Initial characterization and intracellular localization of two suppressors of position effect variegation in *Drosophila melanogaster*, *S2214* and *puckered*

by

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Abstract

Emergence of the higher eukaryotic organisms from their prokaryotic ancestors has been closely associated with an increase of the genetic material. This progression has been dependant on machineries that can package the DNA to various extents, from the levels seen in the 30 nm fibers of interphase nuclei to that of metaphase chromosomes. These evolutionary changes in genome organization have correlated with advancements in regulation of gene expression during development. In eukaryotes, cellular differentiation is partly dependent on the mechanisms that would silence the correct genes in a particular tissue and maintain this silenced state throughout subsequent stages of development. To understand the factors involved in such mechanisms many labs, including ours, have used position effect variegation (PEV) to identify proteins that form or remodel the chromatin fiber. Genetic screens have identified *S2214*, and *puckered* as genes coding for putative modifiers of PEV. The aim of this thesis, is to characterize *S2214*, and *puckered* by addressing two main questions: i) do the mutations in each of these genes modify the phenotype observed in PEV? And ii) do their products localize to the nucleus, and if so to the chromatin? Results show that *P* element mutations in these genes cause dominant and strong suppression of PEV in w^{m4} and Sb^{V}. Moreover, the observed *Su(var)* activity is reverted upon mobilization of the *P* elements. I developed and purified an antibody for each gene. Puc, the product of *puckered*, localized to the nucleus of S2 and KC1cells (which are late embryonic *Drosophila* cell lines), as well as the nuclei of salivary gland cells of *Drosophila melanogaster*, but could not be detected on the polytene chromosomes. In addition, S2214, the product of *S2214*, was found in the nuclear fraction of S2 cells, and could be observed within the nuclei of S2 and KC1 cells as well as those of the salivary glands of *Drosophila melanogaster.*

Furthermore, S2214 was found at several interbands of the polytene chromosomes of these salivary glands. It is our conclusion that gene products of both *S2214* and *puckered* are involved in mechanisms that affect chromatin structure.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

DSPc.....Dual Specificity Phosphatase catalytic domain

ERK.....Extracellular signal Related kinase

GOF.....Gain of Function

JNK.....Jun Kinase Pathway

LOF.....Loss of Function

MAPK.....Mitogen activated Protein kinase

ORF.....Open Reading Frame

PcG..... Polycomb Group

PEV..... Position Effect Variegation

Puc.....Gene product of *puckered*

SAPK.....Stress Activated Protein Kinase

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Chapter 1 Introduction

Evolution of multi-cellular organisms from their single cell predecessors has been accompanied by an enlargement of the genome size. This expansion of genetic material in most eukaryotic cells necessitates: 1) the partitioning of the genome into smaller units to facilitate basic cellular functions, such as DNA replication, chromosome segregation; and 2) the compaction of the DNA, to varying degrees, from the 30 nm fibers seen in interphase nuclei to the compact chromatin of metaphase chromosomes. These advancements in genome organization have correlated with more complex regulations of gene expression. All higher eukaryotes possess various differentiated tissues, each with its unique gene expression pattern and consequently a distinct protein profile which is compulsory for accomplishing its array of tasks. This tissue-specific gene expression is dependent on the mechanisms that activate the correct genes in a particular tissue and maintain this pattern through subsequent cell divisions (Grigliatti, 1991). Furthermore, the illicit activation of genes that should be silent in a particular tissue type may be deleterious. Hence, there must be a different and complementary set of mechanisms that either inactivate or maintain the silenced state of genes in any particular tissue. To understand the latter, many labs including ours, have used assay systems, such as position effect variegation, to address two main questions: how does a cell decide what are the appropriate genes to turn off? And how is this decision passed on from cell to cell?

Although the answers are certainly not completely understood, studies during the last two decades suggest that modifications to structural components of chromatin alter DNA packaging and consequently affect regulation and maintenance of gene expression (reviewed in Fedorova and Zink, 2008).

In this Chapter, I briefly review some of the relevant research on DNA packaging and chromatin modifications, followed by a review of the phenomenon of position effect variegation (PEV) which has been used as an assay system to identify chromatin components. The focus of

this thesis will be two modifiers of PEV, encoded by the genes *puckered* and *S2214* genes. The last portion of this chapter will summarize our current knowledge of these two genes.

1.1 Chromatin structure

In order to manage the enormous size of their genomes, eukaryotic cells package their DNA in the form of chromatin inside their nucleus. Chromatin is a dynamic assemblage of approximately equal amounts of DNA and protein (Hancock, 2004). About half of the proteins associated with chromatin are histones, a group of five highly conserved proteins that collaborate to form nucleosomes, which are the building blocks of all eukaryotic chromatin.

Nucleosomes are formed by wrapping approximately 146 bp of DNA, in a left handed coil, 1.75 times around a histone octamere that is made up of the four core histone proteins: H2A, H2B, H3 and H4. First a H3-H4 heterodimer is formed which is joined by another H3-H4 heterodimer forming a heterotetramer. The H2A-H2B heterodimer binds onto the H3-H4 tetramer due to interactions between H4 and H2B which include the formation of a hydrophobic cluster (Luger et al, 1997). As a result, the histone octamer is formed by a central H3-H4 tetramer sandwiched between two H2A-H2B dimers (Horn and Peterson, 2002; Luger et al., 1997; Turner, 2002). This model of nucleosome is highly conserved and has since been confirmed by crystallography studies of over 20 different nucleosome core particles, including those containing histone variants and histones from different species.

A more detailed look at these crystallographic analyses reveals that the core histones contain two functionally distinct regions: the central, histone-fold, domain and the amino-carboxy-terminal domains (Chakravarthy et al., 2005, Robinson and Rhodes, 2006). The histone fold domains are involved in protein-protein and protein-DNA interactions. These interactions are essential for the stability of nucleosomes. Due to the highly basic charge of all

four core histones, the histone octamer is only stable in the presence of DNA or very high salt concentrations. On the other hand, N terminal tails of the histone proteins do not seem to be involved in nucleosome assembly and stability. In experiments where the N terminal histone tails were removed by trypsin, nucleosomes remained stable. Nonetheless, genes encoding these four histone proteins are amongst the most conserved in eukaryotes (Malik and Henikoff, 2003). The conservation spans all the amino acids from N to C terminus suggesting essential function for the N terminal amino acids. It is thought that these amino acids when modified with certain chemical groups (such as methyl or phosphate groups) participate in secondary contacts with DNA, linker histones, and other chromatin proteins (Carruthers and Hansen, 2000; Hansen, 2002).

In each mammalian cell, about 2 meters of DNA is packaged into the nucleus. Nucleosomes alone are insufficient for such levels of compaction of DNA. Further condensation of chromatin is necessary; however, the mechanism by which this occurs is poorly understood. Current consensus opinion is that nucleosomes linked by a stretch of DNA result in the 10nm fiber. A chain of nucleosomes can be arranged to form the 30 nm fiber, the formation of which is dependent on the interaction of H1 linker histones with both DNA and N- terminal tails of core histones, as well as linker histones of adjacent nucleosomes to produce a more compact chromatin structure (Belmont and Bruce, 1994; Tumbar et al, 1999). In support of this hypothesis, experiments using nucleosomal arrays showed that presence of a linker protein stabilizes an intrinsic tail-mediated condensation (Carruthers and Hansen, 2000).

Beyond the 30-60 nm fiber, the structure of chromatin is even less clear. *In vivo* light microscopy and transmission electron microscopy has given rise to a folded chromonema model where the 30 nm fiber is arranged into loops along a central protein scaffold to form a transcriptionally active 100 nm chromonema fiber. The 100 nm fiber folds to give rise to 200-

300 nm fiber in prophase, which in turn coils to form a metaphase chromosome (Robinson et al., 2006). This model has been supported by other *in vivo* studies, which followed de-condensation of metaphase chromosomes and showed presence of 100 nm wide fibers that occasionally (for short regions of DNA) became 60-80 nm wide, and, still, shorter regions of 30 nm wide of DNA, however no 10 nm fiber was observed (Carruthers and Hansen, 2000).

Regardless of the mechanism of condensation, higher levels of chromatin structure may have both organizational and regulatory benefits. Consequently, gene expression can be regulated based on the presence or absence of complexes that could unravel the chromatin fibers in short gaps (Narlikar et al., 2002). There are two main classes of such chromatin remodeling complexes: 1) those that rely on ATP or NADH (such as SWI/SNF) to provide the energy required for the unraveling of chromatin fibers, and 2) those that modify the core histones (such as histone methyl transferases) or the DNA, causing the nucleosome to dissociate from each other or from the DNA, resulting in a more open chromatin state. These two classes of complexes may not be mutually exclusive but interact in collaboration.

To appreciate the biological significance of the regulatory effects of DNA packaging, one can compare regions of varying chromatin compaction, euchromatin and heterochromatin.

1.2 Different states of chromatin

In 1929, Heitz (as referred to by Horn and Peterson, 2002) described heterochromatin as the portion of the nuclear chromatin which maintains a darkly stained state, with no distinct bands, throughout the cell cycle. The remainder of the nuclear chromatin which he termed euchromatin showed distinct stained bands in interphase chromosomes. These cytological observations have since been correlated with biochemical analysis to suggest that heterochromatin has a higher level of spatial compaction compared to euchromatin. For

instance, nuclease sensitivity experiments revealed that euchromatic DNA is significantly more prone to digestion by nucleases, suggesting euchromatin is more accessible to these enzymes and less protected by chromatin proteins (Grewal and Elgin, 2002; Sun et al., 2001). Subsequent experiments have shown that the nucleosomes in heterochromatin are more regularly spaced and each of the core octameres are associated with a higher portion of DNA than those of the euchromatic nucleosomes.

A number of other structural differences between heterochromatin and euchromatin have been identified that may also be related to, or be a consequence of, the dissimilarity in compaction states (Grewal and Jia, 2007; Talbert and Henikoff, 2006; Richard and Elgin, 2002). For example, heterochromatin contains an abundance of repetitive DNA sequences, most likely remnants of viruses and transposons. However, more than 50 genes have been identified in the pericentric heterochromatin of *Drosophila melanogaster*, (Weiler and Wakimoto, 1995; Eberl et al., 1993). Furthermore, heterochromatin displays low levels of recombination and replicates late in S phase (Richards and Elgin, 2002).

How does a region become heterochromatic? Many have hypothesized that the answer lies within the basic components of chromatin, nucleosomes. Core histones can be modified on their N terminal ends by acetylation, methylation, phosphorylation, as well as other modifications (Kimura et al., 2005; Martin and Zhang, 2005; Fuchs et al., 2006). The importance of these modifications was first realized by Allfrey (1964) who noted a correlation between acetylation of histones and transcriptional activity. Many other correlations between the state of transcription and histone modification have been identified over the years (Grewal and Jia, 2007; Ebert et al., 2006). For example, *Drosophila melanogaster*'s pericentric heterochromatin strongly correlates with mono-, di- and trimethylation of lysine 9 of H3 (H3K9), mono-, di- and trimethylation of lysine 27 of H3 (H3K27) and trimethylation of lysine

20 of H4 (H4K20) (Schotta et al. 2002; Ebert et al. 2004), implying that these post-translational modifications may mark silent regions. In contrast, interbands are enriched with several marks such as methylation of lysine 4 and lysine 36 of H3 (H3K4 and H3K36 respectively) and phosphorylation of serine 10 of H3 (H3S10), which associate with an active chromatin state (Jenuwein and Allis, 2001). Accordingly, it has been postulated that the pattern of modifications, on one or more tails, act sequentially or in combination to form a "histone code" that is interpreted by the cellular machinery to elucidate distinct downstream events (Strahl and Allis, 2000).

Considering all the residues that could be modified on a single nucleosome and the possibility of nucleosome-nucleosome interactions that are also affected by the N-terminal tail modifications, it will be very difficult to unravel the histone code, if such a code exists (Turner 2002). Nonetheless, these interactions are essential to nucleosome assembly and maintenance. It follows that if a mutation renders one of these proteins inactive, the gene expression (that is maintained by the proper DNA packaging) will be altered (Grigliatti, 1991; Eissenberg,1989). In view of this, a number of researchers have used genetic or functional screens in an attempt to identify proteins that affect or comprise the chromatin fiber. One such assay is PEV.

1.3 Position effect variegation

Muller (1930) reported the first example of the phenomenon of PEV, which results in differential gene expression amongst the cells of the same tissue, in *Drosophila* that had been exposed to X-rays. Since its discovery, PEV has been characterized in several species; however, since it has been studied most extensively in *Drosophila* (reviewed by Henikoff, 2006; Grigliatti 1991), this brief review will focus on experiments carried out on *Drosophila melanogaster.*

PEV occurs when a euchromatic region is moved next to a disrupted region of heterochromatin. The repositioned euchromatic gene(s), immediately adjacent to this breakpoint show a mosaic phenotype with respect to their transcriptional status. Perhaps the most striking of these phenotypes can be observed when the white gene is placed juxtaposed to a broken piece of heterochromatin. Flies carrying such mutations show a mottled phenotype where the white gene is expressed in some of the clonal facets of the eye, and silenced in others, resulting in patches of red pigmented facets on a white background (Muller, 1930).

The reason for the variegated phenotype has been debated since the discovery of PEV in 1930s. A number of independent experiments have shown that the variegation observed in PEV is not due to mutation (Judd 1955, Hinton and Goodsmith, 1950). Repositioning of the euchromatic genes by recombination or further chromosomal rearrangement re-establishes the "normal" transcription status. It is therefore, believed that PEV occurs because of the association of the heterochromatic breakpoint with the new euchromatic neighbor. It follows that the distance between the breakpoint and the euchromatic gene should inversely correlate with the likelihood of the mosaic phenotype. Support for this assertion was provided by studies involving rearrangements in which more than one gene were silenced. The genes closest to the breakpoint were inactivated more frequently than those farther away. Some have suggested that this implies the inactivation is "spreading" from a region at or near the breakpoint towards the euchromatin (Cohen, 1962; reviewed by Talbert and Henikoff, 2006). In addition, cytological analyses of polytene chromosomes from variegating strains have revealed that the euchromatic region juxtaposed to the heterochromatin becomes darkly stained and un-banded (similar to regions of β-heterochromatin) (Hartmann-Goldstein, 1967; Schultz, 1936). Although this "spreading" model has been supported by various experiments, alternative models have been proposed.

It has been suggested that the spreading model fails to adequately explain some features of PEV (Talbert and Henikoff, 2006; Grewal and Jia, 2007). In some cases, variegation affects the genes located several mega-bases away from the junction. It is difficult to explain such long distance effects only by linear spreading of heterochromatin. Moreover, it has been noted that the severity of the variegated phenotype can be modified by the position of the break point along the chromosome arm, as well as by its proximity to other heterochromatic regions (Dimitri and Pisano, 1989; Spofford, 1976). These observations point to a role for trans-interactions between different heterochromatic regions in PEV. Talbert and Henikoff (2006) proposed four additional models that could explain how silencing over long distances may occur. In these models, looping or twisting of the DNA as well as sliding or hopping of histone modifying enzyme are responsible for silencing genes that may not be the closest to the breakpoint. Yet another alternative theory, asserts that it is the spatial positioning within the nucleus that determines the fate of the variegating gene. Heterochromatic regions of chromosomes such as centromeres and telomeres occupy the nuclear periphery regions while euchromatin is positioned in the middle (Sedoni et al., 1999). Thus, one can argue that variegating genes will occupy the boundary between the two regions. If they are located in a euchromatic compartment, which contains the factors necessary for gene expression, they will be active; however, if due to the influence of their heterochromatic neighbours, they are positioned in the periphery, they will lack some of the necessary transcription factors and will remain silent. In support of this model, it has been shown that the location of genes in nuclei of B and T lymphocytes has a profound effect on their expression (Brown et al., 1999). However, localization to the nuclear periphery may be the consequence of silencing and not the cause.

Although the mechanism by which PEV occurs may not be completely understood, various groups have shown that factors important for assembly of higher order chromatin, such as that found in heterochromatin, are also important in severity of the associated gene silencing observed in PEV. Hence it would be logical to question whether factors important to heterochromatization during development also affect PEV.

Examinations of mosaic phenotype observed in PEV reveals that the decision regarding the transcriptional fate is made early in development, since the phenotype is manifested in several adjacent cells (and often follows cell lineage specific boundaries) and not singular clones (Spofford, 1976). It can also be concluded that this decision is passed on and remembered with high fidelity (Janning, 1970). Other parallels between PEV and formation of heterochromatin during development have been made. Studies of temperature sensitivity of variegations have revealed two developmentally sensitive periods: one during embryogenesis, and a second during pupation. The former was the most sensitive suggesting that early embryogenesis is the time for the initial decision of PEV (Spofford, 1976). Intriguingly, around the time of blastoderm formation, the embryonic genome is activated, shortly after the chromosomes undergo morphological changes and distinct darkly staining regions appear (Lawrence, 1992). Since PEV is closely dependent on heterochromatin, it is likely that formation of heterochromatin at blastoderm is the early determinative event. It follows that factors which are important for integrity of heterochromatin would modify PEV, and vice versa.

1.4 Factors that modify PEV

Since its discovery in the 1930s, many factors that modify PEV have been identified. These can be categorized in two general groups: i) environmental factors that affect developmental rate, and ii) genetic predisposition.

Factors such as temperature, population density, and various chemicals modify PEV because of their effects on the developmental rate (Michailidis, 1988; Schultz, 1956, Gowen and Gay, 1934). It has been proposed that one or more macromolecule complexes are required to direct the silencing observed in PEV. Delaying the development provides more time for these complexes to form which in return results in a higher incidence of silencing for a particular locus (Zukerkandl, 1974). Insects, like any other cold blooded organisms, are dependent on external temperature for body heat, which is the catalyst for many cellular reactions. A drop in the temperature will reduce the developmental rate and enhances PEV, while an increase in temperature increases the rate of development and suppresses PEV (Gowen and Gay, 1934). Similarly, an increase in population density results in competition for nutrients. As a result, the amount of nutrient available to an individual fly is reduced, leading to lower energy consumption and, thus, longer developmental time which enhances PEV (Hinton, 1949). Finally, presence of certain chemicals (such as DNA synthesis inhibitors) in the media prolongs development and enhances PEV (Schultz, 1956).

