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A regulatory subunit o f Protein Phosphatase 2A, PP2A-B', is dispensable for activation of Sex Comb Reduced activity in Drosophila melanogaster

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A regulatory subunit of Protein Phosphatase 2A, PP2A-B′, is dispensable for activation of Sex Comb Reduced activity in *Drosophila melanogaster*

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Abstract

The Drosophila HOX transcription factor, Sex Combs Reduced (SCR), is required for determining labial and the first thoracic segmental identity. A Protein Phosphatase 2A holoenzyme assembled with the PP2A-B′ regulatory subunit has been proposed to specifically interact with and dephosphorylate SCR homeodomain activating SCR protein activity. To test this hypothesis, a null mutation was created in the *PP2A-B′* gene, *PP2A-B′*$^{\Delta}$, using Flip mediated site-specific recombination. The number of sex comb bristles, salivary gland nuclei and pseudotracheal rows are SCR-dependent and were counted as a measure of SCR activity *in vivo*. Adults and larvae homozygous for *PP2A-B′*$^{\Delta}$ showed no decrease in SCR activity. In addition no evidence of functional redundancy of PP2A-B′ with other regulatory subunits, Twins (TWS) and Widerborst (WDB) for dephosphorylation and activation of SCR activity was observed suggesting that PP2A has no role in activation of SCR activity.

Key words:

Protein Phosphatase 2A (PP2A), PP2A-B′, HOX protein, Widerborst, Twins, dephosphorylation, *PP2A-B′*$^{\Delta}$, gene knockout, and FLP/FRT technique.

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List of Abbreviations:

ANTP: Antennapedia

DAPI: 4',6-diamidino-2-phenylindole

DIG: Digoxigenin

DSH: Dishevelled

FCP: Fyn Carboxyl-Terminal Peptide Phosphatase

FISH: Fluorescent in situ hybridization

FMI: Flamingo

HD: Homeodomain

Mts: Microtubule star

Ph: Polyhomeotic

PKA: Protein Kinase A

PP2A: Protein Phosphatase 2A

PP2A-B′: Protein Phosphatase 2A-B′

PP2A-B′$^{\Delta}$: Protein Phosphatase 2A-B′ deletion allele

PPP: Phosphoprotein Phosphatase

PPM: Phosphoprotein M

SCR: Sex Combs Reduced

S T antigen: Small T Antigen

TWS: Twins

WDB: Widerborst

Chapter One

Introduction

The Central Problem in Development

The central problem in development is often stated as "how is the information stored in linear DNA transformed over time into a three dimensional multicellular organism?" This statement encompasses two important themes in the study of development. First, development is a complex spatial and temporal process through which a single-celled, fertilized egg is transformed into a multicellular organism containing cells specialized for specific biological functions. Second, that the process of development is linked to complex temporal and spatial control of gene expression. The past thirty years have witnessed an explosion in knowledge of the mechanisms that control development.

Drosophila, a Model Organism

Development is studied in many organisms such as mice, frogs, fish, flies and worms. The major advantage of studying development in vertebrate systems is the close relatedness to humans. However, vertebrate systems have a number of disadvantages, such as a long generation time, limited number of progeny and the existence, through at least one genome duplication, of a high number of protein isoforms (McLysaght et al. 2002). Drosophila on the other hand has a short generation time, large number of progeny and a smaller number of protein isoforms. In addition, Drosophila is a more sophisticated genetic system than any vertebrate model system and Drosophila has all the technical advantages that come with the knowledge of the complete sequence of the genome.

Hox Genes

One of the most important sets of mutations identified in Drosophila is mutations in the homeotic genes. In a homeotic mutant, one body part is transformed into the likeliness of another. This mutant phenotype clearly implicates the products of homeotic genes being involved in major developmental decisions. In the early 1980's, the DNA encoding homeotic genes was identified and analysis of the DNA encoding homeotic genes revealed three characteristics. First, the eight homeotic genes shared a common DNA sequence, the homeobox, which encodes the homeodomain (HD). Second, homologues of Drosophila homeotic genes are found in all animals including vertebrates where they are also important for development. Third, the proteins encoded by the homeotic genes are transcription factors that bind DNA through the homeodomain (Gehring and Hiromi 1986).

There are two major problems associated with how HOX proteins function. First, the analysis of the functional structure of HOX protein has proven difficult and often contradictory. For example, the conserved QA motif of Ultrabithorax (UBX) is required for limb repression when the protein is expressed in all cells (Vachon et al. 1992; Galant and Carroll 2002), but is not required when deleted from the *Ubx* locus (Hittinger et al. 2005). Second, although the HD is required for function of all HOX proteins tested thus far, how HOX proteins are able to specify specific morphological structures when the HDs of all HOX proteins recognize the same sequence has proven difficult to explain. Although interaction with the HOX cofactor Extradenticle (EXD) is often used to explain HOX specificity (Chan et al. 1994; Chan and Mann 1996; Pinsonneault et al. 1997), EXD is not required for determination of all structures in *Drosophila melanogaster* (Percival-

Smith and Hayden 1998). Phosphorylation has been proposed to be a mechanism for controlling the activity of the HOX proteins Antennapedia (ANTP) and Sex Comb Reduced (SCR) (Jaffe et al. 1997; Berry and Gehring 2000). In this thesis, I test whether SCR is dephosphorylated and activated by the protein phosphatase PP2A assembled with PP2A-B′ regulatory subunit.

Protein Phosphorylation

Proteins are involved in almost every aspect of cellular life from regulation of gene expression to control of cell division or apoptosis. Precise regulation of protein activity is often maintained by a variety of post-translational modifications such as addition of a phosphate, methyl, glycosyl or carbohydrate group to alter the protein's activity and function (Wold 1981). Phosphorylation, the addition of a phosphate group to proteins, occurs on specific amino acid residues. The most common phosphorylated amino acid residues are serine, threonine and tyrosine. The phosphate group is added by kinase enzymes and removed by phosphatase enzymes. Most proteins are reversibly phosphorylated unless phosphorylation targets the protein for degradation. Reversible phosphorylation of proteins has an important role in different cellular processes such as the control of signal transduction pathways, apoptosis and aging (Shenolikar 1986). Protein kinases and phosphatases are classified into different groups based on their structural and functional properties (Cheek et al. 2005; Barford et al. 1998). Two major groups of phosphatase enzymes are the serine/threonine and tyrosine phosphatases. The serine/threonine phosphatases are divided into distinct groups based on the amino acid sequence similarity of the catalytic subunit (Barford et al. 1998).

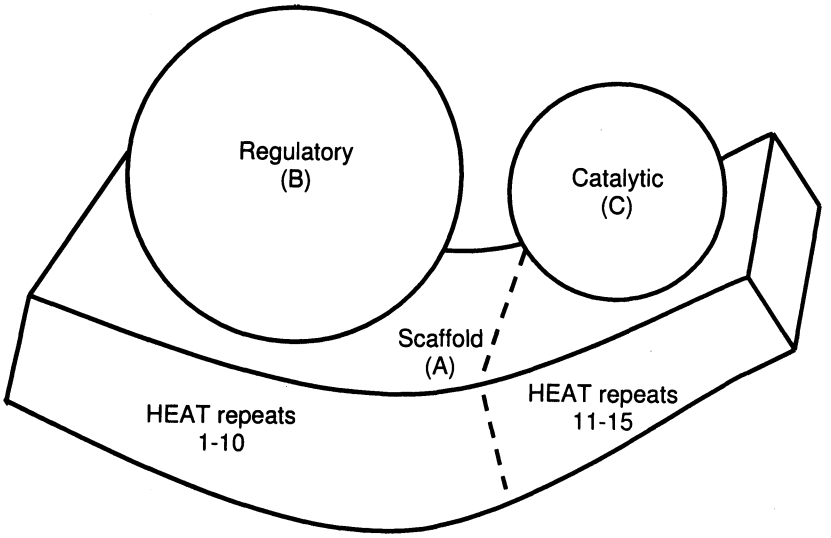
Classification of Protein Phosphatase Family

The serine/threonine phosphatases are classified into three structurally distinct families called Phosphoprotein Phosphatase (PPP), Phosphoprotein M (PPM) and Fyn carboxyl-terminal peptide (FCP) phosphatase (Cohen 2004). This classification is based on the amino acid sequence of their catalytic subunit. The PPP family is the largest serine/threonine phosphatase family composed of seven subfamilies: PPP1, PPP2A (PP2A), PPP3 (PP2B/Calcineurin) and PPP4 to PPP7 (Cohen 2004). With the exception of PPP5 and PPP7, which are monomeric phosphatase enzymes of the PPP family, protein phosphatases of the PPP family are structurally complex enzymes composed of multiple subunits (Cohen 2004). Often each subunit has a variety of different isoforms. The Protein Phosphatase 2A (PP2A) is a multi-subunit enzyme (Tamura et al. 1980; Tung et al. 1985), which is expressed in a variety of tissues (Arino et al. 1988; Hemmings et al. 1990) and evolutionary conserved in eukaryotes (Kinoshita 1990; Sneddon et al. 1990; Orgad et al. 1990; Cormier et al. 1991; Van Hoof et al. 1995; MacKintosh et al. 1990; Arino et al. 1993)

The PP2A Phosphatase

The PP2A phosphatase enzyme is composed of three distinct protein subunits: the scaffold (A), the catalytic (C) and the regulatory subunits (B) (Figure 1-1; Tamura et al. 1980; Tung et al. 1985). The catalytic (C) and scaffold (A) subunits form the core complex (AC), which interacts with a variety of regulatory subunits (B, B′ and B″) (Janssens and Goris 2001). It is thought that different regulatory subunits

Figure 1-1. A schematic of the structure of the PP2A holoenzyme (Cho and Xu 2007). The A subunit is the scaffold subunit, which has a curved structure. The regulatory subunit (B) and catalytic subunit (C) interact with the concave side of the scaffold subunit. The catalytic and regulatory subunits interact with specific HEAT repeats of the A subunit as shown in the figure.



