTTCGCGTGGAGGTCTTCATTAGCCGGAGATCATATCGCTACCCACC
AATCTGGAG

12_013.ab1

14_011.ab1

13_012.ab1

730 740 750 760 770 780 790 800

Untitled_3

GCCGTCGAGGGCAAGCCGTTTGCAGCCAACTGCACAGCAAGAGGCAAACCAGTACCGGAGATTAGTTGGAT
TCGAGATGC

Consensus:

GCCGTCGAGGGCAAGCCGTTTGCAGCCAACTGCACAGCAAGAGGCAAACCAGTACCGGAGATTAGTTGGAT
TCGAGATGC

10_015.ab1

09_016.ab1

GCCGTCGAGGGCAAGCCGTTTGCAGCCAACTGCACAGCAAGAGGCAAACCAGTACCGGAGATTAGTTGGAT
TCGAGATGC

11_014.ab1

GCCGTCGAGGGCAAGCCGTTTGCAGCCAACTGCACAGCAAGAGGCAAACCAGTACCGGAGATTAGTTGGAT
TCGAGATGC

12_013.ab1

14_011.ab1

13_012.ab1

810 820 830 840 850 860 870

Untitled_3 CACACAACCTGAACGTGGCGACCGCCGATCGCTTCCAAGTGAATCCCCAACTGGCCTGG-
TTACCATCAGCTCCGTTAGC

Consensus:

CACACAACCTGAACGTGGCGACCGCCGATCGCTTCCAAGTGAATCCCCAACTGGCCTGGNTTACCATCAGCT
CCGTTAGC

10_015.ab1

09_016.ab1

CACACAACCTGAACGTGGCGACCGCCGATCGCTTCCAAGTGAATCCCCAACTGGCCTGGGTTACCATCAGC
TCCGTTAGC

11_014.ab1 CACACAACCTGAACGTGGCGACCGCCGATCGCTTCCAAGTGAATCCCCAACTGGCCTGG-
TTACCATCAGCTCCGTTAGC

12_013.ab1

14_011.ab1

13_012.ab1

880 890 900 910 920 930 940 950

Untitled_3

CAGGATGATTATGGCACATACACATGCTTGGCCAAGAATAGGGCCGGTGTAGTGGATCAGAAGACCAAGCT
GAATGTTTT

Consensus:

CAGGATGATTATGGCACATACACATGCTTGGCCAAGAATAGGGCCGGTGTAGTGGATCAGAAGACCAAGCT
GAATGTTTT

10_015.ab1

09_016.ab1

CAGGATGATTATGGCACATACACATGCTTGGCCAAGAATAGGGCCGGTGTAGTGGATCAGAAGACCAAGCT
GAATGTTTT

11_014.ab1

CAGGATGATTATGGCACATACACATGCTTGGCCAAGAATAGGGCCGGTGTAGTGGATCAGAAGACCAAGCT
GAATGTTTT

12_013.ab1

14_011.ab1

13_012.ab1

960 970 980 990 1000 1010 1020 1030

Untitled_3

GGTGCGTCCGCAGATCTATGAGTTGTACAATGTGACCGGGGCCAGGACCAAGGAGATTGCCATAACCTGCC
GTGCCAAAG

Consensus:

GGTGCGTCCGCAGATCTATGAGTTGTACAATGTGACCGGGGCCAGGACCAAGGAGATTGCCATAACCTGCC
GTGCCAAAG

10_015.ab1

09_016.ab1

GGTGCGTCCGCAGATCTATGAGTTGTACAATGTGACCGGGGCCAGGACCAAGGAGATTGCCATAACCTGCC
GTGCCAAAG

11_014.ab1

GGTGCGTCCGCAGATCTATGAGTTGTACAATGTGACCGGGGCCAGGACCAAGGAGATTGCCATAACCTGCC
GTGCCAAAG

12_013.ab1

14_011.ab1

13_012.ab1

1040 1050 1060 1070 1080 1090 1100 1110

Untitled_3

GACGTCCGGCACCAGCGATTACCTTCCGTCGTTGGGGAACACAGGAGGAGTACACGAACGGGCAGCAGGA
TGACGATCCC

Consensus:

GACGTCCGGCACCAGCGATTACCTTCCGTCGTTGGGGAACACAGGAGGAGTACACGAACGGGCAGCAGGA
TGACGATCCC

10_015.ab1

09_016.ab1 GACGTCCGGCACCAGCGATTACCTTCCGTCG-TGGGGACCACAGGA-GAGTACACG-
ACGGGCAGCA-GATGACGAT-CC

11_014.ab1

GACGTCCGGCACCAGCGATTACCTTCCGTCGTTGGGGAACACAGGAGGAGTACACGAACGGGCAGCAGGA
TGACGATCCC

12_013.ab1 gACGTCCGGCACCAGCGATTA-CTT-CGTCGTTGGGG-

ACACAGGAGGAGTACACGAACGGGCAGCAGGATGACGATCCC

14_011.ab1

13_012.ab1

1120 1130 1140 1150 1160 1170 1180 1190

Untitled_3

CGCATCATTTTGGAGCCGAATTTTCGATGAGGAGCGCGGCGAGAGCACCGGCACCC-

TTGCGATCTCCAATGCCGAGCGTT

Consensus:

CGCATCATTTTGGAGCCGAATTTTCGATGAGGAGCGCGGCGAGAGCACCGGCACCCNTTCGCATCTCCAATG
CCGAGCGTT

10_015.ab1
09_016.ab1 CGCATCA-TTT-GAG-CG-ATTTTC
11_014.ab1 CGCATCATTTTGGAGCCGAATTTTCGATGAGGAGCGCGGCGAGAGCACCGGCACCC-
TTCGCATCTCCAATGCCGAGCGTT
12_013.ab1
CGCATCATTTTGGAGCCGAATTTTCGATGAGGAGCGCGGCGAGAGCACCGGCACCTTTTCGCATCTCCAATG
CCGAGCGTT
14_011.ab1
13_012.ab1

1200 1210 1220 1230 1240 1250 1260 1270
Untitled_3
CCGACGATGGACTTTACCACTGTATTGCCCCGGAACAAGGGAGCCGATGCCTACAAGACTGGACACATTACC
GTTGAATTT

Consensus:
CCGACGATGGACTTTACCACTGTATTGCCCCGGAACAAGGGAGCCGATGCCTACAAGACTGGACACATTACC
GTTGAATTT

10_015.ab1
09_016.ab1
11_014.ab1
CCGACGATGGACTTTACCACTGTATTGCCCCGGAACAAGGGAGCCGATGCCTACAAGACTGGACACATTACC
GTTGAATTT
12_013.ab1
CCGACGATGGACTTTACCACTGTATTGCCCCGGAACAAGGGAGCCGATGCCTACAAGACTGGACACATTACC
GTTGAATTT
14_011.ab1 gGGAGCCGATGCCTACAAGACTGGACACATTACCGTTGAATTT
13_012.ab1

1280 1290 1300 1310 1320 1330 1340 1350
Untitled_3 GCTCCGGACTTTAGCCACATGAAGGAGCTGCCTCCGG-
TTTTCTCATGGGAGCAGCGCAAGGCGAATCTCAGCTG-CCTG

Consensus: GCTCCGGACTTTAGCCACATGAAGGAGCTGCCTCCGG-
TTTTCTCGTGGGAGCAGCGCAAGGCGAATCTCAGCTG-CCTG

10_015.ab1
09_016.ab1
11_014.ab1
GCTCCGGACTTTAGCCACATGAAGGAGCTGCCTCCGGGTTTTCTCGTGGGAGCAGCGCAAGGCGAATCTCA
GCTG-CCTG
12_013.ab1 GCTCCGGACTTTAGCCACATGAAGGAGCTGCCTCCGG-
TTTTCTCGTGGGAGCAGCGCAAGGCGAATCTCAGCTG-CCTG
14_011.ab1 GCTCCTGACTTTAGCCACATGAAGGAGCTGCCTCCGG-TTTTCTCG-
GGGAGCAGCGCAAGGCGAATCTCAGCTGTCTTG
13_012.ab1