The amount of other heterochromatin could also influence the frequency of PEV. For example, it has been noted that the presence of an extra Y chromosome (which is almost entirely heterochromatic) decreases the incidence of PEV (it is a PEV suppressor), while its absence (in XO males) enhances PEV (Dimitri and Pisano, 1989). Experiments involving duplications or deletions of autosomal heterochromatic regions showed similar results, supporting the

hypothesis that the observed effects are related to the differences in the heterochromatic content (Spofford, 1976).

Chemical agents or genetic mutations affecting the components of chromatin architecture, also affect the extent of silencing caused by PEV. Deficiencies of the histone gene cluster, which encode the building blocks of nucleosomes, cause strong suppression of PEV in w^{m4}. Moreover, flies reared on Na-butyrate, despite having prolonged development, show strong suppression of PEV (Mottus et al. 1980). Recent studies have shown that butyrate is an inhibitor of certain classes of histone deacetylases and therefore affects chromatin structure (Barlow et al., 2001). Several other mutations that modify histones affect the extent of variegation (reviewed in Ebert et al., 2006).

Many of those studying modifiers of PEV have focused on strong dominant mutations, with the underlying assumption that these may be more important since they cause more significant consequences (Grigliatti, 1991). Two such modifiers, *puckered* and *S2214* are the focus of this thesis. Mutations in either of these genes appear to suppress the gene silencing associated with PEV. In the following sections, I will summarize experiments that have contributed to our current knowledge of these genes.

1.5 Introducing *puckered*

Mitogen activated protein kinase (MAPK) signalling pathways are essential for proper differentiation during development. Three distinct classes of these pathways have been identified: 1) p42-p44 extracellular signal related kinases (ERKs), 2) p38 activated protein kinases, and 3) p46-p54 Jun N-terminal kinases (JNKs), also known as stress activated protein kinases (SAPK). As implied by their names, these subfamilies transduce signals in response to different stimuli. The ERKs are generally activated in a Ras-dependent manner in response to

growth factors and hormones, while p38 MAPK and p46-p54 JNK are activated in response to environmental stimuli through Rac1 and Cdc 42 small G proteins (reviewed in Canman and Kastan, 1996). In all of these pathways, the level of active MAPKs is controlled by the balance between MAPK kinases (MAPKKs) and MAPK phosphatases (Mkp). Even slight changes in levels of active MAPKs can have catastrophic consequences during development, causing MAPK phosphorylation and de-phosphorylation to be transient affairs, even in the continuing presence of the stimulus (Canman and Kastan, 1996).

Martin-Blanco et al. (1998) identified Puc, *puckered's* product, as a phosphatase that regulates JNK pathway activity, through a feedback loop. Subsequently, Puc has been shown to affect various cellular processes, such as epithelial differentiation, oogenesis, and apoptosis. In this section, I will briefly discuss our current knowledge of *puckered*, and review the known functions of its product.

1.5.1 Gene structure

puckered maps to 84E12-13 on the right arm of chromosome 3 of *Drosophila melanogaster*. Its ORF of 16967 bp gives rise to a single 2923 bp transcript that encodes a 476 amino acid protein with a predicted molecular weight of 51.3 KD (Martin-Blanco et al., 1998; Tweedie et al., 2009). Martin-Blanco et al. (1998) demonstrated that Puc is a phosphatase capable of dephosphorylating *p*-nitrophenyl phosphate, a substrate structurally related to phosphotyrosine. Further amino acid sequence analysis of Puc has revealed a dual specificity phosphatase catalytic (DSPc) domain between amino acids 133 and 269 (Figure 1-1) (Guan et al., 1991; Martin-Blanco et al., 1998; Letunic et al., 2009).

Over 1500 DSP proteins have been identified in various species ranging from bacteria to higher eukaryotes (Letunic et al., 2009; Juncker et al., 2009). In flies nine such proteins have

been identified through sequence analysis, however, only five have been experimentally characterized (Letunic et al., 2009; Tweedie et al., 2009). All nine proteins have a single, recognized, functional domain, which is their DSPc domain. They all show higher than 35% amino acid identity and higher than 45% amino acid similarity to the 137 amino acid DSPc domain present in *puckered* (Altschul, 1997). This high level of conservation among the DSPc proteins present in a single genome is also often observed in other organisms such as humans, mice, rats, chickens, *C.elegans*, and *Saccharomyces cerevisiae*. Moreover, orthologues of *puckered* also show high levels of conservation between their respective DSPc domains, suggesting an evolutionary important role for these proteins. For example, *puckered* shows 50% amino acid identity and 72% amino acid similarity with human dual specificity protein 10 (DUSP10) that has been shown to be a MAPK5 phosphatase and preferentially acts on JNKs (Altschul, 1997; Theodosiou et al. 1999). Interestingly, three of the five characterized DSPs in *Drosophila melanogaster*, *Mkp3*, *Mkp4*, and *slingshot* (*ssh*), encode proteins that function as members of the p38 MAPK pathway (Kim et al., 2002; Rogers et al., 2005; Sun et al., 2008). The product of the fourth gene, *Mkp1*, functions as both a member of p38 MAPK, as well as a H3S10 phosphatase. The fifth, *puckered,* encodes a phosphatase with a role in the JNK pathway (Martin-Blanco et al., 1998).

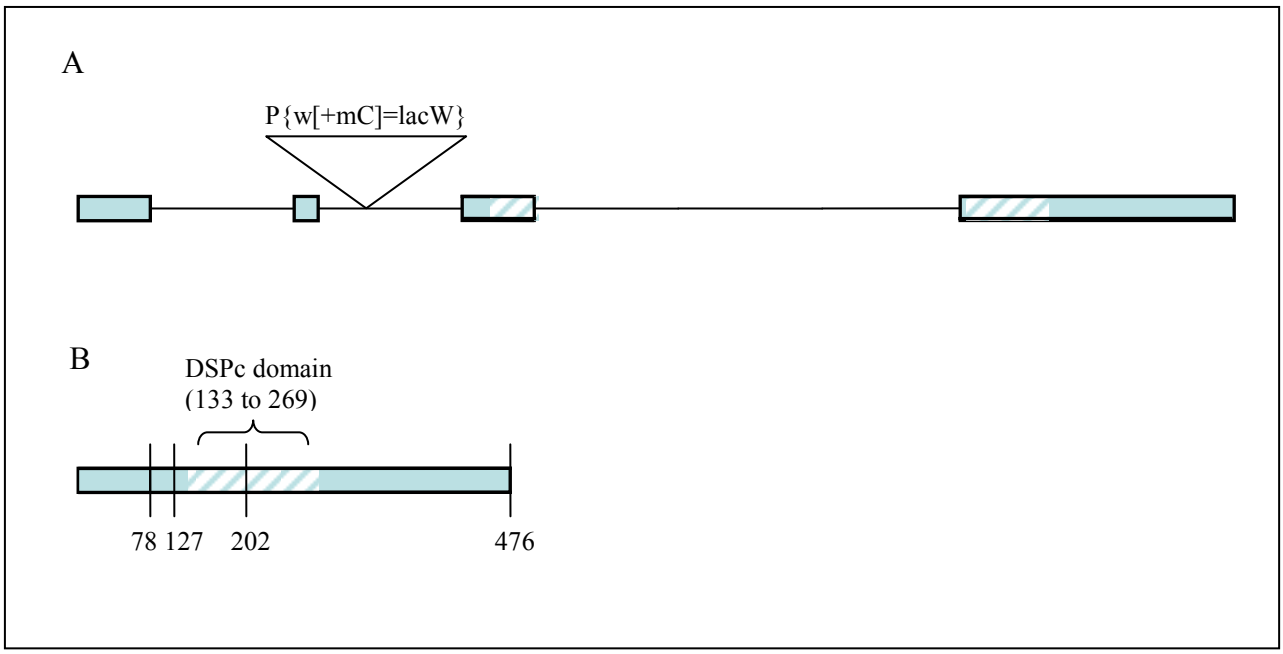


Figure 1-1 Known domains found in *puckered*.

puckered is composed of 4 exons (A), which are all transcribed in the most common 2.9 kb transcript. Minor levels of another 2.1kb transcript have also been detected in some experiments. Exons three and four combine to encode the dual specificity phosphatase catalytic domain (DSPc), shown as shaded area. This domain is the only recognized functional domain (the DSPc), and it spans from amino acids 133 to 269 of the Puc protein (B). The position of the last amino acid of each exon has been marked by a vertical line on the schematic of the protein (B). A *P* element insertion in the second intron produces a strong hypomorph, used in our analysis (A).

1.5.2 Known functions

Sequence analysis of Puc has revealed the presence of motifs with high similarity to those found in the VH1 class of phosphatases, many of which dephosphorylate MAPKs. Further in silico analysis of *puckered* outlined a DSP domain capable of removing both threonine and tyrosine bound phosphates.

In vivo studies showed that loss of function mutations of *puckered* lead to cytoskeletal defects that result in an aberrant dorsal closure, a morphogenetic event that unites the two lateral epidermal sheets (Ring and Martinez-Arias, 1993). This phenotype is both similar and genetically related to that observed in mutations of several members of the JNK pathway in *Drosophila melanogaster*, such as *hemipterous* (*Drosophila* JNK kinase), *basket (Drosophila JNK)*, *Djun, and Dfos*. Subsequent studies demonstrated that *puckered* loss of function (LOF) mutations result in the hyper-activation of DJNK, while overexpression of *puckered* mimics *basket* mutant phenotypes (Ring and Martinez-Arias, 1993). Moreover, Martin-Blanco et al (1998) discovered that *puckered* expression is itself a consequence of the activity of the JNK pathway. Collectively the evidence suggest that *puckered* provides a negative feedback loop that helps regulate the level of activity of JNK pathway and its morphogens during dorsal closure. Since all functions of Puc identified to date, and summarized below, relate to its role in the JNK pathway, a schematic of the relevant portion of this pathway is shown in figure 1-2.

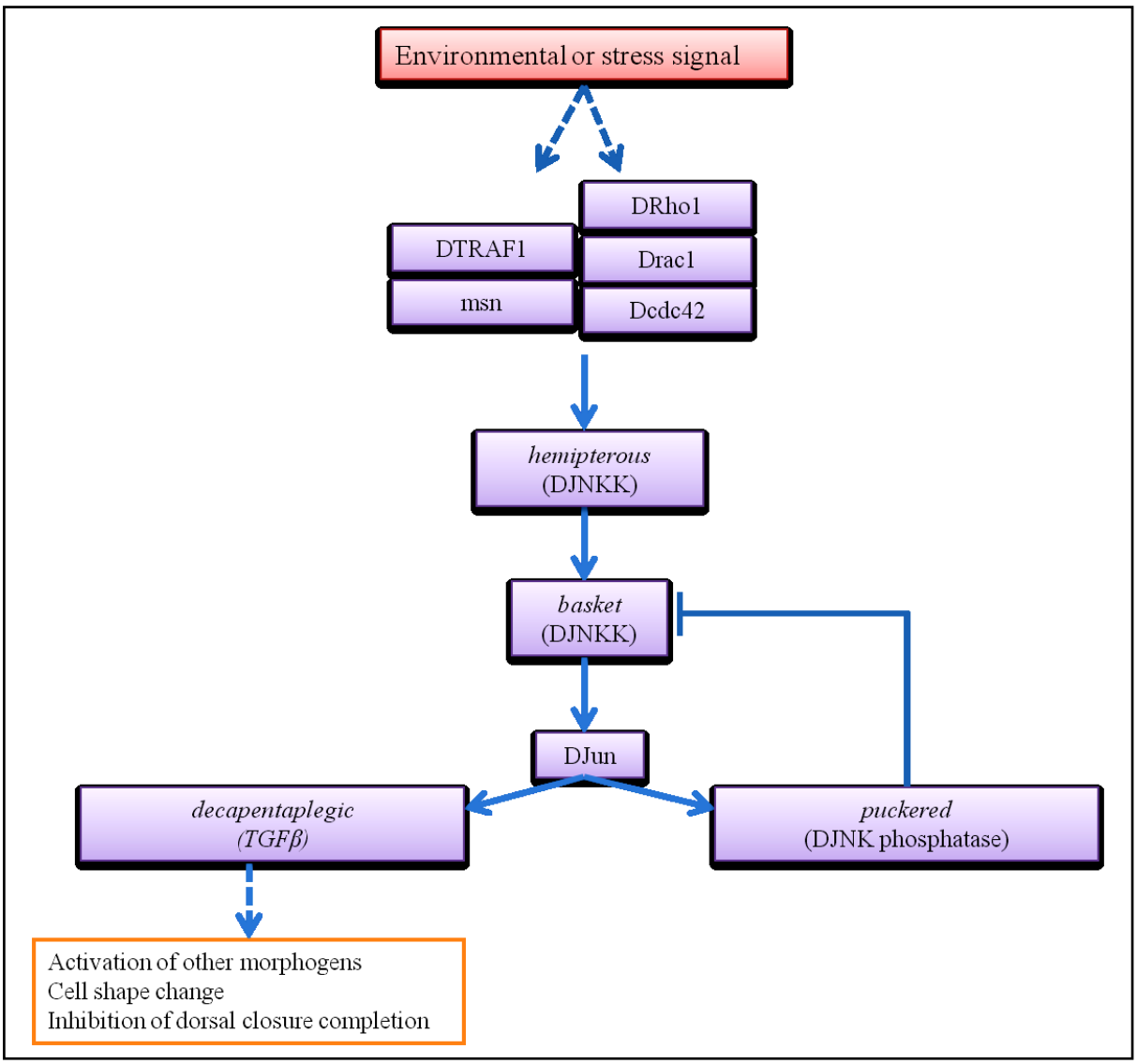


Figure 1-2 a schematic of the core portion of JNK pathway of *Drosophila melanogaster.* During embryogenesis, in the cells at the leading edge of the epidermis, the *hemipterous/basket* pathway becomes activated through products of other genes affected by environmental cues (for example Drac1 and Dcdc42). As a consequence, DJun is activated and gets involved in both the maintenance of *dpp* and *puckered* expression. Puc will drive its own down regulation through inactivation of *bsk,* and it will control the level of expression of dorsal closure effectors such as *dpp.*

Functions of JNK signalling pathway are not limited to embryonic dorsal closure. It has a role in the migrating features of leading edge cells seen in similar processes of disc thoracic closure, and experimental wound healing. JNK pathway mutants such as *hemipterous*, *basket*, *Djun*, and *Dfos* (that demonstrate dorsal open embryos) also show severe defects in disc morphogenesis and thorax formation as well as incomplete wound healing (Glise et al., 1995; Zeitlinger and Bohmann, 1999; Zeitlinger et al., 1997). In support of this, Lee et al. (2005) showed that downregulation of Polycomb-group (PcG) function, which is observed in transdetermined cells, is directly controlled by the JNK signalling pathway, which is activated in cells undergoing regeneration. Accordingly, transdetermination rates are lower in various JNK mutant backgrounds. Moreover, during dorsal and thoracic closures, as well as larval and adult wound healing, the JNK signalling pathway (including *puckered* expression) is selectively activated at the leading edge cells (Martin-Blanco et al., 1998; Agnes et al., 1999; Jacinto et al., 2000, Jacinto et al., 2002; Ramet et al., 2002; Galko and Krasnow, 2004). Taken together, the results suggest that *puckered* expression might also be necessary for proper wound healing of imaginal discs. In support of this, it has been demonstrated that JNK LOF mutants, such as *hemipterous* and *basket*, as well as overexpression of *puckered*, a negative regulator of the pathway, inhibit wound healing and regeneration (Bosch et al., 2005).

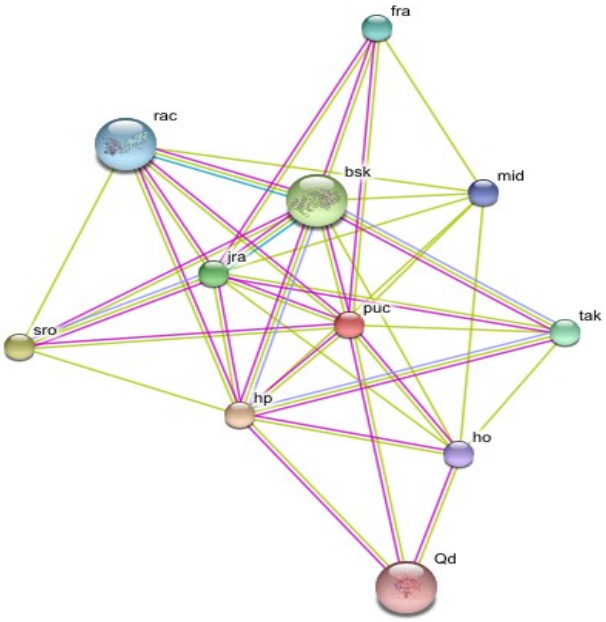
In addition to regulating morphogenesis, JNK signaling is also an important stress response pathway. In this context, JNK activation can initiate apoptosis in response to a variety of affecter molecules such as TNF, TGF, and Myc (Adachi-Yamada et al., 1999; Igaki et al., 2002; Moreno et al., 2002; Adachi-Yamada and O'Connor, 2002; de la Cova et al., 2004). McEwan and Peifer (2005) showed that Puc is both necessary (continuously) and sufficient to antagonize JNK-dependent apoptosis in a cell-autonomous manner. They interpreted their findings to suggest that the basal levels of JNK signaling is just below a lethal threshold in

epithelial cells, and this basal level is maintained due to the inhibitory function of Puc. They also demonstrated that JNK signaling is important in promoting apoptosis in response to both the p53-dependent response to DNA damage and developmentally regulated apoptosis. Others have shown that MST1-mediated activation of JNK is both essential and sufficient for chromatin condensation that leads to apoptosis (Ura et al., 2007). As a result, it has been postulated that JNK signalling can promote apoptosis or proliferation in different cellular contexts, and Puc plays an important role in controlling this balance.

