Analysis of *Drosophila ssp4*

Elizabeth Christine Coates

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In the Faculty of Science, Technology and Medical Studies, The Research School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ UK.

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Contents

ABSTRACT

CAMSAP 1 is a spectrin associated, Calmodulin regulated protein that is a member of a large ubiquitous family of cytoskeletal proteins in the animal kingdom, defined by a novel C-terminal domain, the CKK domain. The role of the CAMSAP proteins is unknown but studies using rat PC12 cells have shown that CAMSAP1 plays a key role in neurite outgrowth. It has been shown to colocalise with microtubules in cultured cells and binds to microtubules *in vitro* via the CKK domain. The CAMSAP family member in the fruit fly *Drosophila melanogastor* is encoded by the gene *short spindles4* (*ssp4*). Little is known about this gene but a role in microtubule dynamics has been shown in cultured cells.

I have interrogated bioinformatics databases and compared Ssp4 with Human CAMSAP proteins and found many similarities, and some differences, between the proteins. Using *in situ* hybridisation I show that *ssp4* transcripts are expressed in the gut, head and central nervous system during embryogenesis and an antibody that recognises the Ssp4 C-terminus reveals expression throughout the development of the gut and nervous system, and in a discrete population of cells in the head.

I have investigated the effects of two independent P-element induced mutant alleles of *ssp4* and show that mutant flies die in late embryonic or early larval stages. Disruptions to the locus do not seem to affect the nervous system but mutants were found to have aberrant head involution. I present preliminary evidence that suggests this defect may be the result of reduced apoptosis in the embryo.

Head involution is a complex process, dependent upon co-ordinated changes to cell shape and the movement of groups of cells from different origins. As Ssp4 is a multi-domain cytoskeletal protein that is required for embryonic development, it may play a role in processes that are common to these morphogenetic events.

Chapter 1

INTRODUCTION

1.1 INTRODUCTION

Through the process of evolution, unicellular animals have given rise to complicated multicellular organisms. This has led to cell differentiation with the development of a wide variety of highly specialised cells. The cytoskeleton has had to evolve to enable cells to take on these specialist roles, from the simple anuclear biconcave red blood cell to neuronal cells with elongated axons. The cytoskeleton is a fibrous dynamic network that shapes, supports and organises the cell. It comprises three different filamentous proteins - actin, microtubules and intermediate filaments - and all their associated proteins. Actin filaments form bundles that increase strength, and allow cell movement through formation of lamellipodia, filopodia and stress fibres. Intermediate filaments are constructed from proteins containing an alpha helical domain, such as keratin, and have a structural role. Microtubules are unbranched arrangements of globular proteins that co-ordinate cell movement, such as nerve axon migration, and intracellular movement such as chromosome separation during meiosis and mitosis.

The erythrocyte, has a well-characterised cytoskeleton that supports the plasma membrane and maintains the cell shape (Yu *et al.,* 1973; Figure 1.1). The membrane cytoskeleton is made up of predominantly actin and spectrin, the spectrin allows the cell to be deformable so that it can move freely in the microcirculation (Petit-Zeman 2004). Spectrin also associates with plasma membrane proteins, and accessory cytoskeletal proteins 4.1, adducin, tropomyosin and tropomodulin. Figure 1.2 shows the spectrin network of 6-sided polygons made from individual molecules attached to actin filaments (reviewed by Bennett and Baines 2000).

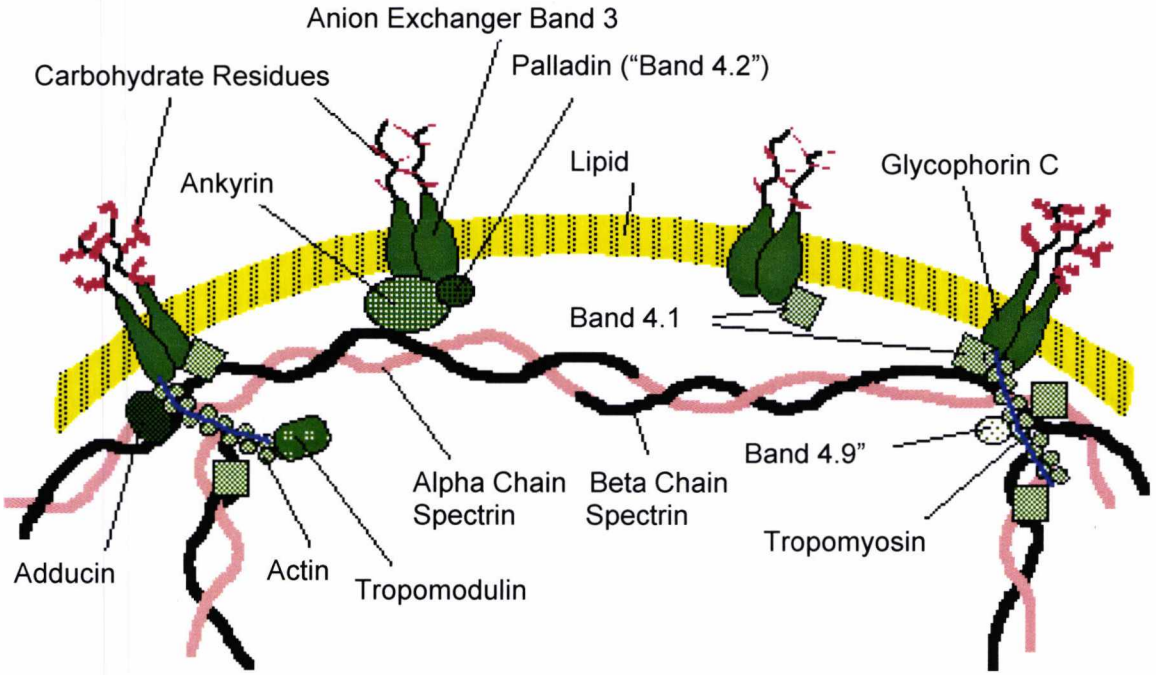


Figure 1.1 – Schematic of The Erythrocyte Cytoskeleton (Taken from Luna and Hitt 1991). *An example of a well characterised cytoskeleton, the erythrocyte cytoskeleton is made up of many proteins, the most abundant of which is spectrin (see below). The cytoskeleton attaches to the plasma membrane via trans membrane proteins, Glycophorin C, and the Anion Exchanger.*

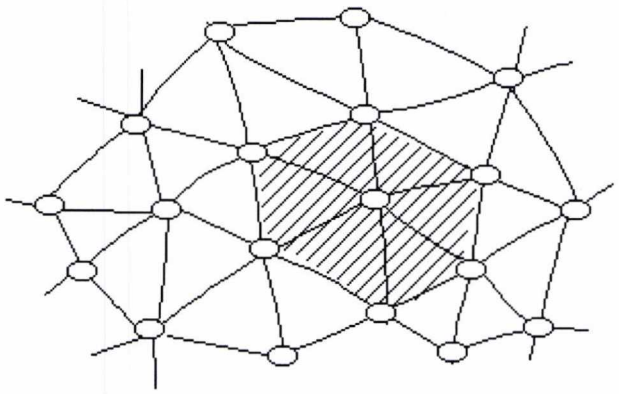


Figure 1.2 - Schematic of arrangement of spectrin molecules in the erythrocyte (Luna and Hitt 1991). *The individual molecules link to actin to form into a basic hexagonal shape, which is fixed to the membrane by ankyrin molecules (circles).*

1.1.1 The Role of the Cytoskeleton.

In nucleated cells, the cytoskeleton connects to junctions in the plasma membrane to link cells to other cells, and to the extracellular matrix (Ramaekers and Bosman 2004). It stabilises the protein composition of the cell membrane, which is essential for cell polarisation and successful development, and for making cell receptors accessible for substrate binding (Dubreuil *et al.,* 2000). These extracellular signals induce physiological changes to the cell to change cell shape and gene expression (Forgacs *et al.,* 2004).

The cytoskeleton is central to both intracellular and extracellular organisation (reviewed by Stein *et al.,* 2002). Polarisation of epithelial cells is established externally through cell/cell and cell/matrix interactions, and maintained by the correct targeting of cell proteins (Figure 1.3). Specialised junctions are then established, which divide the cell into apical and basolateral domains, and these domains are defined by spectrin. Once established, polarity is maintained through the placing of domain specific molecules, mediated by intracellular trafficking pathways (reviewed by Knust 2000). Polarity in the epithelial cell is maintained by the cytoskeleton, with microvilli dependent upon actin for their organisation (Ameen *et al.,* 2001). With polarity established, the cytoskeleton provides a scaffold for signal transducing proteins - a role that was brought about during eukaryotic evolution, which led to the adoption of cytoskeletal components by signal transduction networks (Forgacs *et al.,* 2004).

Whilst some cells such as neurons (Benitez-King *et al.,* 2004) adopt an asymmetric shape and are polarised for their lifetimes, others can become polarised in order to carry out functions such as asymmetric division or migration. Morphogenetic movements, such as gastrulation, can only take place when the cytoskeleton has co-ordinated the polarisation of large numbers of cells, leading to the alteration of their

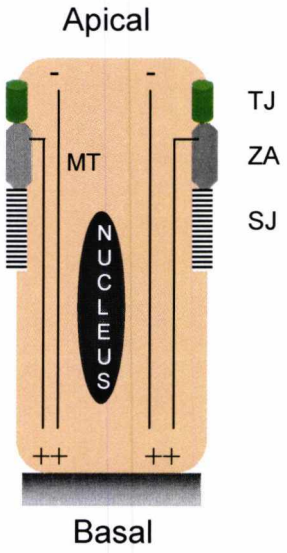


Figure 1.3: Schematic of a Polarised Epithelial Cell. Taken from Akhmanova and Yap 2008. *The epithelial cell is subdivided into the apical and basal domains. This diagram contains details of both the vertebrate and invertebrate epithelial cell, MT, microtubules, ZA, the Zona Adherens is common to both, and contains the Adherens Junction. The SJ is the septate junction, found in invertebrate cells. TJ represents the tight junction in vertebrate cells. This area is the Sub-apical region in the invertebrate. Different arrangements of spectrin tetramers define the apical and basal domains.*

shape. The movement of the cell is then mediated by actin filaments, which bundle together to form protruding structures called lamellipodia and filopodia (reviewed by Yamaguchi and Condeelis 2007). Microtubules co-ordinate cell movements such as flagella motion and nerve axon cell migration, and also transportation of proteins within the cell to maintain polarity. Microtubules form a specialised mitotic spindle to separate chromosomes during meiosis and mitosis, and help mediate apoptosis (programmed cell death). This is a process that regulates cell numbers and removes unnecessary tissues during development. It involves microtubule-mediated formation of blebs in dying cells, and facilitates cell uptake by macrophages (Moss *et al.,* 2006).

1.1.2 Disease States Involving the Cytoskeleton.

As the cytoskeleton is central to so many cellular processes, defects or alterations to cytoskeletal proteins or structure are implicated in many diseases. The aim of ongoing research is to increase knowledge of the complex cytoskeletal apparatus and to produce targeted therapies (Ramaekers and Bosman 2004).

Diseases that affect the structural role of the cytoskeleton lead to fragile skin (Korge and Krieg 1996), and muscle weakness (Reviewed by Clarkson *et al.,* 2004) and are caused by defects to keratins, intermediate filaments which provide cells with physical resilience. Hereditary elliptocytosis is the result of genetic mutations in genes coding for cytoskeletal proteins in the erythrocyte, leading to weakened cells. Aberrant cytoskeletal assembly leads to neurodegenerative diseases with cytoarchitectural abnormalities, and neurofibrillary tangles (Reviewed by Benitez-King *et al.,* 2004). Microvilli inclusion disease is characterised by changes to cytoskeletal structure leading to the loss of epithelial cell polarity, which causes aberrant protein trafficking (Ameen *et al.,* 2001) (Reviewed by Stein *et al.,* 2002).

Cancer cells are able to invade other tissues and undergo metastasis, through the exploitation of cytoskeletal function. Actin polymerisation drives the formation of membrane protrusions needed for migration and invasion, and cytoskeletal proteins involved in protrusion formation are upregulated in cancer cells (Yamaguchi *et al.,* 2005). Highly invasive cancer cells form invadopodia type extensions/podosomes to manoeuvre through the extracellular matrix (Buccione *et al.,* 2004). The Wiskott-Aldrich syndrome proteins associate with actin and are over-expressed in many types of cancer (Sahai 2005). These proteins are also important for normal immune system function as they mediate actin reorganisation, for dendritic cell motility (reviewed by Calle *et al.,* 2006).

Spectrin is a key protein of the membrane cytoskeleton. It plays a central role in cell polarity, signalling and cell movement, and this role is explored in further depth in the following overview.

1.2 SPECTRIN

The protein spectrin is an extended flexible molecule, and a component of the plasma membrane cytoskeleton. It regulates vesicle transport, endocytosis and morphogenesis (reviewed by Bennet and Baines 2001) and links the cell membrane to the components of intracellular transport (Pielage *et al.,* 2005). Spectrin comprises an alpha and beta heterodimer that associate at the head and along the length to form a tetramer (Deng *et al.,* 1995). The alpha and beta chains are made up mainly of repeated 106 amino acid residue motifs which form triple helical repeats (Figure 1.4). Spectrin can unfold and then refold indicating that these triple helical domains have the capacity to act as molecular springs, resisting possible cell deformation from mechanical stress (Rief *et al.,* 1999).

There is 50-60% identity between the spectrin of different species. The variety of alpha and beta subunits, along with variations in their splicing, can lead to the generation of a number of different spectrin proteins, which have different expression patterns.

Mammalian spectrin comprises 2 different alpha subunits (Sahr *et al.,* 1990, Wasenius *et al.,* 1989), and 5 different beta subunits (Berghs *et al.,* 2000, Hu *et al.,* 1992, Mishra *et al.,* 1998, O Hara *et al.,* 1998, Stankewich *et al.,* 1998, Winkelmann *et al.,* 1990). The alpha 2 spectrin contains a calmodulin binding domain, and protease cleavage site (Figure 1.4). Alternative splicing of the beta spectrins produces proteins with short or long C termini. The long termini contain a plekstrin homology domain (Figure 1.4), whilst the short have a substrate site for casein kinase 2 (reviewed by Bennet and Baines 2001). Spectrin functions as a tetramer with 2 alpha and 2 beta subunits joined end to end (see Figure 1.1). The alpha 1/beta 1 subunit combination is found in erythrocyte membranes, striated muscle, and a set of central nervous system neurons,

other combinations of alpha and beta subunits are found in non-erythroid vertebrate tissues (O Hara *et al.,* 1998, Riederer *et al.,* 1986, Lambert and Bennett 1993).

1.2.1 Structural and Physiological Roles of Spectrin.

Spectrin targets proteins to specific cell areas and influences the half-life of cell surface proteins by way of membrane stabilisation. Erythroid spectrin can bind the phospholipid membrane through the plekstrin homology domain of longer beta spectrin isoforms (Hyvonen and Saraste 1995). These interactions give additional support to the lipid bilayer, and also give spectrin a membrane anchor when ankyrin is in short supply. In neurons beta 1 spectrin is responsible for neural cell adhesion molecule (NCAM) redistribution, and partakes in neuron outgrowth (Alberts and Galli 2003). NCAM is a glycoprotein found on the surface of neurons, glia and skeletal muscle.

Spectrin is involved in axonal transport, and has been found in axonally transported proteins (Levine and Willard 1981). Spectrin is also expressed in Purkinje cells in the cerebellum, (Lazarides and Nelson 1983) and in neurons in the retina (Isayama *et al.,* 1991) with different beta subunits expressed in the cell body and axons. Different patterns of beta spectrin are seen during brain development with one isoform appearing only following birth, with maximum expression seen at the tenth day (Hu et al 1992). A neural role for spectrin has also been shown in *Caenorhabditis elegans* since beta spectrin mutant adults are paralysed and dumpy with abnormal axon outgrowth due to disruption of protein anchoring at cell membranes (Hammerlund *et al.,* 2000)

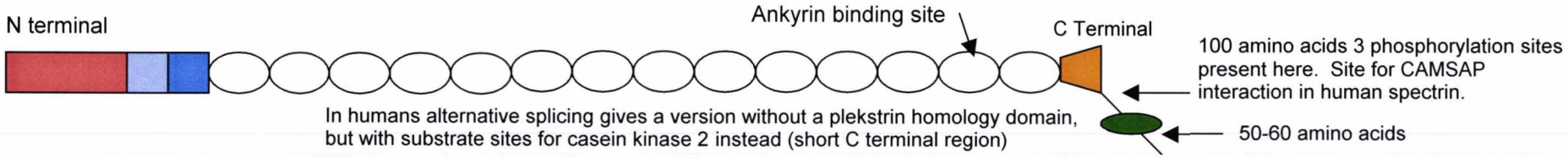
1.2.2 *Drosophila* Spectrin Function

As is the case for vertebrates, invertebrate cells are also reliant upon the cytoskeleton and the spectrin protein. In *Drosophila* there is one form of alpha spectrin, and a beta spectrin and a beta heavy spectrin. *Drosophila* alpha spectrin comprises 22 domains,

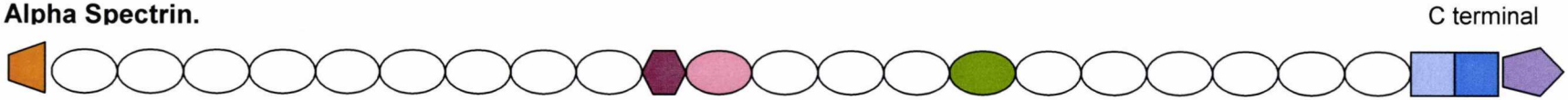
20 of these are triple helical repeats that are found in all spectrin proteins (Figure 1.4). Alpha spectrin has an SH3 domain, and a calmodulin-binding site (Trave *et al.,* 1995, Dubreuil *et al.,* 1989). Phosphorylation of calmodulin causes its release from spectrin, which means any process that involves phosphorylation controls spectrin activity. The C terminal domain comprises EF hands in a calmodulin like structure which bind calcium. Beta spectrin comprises 17 domains, including a pair of actin binding calponin homology domains, an ankyrin binding site and a Plekstrin homology domain at the COOH terminal (Banuelos *et al.,* 1998, Carugo *et al.,* 1997, Sahr *et al.,* 1990, Hayes *et al.,* 2000, Winkelman *et al.,* 1990). Beta heavy spectrin has an N –terminal actin binding domain and an SH_3 domain and 30 triple helical repeats. Unlike beta spectrin, beta heavy spectrin has no ankyrin-binding site (Williams *et al.,* 2004).

Drosophila eggs contain a supply of maternal spectrin, as it is essential for embryonic gut and central nervous system development. Individuals mutant for both alpha and beta spectrin, die during embryogenesis and exhibit disruption in presynaptic neurotransmission. These embryos were the product of adult flies that carried no detectable alpha or beta spectrin, but were rescued to adulthood by the presence of an alpha or beta spectrin minigene, whose expression was mediated by a ubiquitin promoter (Featherstone *et al.,* 2001). Beta spectrin mutants die late in embryogenesis or as young larvae, with midgut stomach morphology and acid secretion defects which are caused by a disturbance to the stable accumulation of NaK-ATPase in the basolateral region of copper cells. (Dubreuil *et al.,* 2000). Beta Spectrin is also needed for maintenance of axonal connections in the CNS, synapse stabilisation, and stabilisation of nerve growth cones (Garbe *et al.,* 2007, Pielage *et al.,* 2005, Hulsmeier *et al.,* 2007). During embryogenesis, spectrin localization appears to follow embryonic cytoskeletal changes rather than instigate them, suggesting a supportive and stabilizing role for spectrin (Pesecreta *et al.,* 1989).

Beta Spectrin.



Alpha Spectrin.



Partial triple helical repeats that form head-head dimers. Comprises 2 helices.

Calmodulin related domain

SH3 domain – Alternative splicing gives a version without an SH3 domain.

Contains a cleavage site for Ca^2 activated protease and a calmodulin binding site in vertebrate alpha spectrin 2.

Drosophila calmodulin binding site

Actin binding tandem calponin homology domain.

Triple helical repeat. Alpha and beta chains comprise 106 amino acid residue motifs that form triple helical repeats.

Plekstrin homology domain – long C terminal region. Major binding site for protein and lipid ligands.

Atypical triple helical repeats.

Drosophila Beta Heavy spectrin has an internal SH3 domain, and no ankyrin binding site.

Figure 1.4: Schematic of Alpha and Beta Spectrin (from a review by Bennett and Baines 2001). *This shows Spectrin subunits with both vertebrate and Drosophila features. Spectrin is a heterotetrameric protein that consists of 2 alpha and 2 beta chains. Three alpha beta subunit interactions give rise to the tetramer. (1) weak lateral association along the length of the antiparallel alpha and beta sub units, (2) The formation of a strong lateral association between the beta subunit N terminal domain and the alpha subunit C terminal domain, (3) connection of the beta C terminal domain with the alpha N terminal domain.*

The alpha spectrin subunit is essential for larval survival, without it larvae die as first or second instars (J Lee *et al.,* 1993). The alpha spectrin subunit facilitates cell/cell contacts essential for cell shape maintenance and tissue organisation (J Lee *et al.,* 1993). Alpha spectrin is also involved in the asymmetric division that leads to oocyte formation, and mediates cellular polarity through its interactions with plasma membrane proteins (Lin and Spradling 1994). Specially generated female flies that are deficient in alpha spectrin in their ovary cells have disrupted cyst formation with egg chambers that often lack an oocyte (De Cuevas *et al.,* 1996).

Beta spectrin influences the half-life of cell surface proteins by way of stabilisation of the membrane. The NaK-ATPase molecule only remains at cell/cell contact sites in the membrane whilst associated with beta spectrin and ankyrin. (Dubreuil *et al.,* 2000). Alpha and beta spectrin stabilise and maintain the *Drosophila* neuromuscular junction in post-embryonic development (Pielage 2005). Spectrin tetramer formation is essential for *Drosophila* development, since alpha spectrin mutations that prevent tetramer formation, lead to larval lethality. Tetramer formation is, therefore, necessary for nucleated cells as well as anuclear erythrocytes. Different tissues have different tetramer requirements for development, with a greater functional demand for oogenesis than for cuprophilic cell formation (Deng *et al.,* 1995).

1.3 CAMSAP 1

One method of studying the cytoskeleton is to disassemble it into its constituent proteins, and this led to the discovery of CAMSAP1, a protein that is found in vertebrates, which binds mammalian beta 2 epsilon I spectrin in a calmodulin regulated manner, and interacts with microtubules *in vitro* (Baines *et al.,* 2009). CAMSAP1 belongs to a large

family ubiquitous in the animal kingdom. The *Drosophila* gene homologous to CAMSAP1 is *short spindles 4 (ssp4).*

Vertebrate CAMSAP1 was discovered during a search for other proteins that associate with different spectrin isoforms (Baines *et al.,* 2009). As the binding of CAMSAP1 to spectrin was regulated by calmodulin, the protein was named CAMSAP1 – **calm**odulin regulated, **s**pectrin **a**ssociated **p**rotein. A protein that binds to spectrin will be involved in some of spectrin's functions, and since CAMSAP's activity was regulated by calmodulin it was thought that it may be involved in processes such as neural development where calcium plays an important role.

CAMSAP 1 is found in astrocyte (star shaped glial cell) lineage cells in rats (Yamamoto *et al.,* 2009). It binds to the last 60 residues of the long C terminal of mammalian beta 2 epsilon I spectrin, which has a plekstrin homology domain in this region (Baines *et al.,* 2009). This particular beta spectrin isoform is expressed in axonal structures in cerebellar granule cells, and in dorsal root ganglia again suggesting that it has a role in neural development. Recent unplublished work in Rat PC12 cells has confirmed this.

Rat PC12 cells are a model system for sympathetic neurones, and antibody labelling shows that CAMSAP 1 is expressed in the cell body, neurite and growing tip. It is essential for neurite outgrowth since 60-70% of serum-starved cells form neurites in the presence of nerve growth factor (NGF), but only 2% of cells form neurites following treatment with short interfering RNA (siRNA) against CAMSAP mRNA. Application of siRNA, targeted against CAMSAP1 transcripts to neurites following exposure to NGF, brings about their collapse. Application of bromodeoxyuridine and subsequent anti Br-dU staining reveals that these cells are synthesising DNA and trying to maintain the cell cycle despite the fact that they are deprived of serum and exposed to NGF, factors that usually prevent cell cycle progression Knockdown of CAMSAP1 in HeLa and COS-7 cells does

not reduce proliferation rates, which suggests that CAMSAP1 does not play a significant role in cell cycle regulation or cell division (Baines and King unpublished data).
In the granule layer CAMSAP 1 is present in high levels where its expression mainly coincides with expression of spectrin although this co-expression is not complete suggesting that either CAMSAP 1 and spectrin co-expression depend upon the presence of another protein, calmodulin perhaps, or that they may each bind other proteins in these areas (Baines et al., unpublished data).

Human CAMSAP 3 associates with the minus end of microtubules in epithelial cells and with PLEKHA7 connecting the microtubule to the zona adherens, which is part of the cadherin based adherens junction and encloses epithelial cells at the apical end (Meng et al., 2008, see Figure 1.3).

1.3.1 The CAMSAP family

The human CAMSAP 1 gene (CAD58627) has been mapped to chromosome 9. Two other CAMSAP related proteins can be identified in the human genome by sequence alignment, gene KIAA1078 on chromosome 1 and gene KIAA1543 on chromosome 19 (Baines *et al.,* 2009). Since these now appear to be members of a gene family, they have been re-annotated as CAMSAP 2 and CAMSAP 3 respectively.

The 3 human CAMSAP proteins all contain an N terminal domain that is analogous to a Calponin Homology domain, identified by the InterPro signature IPR001715. The central region of the proteins contains 3 areas of coiled-coil (Baines *et al.,* 2009). The first coiled coil is adjacent to a calmodulin binding site, identified by the calmodulin target database (Baines *et al.,* 2009). At the C terminal of each protein is a unique domain, Pfam entry DUF1781 (Figures 1.5 and 1.6). This is called the CKK domain and was so named

because it was found in the human **C**AMSAP 1 and the two other CAMSAP related proteins in humans, **K**IAA1078 and **K**IAA1543 (Baines *et al.*, 2009).

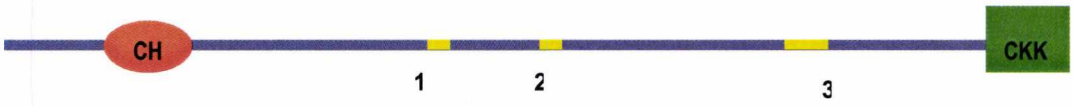


Figure 1.5: Schematic of Human CAMSAP 1 Protein.
Diagram shows the position of the Calponin Homology (CH) domain and the C terminal CKK domain. The three coiled coil regions are shown in yellow. Coiled coil 1contains the calmodulin binding domain.

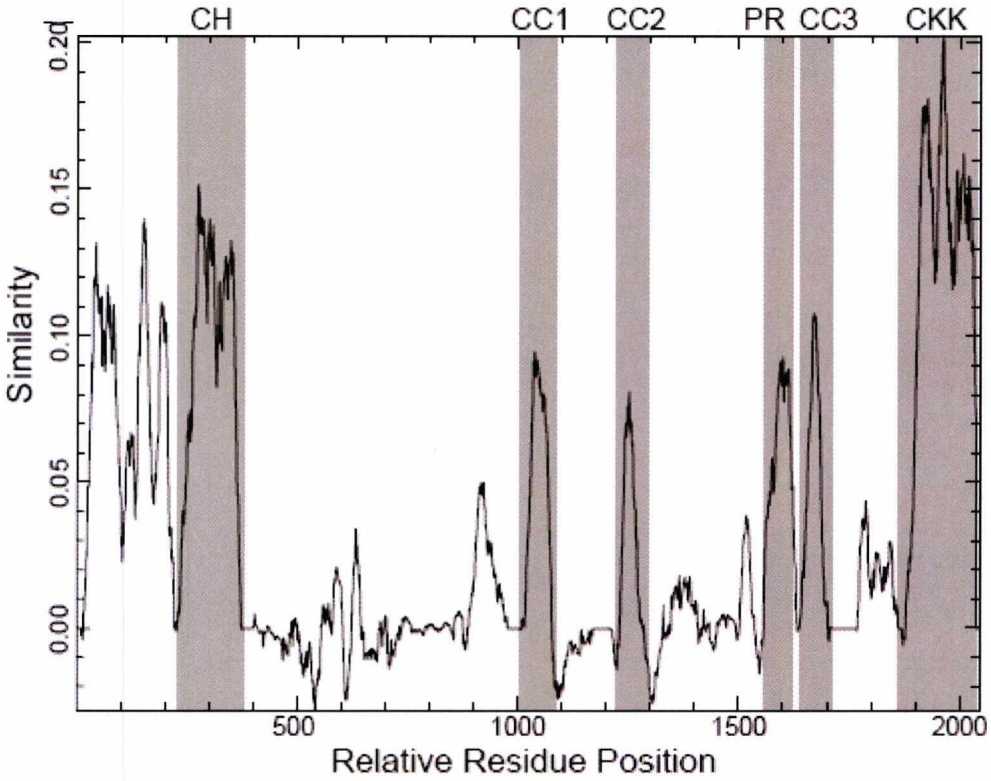


Figure 1.6: Analysis of Alignment of CAMSAP family members using PLOTCON (taken from Baines *et al.*, 2009)
This analysis was carried out using a 20 amino acid window. Sequences from human and frog CAMSAPs 1-3, Drosophila (Ssp4), nematode and sea urchin, were compared. All these species share a CH domain, a CKK domain, and 3 coiled coils (shown as CC1, CC2 and CC3) and a proline rich region, (PR). Note the presence of a possible N terminal domain.

CAMSAP proteins are members of a family of proteins found throughout the animal kingdom, with other family members identified by the presence of the unique CH domain or CKK domain using BLAST analysis of available animal genomes (Baines *et al.*,2009). Most vertebrate organisms have 3 versions of CAMSAP, whereas invertebrate organisms have only one. This is not unusual, as vertebrates possess multiple versions of both spectrin and tubulin, as the result of gene duplication during vertebrate evolution (Holland, 2003). As spliced mRNAs exist for the human and mouse CAMSAP's it is assumed that these copies have gained or developed a function that is complementary to the others (Baines *et al.*, 2009). The genome of the Choanoflagellate *Monsiga brevicollis* had no sequence corresponding to a CKK domain. These free-living, single-cell eukaryotic organisms are an outgroup to metazoans, rather than descendents of them implying CAMSAP genes came about as animals developed differentiated tissues (King 2004). CAMSAP genes that arose in simple animals such as coelenterates (simple multicellular animals whose bodies comprise two primary layers separated by mesoglea) were multiplied during vertebrate evolution, giving existing species multiple copies of CAMSAP family genes.

1.3.2 Domains Common to CAMSAP Family Members

1.3.2.1 The Calponin Homology Domain

The Calponin homology (CH) domain is a sequence motif of approximately 100 amino acids, which allows many cytoskeletal and signalling molecules to bind actin. The CH domains can be divided into eight groups. Types 1,2,3,4,5 fimbrin types 1 and 2 and end binding (EB) type (for a review see Gimona *et al.*, 2002).

Type 1 and 2 CH domains are found in the actinin protein family. The type 3 CH domain is 100 amino acids in length and located at the N terminal of calponin and in Vav proteins where it has a signalling and regulatory role in the Rho signalling pathway. Type 4 and 5

CH domains are found in the actopaxin/parvin family of focal adhesion proteins, which have 2 CH domains arranged in tandem. Type 1 and 2 Fibrin actin binding CH domains consist of 2 domains connected by a linker. Interhelical loop arrangements have given rise to the two different CH domain families (Gimona *et al.,* 2002).

The CH domain in CAMSAP proteins is unique (Baines *et al.,* 2009). A comparison of all *Drosophila melanogastor* CH domain sequences that are held in the Interpro databases produced a tree with proteins falling into discrete CH domain groups. The CH domain for Ssp4 formed a class of its own, closest to the EB (end binding protein) type domain. The Ssp4 CH domain has an insertion and a unique sequence in the loop that connects the first and second helices (Baines *et al.,* 2009). EB proteins attach to microtubule ends and play a role in spindle dynamics and chromosome segregation (Bu and Su, 2003).

There are three different EB proteins in the human, and one in *Drosophila*, EB1 that is spliced in four different ways (Bu and Su, 2003). EB1 has a CH domain in the N terminal, which is well conserved between humans and flies, and binds microtubules through its CH domain. The C terminus of these proteins contains the EB1-C region, which binds other proteins including Adenomatous polyposis coli (APC) a tumour suppressor protein vital for cell cycle regulation. EB1 and APC co-operate to promote microtubule polymerisation in a process regulated by APC phosphorylation (Nakamura *et al.,* 2001).