1360 1370 1380 1390 1400 1410 1420 1430
Untitled_3
GCCATGGGTATTCCGAATGCCACAATCGAATGGCACTGGAATGGTCGTAAGATCAAGGATCTGTACGATAC
CAATCTAAA

Consensus:
GCCATGGGCATCCCGAATGCCACAATCGAATGGCACTGGAATGGTCGTAAGATCAAGGATCTGTACGATAC
CAATCTAAA

10_015.ab1
09_016.ab1
11_014.ab1 GCCATGGGCATCCCG-ATG-CAC-ATCGAATGGCACTGGAATGGTCGT-AGATC-A-GATCTGT
12_013.ab1
GCCATGGGCATCCCGAATGCCACAATCGAATGGCACTGGAATGGTCGTAAGATCAAGGATCTGTACGATAC
CAATCTAAA

14_011.ab1

GCCATGGGCATCCCGAATGCCACAATCGAATGGCACTGGAATGGTCGTAAGATCAAGGATCTGTACGATAC
CAATCTAAA

13_012.ab1

1440 1450 1460 1470 1480 1490 1500 1510

Untitled_3

GATTGTGGCACTGGACCCCGCAGCGATTTGATTGTGCATCCCGTAACGAGGCAGTATTACTCTGGATACA
AGTGCATTG

Consensus:

GATTGTGGCACTGGACCCCGCAGCGATTTGATTGTGCATCCCGTAACGAGGCAGTATTACTCTGGATACA
AGTGCATTG

10_015.ab1

09_016.ab1

11_014.ab1

12_013.ab1

GATTGTGGCACTGGACCCCGCAGCGATTTGATTGTGCATCCCGTAACGAGGCAGTATTACTCTGGATACA
AGTGCATTG

14_011.ab1

GATTGTGGCACTGGACCCCGCAGCGATTTGATTGTGCATCCCGTAACGAGGCAGTATTACTCTGGATACA
AGTGCATTG

13_012.ab1

1520 1530 1540 1550 1560 1570 1580 1590

Untitled_3

CGACCAACATCCATGGAACCGCTGAACATGATATGCAACTGAAGGAGGCACGTGTCCCTGATTTTGTGTCC
GAAGCCAAA

Consensus:

CGACCAACATCCATGGAACCGCTGAACATGATATGCAACTGAAGGAGGCACGTGTCCCTGATTTTGTGTCC
GAAGCCAAA

10_015.ab1

09_016.ab1

11_014.ab1

12_013.ab1

CGACCAACATCCATGGAACCGCTGAACATGATATGCAACTGAAGGAGGCACGTGTCCCTGATTTTGTGTCC
GAAGCCAAA

14_011.ab1

CGACCAACATCCATGGAACCGCTGAACATGATATGCAACTGAAGGAGGCACGTGTCCCTGATTTTGTGTCC
GAAGCCAAA

13_012.ab1

1600 1610 1620 1630 1640 1650 1660 1670

Untitled_3

CCCAGTCAACTGACCGCCACCACGATGACCTTCGACATTCGCGGCCCATCAACCGAACTAGGCCTGCCATT
CTGGCGTA

Consensus:

CCCAGTCAACTGACCGCCACCACGATGACCTTCGACATTCGCGGCCCATCAACCGAACTAGGCCTGCCATT
CTGGCGTA

10_015.ab1

09_016.ab1

11_014.ab1

12_013.ab1

CCCAGTCAACTGACCGCCACCACGATGACCTTCGACATTCGCGGCCCATCAACCGAACTAGGCCTGCCATT
CTGGCGTA

14_011.ab1

CCCAGTCAACTGACCGCCACCACGATGACCTTCGACATTCGCGGCCCATCAACCGAACTAGGCCTGCCATT
CTGGCGTA

13_012.ab1

1680 1690 1700 1710 1720 1730 1740 1750

Untitled_3

CAGTGTGCAGTATAAGGAGGCCCTAAATCCGGACTGGTCGACGGCCTATAACCGCAGTTGGTCACCAGATT
CGCCGTACA

Consensus:

CAGTGTGCAGTATAAGGAGGCCCTAAATCCGGACTGGTCGACGGCCTATAACCGCAGTTGGTCACCAGATT
CGCCGTACA

10_015.ab1

09_016.ab1

11_014.ab1

12_013.ab1

CAGTGTGCAGTATAAGGAGGCCCTAAATCCGGACTGGTCGACGGCCTATAACCGCAGTTGGTCACCAGATT
CGCCGTACA

14_011.ab1

CAGTGTGCAGTATAAGGAGGCCCTAAATCCGGACTGGTCGACGGCCTATAACCGCAGTTGGTCACCAGATT
CGCCGTACA

13_012.ab1

1760 1770 1780 1790 1800 1810 1820 1830

Untitled_3

TTGTGGAGGGACTGCGACCACAGACGGAGTACAGCTTCCGATTTGCCGCCCCGAACCAGGTGGGATTAGG
AAATTGGGGC

Consensus:

TTGTGGAGGGACTGCGACCACAGACGGAGTACAGCTTCCGATTTGCCGCCCCGAACCAGGTGGGATTAGG
AAATTGGGGC

10_015.ab1

09_016.ab1

11_014.ab1

12_013.ab1

TTGTGGAGGGACTGCGACCACAGACGGAGTACAGCTTCCGATTTGCCGCCCCGAACCAGGTGGGATTAGG
AAATTGGGGC

14_011.ab1

TTGTGGAGGGACTGCGACCACAGACGGAGTACAGCTTCCGATTTGCCGCCCCGAACCAGGTGGGATTAGG
AAATTGGGGC

13_012.ab1

1840 1850 1860 1870 1880 1890 1900 1910

Untitled_3

GTCAATCAGCAACAGTCGACACCACGACGATCGGCTCCCGAGGAGCCCAAGCCATTGCATAATCCCGTCCA
GCACGACAA

Consensus:

GTCAATCAGCAACAGTCGACACCACGACGATCGGCTCCCGAGGAGCCCAAGCCATTGCATAATCCCGTCCA
GCACGACAA

10_015.ab1

09_016.ab1

11_014.ab1

12_013.ab1

GTCAATCAGCAACAGTCGACACCACGACGATCGGCTCCCGAGGAGCCCAAGCCATTGCATAATCCCGTCCA
GCACGACAA

14_011.ab1

GTCAATCAGCAACAGTCGACACCACGACGATCGGCTCCCGAGGAGCCCAAGCCATTGCATAATCCCGTCCA
GCACGACAA

13_012.ab1

1920 1930 1940 1950 1960 1970 1980 1990

Untitled_3

AGAGGAACCGGTGGTCGTGTCGCCCTATTCCGATCATTTTCGAGCTGCGCTGGGGCGTGCCCGCCGACAAC
GGAGAGCCTA

Consensus:

AGAGGAACCGGTGGTCGTGTCGCCCTATTCCGATCATTTTCGAGCTGCGTTGGGGCGTGCCCGCCGACAAC
GGAGAGCCTA

10_015.ab1

09_016.ab1

11_014.ab1

12_013.ab1

AGAGGAACCGGTGGTCGTGTCGCCCTATTCCGATCATTTTCGAGCTGCGTTGGGGCGTGCCCGCCGACAAC
GGAGAGCCTA

14_011.ab1

AGAGGAACCGGTGGTCGTGTCGCCCTATTCCGATCATTTTCGAGCTGCGTTGGGGCGTGCCCGCCGACAAC
GGAGAGCCTA

13_012.ab1

2000 2010 2020 2030 2040 2050 2060 2070

Untitled_3

TTGATAGGTACCAGATCAAATACTGTCCGGGCGTTAAGATCAGCGGCACCTGGACGGAAGTGGAGAACTCC
TGCAACACC

Consensus:

TTGATAGGTACCAGATCAAATACTGTCCGGGCGTTAAGATCAGCGGCACCTGGACGGAAGTGGAGAACTCC
TGCAACACC

10_015.ab1

09_016.ab1

11_014.ab1

12_013.ab1

TTGATAGGTACCAGATCAAATACTGTCCGGGCGTTAAGATCAGCGGCACCTGGACGGAAGTGGAGAACTCC
TGCAACACC

14_011.ab1

TTGATAGGTACCAGATCAAATACTGTCCGGGCGTTAAGATCAGCGGCACCTGGACGGAAGTGGAGAACTCC
TGCAACACC

13_012.ab1

2080 2090 2100 2110 2120 2130 2140 2150

Untitled_3

GTTGAGGTGATGGAGACCACATCCTTCGAGATGACCCAGCTGGTGGGCAACACATACTATCGCATTGAACT
GAAGGCGCA

Consensus:

GTTGAGGTGATGGAGACCACATCCTTCGAGATGACCCAGCTGGTGGGCAACACATACTATCGCATTGAACT
GAAGGCGCA

10_015.ab1

09_016.ab1

11_014.ab1

12_013.ab1

14_011.ab1

GTTGAGGTGATGGAGACCACATCCTTCGAGATGACCCAGCTGGTGGGCAACACATACTATCGCATTGAACT
GAAGGCGCA

13_012.ab1

2160 2170 2180 2190 2200 2210 2220 2230

Untitled_3

CAACGCCATCGGCTATTCATCGCCTGCTTCCATTATCATGAAGACGACACGAGATAATCCCCATCCCTCGAC
GAGTGCGC

Consensus:

CAACGCCATCGGCTATTCATCGCCTGCTTCCATTATCATGAAGACGACACGAGATAATCCCCATCCCTCGAC
GAGTGGCG

10_015.ab1

09_016.ab1

11_014.ab1

12_013.ab1

14_011.ab1

CAACGCCATCGGCTATTCATCGCCTGCTTCCATTATCATGAAGACGACACGAGATAATCCCCATCCCTCGAC
GAGTGGCG

13_012.ab1

cGCCATCGGCTATTCATCGCCTGCTTCCATTATCATGAAGACGACACGAGATAATCCCCATCCCTCGACGAG
TGGCG

2240 2250 2260 2270 2280 2290 2300 2310

Untitled_3

CTGCACCCCTGGCCCAACTGCTTGTAAATTTTCACTGCTCTGCCGACAATGCTGCTGATTTTGCCGCCGACGA
CGCACACT

Consensus:

CTGCACCCCTGGCCCAACTGCTTGTAAATTTTCACTGCTCTGCCGACAATGCTGCTGATTTTGCCGCCGACGA
CGCACACT

10_015.ab1

09_016.ab1

11_014.ab1

12_013.ab1

14_011.ab1 CTGCACCCCT

13_012.ab1

CTGCACCCCTGGCCCAACTGCTTGTAAATTTTCACTGCTCTGCCGACAATGCTGCTGATTTTGCCGCCGACGA
CGCACACT

2320 2330 2340 2350 2360

Untitled_3 GCTGGTAAGCCTATCCCTAACCCCTCTCCTCGGTCTCGATTCTACGTAA

Consensus: GCTGGTAAGCCTATCCCTAACCCCTCTCCTCGGTCTCGATTCTACGTAA

10_015.ab1

09_016.ab1

11_014.ab1

12_013.ab1

14_011.ab1

13_012.ab1 GCTGGTAAGCCTATCCCTAACCCCTCTCCTCGGTCTCGATTCTACGTAA

Appendix 4

Full list of proteins extracted from BN-PAGE

Lane 1

Gene > Secondary Identifier	Gene > Symbol	Gene > Name
CG10691	l(2)37Cc	lethal (2) 37Cc Retinoid- and fatty acid-binding glycoprotein
CG11064	Rfabg	Yolk protein 3
CG11129	Yp3	ATP synthase- beta
CG11154	ATPsyn- beta	skpA associated protein
CG11963	skap	
CG12079	CG12079	
CG12203	CG12203	
CG12233	l(1)G0156	lethal (1) G0156
CG13240	l(2)35Di	lethal (2) 35Di sarcolemmal calcium-binding protein
CG1435	CBP	lethal (2) 03709
CG15081	l(2)03709	Sarcolemmal calcium-binding protein 1
CG15848	Scp1	
CG1640	CG1640	Adenine nucleotide translocase 2
CG1683	Ant2	stress-sensitive B
CG16944	sesB	Succinate dehydrogenase A
CG17246	SdhA	Vacuolar H ⁺ - ATPase 55kD B subunit
CG17369	Vha55	Enolase
CG17654	Eno	Triose phosphate isomerase
CG2171	Tpi	NADH:ubiquinone reductase 75kD subunit precursor
CG2286	ND75	Yolk protein 2
CG2979	Yp2	Arginine kinase
CG32031	Argk	mitochondrial ATPase subunit 6
CG34073	mt:ATPase6	Alcohol dehydrogenase
CG3481	Adh	
CG3523	CG3523	
CG3612	blw	bellwether
CG3683	CG3683	
CG3725	Ca-P60A	Calcium ATPase at 60A
CG3747	Eaat1	Excitatory amino acid transporter 1

CG3762	Vha68-2	Vacuolar H[+] ATPase subunit 68-2
CG3861	kdn	knockdown NADH:ubiquinone reductase 23kD
CG3944	ND23	subunit precursor Cytochrome P450-4g1
CG3972	Cyp4g1	
CG4169	CG4169	
CG4769	CG4769	
CG5320	Gdh	Glutamate dehydrogenase
CG5389	CG5389	
CG5670	Atpalpha	Na pump alpha subunit
CG6020	CG6020	
CG6030	ATPsyn-d	ATP synthase, subunit d Elongation factor Tu mitochondrial
CG6050	EfTuM	
CG6058	Ald	Aldolase
CG6647	porin	porin
CG6871	Cat	Catalase
CG7070	PyK	Pyruvate kinase
CG7430	CG7430	
CG7470	CG7470	
CG7610	ATPsyn- gamma	ATP synthase- gamma chain Malate dehydrogenase 2
CG7998	Mdh2	
CG8189	ATPsyn-b	ATP synthase, subunit b Phosphoglucose isomerase
CG8251	Pgi	
CG8844	Pdsw	Pdsw Glyceraldehyde 3 phosphate dehydrogenase 2
CG8893	Gapdh2	Glycerol 3 phosphate dehydrogenase
CG9042	Gpdh	
CG9090	CG9090	
CG9140	CG9140	
CG9277	betaTub56D	beta-Tubulin at 56D Serine protease inhibitor 3
CG9334	Spn3	

Lane 2

Gene > Secondary Identifier	Gene > Symbol	Gene > Name
CG10550	CG10550	
CG1088	Vha26	Vacuolar H[+]-ATPase 26kD E subunit
CG11129	Yp3	Yolk protein 3