JNK signalling affects other cell cycle related processes, of which several may be entwined to its role during the stress response. Dobens et al. (2001) reported a role for Puc in follicle cell morphogenesis during oogenesis. They showed that *puckered* mRNA accumulated in the centripetally migrating follicle cells and cells of elongating appendages. They also demonstrated that increased or decreased Puc activity lead to either incomplete nurse cell dumping or production of aberrant dorsal appendages, resulting in a cup shaped egg chamber due to a lack of coordination between nurse cell dumping and dorsal appendage elongation. In *Drosophila* follicle cells, JNK kinases and Puc collaborate to regulate the transition from mitotic to endoreplication cell cycles (Chen et al., 2007). Mutations in *basket* and *hemipterous* result in the initiation of premature endocycles, while mutations in *puckered* lead to loss of endocycles. Although these experiments were carried out in specialized cells, they highlight at least two points: 1) JNK signalling pathway has a role in regulation of cell cycle; and 2) Puc and JNK kinases have opposing function during cell cycle. Subsequent experiments have shown that Puc may have an important role in the G2-M transition, which can be delayed by activation of stress response pathways (including the JNK signalling pathway) (Pearce and Humphrey, 2001; Petersen and Hagan, 2005). Chen et al. (2007) demonstrated that RNAi knockdown of *puckered* led to a severe accumulation (33%) of large G2 cells, implying that Puc is important for mitotic

entry. A high proportion of RNAi knockdown cells also had reduced copies of centrosomes. This is interesting because human JNK has been reported to localize to centrosomes where it is active from early S through late anaphase (MacCorkle-Chosnek et al., 2001). Consequently, It has been postulated that these results imply possible antagonistic functions for JNK kinase and *puckered* either in regulating centrosome duplication in S phase or separation in mitosis.

Although it has been implicated in many processes, the network of Puc's interacting proteins, shown in figure 1-3, is relatively small (Jensen et al., 2009), perhaps because all of its published functions are facilitated through the JNK pathway.



Protein of Interest:

Puc puckered *(Drosophila melanogaster)*

Predicted Functional Partners:

			Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining
	Hp	Dual specificity mitogen-activated protein kinase kinase hemipterous (MAPKK)				√		√
	Sro	Transcription factor kayak (Fos-related antigen) (dFra) (AP-1)				√		√
	Bsk	Stress-activated protein kinase JNK (dJNK) (Protein basket)				√		√
	Jra	Transcription factor AP-1 (Jun-related antigen) (dJRA) (dJun)				√		√
	Tak	Mitogen-activated protein kinase kinase kinase 7 (TGF-beta-activated kinase1)						√
	fra	frazzled, isoform B				√		√
	rac	Ras-related protein Rac1				√		√
	mid	Transcription factor midline						√
	ho	Protein decapentaplegic precursor (Protein DPP-C)				√		√
	Qd	Optomotor-blind protein (Lethal(1)optomotor-blind)				√		√

Figure 1-3 Known interacting partners of Puc.

This image has been produced using String 8.0 (Jensen et al., 2009). It connects Puc to all its interacting partners that have been either experimentally verified (pink lines), or identified in the literature (yellow lines). All interactions shown, involve members of JNK pathway or proteins that are associated with JNK signalling.

1.6 Introducing *S2214*

1.6.1 Gene structure

S2214, also known as *Dsas-4*, cytologically maps to location 84C6-84C7 on the right arm of chromosome 3 in *Drosophila melanogaster*. One small intron of 50 bp is recognized in its 2810 bp sequence. Its ORF encodes a 901 amino acid protein with a predicted molecular weight of approximately 103 KD (Tweedie et al., 2009; Misra et al., 2002; Adams et al., 2000). In silico analysis has revealed the presence of a single functional domain, the TCP 10-C domain, which spans from amino acids 716 to 897 in the second exon (Letunic et al., 2009; Tweedie et al., 2009) (figure 1-4). This domain has received its name due to its high similarity to the C terminal of t complex protein 10. t complex (also known as T/t complex) refers to an approximately 20cM region of mouse chromosome 17 with many variant forms (referred to as t haplotypes) that have been shown to cause male transmission ratio distortion (TRD) (Schimenti, 2000). However, the function of most domains found in t haplotypes including TCP 10-C domain is unknown.

Approximately 90 proteins with TCP-10C domains have been discovered, all in eukaryotes. Although some organisms such as humans have as many as six proteins with this domain, S2214 is the only such protein in *Drosophila melanogaster* (Letunic et al., 2009). In addition, this domain seems to be well conserved between and within species. For example, the 180 amino acid domain found in S2214 shows more than 40% amino acid identity and higher than 60% amino acid similarity to the TCP-10C domains found in four of the six human proteins, as well as the mouse TCP-10C domain, suggesting an evolutionary important role for this domain (Letunic et al., 2009; Altschul, 1997). The protein with highest similarity to S2214 is human centromere protein J (CenpJ), which has been shown to function as a transcriptional

co-activator for nuclear factor-kappa-B, as well as an inhibitor of microtubule assembly (Koyanagi et al., 2005; Hung et al, 2004).

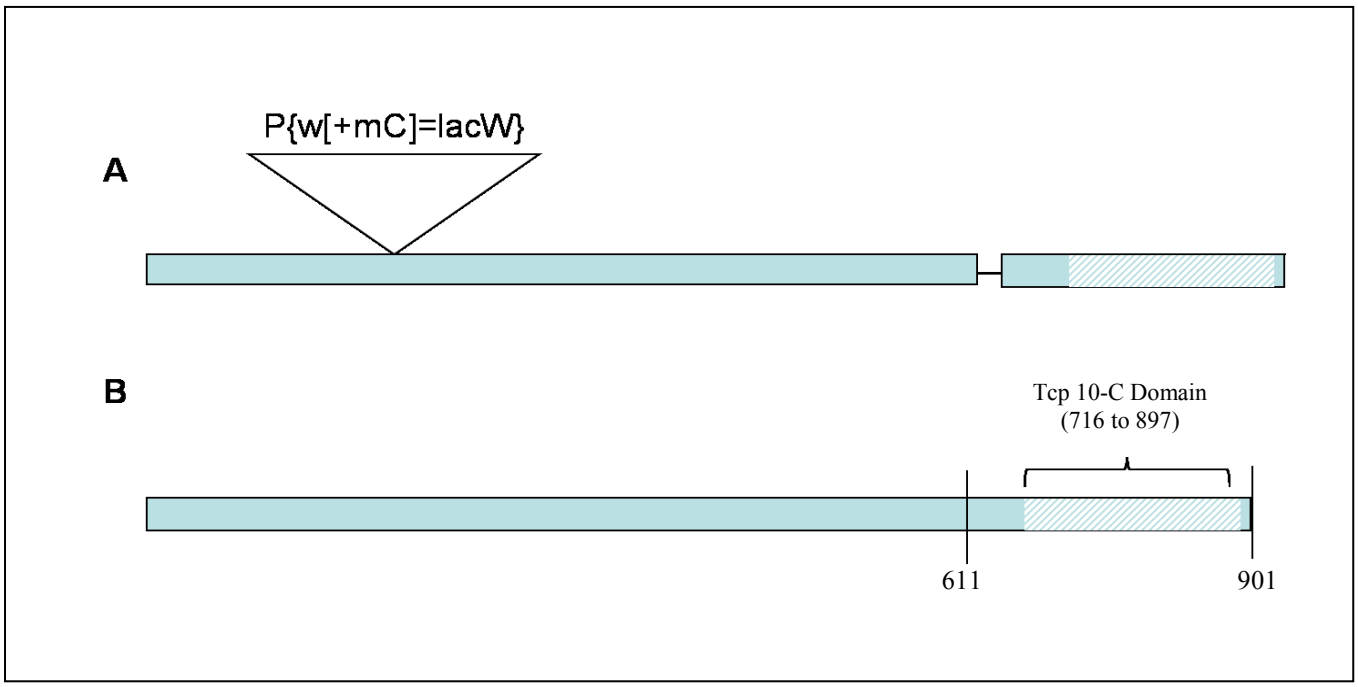


Figure 1-4 Known functional domains found in *S2214*.

S2214 is composed of 2 exons separated by a small (50bp) intron (A). The 901 amino acid long protein shown (B) is the only expected gene product. Exon 2 encodes the only recognized functional domain, a Tcp 10-C domain, which spans amino acids 716 to 897 of the S2214 protein, shown as the shaded area (B). The position of the last amino acid of each exon has been marked by a vertical line on the schematic of the protein (B). A *P* element insertion within the first exon results in a nonsense mutation which significantly truncates the gene product (A).

1.6.2 Known functions

Safronova et al. (2002), in an attempt to explain the transmission ratio distortion observed in t haplotypes, reviewed the available literature of various t complex proteins. They suggested that proteins with Tcp 10-C domain are involved in spermatogenesis. Subsequently, another group, using in-silico screens and phylogenetic analysis to identify genes important for cilia biogenesis, identified S2214 as a member of prototypical cilia gene group (Avidor-Reiss et al., 2004).

More recently, Basto et al. (2006) showed that flies carrying *S2214* mutants lose centrioles during embryonic development, and by the third instar larval stage no centrioles or centrosomes are detected in the brains of these mutants. They further show that an antibody raised to a portion of S2214, localizes to the centrioles in the larval brain cells. They also demonstrated that the mutant flies lack cilia in their sensory neurons but are morphologically normal. However, unlike other centriolar and centrosomal proteins, *S2214* mutants do not affect asymmetric division of neuroblast cells. Interestingly, they also find that these flies do not show significant increases in the rate of chromosomal disjunction. Further research of centriole replication has shown that overexpression of *S2214* results in the formation of hundreds of de novo centriole- like structures, which lack centrioles, in unfertilized eggs (Peel et al., 2007). It is also noteworthy that Basto et al. (2006) have renamed *S2214* (CG10061) to *DSas4* based on the discovery of a "70aa domain that is weakly conserved with the *C. elegans* Sas-4 protein". Consequently, I have performed BLAST searches with various portions of this domain, and I have failed to find any region over 10 amino acids that shows higher than 25% amino acid identity, or 40% amino acid similarity (Altschul et al., 1997; Tweedie et al., 2009). As a result, I have refrained from referring to this gene as Dsas4.

No interacting proteins for S2214 have been discovered experimentally. However, a few potential interacting partners have been identified, by either yeast two hybrid experiments of drosophila proteins or by text mining, and are summarized in table 1-1.

Table 1-1 Putative interacting partners of S2214

Potential S2214 Interacting partner	Functions:	Type of Evidence	Reference
BEAF-2 (also known as *Boundary element-associated factor of 32kD)*	• chromatin insulator sequence binding at scs' boundary element of the hsp70 • Modifier of chromatin structure • regulator of PolII dependant transcription	Yeast 2 hybrid system	Tweedie et al., 2009; Stark et al., 2006; Giot et al., 2003; Zhao et al., 1995.
CtBP (also known as C-terminus binding protein)	• Transcription corepressor/coactivator • Oxidoreductase activity using NAD or NADH as acceptors	Yeast 2 hybrid system	Tweedie et al., 2009; Stark et al., 2006; Giot et al., 2003
Rl (Also known as Rolled)	• MAP kinase activity; • serine/threonine kinase activity; • JUN kinase activity; • ATP binding	Yeast 2 hybrid system	Tweedie et al., 2009; Stark et al., 2006; Giot et al., 2003
Cm (Also known as Carmine)	• synaptic vesicle coating • vesicle coating • lysosome organization • intracellular transport • regulation of alternative nuclear mRNA splicing	Yeast 2 hybrid system	Tweedie et al., 2009; Stark et al., 2006; Giot et al., 2003

Table continued on next page

Potential S2214 Interacting partner	Functions:	Type of Evidence	Reference
CG8010	Protein encoding gene with unidentified function	Yeast 2 hybrid system	Tweedie et al., 2009; Stark et al., 2006; Giot et al., 2003
DSas-6 (also known as Spindle assembly abnormal protein 6)	• Required for centromere duplication • Required for cilia formation • Required for spindle fiber assembly	Text mining	Jensen eta l., 2009; Tweedie et al., 2009.
Cnn (also known as Centrosomin)	• Core component of the centrosome throughout spermatogenesis	Text mining	Jensen eta l., 2009; Tweedie et al., 2009.
Cp190 (also known as centromere protein 190)	• Centrosome-associated zinc finger protein capable of binding centromeres	Text mining	Jensen eta l., 2009; Tweedie et al., 2009.

The aim of this thesis is to initiate the characterization of *S2214*, and *puckered*. To do so, I will employ a two pronged approach. First, through use of genetic tools, I will show that the mutations in these genes cause suppression of PEV. I subsequently test genetic interactions between these genes and a few other suppressors of PEV. Second, by developing and utilizing antibodies and other available molecular biology tools, I will address the intracellular localization of each protein, which may provide clues to their function.

Chapter 2 *S2214* and *puckered* Function as Modifiers of Chromatin

2.1 Introduction

Our lab is interested in identifying and characterizing modifiers of chromatin structure. Several laboratories, including ours, have used EMS and *P* element mutagenesis screens to identify dominant mutations that either suppress [*Su(var*)] or enhance [*E(var)*] PEV, with the hope that such mutations occur in genes that are required for "normal" chromatin assembly. One such gene, the *Su(var 3-4)* has been mapped by Reuter et al. (1986) to 84D14-E1 in cytological location, on the right arm of chromosome 3 of *Drosophila melanogaster*. Previously, a student in our lab attempted to clone *Su(var) 3-4* gene, a recessive lethal and a dominant suppressor of PEV. Although unsuccessful in obtaining a *P* tagged *Su(var) 3-4*, three other genes were identified as possible dominant suppressors of PEV: *Alhambra, S2214,* and *puckered*. *Alhambra*, the homologue of *AF10* in mammals, has since been characterized as a suppressor of PEV and it has been shown to interact with HP1(Perrin et al, 2002). Therefore, I decided to initiate the characterization of the two remaining *Su(var)s* (S*2214* and *puckered)* and generate tools for further analysis of these genes and their products.

In this chapter I will outline experiments that were used to show that *S2214* and *puckered* are strong dominant suppressors of PEV, as well as genetic experiments designed to test possible interactions between these genes and other well characterized *Su(var)s*.

2.2 Effects of *S2214* and *puckered* on PEV

Since *S2214* or *puckered* have never been implicated as modifiers of chromatin or modifiers of gene expression, my first task was to demonstrate that these gene are capable of affecting gene silencing and do so, perhaps, by altering chromatin structure. To accomplish this, I used PEV of the white gene in w^{m4}as an assay system. It has been shown that in PEV the decision regarding the transcriptional activity is made early in development and is transmitted to the subsequent generations with high fidelity (Wallrath and Elgin, 1995; Muller 1930). As such, PEV closely mimics the silencing observed in development and may be a usefull assay system to identify modifiers of chromatin. The underlying assumption is that PEV is caused and maintained by the same silencing mechanisms that determine the fate of a particular cell lineage early in development (Reuter and Spierer, 1992; Grigliatti, 1991; Eissenberjg, 1989). In w^{m4} an inversion on the X chromosome has placed the white gene juxtaposed to a broken portion of heterochromatin. As a result a variegating eye phenotype (mottled) is observed, the eye facets are white in those cells in which the w^{+} allele is silenced, and if the gene is silenced, and in those cells in which the gene is expressed.

2.2.1 *S2214* and *puckered* suppress PEV in w^{m4} and Sb^{V}

To determine whether mutations in *S2214* and *puckered* suppress PEV, males carrying *P* element insertion within each of these genes were crossed (separately) to females showing the w^{m4} phenotype. It should be noted that since *P* element mutations of both *S2214* and *puckered* cause recessive lethality, only dominant suppression of PEV could be observed. The results shown in Figure 2-1, demonstrate that mutations in either *S2214* or *puckered* suppress the w^{m4}phenotype, and restore the level of pigmentation that is observed in the wildtype strain of

Oregon R. Thus (presumably) disruption of their normal expression, by insertion of *P* element DNA, appears to suppress the gene silencing associated with PEV.

However, it could be argued that *S2214* and *puckered* affect the expression of the *white* gene itself and do not influence PEV per se. To address this issue, I asked whether the *P* element mutations in *S2214* and/or *puckered* affected other genes that were silenced due to PEV. S2*214* and *puckered* flies heterozygous for the *P* element were crossed to a strain carrying Stubble variegator (Sb^V). Unlike w^{m4}, Sb^V is a dominant mutant phenotype that when completely active causes 100% of bristles to become stubble; silencing of the Sb^V allele in Sb^V/+ strains results in flies with wildtype bristles. The data, summarized in figure 2-2, show that the number of stubble bristles increased from an average of 42% in the controls (44% for males and 40% for females) to an average of 92% in *S2214* mutants (93% for males and 91% for females) and an average of 84% in *puckered* mutants (86% for males and 82% for females). These results, similar to those from w^{m4} experiments, suggest that both *S2214* and *puckered* are strong dominant *Su(var)s*, and support the hypothesis that they modify chromatin structure.

Since the mutant stocks used have been generated through *P* element mutagenesis, it is possible a second site mutation in the genome, rather than the *P* element insertion itself, causes the *Su(var)* phenotype. To conclusively address this issue, the *P* element in each stock was mobilized and the revertants, $S2214^+$ and $puckered^+$, were examined (separately) for their ability to suppress PEV. In addition, I decided to examine other available *P* element stocks of these genes. Unfortunately, no other mutant stocks of *S2214* have been identified; however, four other *P* element insertions (positioned in introns 2 and 3) were available for *puckered*. These strains were created through 3 different screens using 2 different forms of the *P* elements. Therefore, it would be unlikely that all of these lines have the same second site mutation or that each has a second site mutation in at least one modifier of PEV. All strongly suppressed PEV (>95%,

scored visually), suggesting that suppression observed in *puckered* mutants is due to the insertion in this gene and not a secondary site.