1.3.2.2 The Calmodulin Binding Domain.

Calmodulin is a very significant regulatory protein whose binding to vertebrate CAMSAP1 has been confirmed. Regulatory cellular proteins bind calcium in order to transduce an initial signal into a biological process. The main calcium binding protein is Calmodulin, which can be found in a variety of cells and various subcellular compartments. Calmodulin senses and interacts with kinases and with phosphatases, which govern cell

signalling and cell death. Calcium fluxes are the result of entry through voltage and specialised ligand gated ion channels, discharge from intracellular stores, the uptake of calcium by pumps, and the effects of calcium-activated proteins. These changes to calcium levels can be temporary and spatially specific, or more general. During development in *Drosophila* calmodulin is very important for specification of neural cell fate, and is highly expressed in the developing nerve cord and brain (Hanson-Painton, 1992).

Calmodulin consists of 2 globular calcium ion binding domains connected by a flexible linker that allows clasping of the target protein. Because specific amino acid sequences or structural binding motifs are unnecessary, calmodulin can interact with many targets (Rhoads and Friedberg 1997). Rat CAMSAP 1 has been shown to bind calmodulin with moderate but physiologically significant affinity, in the presence of Ca^{2+} (Baines and King unpublished data). A search of the calmodulin database has predicted a calmodulin binding site in the first coiled coil region located at the centre of each of the human proteins.

1.3.2.3 Coiled Coils

Coiled coils are made up of two or three entwined alpha helices and are found in many structural proteins that are involved in movement and cell shape maintenance. They can be detected from the amino acid sequence because the alpha helix structure repeats every seven residues to form a heptad repeat. This periodicity gives rise to a stripe of hydrophobic residues in a helix allowing it to bind other alpha helices to form the coiled coil. Proteins containing coiled coils also aggregate with identical or different proteins in quaternary structures that assemble and disassemble according to need.

1.3.2.4 Proline rich sequence

The human CAMSAP proteins contain a sequence that is rich in proline residues, the CAMSAP3 protein has a total of 27/110 residues in this sequence that are proline (Baines *et al.,* 2009). This type of region is a very familiar motif, which is found throughout the human proteome (Songyang, 1999). The cyclic side chain of proline causes the protein to kink and/or adopt a type 2 polyproline helix – a structural motif that permits interaction with other proteins. There are possible binding sites in this area for Src homology 3 (SH3) domains, which have a characteristic beta fold, and bind proline rich ligands. This fold is found in proteins that interact with other proteins to assemble protein complexes needed for signalling and regulation of the activity of cellular kinases. WW domains (35-40 amino acids) also bind proline rich sequences. They comprise 2 highly conserved tryptophan residues, 20 – 22 amino acids apart. WW domains are found in splicing and transcription factors. Profilin regulates actin polymerisation through interactions with proline rich ligands (Songyang, 1999).

1.3.2.5 CKK Domain

The CKK domain identifies CAMSAP family members (Figure 1.5). It has a C terminal tryptophan residue common to all classes, and with a well-conserved core comprising Leucine, Arginine and Isoleucine. These conserved residues may be necessary for the folding of the protein. Overall the CKK domain is a beta barrel with an alpha helical hairpin with that binds to microtubules. A search of the uniprot database with an HMM of this domain identified no other proteins with this fold (Baines *et al.,* 2009).

1.3.3 *Drosophila* CAMSAP - *ssp4*

The *Drosophila* member of the CAMSAP family is *short spindles 4* (*ssp4),* and this gene is located on the second chromosome, (also annotated CG33130; *l(2)k07433*). A protein alignment using PLOTCON shows the CH domain, three coiled regions and the CKK C

terminal domain are conserved in all species, although the second coiled coil is not as well conserved in all invertebrates (Figure 1.6). An unknown domain N terminal to the CH domain can also be seen in all species (Baines *et al.,* 2009).

The gene was named *short spindles 4* from a study using a full genome RNAi library and S2 cells, which revealed that 'knockdown' of the transcript led to microtubule severing (Goshima *et al.*, 2007). The microtubules in those cells treated with *ssp4* RNAi show treadmilling behaviour and then disappear which suggests that *ssp4* stabilises growing microtubules. Another study used flow cytometry to analyse the effects of the loss of function of 70% of *Drosophila* genes in S2 cells and revealed a role for *ssp4* in cell cycle regulation (Bjorklund *et al.*, 2006).

In addition to recent RNAi studies the locus has been identified in earlier genetic and biochemical screens. Work carried out by Mutsuddi *et al.,* (2004) showed that *ssp4* interacts with the RNA binding protein Staufen to enhance its interaction with SCA8. SCA8 is a non-coding RNA that causes neurodegeneration, if it contains an expanded CTG region that prevents its interaction with Staufen. Staufen mediates RNA localisation and transport in the oocyte and nervous system, and localises Prospero in larval neuroblasts (Mutsuddi *et al.,* 2004).

A yeast 2 hybrid screen indicated that *ssp4* plays a role in mitosis and female meiosis (L Giot *et al.,* 2003). Three cell cycle/cell division proteins NEDD8, Matrimony and Nucleosomal histone kinase 1 (NHK1), were found to associate with Ssp4. NEDD 8 is involved in cell cycle regulation. Matrimony interacts with polo kinase to prevent premature nuclear envelope breakdown (NEB) in female meiosis and NHK1 is involved in metaphase spindle formation in mitosis, and in mitotic spindle organisation and positioning of chromosomes for the karyosome in female meiosis (Information taken from Flybase).

These studies implicate *ssp4* in a variety of cell processes, but no thorough study of *ssp4* has been undertaken. One of the aims of this work is to undertake a complete study of *ssp4* function and the phenotype produced by its 'knockdown'.

1.4 MICROTUBULES

Microtubules are a major component of the cytoskeleton and dictate cell shape, some methods of cell movement, and movement of intracellular structures for example transport of organelles and separation of chromosomes during mitosis. CAMSAP1 has been shown to bind tubulin *in vitro* via its CKK domain (Baines *et al.,* 2009) and CAMSAP 3 interacts with the Zonula Adherens, the most apical area of the cadherin based adherens junction in epithelial cells. Ssp4 has been shown to have a stabilising effect upon microtubules in interphase cells (Goshima *et al.,* 2007). Since CAMSAP family members all contain a CKK domain the interaction of CAMSAPs with microtubules is likely to be key to their cellular functions. Microtubules are made up of globular tubulin subunits (Weisenberg *et al.,* 1968). Each tubulin subunit is a dimer comprising two 55kd proteins (Burns 1991). These are alpha tubulin and beta tubulin (Figure 1.6) the sequences of which are highly conserved in all eukaryotes (Desai and Mitchison 1997). Each tubulin subunit binds two molecules of GTP. A third tubulin, gamma tubulin, is located in the centrosome where it nucleates the polymerisation of the subunits to form alpha beta microtubules (Hughes *et al.,* 2008). Microtubules function as a scaffold in interphase cells, they are linked by protein bridges and become curved in complex patterns, while isolated microtubules exist as straight rods, in epithelia for example (Figure 1.3).

Some cells, usually non-replicating cells, contain stable microtubule based structures eg sperm tails and axons in neurons. Cell structures composed of microtubules may need to assemble and disassemble quickly, for example, during mitosis (Avila *et al.,* 1990). Microtubule bundles also form the basis of Axopodia, which are used for movement and

feeding. Protists and metazoan cells use lamellipodia and filopodia with cytoskeletal movement regulated by Ca^{2+} fluxes, causing ciliary beat changes in protests and muscle contraction in metazoans (Jennings and Acker, 1970)

Microtubules have a rapidly extending plus end and a minus end that grows far more slowly, and this polarity controls movement along microtubules. This growth is due to GTP binding to beta tubulin, which mediates polymerisation of tubulin dimers. Hydrolysis of GTP to GDP reduces the binding affinity of neighbouring molecules leading to treadmilling, which is the loss of GDP bound tubulin dimers from the minus end with their simultaneous replacement by GTP bound dimers at the plus end (Margolis and Wilson1978). Microtubules undergo cycles of growth and reduction known as dynamic

Taken from Nogales *et al.*, 2000

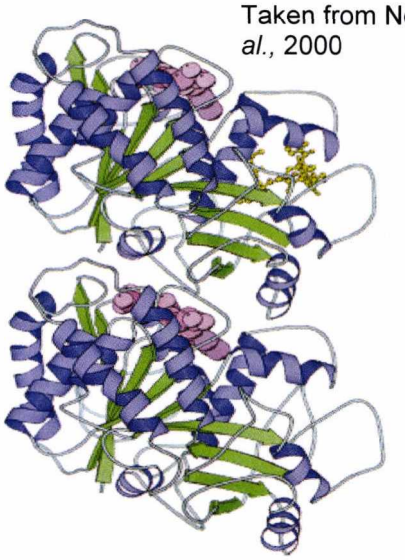


Figure 1.7: Ribbon Diagram of Alpha and Beta Tubulin Dimer.

Beta subunit is at the top. Residues shown in pink are nucleotides. The alpha residue is bound to GTP, and the beta subunit is bound to GDP. The monomers appear similar, but there are differences, which are mainly in the loop regions. In beta tubulin most variation between the different isoforms is seen in the acidic C terminal, which is between 17 and 24 amino acids in length (Reviewed by Nogales et al., 2000).

instability, which is mediated by rates of tubulin addition relative to speed of GTP hydrolysis, and gives microtubules a cellular half-life of just several minutes (Mitchison and Kirschner 1984). Microtubule instability is also affected by cellular regulatory factors such as Ca^{2+} and $p34^{cdc2}$ (Lieuvin, 1994). Microtubule turnover is vital for the cytoskeletal remodelling that is necessary for mitosis.

In an interphase cell the minus ends of microtubules are anchored in a centrosome, which is positioned next to the nucleus. This contains a pair of centrioles, and the pericentriolar material they are surrounded by gives rise to microtubule assembly. During interphase microtubles are long and relatively stable, with a lifespan of about 10 minutes. During mitosis this lifespan is reduced to about 30 seconds with rapid turnover and five to ten times the number of microtubules growing from the centrosome (Avila *et al.,* 1990). The mitotic spindle is formed through the stabilisation of a few of the microtubules that have grown from the centrosome, although for most cell divisions in *Drosophila* centrioles are not required, microtubule nucleation around chromatin is sufficient for bipolar spindle formation (Goshima *et al.,* 2007). At the end of mitosis the spindle disassembles and the interphase MT network reforms.

1.4.1 Structural and Physiological Roles of Microtubules

Drosophila has four isoforms of both alpha (Theurkauf *et al.,*1986) and Beta tubulin (Hoyle and Raff, 1990). Human cells carry approximately 6 genes for both alpha and beta tubulin, producing protein products with different C terminals, which are able to interact with microtubule binding proteins (Reviewed by Nogales, 2000). *Drosophila* alpha tubulins 1 and 3 are constitutively expressed with only two amino acids different between them (Theurkauf *et al.,*1986). Alpha tubulin 2 and 4 are tissue specific with alpha 2 mRNA found only in male adults and the alpha 4 mRNA located in ovarian nurse cells, eggs and early embryos. Beta 1 tubulin is predominant and found in all tissues apart from developing muscle, ovarian follicle cells and male germ line post mitotic cells. Beta 2 tubulin is male testes specific, beta 3 tubulin is expressed during development with spatial specificity, and is the chief variant in developing musculature and ovarian follicle cells, suggesting that this is the preferred isoform for microtubules that are used for cell shape specification or arrangements of tissues (Hoyle and Raff, 1990). Both subunits are subject to a variety of post-translational modifications to the C terminus of alpha and beta

tubulin, and may influence microtubule interactions with cellular factors, localisation, and signalling pathways that involve cytoskeletal interactions (Reviewed by Nogales, 2000).

Microtubule Associated Proteins (MAP) bind microtubules in a phosphorylation regulated manner (Lieuvin, 1994). MAPs prevent microtubule disassembly and are important for the establishment of cell polarity and cell shape (Cooper, 2000). Tau is a MAP that stabilises microtubules and modulates neurite outgrowth (Davis and Johnson 1999). There are proteins that encourage disassembly through cutting of microtubules or by promoting tubulin polymerisation at both ends. Spastin and Fidgetin promote depolymerisation of microtubule minus ends, Katanin induces depolymerisation of microtubule plus ends in anaphase chromosomes (Zhang *et al.,* 2007) and Ssp4 stabilises microtubules in interphase cells (Goshima *et al.,* 2007). Microtubule associated proteins are also responsible for the moving of chromosomes during mitosis.

1.4.2 The Role of Microtubules in Apoptosis.

In *Drosophila* apoptosis or programmed cell death, is needed for embryonic development, nervous system formation and metamorphosis (Bangs and White, 2000). It is induced by extracellular signals such as hormones, survival factors, intercellular interactions, and cell damage (Grether *et al.,* 1995). Cellular interactions and trophic factor levels also induce apoptosis (Reviewed by Nogales, 2000). For example, gut development influences normal apoptosis patterns necessary for successful brain formation (Moss *et al.,* 2006). In the developing nervous system apoptotic cell numbers can vary because of local influences, although the overall pattern of apoptosis remains much the same for any particular developmental stage (Reviewed by Nogales, 2000).

The third *Drosophila* chromosome contains the genes reaper, head involution defective (HID) and grim, which co-operate to induce apoptosis, through the initiation of caspase

activity (Bangs and White, 2000). Following this initiation, apoptotic cells move away from neighbouring cells or the substratum, and the microtubule network disassembles. Cells then undergo surface blebbing (bulging of the plasma membrane) and finally break up. The microtubules re-organise into dynamic bundles that move chromatin and autoantigens into surface blebs and apototic bodies. Markers on the surface of these structures facilitate their recognition and uptake by circulating macrophages, microtubules also construct spikes that help attach apoptotic cells to phagocytes (Moss *et al.,* 2006).

Apoptosis is first seen in the head region at stage 11 and in the epidermal segments at stages 12-14 (Zhang *et al.,* 2007). Germ band retraction also leads to apoptosis at stage 12, in posterior abdominal segments and the procephalic region. Cell death in the dorsal portion of the head is marked at stage 13, and continues into stage 14 when a new ring of dying cells is seen at the dorsally closing tissue. Mid gut apoptosis is seen during stage 15 and also in the head during involution, and large numbers of apoptotic cells are seen as the nerve cord condenses at stage 16 (Valiron *et al.,* 2001). High numbers of glial cells also undergo apoptosis between stages 13 and 17 (Lieuvin *et al.,* 1994).

1.4.3 The Role of Actin and Microtubules in Cell Movement.

The activities of Actin and Microtubules are connected by way of signalling mechanisms or intermediary proteins with a mutual regulation of protein dynamics possibly through steric hindrance (reviewed by Etienne-Manneville 2004). Disruption to the microtubule network enhances neutrophil movement and disturbance of the actin network in differentiating neurons combined with the addition of taxol, leads to vigorous neurite formation (Dehmelt *et al.,* 2003), and this co-operation is co-ordinated by signalling pathways (Palazzo and Gunderson 2002).

In order to move within an embryo, cells need to reach out and withdraw processes, to relinquish contact with other cells, to remove the basal lamina, which would obstruct their path, and prepare a space to migrate to. Prior to movement cells display surface changes resembling blebs, then proceed to stick to the substratum and spread, and finally to move. Movement is guided by endogenous factors, and along known paths for example, neurite migration follows an established route through neural tissue. Cells are also guided by gradients of chemical signals (Armstrong, 1985). The cell has to stick transiently to the substratum so that the actin and microtubules can combine to propel the plasma membrane onwards. Actin is the driving force for cell motility forming filopodia, stress fibres in the cell body and ruffling actin rich lamellipodia for rapid movement at the cell periphery. Slow moving astrocytes use a microtubule skeleton in their long outgrowths with actin providing stress fibres, but it now appears that these roles overlap with bi-directional communication between these two cytoskeletal proteins. Microtubules are not found in filopodia, and only a few are found in lamellipodia, but they may contribute to cell protrusion formation. Inhibition of microtubule dynamics, that leave the network intact, inhibit lamellipodia formation and so microtubules may co-ordinate cell protrusion formation in partnership with actin (Liao *et al.,* 1995).

1.5 PROJECT AIMS

The over arching aim of this project is to study the CAMSAP family of proteins *in vivo* using Drosophila as a model organism. *Drosophila* is ideally suited for the study for the CAMSAPs as it has only one family member, *ssp4*. The *Drosophila* genome is well-characterised. Many of its 15,000 genes are homologous to human genes, and up to 77% of human disease genes conserved (Reiter *et al.,* 2001). Generation of transgenics is straightforward.

In order to establish the function of *ssp4* in *Drosophila* I undertook a comprehensive study of the gene and the results of mutating the locus. Specifically my aims were:

1. To compare the human CAMSAPs and Ssp4 using Bioinformatics.
2. To establish the expression patterns of *ssp4* mRNA and Ssp4 protein. .
3. To analyse disruptions at the *ssp4* locus.
4 To determine the phenotype of flies that lack *ssp4* gene function.

Chapter 2

MATERIALS AND METHODS

2.1 FLY CULTURE AND HUSBANDRY

2.1.1 Food for *Drosophila*

Drosophila were reared on cornmeal-based fly media which comprised agar, cornmeal, yeast, dextrose, sodium potassium tartrate, calcium chloride plus Nipagin – an antibacterial agent. (see 2.4 for protocol).

2.1.2 Stock Maintenance

Stocks were maintained in small vials on cornmeal based media, bottles were used to generate large numbers of flies for embryo production or *en masse* crosses. A proportion of one male to three females was usually adequate to maintain stocks, and these stocks were used to generate healthy offspring to maintain the line. In some cases, for example in lines containing multiple balancers, more adults would be required for stock maintenance.

Stocks were maintained at 18 degrees centigrade, and transferred into fresh food vials daily. For genetic crosses, or amplification of fly numbers, vials and bottles were kept at 25 degrees centigrade the optimum temperature for *Drosophila melanogastor.* .

2.1.2.1 Use of Balancer Chromosomes in Stock Maintenance

Balancer chromosomes contain inversions, which prevent meiotic recombination in female flies. Meiotic recombination does not happen in males. Balancer chromosomes allow specific genotypes to be stably maintained. Balancer chromosomes contain a dominant mutation as a marker, for example, one that changes bristle morphology or number. This can be used to confirm the presence of the balancer chromosome in a given stock or fly.

2.2 HANDLING AND MANIPULATION OF FLY STOCKS

2.2.1 Transfer of Fly Stocks

Stocks were transferred by tapping from one vial, into a fresh vial which contained a little fresh yeast. Vials were then sealed with a cotton wool bung. The escape of flies was prevented by giving the vial a sharp tap on the bench, prior to upending over the new vial and tapping them in.

2.2.2 Anaesthesia of Flies Using Carbon di Oxide

Flies were immobilised using carbon di oxide to allow close examination of their phenotype or to sort males from females for setting up fly crosses. This exposure to carbon di oxide does not harm the flies if kept to a minimum. The gas was supplied from a cylinder (BOC) to a pad at the "fly station". The pad was made from a plastic plate covered in a porous material that let the gas pass through to the flies. The fly station comprised a microscope (Meiji) and a light source (Micro-tech fibre optics MFO-90). Care was taken when working with carbon di oxide, since lengthy exposure can cause headaches.

2.2.3 Setting up and Maintenance of Genetic Crosses

One male to three females was used, wherever possible, to set up genetic crosses in small vials, at 25 degrees centigrade. Once numbers were increased in the vial, these flies would be moved to a bottle to maximise embryo numbers for collection.

2.2.3.1 Sexing Flies

This was carried out under a standard dissecting microscope. There are obvious physical differences between males and females – the females are larger than the males, and have a pointed abdomen and more stripes than the males. Males also have sex combs on their front legs (Flybase).

2.2.4 Monitoring the Development of *ssp4* ^{k07433} and *ssp4*^{EY0525} Flies

Embryos no more than 5 hours old, were collected from laying plates at random, and counted out onto clean laying plates (see protocol 2.4). After being left for 12 hours, first instars were removed to food plates and allowed to continue their development. The remaining embryos were examined using a Leica MZ microscope with a UV light and a GFP filter set to separate mutants from heterozygotes. After 9 days, adult flies were counted, and checked to ensure they were heterozygous for the *ssp4* mutant allele. Wild type embryos were also collected and counted in the same way, as controls, for handling and treatment.

2.3 VISUALISATION OF STRUCTURES IN THE DEVELOPING EMBRYO

2.3.1 Visualisation of the Embryo Using X-Gal Staining.

Embryos containing the Lac Z marker driven by the promoter from the *even-skipped* gene expression were stained with the Lac Z substrate X-Gal (5 bromo-4 chloro-3-indolyl-β-D-galactoside, Melford Labs) in order to discriminate between heterozygous and homozygous mutant flies.

Embryos were collected and dechorionated prior to fixing in a solution of 5% formaldehyde for 10 minutes at room temperature (see protocol in 2.4), and rinsed thoroughly in 1% PBT, allowed to dry and washed again for 2 x 20 minutes. Meanwhile, X-Gal staining solution (recipe in 2.4) was pre-warmed up to 37 degrees centigrade. Crystals of X Gal were added to 1ml of staining solution and left at 37 degrees centigrade until a supersaturated solution had been obtained. The solution was spun to pellet the remaining crystals and the solution removed leaving the crystals in the microcentrifuge tube. The solution was added to the embryos, which were then left overnight at 37 degrees centigrade. All staining steps were carried out in covered watch glasses as the reaction is photosensitive. After staining, the embryos were

washed in PBT, and washed in methanol, prior to devitilinisation in n-heptane and methanol. The embryos were then rinsed thoroughly in PBT. The embryos were then ready for antibody staining if required. If not, they were cleared in 50%,70%,90% and 100% glycerol (BDH) in water and mounted in glycerol on slides that had slightly raised cover slips to prevent squashing. The specimens were examined under the Leica DMR fluorescence microscope and images captured at a magnification of 200X on a Leica DC500 12 mega pixel digital camera.

2.3.2 Preparation of the Ssp4 Primary Antibody

2.3.2.1 Immunisation

All procedures were carried out by staff at the University of Kent under a home office licence and according to guidelines in the Animal Scientific Procedures Act (1986). A synthetic peptide was made using the Shimadzu peptide synthesiser PSSM-8. This peptide represented the C-terminal 14 amino acid residues (RVQLPSKKDMALVI), plus an N terminal cysteine for conjugating to keyhole limpet hemocyanin. Primary injections of 400µg peptide conjugate, in Freund's complete adjuvant (FCA) were given followed by boosts of 400µg of the conjugate in FCA at four weekly intervals. Test bleeds were carried out following the 3rd injection, and the terminal bleed was done after the 4th/5th injection.

2.3.2.2 ELISA (Enzyme-linked Immunosorbent Assay) Test of Serum

A Maxisorp™ plate was coated with the CRVQLPSKKDMALVI peptide used for the immunisation at a final concentration of 10 mg/ml in sodium phosphate buffer (PBS). A dilution series of the serum was made in a solution of 1% BSA in PBS, starting at 1:50 and finishing at 1:3200. A pre-immunized rabbit was used as a control. Four replicates of each concentration were made. The serum was left in the plate for 1 hour, then the plate was rinsed 3 times with a solution of 1 x PBS and 0.05% Tween 20. An anti rabbit secondary, coupled to horseradish peroxidase, was added to the plate at a

concentration of 1:500, diluted in a solution of 1% BSA and PBS. This was left for one hour. The plate was washed 3 times as before and substrate was added. When the wells turned yellow, sulphuric acid was added to stop the reaction. The plate was read on a plate reader to measure absorbance. A strong signal was obtained relative to the control.

2.3.2.3 Peptide Column

The peptide was attached via the cysteine residue to the Sulfolink ® column, via a sulphydrol bond with sulpholink. 3ml Sulfolink ® (Pierce, Illinois, USA) coupling gel suspension was added to a small Bio Rad (California, USA) column and washed with 12ml coupling buffer (50mM Tris-HCl pH8.5 containing 5mM EDTA). 3-6mg of peptide was dissolved in 2ml coupling buffer and added to the column. This was mixed at room temperature for 15 minutes, protected from the light, then left to settle for half an hour. The column was washed with coupling buffer and 1.5ml of blocking buffer (30µl β-mercaptoethanol in 5ml coupling buffer) added. This was mixed for 15 minutes then left to settle for 30 minutes. The column was then washed with 1M NaCl (13ml) and could then be stored in 1 x PBS (with 0.02% w/v azide, 4 degrees centigrade) prior to use.

2.3.2.4 Antibody Affinity Purification.

2µl of leupeptin (5mg/ml), 2 µl Pepstatin (1mg/ml in ethanol) and 2 µl of phenyl methyl sulphonyl fluoride (PMSF) (1mM in isopropanol) were added to 4-6ml of serum, which was the added to the affinity column (flow rate 15ml/h, 4 degrees centigrade, capacity 15mg) pre-equilibrated with 1 x PBS. The column was washed (flow rate 30ml/h) with 15-30ml 1 x PBS and then with 15-30ml 1 x PBS with 1M NaCl. The bound antibody was rinsed from the column using acidic (0.1M glycine, 0.15M NaCl, pH2.8) buffer. The bound antibody was collected as fractions, which were quickly neutralised with unbuffered 2M Tris. The column was then neutralised by rinsing it through with 30ml 0.1M Phosphate buffer pH 7.4. The column was then given a final wash with 1 x PBS

containing 0.02% w/v NaN_3 for storage at 4 degrees centigrade. 2µl of each of the neutralised fractions were spotted onto nitrocellulose which was then blocked in Marvel™ (dried milk) in 1 x PBS at room temperature agitating for 1 hour. Peroxidase conjugated anti rabbit secondary antibody was added, and the fractions that stained the most strongly were combined and purified using dialysis. The remaining fractions were frozen, for possible future use.

2.3.2.5 Western Blot of Ssp4 to Confirm Antibody Specficity.

One overnight collection of embryos was dechorionated and homogenised with a Teflon on ice in pepstatin (10µg/ml) aprotinin (10µg/ml), leupeptin (1µg/ml) and 10µls of protease inhibitor (Roche – 1 tablet dissolved in 10mls of PBS). 4 X SDS buffer was then added (see section 2.4). The embryos were vortexed and left on ice for a further 30 minutes, and spun at 4 degrees centigrade at 4000 rpm. The supernatant was pulled through a syringe to destroy any DNA, and then equal amounts were combined with 4 X SDS buffer and heated to 80 degrees for 5 minutes. 10µl of each sample was run on a 5% acrylamide stacking gel and an 8% acrylamide resolving gel (see 2.4 for protocol) with 10µl of molecular weight marker, at 80 -100V through the stacking gel, and 150-180V through the resolving gel. Coomassie blue was used to check protein levels (see recipe in 2.4). The protein was transferred onto nitrocellulose overnight at 30 milliamps, and at 4 degrees centigrade.

Protein transfer to the nitrocellulose was confirmed by soaking in Ponceau red for 20 minutes, which enabled the transferred proteins to be visualised as pink bands (see recipe in 2.4). The blot was then blocked with Marvel™ (dried milk) and probed with primary Ssp4 antibody at a concentration of 1:1000 in blocker for 3 hours whilst being agitated. Following 3 x 20 minute rinses in PBT the sheet was probed with an anti rabbit IgG horseradish peroxidase conjugate secondary for 1 hour, and then rinsed as above. Thorough rinsing helps minimise background. ECL solution (Promega) was

used to visualise protein staining. The substrate working solution was prepared by mixing equal parts of luminol enhancer solution with peroxide solution, and the membrane was incubated in this for 1 minute at room temperature (Promega). The nitrocellulose was placed in a cassette with a film and exposed for 1 minute in the dark room. Exposure time was sometimes increased as light emission from the nitrocellulose decreased over time.

2.3.3 Visualisation of Protein Expression in the Embryo, using Antibodies

2.3.3.1 Preparation of Embryos for Antibody Staining

Collections of embryos from apple juice agar plates (see section 2.4) were made in the morning (0-15 hour collections) and the evening (0-8 hour collections). The embryos were dechorionated in 100% sodium hypochlorite (Fisher) whilst being regularly monitored through the dissecting scope to ensure that the chorions were removed before rinsing the embryos thoroughly in tap water. The embryos were then fixed in equal volumes of n-heptane (Fisher) and 10% formaldehyde solution (Sigma) for 20 minutes (protocol in 2.4). The lower aqueous phase was removed and methanol added to the embryos and shaken vigorously, for a few minutes, to remove the vitelline membranes. The devitillinised embryos were collected from the bottom of the tube and rinsed several times in methanol. At this point embryos could be dehydrated by rinsing in 50%,70%,90% 100% ethanol and stored at minus 20 degrees centigrade until needed.

2.3.3.2 Antibody Staining of Embryos

Embryos were rehydrated in methanol 1:1 methanol/PBT. They were then rinsed in PBT alone. At this point, embryos could be incubated in a 10% bovine serum albumin solution (Acros organics) for 30 minutes to block non-specific antibody binding, if required. Primary antibody was then added in PBS, at the following concentrations:

Ssp4	(rabbit polyclonal)	1:5000
Anti Beta Galactosidase (β Gal)	(mouse monoclonal)	1:5000
ELAV	(mouse monoclonal)	1:1000

All other antibodies used in this study were mouse monoclonal and used at a concentration of 1:250. All mouse monoclonal antibodies, apart from Anti β Gal, were obtained from the Developmental Studies Hybridoma Bank, and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

Serum (Vector Labs) was added to the primary antibody, to a final concentration of 5%, if required. This was goat serum if the primary was raised in rabbit, or horse serum if the primary was raised in mouse. Embryos were then left agitating overnight at 4 degrees centigrade.

Following washing in PBT (3 x 3 minutes, 3 x 20 minutes) secondary antibody was added at a concentration of 1:500 and incubated as for primary.

2.3.3.3 Vector ABC Complex & Visualisation using Diaminobenzidine (DAB)

Embryos were rinsed in PBT (3 x 3 minutes, 3 x 20 minutes). The Vector ABC complex was prepared (16µl A, 16µlB in 1ml PBT) and incubated at room temperature for one hour to allow complex formation. The embryos were added to the ABC for 30 minutes, washed for 1 minute and then given 3 x 20 minute washes in PBT. DAB was prepared with hydrogen peroxide as part of a kit, according to the instructions. Care had to be exercised due to its carcinogenic nature. DAB was added to the embryos, and this reaction was closely observed through the dissecting scope, and stopped using PBT. After a series of washes in PBT the embryos were dehydrated in 30%, 50%, 70%, 90%, and 100% ethanol in water. These dehydrating incubations were of 15 minutes duration, and the 100% incubation was carried out 3 times. The dehydrated embryos were cleared in Histoclear (National Diagnostics) and mounted in DePex (BDH) on

microscope slides. Images were obtained using the Leica DMR microscope as described above.

When staining to embryos was carried out using 2 antibodies, both primaries would be added together, and then one secondary. After the DAB reaction, instead of dehydrating the embryos, they would have the second secondary antibody added and the visualizing reaction carried out with Alakaline Phosphatase (Vector Labs). These embryos could not be dehydrated in ethanol as this removed the blue colour of the alkaline phosphatase. Instead, they were dehydrated in 30%, 50%, 70%, 90%, and 100% glycerol in water. These incubations were carried out at 4 degrees centigrade for a minumum of two hours each, with the final incubation taking place overnight.

2.3.4 Visualisation of Protein Expression in the Embryo, using an *In Situ* Probe

2.3.4.1 Preparation of the *ssp4* DNA for Probe Production

A probe complementary to ssp4 mRNA was produced, using a cDNA (AT18914) from the Berkeley Drosophila Genome Project (Stapleton et al., 2002). This cDNA is complimentary to *ssp4* and represents a full-length transcript that includes the exons that make up *ssp4* transcript A (Flybase). The cDNA was inserted into a pOTB7 plasmid. As well as the *ssp4* DNA this vector also contained a gene that coded for chloramphenicol acetyltransferase, conferring resistance to chloramphenicol upon cells carrying the vector (See Figure 2.1).

The *E. coli* carrying the *ssp4* were streaked onto agar plates, using the aseptic technique, and then left overnight at 37 degrees centigrade. 12 individual colonies were isolated, and placed into autoclaved LB broth (see 2.4 for protocol), and left overnight at 37 degrees centigrade. The Qiagen Mini Spin Kit, was used to isolate the plasmid containing the *ssp4* DNA from the bacteria.