PP2A Holoenzyme

confer substrate specificity to PP2A such that PP2A can make a broad contribution to many cellular processes, anywhere from regulating cytoskeleton protein assembly (Lin and Arndt 1995; Nunbhakdi-Craig et al. 2003), signal transduction pathways (Millward et al. 1999) to control of the cell cycle (Picard et al. 1989). The characteristics of the three subunits of PP2A will be discussed in the following.

1) The Scaffold Subunit (PR65)

The scaffold protein, protein A subunit or PR65, is a 65 kDa protein that acts as a scaffold protein to allow the interaction between the regulatory and catalytic subunits. The scaffold protein has a hook-like structure (Groves et al. 1999) and is composed of 15 HEAT repeats. HEAT repeats were first found in Huntingtin, Elongation factor 3, the A subunit of PP2A and TOR1 (Andrade and Bork 1995). HEAT repeats fold to form a structure consisting of two anti-parallel α-helixes (Groves et al. 1999). The common feature of these repeats is a specific arrangement of hydrophobic and uncharged amino acids (Andrade and Bork 1995). These hydrophobic sites facilitate the interaction of the catalytic and regulatory protein subunits with protein A (Groves et al. 1999). The catalytic and regulatory subunits interact with the inner part of the curved protein A. The regulatory subunit interacts with the first ten HEAT repeats and the catalytic subunit interacts with the last 5 HEAT repeats (Ruediger et al. 1994). Two genes encode two isoforms for the A subunit in mammals (Aα and Aß) (Hemmings et al. 1990), but only one gene encodes protein A subunit in yeast (van Zyl et al. 1992; Stark 1996) and Drosophila (Mayer-Jaekel et al. 1992).

2) The Catalytic Subunit (PP2A-C)

All serine/threonine protein phosphatases of the PPP family share high degree of amino acid sequence similarity in the catalytic subunit (Goldberg et al. 1995; Cohen 2004). High degree of amino acid sequence conservation of the catalytic subunit of each PPP subfamilies (such as PP2A) is also observed among species (Sneddon et al. 1990; Stone et al. 1987; Arino et al. 1988). The catalytic subunit of the PP2A enzyme is composed of a series of α helixes and ß-pleated sheets. Interaction of two manganese ions with the catalytic subunit of PP2A-C generates a functional catalytic subunit. Despite high structural and amino acid sequence similarities between the catalytic subunit of PP2A with PPP1, 5 and 7 there are significant local differences in the structure of the catalytic subunit which makes interaction of the scaffold and regulatory subunits highly specific to PP2A-C subunit (Goldberg et al. 1995; Xing et al. 2006).

Post-translational modifications such as phosphorylation and methylation of the catalytic subunit regulate PP2A activity. Phosphorylation of the catalytic subunit by tyrosine kinases inhibits the phosphatase activity of PP2A (Chen et al. 1992). Methylation of the C terminal sub-domain of the catalytic subunit alters the ability to interact with different regulatory subunits. Interaction of the regulatory B subunit (PR55) with the catalytic subunit is dependent on methylation of six highly conserved residues on the C terminal domain of the catalytic subunit (Orgis et al. 1997; Longin et al. 2007). However, interaction of the B′ regulatory subunits with the catalytic subunit is not dependent on methylation or presence of the six conserved residues on the C terminal arm of the catalytic subunit (Longin et al. 2007; Xu et al. 2006). The results are

conflicting for the interaction of the B″ regulatory subunits with methylated or unmethylated catalytic subunit (Longin et al. 2007; Xing et al. 2006).

In contrast to mammals and yeast, which have two genes that encode PP2A catalytic subunits (Arino et al. 1988; Khew-Goodall et al. 1991; Sneddon et al. 1990), only one gene encodes the catalytic subunit in *Drosophila melanogaster* (Orgad et al. 1990; Mayer-Jaekel et al. 1992). The gene encoding the PP2A-C subunit in *Drosophila melanogaster* is called *microtubule star (mts)* (Snaith et al. 1996). *MTS* mRNA transcribed from the maternal genome during oogenesis and deposited in the egg is present at stages 1 to 3 of embryogenesis before initiation of transcription of the zygotic genome. The maternal mRNA is partially degraded during stages 7 to 9 of embryogenesis. *MTS* mRNA is then expressed from the zygotic genome (Orgad et al. 1990; Lecuyer et al. 2007). Expression of *microtubule star* is essential for the proper cell division; the *mts* mutants are embryonic lethal due to disruption in the formation of the mitotic plate and spindle and arrest of mitosis (Snaith et al. 1996).

3) The Regulatory Subunits of PP2A

A variety of protein subunits interact with the AC dimer of PP2A enzyme to regulate its function. These regulatory subunits are classified into three groups based on their sequence similarity: PR55 (B), PR61 (B′) and PR72 (B″) (Janssens and Gris 2001). In mammals, each regulatory subunit has multiple isoforms. Regulatory subunit isoforms in mammals and Drosophila are listed in Table 1-1. Interaction of the regulatory proteins with the AC complex is essential for the formation of a functional and

Table 1-1. Classification of PP2A subunits in humans and *Drosophila melanogaster*.

Subunit Family	Isoform(s) in humans	Isoform(s) in *Drosophila melanogaster*
PP2A C	C	Microtubule star
PR65	Aα	Pp2A-29B
	Aβ	
B/ PR55	Bα	Twins
	Bβ	
	Bγ	
	Bδ	
B′ /PR61	B′ α	Widerborst
	B′ β	PP2-A-B′
	B′ γ 1,2,3	CG32568
	B′ δ 1,2,3	
	B′ ε	
B″/PR72	PR72, PR130	CG 4733
	PR70	
	PR48	
	G5PR	
	mPR59	

stable PP2A holoenzyme. In Drosophila S2 culture cells, loss of any of the regulatory subunits results in loss of the catalytic and scaffold subunit of PP2A, which implies that interaction of regulatory subunits with the AC dimer increases the stability of the protein complex (Silverstein et al. 2002; Strack et al. 2002). Also, it has been shown that regulatory subunits that fail to interact with the AC dimer are quickly degraded by ubiquitin-dependent proteolysis (Strack et al. 2002). In the following, individual members of the B subunit sub-families are described in detail.

3-1) The PR55 Regulatory Subunit (B)

In mammals, the PR55 (B) family has 4 isoforms (α, β, γ and δ). Although PR55 isoforms are almost 80% identical, they have distinct functions and expression patterns. While the Bα and Bδ isoforms are expressed ubiquitously, Bγ is expressed specifically in brain and the Bβ isoform is expressed in brain and testis of mice (Schmidt et al. 2002). Studying the primary structure of the B regulatory subunit revealed several WD40 amino acid repeats. The WD40 repeats are about 40 amino acids long that typically end with the tryptophan–aspartate (W-D) amino acids (Smith et al. 1999; Schmidt et al. 2002). Each WD40 repeat forms four-stranded anti-parallel β sheet and each four-stranded β sheet forms a blade. All seven beta-sheet blades are arranged around a central axis, forming a doughnut-shape protein (Strack et al. 2002; Xu et al. 2008). Protein mutagenesis studies and the molecular structure of the B regulatory subunit revealed that the critical sites for the B subunit interaction with the AC dimer is within WD40 repeats 2-4 (Strack et al. 2002; Xu et al. 2008).

The gene encoding the B regulatory subunit in fruit flies was identified in a genetic screen for mutants that disrupt the formation of imaginal discs (Uemura et al. 1993). In

fruit fly larvae homozygous for a mutation in the gene encoding the B regulatory subunit, wing discs are duplicated and the larvae die soon after pupal stage. Because of the duplication of the wing imaginal discs, the gene encoding the B regulatory subunit is called *twins (tws).* A second allele in the *twins* gene was identified independently and named abnormal anaphase resolution (tws^{aar}) (Gomes 1993). Flies homozygous for the tws^{aar1} mutation fail to complete mitosis due to several mitotic abnormalities during metaphase and anaphase. More *tws* mutant alleles have been isolated by mobilizing the P element in the *tws* gene (Shiomi et al. 1994). The tws^{55} allele is an adult lethal mutation. Most of the flies with the tws^{55} mutation emerge from the pupae but die immediately because of walking, jumping and flying defects. These flies also exhibited duplication of the external sensory organs. The external sensory organs cover most of the fruit fly body; the bristles on the fly are part of external sensory organs. The majority of external sensory organs in flies are composed of three support cells surrounding a sensory neuron cell (Jan and Jan 1990). A pair of the support cells forms the external sensory organ structures, the shaft and the socket, and the other support cell wraps around the sensory neuron cell. The shaft is the hair or bristle and the socket is a ring-shaped cuticular structure that wraps around the shaft base. In homozygous tws^{55} flies, often two bristles grow out of a sensory organ and the shafts are wrapped with two fused sockets. This phenotype is probably caused by transformation of the neuron and sheath cell to support cells. The molecular mechanism of this transformation is not known yet (Shiomi et al. 1994). In summary, PR55 or the B regulatory subunit activity is required for imaginal disc patterning, control of the cell cycle, mitosis and external sensory organ development.