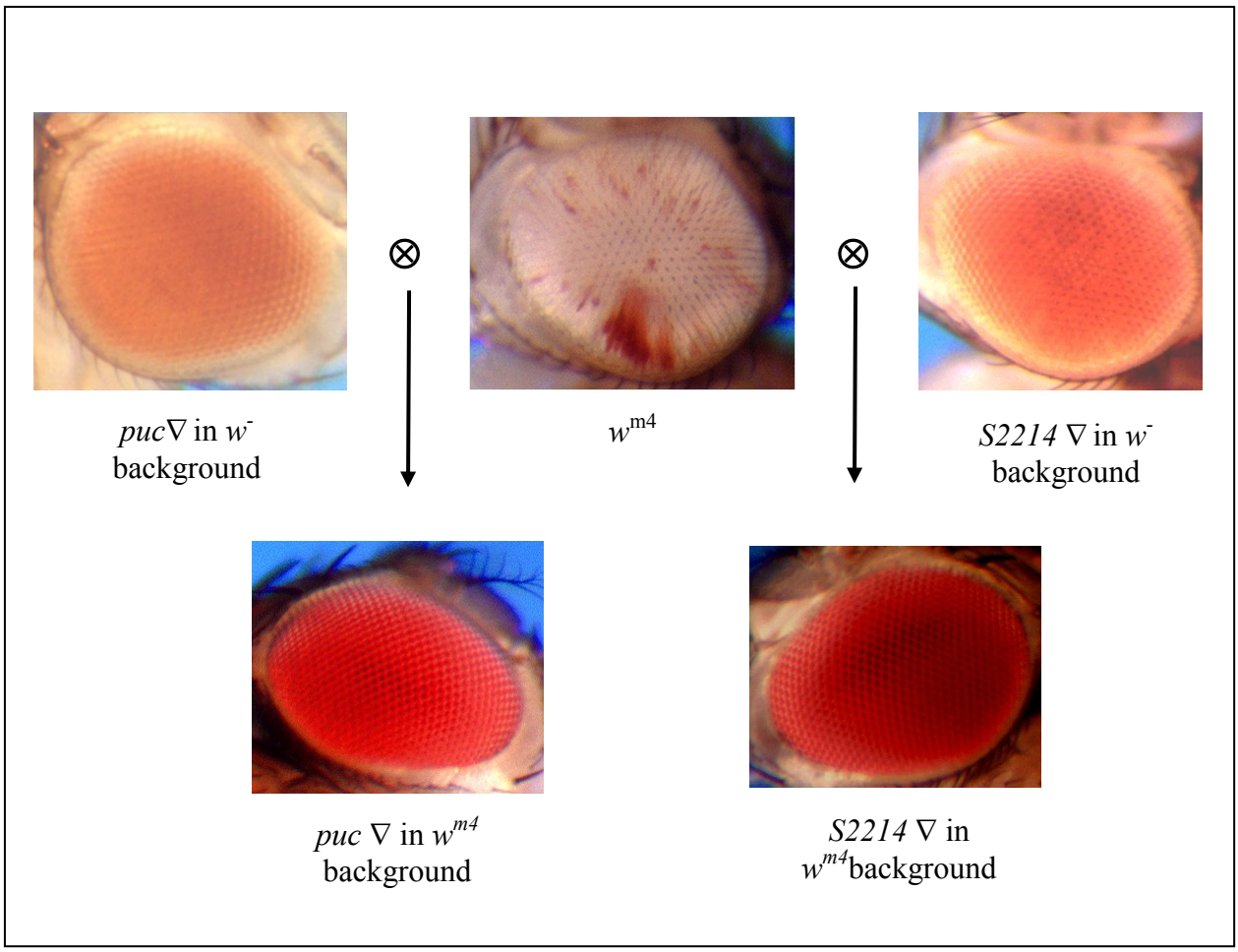


Figure 2-1 *S2214* and *puckered* are strong dominant suppressors of PEV in W^{m4}. Crosses between w^{m4} and the *P* element mutant *puckered* and *S2214* (each carrying *mini white*) were made (upper half of the figure). Resulting progeny have strongly suppressed eye phenotypes (shown as red eyes in the bottom half of the figure), which would be indistinguishable from the eyes of a wildtype fly.

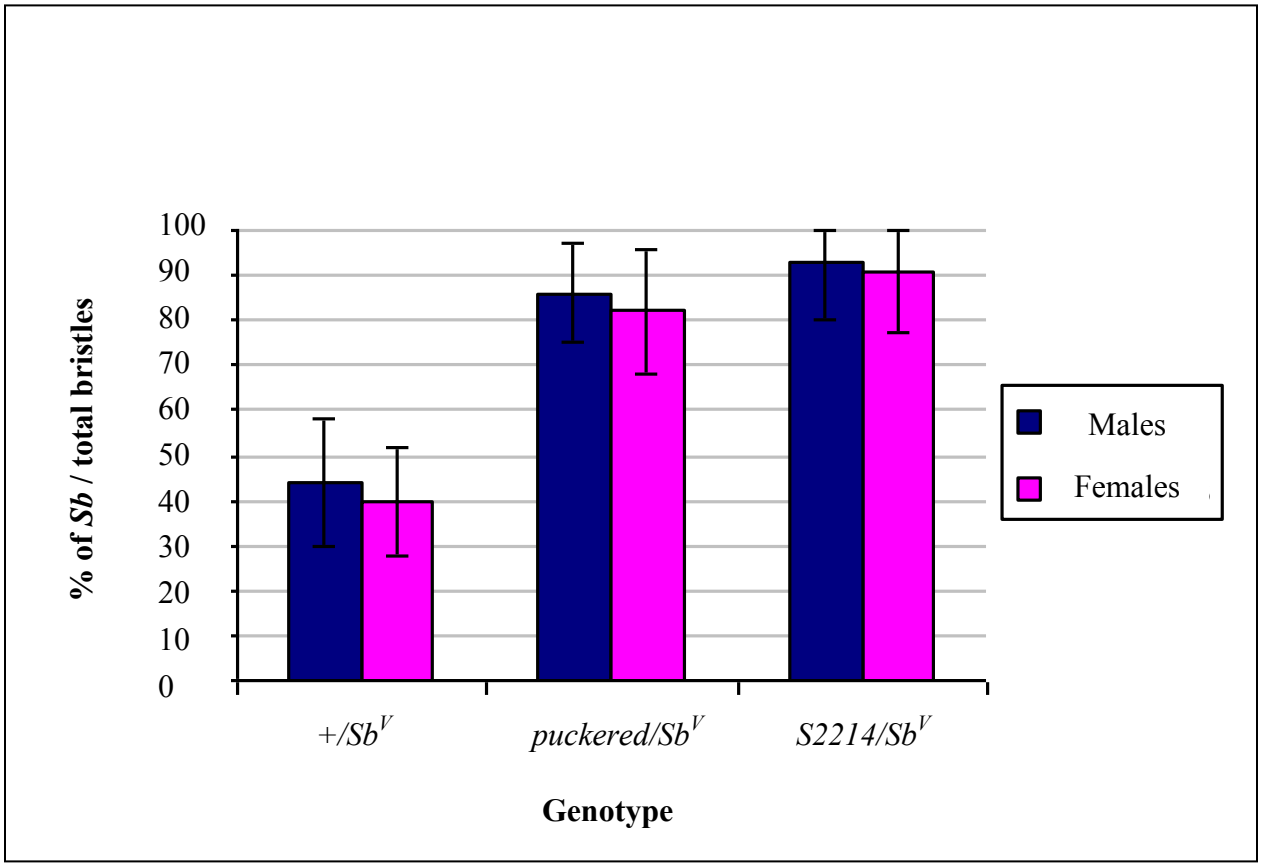


Figure 2-2 *S2214* and *puckered* are strong dominant suppressors of PEV in Sb^V

Sb^V was crossed to the *P* element mutants of *puckered* and *S2214*. Fourteen bristles on the dorsal thorax of 300 male and 300 female flies were scored. Since *Sb* in this variegator is a dominant mutation, suppression of the variegating phenotype would result in an increase in the number of stubble bristles. Both *puckered* and *S2214* in the variegating background show an increase in the number of stubble bristles, from ~ 42% in the controls to ~ 84% in *puckered* mutants and ~92% in *S2214* mutant. Vertical bars on the graph denote one standard deviation observed in the population.

2.2.2 Revertants of *S2214* and *puckered* no longer suppress PEV

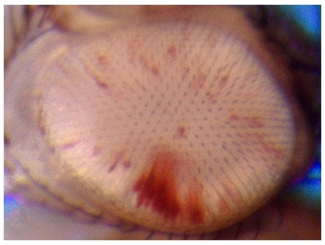
The *P* element in *S2214* and *puckered* were mobilized through crosses with a strain *Δ 2-3* that expresses the transposase which is necessary for mobilizing these *P* elements. The *P* elements are marked with *mini white* gene, which produces phenotypically orange eyes, thus the re-mobilizations can be inferred from loss of this orange eye phenotype. Subsequently, stocks of individual re-mobilizations were made. Putative perfect excisions were identified based on phenotypic markers. These were further analyzed by PCR and sequenced to identify the true revertants (data summarized in table 2-1).

Table 2-1 Result of *P* element mobilization of *S2214* and *puckered* mutants

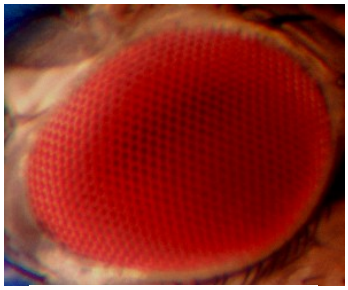
	S2214	*puckered*
# of males counted (in total of 3 rounds of mobilizations)	3172	1335
Average rate of *P* element mobilization (in 3 rounds)	7.9%	11.4%
Number of putative perfect excisions	14	12
Strains with perfect excision of the *P* element (based on PCR analysis)	7	4
Number of perfect excisions verified by sequencing	2	2

Two perfect excision lines from each strain (verified by sequencing) were crossed (separately) to w^{m4} to assess their ability to suppress PEV. The results shown in Figure 2-3, demonstrate that all perfect excisions tested for both *S2214* and *puckered* revert the *Su(var)* phenotype. In all cases, the phenotype was similar to that of the w^{m4} control This suggest that the suppression of PEV observed in *S2214* and *puckered* strains resulted from mutations in these genes. Taken together the data from this section suggests that *S2214* and *puckered* are strong

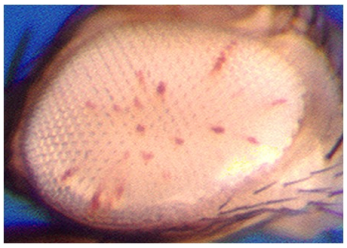
dominant suppressors of PEV. However, the question of how they suppress the gene silencing associated with PEV still remains.



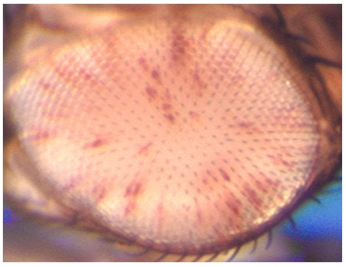
w^{m4}



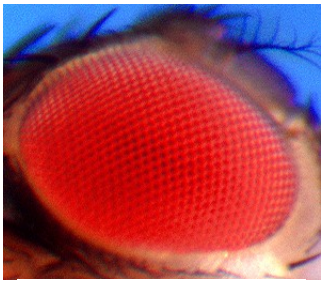
S2214 ∇ in
w^{m4}background



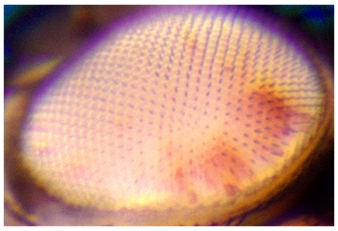
S1-13(a *S2214* revertant)
in w^{m4} background



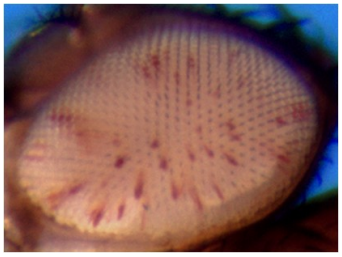
S2-51(a *S2214* revertant)
in w^{m4} background



puc ∇ in
w^{m4}background



J2-4(a *puc* revertant)
in w^{m4} background



J2-20(a *puc* revertant)
in w^{m4} background

Figure 2-3 *S2214* and *puckered* are strong dominant suppressors of PEV in w^{m4}

Crosses between w^{m4} and the revertants of *puckered* and *S2214 (*where the *P* element has been mobilized) were made. Comparisons of the phenotypes of *S2214* mutants (*S2214* ∇) in w^{m4} background and those of its revertants (left panel of the figure) show that upon mobilization of the *P* element, suppression of the PEV in w^{m4} is reverted, back to the levels observed in the parental w^{m4}. Similarly, *puckered* revertants are indistinguishable from the parental w^{m4}, suggesting the mutation caused by the *P* element was responsible for the suppression (right panel of the figure).

2.3 General characterization of *S2214* and *puckered*

Since both *puckered* and *S2214* are strong modifiers of PEV, it is possible that they interact with other strong *Su(var)s* known to encode non-histone chromatin proteins. However, designing informative genetic assays to test interaction between chromatin modifiers has been challenging. Many variegator stocks used to identify modifiers are "too strongly" suppressed, and thus double mutant analysis is not informative. Moreover, uncertainties with regards to the central dogma of establishment and maintenance of chromatin structure make it difficult to select putative interacting partners.

It may be possible that chromatin structure and its modifications are established or maintained through a main pathway that acts in a genome wide fashion. On the other hand, different pathways may be responsible for establishing or maintaining different types of chromatin in various regions of the genome. Moreover, some researchers have argued that although some factors are shared among different types of chromatin (and more specifically heterochromatin), the overall constitution of different chromatin neighborhood maybe very different (Ebert et al., 2006; Futchs et al., 2006; Gelato fischle, 2008, and Doheny et al., 2008).

PEV modifiers such as *Hp1*, *Hdac1*, and *Su(var) 3-9* have been localized to most regions of the genome and their mutations affect both chromatin structure and gene expression in various loci (Singh et al., 2002; Ner et al., 2002; Schotta et al., 2002; Schotta et al., 2004; Badgu et al., 2005; Kimura et al., 2005; Foglietti et al., 2006). Although, their effect may vary, they have a more global effect, and hence may interact with a wider range of other chromatin modifiers. As a result, I decided to test whether *S2214* and *puckered* show genetic interactions with *Hp1, Hdac1,* or *Su(var)3-9*.

I decided to ascertain whether mutations in *puckered* and *S2214* interact with those of *HP1*, *Hdac1*, and *Su(var)3-9* to cause:

a. Higher synthetic lethality

b. Higher frequency of non-disjunction of X chromosomes

To address questions regarding synthetic lethality, first one must establish the survival rates of *puckered* and *S2214* mutants.

2.3.1 Lethal phase analysis of *puckered*; a literature review

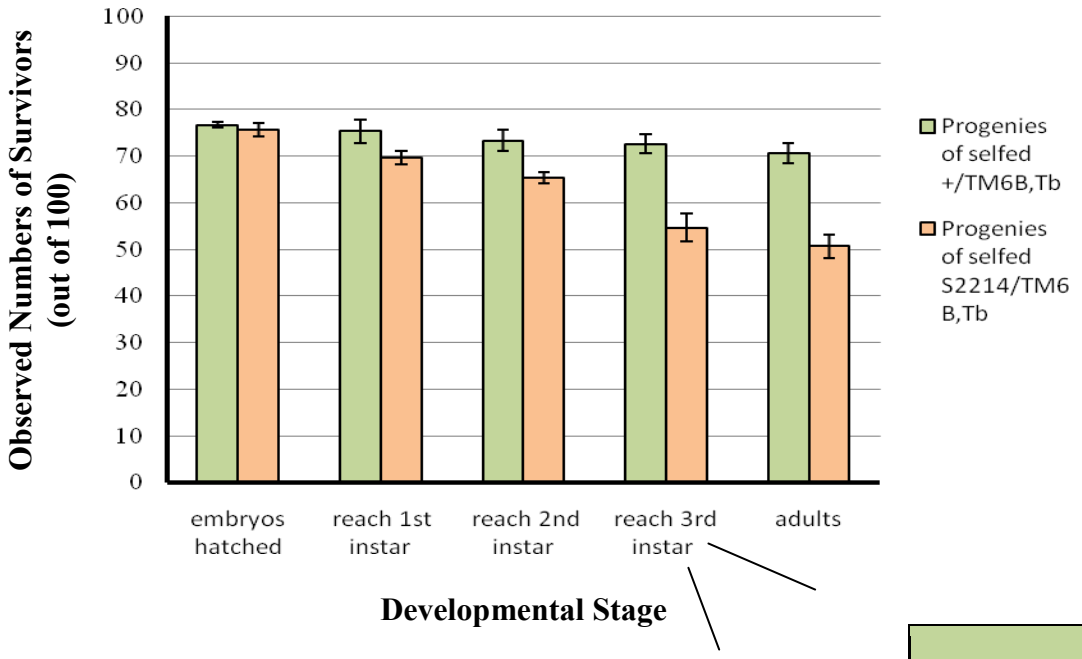
Ring and Martinez-Arias (1993) showed that *puckered* mutants develop defects along the dorsal midline during dorsal closure. These defects vary in intensity depending on the severity of the mutation, ranging from misaligned segments in the weakest allele to absence of dorsal hairs along the midline and strong puckering of the epidermis seen in puc^{E69} and $puc^{A251.1}$(the *P* element mutation used in my experiments).

Lethal phase analysis of *puckered* embryos homozygous for strong mutant alleles, such as $puc^{A251.1}$, have shown that no stage 16 embryos (~ 14 hours post fertilization) were present, implying that all homozygous embryos have lethal complications resulting from aberrant dorsal closure, which in wildtype embryos is completed in stage 15 at approximately 13 hours (Ring and Martinez-Aria, 1993).

2.3.2 Lethal Phase analysis of *S2214*

Unlike *puckered*, little is known about *S2214's* mutant phenotype or phase of lethality. Besto et al. (2006) carried out experiments to measure developmental and survival rates of the *S2214 P* element mutant flies, and found that "development proceeded with near normal timing and morphologically normal flies hatched at near normal rates". My experience with the survival rates of these mutants has been different. Lethal phase analysis carried out (in 3 independant replicates), is summarized in figure 2-4. These data suggest that *S2214* is a larval lethal. Although on average ~20% (5% out of the expected 25%) of the homozygous mutant embryos survived passed third instar larvae and into adulthood, a significant drop in survival rates were observed between 2nd and third instar larval stages. I have no explanations for the discrepancy between my results and those observed by Besto et al. (2006); however, I am confident that under our environmental conditions, my results are reproducible.

In the course of their experiments, Basto et al. (2006) also noted that adult flies homozygous for *S2214 P* element mutation were morphologically normal but "uncoordinated" due to the lack of cilia in their chemosensory neurons. Similarly, my observations suggest that *S2214* mutant flies that survive to adulthood are morphologically normal. However, in my experience they show no signs of reduced coordination.