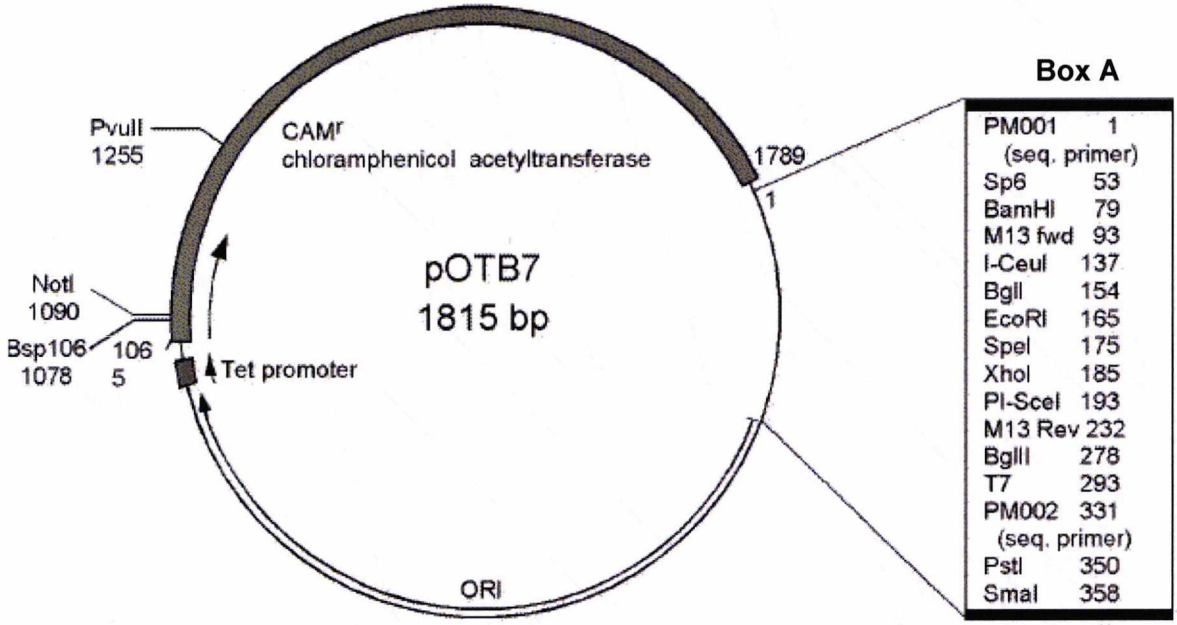


Figure 2.1 The pOTB7 Vector Containing *ssp4* cDNA *(Stapleton* et al 2002*).*

Tet promoter – tetracycline resistance promoter. In the presence of tetracycline this promoter is inactive. It drives expression of chloramphenicol acetyltransferase, conferring resistance to chloramphenicol. Box A – PM001 and PM002, M13 5' fwd and M13 3' rev are primers for ssp4 DNA. Sp6 and T7 are sequencing primers, and sites for restriction enzymes. The ssp4 cDNA is located between the Eco RI and Bgl II restriction sites. Sp6 and T7 are the sites for the polymerase that produces the sense and antisense strand, respectively.

An alkaline buffer broke open the bacterial cells, and denatured the bacterial DNA, and linearised the plasmid. This buffer was then neutralised to allow the plasmid to reform. The bacterial DNA pellet and unwanted proteins were discarded, following subsequent spins, then the plasmid DNA was washed through the column with elution buffer pH 7 – 8.5, the optimum pH for elution. A restriction enzyme was used in a DNA digest of 2µl from each plasmid extraction, to linearise the plasmid. These were then run on a DNA gel, and the 2 best samples were selected for purification using the QIAquick PCR purification kit (Qiagen). A little of each of these samples was sent away to Lark Laboratories for sequencing, and to confirm the position and orientation of *ssp4* DNA in the plasmid. The DNA sent for sequencing was confirmed to be of very good quality.

The 2 samples were then grown up in broth (see 2.4 for protocol), and a maxi prep kit (Qiagen) used to isolate large quantities of plasmid, to provide sufficient DNA for the entire project. A small amount of this plasmid DNA was linearised using EcoRI (Roche) and Bgl 2 (Roche) restriction endonuclease to provide the template for the sense and anti-sense strand, respectively (Figure 2.1) and checked on a DNA gel (see 2.4 for protocol). The rest was frozen at minus 20 degrees centigrade.

2.3.4.2 Probe Production

The plasmid DNA was linearised with the restriction endonucoleases EcoRI and Bgl2. The DNA was then purified, and the probe generated. 1µg of the linearised DNA was combined with 2µl of DIG (digoxygenin)/NTP mix, 2µl of 10 times transcription buffer, and 1µl RNAse inhibitor (40µg/µl). 2µl of either T7 polymerase or SP6 polymerase was then added, according to whether an antisense or sense probe was being generated. All the reagents used came from Roche. This reaction was left at 37 degrees centigrade for 2 hours. 2µl of DNAse (Roche) was then added to remove the DNA template, and left for 15 minutes. The reaction was stopped using 2µl of 0.2M EDTA (BDH) pH 8.0 or by heating for 20 minutes at 65 degrees centigrade. The probe was then aliquoted and stored at minus 20 degrees centigrade. To determine the probe concentration and labelling efficiency ethanol was used to pellet the probe which was then re-suspended in RNAse free water. A serial dilution of 1/10,1/100,1/1000, 1/10,000, and 1/100,000 was made of the experimental probe and of the control RNA provided in the kit (Roche). All these samples were spotted onto a nylon membrane in separate rows and fixed by exposure to ultra violet light. The membrane was then washed in buffer and incubated with blocking solution for 30 minutes prior to incubation with 1:5000 anti digoxygenin antibody, conjugated to alkaline phosphatase (Roche). After washing, colour detection buffer (4.5µl of NBTand 3.5 µl BCIP per ml of buffer), and then colour substrate solution were added. The reaction was stopped by washing the membrane in sterile water. A

comparison of the spot intensities with the control revealed the probe concentration. Probe integrity was confirmed using a DNA gel.

2.3.4.3 Preparation of Embryos for *In Situ* Staining

Embryos were prepared in the same way as for antibody staining (see 2.3.3.1). They are then rehydrated for 5 minutes 7:3 (Methanol :4% paraformaldehyde), then 5 minutes 5:5 (Methanol: 4% paraformaldehyde) and 5 minutes 3:7 (Methanol: 4% paraformaldehyde) and then fixed again for 20minutes in 4% paraformaldehyde.

2.3.4.4 *In Situ* Staining of Embryos

Embryos were washed for 3 x 10minutes in PBT, and incubated for exactly 3 minutes with 25µg/ml proteinase K in PBT (Sigma), then rinsed for 2 x 1 min with 2mg/ml glycine in PBT. They were then washed for 2 x 5 minutes with PBT and post-fixed for 20 minutes in 4% formaldehyde (Sigma), and washed for 6 x 5 minutes in PBT. They were then incubated for 10 minutes in 500µl 1:1 (hybrix:PBT)(2.4). Meanwhile, 200µl hybrix was preheated to 56°C, and added to the embryos to pre-hybridise at 56° for 1-4 hours. This hybrix was then replaced with fresh pre-heated hybrix, with heat denatured cRNA probe diluted in RNase free water at a final concentration of ~0.25-0.5ng/µl. The embryos are left to hybridise overnight at 56 degrees centigrade.

The embryos were rinsed briefly with 500µl hybrix at 56°C, then incubated for 2 x 20 minutes with 500µl hybrix at 56°C, followed by an incubation of 1 x 20 minutes in 1ml 1:1 PBT:hybrix at 56°C, and then for 1 x 10 minutes in 1ml PBT at 56°C. The solution was removed and 1ml of PBT added. All procedures from this point were carried out at room temperature. The embryos were washed 4 x 20 minutes in PBT, and then for 3 x 20 minutes in PBT+0.2mg/ml BSA to block the tissue, prior to a 2 hour incubation in PBT+0.2mg/ml BSA +1:2000 to 1:2500 DIG antibody. They were then rinsed for 5minutes in PBT, and for 4 x 20 minutes in PBT, with a final rinse in PBS.

2.3.4.5 Visualisation of *In Situ* Staining of Embryos

The embryos were washed for 3 x 5 minutes in alkaline phosphatase staining solution (2.4) to equilibrate tissue, and transferred to a glass dish for colour development. The embryos were kept in the dark with 3.5-4.0µl/ml NBT (100mg/ml in 70%v/v DMF) + 3.5µl/ml X-phosphate (50mg/ml in DMF). The reaction can take from minutes to several hours, and was photosensitive, so the embryos were observed regularly during colour development. The reaction was stopped using 3 x 5 minute washes in PBT and the embryos dehydrated and mounted in glycerol, as detailed in section 2.3.3.3.

2.3.5 Use of Acridine Orange to Label Apoptotic Cells.

Embryos were dechorionated in a solution of 50% sterile water and 50% sodium hypochlorite (Fisher), and then rinsed well with water. They were then shaken for 5 minutes in a solution of 1:1 heptane, 5µg/ml acridine organge in 0.1M sodium phosphate buffer, pH 7.2. The stock solution of Acridine Orange was made up in ethanol as follows: 1mM stock – 1.85mg of Acridine orange in 5ml of ethanol – and stored in the dark at room temperature. It was diluted in sodium phosphate buffer just prior to use. The embryos were then rinsed in N heptane and viewed under halocarbon oil on the fluorescence microscope (see 2.3.1) using green fluorescence filters. Because this procedure is carried out on live tissue, visualisation must be undertaken immediately (Denton *et al.*,2008).

2.4 STANDARD SOLUTIONS USED IN EXPERIMENTAL WORK

Unless stated otherwise all reagents are of a standard laboratory grade.

Preparation of Fly Media

This protocol will produce 6 litres of cornmeal based fly media.

Agar	55.0g
Cornmeal	450.0g
Yeast	189.0g
Dextrose	558.0g
Sodium Potassium Tartrate	51.6g
Calcium Chloride ($2H_2O$) (BDH)	4.2g
* p-Hydrobenzoic Acid Methyl Ester (Nipagin –irritant)	15.0g
Ethanol	56.0ml

After preparation dispense the cornmeal media immediately into culture vials (7-8mls per vial) or bottles (70mls per bottle).

Laying Plates

1. Add 9 grams of Agar (Beckton, Dickenson and Co, UK) to 250ml of apple juice.
2. Bring to the boil in the microwave.
3. Add 6ml of acetic acid and 6ml of ethanol to the apple juice and agar mixture.
4. Add 250ml of apple juice.
5. Cool and pour into petri dishes.

Fly Work Solutions

PBS – 10X STOCK SOLUTION

1 Add 86.77g of NaCl to 100ml of 1M $NaPO_4$ (sodium phosphate buffer) pH 6.8.

2 Make up to 1 litre using de-ionised water (dH_20).

3 Autoclave and dilute with dH_20 when needed.

PBT

1 x PBS + 0.1% of the detergent Triton X – 100 (Acros, USA).

X Gal Staining Solutions

Embryo Fixing Solution

50µl of Formaldehyde (36.5%)

400µl of Citrate buffer (9 vol of Na_2HPO_4 0.2 M,1 vol of citric acid 0.1M,10 vol water)

500µl of N Heptane (Fisher Scientific).

X Gal Staining Solution

10mM sodium phosphate (pH 7.2)

150mM NaCl

1mM $MgCl_2$

3mM $K_4[Fe(CN)_6]$

3mM $K_3[Fe(CN)_6]$

0.3% Triton X -100

Solutions for Western Blotting

4x SDS sample buffer

0.25M Tris-HCl pH 6.8

8% SDS

30% Glycerol

0.02% Bromophenol Blue)

0.3M DTT

8% Resolving Gel (sufficient for 2 gels)

5.3ml of distilled water

2ml of 40% acrylamide mix (Biorad)

2.5ml of 1.5 M Tris (pH 8.8)

1ml of 10% SDS,

1 ml of 10% APS

10µl of Temed (Sigma).

The gels were poured into the Protean II glass sandwich on a clean surface, and fitted into the clamp assembly. After pouring the resolving gel was overlaid with water and left to set. When set, the water was poured away from the resolving gel, the stacking gel was added, and a comb inserted.

5% Stacking Gel (sufficient for 2 gels)

3.64 ml of distilled water

0.625ml of 40% acrylamide mix

0.630 ml of 1M Tris (pH 6.8)

50 µl of 10% SDS

50µl of 10% APS

5µl of Temed.

Running Buffer - pH 8.3

25 mM Tris base

190 mM glycine

0.1% SDS

Transfer Buffer – pH 8.3

25 mM Tris base

190 mM glycine

0.037% SDS

20% Methanol

Cool to 4 degrees centigrade prior to use.

Ponceau Red

2% Ponceau S in 5% Acetic Acid. Destain by rinsing with water.

Coommassie Blue

0.25g Coomassie Brilliant Blue in 90ml methanol:water (1:1 v/v) and 10ml glacial acetic acid. Filter through Whatman™ paper.

Coommassie Blue Destain.

Destain for Coomassie Blue is methanol:water:acetic acid mix as for stain omitting the Coomassie.

Embryo Fixing Solution for Antibody Staining

1.5ml of fixation buffer. (1.3 x PBS, 67nM EGTA pH 8.0).

0.5ml 37% formaldehyde (Sigma).

Add 2.5ml of n-Heptane (Fisher Scientific).

***In Situ* Hybridisation Solutions**

LB (Luria-Bertani) Broth

950 ml de-ionized water

10 g Tryptone (Oxoid Ltd)

10 g NaCl (Fisher Chemicals)

5g Yeast Extract (Oxoid Ltd)

DNA Gel

1.2g Agarose (Melford Laboratories)

Ethidium Bromide (Sigma)

50ml 1 X TAE

1X TAE (1 litre)

4.84g tris base (Melford Labs),

1.14g of glacial acetic acid (Fisher Scientific),

2ml of 0.5M EDTA (BDH).

Make up to 1 litre with de-ionised water

Hybrix (Store in aliquots at minus 20 degrees centigrade)

50% deionised formamide

(molecular biology grade, BDH. Store in aliquots at minus 20°C)

5 x SSC

100µg/ml tRNA

50µg/ml Heparin

0.1% Tween-20

Alkaline Phosphatase Staining Solution for 10 mls

100mM NaCl	1ml of 1M
50mM $MgCl_2$	1ml of 0.5M
100mM Tris.HCl pH9.5	1ml of 1M
0.1% Tween-20	10 µl
1mM Levamisole*	0.024 g/ml = 100 mM. Add 100µl

*must be made fresh every day, inhibits endogenous phosphatases.

Make up to 10mls with sterile water.

Chapter 3

COMPARISON OF DROSOPHILA SSP4 TO HUMAN CAMSAP PROTEINS USING BIOINFORMATICS

3.1 CHAPTER OVERVIEW

CAMSAP1 is a calmodulin-associated protein, and this association regulates CAMSAP 1 spectrin binding. CAMSAP1 has a novel calponin homology (CH) domain at the N terminal, and a CKK domain, which binds microtubules, at the C terminal. The protein contains three coiled coils, one of which contains the calmodulin binding domain (CaMB), and putative binding sites for SH3 domains. (Baines *et al.,* unpublished data).

The *ssp4* gene is the *Drosophila melanogaster* version of the vertebrate CAMSAPs. In this chapter Bioinformatics (a combination of biology and information technology) is used to compare s*sp4* and the human CAMSAPs.

3.2 BIOINFORMATICS

The sequencing of the *Drosophila* genome in March 2000 (Adams *et al,* 2000), and the publishing of the complete human genome in 2003 allowed the creation of Bioinformatics databases with data for these two organisms, and subsequently many others. It is possible to find gene sequences, and transcripts, and scan a peptide sequence to predict protein structure and function, such as the secondary structure, putative sites for protein/protein interactions and motifs (Tweedie *et al.,* Pagni *et al.,* 2001). This information can be combined with details of gene expression reports to inform the nature of the protein function. Blast searches locate homologous sequences in other genomes and the Clustal search facility can then be used to compare them.

3.2.1 Results of Transcript Analysis

ssp4 genomic sequences and protein sequences were retrieved from Flybase version R5.8. Flybase is an online bioinformatics database of the genomics and molecular biology of *Drosophila melanogastor* and related *Drosophilid* dipterans. The *ssp4* gene (CG33130) is located on the 2R chromosome, between bases 13034543 and 13050512 and has eight known splice variants predicted through comparison to either protein or cDNA sequences (Figure 3.1). The amino acid sequences were scanned to produce a schematic with their final protein products. Features shown include protein binding domains and areas containing high levels of a single amino acid (Figure 3.2).

Isoforms A – F share the first eight exons, which give rise to an N terminal domain and CH domain. These two domains are absent in the G isoform which consists of solely the CKK domain and truncated H isoform. Whilst there is variation between the third coiled coil and the CKK domain in isoforms A – F the CKK domain is common to all eight, and most have three coiled coils. Scores are allocated by Flybase to transcripts to demonstrate a level of confidence in the transcript annotation. Isoforms C, D, E and H are weakly supported, and isoforms A,B, G and F are strongly supported (Flybase version R5.8), with isoform A being fully consistent with the annotated transcript. Unless otherwise stated, isoform A will be used as a comparison with the human CAMSAP proteins in this chapter. Clustal analysis which compares protein sequences, showed the closest match for *ssp4* was the human CAMSAP3, followed by CAMSAP 2 (Clustal W2, EBI, EMBL).

3.2.2 Results of Comparison of Ssp4 and Human CAMSAP Proteins.

Human CAMSAP protein sequences were retrieved from Ensembl (Spudich *et al* 2007). The positions of the protein features were calculated relative to protein length (Figure 3.2).

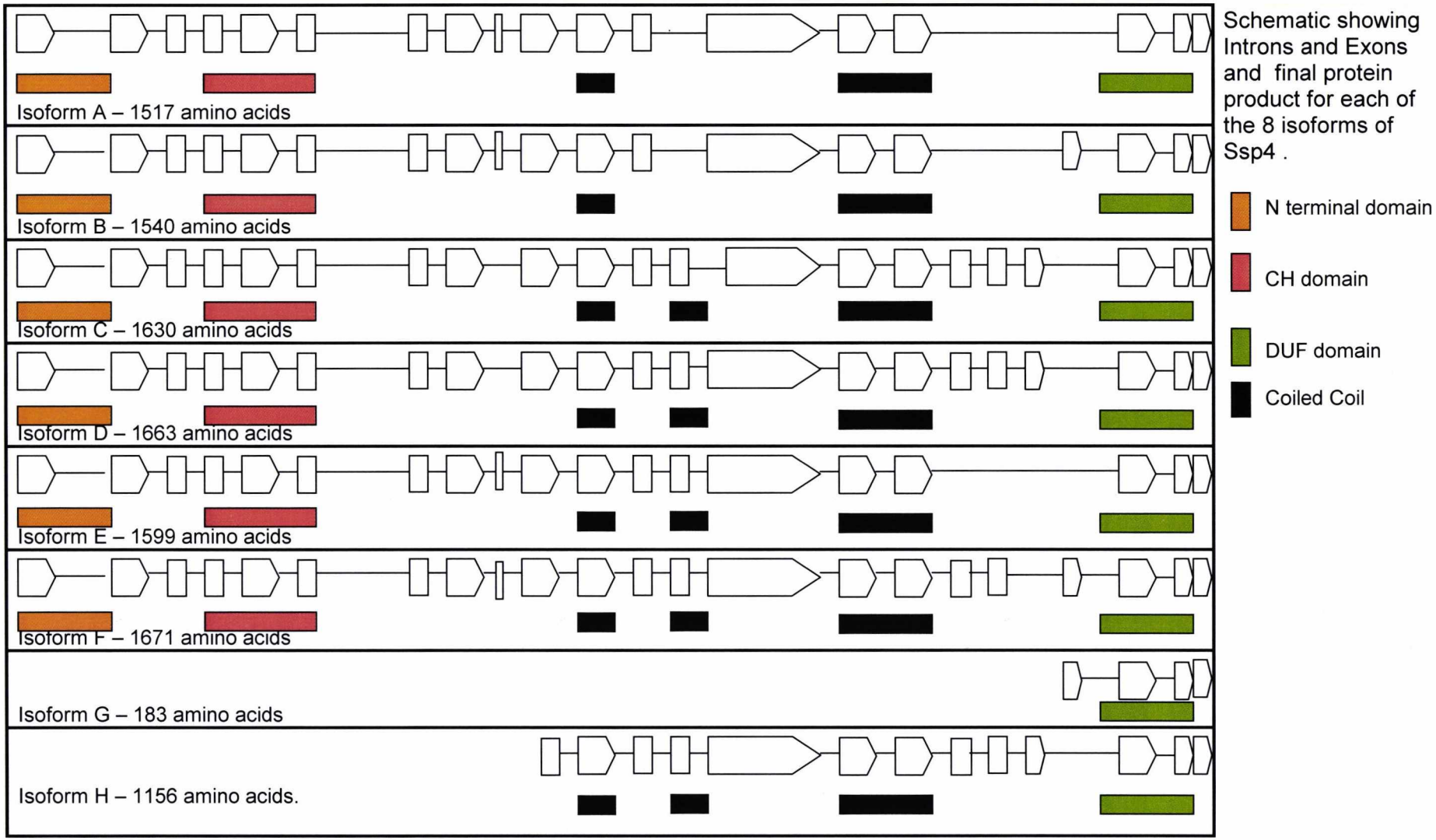
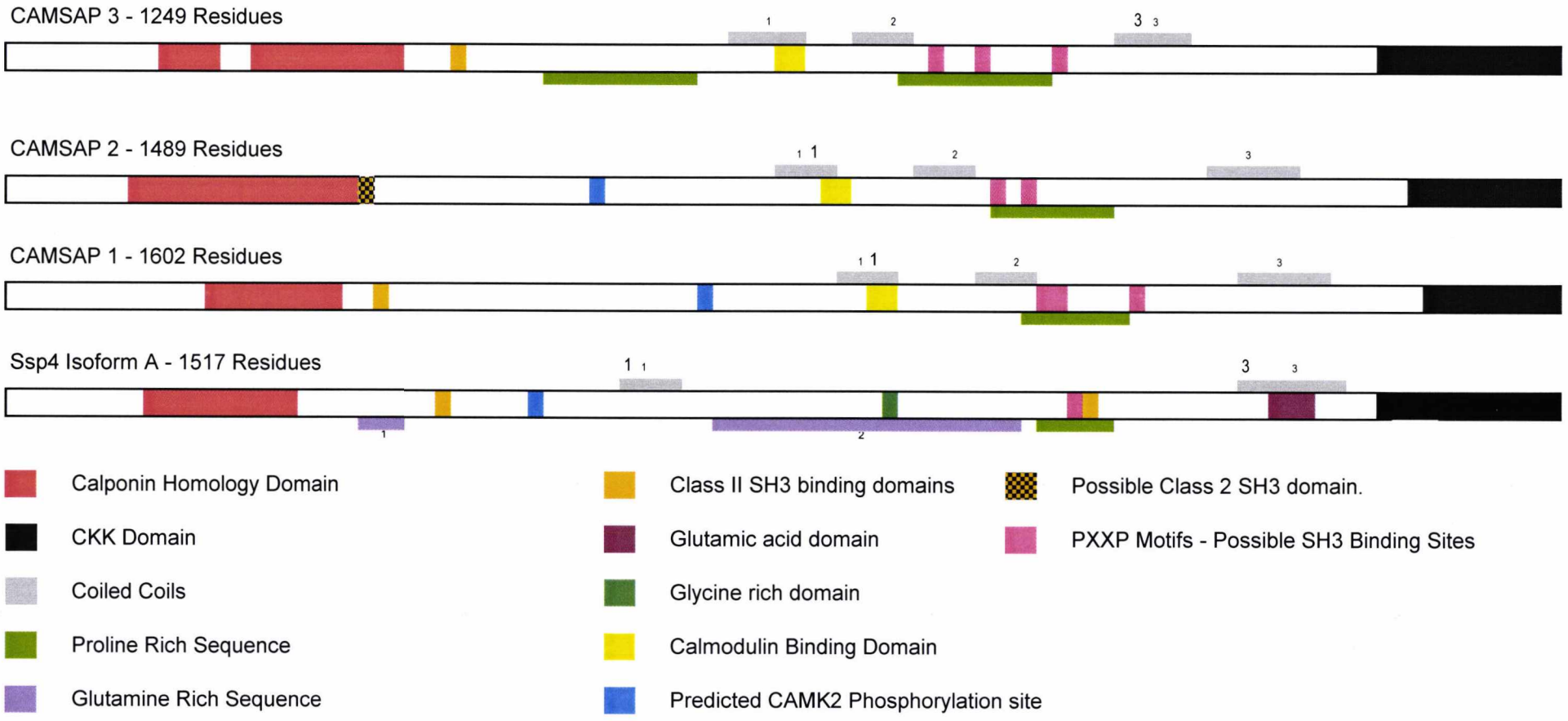


Figure 3.1: Splice variants for Ssp4 isoforms.

The putative calmodulin binding sites and extended glutamine rich domains will be discussed in sections 1.3.3 and 1.3.4. Exon numbers might be the same but because of differences in these exons, overall protein is different. Amino acid number does not correlate to increase in protein complexity.

Figure 3.2 Positions of the Proteins Features Calculated Relative to Protein Length



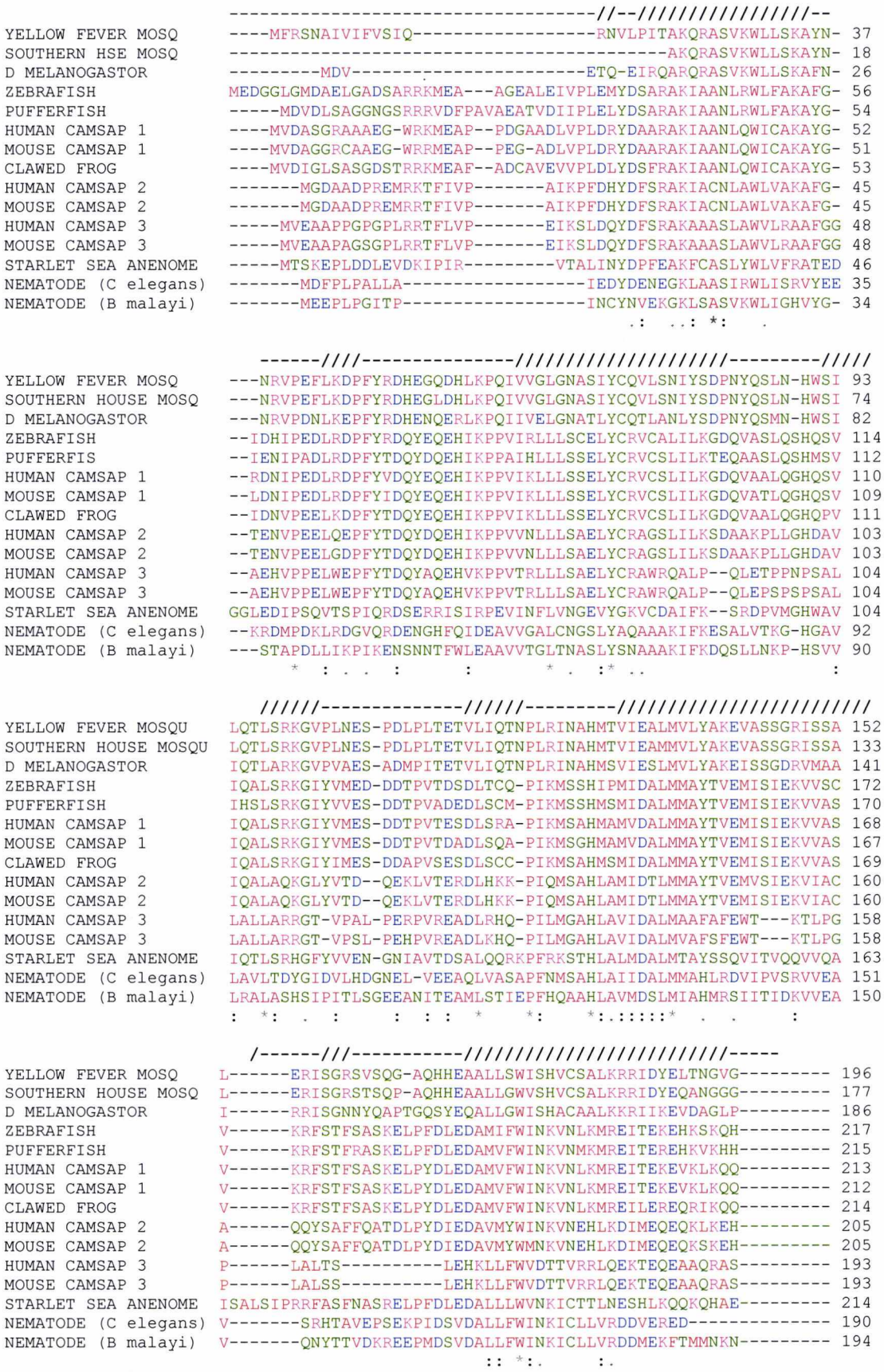
The amino acid sequences for the three human CAMSAPs were taken from Ensembl, and the sequence for the A isoform of Ssp4 were taken from Flybase. Positions of domains etc have been calculated relative to the overall protein length. The databases at Pfam, Scansite, and Embnet supplied details of protein features. NB Glutamine rich sequences in Ssp4 are absent in human CAMSAPs.

The CH and CKK domains have already been discussed (see introduction) and Clustal analysis revealed an N -Terminal domain common to Ssp4 and the Human CAMSAPS. There appears to be no CaMB in Ssp4, but Ssp4 has glutamine rich region not present in the human CAMSAPs. CAMSAP 3 has a proline rich region containing possible binding sites for SH3 domains, and similar sequences are seen in CAMSAPs 1 and 2 and in Ssp4. The Pfam database (Finn *et al.,* 2008) identified a strong match for an Inosine Monophosphate Dehydrogenase (IMPDH) domain, in all the CAMSAP proteins. IMPDH is a member of the Tim Barrel Family, which has an 8 fold βα barrel structure and so it may be that the CAMSAP protein structure resembles a Tim Barrel. Both species have a conserved 1st and 3rd coil, although the first coiled coil begins at an earlier point in the protein in the fly. The human CAMSAPs also have a second coiled coil, as do 5 isoforms of Ssp4 (Figure 3.1).

3.2.2.1 The N Terminal Domain

An alignment of the complete CAMSAP protein sequences of many family members was carried out using the PLOTCON programme, in the EMBOSS suite (see introduction, Figure 1.6). This revealed a possible domain of approximately 170 residues in length, beginning close to the N terminal and continuing up CH domain. Figure 3.3 shows a Clustal W2 alignment of the human proteins, Ssp4 and 11 other CAMSAP family members (Clustal W2, EBI, EMBL, Larkin *et al.,* 2007). The correlation between them confirmed the presence of a possible N terminal domain. A secondary structure prediction for this domain was made using PsiPred for each family member and is shown above the Clustal W2 result, (PsiPred Protein Structure Prediction Server, McGuffin *et al.,* 2000 Jones *et al.,* 1999. Figure 3.3). The broken line represents protein coils and the forward slash represents helix. Although there is a better match between the vertebrate amino acid sequences, the secondary structure for all species

Figure 3.3: CAMSAP N terminal domain secondary structure prediction.
Alignment of n terminal domain of various CAMSAP family members. Conserved secondary structure (PsiPred) is also shown above protein sequences.



was almost identical, despite differences in the amino acid sequences. Of the total number of residues only 10 were common. The conserved residues (2 Tryptophan and Proline, 1 Tyrosine, Histidine and Methionine, and 3 Leucine) are bulky and hydrophobic, and maintain protein shape from within.

3.2.2.2 Coiled Coil Regions

There are three conserved coiled coils in all vertebrate examples of CAMSAP. These were revealed using the Coils programme (The Coils Server, Lupas *et al.,* 1991), which predicts the coiled-coil forming ability of a protein sequence. The window of 28 residues was used to predict coiled coils, as recommended by the program. The same criteria were used to to scan isoform A of Ssp4 and two definite coiled coils were predicted. This is also the case in other invertebrates for example in one isoform of *Aedes aegypti (A aegypti)*, and in *Caenorhabditis elegans (C elegans)* (YB Yu, 2002) (data not shown). However, three definite coiled coils were present in Ssp4 isoforms, C,D,E,F and H (Figure 3.1).

Figure 3.4 shows a comparison of the first coiled coils in CAMSAP1 and Ssp4. The LEEKRR sequence is believed to be within the spectrin-binding domain (Baines *et al.,* unpublished data). The sequence shown in red, in CAMSAP1 is the CaMB, whose binding leads to the dissociation of CAMSAP from Spectrin.

Figure 3.4: Clustal W2 alignment of the first coiled coil of Ssp4 and CAMSAP1.

```
CAMSAP 1      SLL--ASELVQLHMQLEEKRRAIEAQKKKMEALSARQRLKLGKAAFLHVVKKGK
SSP4          RSLENASKLSTIRMKLEEKRRRIEQDKRKIEMALLRHQEKEDLESCPDVMKWET
                *  **::  ::*:****** ** :*::::*    *:: * .  :   * *
```

The sequence LEEKRR, common to all three species, is believed to be within a possible spectrin binding domain (25). The sequence in red, starting LSAR is the CaMB

The first coiled coil in Ssp4 starts at a point that is 0.36 of the protein length in the human CAMSAPs it starts at just over the half way point (Figure 3.2) and has the CaMB

at its C terminal giving this coil a role in protein binding. The third coiled coil is conserved between both species and compared with a selection of invertebrate family members, its position varied from 0.72 to 0.79 of the total protein length.

3.2.2.3 Calmodulin Binding Domain.

CAMSAP1 is known to bind calmodulin and the binding domain has been confirmed. A scan of Ssp4 protein using the Calmodulin Target database (Ikura Lab) identified 2 possible CaMB domains, 1 in the N terminal domain and 1 in the CKK domain. I believe these CaMB domains are probably coincidental for 2 reasons -firstly because the database predictions are not always accurate and secondly because the CaMB would be expected to be located closer to the interaction that it is inhibiting, as is the case in the human CAMSAPs where the CaMB is located close to the putative spectrin-binding domain (see Figure 3.4).