3-2) The PR 72 Regulatory Subunit (B″)

The B″ regulatory subunit family is represented by six protein isoforms in mammals (Table 1-1). Members of the B″ protein family share two conserved domains that interact with the A subunit of PP2A, EF-1 and EF-2 (Janssens et al. 2003). These conserved domains are consensus Ca^{2+} binding EF-hand motifs. Mutation in the EF-2, the EF-hand motifs of PR72, resulted in both loss of Ca^{2+} binding affinity and loss of interaction with the A subunit of PP2A. Therefore, it is proposed that Ca^{2+} is required for assembly of the regulatory subunit B″ with the PP2A core enzyme (Janssens et al. 2003). The PR72/B″ protein family is involved in a variety of different pathways in mammals such as regulation of the Wnt pathway (Creyghton et al. 2005; Creyghton et al. 2006), regulation of the cell cycle (Yan et al. 2000), control of cell differentiation (Miyabayashi et al. 2007) and interacting with calcium channels in the cardiovascular system and brain cells (Marx et al. 2001; Hall et al. 2006). A homologue of PR72, *CG4733,* was identified in *Drosophila melanogaster* (Li et al. 2002); however, not much is known about the role of *CG4733* in Drosophila.

3-3) The PR61 Regulatory Subunit (B′ or B56)

The nine mammalian PR 61 isoforms (α, β, γ1,2,3, δ1,2,3 and ε) are encoded by 5 genes (Table 1) (McCright and Virshup 1995; Csortos et al. 1996; Tehrani 1996; McCright et al. 1996; Zolnierowicz et al. 1996; Eichhorn et al. 2009). These isoforms are expressed in different tissues and the proteins are localized in the nucleolus or cytoplasm. The regulatory subunit B′ α and B′ γ are expressed in heart and muscles while B′ β and B′ δ and B′ ε are expressed in brain (McCright and virshup 1995; Tehrani et al. 1996; McCright et al. 1996a; McCright et al. 1996b).

The crystal structure of the B′ regulatory subunit and the structure of B′ in association with PP2A core subunits have been determined. The B′ regulatory subunits have eight HEAT like repeats in their amino acid sequence alignment (Xu et al. 2006; Cho and Xu 2007). The crystal structure of the B′ γ-1 regulatory subunit protein revealed 18 α-helixes that interact to form a cupped structure. The concave pocket of the B′ γ-1 regulatory protein is mainly composed of negatively charged amino acids and the convex side of the protein is composed of hydrophobic amino acids, which interact with the scaffold A subunit (Xu et al. 2006).

The regulatory subunit of B′ or B56 is involved in different cellular processes. The PP2A complex associated with the regulatory subunit B56γ acts as a tumour suppressor upon infection with SV40 virus (Chen et al. 2004). The small t (ST) antigen of SV40 induces cell transformation by targeting and replacing B′ /B56γ in the PP2A complex. The other isoform of PR61, B56ε, is required for dorsal development and Wnt-dependent accumulation of β-catenin protein in Xenopus. Loss of B56ε causes disruption in the formation of midbrain-hindbrain boundary, neural tube closure and head formation of Xenopus (Yang et al. 2003). In Drosophila the B′ regulatory subunits are encoded by three distinct genes called *widerborst (wdb), PP2A-B′* and *CG32568* (Table 1-1). Several studies have reported specific roles for WDB and PP2A-B′, but not much is known about the *CG32568* gene product which was first identified in a microarray analysis screening for genes expressed in Drosophila testis (Andrews et al. 2000).

3-3-1) Widerborst (B56-2):

widerborst (wdb) was identified in a genetic screen for the genes affecting formation of cell planar polarity in *Drosophila melanogaster* (Hannus et al. 2002). Planar

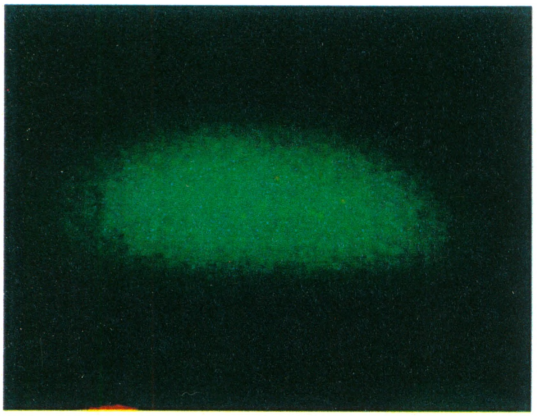
cell polarity, or tissue polarity, is essential for development of an organism during gastrulation (Heisenberg et al. 2000; Wallingford et al. 2000) and also for differentiation of tissues with specific cell orientation (Eaton 1997). Cellular studies revealed that the planar cell polarity mechanism is conserved from flies to humans (Fanto and McNeill 2004). One example of planar cell polarity in fruit flies is the formation of distally pointed bristles on the body and the wings (Adler 1992). WDB is an important factor for formation of polarized hairs. Over expression of WDB results in cells growing multiple hairs with disrupted polarity (Hannus et al. 2002). It is suggested that interaction of WDB with the AC dimer of PP2A results in dephosphorylation of a factor that is essential for the correct polarization of microtubules at the distal side of cells. WDB is distally localized along with other polarity proteins such as Flamingo (FMI) and Dishevelled (DSH). Disruption of FMI protein localization does not disrupt the correct localization of WDB. Indeed, observation of the protein localization pattern in wing cells revealed that polarization of FMI and DSH depends on WDB localization. In general, the B′ regulatory subunit of PP2A, WDB, is suggested to be critical for planar cell polarity (Hannus et al. 2002).

3-3-2) PP2A-B′ (B56-1):

A Drosophila homolog of mammalian B56 subunit, PP2A-B′, was identified in a yeast two-hybrid screen for proteins that interact with Sex Combs Reduced (SCR) (Berry and Gehring 2000). *PP2A-B′* mRNA is transcribed from the maternal genome and deposited in the egg (Lecuyer et al. 2007). This maternal mRNA is present during the early embryonic stages of development, but is degraded after the fifth embryonic stage (Figure 1-2). Despite the protein sequence similarity of PP2A-B′ and Widerborst (Hannus

Fig 1-2. Expression pattern of *PP2A-B'* in the wild type *Drosophila melanogaster* embryo, using fluorescent *in situ* hybridization (FISH). In all pictures the anterior side of the embryos is toward the right. The dorsal side of embryo in the upper images is upward and in the lower images is downward. *PP2A-B'* mRNA is expressed during stages one to three of embryogenesis. *PP2A-B'* mRNA accumulation is reduced during the next stages and is gone by the fifth embryonic stage. Red signal indicates nuclei (detected by DAPI) and green signal indicates mRNA (detected by a primary biotinylated anti-DIG antibody and a secondary streptavidin-HRP antibody) (available in Drosophila embryo mRNA localization pattern data base: http://fly-fish.ccbr.utoronto.ca/; Lecuyer et al. 2007).

Stage 1-3



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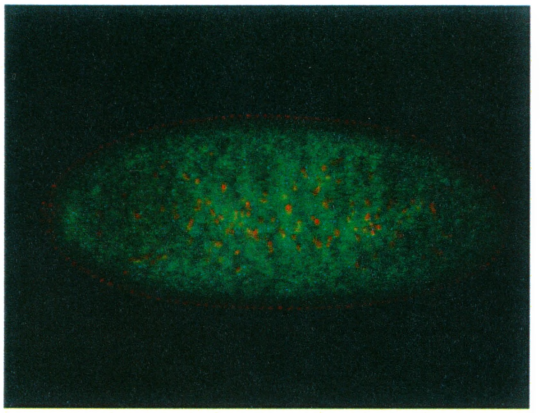

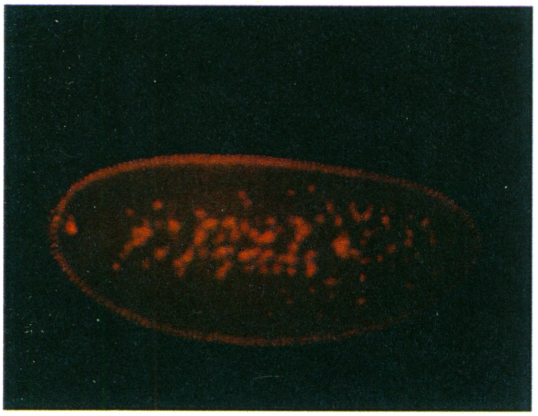

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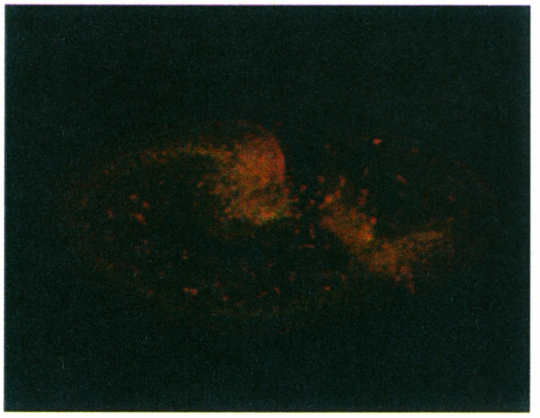