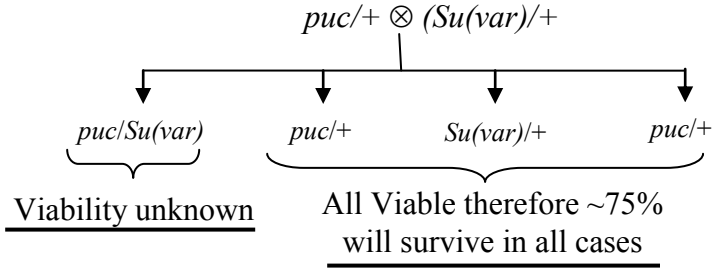
		Progenies of selfed +/TM6B,Tb	Progenies of selfed *S2214*/TM6B,Tb
Phenotypically wildtype larvae that reached third instar	Average %	26.7% (+/-0.6%)	5.33% (+/-2.3%)
	Genotype	+/+	*S2214/S2214*
Phenotypically Tubby larvae that reached third instar	Average %	46.00% (+/- 2.6%)	49.3% (+/- 1.6%)
	Genotype	+/TM6B,Tb	+/TM6B,Tb

Figure 2-4 *S2214 P* element mutant demonstrates larval lethality

Flies with either wild-type (+) third chromosome or *S2214* mutant third chromosome heterozygous with a TM6B balancer that contained the dominant marker Tubby (Tb) were obtained through standard genetic crosses. +/TM6B,*Tb* or *S2214*/TM6B,*Tb* flies were self crossed. 100 early embryos (< 3 hours old) were placed on a 2 cm^2 blue paper and placed in a vial for 48 hours (this process was repeated 3 times). Subsequently, the numbers of embryos that hatched in each vial, as well as the numbers of larvae/flies present in each developmental stage were recorded. The survival rates of the homozygous wildtype and homozygous S2214 larvae were measured by counting the number of non tubby larvae at each stage. Results show that fewer progeny of *S2214*/TM6B,*Tb* selfings survive to the pupae stage (graph). Further analysis show that this observed decrease in the survival rates is due to the lethality of the homozygous *S2214* mutation (table). On average only ~20% of the homozygous mutant embryos survived passed third instar larvae and into adulthood. The highest drop in survival rates were observed between 2^{nd} and third instar larval stages.

2.3.3 Synthetic lethality

This genetic interaction occurs when two strains with viable mutations result in reduced or no growth when combined in a double mutant. In my analysis, all heterozygous mutant strains are viable; the experiments test whether any of the double mutants show a significant reduction in survival rates. The following sample cross outlines the expected ratios among the progeny:



Results suggest that neither *puckered* nor *S2214 P* element mutants cause synthetic lethality with *Su(var)2-5*^{04} (*Hp1* mutant), *Hdac1*^{326}, *Su(var)3-9*^{06}, or with each other (table 2-2).

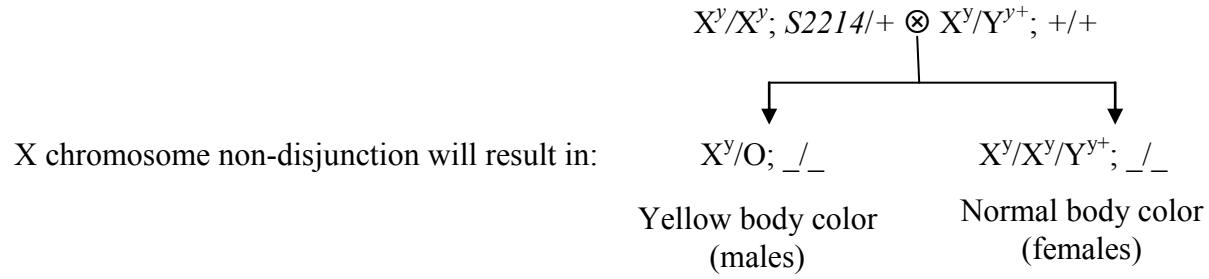
Table 2-2 Synthetic lethality results of *S2214* and *puckered* with other modifiers of PEV

Cross *			% of flies that reached adulthood	Standard deviation
+/+	⊗	+/+	94.67	+/- 1.37
puc/+	⊗	+/+	94.33	+/- 1.63
S2214/+	⊗	+/+	93.67	+/- 2.25
Hdac1^{326}/+	⊗	+/+	95.17	+/- 0.98
Su(var)2-5^{04}/+	⊗	+/+	94.50	+/- 1.87
Su(var)3-9^{06}/+	⊗	+/+	95.17	+/- 1.47
puc/+	⊗	*S2214*/+	94.50	+/- 1.38
puc/+	⊗	*Hdac1*^{326}/+	94.00	+/- 1.26
puc/+	⊗	*Su(var)2-5*^{04}/+	94.67	+/- 1.21
puc/+	⊗	*Su(var)3-9*^{06}/+	94.33	+/- 2.16
S2214/+	⊗	*Hdac1*^{326}/+	94.83	+/- 1.72
S2214/+	⊗	*Su(var)2-5*^{04}/+	95.17	+/- 2.56
S2214/+	⊗	*Su(var)3-9*^{06}/+	94.83	+/- 1.47

* Reciprocal crosses were carried out for each of the pairings. Survival rates of 600 embryos for each set of crosses were tested (3 x 100 for each cross and 3 x 100 for each reciprocal cross).

2.3.4 Non-disjunction of X chromosomes

Some of the other modifiers of chromatin such as *Hp1*, and some PcG genes affect the rate of non-disjunction. It has been suggested that this maybe due to abnormal chromatin condensation and/or compaction (Badugu et al., 2005; Singh et al., 2002; Aasland et al., 1995). Therefore, other modifiers of chromatin structure (such as *S2214* and *puc*kered) may either cause non-disjunction on their own, or enhance the rates of non-disjunction observed with other mutants. To test for this possibility, I decided to measure the rate of X chromosome non-disjunction, as a representation of overall non-disjunction. In *Drosophila*, sex determination is dependent on the number of X chromosomes, and not the presence of Y chromosome. As a result, non-disjunction of the X chromosome can result in XO males and XXY females. Thus a cross such as the one shown below can be used to calculate the rate of X chromosome non-disjunction.



Results, summarized in table 2-3, reveal that neither *S2214* nor *puckered* affect the rates of X chromosome non-disjunction as single mutants, or enhance the rates of non-disjunction in *Su(var)2-5*^{04} as heterozygous double mutants.

Table 2-3 Effects of *S2214* and *puckered* on rate of X chromosomes non-disjunction as single mutants or as second mutations in a *Su(var)2-5^{04} background*

Cross		Female (X^y/X^y ;) ⊗ Male (X^y/Y^{y+};)	Frequency of X^y/O; _/_ Yellow bodied males	Frequency of $X^y/X^y/Y^{y+}$; _/_ White bodied females	Rate of non-disjunction (%)
1	A	+/+ ⊗ +/+	13/13076	14/13396	0.1020 %
2	A	*puc*/+ ⊗ +/+	7/7138	7/7196	0.0977 %
	B	+/+ ⊗ *puc*/+	7/6997	7/7211	0.0985 %
3	A	*S2214* /+ ⊗ +/+	7/7011	7/7113	0.0991 %
	B	+/+ ⊗ *S2214*/+	7/7115	8/7228	0.1046 %
4	A	*Su(var)2-5^{04}*/+ ⊗ +/+	14/7057	13/6999	0.1921 %
	B	+/+ ⊗ *Su(var)2-5^{04}* /+	18/7088	15/7200	0.2310 %
5	A	*puc,+/+,S2214* ⊗ +,+/+,+	7/7068	7/7160	0.0984 %
	B	+,+/+,+ ⊗ *puc,+/+,S2214*	6/7144	7/7041	0.0916 %
6	A	*puc; + / +;Su(var)2-5^{04}* ⊗ +/+;+/+	14/7101	13/7214	0.1886 %
	B	+/+;+/+ ⊗ *puc;+/+;Su(var)2-5^{04}*	17/7135	18/7341	0.2418 %
7	A	*S2214/ + ;+/Su(var)2-5^{04}* ⊗ +/+;+/+	13/7197	13/7137	0.1814 %
	B	+/+;+/+ ⊗ *S2214/+ ; Su(var)2-5^{04}*/+	16/7084	18/7271	0.2369 %

2.3.5 Effects of JNK signaling on PEV

Since its discovery as a member of JNK pathway, all of known functions of *puckered* relate to its role in this pathway. Therefore, one cannot ignore the possibility that JNK pathway (and not *puckered* alone) is involved in modifying chromatin structure.

To test this hypothesis, LOF mutant stocks of *basket, Djun,* and *decapentaplegic* genes were obtained. Since Puc is a negative regulator of JNK signaling pathway, it should be expected that LOF mutations in all three selected genes would have an effect opposite of *puckered* mutants, and enhance PEV. In other words, they should increase the number of white eye facets present in w^{m4} strains. Moreover, due to their positions in the pathway (figure 1-2), one would expect *basket* and *Djun* to be epistatic to *puckered*. Thus, a double mutant containing LOF mutations in *puckered* and either *Djun* or *basket* should also enhance PEV.

Loss of function mutant stocks of *basket, Djun,* and *decapentaplegic* were first crossed to a w^{m4} (5% - 15% pigment) and their progeny were scored visually for the amount of pigment present. In addition, they were crossed to suppressed w^{m4}, in *puc*kered background. In each case at least 300 male and 300 female flies were scored. There were no observed differences between the controls and the experimental samples. Based on the results of these experiments, it can be concluded that *basket, Djun,* and *decapentaplegic* are not strong dominant modifiers (enhancers or suppressors) of PEV in w^{m4}. Although these results do not eliminate the possibility of these genes being weak or recessive modifiers, I believe they suggest that the role of *puckered* in modifying chromatin structure is distinct from its function in JNK pathway.

2.4 Summary of results

Experiments described in this chapter have shown that *P* element mutations in previously uncharacterized *Su(var)s*, *puckered* and *S2214*, are responsible for the strong suppression of PEV observed in both w^{m4} and *Stubble* variegators. However, I could not detect any interactions between *puckered* and *S2214* and other strong *Su(var)s.* It is possible that *S2214* and *puckered* are haplo-sufficient, and the experiments selected were not sensitive enough to demonstrate the double mutant phenotypes.

In the case of *puckered*, analysis has revealed that the function of Puc as a modifier of PEV and, by extension, chromatin structure, may be distinct from its role in the JNK signaling pathway. It is also plausible that other member of JNK are also involved in this process, however, none of the mutations in these genes had detectable affects on PEV.

Analysis of *S2214* survival rates has revealed that a significant number of *S2214* homozygous mutants do not survive to adulthood, suggesting its product has an important role during the larval stages.

Since *S2214* and *puckered* are strong dominant *Su(var)s*, and PEV is closely associated with heterochromatin, it is likely that both of these genes are involved in pathways for establishment or maintenance of chromatin structure.

Chapter 3 Intracellular Localization of S2214 and Puc

3.1 Introduction

Many factors that influence chromatin structure have been identified, but the transcriptional programs in which they participate are still poorly understood. Factors that affect establishment or maintenance of higher order chromatin structure can be categorized into two general groups:

i) Those that interact with chromatin directly and as such localize to the chromatin fibre. Mutations in many of the characterized modifiers in this group, such as *Hp1, Su(var)3-9, Jil1, Pc,* and *trx*, have profound effects on the regional and global transcription levels.

ii) Those that indirectly modify chromatin structure, which may (or may not) localize to the nucleus. The exact role, as it relates to chromatin structure, of the members of this group (that include kinases, phosphatases, ubiquitinases, protein chaperon, and receptor proteins) have been more difficult to elucidate.

For a protein to be a direct modifier of chromatin structure, it has to localize to the chromatin, at least transiently. As such the next logical step in characterization of *S2214* and *puckered* as *Su(var)s* would be to address their localization within the cell.

Groups concerned with localization of proteins *in vivo* have generally employed two methods. Some have created fusion and tagged proteins whose localization can be ascertained once it is expressed, under native or ectopic conditions. Others have used antibodies to determine the localization of proteins within the cell or cellular fractions through immuno-staining. Although both methods have been used successfully by many, antibodies can be used in more applications, and thus I have chosen to raise and purify antibodies for both S2214 and Puc, in order to elucidate their localization within the cell.

3.2 S2214 antigen production, antibody purification, and immuno-staining

The optimal length of the peptide antigen for generating antibodies is dependent on the specific aim of the project. It is believed that shorter sequences (10-20 amino acids) offer slightly better specificity and are easier to purify, but usually have lower immunogenicity; while longer peptides, 50 to 80 amino acids, are more likely to allow for proper folding of the peptide sequence and therefore are more advantageous for raising antibodies that recognize the native protein (Van Regenmortel 1988; Halow, 1988).

In addition, one should attempt to identify regions specific to the protein of interest that have the highest antigenic potential. In general, sequences that are hydrophilic, surface-oriented, and flexible provide high immunogenicity (Van Regenmortel, 1988; Westof, 1984; Hopp and Woods, 1982). Surface regions or regions of high accessibility, which often border helical or extended secondary structures, have also been shown to have a high antigenic index (Parker and Hodges, 1991; Chou and Fasman, 1974; Lim,1974).

To identify S2214's most suitable region of antigenesity, I used bioinformatics software that employ algorithms to identify the aforementioned characteristics. Four putative regions of high antigenecity were identified. However, only two spanning from amino acids 88 to 160 and 706 to 737 respectively) were specific to both *Drosophila melanogaster* and S2214 (figure 3-1A). To increase the likelihood of obtaining a reactive antibody, the larger of the two regions (from amino acids 88 to a160) denoted as S2214 antigenic region 1 (SAR1), was selected and used in all of the experiments described below.

3.2.1 S2214 antigen production

Once selected, the antigenic region of S2214 was amplified by PCR from genomic DNA. Since the antibody raised to this peptide will be used to localize S2214 *in vivo*, it would be desirable to express and purify the protein under native conditions. In order to facilitate the purification process, the fragment was fused at its N terminal to a glutathione *S*-transferase (GST) protein. This tag may have the secondary benefit of helping to elicit an immune response once the antigen is injected into rabbits.

This fusion peptide was expressed in abundant levels in BL21 bacterial expression cell-lines, but attempts at its extraction under native conditions were unsuccessful. Subsequently, various conditions during both expression and extraction processes (such as temperature, inducer concentration, induction time, buffering agents, PH, etc.) were modified; however, none of the native conditions yielded soluble SAR1 fusion peptide. I therefore had no choice but to extract and purify this peptide under denaturing conditions. Results of these purifications (figure 3-1B) demonstrate that even at high concentrations (>20 μg) only one product corresponding to the correct size of GST-SAR1 (53KD) was present; no other contaminating bands could be detected. Therefore, this purified peptide was used as antigen to raise polyclonal antibody in rabbits.

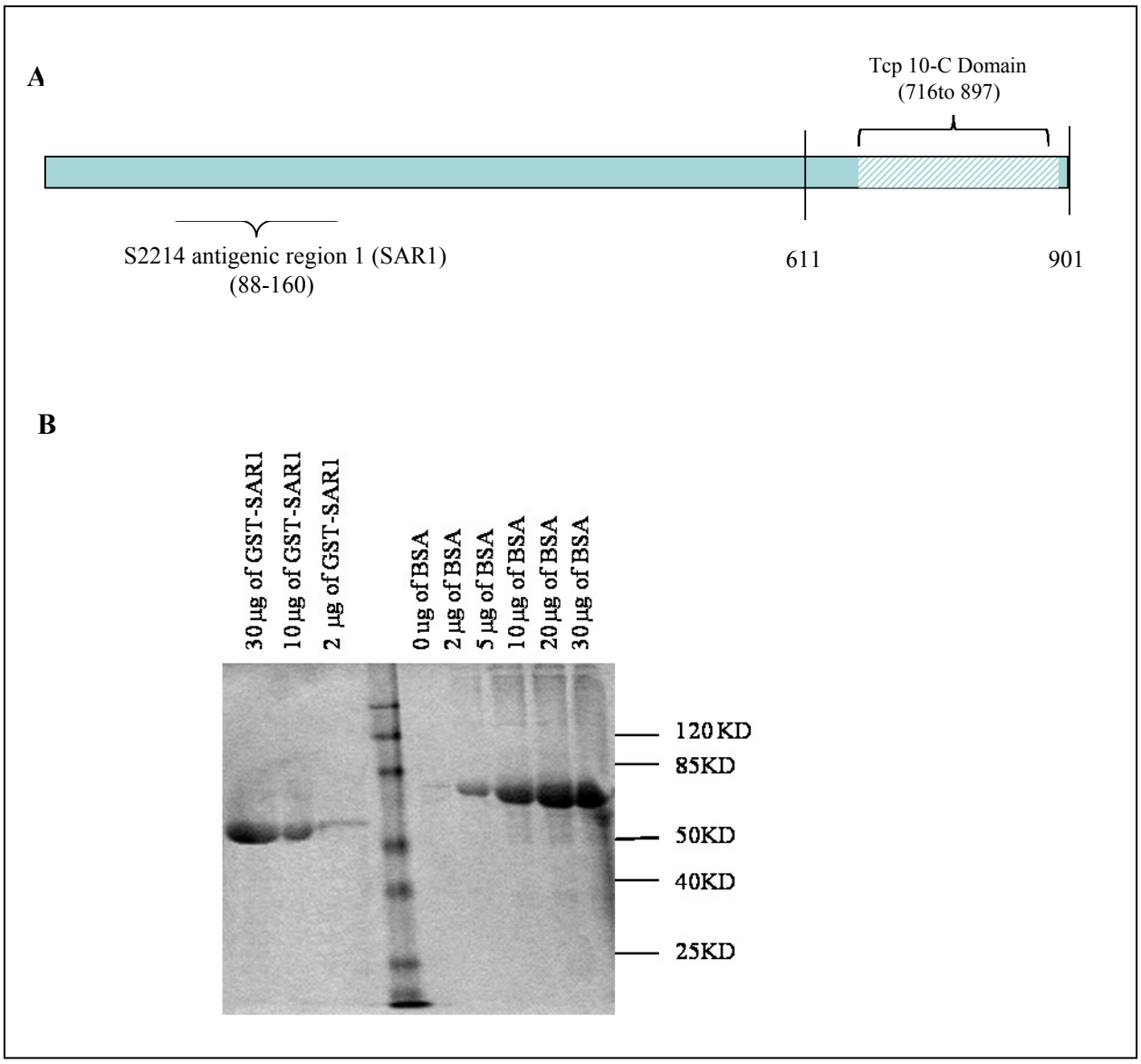


Figure 3-1 Expression and purification of GST-SAR1 peptide

A region unique to S2214 and *Drosophila* with high antigenic potential (based on scores of hydrophilicity, surface potential, and flexibility) was selected (A). This S2214 antigenic region (SAR1), spanning from amino acids 88 to 160, was fused at its N terminal with a GST protein tag. This fusion peptide was expressed in *E.coli,* and subsequently extracted and purified under denaturing conditions. The purity of the antigen was analyzed through SDS-PAGE (B). Results show that no contaminating bands can be detected when solutions of 2, 10, and 30 µg of purified protein are separated on a 10% acrylamide gel.