3.2.2.4 Sequences Rich in Certain Amino Acids.

Proline Rich Region

Proline rich regions are among the most common motifs identified in proteins and the most common sequence motif in *D melanogaster*. CAMSAP3 has two predicted proline rich regions (Expasy Proteomics Server, Pagni *et al.,* 2001). However, CAMSAPs 1 and 2, and Ssp4 all have enhanced numbers of proline residues in corresponding areas (see Figure 3.2). Proline rich regions often contain binding domains for SH3 proteins. There are more than 300 proteins with SH3 domains in the human proteome, and 63 in *Drosophila*. These 60 residue domains bind proline rich regions via a sequence of PXXP where X is any amino acid (Songyang, 1999). See Figure 3.5.

Figure 3.5: Table Showing Proline Rich Regions in Human and Ssp4

CAMSAP	Domain length.	% proline residues	Potential Recognition Sites for SH3 domains.
CAMSAP 1	97	20	1052-1055,1078-1081,1142-1145
CAMSAP 2	79	23	932-935, 966-969
CAMSAP 3	111	24	731-734, 778-781,834-837
Ssp4	62	21	1035-1038,1043-1046,1046-1049

PXXP motifs that may be potential binding sties for SH3 domains.

The SH3 domain was first discovered in the Src protein, where it has an autoinhibitory role. It is about 60 amino acids long with a distinctive beta barrel fold, and is in proteins that create transitory complexes through binding other proteins by way of proline-rich motifs. SH3 binding motifs comprise two classes, I and II. There are 2 predicted binding sites for SH3 class II binding (Pagni *et al.,* 2001) at the Ssp4 C terminal, and predicted binding sites for SH3 domains at the N terminal of Ssp4 and CAMSAPs 1 and 3, where there is also a possible class II SH3 binding domain in CAMSAP2. The binding motif for SH3 class II is PXXPXR (Songyang, 1999).

Glycine Rich Region

Embnet predicted a glycine rich region in Ssp4 at 875-885 (Pagni *et al.,* 2001). Glycine has conformational flexibility (Creighton, 1997) and glycine-rich regions most likely represent flexible linkers between protein domains. A search of Ssp4 using the domain prediction programme (Marsden *et al.,* 2002) did identify a domain boundary at residue 888 (data not shown).

Glutamine Rich Region

Two Glutamine rich regions at residues 360-394 and 712-999, were predicted by Embnet (Pagni *et al.,* 2001), which are between the first and third coiled coils. These domains are completely absent in the human CAMSAPs. The first region consists of 34 residues of which 23 are glutamine (20 coded by CAG and 3 coded by CAA) clustered into groups separated by one or two other residues (see Figure 3.6).

The second region contained a few clusters of purely glutamine residues, most are single or in pairs interspersed with other amino acids (see Figure 3.7). Of the few that are in groups of four, some are from codons CAG and some from CAA. This random distribution is characteristic of these kinds of sequences that are found in *D melanogaster* genes (Hancock and Simon, 2005). The DNA for this region comprised

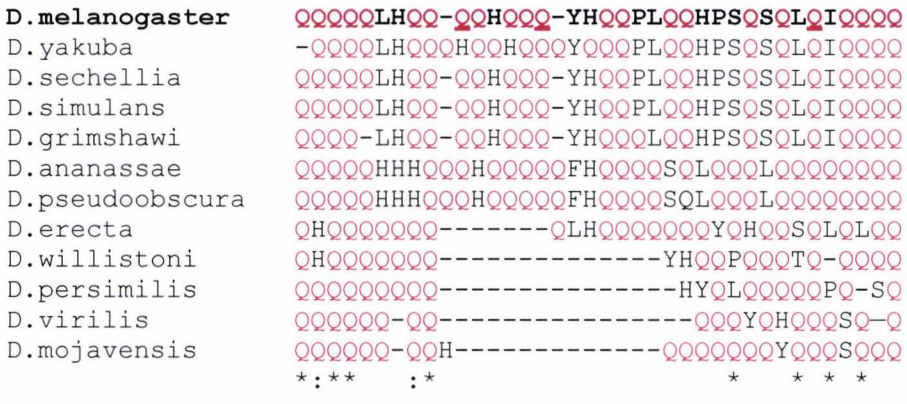


Figure 3.6: Clustal Analysis of First Glutamine Rich Region of Ssp4.
Results of a Clustal analysis comparing the first glutamine rich domain of Ssp4 with homologous proteins in other 11 other Drosophila species. The sequence is well conserved in the first 5 species. in D. ananassae and D.pseudoobscura there are higher numbers of glutamine residues relative to other amino acids whilst in the remaining species glutamine residues are reduced in number.

an approximate 40/60 split of CAA and CAG codons (data not shown). This suggests that their presence is deliberate rather than the result of replication errors, which would give rise to a high proportion of just one of these codons (Michelitsch and Weissman 2000). These regions were conserved in other members of the *Drosophila* family. Figure 3.6 shows an alignment of the first glutamine rich region for 12 different species of *Drosophila.* The 3 underlined residues in the *D.melanogaster* sequence are CAA, the rest are CAG (data not shown). Although this region is conserved in all species there are variations, *D yakuba, D sechellia, D simulans and D grimshawi* all resemble *D melanogaster* but the remaining groups have fewer glutamine residues. Whilst this region is common to all species the fact that such variation is tolerated suggests that it is not essential for Ssp4 functioning and because 87% of the glutamine residues are CAG, this region is probably the result of DNA duplication errors.

Figure 3.7 compares the second glutamine rich region of Ssp4 with the homologous proteins in 12 different species of *Drosophila* (glutamine residues highlighted in red). There are some differences in glutamine residue distribution between species but generally glutamine residue positions are more highly conserved than in Figure 3.6. Large groups of glutamine residues are scarce, instead individual and pairs of residues are seen at regular intervals throughout the region. The size of the first and last