Stage 8-9

et al. 2002), these two proteins have distinct functions. RNA interference studies showed depletion of PP2A-B′ results in up regulation of genes involved in defence mechanisms during oxidative stress (Liu et al. 2007). Also, PP2A-B′ is suggested to have a negative effect on cell apoptosis in S2 tissue culture cells. Knockdown of PP2A-B′ leads to an increase in the expression of genes that enhance apoptosis, *reaper* and *sickle*. Comparing the genes that were affected by knockdown of PP2A-B′ with the genes that were affected by WDB knockdown indicates that these two B′ subunits have distinct functions and are not involved in the same pathways. The PP2A-B′ regulatory subunit is proposed to be required for regulation of the developmentally important HOX transcription factor, SCR. PP2A-B′ is proposed to interact specifically with the N-terminal arm of the SCR homeodomain and result in dephosphorylation of a serine and a threonine residue in the N-terminal arm of SCR homeodomain (Berry and Gehring 2000).

HOX Proteins

HOX proteins are a group of highly conserved transcription factors that are expressed in all animal species from nematode to human (McGinnis and Krumlauf 1992; Kenyon 1994; Krumlauf 1994). There are eight HOX transcription factors in *Drosophila melanogaster* which confer segmental identity along the anterior-posterior body axis. Mutation in *hox* genes cause transformation of one body part to another, for example transformation of antennae into legs in *Antennapedia (Antp)* mutants (Schneuwly and Gehring 1985) or replacement of halteres with a pair of wings in *Ultrabithorax* mutants (Lewis 1978). Based on these observations, HOX proteins are transcription factors that regulate expression of

specific downstream genes to confer segmental identity. This precise regulation of gene expression requires high degree of functional specificity.

HOX proteins interact with DNA binding sites through a highly conserved protein domain, the homeodomain (HD) (McGinnis et al. 1984; Scott and Weiner 1984). The Homeodomain is a 60 amino acid protein domain folded into three α-helixes. The third α-helix interacts with specific bases of the DNA major groove, while the N-terminal arm interacts with the DNA minor groove (Kissinger et al. 1990; Mann 1995; Joshi et al. 2007). Due to the high degree of sequence similarity between HOX homeodomains, all HOX homeodomains interact with similar DNA binding sites *in vitro* (Gehring et al. 1994). Thus, functional specificity of HOX proteins can not be explained by specific interaction of the third α-helix of HD with DNA. Genetic evidence suggests that the N-terminal arm is involved in determining specificity of HOX function. Replacing the amino acid sequence of SCR N-terminal arm with ANTP N-terminal amino acid sequences results in a SCR protein with ANTP function (Furukubo-Tokunaga et al. 1993; Zeng et al. 1993).

Berry and Gehring (2000) screened for proteins that interact with the N-terminal arm of SCR homeodomain in a yeast two-hybrid screen. The B′ regulatory subunit of PP2A, PP2A-B′, was found to interact specifically with the N-terminal arm of the SCR homeodomain and not with the N-terminal arm of the Antennapedia homeodomain. The N-terminal arm of the SCR homeodomain includes two serine/threonine amino acid residues. SCR is a phosphoprotein in tissue culture cells and its N-terminal arm peptide sequence was phosphorylated *in vitro* by protein kinase A and dephosphorylated by PP2A. To test whether phosphorylation of the N-terminal arm was important for SCR function, aspartate and alanine where substituted for the Ser/Thr residues to mimic constitutive phosphorylation and

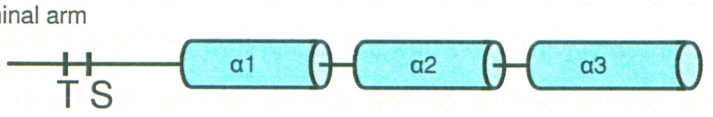
constitutive dephosphorylation of SCR. The mutant SCR protein that mimicked constitutive phosphorylation state is inactive. The negatively charged aspartic acid residues on the N-terminal arm of SCR inhibited interaction of the N-terminal arm with the minor groove of the DNA binding site. SCR is required for the formation of larvae and adults salivary glands (Panzer et al. 1992); using RNAi to deplete *PP2A-B'* mRNA, Drosophila embryos developed lacking salivary glands (Berry and Gehring 2000). These experiments lead to a model for regulation of SCR activity (Figure 1-3).

In the model presented by Berry and Gehring, SCR is regulated by phosphorylation and dephosphorylation of the N-terminal arm of the SCR HD. Protein phosphatase 2A and Protein kinase A were proposed to activate and deactivate SCR activity, respectively. Phosphorylated SCR interacts with the B′ regulatory subunit of PP2A and the phospho-serine and phospho-threonine residues of the N-terminal arm of SCR homeodomain lose their phosphate groups. The dephosphorylated N-terminal arm of the SCR homeodomain can interact with the minor grove of DNA.

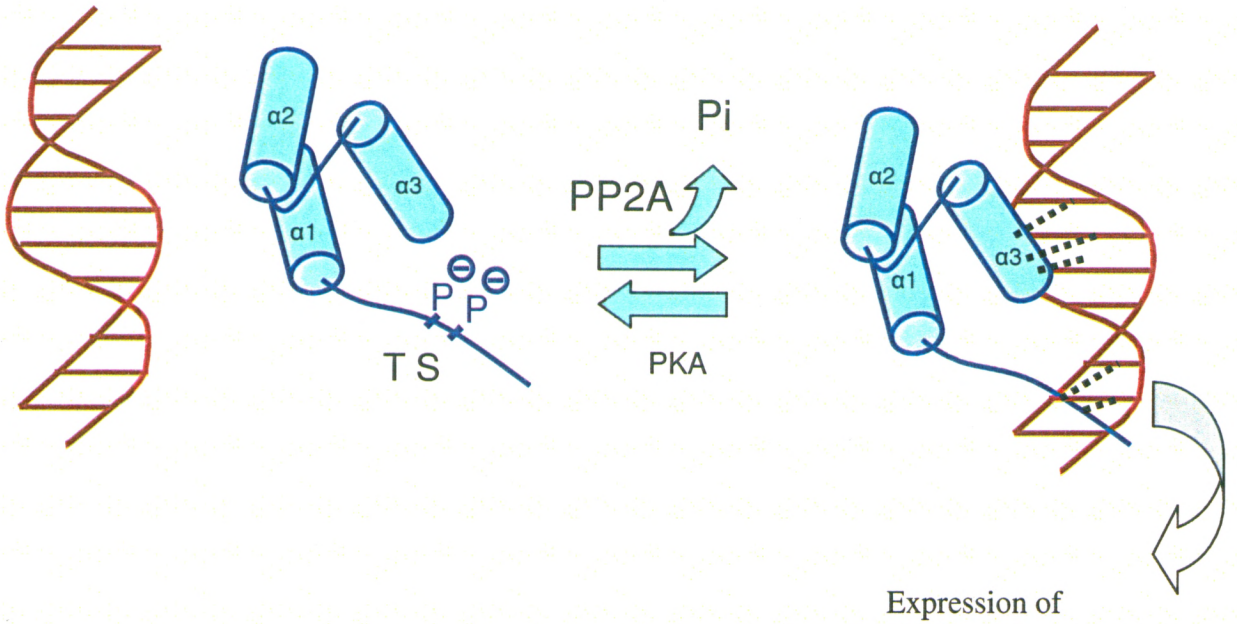
In order to test the model presented by Berry and Gehring (2000) and test the possibility of dephosphorylation of other HOX proteins such as Ultrabithorax (UBX) by PP2A; I created a null mutation in the *PP2A-B'* gene that removed most of the coding sequence of *PP2A-B'*. SCR is required for development of the larval and adult first thoracic and labial segments of fruit flies (Mahaffey and Kaufman 1987; LeMotte et al. 1989). SCR expression is required for formation of salivary glands (Panzer et al. 1992) through interaction with *fork head* gene regulatory region and activation of *fork head* transcription (Ryoo and Mann 1999). If PP2A-B′ function is required for SCR activity, loss of *PP2A-B'*

Figure 1-3. The model for regulation of SCR activity by PP2A (Berry and Gehring 2000). A) The SCR homeodomain is composed of three α-helixes and an N-terminal arm. (B) The model of regulation of SCR activity by phosphorylation/ dephosphorylation. Phosphorylation of Ser/Thr amino acid residues by protein kinase A (PKA) on the N-terminal arm inhibits SCR homeodomain interaction with the minor groove. PP2A holoenzyme specifically dephosphorylates the N-terminal arm of SCR, which results in interaction of the third alpha-helix and the N-terminal arm with DNA. In the schematic, S and T indicate serine and threonine amino acid residues.