3.2.2 Enrichment of anti-S2214 in the serum

Since the GST protein had not been cleaved prior to injection, the serum would contain antibodies to GST, S2214, as well as any bacterial contaminants that may have carried over. Therefore, prior to further experiments, either anti-S2214 needed to be affinity purified or other "unwanted" antibodies, such as anti-GST should be depleted from the serum. Although affinity purifications of antibodies have been used by many groups to specifically distil the desired antibody, it can be argued that this method also eliminates highly reactive "desired" antibodies, because of their high affinity to the matrix. For this reason, I decided to deplete the serum of other antibodies. To accomplish this, GST was expressed in BL21 expression cells, these cells were made into acetone powder, which were then used to deplete the serum from anti-GST as well as antibodies to any other bacterial proteins that may have been present. Lastly, all remaining IgGs were purified from the serum and concentrated.

To test the success of the depletion process, I compared the ability of the purified IgGs from the serum to immunostain GST and GST-SAR1 fusion peptide, which was used as the antigen. To ensure that the antibody present in the serum can recognize S2214, serum was also used to immuno-stain a different expressed fusion protein, consisting of an N terminal T7 tag and full length S2214. Results demonstrate that purified IgGs can recognize a protein, corresponding to the correct size (53KD) of the GST-S2214, while GST alone was not recognized (Figure 3-2). This implies that the depletion process has successfully removed anti-GST as well as other antibodies raised to bacterial contaminants. Furthermore, Anti-S2214 present in the serum also recognizes a band of correct size (105 KD) for the T7 tagged full length S2214 protein, suggesting the purified IgGs are specific to S2214.

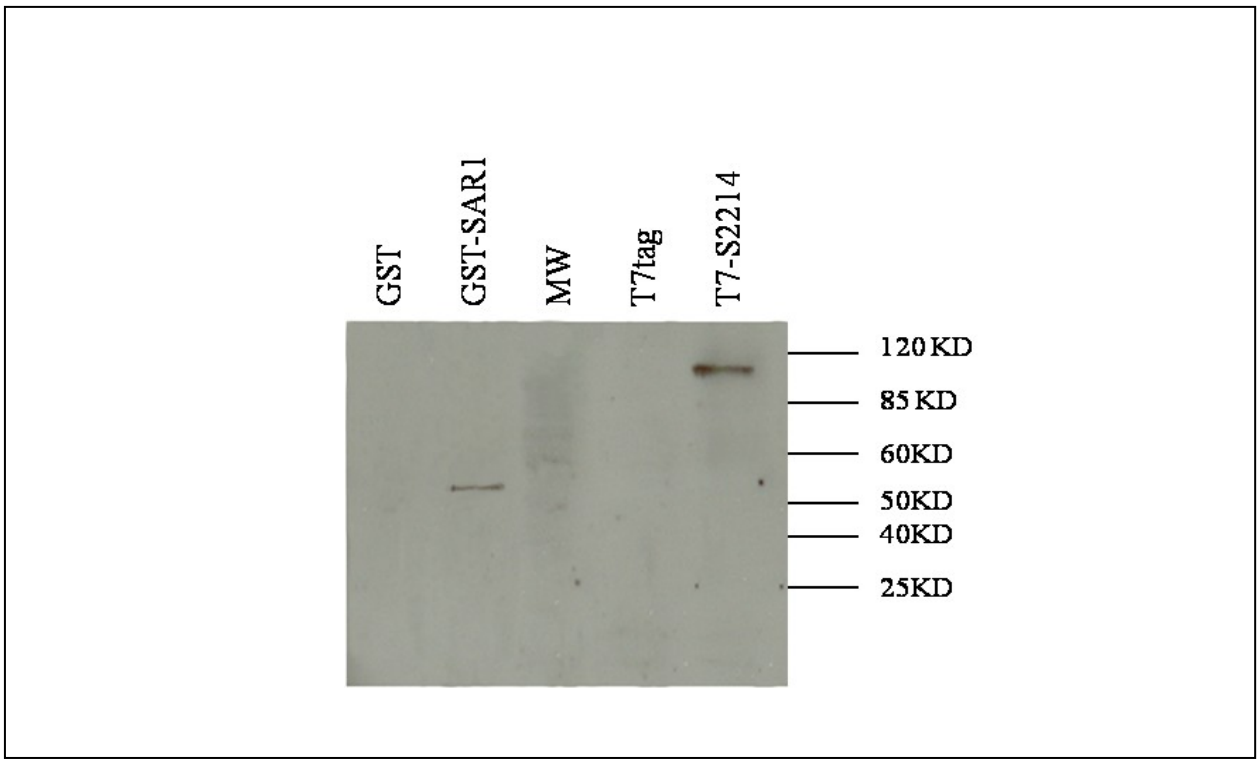


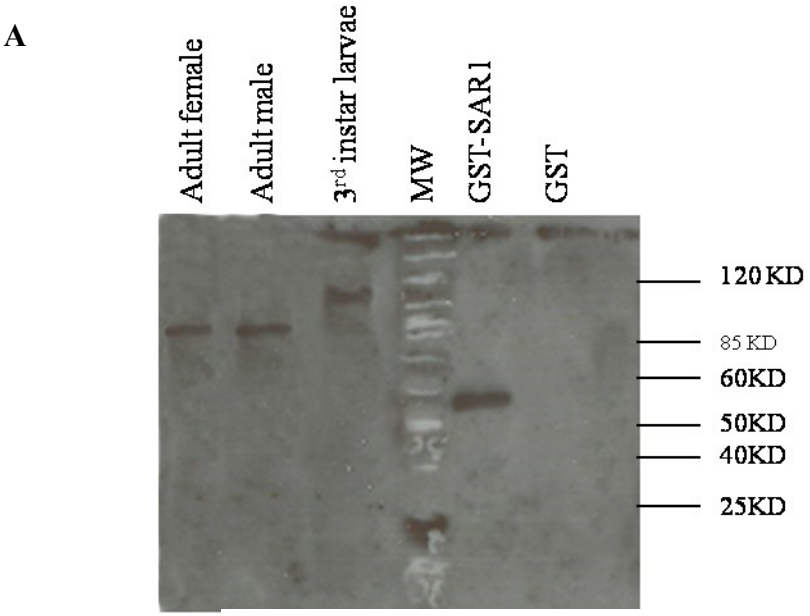
Figure 3-2 Anti-GST depleted serum contains anti-S2214 IgG

Purified IgGs from the serum were tested for their ability to immunostain a western blot of bacterial extract from cultures that have expressed GST, GST-SAR1 fusion peptide, T7 tag, and T7-S2214 fusion protein. No products interact with any of the purified IgGs in the GST lane, while a single product corresponding to the correct size of GST-SAR1 (~53 KD) can be detected in the corresponding lane. In addition, the antibodies purified from the serum can also detect a protein matching the expected ~105 KD size of T7-S2214 (T7 tagged full length S2214 protein); while T7 tag alone is not reacting with any antibodies. These imply that the depletion process has successfully removed anti-GST as well as other antibodies raised to bacterial contaminants.

3.2.3 Detection of S2214 at various developmental stages

Anti-S2214 successfully immunostained expressed fusion proteins, however, its ability to recognize the native protein in extracts from *Drosophila melanogaster* was unknown. To address this issue, extracted proteins from wild type third instar larvae, male and female flies were separated by SDS-PAGE and transferred to a nitrocellulose membrane. This membrane was then immunostained using the serum (figure 3-3A). The results demonstrate that the antibody directed against S2214 recognizes a single product, corresponding to the correct size of S2214 (~103 KD), in both male and female wildtype flies.

My findings with respect to the third instar larvae were unanticipated. A slightly larger product is recognized by the anti-S2214 antibody, during this stage. To find out whether both of these proteins (the product found in adults and that found in larvae) are produced by *S2214*, the western blot profile of the wildtype third instar larvae was compared to that of a homozygous mutant *S2214* third instar larvae. Figure 3-3B shows that in the mutant flies, no protein is recognized by the antibody of the serum. Therefore, one can postulate that either both products are encoded by S2214, or a non-specific cross reaction, present in the wildtype third instar larvae, is coincidently abolished in the mutant larvae. The latter hypothesis seems quite unlikely, implying that anti-S2214 is specific to products of *S2214*, and it recognizes proteins produced at various stages of fly development.


A
Adult female
Adult male
3rd instar larvae
MW
GST-SAR1
GST
120 KD
85 KD
60KD
50KD
40KD
25KD

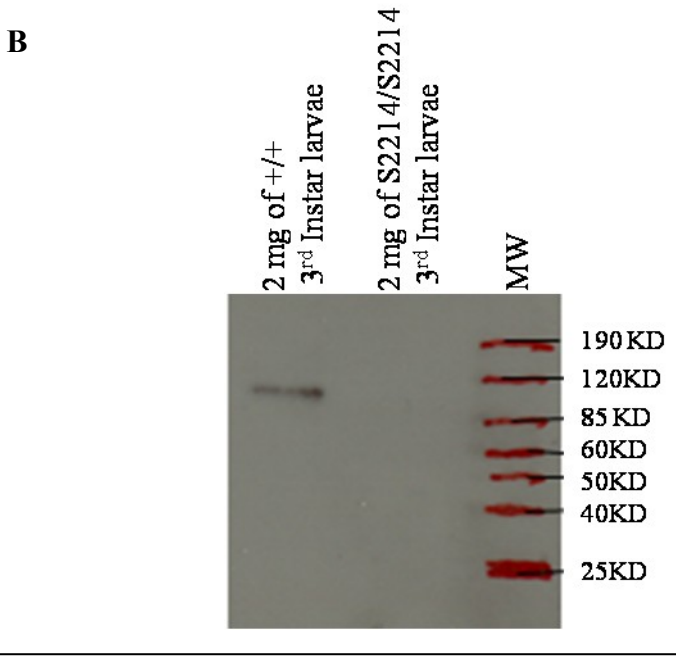

B
2 mg of +/+ 3rd Instar larvae
2 mg of S2214/S2214 3rd Instar larvae
MW
190 KD
120KD
85 KD
60KD
50KD
40KD
25KD

Figure 3-3 Anti-S2214 recognizes products at various stages of development

Proteins from whole organism extracts of wild type third instar larvae, male, and female flies were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. This membrane was then immunostained using the purified IgGs from the serum (A). A single product is detected in all stages. In male and female flies, the size of this detected protein corresponds to the expected size of S2214 (~103 KD), however, in larvae a product of slightly larger size (~115 KD) reacts to the purified IgGs (presumably anti-S2214). The latter product is absent in homozygous *S2214* third instar larvae, as evident on a western blot of larval extract, immunostained by purified IgGs from the serum (B).

3.2.4 Intracellular localization of S2214

Nuclear and cytoplasmic fractions of S2 cells (a late embryonic cell line), were prepared and their products were analyzed by Immuno-staining with the S2214 antibody. Western blot analyses revealed that S2214 is present in the nuclear fraction and can be detected by the antibody (figure 3-4A). Similarly, *in situ* staining of the salivary glands of wildtype third instar larvae also shows higher than average accumulation of anti-S2214 within the nuclei (figure 3-4B).

To be a direct modifier of chromatin structure, S2214 should be in contact with the chromatin fiber. It follows that if this interaction is not transient, one may expect to detect S2214 at specific loci on the chromatin. To test this assertion, I took advantage of the giant polytene chromosomes present in the salivary gland cells of *Drosophila*. These cells undergo repeated rounds of DNA replication without cell division, resulting in polytene chromosomes that consist of many sister chromatids that remain synapsed together. Due to their size, it is easier to detect proteins that may interact with chromatin.

Polytene chromosomes of wildtype female third instar larvae were immunostained with the serum containing anti-S2214. Results clearly demonstrate that S2214 localizes to interbands at many loci on the polytene chromosomes (figure 3-4 C and D). In addition to intense accumulation at some interband sites, less intense staining of S2214 appears to occur as dispersed staining throughout chromatin. The S2214 staining does not co-localize with HP1, an influential chromatin modifier. Nonetheless, it can be argued that S2214 fulfills the basic criteria of a direct modifier of chromatin.

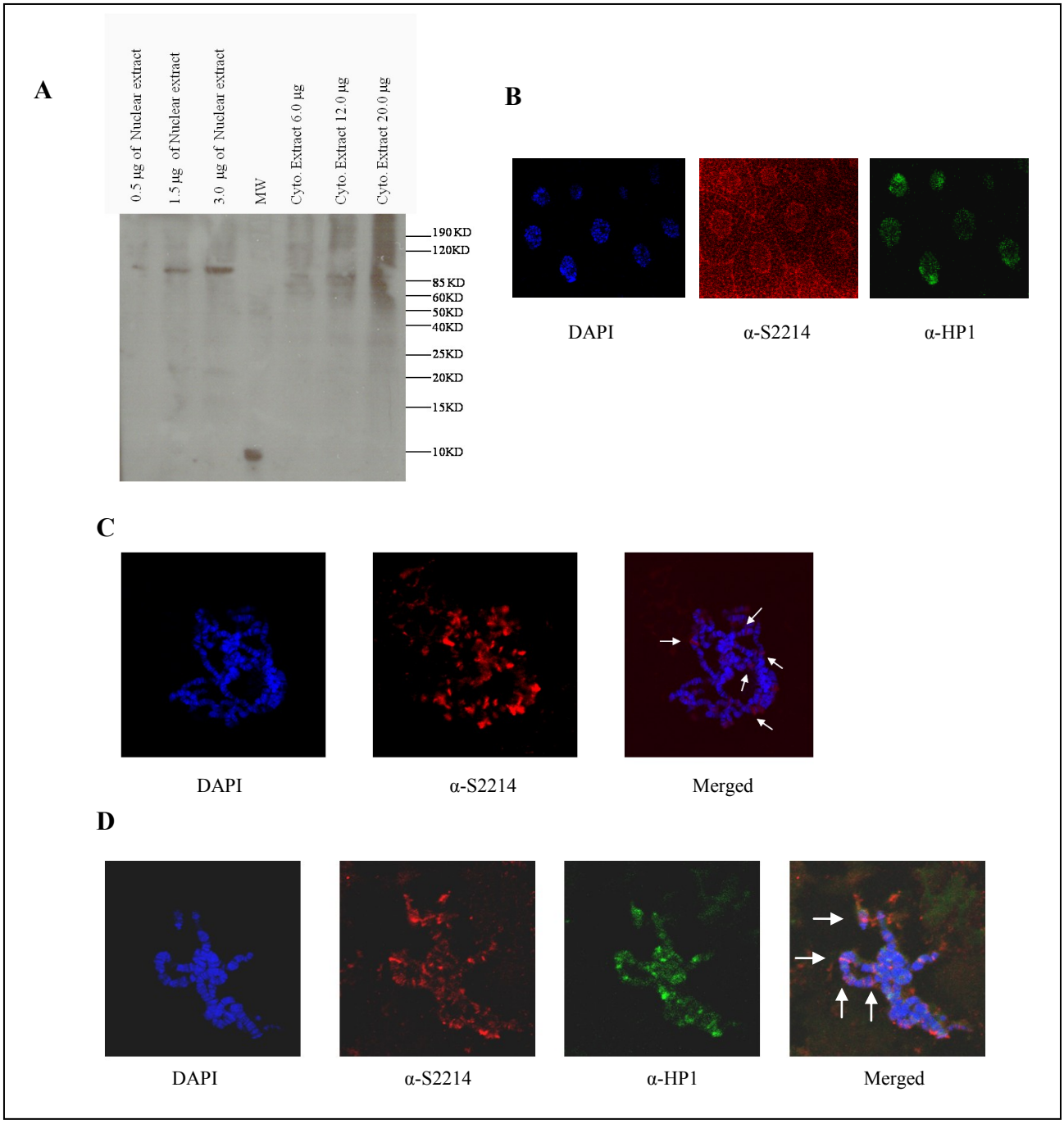

A
0.5 µg of Nuclear extract
1.5 µg of Nuclear extract
3.0 µg of Nuclear extract
MW
Cyto. Extract 6.0 µg
Cyto. Extract 12.0 µg
Cyto. Extract 20.0 µg
190 KD
120KD
85 KD
60KD
50KD
40KD
25KD
20KD
15KD
10KD
B
DAPI
α-S2214
α-HP1
C
DAPI
α-S2214
Merged
D
DAPI
α-S2214
α-HP1
Merged

Figure3-4 S2214 is present in the nucleus and localizes to the interbands of polytene chromosomes

Nuclear and cytoplasmic fractions of S2 cells, were separated by SDS-PAGE and blotted onto a membrane, and analyzed by Immuno-staining with the anti-S2214 IgGs in the serum, on a western blot. A product corresponding to the expected size of S2214 was detected in the nuclear fraction (A). Immuno-staining of the salivary glands of wildtype third instar larvae also shows accumulation of anti-S2214 within the nuclei (B). This distribution seem to coincide with the DAPI staining of the chromosomes, however, it does not show the same pattern of distribution as the HP1 (B,C, and D). In support of these, Immuno-staining of polytene chromosomes of wildtype third instar larvae demonstrate that S2214 localize to several interbands (identified by lower levels of DAPI staining; some highlighted by white arrows) and can be detected in lower amounts dispersed throughout chromatin (C and D). However, regions with high anti-S2214 signal do not overlap those of HP1.