Figure 3.7: Clustal Analysis of Second Glutamine Rich Region of Ssp4.

```
                                          ____1 ____
D.ananassae         QAYNAPVSAYSSRPPSRDPYQQQ------HQQQQPMAMPQP
D.pseudoobscura     QAYNAPVSAYSSRPPSRDPYQQQ------HQQQQPMAMPQP
D.yakuba            QAYNAPVSAYSSRPPSRDPYQQQLH----HQQQQPMPMPQP
D.melanogaster      QAYNAPVSAYSSRPPSRDPYQQQLH----HQQQQPMPMPQP
D.sechellia         QAYNAPVSAYSSRPPSRDPYQQQLH----HQQQQPMPMPQQ
D.simulans          QAYNAPVSAYSSRPPSRDPYQQQLH----HQQQQPMPMPQQ
D.grimshawi         QAYNAPVSAYSSRPPSRDPYQQQLH----HQQQQPMPMPQP
D.erecta            QAYN-----YSSRPPSRDPYQQH------QQQQQPMAMPQP
D.willistoni        QAYNAPVSTYSSRPPSRDPYQQQ-------QAPPPMAMPQP
D.persimilis        QAFNAPVSAYNSRPPSRDPYQQQQQ--HHPH-QQPMQMP-P
D.virilis           QAFNAPVSAYNSRPPSRDPYQQQQQQ-HHSHQQQPMQMP-P
D.mojavensis        QAFNAPVSAYNSRPPSRDPYQQQQQQQQQHHHPQPMQMP-P
                    **:*     *.***********:       :   ** **

D.ananassae    MQFVNEHGQYMSPPQPSHYQP--------QSIYSDNG-APYNNHS-PHYGAAAP-----PQ-
D.yakuba       MQYVNEHGQYMSPPQPAHYMP--QQTQQPQSIYSDNG-AAYNHSNHSPYGGAP-------Q-
D.melanogasterMQYVNEHGQYMSPPQPAHYMP--QQAQQPQSIYSDNG-AAYNHSNHSPYGGTP-------Q-
D.sechellia    MQYVNEHGQYMSPPQPAHYMP--QQAQQPQSIYSDNG-AAYNHSNHSPYGGGP-------Q-
D.simulans     MQYVNEHGQYMSPPQPAHYMP--QQAQQPQSIYSDNG-AAYNHSNHSPYGGAP-------Q-
D.grimshawi    MQYVNEHGQYMSPPQPAHYMP--QQTQQPQSIYSDNG-AAYNHSNHSPYGGAP-------Q-
D.erecta       MQFVNEHGQYMSPPQPAHYMSPQQHQQQPQSIYSDNG-AAYNHS---PYGGAPP-----PQ-
D.willistoni   MQYVNEHGQYMSPPQPAHAYMQ------PQSLYSDNGGGPYNHNHNHSPYGAPP-----PQ-
D.persimilis   MQYVNEHGQYMSP--PAHYMQ-------PQSIYSDNGGAPYNNHS--PYG-APPMQQYQQQQ
D.virilis      MQYVNEHGQYMSP--PAHYMQ-------PQSIYSDNG-APYNNHS--PYG-APPMPQY-QQQ
D.mojavensis   MQYVNEHGQYMSP--PAHYMQ-------PQSLYSDNG-APYN-HS--PYGAAPPQPQY--QQ
D.             MQFVNEHGQYMSPPQPSHYQP--------QSIYSDNG-APYNNHS-PHYGAAAP-----PQ-
pseudoobscura
               **:**********  *:*           **:***** ..**         .       *

                                                          ___2___
D.ananassae   YRSSVVFDDYGQPTNHFYLHESSPQA---PQGHPQRRTWAHSAAAAAYEQQQQIQQP----MV
D.yakuba      YRSSVVYDDYGQPTNHFYLHESSPQP---AHPHPQRRTWAHSAAAAAYEQQQQIQPS----LV
D.melanogasteYRSSVVYDDYGQPTNHFYLHESSPQP--QAHQHPQRRTWAHSAAAAAYEQQQQIQPS----LV
D.sechellia   YRSSVVYDDYGQPTNHFYLHESSPQP--QAHPHPQRRTWAHSAAAAAYEQQQQIQPS----LV
D.simulans    YRSSVVYDDYGQPTNHFYLHESSPQP--QAHPHPQRRTWAHSAAAAAYEQQQQIQPS----LV
D.grimshawi   YRSSVVYDDYGQPTNHFYLHESSPQP--QAHPHPQRRTWAHSAAAAAYEQQQQIQPS----LV
D.erecta      YRSSVVYDDYGQPTNHFYLHESSPQPPHPQQQHPQRRTWAHSAAAAAYEQQQHQQMS-----V
D.willistoni YRNSVVYDEYGQPTNHFYLHETSPQP------PPQRRTWAHSAAAAAYEQQQHQQPQVQQPLV
D.persimilis PQRNSVYDEYGQPANHFYLHESPPQPL----AHPQRRTWAHSAAAAAYEQQQQQQAQQQQPLL
D.virilis     QQRNSVYDEYGQPANHFYLHESPPQP------HPQRRTWAHSAAAAAYEQQQQAQQQQQPLL
D.mojavensis PQRNSVYDEYGQPANHFYLHESPQQPP-PQHSHPQRRTWAHSAAAAAYEQQQQQQQQ---PLL
D.            YRSSVVFDDYGQPTNHFYLHESSPQA---PQVHPQRRTWAHSAAAAAYEQQQQIQQP----MV
pseudoobscura
                : .  *:*:****:*******:*.       ********************: *
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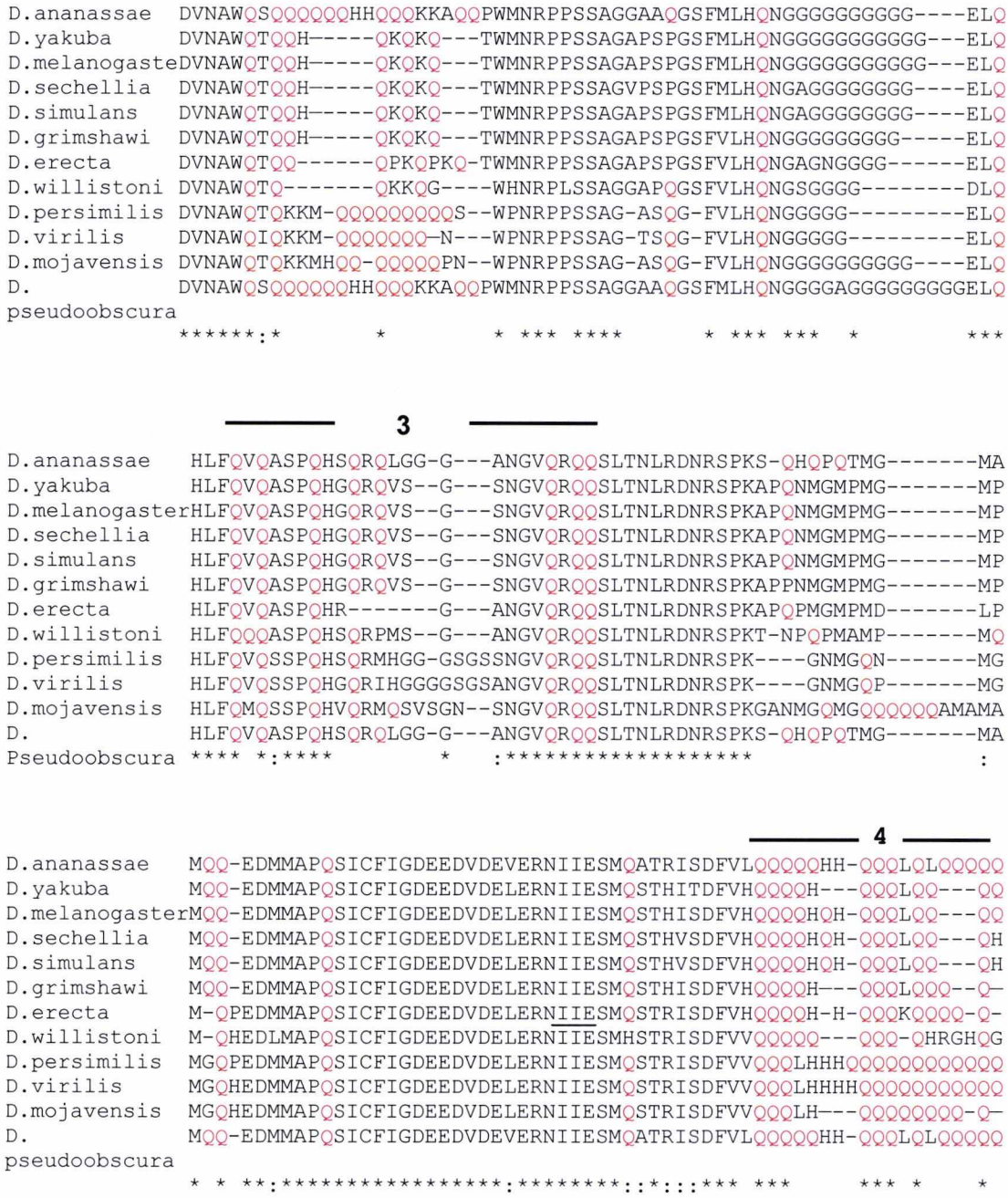


Figure 3.7: Clustal Analysis of Second Glutamine Rich Region of Ssp4.
Results of a Clustal analysis comparing the second glutamine rich domain of Ssp4 with homologous proteins in other Drosophila species. The sequence is well conserved in all species. Large groups of glutamine residues are absent but four small groups are numbered 1-4. Variation between species is highest in groups 1 and 4.

sequences (marked 1 and 4) has the greatest amount of variation between the different species, which implies that these are not as important for the purpose of this region, as areas 2 and 3 in the centre of the protein, where there is closer correlation between the different family members.

3.2.2.5 Phosphorylation Sites

Because CAMSAP1 binds spectrin, and spectrin binding to calmodulin is regulated by phosphorylation, I decided to see if *ssp4* and the mammalian CAMSAPs had a common phosphorylation site, which might regulate their activity.

There are hundreds of kinases and phosphatases in eukaryotes that reversibly phosphorylate proteins involved in various cellular activities. Protein phosphorylation leads to conformational change altering entire protein structure through mediation of interactions between hydrophobic and hydrophilic domains (for a review see Knippschild *et al,* 2005). Kinase activity is regulated by various factors such as kinase subcellular location, availability of substrate and the overall phosphorylation state of the cell. Serine, threeonine and tyrosine are subject to phosphorylation. The phosida database was used to scan the human CAMSAPs and identify phosphorylation sites, Ssp4 was scanned using Scansite as the phosida database did not have the facility to scan *Drosophila* proteins. A common CaMK2 (Calmodulin dependent kinase 2) substrate-binding site was identified in CAMSAPs 1 and 2 and Ssp4 (See Figure 3.2). This substrate-binding site in CAMSAP1 and Ssp4 is located at 86% of the distance between the N terminal and the first coiled coil. In CAMSAP2 it is closer to the N terminal, at 60% of the distance. The fact that this phosphorylation site is located in a similar position in all three proteins suggests that phosphorylation is necessary for their common functioning.

CaM Kinase 2 is a multifunctional kinase, which phosphorylates a variety of proteins including MAPs (microtubule associated proteins). It has a catalytic domain at its N terminal, which is subject to inhibition by the CaMK2 regulatory domain. A transitory rise in calcium activates calmodulin, which then activates CaMK2, conferring memory of the effect of this brief calcium flux, converting it into a longer-lived signal.

3.3 DISCUSSION

The results of the comparison of human and Ssp4 revealed more similarities than differences, and confirmed what was already known as well as suggesting other possible roles for the protein.

A new domain was investigated at the N terminal adjacent to the CH domain. CH domains have regulatory roles in many signalling proteins, and are usually are found in pairs for example, spectrin has tandem CH domains that combine to form an actin-binding domain (ABD)(Korenbaum and Rivero 2002). The CH domains that make up the ABD work synergistically to produce a tenfold increase in binding affinity, with one as a positioning device or anchor that allows the other CH domain to bind effectively (For a review see Gimona *et al.,* 2002).

Unlike the predicted phosphorylation sites and calmodulin binding domain, the N terminal domain is common to all three human proteins and Ssp4, and many other family members and so must play a significant role. It may act in partnership with the CH domain in CAMSAP and Ssp4, it was not possible to predict its functioning from the data collected, but a synergistic or anchoring function could not be ruled out (For a review see Gimona *et al.,* 2002).

Calcium fluctuations inhibit Spectrin binding in Human CAMSAP1 and alterations to cellular calcium levels activate CaMK2, which may lead to possible conformational changes to CAMSAPs1 and 2 and Ssp4, through phosphorylation of predicted CaMK2 domains mediating protein/protein interactions, possibly through positioning of domains, and exposing motifs such as the SH3 binding domains.

The 3 Human CAMSAPs and Ssp4 all share common SH3 binding domains, which collect together proteins with modest affinity and low specificity, governed by concentration and location of available interacting proteins (For a review see Mayer, 2001). These complexes are rapidly formed and short-lived as a transitory response to an environmental stimulus. A binding site for an SH3 domain can interact with many different partners, leading to non linear cellular pathways in which the numbers of possible interactions exceed the numbers of participating proteins (For a review see Mayer, 2001), and it may be that CAMSAP forms a variety of different complexes according to the nature of the stimulus involved.

With many features common to both species, it is likely that a CaMB in Ssp4 would be placed in a similar position to that of the vertebrates, and an unidentified CaMB might exist in the first coiled coil of Ssp4. Spectrin binding regulation would logically be undertaken closer to the predicted spectrin-binding site than the Ssp4 N terminal. In human alpha II spectrin, the CaMB protects the protein from caspase binding from 6 residues away (Simonovic *et al.,* 2006), and the *Drosophila* alpha spectrin CaMB is close to the protein centre like its human counterpart, and not at the C or N termini (Dubreuil *et al.,* 1989),

Invertebrates may bind proteins by way of their glutamine rich regions - calcium may still regulate this association, but the regulation mechanism would be different. Perutz has suggested glutamine rich regions might to form a polar zipper, conferring an ability to interact with other proteins (Perutz *et al.,* 1994). Glutamine residues can form hydrogen bonds between main chain amides, and between their polar side chains, creating beta strands, which could make sheets or barrels, to interact with other proteins. In yeast, glutamine rich regions mediate functions including cytoskeletal organisation, cellular metabolic pathways and kinase activity inhibition. In *Drosophila*

the tanscription factor Sp1 binds the DTAFII110 element of the TFIID complex via a glutamine rich region (Michelitsch and Weissman, 2000).

Proteins in *S cerevisiae* use glutamine/asparagine rich regions to adopt a prion state in response to temperature changes (Mayer, 2001), although this type of domain is unfavourable in hot temperatures and selected against by thermophiles (Michelitsch and Weissman, 2000). Areas of charged residues discourage protein aggregation due to glutamine rich regions and Ssp4 has a glutamate rich domain in its third coiled coil (Michelitsch and Weissman, 2000).

3.4 CONCLUSION

Ssp4 shares many common features with the Human CAMSAPs, although there are some differences. The CaMB bearing second coiled coil in the human CAMSAPs appears to have been replaced with a Glutamine rich domain in *Drosophila*, and the CaMK2 phosphorylation site in Ssp4 is matched in only human CAMSAP 1 and 2. However, there are shared putative binding sites for SH3 domains, and an N terminal domain is that common to a variety of CAMSAP family members. The CAMSAPs and Ssp4 appear to be equipped to respond to extracellular signals, which could alter their structure, allowing them to interact with other proteins, and these interactions may be common to both species.

Having carried out a comparison between vertebrate CAMSAP and *Drosophila* Ssp4 an antibody and *in situ* probe were generated to examine protein and mRNA expression in developing *Drosophila* embryos, and the results of this investigation are shown in chapter four.

Chapter 4

THE EXPRESSION OF SSP4 IN A DEVELOPING EMBRYO

4.1 INTRODUCTION

Bioinformatic analysis and genome annotation indicate that *ssp4* is a functioning gene (chapter 3). Indeed the presence of expressed sequence tags (EST's) isolated from embryos, testes and nervous tissue indicate that this gene is probably involved in many aspects of *Drosophila* biology. Microarray studies have indicated that *ssp4* is expressed throughout development (Arbeitman *et al* 2002) (Gauhar *et al,* 2008).

As a first step to elucidation of the function of *ssp4* it was necessary to confirm that it is expressed in *Drosophila* during embryogenesis. To do this a single stranded RNA probe was used to locate the *ssp4* mRNA, and a polyclonal antibody raised against an Ssp4 peptide was used to detect protein expression. Both the antibody and the *in situ* probe were designed to label all *ssp4* isoforms.

4.2 *IN SITU* HYBRIDISATION TO SSP4 TRANSCRIPTS.

4.2.1 Introduction

In situ hybridisation establishes the presence of mRNA in any cell at any particular time. A digoxygenin labelled RNA probe, which is complementary to cellular mRNA, binds in the tissues. After rinsing away any excess probe, an alkaline phosphatase conjugated antibody against digoxygenin, is bound to the probe where it has hybridised. An alkaline phosphatase substrate that produces a bluish dye when dephosphorylated is added and indicates in which cells the mRNA is located.

4.2.2 Probe Production

To generate a probe to *ssp4* mRNA a cDNA (AT18914) was obtained from the Berkeley Drosophila Genome Project (Stapleton *et al*, 2002). This cDNA represents a full-length transcript that includes the exons that make up *ssp4* transcript A (obtained from an EST

library generated from testes and seminal vesicles removed from 0-3 day old Oregon R (wild type) males, and contained in the POTB7 vector (Stapleton *et al 2006*). By cutting with appropriate enzymes (see materials and methods) and using Sp6 and T7 polymerase, an antisense and sense (control) RNA probe were generated *in vitro*. Both probes were labelled with digoxigenin, a steroid hapten (a molecule that binds antibody when attached to a carrier molecule) that is coupled to dUTP.

4.2.3 *In situ* Hybridisation to Whole Mount Embryos.

An *in situ* hybridisation was carried out on whole mount wild type embryos (Tautz and Pfeifle 1989). A consistent spatially and temporally regulated *ssp4* expression pattern was observed throughout embryonic development (Figure 4.1). Stages shown are according to the Campos-Ortega staging system (Campos-Ortega, 1997). The sense control probe did not detect staining at any stage.

During stages 1 - 4 the nucleus of the fertilised embryo undergoes 13 rapid divisions. The resultant nuclei arrange themselves in a single layer beneath the egg surface and cell membranes form around them, leading to the production of a cellular blastoderm at stage 5 (Figure 4.1B). At stage 5 the nuclei undergo four further rounds of division, and elongate. *ssp4* mRNA is present in the egg plasma, which has moved to the edge of the embryo, and cellularisation takes place (Figure 4.1B). Stage 8 (Figure 4.1C) follows gastrulation (invagination of cells to form a gut cavity and three cell layers). *ssp4* expression is in the mid-gut endoderm and germ band (consists of an inner mesodermal layer and an outer ectodermal layer), which is almost fully extended.

During stage 9 the first neuroblasts delaminate from the ectoderm, to produce a clearly three layered germ band comprising mesoderm, neuroblasts and outer ectoderm. Expression at stage 10 (2 D) can be seen in the fully extended germband (arrow) and in

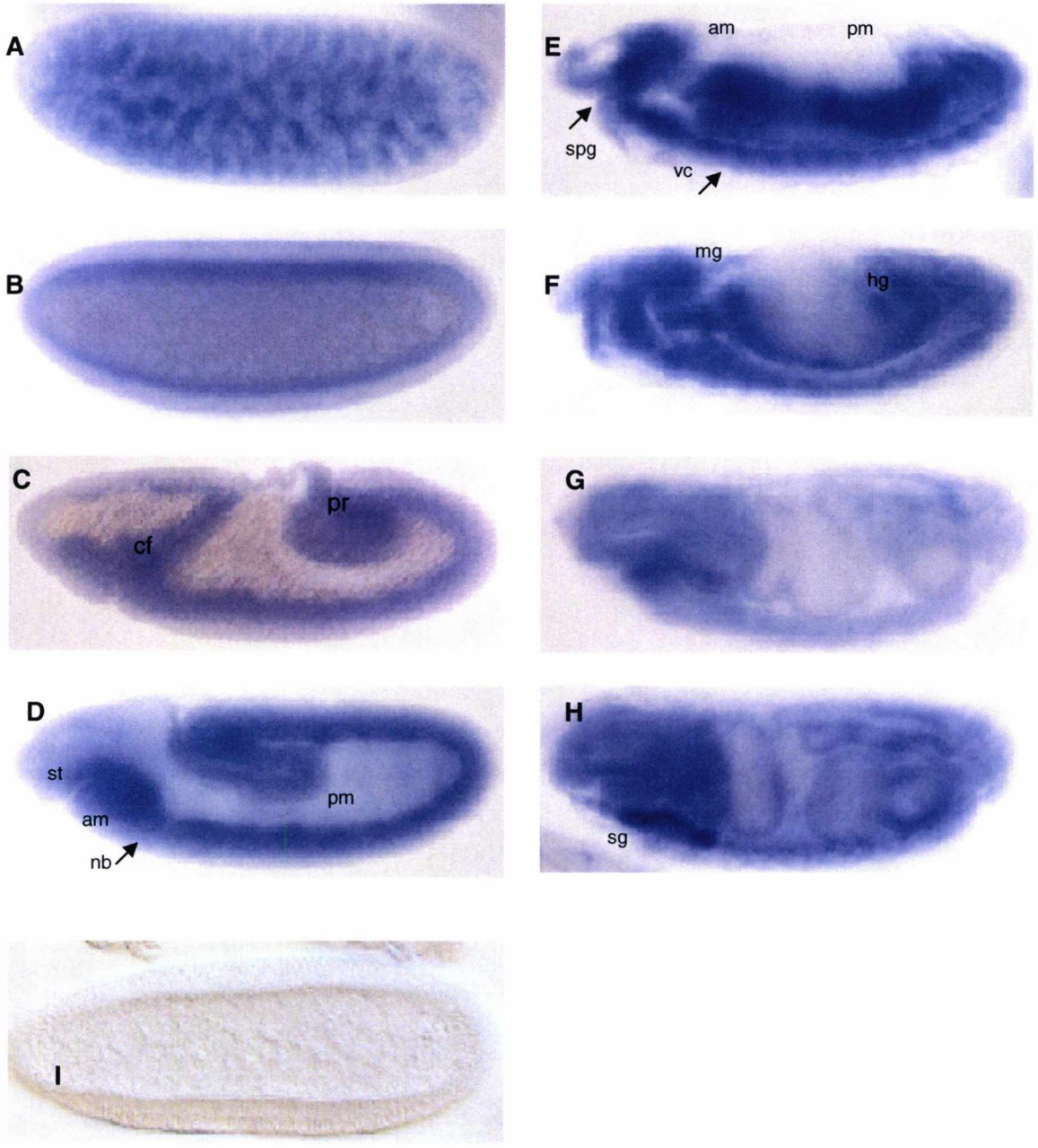


Figure 4.1*: In Situ* Expression of Ssp4 in Oregon R Embryos up to stage 16.

Oregon R whole mount embryos in lateral view (anterior to the left) at a magnification of 200X. The mRNA is labelled with a digoxygenin labelled antisense probe, and a dark blue dye reveals its expression pattern at developmental stages ranging from stage 3 to late stage 16. Expression is seen in the blastoderm (A) then during cellularisation (B.) At stage 8 (C) the extending germ band is labelled with the gut and cephalic furrow, and this expression remains at stage 10. Staining of the developing midgut and CNS are apparent at stage 14 with increased expression in the head. At stage 15 expression spreads to the developing hind gut, and by stage 16 staining to a discrete set of cells in the CNS can be seen. This CNS staining is no longer seen in the final stage of embryogenesis (H), although other staining patterns persist. I - sense probe control. Abbreviations: am – anterior midgut, pm – posterior midgut, nb – neuroblasts, hg – hindgut, sg – salivary gland pr – proctodeal opening, cf cephalic furrow, st - stomodeum

the developing posterior and anterior midgut. In the germ band the neuroblasts divide to produce ganglion mother cells and the ventral and procephalic neuroblasts of the central nervous system. Expression here is seen in the anterior and posterior midgut structures as they fuse at stage 14 (Figure 4.1E), in the supraesophageal ganglion (the brain) and in the condensing central nervous system (arrow), this condensation continues into stage 15 (Figure 4.1F) when a constriction forms in the midgut. The head is the most clearly labelled structure in stage 16, midgut and CNS labelling are reduced (Figure 4.1G). A clearly labelled set of cells are visible in the CNS. Late into stage 16 (Figure 4.1H), which is very close to the end of embryogenesis, *ssp4* expression increases in the midgut, hindgut and is now clearly visible in the salivary glands. In the central nervous system expression is increased and more general than in the early part of this stage with no staining seen in specific cells.

4.3 ANTIBODY STAINING TO IDENTIFY EXPRESSION PATTERNS OF SSP4 DURING EMBRYOGENESIS.

4.3.1 Introduction

In situ hybridisation showed *ssp4* mRNA to be present in the central nervous system and gut from embryonic developmental stage 10. An antibody was raised and its specificity was established using a western blot. This was used to reveal protein expression patterns, and to confirm whether the *in situ* was an accurate representation of *ssp4* expression.

4.3.2 Antibody Production

A peptide was designed *in silico* to be used to raise an antibody to Ssp4 as there was no antibody commercially available. This polyclonal antibody was raised in rabbit against the C terminal fourteen amino acids (RVQLPSKKDMALVI), which are common

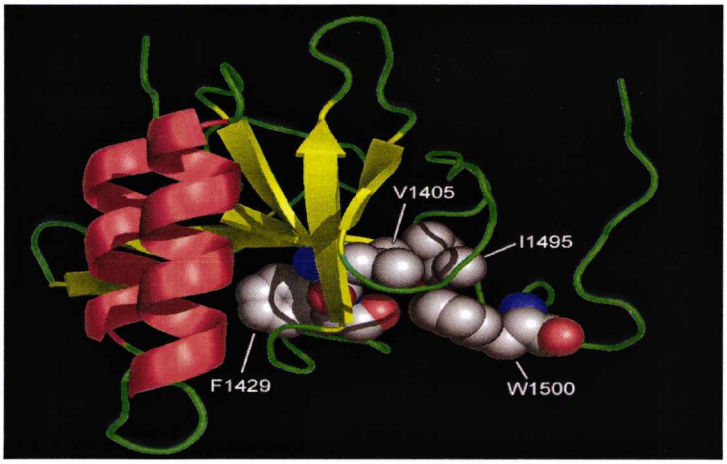


Figure 4.2: Molecular model of the Ssp4 C-terminal domain.
The structure of the C-terminal domain of Ssp4 was modelled using the programme Phyre, with PDB:IUGJ as the template. NB the 3' end of the protein is predicted to be free for antibody binding (5) shown with green bracket.

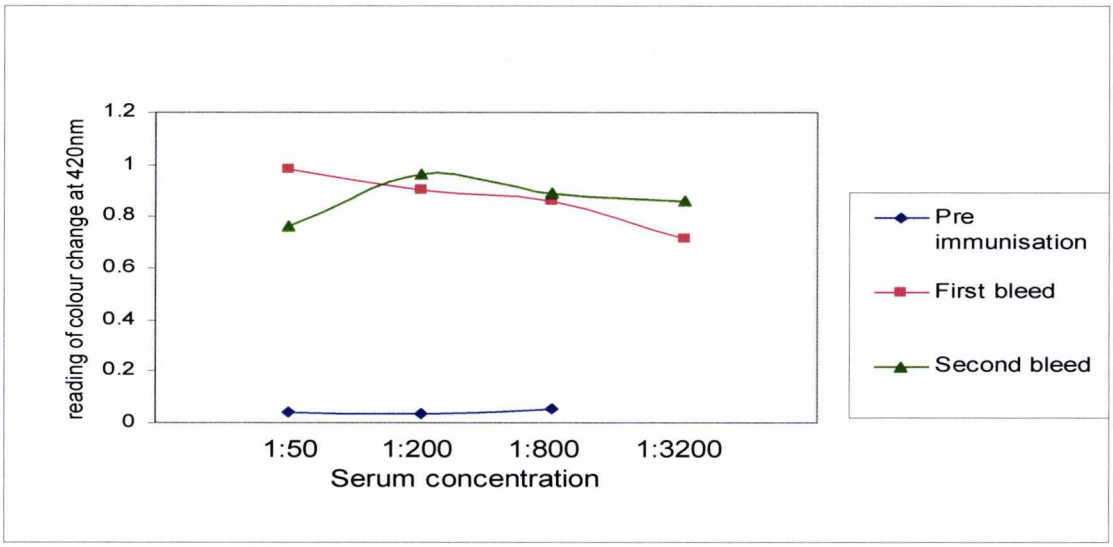


Figure 4.3: Results of Elisa Test of Rabbit Serum Ssp4 Antibody
Levels of ssp4 antibody in Rabbit serum relative to pre immunisation.

to each of the putative Drosophila isoforms and chosen because the C terminal end of the protein may be more likely to be exposed in a biological and cellular context (Figure 4.2). In addition, an effective antibody had already been produced to the corresponding region in vertebrate CAMSAP (Baines et al 2009). The peptide sequence was compared with the Drosophila proteome and no match was found. Although this was no guarantee of specificity it indicated that this region may be a good candidate for the antibody.

4.3.2 ELISA (Enzyme-linked Immunosorbent Antibody) Test of Rabbit Serum

An ELISA assay confirmed the presence of the Ssp4 antibody in rabbit serum. There was an average increase of approximately 1875% and 1885% in antibody binding in the first and second bleeds respectively, relative to pre immunisation levels, indicating high levels of antibody coupled with effective binding to the Ssp4 peptide (Figure 4.3).

The serum was then passed through a peptide column to produce fractions of Ssp4 antibody (see Chapter 2 Materials and Methods). Diaminobenzidine staining was used to establish levels of antibody in each of these fractions (data not shown) and the fractions that stained the most strongly were combined, and purified using dialysis. The purified antibody was then used for ssp4 detection by western blotting and immunocytochemistry on whole mount Oregon R embryos.

Western blot analysis was carried out to confirm that the antibody raised against the ssp4 C terminal bound to proteins from *Drosophila* embryos (Figure 4.4). Proteins were extracted from embryos and subjected to SDS-PAGE. Resulting gels were transferred onto nitrocellulose and probed with Ssp4 primary antibody and a horseradish peroxidase secondary (see materials and methods). Predicted weights of the isoforms

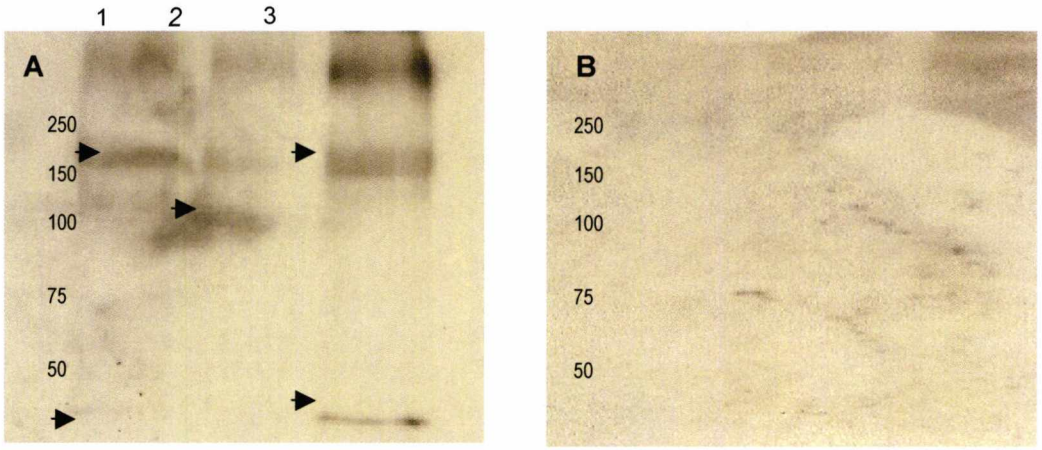


Figure 4.4: Antibodies raised against the C-terminus of Ssp4 bind to specific protein bands from Oregon R embryos.
Western blot of Drosophila Oregon R cellular proteins allowed to react with Ssp4 purified antibody in wells 1-3. A – [primary antibody] 1:500, Ssp4 antibody detected a band at just under 250Kda in lanes 1 and 3(arrow), which may represent non-truncated isoforms, which are between 171 and 188 kDa. The antibody detected a further band at below 50kDa in wells 3 and 1(arrow) which may represent a truncated 20kDa isoform, and a further band at just over 100kDa in lane 2 (arrow) and faintly in well 1was also detected and may represent the H isoform of 130kDa. All lanes in image A had a band of antibody binding at the very top, which was probably due to aggregation in the lanes. Image B – secondary antibody only control. [Secondary antibody] 1:1000. There were no signs of binding in the secondary only control

of Ssp4 in kDa, are as follows: Isoform A: 170.9, Isoform B: 173.2, Isoform C: 183.6, Isoform D: 187.5, Isoform E:180.5, Isoform F: 188.1, Isoform G: 20, Isoform H: 130.1. A clear band is visible between 150 and 250kDa (Figure 4.4A). Isoforms A to F would fall into this band, the other two isoforms may be represented by the band at the bottom of the well 3 and the possible and at just over 100kDa in the well 2. The primary antibody appears to be binding to proteins of expected sizes to be Ssp4 proteins.

4.3.4 Antibody staining of Whole Mount Oregon R Embryos.

Whole mount embryos were treated and stained for Ssp4 expression using a standard protocol, starting at stage 3 and continuing to the end of embryogenesis. (Figure 4.5). Controls using the secondary antibody only showed no staining at all. (Figure 4.5I). The stage 3 embryo is almost uniform in colour with expression ubiquitous (data not shown) however at the time of cellularisation (stage 5) the protein appears to be

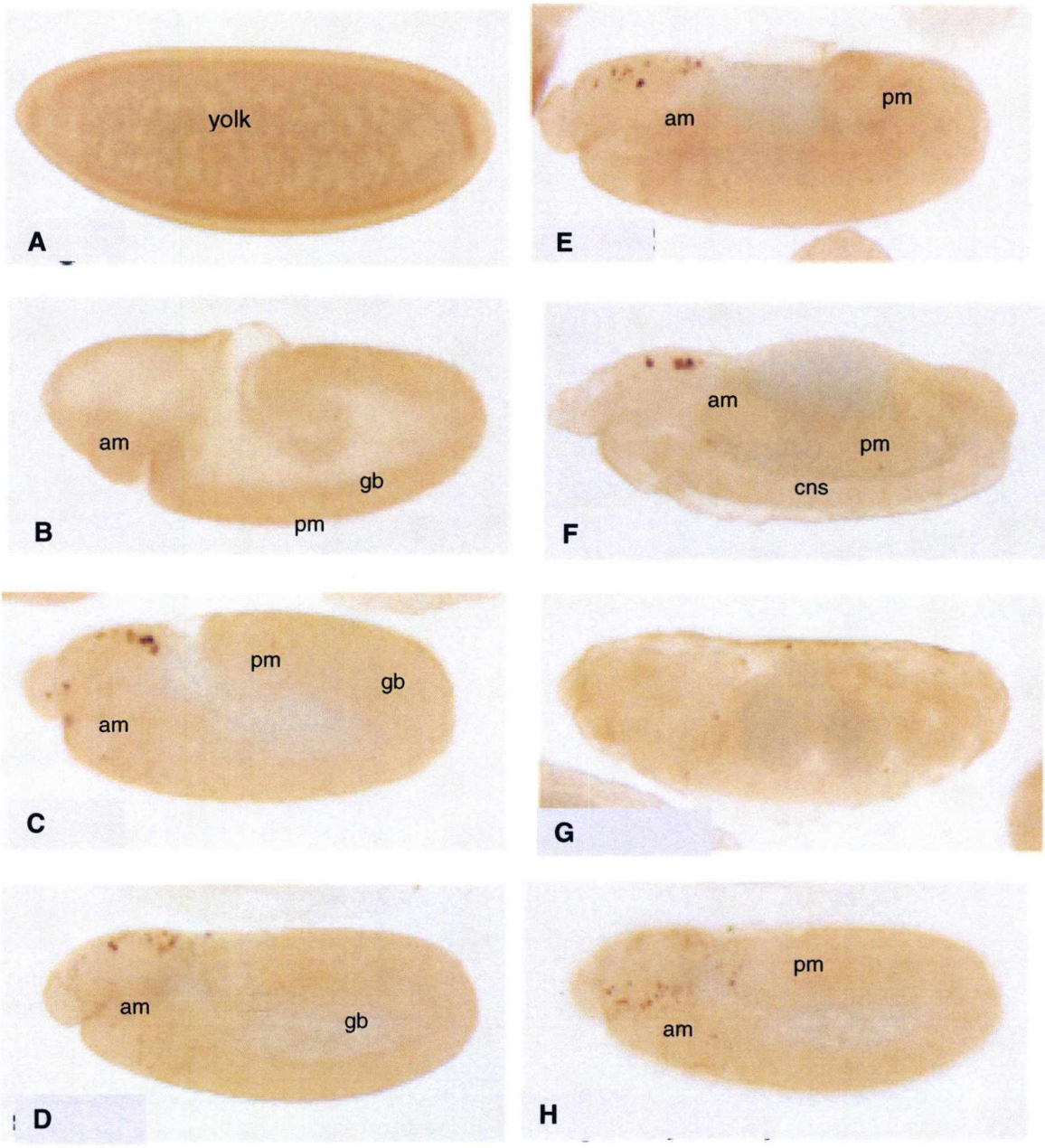


Figure 4.5: Expression Patterns of Ssp4 in Oregon R Embryos

Primary antibody(1:5000) has been bound by biotinylated secondary (1:1000) to give a stable brown dye.

At stage 5 (A) a slightly darker band is emerging at the embryo edge where the blastoderm is undergoing cellularisation, the contrast between the stained area and the rest of the embryos is much clearer at stage 8(B) when the extending germ band is highlighted along with the gut and cephalic furrow. At stage 9(C) a set of stained cells in the dorsal area of the head can be seen, and the numbers of these cells increase at stage 10(D and H, D shows a saggital view) although they are more diffuse. At stage 13 expression is mainly confined to the dorsal head area (E), with a few cells scattered throughout the embryo, by stage 16 (G) there is no longer any expression in the head, as these cells appear to move to the posterior of the embryo. Secondary only control shows no staining (compare I with F). anterior midgut-am, posterior midgut-pm, germ band- gb, cns-central nervous system.

concentrated within the yolk, on the basal side of the cells (Figure 4.5A). The overall staining in the stage 3 embryo may be due to a maternal component providing protein necessary for the very first stages of embryonic development as the first evidence of zygotic transcription is not seen until the end of nuclear migration (Pritchard1996). By stage 8 (Figure 4.5B) low levels of Ssp4 expression can be seen in the fully extended germ band, and the posterior and anterior midgut.

At embryonic stage 9 (3.40 – 4.20 hours) it was possible to see distinct staining in small groups of cells in the head of the developing embryo and continuing up until late embryogenesis (Figure 4.5D). These embryos had paler overall staining with a strongly contrasting stained cell subset in the head, in the middle of the head, and in the ventral germ band, at the head end. Figure 4.5H shows a view of the surface of the same embryo, where the distribution of stained cells is the same except for the dorsal head area. There also appears to be some stained cells on the ventral side of the end of the germ band, just behind the head. At stages 12 and 13 (Figure 4.5 E and F) the staining is only in the head and the cells are larger, darker and fewer in number. By stage 16 (Figure 4.5G) there is no staining to any group cells in the head but there are individually stained cells throughout the embryo. In addition to the staining of individual cells there was a paler staining pattern that mirrored that of the *in situ* with labelling to the germ band, CNS, and anterior and posterior mid gut. During stages 14 and 15 the individually stained cells remain in the head but the retracting germ band is labelled, with antibody and *in situ* probe and by stage 15 this staining is seen in the gut and CNS. At stage 16 the embryonic head has high levels of Ssp4 protein and mRNA, although staining in the CNS has declined by stage 16 (Figure 4.1H and Figure 4.5G).

To determine whether the antibody was specific to Ssp4 protein I tested the serum on embryos that were mutant for *ssp4* (see chapters 5 and 6). The prediction being that

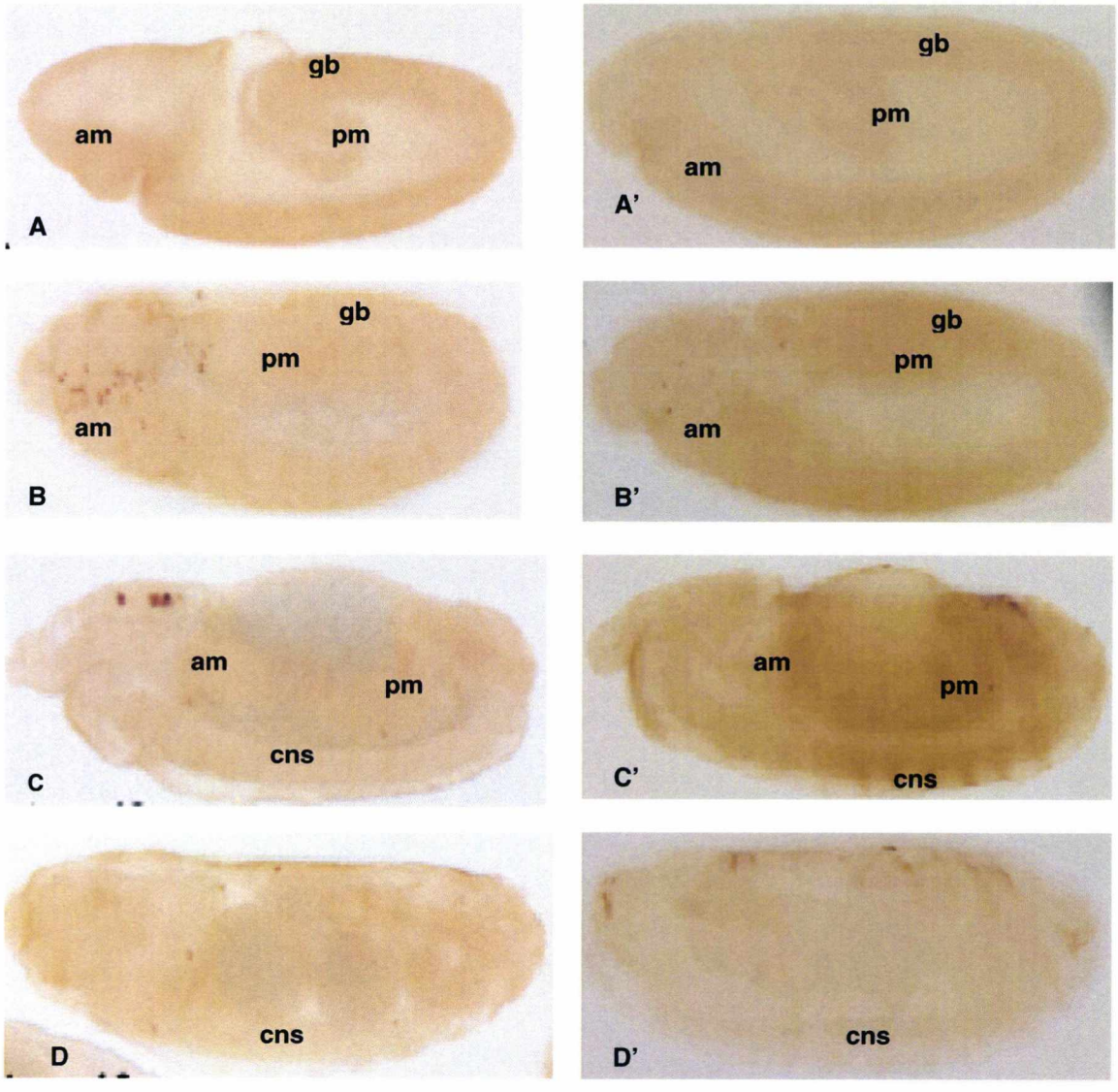


Figure 4.6: Comparison Ssp4 Expression in Wild Type and Homozygous Ssp4^{k07433} Embryos.

A-D are Oregon R, A'-D' are homozygous Ssp4 mutants. whole mount embryos up to approximately 16 hours in lateral view (anterior to the left) at a magnification of 200X, stained with anti Ssp4.. Stage 8 embryos (A and A') have no staining to discrete cells, however, this pattern of staining seen in the wild type is not seen in the homozygous mutants. Stage 10 embryos (B and B') have similar expression in the germ band, head and gut with some discrete cells labelled in the mutant. At stage 14 (C and C') the mutant has no individual cells labelled, and the labelling of the CNS and gut is far more distinct in the wild type than the mutant. The wild type stage 16 embryo (D) has clear staining to the head, and around the gut that is not seen in the mutant (D'). For abbreviations see Figure 4.6.

they should show reduced or absent staining in the cells identified by wild type embryos. There is no staining to individual cells in either of the stage 8 embryos (Figure 4.6 A and A'). There does appear to be some staining in the mutant germ band and gut, however, this is more diffuse than the staining in the wild type. At stage 10 (Figure 4.6 B and B') the staining to the individual cells in the head is not seen in the mutant although there are one or two individual cells stained brown, in the head and the end of the germ band. This suggests that perhaps Ssp4 expression is not totally knocked down in individuals carrying two copies of the $Ssp4^{k07433}$ allele. In the germ band and gut the mutant has a similar Ssp4 expression pattern as the wild type embryo, which might also be due to the presence of a maternal component. It may also be non-specific binding but this is unlikely as it is matched by the *in situ* labelling.

At stage 14 (Figure 4.6 C and C'), the staining in the anterior and posterior mid gut and CNS, and the individually labelled cells in the wild type head are not seen in the mutant, by stage 16 (Figure 4.6 D and D') the mutant has no Ssp4 staining at all, whilst the wild type shows staining to discrete cells throughout the body and patches of staining in the head, as well as the edges of the gut.

4.4 DISCUSSION

The expression of ssp4 mRNA is clear from early development, following formation of the gut and central nervous system, and highlights discrete groups of the neuronal cells. The in situ data shows ssp4 mRNA is present from the start of development, and this was also found in a DNA microaray study, carried out by Gauhar et al (2008). This expression must be due to a maternal component as zygotic expression only starts at about cellular blastoderm (stage 5) (Luengo Hendriks et al 2006). The translated protein is seen in the stage 3 embryo and then at blastoderm cellularisation.

The mRNA expression levels of *ssp4* match the Ssp4 protein expression patterns for the germ band, and developing gut, for the developmental period starting stage 3 and continuing through to stage 16 (13 – 16 hours), which is very close to the end of embryogenesis. There is one significant difference between these two results, and that is relating to the antibody staining of the discrete subset of cells in the embryonic head, which are then seen throughout the embryo at stage 16.