A)


N-terminal arm
T S
α1
α2
α3

B)


α2
α3
α1
P P
T S
Pi
PP2A
PKA
α2
α3
α1
Expression of
SCR regulated genes

should result in loss of sex combs bristles pseudotrachea and salivary glands because SCR remains in its phosphorylated and inactive form.

Chapter Two

Introduction

Protein Phosphatase 2A (PP2A) is a serine/threonine phosphatase that regulates dephosphorylation of many proteins and has major roles in cell signalling and growth (Janssens and Goris 2001). The PP2A holoenzyme is composed of three subunits: the scaffold, catalytic and regulatory subunits. In *Drosophila melanogaster,* the scaffold and catalytic subunits are each encoded by a single gene but the regulatory subunits are encoded by five genes. The regulatory subunits fall into three structurally distinct groups: B, B′ and B″ subunits. The regulatory subunits B and B″ are encoded by *twins* and *CG4733,* respectively (Uemura et al. 1993; Li et al. 2002). The B′ regulatory subunits are encoded by three genes: *widerborst, PP2A-B′* and *CG32568* (Hannus et al. 2002; Berry and Gehring 2000; Andrews et al. 2000).

The B′ regulatory subunit, PP2A-B′, is proposed to be required for the regulation of Sex Combs Reduced (SCR) activity (Berry and Gehring 2000). SCR is a Drosophila HOX transcription factor that is required for formation of the first thoracic and labial segments in Drosophila larvae and adults (Mahaffey and Kaufman 1987; LeMotte et al. 1989). SCR interaction through its homeodomain with specific DNA binding sites regulates expression of downstream genes (Hueber et al. 2007). One SCR regulated downstream gene, *fork head* is required for salivary gland formation (Panzer et al. 1992; Ryoo and Mann 1999). The homeodomain is composed of 60 amino acid residues, which folds into three α-helixes. For recognition of DNA binding sites the third α-helix makes protein-DNA contacts with the

major groove and the N-terminal arm contacts with bases in the minor groove (Kissinger et al. 1990; Gehring et al. 1990).

Berry and Gehring (2000) presented a model for regulation of SCR activity by phosphorylation/dephosphorylation of serine and threonine residues in the N-terminal arm of the SCR homeodomain. The model proposes that phosphorylation of the N-terminal arm of the SCR homeodomain prevents SCR from interacting with DNA. Substitution of the serine and threonine residues with aspartic acid to mimic constitutive phosphorylation results *in vitro* in an inability of the SCR homeodomain to interact with DNA, and *in vivo* in an inability to induce ectopic T1 beard and salivary gland formation. In COS-1 mammalian tissue culture cells, SCR is a phosphoprotein and *in vitro* an SCR HD N-terminal arm peptide is phosphorylated at the serine and threonine residues by protein kinase A. The phosphorylated N-terminal arm sequence is dephosphorylated *in vitro* by PP2A. The PP2A holoenzyme containing the PP2A-B′ regulatory subunit is proposed to specifically mediate SCR dephosphorylation because the PP2A-B′ regulatory subunit interacts specifically with the SCR N-terminal arm of the HD in a yeast two-hybrid assay. Also, chromosomal deficiencies lacking *PP2A-B′* and an RNAi against *PP2A-B′* result in the loss of salivary glands (Berry and Gehring 2000). We generated a null mutation in the *PP2A-B′* gene and observed no evidence for a role of PP2A-B′ in dephosphorylation and activation of SCR activity: flies homozygous for the $PP2A\text{-}B^{\Delta}$ allele exhibited a wild type phenotype.

Material and Methods

Drosophila Stocks and Crosses. The stocks used were obtained from the Bloomington and Harvard Stock Centers, and ph^{410} was provided by Hugh Brock. The *PBac*

insertions *PBac{WH}*^{f05278}, *PBac{WH}*^{f07111} and *PBac{RB}*^{e03725} were used to generate a deficiency *Df PP2A-B'-CG7208* and *PP2A-B'*$^{\Delta}$ gene deletion as described in Parks et al. 2004 (see Appendix A for the crossing scheme). PCR was used to confirm the structure of the chromosomal deletions (see Appendix B for the table of primers used). Standard Drosophila crosses were used to recombine the deficiencies *Df(3R) ED6265* and *Df(3R) ED5474* with *PP2A-B'*$^{\Delta}$. Because the two deficiencies and *PP2A-B'*$^{\Delta}$ are marked with a mini-white gene, F2 males were screened for dark eye color and subsequently the presence of the deficiency and *PP2A-B'*$^{\Delta}$ were confirmed by PCR.

Phenotypic Observation. For scanning electron microscopy, flies were dehydrated in ethanol, critical point dried and sputter gold coated. Images were collected on a Hitachi 3400 N VP scanning electron microscopy (Biotron Imaging Unit, The University of Western Ontario). To count the number of larval salivary gland nuclei, salivary glands were dissected in Drosophila Ringers solution, fixed in PBS containing 4% formaldehyde for 20 minutes and the DNA stained with DAPI. To count the number of ectopic sex combs bristles, legs were removed and mounted on slides in Hoyer's mountant (Wieschaus and Nusslein-Volhard 1986).

Statistical Analysis. Data were statistically analyzed using SPSS v.16.0 (Statistical Package for the Social Sciences; SPSS Inc. 2007). Formation of the sex comb bristles on the first leg was analyzed with one-way Analysis of Variance (ANOVA). Multiple pair-wise comparisons were made using a Tukey test. Analyses of ectopic sex comb bristle formation on the second and third leg were performed using a Kruskal-Wallace test; followed by multiple pair-wise comparisons using a Dunnett T3. The numbers of salivary gland nuclei were analyzed using one-way ANOVA.

Results

PP2A-B′ is Dispensable for Development

FLP mediated site specific recombination was used to create two specific deletions of the *PP2A-B′* locus (Figure 2-1; Figure 2-2). *PBac* insertions *PBac{WH}^{f05278}* and *PBac{WH}^{f07111}* were used to create a deficiency that deletes all the coding DNA of *PP2A-B′*, as well as the genes *cdm* and *CG7208.* This deletion was called *Df PP2A-B′-CG7208. PBac* insertions *PBac{WH}^{f05278}* and *PBac{RB}^{e03725}* were used to create a specific deletion of *PP2A-B′*, *PP2A-B′$^{\Delta}$*. The primary *PP2A-B′* transcript is spliced to provide at least two transcripts (*PP2A-B′-RA* and *PP2A-B′-RK)* and potentially a total of eight transcripts (Tweedie et al. 2009). The *PP2A-B′$^{\Delta}$* encompasses all exons that encode the conserved protein domains of PP2A-B′ and the only coding region that remains is a 3′ exon of the alternatively spliced transcript, *PP2A-B′-RK*. The deficiency *Df PP2A-B′-CG7208* was homozygous lethal, but *PP2A-B′$^{\Delta}$* was homozygous viable and was not male or female sterile. The effect of *PP2A-B′* removal on the development of SCR-dependent body parts was examined. Sex comb bristle formation is very sensitive to the level of SCR activity, a two fold reduction in SCR activity results in a significant loss of sex comb bristles (Lewis et al. 1980a). Relative to wild type, *PP2A-B′$^{\Delta}$* mutants showed no significant decrease in the sex comb bristles number (P=0.996) (Table 2-1). In *Scr* mutants the proboscis is transformed to a maxillary palp and all the pseudotracheal rows are lost (Lewis et al. 1980b; Struhl 1982; Percival-Smith et al. 1997). The number of pseudotracheal rows did not decrease in *PP2A-B′$^{\Delta}$* mutants (P=0.994). In *Scr* null mutants, the salivary glands do not form (Panzer et al. 1992). The number of larval salivary gland nuclei in a *PP2A-B′$^{\Delta}$* mutant of 111± 2 were not significantly lower than the wild type number of 113± 3 (P=0.280). Overall, removal of

Figure 2-1. The structure of two mutations in the *PP2A-B'* locus. A) Physical map of the *PP2A-B'* locus and the surrounding region. The coordinates are derived from the R5.13 sequence database (Adams et al. 2000). B) The *PBac* insertion elements used to create the deletions are *PBac{WH}*^{f05278} at 3R: 13995122, *PBac{RB}*^{e03725} at 3R:14002909 and *PBac{WH}CG7208*^{f07111} at 3R:14010659-796. The insertion elements are indicated by triangles and the FRT sites within the elements are indicated by boxes. The relative position of each insertion element on the third chromosome is indicated below each insertion element. C) The structure of the two deletions *Df PP2A-B'–CG7208* (created by Robin Rosenfeld) and *PP2A-B*$^{\Delta}$. The relative position and orientation of the five primers used to confirm the structure of *PP2A-B*$^{\Delta}$ are indicated by arrows.