CG11963	skap	skpA associated protein
CG12233	l(1)G0156	lethal (1) G0156
CG12403	Vha68-1	Vacuolar H ⁺ ATPase subunit 68-1
CG15848	Scp1	Sarcoplasmic calcium-binding protein 1
CG16936	CG16936	
CG16944	sesB	stress-sensitive B
CG1721	Pglym78	Phosphoglyceromutase
CG17369	Vha55	Vacuolar H ⁺ -ATPase 55kD B subunit
CG1743	Gs2	Glutamine synthetase 2
CG17870	14-3-3zeta	14-3-3zeta
CG1803	regucalcin	regucalcin
CG1913	alphaTub84B	alpha-Tubulin at 84B
CG2985	Yp1	Yolk protein 1
CG3001	Hex-A	Hexokinase A
CG31196	14-3-3epsilon	14-3-3epsilon
CG3127	Pgk	Phosphoglycerate kinase
CG3481	Adh	Alcohol dehydrogenase
CG3731	CG3731	
CG3835	CG3835	
CG4665	Dhpr	Dihydropteridine reductase
CG4696	Mp20	Muscle protein 20
CG4847	CG4847	
CG4869	betaTub97EF	beta-Tubulin at 97EF
CG5028	CG5028	
CG6084	CG6084	
CG6988	Pdi	Protein disulfide isomerase
CG8280	Ef1alpha48D	Elongation factor 1alpha48D
CG8938	GstS1	Glutathione S transferase S1
CG9042	Gpdh	Glycerol 3 phosphate dehydrogenase
CG9277	betaTub56D	beta-Tubulin at 56D
CG9364	Treh	Trehalase
CG9429	Crc	Calreticulin
CG9466	CG9466	
CG9476	alphaTub85E	alpha-Tubulin at 85E
CG9579	AnnX	Annexin X
CG9916	Cyp1	Cyclophilin 1

Lane 3

Gene >	Gene >	Gene >
Secondary	Symbol	Name
Identifier		

CG10664	CG10664	Retinoid- and fatty acid-binding glycoprotein
CG11064	Rfabg	ATP synthase-beta
CG11154	ATPsyn-beta	lethal (2) 03709
CG15081	I(2)03709	
CG1640	CG1640	
CG16944	sesB	stress-sensitive B Succinate dehydrogenase A
CG17246	SdhA	Enolase
CG17654	Eno	
CG1970	CG1970	NADH:ubiquinone reductase 75kD subunit precursor
CG2286	ND75	lethal (1) G0230
CG2968	I(1)G0230	Vacuolar H[+] ATPase subunit
CG3161	Vha16-1	16-1
CG3446	CG3446	Alcohol dehydrogenase
CG3481	Adh	
CG3523	CG3523	
CG3524	v(2)k05816	v(2)k05816
CG3612	blw	bellwether
CG3725	Ca-P60A	Calcium ATPase at 60A
CG3861	kdn	knockdown
CG4169	CG4169	
CG4264	Hsc70-4	Heat shock protein cognate 4
CG4307	Oscp	Oligomycin sensitivity-conferring protein
CG4692	CG4692	
CG4869	betaTub97EF	beta-Tubulin at 97EF
CG5548	CG5548	
CG5670	Atpalpha	Na pump alpha subunit
CG6020	CG6020	
CG6058	Ald	Aldolase
CG6105	I(2)06225	lethal (2) 06225
CG6186	Tsf1	Transferrin 1
CG6343	ND42	NADH:ubiquinone reductase 42kD subunit precursor
CG7070	PyK	Pyruvate kinase
CG7145	CG7145	
CG7176	Idh	Isocitrate dehydrogenase
CG7430	CG7430	
CG7610	ATPsyn-gamma	ATP synthase-gamma chain
CG7998	Mdh2	Malate dehydrogenase 2
CG8251	Pgi	Phosphoglucose

		isomerase
		Glyceraldehyde 3
		phosphate
CG8893	Gapdh2	dehydrogenase 2
		Glycerol 3
		phosphate
CG9042	Gpdh	dehydrogenase
		beta-Tubulin at
CG9277	betaTub56D	56D
CG9762	l(3)neo18	lethal (3) neo18
CG9916	Cyp1	Cyclophilin 1