At stage 8 the *ssp4* mRNA is expressed in the dorsal area of the head, and this expression is matched in the stage 8 antibody stained embryo. At stage 9 these cells are darkly stained with antibody and remain so until stage 15. However, the stage 10 embryo has no mRNA expression in this area. The presence of the protein could be explained by a lag time between mRNA production at stage 8 (3 hours ten minutes – 3 hours 40 minutes) and protein production at stage 9 (3 hours 40 minutes to 4 hours 20 minutes), or because *ssp4* is being expressed in migrating cells.

Hemocytes are migrating cells that originate from procephalic mesodermal stem cells and are seen in the embryonic head late in stage 10 (Tepass *et al* 1994). They are of two types - the crystal cell (targets pathogens) and the plasmatocyte (a small round phagocytic cell), which is analogous to the mammalian monocyte/macrophage (Williams 2007). They are either sessile (tissue-associated) or circulating (found in the haemolymph) and phagycytose harmful foreign bodies and apoptotic cells. At the start of stage 12 they begin to travel through the embryo, moving anteriorly into the head, and posteriorly to the edge of the germ band (see Figure 4.5H) with a large proportion of them remaining in the dorsal head area. As the germ band retracts it takes the haemocytes at its edge through the embryo, they then move towards the embryonic centre (U Tepass *et al* 1994). In Figure 4.5C stained cells are seen to move into the front of the embryonic head, and in Figure 4.5H the have moved ventrally and also into the edge of the germ band behind the head. From stage 12 some of these cells display

phagocytic activity, particularly those in the brain and ventral nerve cord (Tepass *et al* 1994). There are no individually antibody labelled ventral nerve cord cells, but there is general CNS staining (Figure 4.5E and F). The *in situ* data also shows labelling of individual CNS cells in the stage 16 embryo.

The group of individually labelled cells in the head might be macrophages that are phagocytosing cells expressing Ssp4, through programmed cell death (PCD), which is needed to reduce the head epidermis for eye formation and head structure fusion (Rusconi *et al*, 2000). This would explain why these cells were uneven in size and rough looking at the edges and why *ssp4* mRNA is not seen in individual cells in the head, but in patches or ubiquitously expressed. Labelling with a haemocyte marker would unequivocally identify these cells as being phagocytotic.

PCD reduces cell numbers, facilitates the separation of layers of tissues, organogenesis, and the tightening of borders between developing organs (Page, 2008). It begins in the head at stage 11 (Abrams *et al*, 1993), and between stages 12 –14 continues in the CNS as it separates from the epidermis to allow germ band retraction and condensation (Mutsuddi, *et al* 2004). *ssp4* may be involved in apoptosis, through its interaction with microtubules (see intro section 1.5.2), but macrophages are not seen until late embryonic stage 10 and haemocytes do not begin actively phagocytosing until stage 12 (Tepass *et al* 1994). *ssp4* mRNA and protein are expressed from the start of embryogenesis, so *ssp4* may be involved in one or several processes that are ongoing throughout development, for example, in the co-ordination of cell movements needed for gastrulation (Figure 4.1).

4.5 CONCLUSION

The use of antibodies and *in situ* probes revealed that Ssp4 is expressed throughout embryogenesis in the CNS, gut and head. Distinct staining was seen in a sub-set of

cells in the head, and this expression spreads to other parts of the embryo by the end of development. The role of *ssp4* might be in apoptosis, or in macrophage activity, but these processes do not occur until stage 11 and 12 respectively and *ssp4* expression is seen to start at the beginning embryogenesis, and this result is supported by published data (Gauhar *et al* 2008). It may be that *ssp4* plays a role in the cytoskeletal processes that are continuous throughout development, which include alterations to cell shape, polarity and adhesive properties. As CAMSAP1 is known to be essential for neurite outgrowth, and is expressed in astrocyte lineage cells (Yamamoto *et al* 2009) the CNS development in mutant individuals will be compared to that of heterozygotes to assess the effects of expression knockdown on the developing embryonic central nervous system.

Chapter 5

CHARACTERISTICS OF *SSP4* MUTANTS

5.1 INTRODUCTION

Antibody staining and *in situ* hybridisation show expression of *ssp4* throughout development, with staining in the gut and CNS and head areas, and possible specific labelling of a subset of glial cells. There is also staining of a discrete group of cells in the head, which might possibly be undergoing phagocytosis following apoptosis. In this chapter two lines of flies that bear a P-Element insertion in *ssp4* were studied to see what the effects of reduced *ssp4* expression were on overall embryonic development.

5.1.1 P-Element Insertions in *ssp4*

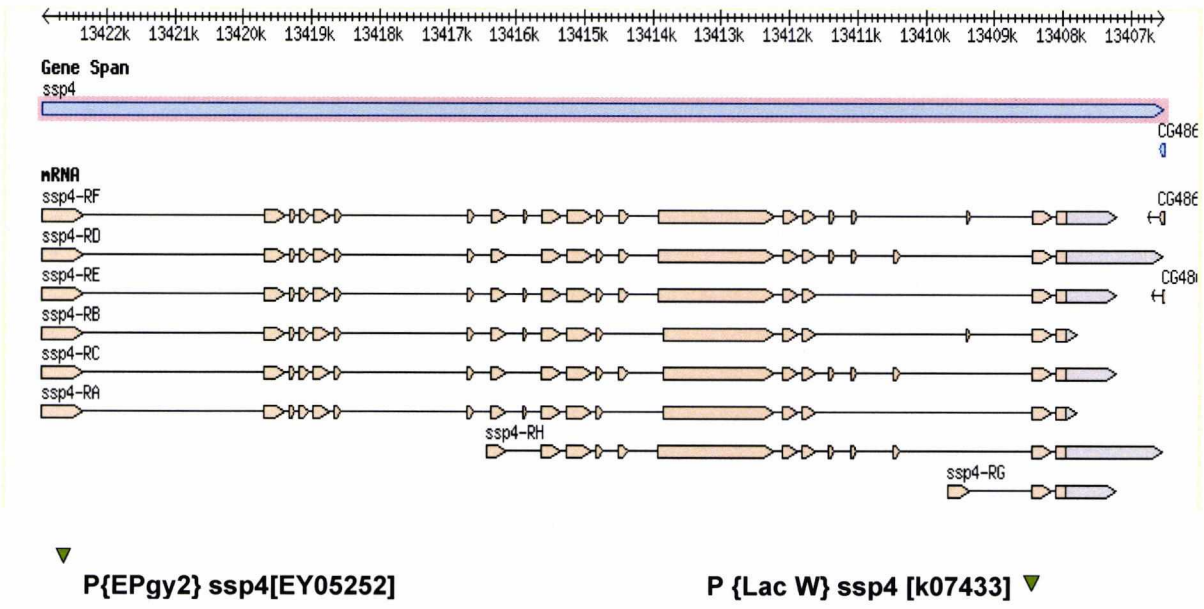


Figure 5.1: Insertion Sites of P-Elements into *ssp4* (*Flybase version R5.8*))
The above Figure is a screen shot taken from Flybase. The EY05252 P-element is inserted into a non-coding exon at the 5' end whilst the k077433 element is located at the 3' end. Their exact positions are shown by the green triangles. P- element disruption may lead to the production of functioning or non-functioning truncated protein products or of no protein at all. Both insertions will partially disrupt ssp4 function.

ssp4 is located on the 2R chromosome arm, between base numbers 13,406,523 and 13,422, 942 and is 16,419 bases in length. Annotation of *ssp4* indicates that 12 P-element insertions have been identified in *ssp4* two near the 3' end and 10 in or near

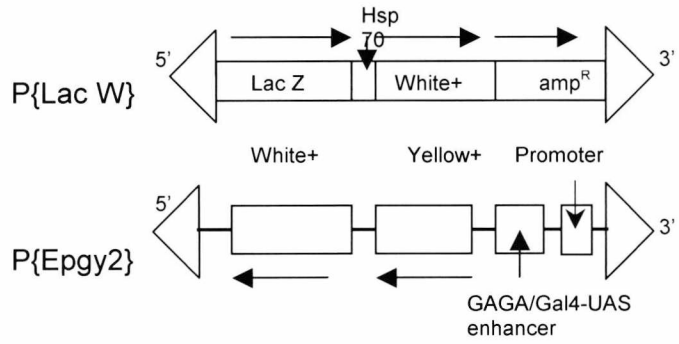
the 5' end of the gene. Of these the EY05252 (5) and the k07433 (3) lines were chosen because the P-elements in these lines were positioned well within *ssp4*, and because they are located in different parts of the gene (see Figure 5.1). In the s*sp4*^{EY05252} allele the P-element is inserted at the 5' end of the gene in an exon that encodes the 5' UTR. The presence of the P-element most likely either disrupts correct splicing of the *ssp4* transcript or results in an aberrant 5'UTR that is unable to function normally.

In the s*sp4*^{k07437} allele the P-element is located in exon 17 just prior to the exons that encode the CKK domain. It disrupts *ssp4* either by disrupting the correct splicing of the *ssp4* transcript or through the production of a truncated transcript that might lead to no protein product or a truncated non-functioning protein. It is also possible that a protein with some functioning could be produced.

Stocks of these flies were available from Bloomington, and were classified as being homozygous lethal. These two P-element bearing lines were generated by different projects in different laboratories and are described below.

5.1.1.1 Dmel\P{EPgy2}1(2)*ssp4*^{EY05252} Insertion (Figure 5.2)

The chromosomal location of this P-Element insertion is 2R:13,422,859 and is 83 bases from the 5' end of the gene. The transposon {Epgy2} is 10.908 Kb, carries the mini white marker gene in the CaSpeR segment, and the yellow+ marker is carried on the y$^{+mDint2}$5.2(S,S) segment. The {EPgy2} construct also carries a GAGA/Gal4-UAS enhancer, and promoter. The P- element is balanced over *CyO*, the Curly of Oster chromosome, which confers curly wings on adult flies. Because of the w^{67c23} deletion, the line containing the P-element has orange eyes since it carries the mini white gene (Bellen *et al,* 2004; Wilson *et al,* 1989).



P-Element constructs used to disrupt expression of ssp4. Horizontal arrows show the direction of translation, both constructs carry a White+ minigene as a marker.

Figure 5.2: Schematic showing the structure of P{lac W} and P{Epgy2}

5.1.1.2 Dmel/P{lacW}*ssp4*^{k07433} insertion (Figure 5.2)

In this line the P{lacW} synthetic construct is located at 2R 13,409,159 which is 2,636 bases from the 3' end of *ssp4*. The transposon P{lacW} contains a Lac Z segment, and a white+ minigene. The second chromosome also carries the *CyO* balancer to prevent loss of the P-element through recombination. The *white+* minigene in the P-element gives the eyes an orange colour allowing identification of flies carrying the insertion (Spradling *et al.*, 1999; Torok *et al.*,1993)

5.2 DETERMINING THE EARLIEST LETHAL PHASE OF *SSP4* MUTANT FLIES.

In Flybase the alleles *ssp4*^{EY0525} and *ssp4*^{k07433} are both annotated as homozygous lethal. Observation of both stocks confirmed this as all adults carried the *CyO* balancer chromosome and therefore were heterozygous for the *ssp4* mutation. Crossing the two independent alleles also resulted in all adults containing the balancer and equal proportions of the insertions as judged by eye colour. It was not known at which stage *ssp4* homozygous mutants died, so collection and careful monitoring of embryonic development were undertaken in order to investigate possible embryonic lethality and to ascertain the lethal stage.

To determine the earliest lethal phase (ELP) of *ssp4* mutants, young adults were put into cages with laying plates, and collections of embryos made after 8 hours from the stocks containing the two *ssp4* alleles. The expected progeny are shown in Figure 5.3.

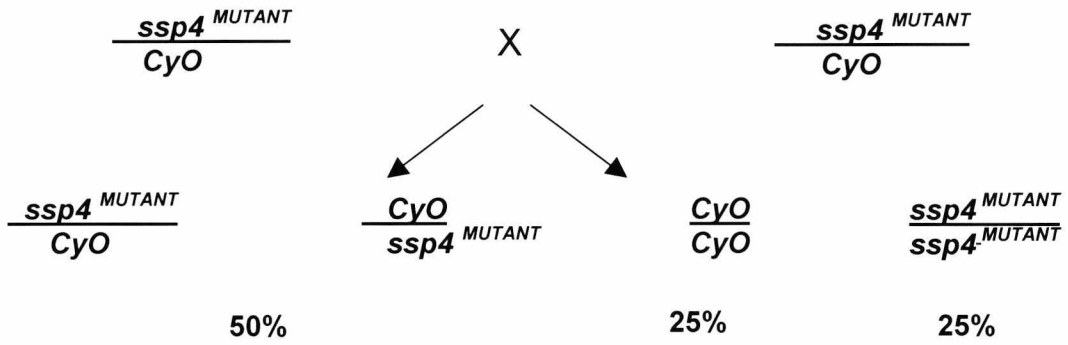


Figure 5.3: Crossing Scheme For Monitoring of Lethal Phase in Homozygous *ssp4*^{k07433} and *ssp4*^{EY0525} Individuals.

Crossing scheme shows 50% heterozygotes and 25% of CyO homozygous individuals, which die as embryos, and 25% homozygous ssp4^{MUTANT}. (ssp4 ^{MUTANT} represents either ssp4^{EY05252} or ssp4^{k07433}).

After collection the embryos were counted onto fresh plates so their development could be monitored. Of the 3508 embryos from both lines that were treated in this way, 1,614 (46%) failed to hatch after 30 hours, and 1789 (51%) gave rise to first instar larvae. These larvae were collected and transferred to fresh vials to continue development, of these 1252 (70%) developed through pupation and gave rise to adult flies. The 70% survival rate may have been the result of damage inadvertently inflicted upon first instars during their transfer into vials. All of these adults had curly wings and the distinctive orange eye confirming that they were heterozygous for either *ssp4* ^{k07433}or *ssp4* ^{EY0525}. 105 (3%) embryos were unaccounted for. These might have hatched and crawled away, or may have remained unhatched but overlooked in the dish. As the surviving adults were heterozygous, and 50% of the unhatched embryos were *CyO* homozygotes, which die during embryogenesis, the ELP of homozygous *ssp4* ^{k07433} and *ssp4* ^{EY0525} individuals appeared to be during embryogenesis. After four days the

embryo cases began to disintegrate on the plate, after six days it was possible to tell unfertilised eggs from embryos since the latter turned brown, while the unfertilised eggs retained a uniform white appearance. In an attempt to reduce numbers of unaccounted for individuals, the apple juice laying plates that had been used, were substituted with grape juice (dark red laying plates) and the exercise repeated with 3,300 embryos. Switching to red grape juice plates, helped detection of unhatched embryos and reduced the 'unaccounted for' numbers by a third. It was assumed that the remaining unhatched fertilised embryos comprised an equal number of homozygous *CyO* and homozygous mutant *ssp4* individuals. See Figure 5.3.

As a control, 718 wild type flies were monitored, 91% hatched into first instars, 4% were unaccounted for and 5% remained unhatched. The higher numbers unaccounted for may have been due to a possible increased fitness of wildtype first instars, which might have been better able to crawl away.

5.2.1 Use of *CyO* 'Green' Balancer Chromosome to Identify Mutants

In order to identify and monitor more closely homozygous *ssp4* mutants I generated stocks in which the two alleles are over a *CyO* "green" balancer chromosome - w/w; *ssp4* $^{P\text{-ELEMENT}}$ / *cyo* P{GAL4-Kr.C}DC3, P{UAS-GFP.S65T}DC7 - that express GFP in a *kruppel* (*Kr*) expression pattern (Brand and Perrimon., 1993; Vef *et al.*, 2006).
This allowed me to unequivocally identify all flies containing the *CyO* chromosome, although this was tricky at stages where *Kr* expression was low.

Green fluorescent protein expression starts in the blastoderm (Hoch *et al*, 1990) and is seen in the posterior ectoderm and amnioserosa and in the extended germ band at stages 9 -12 before fading during stage 13 -14. From stage 16 onwards it is expressed strongly in the macrophages and Bolwig's organ. Embryos of all ages were collected and checked every thirty minutes. It was anticipated that approximately 50% would be

heterozygous GFP expressing embryos that would go on to become viable adults, and 50% homozygous for either *CyO* or *ssp4* ^{MUTANT}, differentiation between these two groups would be straightforward, with high GFP levels in the former, and no GFP expressed in the latter. 1st instars were kept at 25 degrees centigrade and fed on a diet of standard cornmeal/agar media (Ashburner 2004, and see Chapter 2). The homozygous *ssp4* ^{MUTANT} individuals were closely monitored. Collections of wild type embryos were also made, to establish rates of normal embryonic death. Care had to be taken in distinguishing between the yellow embryonic auto fluorescence of the yolk from the GFP fluorescence of the reporter.

5.2.2 Monitoring Development Using a GFP Balancer Chromosome

Embryos from the *ssp4* ^{k07433}/ *CyO* ^{KrGFP} line were collected and laid out on grape juice plates as previously described. Of the 3,300 that were monitored only 3 non-glowing larvae were seen. By phenotype these were the only homozygous animals that had completed embryonic development. They hatched after about 4 days, they trembled, their guts were swollen with a dark brown line through them. They lived for only a few hours, able to crawl a little but unable to move away from a prod with a pipette tip. Some *ssp4* homozygous first instars were seen to poke their heads through their cases but were unable to wriggle free. Case deterioration may have released a few of these non-glowing, possible homozygous *ssp4* mutant individuals, which would explain why these larvae were not seen until a few days after heterozygous larvae had hatched.

Homozygous mutants, identified by the lack of GFP protein, were closely monitored and appeared to die late in development, at approximately stage 16, whilst heterozygotes continued to develop normally. These mutants showed no movement.

Some stage 14 mutants had their chorions removed, using double-sided tape, and were than allowed to continue their development under oil, to prevent their drying out, to see if they would live longer outside the chorion but they died in the final embryonic stages.

A further cross was set up between s*sp4*^{EY05252}/*CyO KrGFP* and s*sp4*^{k07433}/*CyO KrGFP* individuals to confirm that lethality was due to the disruption to the *ssp4* gene. Virgin female *ssp4*^{k07433} were collected, crossed to male *ssp4*^{EY05252}and left for 24 hours. They were then put in a cage and embryos collected 8 hours later. This cross was repeated 4 times and 1300 embryos were counted altogether. In these crosses, 52% hatched as larvae, and 48% did not hatch, of these approximately 50% were homozygous mutants. There were very few unfertilised embryos, and this may be because flies were all newly eclosed and mated at the same time whereas the flies used in the previous crosses were kept in bottles, and were of mixed ages.

5.3 EXCISION SCREEN

In order to confirm that the lethality in homozygous *ssp4* mutants is due to the P-element insertion in the *ssp4* gene and not the result of an undetected mutation elsewhere on chromosome two, an excision screen was undertaken to remove the P-element from the *ssp4*^{k07433} line to see if this restored viability. This stock was chosen because the P-element is positioned within the gene and its imprecise excision may generate more alleles of *ssp4* that could be useful for genetic analysis since there is no deletion that covers this region. The P-element on the *ssp4*^{EY05252} allele is close to the 5'end of *ssp4* and since removal of DNA through imprecise excision is essentially random in direction there is an equal chance of disruption to CG4878 that lies just 5' to *ssp4*.

5.3.1 P-Element Mobilisation

Native P-elements are 2.9kb with 3 splice positions the third of which can only be processed in germ line cells (O'Hare, 1983). The product is a 87kDa protein which is a transposase. The transposase binds to recognition sequences on the P-element to mediate its excision from one site and its random reinsertion. The end of the P-element comprises inverted repeats of 31base pairs (bp). It has been used as a method of classifying gene functioning in Drosophila as it inserts itself into the genome at very high rates (Bellen et al, 2004) however, the establishment of the effect of mutating a particular gene requires it to be fixed. A plasmid that contains a reporter but cannot produce transposase, can be constructed and injected into early stage embryos, a few of which will take the P-element up. These flies can then be studied to establish the effect of disruption to their genes. The insertion of the P-element occurrs preferentially near regularly transcribed genes as this is where the chromatin is loosest. This is known as a non-autonomous plasmid as it is incapable of excising and reinstating itself. It is possible to excise a non-autonomous fixed plasmid by adding a P-element that supplies the transposase. This was achieved using a modified P-element, called P{ry+ Δ2-3} (99B), that is an efficient producer of transposase leading to mobilisation of P-elements while it remains stably integrated itself (Robertson et al , 1988).

5.3.1.1 Fly Crossing Scheme for P-Element Removal from *ssp4* ^{k07433}.

A fly line y^1/w*; +/+; ry[506] Sb1 P{delta2-3} 99B/TM6 was obtained from Bloomington. This line contains P{ry+ Δ2-3} on a chromosome marked with *sb*.

Figure 5.4 shows the crossing scheme for the P-element mobilisation of k07433. The F1 generation produced 30 male progeny were selected and crossed with 50 females, to get the *ssp4* P-element onto their second chromosome. 100 F2 generation males,

bearing the *ssp4* ^{k07433} allele and the transposase in their germ line were crossed *en masse* to 200-300 female virgin double balancer flies. 74 male F3 offspring, with white eyes were recovered. They were white eyed because the P-element had been excised, along with the white plus minigene. These males were crossed to double balancer females in order to rescue the chromosome from the males.

Of these 74 males, three were infertile. The others produced offspring that were crossed *inter se* to produce lines. Of the 71 lines the vast majority (79%) produced straight wing flies indicating that the *ssp4* gene was no longer disrupted. This was presumably due to a precise or near precise excision of the k07433 P-element.

Fifteen lines, however, provided only curly-winged adults, indicating that the chromosome from which the k07433 P- element had been excised still contained a lethal mutation. There are three likely reasons for this.

1 An internal deletion in k07433 had removed the white plus gene but most of the P-element remains to cause disruption to the gene.
2 Surrounding genomic DNA had been removed causing disruption to *ssp4.*
3 An unusual genetic event had occurred, for example, a precise excision of K07433 followed by integration and subsequent imprecise excision causing disruption elsewhere in chromosome two.

To start to distinguish these possibilities males from these lines were crossed to ssp4^{EY0525} / *CyO* females. 14 of these lines produced *CyO* flies only indicating that the excision chromosome still had a disrupting effect at the *ssp4* locus. This is because a significant amount of the P-element was still causing disruption of *ssp4*, or that genomic DNA had been removed following an imprecise excision.

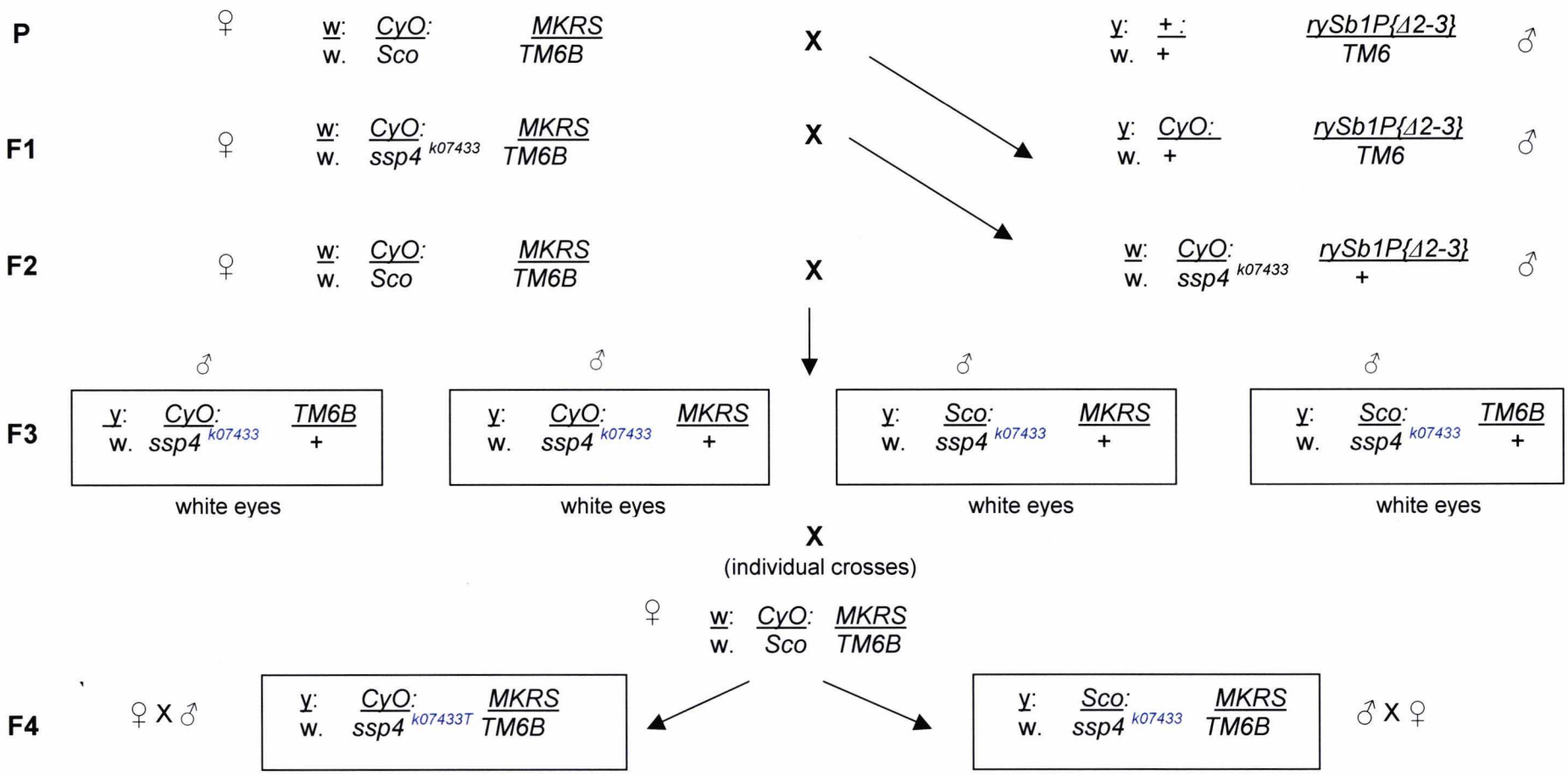


Figure 5.4: Crossing Scheme for P-Element Removal

P – Initial cross of double balancer females to males to generate males bearing the CyO gene and transposase. These were crossed to Ssp4 ^{KO7433} females, F1, to generate males bearing the P element and transposase, F2. Males produced (F3), were white eyed were selected and crossed to double balancer females to produce double balanced offspring that have undergone an excision event. In F4 these were sibling crossed to create a stock.

Primers were generated that recognised DNA on either side of the P-element site, and also within the P-element site to determine if a complete excision had occurred, by PCR analysis. This exercise is due to be carried out at a later date.

5.4 DISCUSSION

The use of the *ssp4*^{k07433} and *ssp4*^{EY0525} alleles has shown that defects to this gene cause death late in embryogenesis. These lines were each from different laboratories, and caused disruption at different points of the *ssp4* gene but gave a similar ELP. *ssp4* is expressed in the nervous system, gut and in head cells that might be undergoing apoptosis (chapter 4). The mutant phenotype could be the result of a defect in one or several of these areas as they are all involved in the process of embryogenesis.

5.4.1 Monitoring Development of *ssp4* Mutant Flies.

I initially carried out some observational exercises, to establish the effects of the mutation on embryonic development. There were problems with embryo collection that had to be resolved such as food drying out and high embryo numbers, leading to variation in first instar numbers. Low embryo numbers and increased humidity solved these problems and also increased adult fly numbers. An embryo-washing step was introduced to remove yeast, which obscured larvae and embryos on the laying plates. Small scratches in the jelly surface from embryo placing gave instars a place to hide, but this was fixed by first coating the dish with a thin film of water.

The GAL4/UAS GFP system permitted identification of embryos of different genotypes, once initial issues with autofluorescence were overcome. This system has been shown lead to apoptosis and developmental defects but only when expressed in the eye (Kramer, 2003) and so is a reliable system for embryo study.

The presence of the GFP meant that it was not necessary to rely totally on larval numbers to establish rates of lethality. This was helpful as it allowed differentiation

between homozygous *ssp4* mutants and *CyO* balancer homozygotes, the latter of which sometimes escape and hatch as first instars. GFP protein remains glowing in cells 96 hours after cyclohexamide treatment (Kamau *et al* 2001*)*. As the embryos had to be left overnight this long half-life allowed the correct assignment of genotype to dead embryos.

The individuals homozygous for $ssp4^{EY05252}$ or $ssp4^{k07433}$ develop to a late stage but are unable to hatch, and the mutational effect varies in severity in this respect. Most died inside their eggs, some appeared to partly hatch. Only a very few were seen free of their cases. When the chorion was manually removed from mutant embryos they did not live long, so their deaths were not the consequence of being trapped inside their eggs. The mutant first instars were almost completely immobile, and were only seen because the egg cases had melted away from them, which is why there were so few and why they were not seen until some days after the normal larvae had hatched. Most deteriorated egg cases had dead larval remains associated with them. In the majority of cases mutant larval heads did poke out from their eggs without going on to hatch. This may have been because chorion intergrity is lowest at the head end, causing it to disintegrate first (Siekhaus and Fuller, 1999), or due of some limited head movement, or perhaps a combination of both factors.

A major fault in head development might have arisen as a consequence of low apoptosis levels in the embryonic head preventing normal formation of the head or its components (Chapter 4). In normal larvae 4 – 5 hours prior to hatching, the mouth-hooks are scraped against the vitelline membrane and chorion in a semi circular arc in the hatching region (Siekhaus and Fuller, 1999). This manoeuvre would be impossible if the head or mouth hooks were absent or malformed.

Abnormal gut development could prevent the breakdown of yolk in the mid gut of late stage embryos, starving homozygous *ssp4* individuals of the nourishment and proteins

needed to complete development. Yolk is the food supply that embryos depend on for development. Yolk granules release proteins with a pH regulated temporal and spacial specificity (Fagotto 1996). A gut defect would explain the swollen, discoloured mutant larval guts, and lack of feeding.

A defect in the development of the nervous system would lead to the immobility seen in homozygous mutants, as the nervous system co-ordinates the muscular movements that lead to hatching. Egg hatching consists of several minutes of extension and retraction of the larval head, which then extends further and moves about for a minute, before being followed by the rest of the body. The peristaltic waves of muscular movement, necessary for hatching, are the result of the connecting of the musculature with the sensilla, and are also mediated by the protocerebrum and glial cells (Pereanu *et al,* 2007). Lack of innervation of the musculature would account for larval immobility and weakness, and the fact that they were completely unable to feed even when liquid yeast paste was placed very close to their mouths.

5.4.2 Excision of P-Element from *ssp4*^{k07433}

To be certain that embryonic lethality in homozygous *ssp4* mutants was due to the P-element insertion in the *ssp4* gene and not the result of an undetected mutation elsewhere on chromosome two, the P-element from the *ssp4*^{k07433} line was excised to restore viability. The vast majority of lines, 80%, were homozygous viable indicating that the precise or near precise excision of the P-element had removed the lethal effect. 19% were homozygous lethal lines, and 1% infertile. This infertility was probably the result of hybrid dysgenesis, due to P-element transposition in germ line cells. These percentages are consistent with those obtained in similar screens (Roberts, 1980).

The P-element is positioned in an intron and its method of disruption is unknown. It might be that it shifts the reading frame disrupting translation or that it interferes with

splicing or inserts a stop codon. It might be that its physical presence alone makes the mRNA too long and unstable. Some pilot data recently generated by PCR has identified a viable line from this screen, which is still carrying the P-element in *ssp4* although the exact P-element length is not known. It may be enough to just remove some of the P-element in order to remove its lethal effect.

The expression of *ssp4* from very early in development may be due to a maternal effect. The reason the phenotype is not seen until late in development may be because the effect persists until this point. Alternatively it could be that *ssp4* is not needed until this developmental stage although this seems unlikely as zygotic *ssp4* could be produced from stage 5 onwards. This maternal effect may prevent the establishment of *ssp4* zygotic function, the stage at which it is needed, and the developmental processes it takes part in.