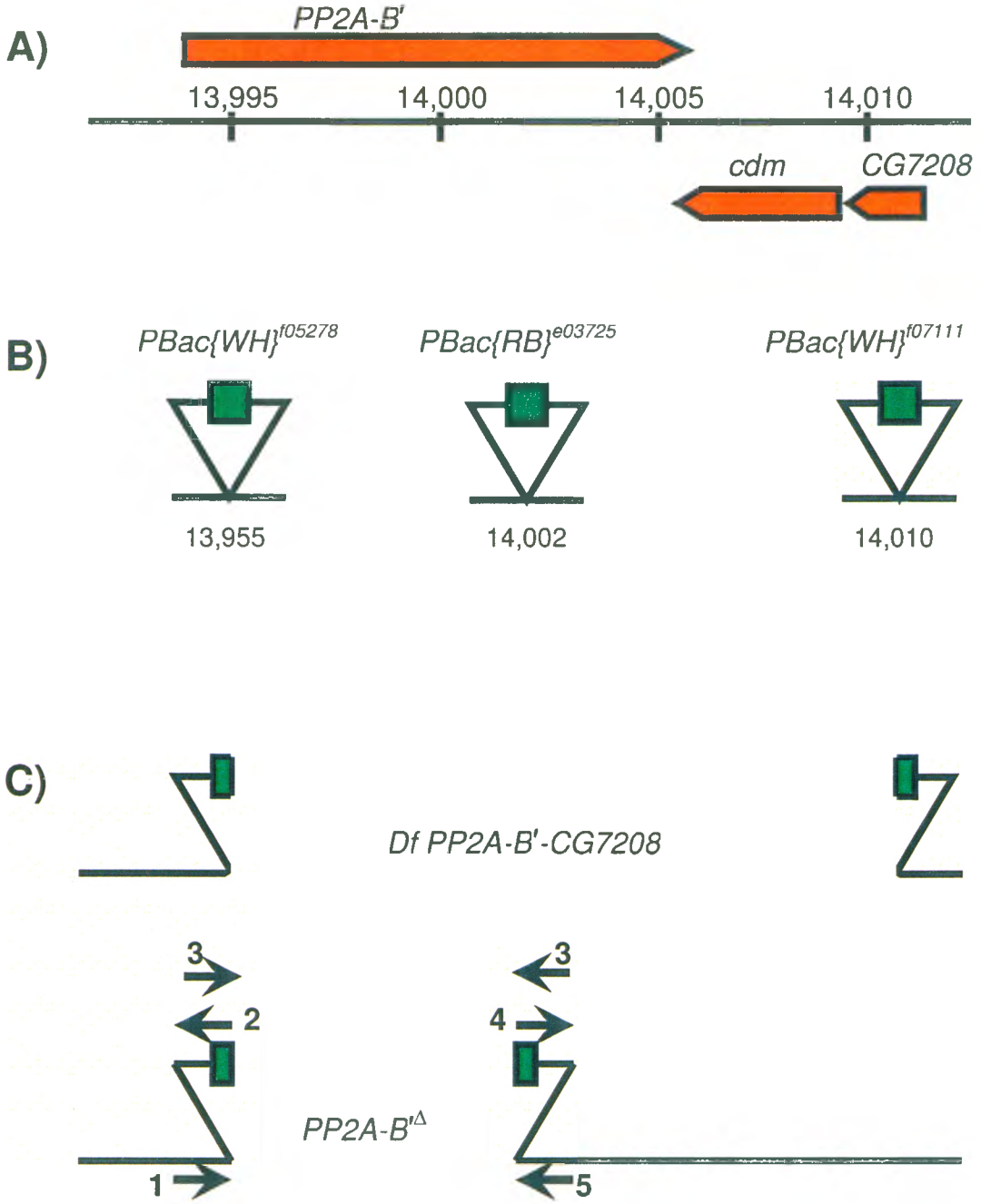

A)
PP2A-B'
13,995
14,000
14,005
14,010
cdm
CG7208
B)
PBac{WH}f05278
PBac{RB}e03725
PBac{WH}f07111
13,955
14,002
14,010
C)
Df PP2A-B'-CG7208
3
2
1
PP2A-B'Δ
3
4
5

Figure 2-2. Confirmation of the structure of *PP2A-B*$^{\Delta}$ by PCR analysis. DNA extracted from *PBac{WH}*^{f05278}(lanes 1-4), *PBac{RB}*^{e03725} (lanes 5-8) and *PP2A-B*$^{\Delta}$ (lanes 9-12) flies were used in PCRs with 4 sets of primers, to review the corresponding transposon elements and primers consult figure 2-1.C. The first reaction detects the 5′ boundary of the insertions of *PBac{WH}*^{f05278} (lanes 1, 5, 9). The second reaction detects the 5′ boundary of the insertion of *PBac{RB}*^{e03725} (lanes 2, 6, 10). The third reaction detects the formation of a hybrid WH-RB element (lanes 3, 7, 11). The fourth reaction detects formation of WH and RB element with coincident of deletion of 7,787 bp.

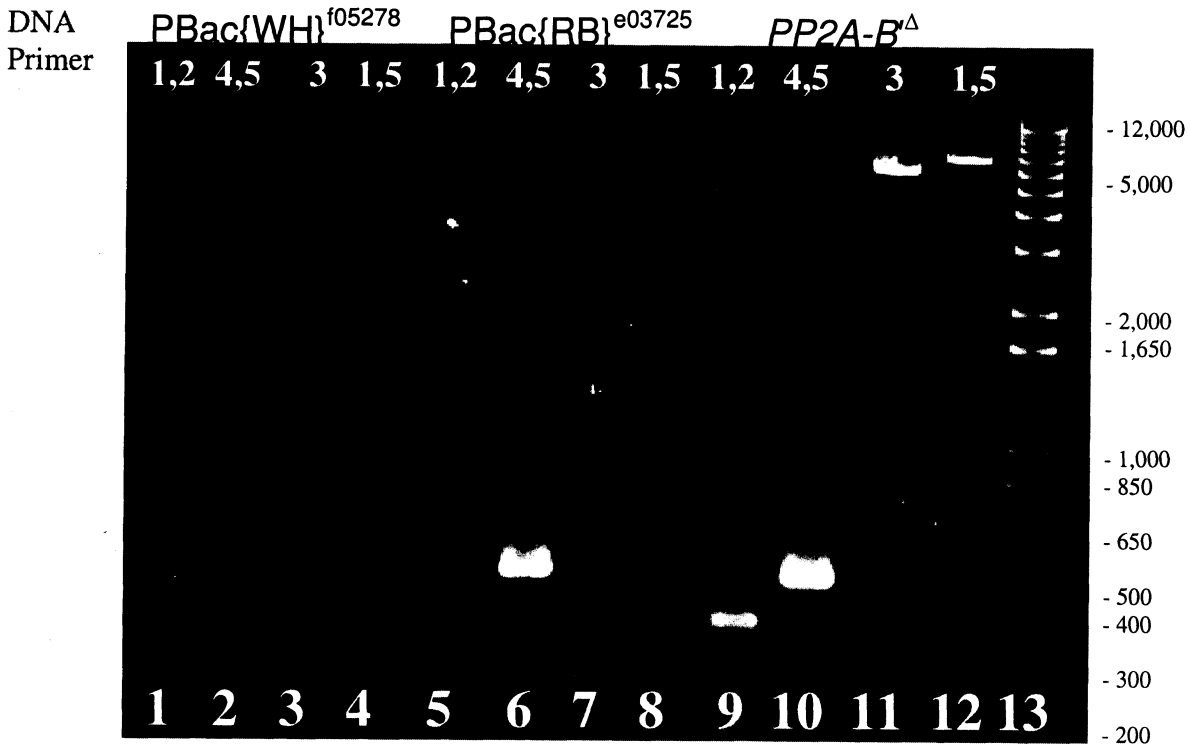

DNA
Primer
PBac{WH}f05278
PBac{RB}e03725
PP2A-B'Δ
1,2 4,5 3 1,5 1,2 4,5 3 1,5 1,2 4,5 3 1,5
- 12,000
- 5,000
- 2,000
- 1,650
- 1,000
- 850
- 650
- 500
- 400
- 300
- 200
1 2 3 4 5 6 7 8 9 10 11 12 13

Table 2-1. Sex comb bristles and pseudotracheae formation in flies with mutations in different regulatory subunits of PP2A. Data in the same column with the same letters are not significantly different, $p>0.05$. The number of samples used for statistic analysis is indicated in parenthesis (n).

Genotype	Number of sex comb bristles	Number of pseudotracheal rows
wild type	9.3±0.1 ^{b} (13)	6±0.0 ^{a} (8)
PP2A-B'$^{\Delta}$	9.6±0.2 ^{b} (25)	6.1±0.1 ^{a} (10)
Df wdb ‡/+	9.3±0.2 ^{b} (13)	6±0.0 ^{a} (18)
PP2A-B'$^{\Delta}$ Df wdb /PP2A-B'$^{\Delta}$	10.9±0.1 ^{a} (16)	6±0.0 ^{a} (11)
Df tws ¶ /+	10.4±0.3 ^{ab} (14)	6±0.0 ^{a} (8)
Df tws, PP2A-B'$^{\Delta}$/PP2A-B'$^{\Delta}$	11.0±0.2 ^{a} (15)	6±0.0 ^{a} (12)
Df tws/Df wdb	10.7±0.2 ^{ab} (11)	6±0.0 ^{a} (8)
Df tws PP2A-B'$^{\Delta}$/PP2A-B'$^{\Delta}$ Df wdb	11.5±0.2 ^{a} (12)	6.2±0.1 ^{a} (12)

‡ Df(3R)ED5474

¶ Df(3R)ED6265

PP2A-B' had no effect on the formation of SCR-dependent body parts (Figure 2-3).