Lane 4

Gene > Secondary Identifier	Gene > Symbol	Gene > Name
CG10664	CG10664	
		Vacuolar H[+]-
		ATPase 26kD E
CG1088	Vha26	subunit
		Retinoid- and
		fatty acid-binding
CG11064	Rfabg	glycoprotein
CG11129	Yp3	Yolk protein 3
		ATP synthase-
CG11154	ATPsyn-beta	beta
		Superoxide
CG11793	Sod	dismutase
		Glyceraldehyde 3
		phosphate
CG12055	Gapdh1	dehydrogenase 1
		Vacuolar H[+]
		ATPase subunit
CG12403	Vha68-1	68-1
		Ferritin 2 light
CG1469	Fer2LCH	chain homologue
CG1516	CG1516	
		Sarcoplasmic
		calcium-binding
CG15848	Scp1	protein 1
CG1640	CG1640	
CG16944	sesB	stress-sensitive B
		Vacuolar H[+]-
		ATPase 55kD B
CG17369	Vha55	subunit
CG17654	Eno	Enolase
		Proteasome
CG18495	Prosalpha1	alpha1 subunit
		alpha-Tubulin at
CG1913	alphaTub84B	84B
		Triose phosphate
CG2171	Tpi	isomerase
		Ferritin 1 heavy
CG2216	Fer1HCH	chain homologue
CG2968	l(1)G0230	lethal (1) G0230

CG30191	Gr59b	Gustatory receptor 59b
CG32031	Argk	Arginine kinase
CG3446	CG3446	
CG3481	Adh	Alcohol dehydrogenase
CG3523	CG3523	
CG3612	blw	bellwether
CG3725	Ca-P60A	Calcium ATPase at 60A
CG3747	Eaat1	Excitatory amino acid transporter 1
CG3861	kdn	knockdown
CG3944	ND23	NADH:ubiquinone reductase 23kD subunit precursor
CG3972	Cyp4g1	Cytochrome P450-4g1
CG4233	Got2	Glutamate oxaloacetate transaminase 2
CG4307	Oscp	Oligomycin sensitivity-conferring protein
CG4347	UGP	UGP
CG4692	CG4692	
CG4869	betaTub97EF	beta-Tubulin at 97EF
CG5670	Atpalpha	Na pump alpha subunit
CG6020	CG6020	
CG6030	ATPsyn-d	ATP synthase, subunit d
CG6058	Ald	Aldolase
CG6186	Tsf1	Transferrin 1
CG7070	PyK	Pyruvate kinase
CG7254	GlyP	Glycogen phosphorylase
CG7610	ATPsyn-gamma	ATP synthase-gamma chain
CG7920	CG7920	
CG8189	ATPsyn-b	ATP synthase, subunit b
CG8251	Pgi	Phosphoglucose isomerase
CG8256	Gpo-1	Glycerophosphate oxidase-1
CG8280	Ef1alpha48D	Elongation factor 1alpha48D
CG8893	Gapdh2	Glyceraldehyde 3 phosphate dehydrogenase 2
CG9042	Gpdh	Glycerol 3 phosphate dehydrogenase
CG9090	CG9090	
CG9277	betaTub56D	beta-Tubulin at 56D
CG9334	Spn3	Serine protease

		inhibitor 3
CG9762	l(3)neo18	lethal (3) neo18

Random protein selection

Gene >
Secondary
Identifier
CG11290
CG14000
CG14005
CG14006
CG14007
CG14010
CG14011
CG14013
CG14014
CG14015
CG14017
CG17002
CG17003
CG17005
CG1701
CG17010
CG17012
CG17018
CG17019
CG1702
CG17024
CG2200
CG2201
CG3342
CG3344
CG3345
CG33453
CG33454
CG33458
CG5315
CG5316
CG5321
CG5322
CG5323
CG5325
CG5326
CG5327
CG5334

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