One way to find out the nature of *ssp 4* would be to use the FLP/FRT system, to create a mosaic individual, which would allow comparison between homozygous mutant and normal cells at all developmental stages, and in all tissues (Theodosiou and Xu,1998). The FLP recombinase gene has been taken from *Saccharomyces cerevisiae* and its protein product recognises a FLP recombinase target (FRT) site. An FRT is a DNA sequence located near to the centromere on each chromosome arm. Production of the recombinase, which is controlled by either a heat shock promoter or the GAL 4 UAS system, leads to genetic recombination at these sites, during mitosis. If the FLP recombinase is activated during a period of high levels of cell division, clones of these cells will be *ssp4* homozygous. This will create a mosaic individual, in whom some cells will continue to develop as normal whilst others will not, allowing the effects of knock down of gene expression to be seen. The homozygous mutant cells will be easily identifiable using the GAL4 UAS GFP system, as they will not glow under the

fluorescence microscope. Antibody staining will then show the effects of *ssp4* knockdown on normal developing tissues.

The FLP/FRT system can also be used to generate female germline clones, unable to pass on functioning *ssp4* to their offspring. This would remove any maternal interference with establishment of the zygotic functioning of *ssp4* and would show when *ssp4* was needed, which would tell us the processes it was necessary for. These clones are produced through using dominant female sterile (DFS) mutations. Females heterozygous for a DFS mutation, do not produce eggs. Mitotic germline recombination can lead to loss of this mutation in some cells, leading to egg production. Ovo^{D1} is a DFS mutation, which has been inserted onto the arms of the X, 2nd and 3rd chromosomes. Heat shocking of third instar larvae and adults, who are heterozygous for Ovo^{D1} and *ssp4* leads to activation of the FLP recombinase. As the target for the recombinase is on the same chromosome arm as *ssp4,* recombination in some germline cells leads to the production of eggs carrying two mutant copies of *ssp4.* As these eggs have no copy of Ovo^{D1} they are the only ones that will be produced.

It may be that the P-elements do not completely suppress production of *ssp4* in the two mutant fly lines, and that they can produce small amounts of functioning protein which is just sufficient to get them to the later developmental stages but inadequate for complete development. If this was the case, then using the FLP/FRT system with $ssp4^{k07433}$ or $ssp4^{EY05353}$ would not completely eradicate all traces of *ssp4*. It was hoped that the screen would create a fly line carrying a carrying a damaged and completely non-functioning copy of *ssp4* through an imprecise excision. This line could have been used to reveal whether $ssp4^{k07433}$ or $ssp4^{EY05252}$ were hypomorphs or null alleles. It may also have shown whether *ssp4* was needed at an earlier developmental stage. Unfortunately time constraints prevented the PCR analysis that was necessary to identify such a line.

RNA interference could be used to knockdown expression of *ssp4* in a particular tissue at a particular time. RNA interference was a term coined by Fire and Mello in 1998 when they discovered that double sense RNA (dsRNA) could suppress gene expression. In 2000, Zamore and colleagues found drosophila embryo lysates produced small interfering RNAs (21-23 nucleotides long) from dsRNA, which cleaved homologous cellular mRNA in the region corresponding to these dsRNA nucleotide lengths. Because RNA interference uses the complimentary copy of its target mRNA it is very specific. Inducible RNAi can be driven by the GAL 4 UAS system, which is carried in many fly lines, and would allow *ssp4* mRNA to be destroyed with temporal and spatial specificity. A search of flybase identified 2 fly lines from the Vienna Drosophila RNAi Centre (VDRC) that were specific for *ssp4* (33677 and 27654). These double stranded hairpin RNA's, of 300-400 base pairs, are processed by the cells own machinery into short interfering RNAs (siRNA) which target the mRNA of the gene of interest.

RNAi is a powerful tool for investigation of gene function. However, it carries a risk of off target effects. Ma *et al* (Nature 2006) carried out an RNAi screen for novel components of Wingless (Wg) signal transduction, and found few legitimate candidates, with off target effects influencing the Wg response. A computational study involving *Caenorhabditis elegans, Schizosaccharomyces pombe and Homo sapiens,* found off target effects ranged from 5%-80% for each organism (S Qiu *et al* 2005).

I decided that the most direct way forward would be to use a label to distinguish homozygous mutants from heterozygote individuals and carry out a close study of their gross morphology, particularly the head and gut, to get a clearer idea of what the nature of the mutation was. As CAMSAP 1 has a significant role in neurite outgrowth in

vertebrates, and *ssp4* is expressed in the embryonic nervous system, I decided to use antibodies to label the nervous system, to show the effects of a knockdown of *ssp4.*

5.5. CONCLUSION

This chapter has shown that the *ssp4* mutation is homozygous embryonic lethal. The mutants die late in embryogenesis, although the mutation itself may begin to exert its effects earlier. This lethality may be the consequence of a fault with the embryonic gut, nervous system, or head development, or perhaps all three, as this is where *ssp4* is expressed in the embryo. Removal of the P-element, from $ssp4^{k07433}$ restored embryonic viability.

The presence of a possible maternal component is hampering the identification of the role of *ssp4.* A maternal clone might remove this constraint but if *ssp4* is needed at very early developmental stages, its specific role would still be unknown. A mosaic individual would allow the comparison of mutant *ssp4* cells with normal cells. RNAi could be used to knockdown expression of *ssp4*, but is prone to off target effects, which may result in an ambiguous or incorrect result.

Instead, in chapter 4 I shall study the gross physiology of *ssp4* mutant embryos, paying particular attention to the gut and head development. I shall also label the central nervous system, as CAMSAP 1 is vital for neurite outgrowth in vertebrates. The most significant embryonic stages will probably be towards the end of embryogenesis.

Chapter 6

ANALYSIS OF THE SSP4 MUTANT PHENOTYPE

6.1 INTRODUCTION

Ssp4 is expressed in the central and nervous system, and is highly expressed in cells in the head that may be involved in apoptosis. P-element insertions into *ssp4* revealed that it was an essential gene for development with embryonic lethality occurring late in embryogenesis.

Work carried out in cultured mammalian cells has shown that CAMSAP 1 is necessary to sustain neurite outgrowth. Therefore as a first step *Drosophila* embryonic nervous system development was studied in mutant animals to see if a nervous system defect was the cause of lethality. The gross morphology of embryos at various stages was examined and monoclonal antibodies raised against proteins expressed in the nervous system were used to observe neuronal morphology.

6.2 DOUBLE LABELLING TO IDENTIFY *ssp4*^{K07433} AND *ssp4*^{EY05252} HOMOZYGOTES.

The two *ssp4* mutant alleles *ssp4*^{k07433} and *ssp4*^{EY05252} were put over a *CyO* balancer coupled to a marker (O Kane *et al,* 1987). This chromosome contains the *lacZ* gene from *Escherichia coli* fused to the *even-skipped* promoter. The *lac Z* gene encodes beta galactosidase. *even-skipped* (*eve)* is a pair rule gene that acts as a transcription repressor. It is switched on 2.5 hours into development and is expressed in the embryo until the end of embryogenesis. After germ band retraction Eve expression is seen in aCC, pCC, CQ, EL and RP2 neurons (Fujioka *et al.,*1996). Expression of Beta-galactosidase (Lac Z) was observed using immunohistochemistry.

Embryos were initially stained using X Gal as a substrate but high background staining coupled with low levels of antibody binding to nervous system proteins, made this method unsuitable for study of nervous system development (see Figure 6.1). So antibodies to Lac Z were used to allow identification of heterozygous individuals.

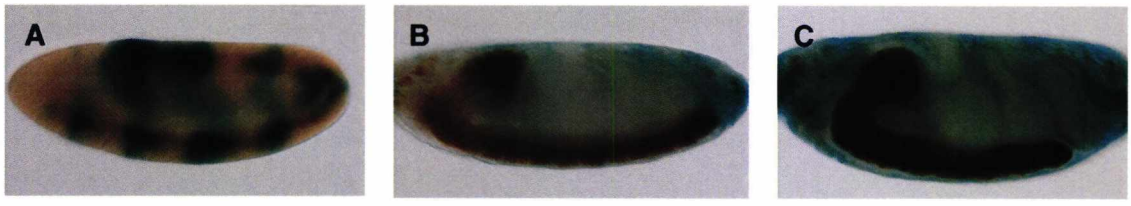


Fig 6.1: Images of Whole Mount *ssp4*^{k07433} /*CyO* $^{EVE\ LACZ}$ Heterozygous Embryos.
Heterozygous ssp4^{k07433} *whole mount embryos in lateral view (anterior to the left) at a magnification of 200X. Embryos that have all been stained with X Gal, and then with ELAV. The ELAV antibody is bound by a biotinylated secondary which produces a stable brown dye. Fig A; Stage 12: Neuroblasts are undergoing waves of mitosis, with emergence GMC's, seen as a brown strip of ELAV staining which can be seen through the blue stripes, along the bottom edge (ventrally) of the embryo. Figs B & C; Stages 14 and 16: Neuronal differentiation, with the brain visible on the left hand side, followed by CNS condensation. The X Gal blue staining is diffuse and the ELAV staining is not a clear enough to reveal staining of individual CNS or PNS cells.*

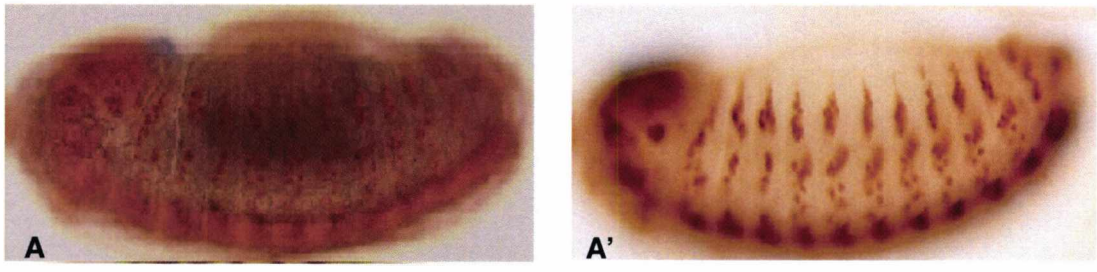


Figure 6.2: A Comparison of Nervous System Development in *ssp4*^{k07433} Heterozygote and *ssp4*^{k07433} Homozygote.

Whole mount stage 14 heterozygous (A) and homozygous (A') mutant embryos in lateral view (anterior to the left) at a magnification of 200X. The ELAV primary antibody is bound by a biotinylated secondary, which produces a stable brown dye. The Heterozygote has a blue tinge in the gut and head area from the alkaline phosphatase secondary that was used to bind the Lac Z antibody. The CNS and individual cells of the PNS can be seen clearly enough to compare and at a gross level these two embryos appear to be indistinguishable.

Homozygous Ssp4 mutants were identified by their lack of staining. A rabbit polyclonal antibody to Lac Z was used in conjunction with mouse monoclonal antibodies to nervous system proteins. A monoclonal anti Lac Z antibody was used in conjunction with the Ssp4 antibody to identify homozygous mutants (Figure 4.7, chapter 4) Lac Z expression was visualised using Diaminobenzidine (DAB) detection, but it was necessary to label Eve with alkaline phosphatase, when the DAB system was used to label the nervous system proteins. Fluorescent antibodies were also used to label some embryos to see if the labelling quality could be improved.

6.2.1 Mouse Monoclonal Antibodies Against Neural Proteins to Identify *ssp4* Mutational Effects.

Neurogenesis begins at stage 5 with the formation of the ventral neurogenic region, and ends with condensation of the CNS at the end of embryonic development. Ssp4 protein expression is seen in the cells of the ventral neurogenic region at stage 8 and continues throughout the subsequent development of the CNS and PNS.

CNS formation begins with delamination of 30 neuroblasts (NB) per hemisegment, from the neurectoderm between stages 8 until 11, to produce 3 columns of cells in the NB layer (Bossing *et al,* 1996). NB's then undergo 8 rounds of mitosis to produce ganglion mother cells (GMCs) during stages 9-13, which divide to produce neurons. This process is conserved so it is possible to identify individual NBs and study their specification. Four mouse monoclonal antibodies from the Developmental Studies Hybridoma Bank in Iowa, were used to compare the developing CNS between homozygous mutants and heterozygotes to elucidate the role of *ssp4*.

Embryonic lethal abnormal vision (ELAV) is an mRNA binding protein that is necessary for central and peripheral nervous system differentiation and maintenance. It is expressed in all neurons after their differentiation. Staining with an antibody against this

protein will reveal any defects in division and number of nerves within the CNS. The antibody 22C10 recognises the microtubule-associated protein Futsch. It is expressed in the PNS and CNS, in post mitotic neurons prior to axonogenesis (Hummel *et al,* 2000), interneurons, motoneurons and all sensory neurons in the axon, dendrite and cell body. Reversed polarity (REPO) protein is expressed in glial nuclei, excepting midline glia, which are of mesectodermal origin. Other glia arise from ventral neuroectoderm. All glial cells except midline glia need the *glial cells missing* for their specification, as transient expression of *glial cells missing* leads to sustained REPO expression (Yuasa *et al,* 2003). Although it will not be possible to see any mutational effects upon midline glial cells, antibody labelling of REPO will show if there are any faults in specification of cell function, or glial cell number.

The antibody BP102, developed by C Goodman at the University of California, recognises a carbohydrate epitope, and stains CNS axons clearly showing commissures and connectives as a ladder like pattern (Carney *et al* 1997). Disruption to axon guidance during CNS formation leads to fused anterior and posterior commissures (Garbe *et al* 2007).

6.2.1.1 Staining for ELAV Protein

Figure 6.2 shows labelling of embryos using the mouse monoclonal antibody to ELAV. The control embryo has a blue wash from the alkaline phosphate labelling of the Lac Z but this did not obscure the pattern of PNS cells in the 10 embryonic segments and head although they were easier to make out in the mutant. All segments have 3 distinct areas of staining, a section that is dorsal, and a section half way down the embryo. Segments 3-9 have an additional group of either 3 or 4 cells abutting the CNS. These distinct groups are present in the heterozygotes and mutants but are much clearer in the heterozygotes.

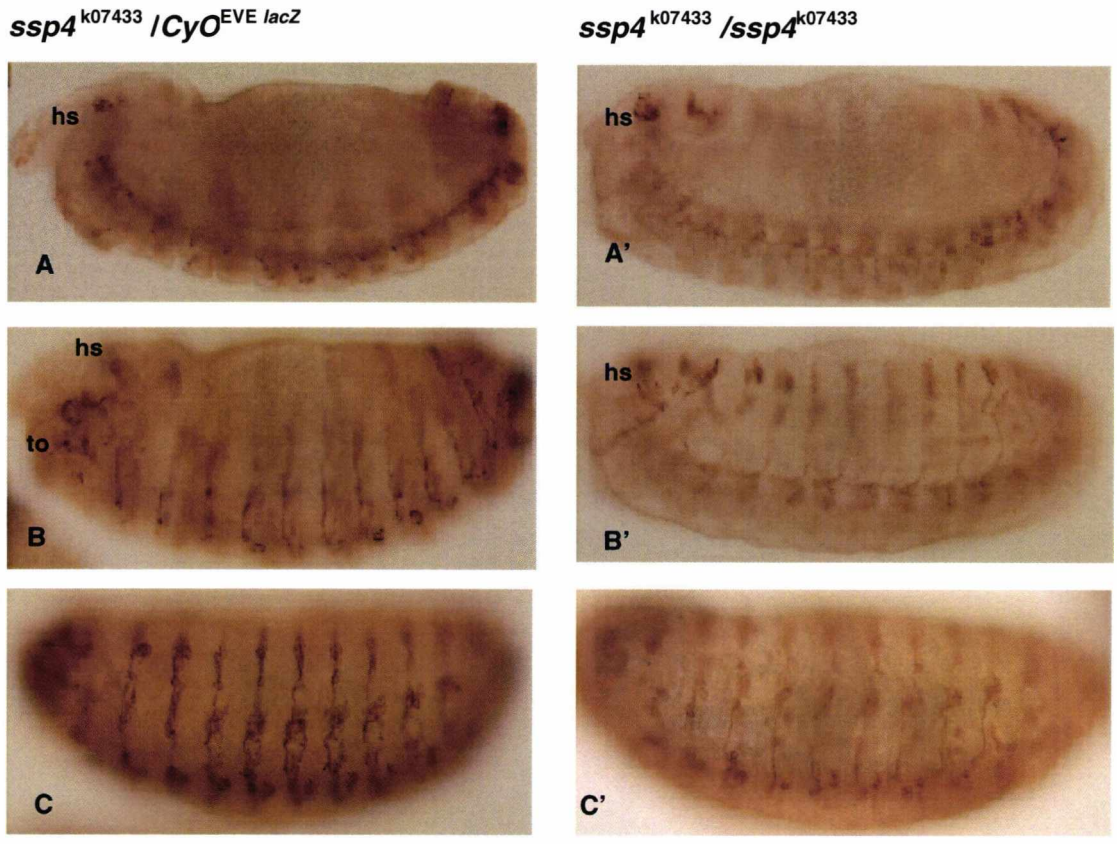


Figure 6.3: A Comparison of PNS Development in *ssp4*^{k07433} Heterozygotes and *ssp4*^{k07433} Homozygotes.

Whole mount stage 14 and late stage heterozygous and homozygous mutant embryos in lateral view (anterior to the left) at a magnification of 200X, stained with 22C10 and Lac Z. The primary antibodies are bound by a biotinylated secondary, which produces a stable brown dye. Both mutant and heterozygote have 10 sensory neurons (panels B, B', C, C') head spot (hs)(panels A, A', B, B') labelled and gross neuronal morphology is indistinguishable. to – terminal organ.

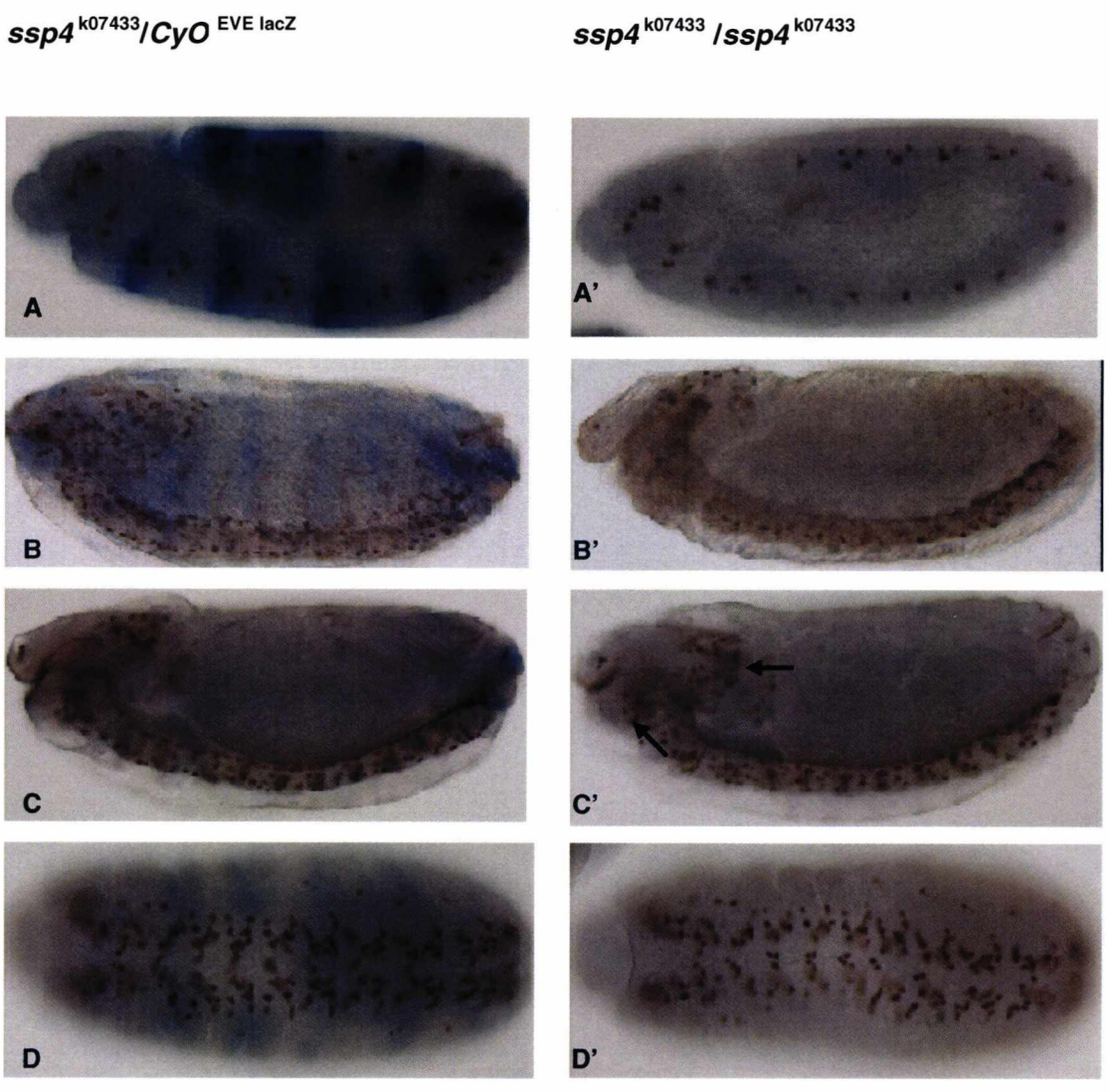


Figure 6.4: A Comparison of CNS Development in *ssp4* k07433 Heterozygotes and *ssp4* k07433 Homozygotes.

Whole mount mixed stage heterozygous and homozygous mutant embryos in lateral view (anterior left) at a magnification of 200X. The REPO antibody is bound by a biotinylated secondary, which produces a stable brown dye. The Lac Z antibody is bound by an Alkaline Phosphatase to give a stable blue dye. Stained cell numbers appear to be higher in the heterozygotes in stages 9 (A and A') and 13 (B and B'). In stage 15 (C and C'), the glial cell number is higher in the mutant (arrows). Stage 12 (D and D') embryos are a ventral view, to show that glia in the midline region are unaffected by Ssp4 knockdown, however, anti REPO does not label the actual midline glia.

6.2.1.2 Staining of Futsch Protein (22C10).

Figure 6.3 shows the labelling of the CNS (A, A' and B.B') and the PNS (C,C') in stage 14 embryos. The thick stripes of B-galactosidase staining in the heterozygotes slightly obscure PNS axon staining, except for in the late stage embryo where sensory neuron structure is clear in both examples (C, C'). At a gross morphological level the structure of the CNS and PNS appear to be comparable. However, there is a difference in the shape of the embryos with the mutants having a less curved ventral edge and a flatter front edge to the head. The terminal organ (to) position also appears to be altered or missing in the mutant (see B'), although the position of the head spot is unchanged.

6.2.1.3 Staining of Glial Cells Using Reversed Polarity Protein (REPO)

Figure 6.4 shows a comparison between glial cell numbers in heterozygotes and homozygous mutants, using REPO. This antibody labels all glial cells except for midline glia from stage 8 until the end of embryogenesis. In the stage 9 mutant embryo (A') there appear to be fewer glial cells in the different embryonic segments particularly in the ventral area. Cell numbers in the head appear to be lower in the mutant at stage 13 but this might be due to a slight difference in the focus (compare outline of CNS between B and B'). At stage 15 there are more glial cells in the posterior of the head, and in the ventral area to the front of the head, which are shown with arrows in C'. The stage 12 ventral view shows the pattern of expression in the CNS is the same for both (D and D'). The overall shape of mutants and heterozygotes appears to be the same until stage 15, where the mutant appears to be slightly flattened ventrally.

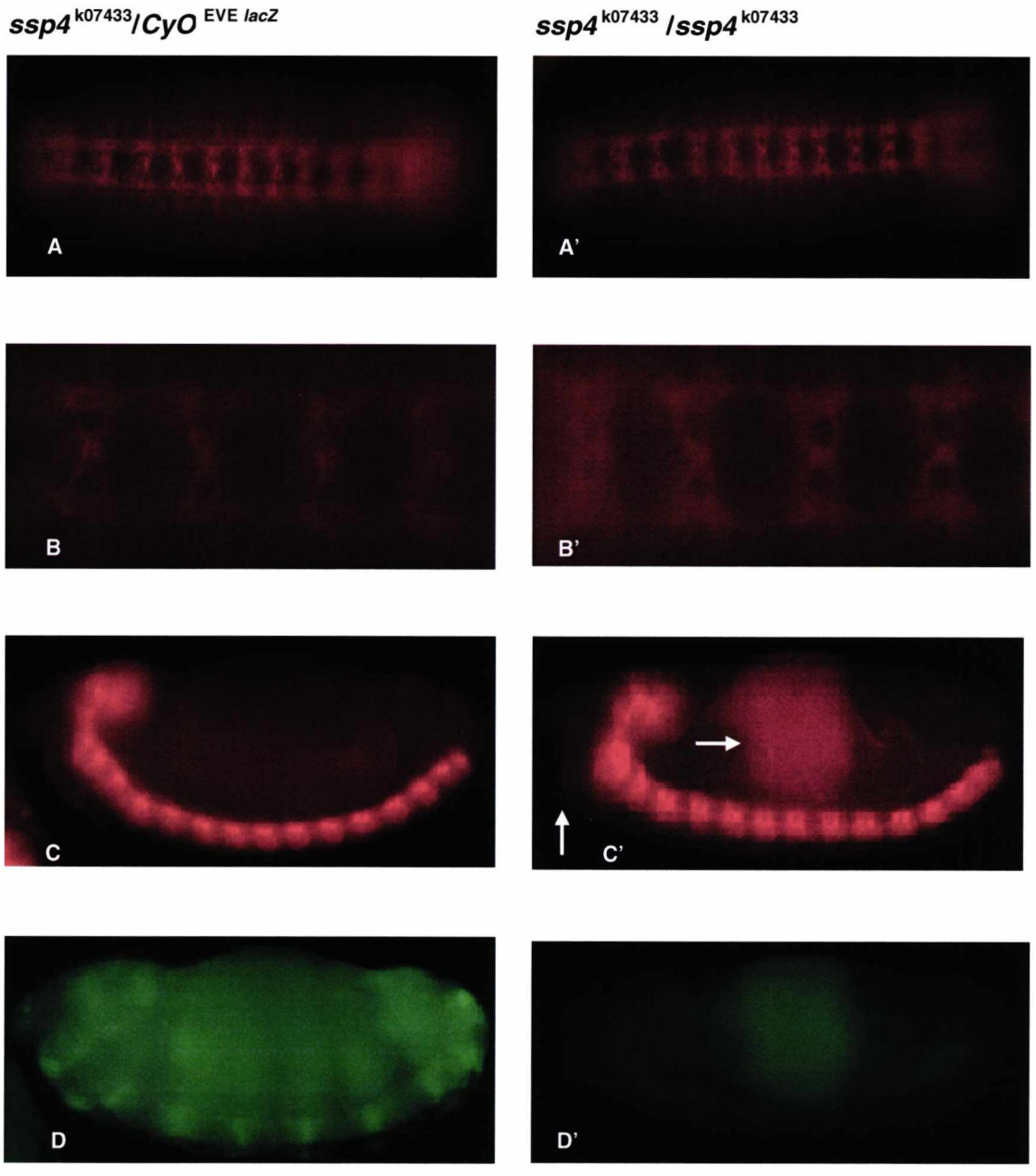


Figure 6.5: A Comparison of CNS Development in *ssp4*^{k07433}Heterozygotes and *ssp4*^{k07433} Homozygotes.

Embryos double labelled to identify mutants. Magnification is 200X except for panels B and B', anterior is to the left. A,A',B,B' are dorsal view and C,C',D,D' are lateral view. A and A' – stage 16 embryos stained with Mab BP102, A is the control and A' the mutant. B and B' are enlargements (400X) showing the ladder arrangement of the CNS matrix. Note the thick commissures and the longitudinal tracts in A,A',B,B' (D Garve 2007). C and C' are stage 14 embryos showing labelled condensing CNS. C' - stage 14 embryo stained with antibody to Lac Z, the label for identifying heterozygous ssp4^{k07433} *individuals. D' shows a homozygous ssp4*^{k07433} *embryo at stage 14. Note the obvious difference in gut shape between heterozygote and homozygote mutant, C and C', D and D'. The difference in staining pattern and levels that make it easy to identify mutant embryos, D and D'. The faint green staining in D' may be autofluorescence.*

6.2.1.4 Staining of BP102

Staining to the CNS using fluorescent antibodies, binding to BP102 is shown in red, and Lac Z binding is shown in green (Figure 6.5). The Lac Z antibody successfully identifies mutant embryos. Both mutants and heterozygotes show axons as a regular ladder like pattern (A and A') with a pair of commissures in every segment (B and B'). Figs C and C' show that CNS structure is the same in both embryo examples. However, the stage 14 embryos (C and C') are different in overall structure with the mutant C' having a less rounded appearance overall, and a flattened head end. The gut in the mutant also appeared to be a more rounded shape than in the heterozygote.

To summarise, staining of the central nervous system revealed no gross morphological defects in the mutant embryos, except for possible differences in glial cell number. However, it appeared that the mutant embryos differed to the heterozygotes in their overall shape, and the development of their gut and head.

6.2.2 Embryonic Development of *ssp4*^{k07433} mutants.

I decided to examine the different developmental stages, labelling for the presence of the balancer chromosome, in order to identify homozygous mutants. Whole mount *ssp4* ^{k07433}/ *CyO* $^{EVE\ lacZ}$ and *ssp4* ^{k07433}/ *ssp4* ^{k07433} embryos were stained with a polyclonal antibody to Lac Z (Figure 6.6). The Eve pattern is shown clearly in the cellular blastoderm of the *ssp4* ^{k07433}/ *CyO* $^{EVE\ lacZ}$ embryos and in the developing segments of the stage 7 embryo (B). Expression is far stronger at stage 8 (C) and the extended germ band is clearly labelled. In the stage 14 embryo (D) staining is clear in the CNS and appears as faint, broad vertical stripes in the embryo. As embryogenesis continues the staining in the CNS becomes more specific with individual groups of neurons clearly visible. This staining pattern continues until larval hatching.

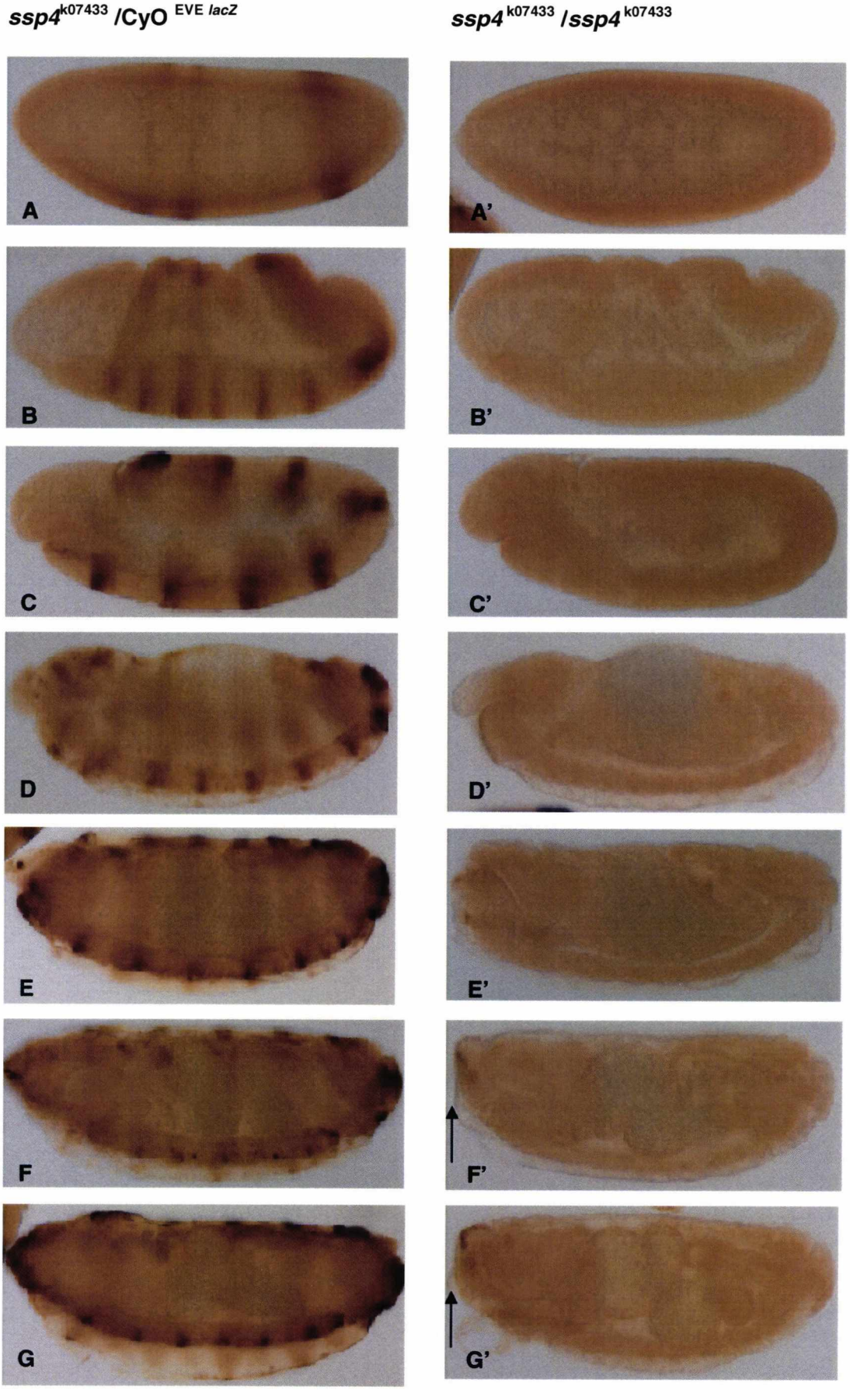

ssp4k07433 /CyO EVE lacZ
ssp4k07433 /ssp4k07433
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
G
G'

Figure 6.6: Embryonic Development of *ssp4*^{k07433} Mutants (previous page).

Whole mount embryos in lateral view (anterior left) at a magnification of 200X. Embryos in the left hand column are heterozygous for ssp4^{k07433}*. Staining is to Lac Z expressed in the pattern of Eve. Embryos in the right hand column are homozygous ssp4*^{k07433}*. A – cellular blastoderm, B – stage 7, C – stage 9, D – stage 14. The first morphological difference between heterozygote and homozygote is at stage 15 (Fig E and E'), where the front of the mutant head is flattened (arrows). Stage 16 – F and G – final stage.*

The mutant *ssp4*^{k07433} embryos showed some signs of non-specific staining, which may have been due to primary or secondary antibody binding. This effect was not seen in figure 6.7, where a mouse monoclonal antibody was used. However, it did not prevent morphological features being seen in the heterozygotes, and allowed mutant identification. The anterior end of the embryo is altered in the mutant individuals from stage 15 onwards giving the head a much flatter and squarer appearance (see Figures 6.6,E',F',G'). The alteration to the front end appears to be the only obvious effect of the mutation, with gut development and CNS condensation completed as normal.

Figure 6.7 shows a comparison of *ssp4*^{EY05252} heterozygotes and *ssp4*^{EY05252} homozygous mutants, stained using a mouse monoclonal anti Lac Z antibody. Although the overall embryo colour is paler in these examples than in the *ssp4*^{k07433} homozygotes and *ssp4*^{k07433}/ CyO $^{EVE\ LacZ}$ examples, staining levels are very similar, and the mutants have reduced non-specific binding, with clear staining to the hind-gut and salivary gland only, (Figure 6.7 C' and C''). This 'cleaner' look may be due to the use of a mouse monoclonal Lac Z antibody rather than a rabbit polyclonal (Figure 6.6). The flattened front end is apparent in the stage 14 embryos, and C' also appears to have a swollen gut.

I decided to cross virgin *ssp4*^{EY05252}/ *CyO* $^{EVE\ lacZ}$ females with *ssp4*^{k07433}/ *CyO* $^{EVE\ lacZ}$ males, to observe the phenotype in *ssp4*^{EY05252}/ *ssp4*^{k07433} heterozygotes. I was only able to perform this on a small scale. However, I was able to see the see the same flat end to the front of the head (data not shown).

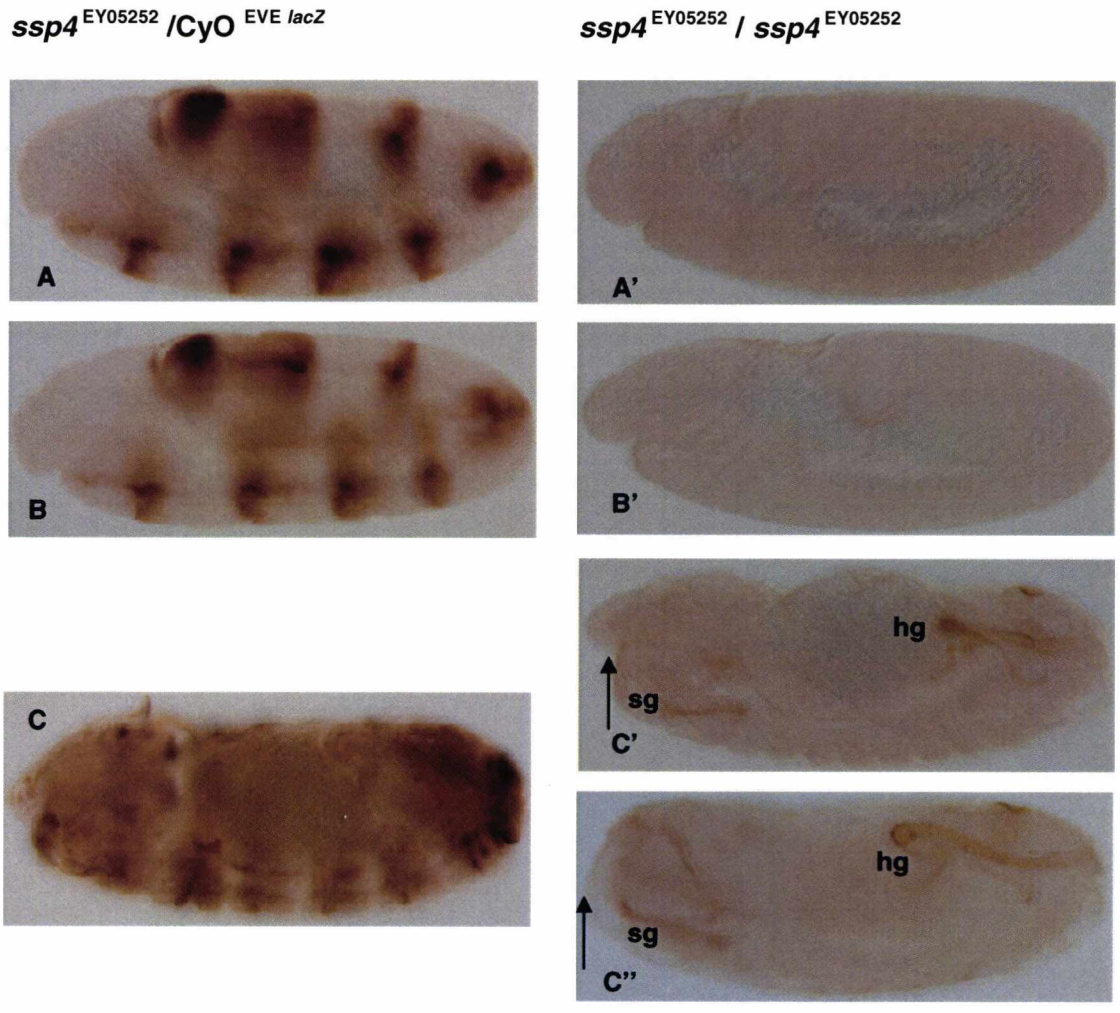


Figure 6.7: Embryonic Development of *ssp4*^{EY05252} Mutants.

Heterozygous and homozygous mutant ssp4^{EY05252} *whole mount embryos in lateral view (anterior to the left) at a magnification of 200X. Embryos in the left hand column are heterozygous for ssp4*^{EY05252}*. Staining is to Lac Z expressed in the pattern of Eve. Embryos in the right hand column are homozygous ssp4*^{EY05252} *mutants. A –stage 9, B – stage 11. The first sign of the mutant phenotype is in the stage 14 embryo C. C' has a flattened front end (arrow), and swollen gut. C" is so badly deformed (arrow) it is difficult to identify an exact developmental stage although the presence of the hindgut and salivary gland suggest it is late in embryogenesis.*

6.2.3 Labelling with an Antibody to Engrailed Shows a Head Involution Defect in Mutant Embryos.

A closer examination of the heads of mutant individuals was undertaken, using anti Engrailed. Engrailed is a transcription factor that is expressed as 14 stripes in the embryo where it divides the developing fly into segments. It is also expressed in late stage embryonic heads allowing the identification of morphological defects caused by an absence of apoptosis (Nassif *et al,* 1998). The anti engrailed antibody (4D9) labels NBs, GMCs and neurons. It labels commissural axons without the longitudinal tracts on either side (Flybase).

Heterozygotes in Figure 6.8 have been stained with engrailed as well as Lac Z antibody, to identify mutants and to compare patterns of engrailed expression. The blue staining of the Lac Z is clear in the stage 10 embryo but as development proceeds this marker shows as a blue wash rather than specific staining for example, individual cells are not stained in the CNS in the stage 16 embryo (Figure 6.8C) as they were in Figure 6.6 G. Staining patterns in the embryonic heads revealed differences between mutants and heterozygotes (B Rogers 1996). Figure 6.9 shows these differences in greater detail.

The head is made up of six segments, the most anterior are the ocular, antennal and intercalary and the posterior ones are the mandibular, maxillary and labial and the staining pattern of engrailed was analysed for each of these segments. The mandibular, maxillary and labial segment staining was identical in heterozygotes and mutants in stage 12 but by stage 16 the maxillary, mandibular and antennal segments were positioned more dorsally in the mutant (Figure 6.9 B and B'). The most dramatic difference at stage 16 was with the dorsal ridge, which was positioned upon the top of

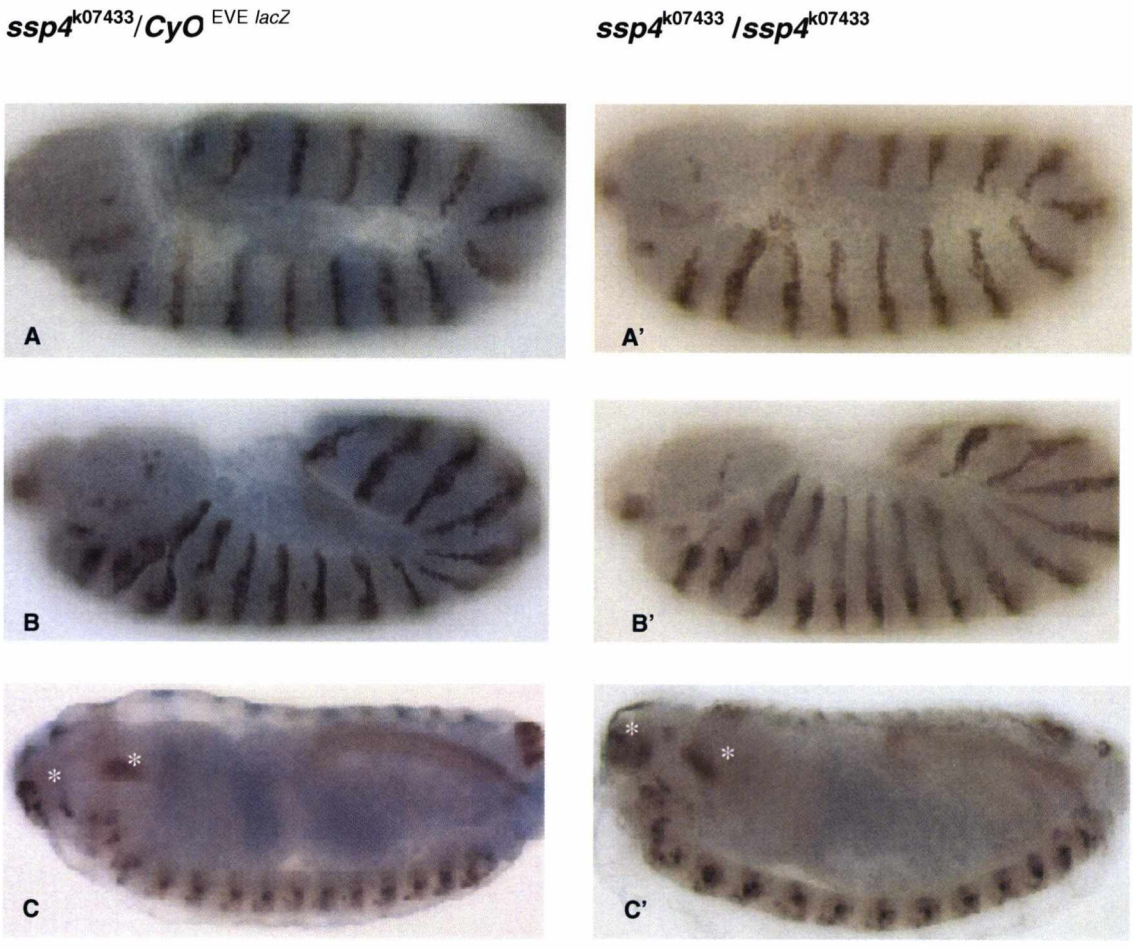


Figure 6.8: Comparison of *ssp4*^{k07433}Heterozygotes and *ssp4*^{k07433} Homozygotes using Engrailed

Engrailed expression reveals morphological differences between heterozygous and homozygous mutant individuals whole mount embryos in lateral view (anterior to the left) at a magnification of 200X. The Engrailed antibody is bound by a biotinylated secondary, which produces a stable brown dye. The Lac Z antibody is bound by an Alkaline Phosphatase to give a stable blue dye. The segmental staining is the same in both mutants and heterozygotes, A and A' are stage 8, B and B' are stage 10. There is a difference in the staining of the head at stage 16 (C and C') indicated by white stars.

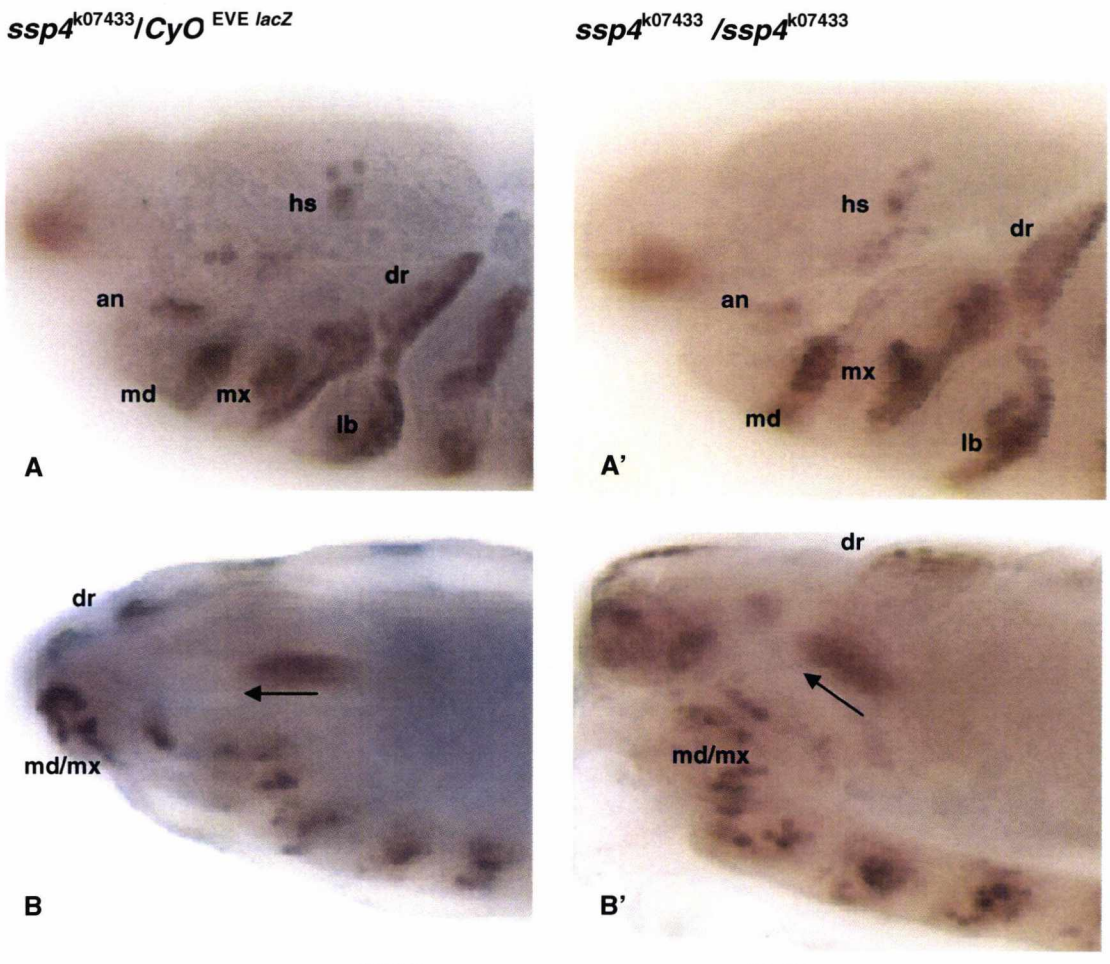


Figure 6.9: Detail of *ssp4*^{k07433} Heterozygote and *ssp4*^{k07433} Homozygote Heads.

Engrailed expression reveals morphological differences between heterozygous and homozygous individuals whole mount embryos in lateral view (anterior to the left) at a magnification of 400X. The Engrailed antibody is bound by a biotinylated secondary (brown dye,) the Lac Z antibody is bound by an Alkaline Phosphatase (blue dye). A and A' are stage 12 the head spot, antenna and intercalary areas have moved dorsally relative to stage 10 in both heterozygote and mutant (B and B'). By stage 16 (B and B') head involution has successfully taken place in the heterozygote but the dorsal ridge has been unable to move over the clypeolabrum in the mutant, the pharynx (arrow) tilts dorsally in the mutant. The mandibular and maxillary segments are positioned more ventrally in the heteroxygote, than in the mutant. Key an-antennal, md-mandibular, mx maxillary, lb-labial, dr dorsal ridge, hs– head spot.

the head in the mutant where it had not been able to move over the clypeolabrum to complete head involution (Figure 6.9 B'). This had led a change to the orientation of the pharynx, which ended up at a sharp angle in the mutant and not laying flat as in the heterozygote as well as forcing up the mandibular and maxillary segments, leading to the characteristic flat-ended appearance of the homozygous mutant. In Figure 6.10 the white arrowheads on the stage 16 embryos indicate other alterations to the shape of the head. The engrailed antibody stains just a few cells in the pharynx, and the pharynx position has been altered in the mutant.

6.3 THE POSSIBLE ROLE OF APOPTOSIS IN THE MUTANT PHENOTYPE

Engrailed staining shows that at least one of the defects in *ssp4* mutants was failure of head involution. This phenotype is seen in mutants that are defective in programmed cell death (M Grether *et al* 1995). The first gene identified in the PCD pathway in *Drosophila* was head involution defective (hid) after the phenotype when mutant. Because of the Ssp4 antibody staining to a specific subset of cells in the head (Figure 4.7 chapter 4), and the aberrant head involution seen in *ssp4*^{k07433} and *ssp4*^{EY05252} homozygous mutants (Figures 6.6 - 6.9 this chapter) I decided to examine apoptosis levels in the developing head.