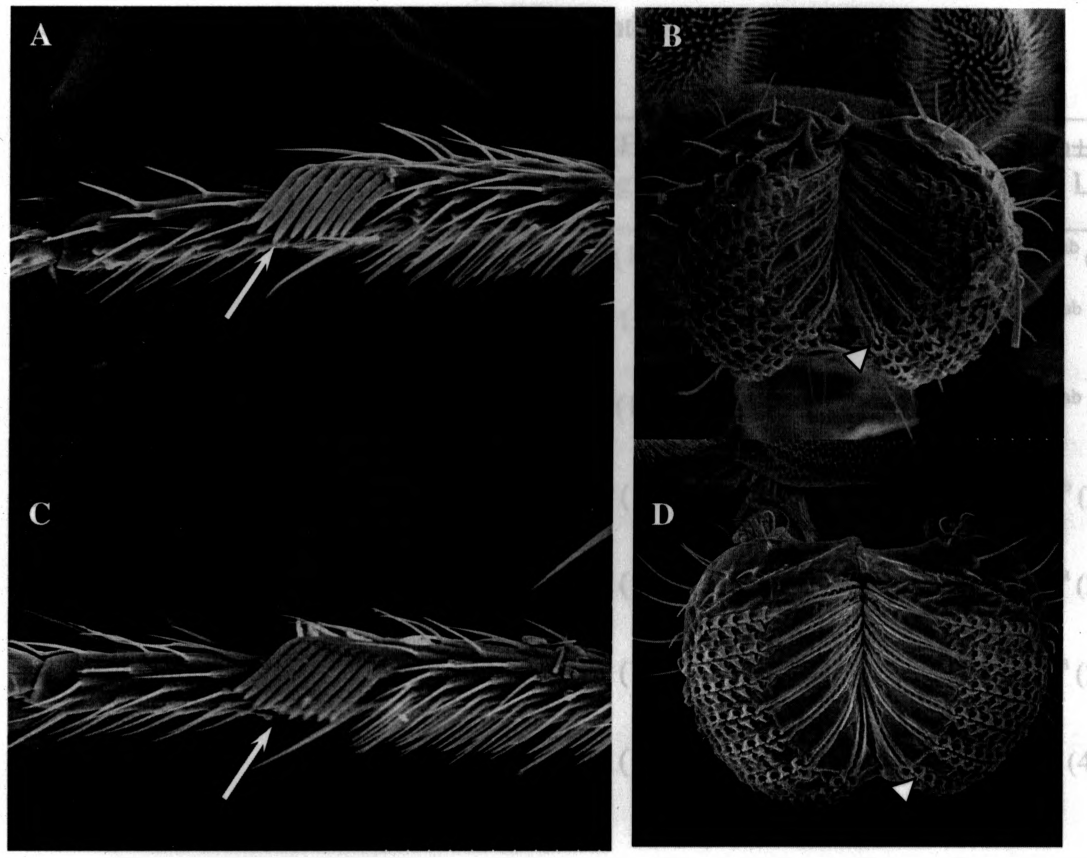
Functional Redundancy of Regulatory Subunits

In Drosophila tissue culture cells, PP2A-B' and the WDB appear to be redundant for regulation of the mitotic cycle and apoptosis (Chen et al. 2007; Li et al. 2002). Because *wdb* null alleles are cell lethal, we tested whether a reduction in the number of sex comb bristles and the number of pseudotracheal rows would be detected when PP2A-B' activity was removed along with a reduction in *wdb* and/or *tws* expression. Two deficiencies encompassing either the *tws* gene or *wdb* gene were recombined onto a chromosome containing $PP2A\text{-}B'^{\Delta}$. In all cases no decrease in the number of sex comb bristles and number of pseudotracheal rows was observed (Table 2-1). Indeed most surprisingly, a significant increase in the number of sex combs bristles was observed when one copy of *tws* or *wdb* was removed, and when both a copy of *tws* and *wdb* were removed ($p=0.005$, $=0.001$, <0.001), respectively. Clearly, PP2A holoenzymes containing TWS or WDB are not compensating for the lack of PP2A-B' activity in the dephosphorylation and activation of SCR.

Ectopic Sex Comb Formation

Mutations in the Polycomb set of genes result in the formation of ectopic sex combs on the second and third leg by derepressing SCR expression in the second and third imaginal discs (Dura and Ingham 1988). Loss of one copy of *PP2A-B'* is suggested to result in the formation of fewer ectopic sex combs in the ph^{410} mutants, because a greater proportion of SCR is phosphorylated and inactive (Berry and Gehring 2000). We tested this hypothesis with the $PP2A\text{-}B'^{\Delta}$ allele. The loss of *PP2A-B'* did not significantly affect the number of ectopic sex comb bristles that formed on the second and third leg of ph^{410} mutants (Table 2-2). In addition, reduction in the expression level of either *microtubule star (mts),*

Figure 2-3. Sex comb bristles and the proboscis form normally in flies homozygous for *PP2A-B'*$^{\Delta}$. Panels A and B are wild type flies and panel C and D are *PP2A-B'*$^{\Delta}$ flies. The male sex comb bristles are indicated by an arrow and the pseudotracheae by an arrow head.



Wild type

PP2A-B'$^{\Delta}$

First leg sex combs **Labial palp**

Table 2-2. Ectopic sex comb bristles formation in ph^{410} mutant flies. Data in the same column with the same letters are not significantly different, p>0.05. The number of samples used for statistical analysis is indicated in parenthesis.

Deficiency	Affected gene	Mean±SEM 1st Leg	Mean±SEM 2nd Leg	Mean±SEM 3rd Leg
None	None	10.5±0.2[a] (10)	0.8±0.2 [ab] (42)	0.2±0.1 [ab] (36)
Df(3R)P14	*PP2A-B'*$^{-}$	10.3±0.3 [a] (14)	1.0±0.3 [ab] (30)	0.6±0.2 [ab] (28)
Df(3R)DG4	*PP2A-B'*$^{+}$	10.5±0.3 [a] (17)	0.5±0.1 [b] (19)	0.2±0.1 [ab] (36)
Df(3R) Exel6178	*PP2A-B'*$^{-}$	11.5±0.1 [a] (10)	0.2±0.1 [b] (28)	0.0±0.0 [b] (26)
Df(3R)BSC509	*PP2A-B'*$^{+}$	11.2±0.2 [a] (18)	1.3±0.2 [ab] (25)	1.2±0.2 [a] (29)
Df(3R)BSC510	*PP2A-B'*$^{+}$	10.4±0.2 [a] (16)	1.7±0.2 [ab] (28)	1.7±0.2 [a] (24)
Df PP2A-B'-CG7208	*PP2A-B'*$^{-}$	10.5±0.2 [a] (17)	1.1±0.2 [ab] (34)	0.6±0.1[a] (42)
PP2A-B'$^{\Delta}$	*PP2A-B'*$^{-}$	10.3±0.1 [a] (19)	1.0±0.2 [ab] (26)	0.2±0.2 [ab] (16)
Df(2L)ED12527	*mts* $^{-}$	09.8±0.4 [a] (11)	1.6±0.4 [ab] (18)	0.6±0.2 [ab] (25)
Df(3R)ED5474	*tws* $^{-}$	12.3±0.3 [a] (19)	2.1±0.3 [a] (24)	1.5±0.3 [a] (22)
Df(3R)ED6265	*wdb* $^{-}$	11.1±0.2 [a] (11)	1.3±0.2 [ab] (16)	0.3±0.1 [ab] (19)
Df(3R)ED6027	*B''* $^{-}$	11.2±0.2 [a] (12)	1.1±0.3 [ab] (21)	0.8±0.2 [ab] (25)
Scr ^{13A}	Scr $^{-}$	06.4±0.1 [b] (19)	0.4±0.1 [b] (27)	0.0±0.2 [ab] (25)

which encodes PP2A catalytic subunit, or the genes that encode other PP2A regulatory subunits did not significantly reduce the formation of ectopic sex comb bristles (Table 2-2). There is no evidence that PP2A-B′ activates SCR activity by dephosphorylation for ectopic sex comb formation.