3.3 Puc antigen production, antibody purification, and immuno-staining

Regions of Puc with high antigenesity were identified, through the use of bioinformatics softwares that employ algorithms to identify characteristics described in section 3.2 of this chapter. Three putative regions with high immunogenicity were identified. However, only one, denoted as Puc antigenic region 1 (PAR1), spanning from amino acids 258 to 308 was specific to both *Drosophila melanogaster* and Puc (figure 3-5A).

3.3.1 Puc antigen production

PAR1-GST fusion peptide was expressed in high levels in BL21 bacterial cell-lines, and was purified under native conditions using matrices with high affinity for the GST protein tag. Two proteins appear to be made by the engineered BL21 cells. The most prominent is a 33-34 KD protein (figure 3-5B). But a Second smaller, less plentiful protein (~27KD) is also present. Concentration of this protein seems to be dependent on the amount of purified Puc. All attempts to re-purify the 33KD PAR1 fusion peptide away from this smaller product have been unsuccessful. Therefore, I believe that this is a breakdown product of PAR and not a contaminant. No other contaminating proteins were detected even when high concentrations (>40 µg) of the purified peptide was analyzed. Hence the mixture of these proteins was used to elicit an immune response in rabbits.

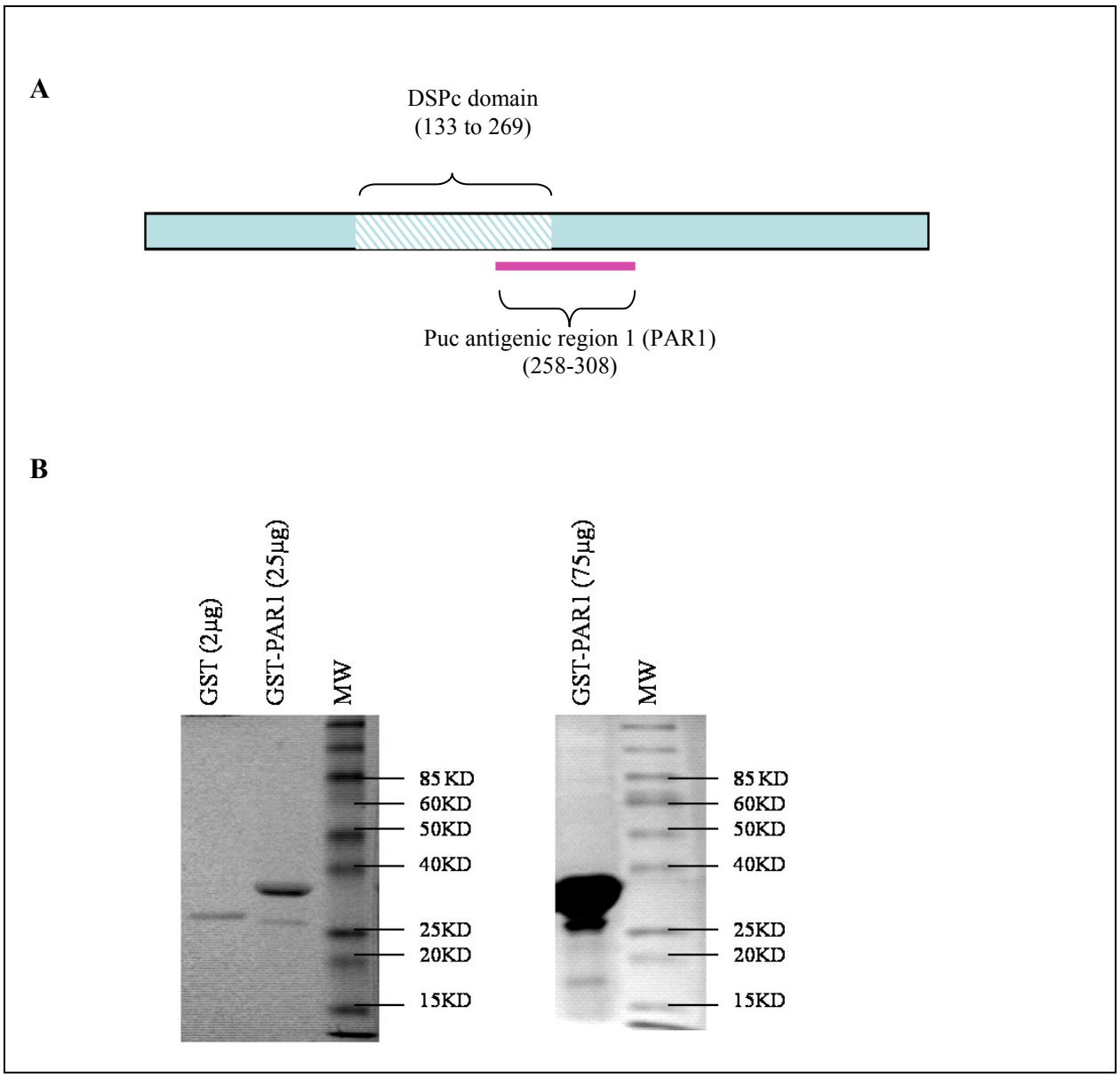


Figure 3-5 Expression and purification of GST-PAR1 peptide

A region unique to Puc and *Drosophila* with high antigenic potential (based on scores of hydrophilicity, surface potential, and flexibility) was selected (A). This Puc antigenic region (PAR1) spanning from amino acids 258 to 308 was fused at its N terminal with a GST protein tag. This fusion peptide was expressed in *E.coli,* and subsequently extracted and purified under native conditions. The purity of the antigen was analyzed through SDS-PAGE (B). Results show that even at high protein concentrations (>70 µg), no contaminating bands can be detected.

3.3.2 Purification of anti-Puc from the serum

Similar to the case of S2214, since the GST protein had not been cleaved prior to injection, the anti-Puc serum would contain antibodies to GST, S2214, as well as any bacterial contaminants that may have been carried over. Therefore, these unwanted antibodies were depleted the serum from using acetone powder prepared from BL21 cells that have expressed only GST. Subsequently, IgGs were purified from the serum and concentrated.

To test the success of the depletion process, I compared the ability of the serum to immuno-stain GST and GST-PAR1 fusion peptide, which was used as antigen. In addition, the ability of the depleted serum to recognize a different expressed fusion protein, consisting of an N terminal T7 tag and full length Puc protein, was also examined. The results of this set of experiments demonstrate that antibodies present in the serum recognize proteins whose sizes correspond to the GST-PAR1 (~33KD), and T7-Puc (~60 KD), while GST and T7 tags alone are not recognized (Figure 3-6).

In support of these results, a single product corresponding to the correct size of Puc (~51 KD) was recognized in wildtype third instar larvae (figure3-6). Taken together, the results suggest that the depletion process has successfully removed anti-GST as well as other antibodies raised to bacterial contaminants.

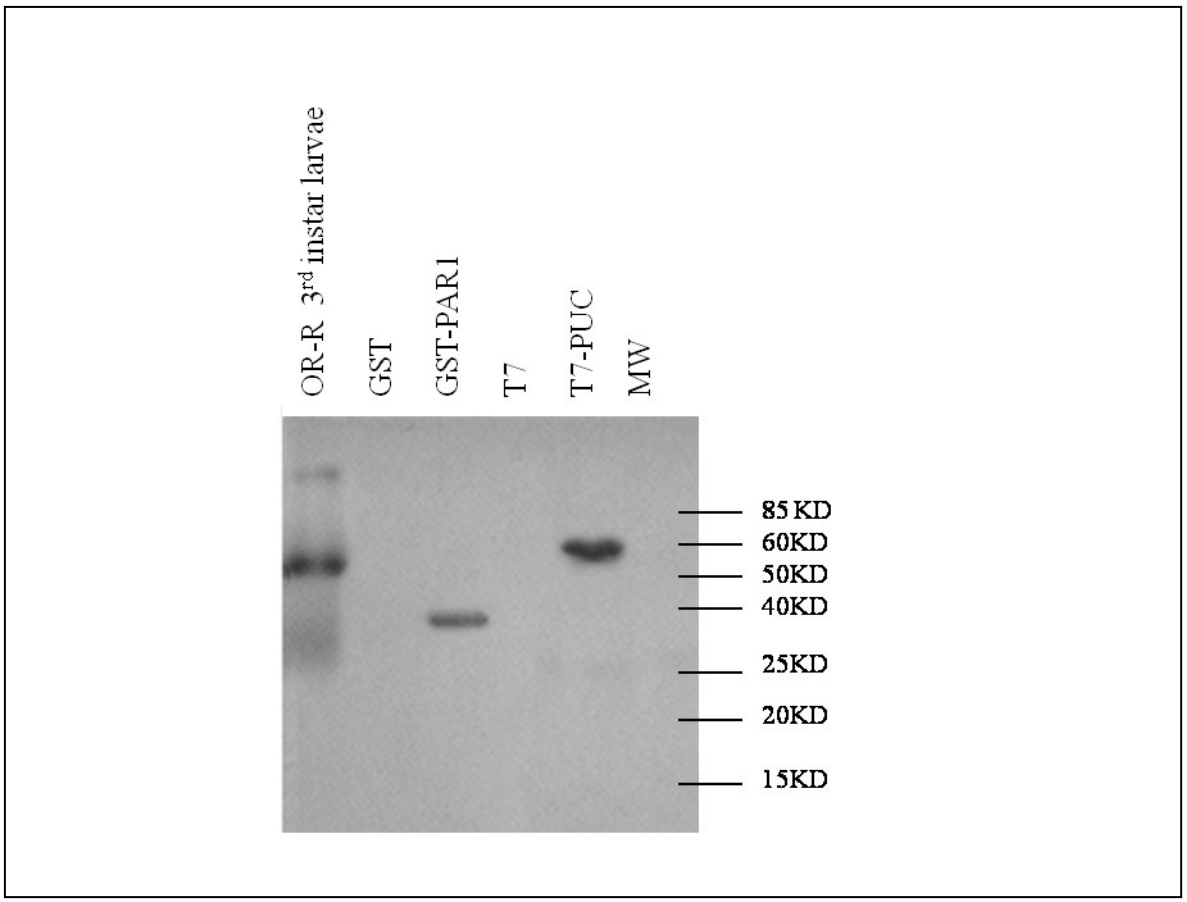


Figure 3-6 Anti GST depleted serum contains anti-Puc IgGs

Purified IgGs from the serum were tested for their ability to immunostain a western blot of bacterial extract from cultures that have expressed GST, GST-PAR1 fusion peptide, T7 tag, T7-Puc fusion protein, as well as larval extracts of a wildtype third instar larvae. No products interact with any of the purified IgGs in the GST and T7 lane. A single product corresponding to the correct size of GST-PAR1 (~33 KD) and T7-Puc (~60 KD) can be detected in the corresponding lane. Similarly, the antibodies purified from the serum react to a protein matching the expected 51KD size of Puc in the larval extract. Results suggest that the purified IgGs contain antibodies specific to Puc and are depleted from anti-GST as well as other unwanted bacterial antibodies.

3.3.3 Intracellular localization of Puc

To determine whether Puc can be found in the nucleus, immuno-localization experiments were carried out using S2 cells (grown in tissue culture), as well as salivary glands from wildtype third instar larvae. The results summarized in Figure 3-7 show that IgGs purified from the serum accumulate in the nucleus in both cell types. Moreover, regions of punctate staining within the nucleus can be observed, which may imply an enrichment of the Puc protein in some nuclear compartments. However, unlike HP1, Puc distribution does not seem to be focused around the DNA, instead, it is present throughout the nucleus. In support of this, I have been unable to detect Puc on the polytene chromosomes of the salivary glands of the wildtype third instar larvae, under a variety of immuno-staining conditions. These results suggest that either Puc does not directly interact with the chromatin fiber or that its interactions with chromatin are transient and as such escaped our detection.

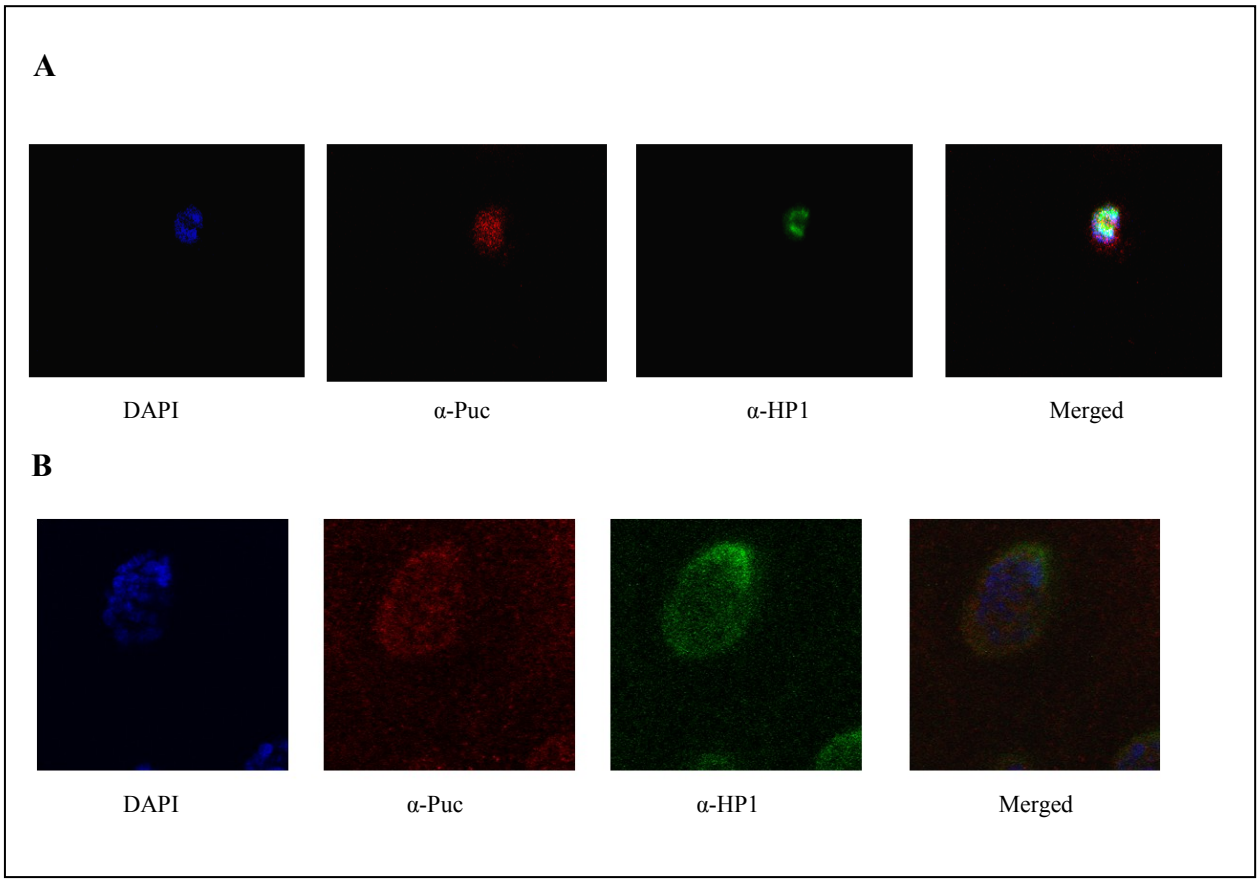


Figure 3-7 Puc is present in the nucleus

To ascertain whether Puc can be found in the nucleus, immuno-localization s of S2 cells (A), as well as salivary gland cells of wildtype third instar larvae (B) were carried out. IgGs purified from the serum accumulate in the nucleus in both cell types, and can be detected as punctate staining at various locations within the nucleus (A and B). However, unlike HP1, Puc distribution does not seem to be focused around the DNA (which is stained by DAPI).

3.4 Summary of results

Results presented in this chapter showed that antibodies to specific antigens of two Su(var) proteins were raised and purified successfully. Furthermore, these antibodies show good reactivity against denatured and native proteins with minimal background.

Furthermore, it was discovered that Puc localizes to the nucleus in late *Drosophila* tissue culture cells as well as in the nuclei of the *Drosophila* salivary glands. Although, it has not been detected on the chromatin fiber, several regions can be identified within the nucleus with punctate Puc accumulation. Further experiments will be necessary to dissect the exact location and function of Puc within the nucleus.

Data obtained using the S2214 antibody have been intriguing. It produces a single product in all stages of development; however, the protein found in the larval stage was slightly larger than that found in the adult. This protein is absent in the homozygous mutant larvae, and therefore, is likely not a contaminant.

Western blot analysis show that S2214 localizes to the nuclear fraction of S2 cells. Immuno-localization experiments show that S2214 is detected within the nucleus of both KC1 and S2 cells, as well as the nuclei of the wildtype third instar larvae. Furthermore, I found an intriguing distribution pattern for S2214 on the polytene chromosomes. Unlike, most other *Su(var)s,* S2214 predominantly localized to the interbands on poletene chromosomes. It should be noted that small amounts of protein at heterochromatic regions are also recognized by the antibody.

Chapter 4 Discussion and Future Directions

The experiments presented here have demonstrated that *S2214* and *puckered* are strong dominant suppressors of PEV. Although the mechanism by which PEV occurs may not be completely understood, various groups have shown that factors important in the gene silencing as was with PEV, such as HP1, Su(var)3-9, Hdac1, etc., are also important for the assembly of higher order chromatin. It follows that the products of *S2214* and *puckered* may also be important in either establishment or maintenance of chromatin structure and thus interact with other known modifiers of these mechanisms. However, we could not detect any interactions between *puckered* or *S2214* and other strong *Su(var)s.* It should be noted that we have only tested 3 other *Su(var)* mutants [$Su(var)2\text{-}5^{04}$, $Hdac^{326}$, and $Su(var)3\text{-}9^{06}$]. Although these mutants were selected due to their well defined effect on the chromatin, it is possible that either the assays selected were not sensitive enough to detect of the double mutant phenotypes, or *S2214* and *puckered* do not interact with these particular members of the chromatin architectural machinery. Alternately, *S2214* or *puckered* may act through pathways that require a different set of complexes than the modifiers tested. No specific conclusions can be made due to the limited scope of our analysis. Instead, I attempted to deduce the mode of action of these newly identified *Su(var)s* through their intracellular localization.