Acridine orange is a green fluorescent marker that binds to and differentiates between DNA and RNA once it has diffused to the interior of a cell. This makes it useful for distinguishing between living and dead cells, and labels cells undergoing apoptosis with an impermanent stain in living tissue. It was used on embryos to reveal whether or not the *ssp4* mutant phenotype was the result of a lack of apoptosis in the head. It was not possible to stain mutants older than stage 14 as they were very rare and dying.

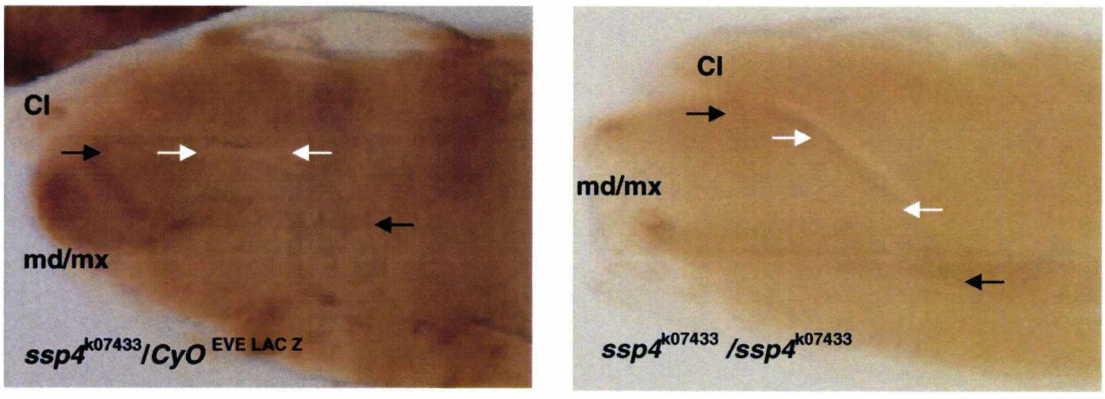


Figure 6.10: Study of Pharynx Position In *ssp4*^{k07433}Heterozygote and Mutant.

Heterozygous and homozygous mutant ssp4^{k07433} *whole mount stage 16 embryos in lateral view (anterior to the left) at a magnification of 400X. Staining is to Lac Z expressed in the pattern of Eve. The pharynx opening is positioned between the clypeolabrum (cl) and the mandibular/maxillary segments (md/mx) in both heterozygote and mutant. However, the position of all these structures is more dorsal in the mutant. The pharynx length is the same in both subjects but its angle is steeper in the mutant head and its end is more ventral (black arrowheads). The section that is labelled by engrailed is most probably between the white arrowheads.*

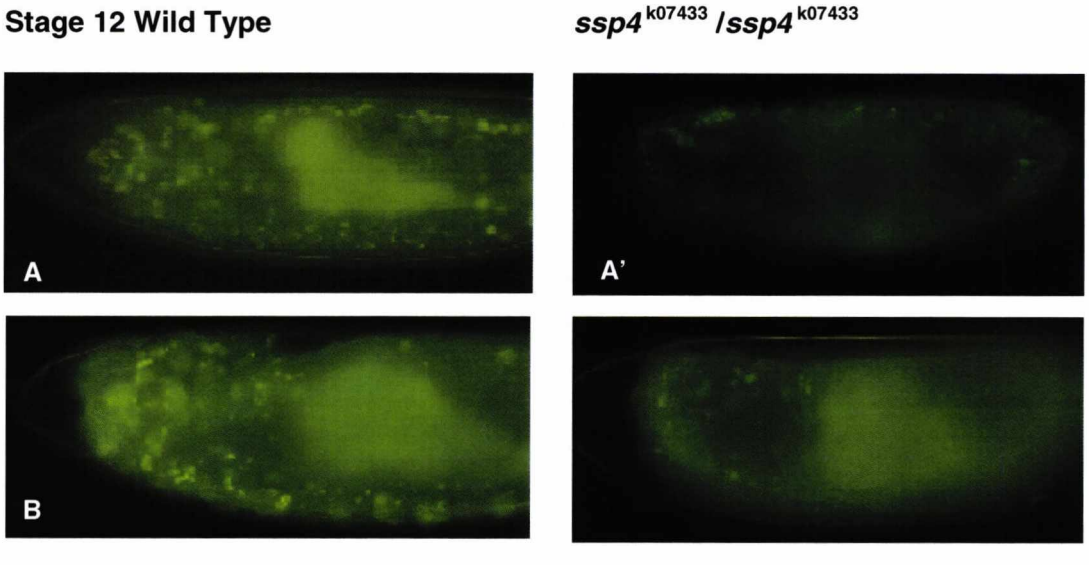


Figure 6.11: Staining of Wild Type and Mutant Embryos Using Acridine Orange

Live whole mount embryos stained with Acridine Orange to label apoptotic cells in lateral view (anterior to the left) at a magnification of 200X. Embryos A and B are Oregon R and A' and B' are ssp4^{k07433} *Homozygotes. Fewer cells appear to be undergoing apoptosis in the mutant embryos than the wild type.*

6.3.1 Acridine Orange Staining.

Figure 6.11 shows the results of acridine orange staining to Oregon R embryos and homozygous *ssp4* mutant embryos. Although it was only possible to compare one developmental point, due to scarcity of mutant embryos that were suitable for treatment after stage 14, these results suggest that the knockdown of *ssp4* expression has a negative effect upon rates of apoptosis. Acridine orange is a fluorochrome that binds DNA in all cells, making the nuclei appear green. In cells that are undergoing apoptosis the nuclei are bright green, due to condensed or fragmented chromatin, and it is these cells that are visible in Figure 6.11, A and B, in the wild type head and CNS. In the mutants there are very few bright green cells, which suggests that apoptosis has been reduced here.

6.4 DISCUSSION

The double labelling of embryos using the Lac Z antibody led to the identification of a defective head phenotype in stage 14 mutants. The monoclonal antibodies against different proteins specific to the nervous system revealed that there were no obvious defects in the nervous systems of homozygous mutants with the gross architecture and the patterns and numbers of individual groups of cells comparable to those in the heterozygotes. The most obvious mutational effect was that stage 14 embryos consistently had a flatter, squarer head end shape. An investigation using the engrailed antibody revealed that this is probably due to a head involution defect. Head involution is a complex process of cell rearrangement such as delamination, invagination, intercalation and breaking of contacts between cells. All these cell movements rely upon dynamic changes in the cytoskeleton.

6.4.1 An Overview of Head Involution

The larval head is the product of the gnathal segments, the procephalon, and area anterior to the cephalic furrow (Figure 6.12). Head formation depends upon head involution. This process combines segment internalisation with the formation of the internal head structures and skeleton, and the concomitant movement of epidermis over the head (Campos-Ortega and Hartenstein 1997).

The embryonic head is formed from 6 head segments. The mandibular, maxillary and labial metameres are the gnathal segments and are the precursors for the adult mouthparts. Together they form the gnathocephalon. The acron, antennal and intercalary segments make up the procephalon. The procephalon with the clypeolabrum make up the procephalic lobe. These segments move through the stomodeum, the mouth precursor, during head involution to form the atrium, pharynx

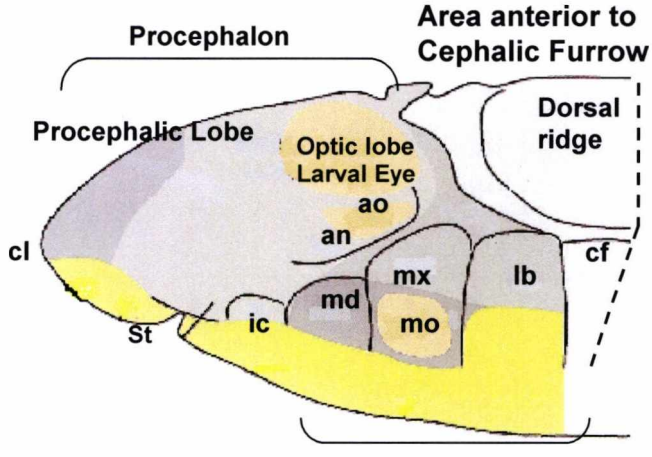
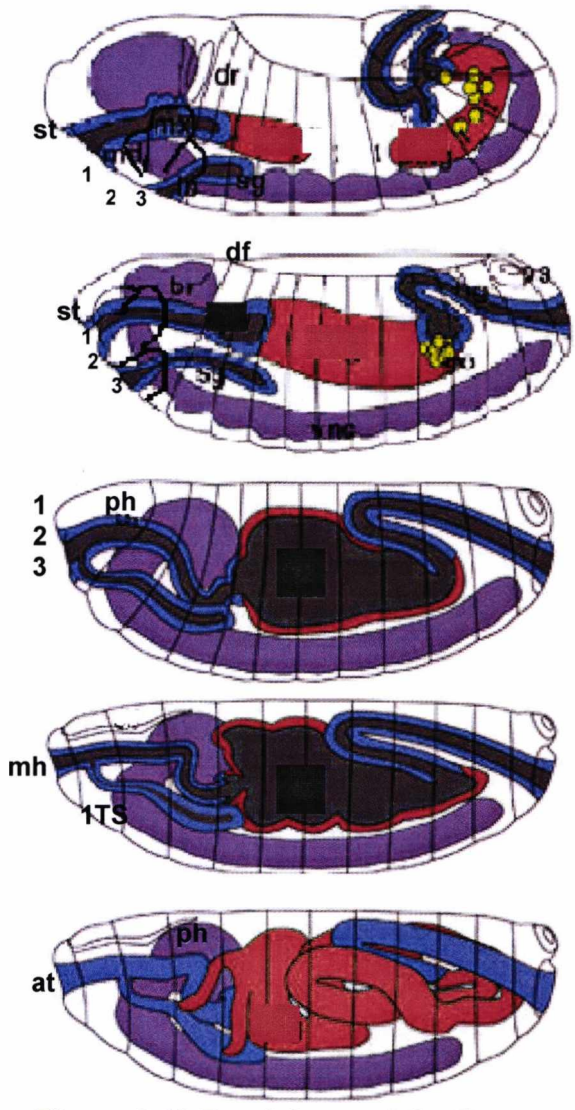


Figure 6.12 Schematic of Head Stage 10-11. *Taken from Nassif et al, 1998.*
Stomodeum (St), mandibular (md), maxillary (mx) maxillary organ(mo) and labial (lb) comprise the gnathal segments. Antennapedia (an) intercalary (ic)antennal organ(ao) and clypeolabrum (cl) make up the procephalon. The position of the cephalic furrow (cf) is marked with a broken line.

and foregut (Figure 6.13). The epidermis is drawn over the head by the dorsal fold, which is formed from the dorsal ridge, a group of cells on either side of the embryonic head (Figure 6.12). Once these segments are correctly placed within the embryo, they secrete the cephlopharyngeal skeleton, which provides the framework for attachment of muscles and foregut (Vann Hook and Letsou 2008).

Head involution begins at stage 13. Oral displacement of the gnathal structure takes place along with fusion of salivary gland pits on the ventral midline to make a common duct, and the dorsal ridge becomes visible over the labial bud (Figure 6.13).



Stage 12 – 1,2 & 3 show the mandibular (md), maxillary (mx) and labial (lb) segments, respectively; st – stomodeum, dr – dorsal ridge.

Stage 14 – Dorsal closure (the 'zipping up' of epidermis along the dorsal edge) is almost complete, lb – moves inwards, md,mx move towards the stomodeum, the dorsal fold (df) starts to move the epidermis over the head.

Stage 15 – Dorsal closure is completed, and head involution is well underway. Widening of the embryonic trunk leads to movement of epidermis over the head. md, mx & lb are internalised and the pharynx (ph) has now formed.

Stage 16 – Head involution is almost complete. Mouth hooks (mh) can be seen with the first thoracic segment (1TS) just behind them.

Stage 17
The fully developed embryo. The pharynx is in its final position, and the atrium (at) has now formed.

Figure 6.13 Breakdown of the Process of Head Involution

Schematic showing the stages of head involution, taken from 'The Drosophila Atlas of Development' J Campos-Ortega and V Hartenstein.
Key mandibular (md), maxillary (mx) and labial (lb) segments,dorsal ridge (dr), salivay gland (sg) anterior mid gut (amg)posterior midgut(pmg),brain (br,) proventriculus (pv),midgut (mg), hind gut (hg)posterior spiracles (ps) stomodeum (st) mouth hooks (mh) pharynx (ph) atrium (at) first thoracic segment (1TS)dorsal fold (df)

The dorsal ridge joins its partner across the embryonic midline to form a dorsal fold, and during stage 14 it starts to slide over the procephalon, producing the frontal sac, and halting at the end of involution, at stage 17. Simultaneously, the six head segments

move towards the stomodeum where they turn and move inside the embryo (Figure 6.13).

Dorsal closure can be used as a model to explain some aspects of head involution, where leading edge epithelial cells elongate, followed by elongation of lateral epithelial cells, and spreading of epithelium (Jacinto *et al* 2002). Cell junctions link the cytoskeleton to adhesion and signalling molecules, and mutations to elements necessary for adherens and septate cell junctions lead to faults in head involution. Mammalian CAMSAP 3 is known to interact with microtubules and adherens junctions through the N terminal domain (Meng *et al* 2008) and CAMSAP3 is the human CAMSAP that is most homologous to *ssp4* (EMBL EBI).

6.4.2 Role of Apoptosis in Successful Head Involution

Cell death is important for successful head development. In Figure 6.11 the highest numbers of cells undergoing apoptosis were in groups in the head region. The apoptosis patterns in the head differ from those in the trunk in that death occurs in prominent groups and these areas are significant for the morphogenetic movements necessary for head involution such as dorsal ridge formation, fusion and involution of the gnathocephalon, clypeolabrum retraction, organisation of the progenitor cells of the brain and involution itself (Nassif *et al* 1998). Blocking of apoptosis does not affect events that happen early in head development such as dorsal ridge and pharynx formation, but does disrupt head involution, which occurs later in development (Nassif *et al* 1998). The pharynx and the dorsal ridge both form as normal in the mutant, so the defective head involution could be the result of reduced apoptosis or the failure of other morphogenetic processes linked to diminishing maternal component.

6.4.3 Use of Acridine Orange to Study the Mutant Phenotype.

Ssp4 is believed to be involved in cell cycle regulation and has been shown to interact with microtubules. During apoptosis microtubule networks are extensively remodelled to form spikes that allow uptake of dying cells by macrophages, and to allocate cell contents into blebs and apoptotic bodies.

A comparison of wild type and mutant embryos showed that apoptosis levels of below normal in mutant embryos in the CNS and developing head (Figure 6.11). Staining cells with acridine orange is a straightforward process but care is needed with interpretation of the results, as there is no clear delineation between a normal nucleus and one that is in the early stages of apoptosis, making it difficult to quantify apoptotic cell numbers (Ribble *et al,* 2005). However, a comparison of the mutant against a wild type embryo shows an obvious difference. TUNEL labeling (which detects cells undergoing apoptosis by attaching a label to the end of fragemented DNA) could be used to confirm the results seen in Figure 6.11.

6.5 Conclusion

The *ssp4* mutation appears to specifically affect head involution in the embryo. Although the acridine orange system is not perfect for identifying apoptotic cells with complete accuracy, mutant embryos show significantly lower levels of apoptosis than the wild type and a lack of apoptosis would explain the failure of involution of the head segments and subsequent failure of the anterior movement of the dorsal ridge.

Head involution is also dependent upon cell movement. This requires cytoskeletal remodelling, cell polarisation, and alterations to the adhesive properties of cells, which may require Ssp4.

Chapter 7

DISCUSSION

7.1 INTRODUCTION

In this thesis I have undertaken the study of *Drosophila* CAMSAP, which is encoded by the gene *ssp4*. All previous knowledge of the CAMSAP proteins had been acquired through use of cell culture systems, and previous studies postulate a role for *ssp4* in cell protein localisation, regulation of cell division and the cell cycle, and neurite outgrowth. This study has looked at the role of a CAMSAP family member in a developing organism.

7.1.1 Bioinformatic Analysis of Ssp4

Using bio-informatics I have confirmed the data of Baines *et al.*, that Ssp4 is predicted to contain the same domains as its vertebrate counterparts and that it is most similar to human CAMSAP3. All CAMSAP family members contain one definite CH domain. I found that they also have a novel N terminal domain with unknown function but it may have a stabilising role, to facilitate interactions between the CAMSAP CH domain and other proteins, working in a similar way to the tandem CH domains that comprise the actin-binding domain in Spectrin (reviewed by Gimona *et al* 2002).

Since CAMSAP proteins interact with other cytoskeletal proteins, their activity may be subject to regulation by phosphorylation. A possible binding site for CaMK2 was identified in the human CAMSAPs 1 and 2 and in Ssp4. CaMK2 is involved with neurite outgrowth by way of interaction with activity regulated cytoskeleton associated protein (Arc) (Donai *et al.*,2003). Interestingly, CAMSAP1 has been shown to be essential for neurite outgrowth, and this role may well be regulated by phosphorylation.

The activity of the human CAMSAPs is regulated by a calmodulin binding domain, which is absent in *ssp4*. However the Glutamine rich domains in *Drosophila* may provide an alternative mechanism of protein activity regulation, as they do in

cytoskeletal organisation in yeast (Michelitsch and Weissman, 2000). SH3 domains are used to collect together proteins of modest affinity for a short -lived response to an extracellular signal. These domains are found in the human CAMSAPs and are conserved in Ssp4.

7.1.2 Embryonic Expression of Ssp4

Ssp4 mRNA and protein expression are ubiquitous throughout development although levels increase as development proceeds, with enhanced expression in the developing nervous system, gut and head (Chapter 4). Generally, the *in situ* and antibody staining were identical apart from a subgroup of cells in the head that were labelled with antibody, but did not appear as individually labelled cells by *in situ* hybridisation to *ssp4* mRNA. These cells were large and deeply stained. By their size and position within the embryo it is likely that they are cells, or aggregates of cells, that are being phagocytosed by haemocytes, a process required during embryogenesis particularly to allow morphogenetic movements (Nassif *et al.,* 1998). This would be consistent with Ssp4 protein accumulating and staining clearly yet mRNA not showing this pattern. To confirm this embryos would need to be double-labelled for Ssp4 and a haemocyte-specific marker. *ssp4* is also expressed in areas of mitotic activity, in the germ band and the cephalic furrow for example, with expression absent in areas where mitosis does not take place such as the amnioserosa. Expression was also high in cells that were undergoing morphogenetic movements (Chapter 4 Figure 4.1).

I have shown by western blot techniques that the anti-Ssp4 antibody binds to proteins of the predicted size of Ssp4 isoforms. This taken together with the fact that ssp4 mutant embryos show a much reduced level of staining indicates that the antibody is specific for Ssp4. There is staining seen in homozygous mutants up to stage 16 but this may have been due low level non-specific primary antibody binding, or because of the presence of Ssp4 protein (chapter 4, Figure 4.7). This presence might be due to a

maternal component of *ssp4* function. A maternal component comprises the chemical cues, including maternal RNAs, which are placed into the egg by the mother during embryogenesis. These are responsible for a variety of functions including establishment of anterior and posterior ends and dorsal ventral polarity in *Drosophila* oocytes. The transport and positioning of RNAs in the oocyte, during oogenesis, is mediated by microtubules (Pokrywka *et al.,*1995). A yeast two hybrid study has shown Ssp4 to interact with Matrimony (required for chromosome compaction into a karyosome prior to release onto the mitotic spindle) and NHK1 (which maintains oocyte architecture) (L Giot *et al.,* 2003). It is therefore likely that the *ssp4* mRNA is part of the maternal component provided by the heterozygous mother that is able to provide some Ssp4 protein to the developing embryo.

7.1.3 Knockdown Embryonic Expression of *ssp4*.

To analyse the role of *ssp4* during development I used two independent P-element mutant alleles. The insertions had been molecularly mapped to two different sites within the locus and both had been annotated as causing lethality. I confirmed this and also confirmed that they were lethal *in trans* indicating that both P-elements were indeed disrupting the *ssp4* gene. In addition, I excised one of the P-elements and showed that it was the cause of the lethality since I recovered viable revertants from precise, or near precise, excision events. From this excision I also recovered some new mutant alleles of *ssp4* but these are yet to be characterised. The evidence presented suggests that the two alleles are nulls or strong hypomorphs. This is because zygotic production of functioning *ssp4* is essential for successful head involution, and completion of embryogenesis. In addition, the phenotypes of both *ssp4* ^{k07433} and *ssp4* ^{EY05252} mutants and the transheterozygotes were all the same something that would not be expected if they reduced *ssp4* function by different amounts. The fact that Ssp4 protein is seen in the embryo at stage 10 may suggest that the P-element

allele is a hypomorph rather than a null, although this expression might be due to some residual maternal component.

Sometimes however, protein can be produced even with the presence of a P-element. For example, *arc* has a P-element inserted 11 bases downstream of the transcription initiation site for its three transcripts, which has not eradicated *arc,* but has reduced it to 15% of that in wild type individuals with a reduction of expression that was greater in isoform I and II than it was in III (Liu *et al.,* 2000). *ssp4* exists as 8 different isoforms and some of these might have been expressed preferentially. It could be that in some systems there is redundancy, which allows development to proceed in the absence of the required isoform. Alternative proteins or processes may rescue some absent or reduced Ssp4 isoforms for example the CNS can regulate its own programmed cell death despite deficiencies in the pro-apoptotic genes hid, grim and reaper, giving rise to viable adults (Page *et al.,* 2008).

In order to be certain that the *ssp4* ^{k07433} and *ssp4* ^{EY05252} alleles were null, their mutant phenotypes would need to be compared to the homozygous mutants from a line that was known to carry a null allele. Indeed, it was hoped that the screen would create a fly line carrying a carrying a damaged and completely non-functioning copy of ssp4 through an imprecise excision. An identical phenotype would confirm that the *ssp4* ^{k07433} and *ssp4* ^{EY05252} alleles were null. A more severe phenotype would indicate that *ssp4* ^{k07433} and *ssp4* ^{EY05252} were hypomorphic as a null allelic combination has a more severe phenotype than a hypomorph. Western blot and *in situ* analysis of late stage homozygous mutant embryos could be used for confirmation. The fact that the P-elements disrupt *ssp4* in different parts of the gene would make it more likely to produce mutant offspring with varying degrees of severity of phenotype, as is the case with *arc*, but not the case with *ssp4.*

Embryos homozygous for mutant ssp4 were monitored during their development (Chapter 5). *ssp4*^{k07433} was found to be embryonic lethal with death occurring at the end of embryogenesis. However, the fact that the effects of ssp4 knockdown are not seen until late in development does not mean that *ssp4* does not play a role in earlier developmental stages. The fact that mRNA and protein are expressed from the start of embryogenesis suggests that it is needed for many developmental processes. A possible maternal component may have kept these embryos alive until this point (Chapter 4, figure 4.6). However, this might also have been because the disrupted gene was hypomorphic.

7.1.4 The Mutant Phenotype of *ssp4.*

I have established the mutant phenotype as a defect in head involution. This occurred between stages 14-16 confirming the monitoring results in chapter 5. The phenotype may have been caused by a lack of apoptosis in the head region, and the data collected supported this view (chapter 6). A reduction of apoptosis would be lethal as it is necessary for creating space for the head segments to move into, and is essential for other aspects of embryonic development.

Head involution also requires a number of morphogenetic processes that take place throughout embryogenesis which are marked by Ssp4 expression, making it a likely candidate for involvement in other major morphogenetic events. It is also highly expressed in areas of cell division and it may be expressed in cells undergoing apoptosis, and so probably has a role in processes that are common to all these events. All of these events involve remodelling of the cytoskeleton in response to extracellular messages, co-ordinated with cellular re-organisation. Cells involved in morphogenesis, would also need to become polarised and alter their shape and adhesive properties to be able to move. ssp4 would be expected to play a role in these events through its association with other cytoskeletal proteins. Spectrin is needed for polarisation and for

positioning receptors in the membrane that would allow the cell to follow chemical cues, microtubules also regulate cell polarity and are needed for formation of cell protrusions and outgrowths.

7.1.5 Possible roles for *ssp4*

The data from the study did not support a role for Ssp4 in neurite outgrowth. This was surprising as knockdown of CAMSAP1 had led to neurite collapse, and also the human CAMSAPs 2 and 3 and *ssp4* contain possible substrate binding sites for CaM kinase II, which is involved in neurite extension. However this may be because the share of a maternally derived Ssp4 protein and/or mRNA varies according to tissue, depending upon whether cell division was exponential or at lower levels. The reason why the CNS was unaffected by Ssp4 knockdown may be because it derived directly from stem cells that had not undergone a cycle of division, and this was not the case for the structures of the head which was the site of the first embryonic mitotic divisions. Alternatively nervous system cells may also have a different requirement from Ssp4 for neurite outgrowth, than the cells in the head have for the morphogenetic processes of involution.

It may be that in some tissues Ssp4 has a more minor role, or that some tissues could manage with a truncated version of Ssp4 that has not CKK domain, and therefore, no microtubule binding capability. Alternatively, the very low levels of protein produced and seen in the antibody staining were sufficient to get embryos to the final stages of development (Chapter 4, figure 4.6). These issues could not be addressed as this study used an antibody and an *in situ* probe that detected all isoforms.

Beta spectrin plays an important part in establishment and maintenance of polarity in gut epithelial cells, and beta spectrin mutants die as embryos. In most embryos the midgut develops normally but in a few there is a mis-shapen gut phenotype that differs

in degrees of severity (data not shown). This could be due to the presence of very low levels of an isoform needed for gut development in $Ssp4^{k07433}$ homozygous mutants, which are too low for some individuals to complete midgut development. Alternatively the midgut invaginations take place during stage 7, following the start of gastrulation, and at this point there may well be some Ssp4 maternally derived protein available. This may have been sufficient to take midgut development to the end of embryogenesis, depending upon protein half-life, making differing levels of maternal component responsible for varying phenotype severity.

Ssp4 could be involved in alteration to cell shape or in cytoskeletal remodelling to permit cell division or movement, for example microtubules communicate instructions to change shape to the actin cytoskeleton which then makes cells wedge shaped to allow invagination. Alternatively Ssp4 has at least one calponin homology domain (or possibly a tandem domain), which may bind actin directly. The possible capacity for Ssp4 to form transient complexes with other proteins via SH3 domains may place Ssp4 in a pathway linked to cytoskeletal remodelling. Ssp4 may also remodel the spectrin network to facilitate changes to cell shape, and to polarise cells and alter their adhesive properties. CAMSAP3, the human protein most similar to ssp4 has been shown to interact with adherens domains in epithelial cells (Meng et al., 2008).

The presence of a possible maternal component may be masking the extent of the role of *ssp4* in a developing embryo. To get around this, systems such as RNAi or FLIP/FRT could be used to knockdown mRNA/protein production at specific developmental points and in the case of FLIP/FRT would allow a direct comparison of mutant and normal tissues (Theodosiou and Xu 1998). Pulse chase analysis and/or cyclohexamide blocking could be used to measure the turn over Ssp4 in cultured cells (Zhou 2004). It is also possible to 'rescue' individuals using temporally and spatially controlled systems of protein production, similar in principle to the system that labelled

the heterozygous embryos with Lac Z. A female germline clone, could be generated, however if the effects of Ssp4 knockdown caused very early embryonic lethality this may not reveal a great deal about its functioning.

Previous studies, using S2 cells and the yeast two-hybrid system, had suggested a role for Ssp4 in mitosis, cell polarisation, and cell cycle regulation. The results of such studies need to be treated with caution. S2 cells are a reliable cell line (Schneider, 1972). When they are used in RNAi studies off target effects are common due to the presence of tandem trinucleotide repeats, found in many genes and dsRNAs. Genes containing sections of tandem repeats are common in candidate lists in such screens (Ma et al., 2006), and Ssp4 does contain one extensive, and one short glutamine rich domain. The yeast two hybrid system is straightforward, quick and cheap, but for unknown reasons, gene activity has been reported where no protein/protein intearction has taken place (Vollert and Uetz, 2006).

7.2 CONCLUSION

The aim of this project was to carry out a functional analysis of Ssp4. At the end of this study, is the role of *Ssp4* now known? It is known that knockdown of *Ssp4* leads to aberrant head involution, and that it is linked to unsuccessful apoptosis. There may be other effects that are hidden because of the presence of maternal component. It is also possible that $ssp4^{KO7433}$ and $ssp4^{EY05252}$ are hypomorphic. The pattern of expression also gives an indication of its role, but the question of what that role is cannot yet be answered completely. In vertebrate cell culture CAMSAP was found to be necessary for neurite outgrowth but this role could not be confirmed or disproved in *Drosophila* despite the fact that the Ssp4 protein is comparable to CAMSAP1, not only because of the maternal component but because in a living organism there may be some redundancy. For example, blocking cell death in midline glial cells does not prevent

normal nervous system development as the embryo has repair mechanisms (Page *et al.*, 2008)

In conclusion the identification of the exact nature of the role of Ssp4 is complicated by the presence of a maternal component, which may rescue homozygous mutants and mask the complete effect of Ssp4 knockdown. However, this maternal component did allow the embryo to reach an advanced stage of development and to reveal the head involution phenotype. Expression of the pro apoptotic head involution defective gene is not seen until stage 11, and *ssp4* is expressed from the beginning of development and so is probably involved in developmental processes common to apoptosis such as microtubule re-organisation. *ssp4* is known to interact with spectrin and microtubules and these two proteins co-operate to establish and maintain cell polarity, and a role in polarity has also been suggested by other studies (L Giot *et al.*, 2003). Involution also requires cells to become less adhesive and move, and human CAMSAP3 has been shown to interact with the adherens junction. Cell division requires *ssp4* to interact with the mitotic spindle, and *ssp4* expression has been seen in areas of cell division, especially in the very early embryo. It may be that *ssp4* has several roles that are dependent upon developmental stage and tissue.

Chapter 8

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