Discussion

Analysis of the phenotype of a *PP2A-B′* gene deletion allele, *PP2A-B′*$^{\Delta}$, clearly showed that a PP2A holoenzyme containing the PP2A-B′ regulatory subunit does not dephosphorylate and activate SCR activity required for the formation of larval salivary glands, adult sex comb bristles, adult pseudotracheal rows, and ectopic sex comb bristles. No evidence was found for a role of PP2A holoenzyme in activating SCR for formation of sex comb bristles; indeed, the genetic analysis of redundancy may suggest that PP2A activity has a role in the dephosphorylation and repression of SCR activity (Table 2-1). Berry and Gehring (2000) were screening for a protein that interacts with the N-terminal arm of the SCR homeodomain, because the N-terminal arm determines functional specificity of SCR (Furukubo-Tokunaga et al. 1993; Zeng et al. 1993). One of the proteins identified in the yeast two-hybrid screen was a fragment of PP2A-B′; however, a subsequent systematic screen of the Drosophila proteome did not detect the SCR-PP2A-B′ interaction leading to the suggestion that the interaction of the PP2A-B′ fragment with the SCR N-terminal arm may be an artefact (Giot et al. 2003). *In vitro*, the N-terminal arm peptide is phosphorylated by protein kinase A, and dephosphorylated by the PP2A catalytic subunit. SCR is a phosphoprotein in tissue culture cells; however, the technically difficult analysis of directly demonstrating SCR phosphorylation in the N-terminal arm of homeodomain has not been

performed. The N-terminal arm of the HD is required for DNA binding (Tayyab et al. 2004; Percival-Smith et al. 1990); therefore, although substitution of the serine and threonine residues with aspartic acid results in an inactive homeodomain and an inactive SCR protein, the aspartic acid may just be interfering with DNA binding rather than mimicking constitutive phosphorylation of SCR. The genetic analysis with large chromosomal deficiencies containing or deleting the *PP2A-B′* locus may be detecting the role of other genes encompassed by these deficiencies for salivary gland formation. The suppression of salivary gland formation with an RNAi molecule targeting the *PP2A-B′* mRNA may be an example of an off target effect (Ma et al. 2006; Kulkarni et al. 2006).

Although the data presented in Berry and Gehring 2000 supported a model for the regulation of SCR, the viable $PP2A\text{-}B^{\Delta}$ mutant flies rule out the requirement of PP2A-B′ for SCR activity. The fact that the gene that encodes PP2A-B′ is dispensable for viability does not mean that PP2A-B′ is not functional. The analysis of functional redundancy between PP2A-B′ and TWS/WDB showed that removal of *PP2A-B′* in a genetic background that had a deficiency in one or both of the *tws* and *wdb* loci significantly increased the number of sex comb bristles. This may suggest that the PP2A holoenzyme containing either TWS, the B regulatory subunit, or WDB, the B′ regulatory subunit, can functionally substitute for the loss of PP2A-B′. Generating viable flies with loss of *PP2A-B′* expression will be useful for investigating whether PP2A-B′ is required for regulation of apoptosis (Liu et al. 2007), control of mitotic cycle (Chen et al. 2007) or effecting circadian rhythm and behaviour (Sathyanaryanan et al. 2004).

Chapter Three

Future Studies

To determine the role of the PP2A holoenzyme, reduction of the expression level of the individual subunits of PP2A have been examined in tissue culture cells. Examination of the role of a protein in tissue culture cells has a number of drawbacks. First, tissue culture cells are immortal highly selected cells that are distinct from developing cells of an organism, second, tissue culture cells do not develop into and have the morphology of the whole organism, third, tissue culture cells do not exhibit complex behaviours like courtship. Therefore, the null *PP2A-B′*$^{\Delta}$ mutation may assist in investigating the role of PP2A-B′ activity in complex cellular and behavioural processes.

Whether PP2A regulates cellular apoptosis positively or negatively is a controversial issue. In mammalian tissue culture cells, PP2A activates cellular apoptosis; however, in Drosophila tissue culture cells PP2A represses cellular apoptosis. In HL60 (Human promyelocytic leukemia cells) and the murine interleukin-3-dependent NSF/N1.H7 cell lines, apoptosis is stimulated by ceramide which results in dephosphorylation and inactivation of Bcl2, a protein with antiapoptotic activity (Ruvolo et al. 1999; Ruvolo et al. 2002). In these cell lines, the PP2A enzyme dephosphorylates and inactivates Bcl2 antiapoptotic activity, thus stimulating apoptosis. The positive effect of PP2A on apoptosis has been also reported in interleukin-3-dependent FL5.12 lymphoid cells. In this case, the regulatory subunit of B56α in association with PP2A dephosphorylates and activates the proapoptotic protein, Bad (Chiang et al. 2001; Chiang et al. 2003). In the Schneider (S2) Drosophila cell line, PP2A is a negative regulator of apoptosis. In an RNAi approach, loss of both B56 regulatory subunits

(WDB and PP2A-B′) resulted in apoptosis and an increase in caspase activity (Li et al. 2002). In another experiment, depletion of PP2A-B′ regulatory subunit in a S2 Drosophila cell line resulted in an increase in the expression level of apoptosis inducers, *reaper* and *sickle* (Liu et al. 2007). A better approach to examine the role of PP2A-B′ in apoptosis would be using *PP2A-B′*$^{\Delta}$ mutant flies. This approach would provide a more accurate picture of the effect of loss of PP2A-B′ because it is been investigated in the whole organism and not in tissue culture cells. Functional redundancy of PP2A-B′ with WDB or TWS for regulation of apoptosis could be also examined by using the chromosomes created in my project.

PP2A holoenzyme is reported to affect circadian rhythm in Drosophila by dephosphorylating and stabilizing Period protein (PER), a regulator of circadian rhythm (Sathyanarayanan et al. 2004). Both WDB and TWS regulatory subunits of PP2A, are involved in regulation of circadian rhythm; however, there is no report on the effects of PP2A-B′ on circadian rhythm in Drosophila. Over expression of *wdb* results in an increase in the level of *per* expression which causes an increase of circadian rhythm length and loss of behavioural rhythm. Studying circadian rhythm in flies with loss of *PP2A-B′* expression alone or in combination with *wdb* or *tws* deficiencies may reveal a role for PP2A-B′ on circadian rhythm and if the regulatory subunits of PP2A are functionally redundant in circadian rhythm.

Knockdown of *PP2A-B′* along with *WDB* resulted in disruption of microtubule spindle formation and organization, thus resulting in an unsuccessful mitosis; this phenotype is quite similar to what was observed in cells lacking the catalytic subunit of PP2A (MTS) (Chen et al. 2007). However, since loss of the expression of both B′ subunit results in lower PP2A-C expression; mitotic defects in cells with loss of both B′ subunits might be due to lower

expression of PP2A-C rather than functional redundancy of B′ subunit of PP2A for regulation of mitotic cycle. Flies carrying a null *PP2A-B′* chromosome along with a deficiency in *wdb* gene may help us to investigate formation of microtubule spindle during mitosis.

Appendix A

Genetic crosses for generation of FLP-FRT deletion

To recover deletions in the *PP2A-B'* locus, male flies containing a transposon element were mated to the female virgin flies carrying a heat-shock induced FLP recombinase gene (Figure 3-1). Male progeny of the first generation carrying both the FLP and the insertion element were crossed to the female flies containing the second transposon element. These G1 flies were allowed to lay eggs for two days. Afterwards, the progeny were subjected to one hour heat shock at 37 ° C. The heat shock was repeated the next day. Female progeny of generation two were collected and crossed to male flies carrying a third chromosome balancer. At least fifty males of the generation three, which are suspected to carry a deletion, were back crossed to female virgins carrying the balancer on the third chromosome (one male mated to three females). These males were collected after 5 days of mating for single fly PCR analysis. When a site specific recombination event occurs between two transposon elements, the genome sequence right of the right element is maintained as is the genomic sequence left of the left element. Both the right and left genomic sequences flanking the transposon elements are detected by PCR using primers specific to the transposable element and the flanking DNA.

Figure 3-1. Crossing scheme for creating *PP2A-B*$^{\Delta}$ null allele, using FLP mediated recombination technique. The deletion is created in four generations. The genotype of selected progeny of each generation is underlined. Genetic abbreviations used in the picture are: *hsFLP* (heat shock induced FLP recombinase), *TM3* (Balancer chromosome) and genetic markers are: *D* (Dichaete wings), *Dr* (Drop eye), *Sb* (stubbled bristles), *Ser* (Serrate wings), *y* (yellow body), and *w* (white eye).

♀ *y w hsFLP; Dr/TM3 Sb* X ♂ y^+ *w; PBac{WH}*^{f05278}

↓

G1 ♂ *y w hsFLP; PBac{WH}*^{f05278} */ TM3, Sb* X ♀ y^+ *w; PBac{RB}*^{e03725}

↓ Heat shock progeny

G2 ♂ *y w; D/TM3, Sb Ser* X ♀ *y w hsFlp; PBac{WH}*^{f05278}*/ PBac{RB}*^{e03725}

↓

G3 ♂ *y w hsFLP; PP2A-B*$^{\Delta}$*/ TM3* X ♀ *y w; D/TM3, Sb Ser*

↓

G4 *y w; PP2A-B*$^{\Delta}$ */ TM3, Sb*

Stock

Appendix B

Table 3-1. Table of primers. Primers were used for confirmation of a deletion in the *PP2A-B'* locus (*PP2A-B*$^{\Delta}$) and confirmation of recombination between the *PP2A-B*$^{\Delta}$ and the deficiencies *Df(3R)ED5474* and *Df(3R)ED6265.*

Primer Name	Primer Sequence
Primer 1	GGTAATGTGTTGTTTACAGTTATGG
Primer 2	CCTCGATATACAGACCGATAAAAC
Primer 3	GACGCATGATTATCTTTTACGTGAC
Primer 4	TCCAAGCGGCGACTGAGATG
Primer 5	TAAAAATGGTGATTCACTTGAGG
TWS	TCGCGTTCCTGCTTCTAGTTCC
WDB	TTCCGTTGGTCTTGGCATACCTT

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