In the case of *puckered*, our analysis has revealed that the function of Puc as a modifier of PEV and may be distinct from its role in the JNK signaling pathway, since mutation in other members of the pathway did not show a dominant effect on PEV. A less likely alternative maybe that other members of JNK also influence this process, but the effects of their mutations are not as influential as that of *puckered.* In any case, these experiment highlight a new role for Puc.

Immuno-staining studies of Puc have demonstrated that it can be found throughout the nucleus but it cannot be detected on the polytene chromosomes. Furthermore, the punctate staining observed does not match that of HP1 (which is found on the DNA), instead they seem to localize to the nuclear periphery, which is also occupied by heterochromatic regions of chromosomes (Sedoni et al., 1999). It may be possible that Puc interacts with either chromatin or proteins involved in chromatin architecture at these regions of punctate staining. Some researchers have suggested that MAPK phosphorylation and de-phosphorylation is a transient affair, even in the continuing presence of the stimulus (Canman and Kastan, 1996). Thus, it may be plausible that Puc interacts with chromatin transiently.

Future experiments on Puc, will likely focus on identifying its targets among the members of chromatin machinery. Many kinases and phosphatases are present in the nucleus and modify histones and non-histone chromatin protein. However, relatively few phosphatases are capable of removing both serine/threonine and tyrosine bound phosphates. Kinney et al. (2008) discovered that MKP-1, a MAP kinase dual specificity phosphatase, de-phosphorylates H3S10 *in vitro*. Moreover, through substrate trap experiments, they also found that MKP-1 also shows high affinity for H2A, H2B, and H4, but was incapable of dephosphorylating any of their residues. It is likely, that another protein, such as Puc, with a DSP domain that has high similarity to that of MKP-1, also recognizes these histones and may be involved in modifying them. In addition to histones, many non-histone chromatin proteins are phosphorylated/de-phosphorylated. It follows that a phosphatase capable of modifying such non-histone proteins can affect higher order chromatin structure. Therefore, it would be logical to assay nuclear extracts for Puc interacting partners using immuno-precipitation. Moreover, since the catabolic domain of Puc has been characterized, mutant analysis of select residues may provide a valuable mutant enzyme that can be used for substrate traps.

Genetic analysis of *S2214,* I demonstrated that a significant proportion of *S2214* homozygous mutants do not survive to adulthood, suggesting an important role for this protein during the larval stages. These results, differ from those reported by Besto et al. (2006), where experiments to measure developmental and survival rates of the *S2214* mutants found that: "development proceeded with near normal timing and morphologically normal flies hatched at near normal rates". I cannot explain the difference in the data, however, examination of their data suggests that their primary objective has been to compare the developmental rates of the mutant and wildtype flies; as such our methods differ slightly.

In the course of their experiments, Basto et al. (2006) also noted that adult flies homozygous for *S2214 P* element mutation were morphologically normal but "uncoordinated" . Similarly, my observations suggest that *S2214* mutant flies that survive to adulthood are morphologically normal. However, the S2214 homozygous adults had no detectable coordination problems.

In an attempt to address the intracellular distribution of S2214, an antibody to a unique region of S2214 was developed. Subsequent Immuno-staining using this antibody revealed that S2214 can be detected in the nuclear fraction of S2 cells as well as the nuclei of the polytene chromosomes. Furthermore, a single product is present in larvae and adult stages. However, A slightly larger than expected product is presnt at the larval stage. This product is absent in the flies homozygous for the *P* element mutation. Therefore, one can postulate that either both products are encoded by S2214, or a non-specific cross reaction, present in the wildtype third instar larvae, is coincidently abolished in the mutant larvae. The latter hypothesis seems very unlikely, implying that anti-S2214 is specific to products of *S2214.* Moreover, further analysis of the genomic sequence with in-silico tools cannot detect any putative splice variants. In

addition, the S2214 amino acid sequence consists of residues that can be phophorylated, methylated, sumolyated, etc.; Therefore, it seems plausible that the apparent S2214 size difference observed between third instar and adults is due to post-translational modifications. It is noteworthy that Basto et al. (2006) have also reported that no protein could be detected in the brain of the third instar homozygous mutant. Curiously, Basto et al. (2006) report that the antibody they developed cannot detect any products on western blots. Therefore they have been unable to comment on the size of the detected protein(s).

Perhaps the most interesting discovery, outlined in this thesis, is the distribution pattern of the S2214 on the polytene chromosomes. It localizes to several interbands as well as other regions of the chromatin, suggesting s2214 may be a direct modifier of chromatin. Generally speaking, interband DNA appears to be decondensed and dispersed. Molecular analyses of several putative interbands indicate that these regions are rich in recognition sites for topoisomerases (Demakov et al. 1993). Moreover, several proteins that have been associated with interbands [such as H3S10 kinase (Jil-1), the elongating form of RNA Pol II, and various remodelling complexes] are factors that either help activate gene expression and/or maintain the active chromatin (Armstrong et al. 2002; Kaplan et al. 2000; Wang et al. 2001). Why would a suppressor of PEV keep such company?

One possibility is that S2214, similar to other Su(var)s that associate with euchromatin, such as Hdac1, performs a function that precedes the activity of other modifiers. However, an alternative theory may be derived from S2214 putative interacting partners. In the introduction to S2214 in chapter 1 of this thesis, I referred to the results of yeast two hybrid experiments, which have identified BEAF 32D, a boundary element associated factor, as a potential interacting partner for S2214. Intriguingly, BEAF32D also localizes to several interbands as

well as puff borders on the polytene chromosomes. It is possible, that the actions of S2214 are facilitated through interactions with BEAF 32D or other boundary associated proteins. Thus, further experiments of S2214 should explore the possibility of such interaction, perhaps through immuno-precipitation of nuclear fraction proteins.

Chapter 5 Materials and Methods

5.1 Fly crosses

All flies were crossed and experiments were kept at 25ºC unless otherwise specified.

P element stocks were obtained from Bloomington stock center (Indiana University, Bloomington, IN). *Su(var) 2-5*^{04}, and *Su(var)3-9*^{06} , and *Hdac*^{326}were obtained from colleagues at the lab. *P* element excision was performed using standard genetic methods and precise excisions were confirmed by sequencing.

5.2 Measurements of fly survival rates

Fly stocks were generated that contained either a wild-type (+) third chromosome or *S2214* mutant third chromosome heterozygous with a TM6B balancer that contained the dominant marker *Tubby* (*Tb*). +/TM6B,*Tb* or *S2214*/TM6B,*Tb* flies were self crossed and left at 25ºC for 5 days. At which time three sets of 5 males and 20 females for each genotype were placed in a cage. 100 eggs were placed on a 2 cm^2 blue paper and left in a vial for 48 hours. After removal, the number of embryos on the paper was counted to establish the ratio of embryonic lethality. The survival rates of the +/+ and *S2214/ S2214* larvae were measured by counting the number of non tubby larvae at each stage.

5.3 Bacterial culture growth

All bacterial cultures were grown in LB broth (10 g tryptone, 5 g of yeast extract, and 5 g of NaCl per 1 L of dH_2O) at 37°C. They were placed on orbital shakers, set at 250 RPM.

5.4 Transformation of bacterial competent cells

After the competent cells (DH10B for sub-cloning or BL21 for expression) have been thawed (on ice), an appropriate concentration of DNA is added to the cells and left on ice for 30 minutes. The cells were then heat shocked at 42°C for 90 seconds and immediately transferred to 37°C incubator for 30 minutes. Finally, the cells were spread on appropriate selection plates and left for 14-16 hours.

5.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All SDS-PAGE gels, unless specifically mentioned, were made so that they contained 10% acrylamide in the running portion and 4% acrylamide in the stacking portion. Electrophoresis was carried out at constant voltage of 100 volts for 90-120 minutes.

5.6 Western blotting and protein visualization

Solutions:

Transfer buffer:	10X PBS:	PBT:	All PBS used in this protocol is 1X PBS.
2.42 g Tris +11.17 g glycine + dH_2O to 667 ml with HCl bring PH to 8.0 + 200 ml methanol +dH_2O to 1 L	80 g NaCl + 2 g KCl + 11.5 g $Na_2HPO_4.7H_2O$ + 2g KH_2PO_4 + dH_2O to 1 L	0.1% Tween in 1 X PBS	

The protein bands from the SDS-PAGE gel were transferred to a "Hybond-C^+" membrane (from Amersham sciences) at a constant voltage of 21V overnight (~14 hours). Subsequently, the membrane was washed with dH_2O and placed in the blocking solution (5% skimmed milk in PBS) for 2 hours at room temperature. After a quick rinse in PBS, the 1° antibody was added, at 1:4000 of purified IgGs for anti-Puc and at 1:10000 for anti-S2214, in

20 mls of PBS + 0.25% skimmed milk and incubated for 2 hours at room temperature. The membrane was then washed in PBT 5 times, for 5 minutes each. The 2° antibody (Goat anti-rabbit HRP) was added, at a dilution of 1:5000, in 20 mls of PBS + 0.25% skimmed milk and incubated for 2 hours at room temperature. The membrane was washed 10 times for 5 minutes each in PBT, followed by three washes in PBS, and finally by two washes in dH_2O for 5 minutes each. The results were visualized through chemiluminescence.

5.7 Antigen purification

5.7.1 Preparation of bacterial sonicates

The BL21 cells carrying the pGEX-5x1 fusion plasmid was grown at 37°C for 12 hours at which time GST expression was induced by 0.15mM of IPTG. After 8 hours of expression, the culture was centrifuged 8,000 rpm in Beckman JA20 rotor for 10 minutes at 4°C to sediment the cells. The pellet was placed on ice and immediately completely re-suspended in 1.5 ml of ice-cold 1X PBS (recipe given above) per 1ml of culture. The cells were sonicated (on ice) for 3 x 15 seconds 15% until cell disruption was evident by partial clearing of the suspension. Subsequently, Triton X-100 was added to a final concentration of 1%. The solution was left on a lab shaker to mix gently for 30 minutes to aid in solubilization of the fusion protein. It was then centrifuged at 10,000 rpm in a Beckman JA20 rotor for 10 minutes at 4°C. The supernatant was transferred to a fresh container and saved for the batch purification.

5.7.2 Preparation of 50% slurry of glutathione sepharose 4B

1.33 ml of the original Glutathione Sepharose 4B slurry was added per ml of bed volume required. The gel was then sedimented by centrifugation at 500 x g for 5 minutes. The

supernatant was removed and the Glutathione Sepharose 4B was washed by the addition of 10 ml of cold 1X PBS per 1.33 ml of the original slurry. The gel was sedimented again by centrifugation at 500 x g for 5 minutes. Subsequently the supernatant was carefully removed. This resulted in a 50% slurry used in the next step.

5.7.3 Batch purification of glutathione S-transferase proteins

2 ml of the 50% slurry of Glutathione Sepharose 4B was added per 100 ml of bacterial sonicate. The mixture was left to gently rotate at room temperature for 30 minutes. The suspension was then centrifuged at 500 rpm for 5 minutes to sediment the gel. The supernatant was removed and the beads were washed three times with 10 bed volumes of 1X PBS. At this stage, 1.0 ml of Glutathione Elution Buffer (0.154 g of reduced glutathione dissolved in 50 ml of 50 mM Tris-HCl , pH 8.0) was added per ml of bed volume. The suspension was mixed gently at room temperature for 10 minutes to liberate the fusion protein from the gel, and centrifuged at 500 rpm for 5 minutes to sediment the gel. The supernatant (eluate) was transferred to a fresh centrifuge tube. This elution step was repeated 2 more times and all the eluates were pooled.

5.8 Antigen injection

The antigens were concentrated to 1 mg/ml in 1 X PBS. 1 ml of the antigen was added to a vial of RIBI adjuvant (currently known as MPL + TDM + CWS) and vortexed for 3 minutes. The rabbits were injected with the resulting emulsion. The injection protocol, administered by a representative from the UBC animal care unit, was 400 µl intradermally (100µl in each of 4 locations), 400 µl intramuscularly (200µl in each hind leg), 200 µl subcutaneously (neck region). Each rabbit was injected 4 times 28 days apart. This protocol was used for the initial injection as well as the three boosts.

5.9 Antibody purification

5.9.1 Preparation of the acetone powder

A 100 ml culture of BL21 *E.coli* containing a pGEX 5x1 (with a functioning *GST* gene) was grown at 37° C for 12 hours at which time *GST* expression was induced by 0.15mM of IPTG. After 8 hours of expression, the culture was centrifuged at 8,000 rpm in Beckman JA20 rotor for 10 minutes at 4°C to sediment the cells. The pellet was placed on ice and immediately completely re-suspended in 2 ml of ice-cold 1X PBS (recipe given above) per 1ml of culture. The cells were sonicated (on ice) for 3 x 20 seconds 15% until cell disruption was evident by partial clearing of the suspension. The suspension were transferred to ice for 5 minutes. Subsequently, 4 x volumes of acetone (pre-chilled to -20 ° C) was added to the suspension, vortexed for 2 minutes, and left on ice for 1 hour (with intermittent vigorous mixing). The pellet was precipitated at 10,000 rpm for 10 minutes. 4 x volumes of acetone were added again, the mixture was left on ice for 10-15 minutes prior to centrifugation at 10,000 rpm for 10 minutes. The resulting pellet was spread on a filter paper to dry. Once dry, the clumps are made into a fine powder with mortar and pestle.

5.9.2 Depletion of serum from anti-GST

5 g of acetone powder (of BL21 induced for GST expression) was added to 10 ml of serum. The mixture was diluted to 50 ml with 1x PBS and left rotating gently at 4°C for 3 hours, before centrifugation at 8,000 rpm in Beckman (JA20 rotor) for 10 minutes. The pellet (containing bacterial proteins and antibodies) were removed. This process was repeated 3 times with the supernatant.

5.9.3 Fractionation of rabbit IgG

Diluted and depleted serum was placed on ice for 30 minutes to separate the lipid phase. 5 g of ammonium sulphate was added to 15 ml of the serum, in small batches, and mixed at 4°C. The mixture was neutralized with 5 µl of 1M NaOH. Solution continued mixing for 2 hours at 4 °C, prior to centrifugation at 10,000 rpm in Beckman (JA20 rotor) for 20 minutes. The precipitate was solubilized in 1.5 ml of ddH_2O, and dialyzed against 2L of 1xPBS containing 5% glycerol overnight at 4°C. The dialysate was centrifuged at 10,000 rpm for 10 minutes at 4°C. 18% (w/v) Na_2SO_4 was added to the supernatant and left rotating at room temperature for 30 minutes. The mixture was centrifuged at 8,000 rpm for 20 minutes at room temperature. The precipitate was solubilized in 1.5 ml of ddH_2O, and dialized against 1L of 1xPBS containing 25%glycerol and 0.04% sodium azide. Finally the dialysate was centrifuged at 10,000 rpm for 10 minutes at 4°C to remove any impurities. The IgGs were stored at -80°C for future use.

5.10 Immunostaining

5.10.1 Cell lines

Cells were grown on a coverslip (3 wells) to a density of 5,000-10,000 per well. They were washed twice with PBS^{2+}(containing 0.9 mMCaCl2, 0.52 mMMgCl2 and 0.16 mM MgSO4). Cells were then fixed for 20 minutes with 4% formaldehyde at room temperature and washed 3 times with PBS^{2+}. Subsequently, the cells were treated by 0.2 % Triton-X-100 for 10 minutes to permeabilize them. They were washed 3 more times with PBS^{2+}. Cells were then blocked in PBS^{2+} with 1%BSA for 20 minutes, and then incubated for 1 hour with100 µl of primary antibody dilution inPBS^{2+} with 1%BSA (1/500 for anti-HP1 (made in mouse), 1/500 for

anti-Puc, 1/1000 for anti-S2214). Following another three times wash with PBS^{2+}, the cells were incubated for 1 hour with 100 µl of secondary antibody dilution in PBS^{2+} with 1%BSA (1/1000 Alexa 488 goat anti-rabbit for green florescence, and 1/1000 Alexa 568 for red goat anti-mouse florescence). The cells were washed five times with PBS^{2+} and covered by Invitrogen's VECTASHIELD and examined by a confocal microscope.

5.10.2 Salivary glands

Glands were dissected in 1xPBS containing 1% triton and protease inhibitors. The glands were fixed in a 5.4% formaldehyde solution (diluted with 1xPBS) for 20 minutes at room temperature. The glands were washed five times with PBS^{2+}. They were then blocked in $PBS^{2+}$with 1%BSA for 1 hour at room temperature. Subsequently, the glands were incubated for 1 hour with100 µl of primary antibody dilution inPBS^{2+} with 1%BSA (1/500 for anti-HP1 (made in mouse), 1/500 for anti-Puc, 1/1000 for anti-S2214). They were washed five times with PBS^{2+}, and were then incubated for 1 hour with 100 µl of secondary antibody dilution in PBS^{2+} with 1%BSA (1/1000 Alexa 488 goat anti-rabbit for green florescence, and 1/1000 Alexa 568 for red goat anti-mouse florescence). The glands were placed on a slide, surrounded by a 1 mm spacer (made of cutting from a coverslip). They were covered with Invitrogen's VECTASHIELD, placed under a coverslip and examined by a confocal microscope.

5.10.3 Polytene chromosomes

Wandering third instar larvae were selected and chromosome squashes were prepared as previously described by Cavalli (http://www.igh.cnrs.fr/equip/cavalli/link.labgoodies.html; adapted from Zink and Paro, 1995). Slides with the chromosomes fixed to them, were washed twice with 1x PBS, and blocked for 1 hour in 1xPBS with 1%BSA. 20 µl dilution of the 1º

antibody was applied to each slide in the vicinity of the chromosome squashes (1:100 for anti-Puc and anti-HP1, 1:300 for anti-S2214). They were incubated with the 1º antibody for 20 minutes at room temperature. The slides were washed three times with 1x PBS containing 300mM, 400mM, and 400mM respectively. They were then stained with a 20 µl dilution of the 2º antibody for 20 minutes at room temperature (1/300 Alexa 488 goat anti-rabbit for green florescence, and 1/300 Alexa 568 for red goat anti-mouse florescence). This was followed by three 1x PBS washes containing 300mM, 400mM, and 400mM respectively. The slides were covered with Invitrogen's VECTASHIELD, placed under a coverslip and examined by a confocal microscope.

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