A2BP1: a novel component of Notch Pathway during Nervous System development in *Drosophila*

A thesis

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by

Jay Prakash Shukla

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Dedicated to my Parents

CERTIFICATE

Certified that the work incorporated in the thesis entitled "**A2BP1: a novel component of Notch Pathway during Nervous System development in *Drosophila***" submitted by **Jay Prakash Shukla** was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

Prof. L S Shashidhara
Supervisor

DECLARATION

I declare that this written submission represents my ideas in my own words and where others ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

Jay Prakash Shukla
20093029

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List of Abbreviations

A/P	Anterior/Posterior
D/V	Dorso-ventral
Ac/Sc	Achaete/Scute Complex
E(spl)-C	Enhancer of Split complex
Ac	Achaete
Sc	Scut
PNS	Peripheral Nervous System
CNS	Central Nervous System
RBD	RNA binding domain
RRM	RNA recognition Motif
SOP	Sensory Organ Precursor
PNC	Proneural cluster
SCA2	Spinocerebellar ataxia2
Poly Q	Poly Glutamine
Fox	Feminizing locus on X
UTR	Un-translated region
G4	GAL4
Nicd/NICD	Notch intracellular domain
NDN	Notch dominant Negative

Synopsis

Title: "dA2BP1: a novel component of Notch Pathway during Nervous System development in *Drosophila*"

Name of the Student: **Jay Prakash Shukla**

Name of the thesis advisor: **Prof. LS Shashidhara**

Indian Institute of Science Education and Research (IISER), Pune

Introduction:

Spinocerebellar ataxia 2 (SCA2) is a progressive neurodegenerative disease, which leads to severe loss of cerebellar function and severely affects muscle movement (Taroni and DiDonato, 2004). This is attributed to the triplet, CAG, repeat expansion (resulting in poly-Q domains) in a protein named Ataxin-2. Normally, Ataxin-2 is distributed in the nervous system and after Poly Q expansion, it accumulates in Purkinje cells and its aggregation increases with age. Ataxin-2 binding protein (A2BP1) was identified as an interactive partner of Ataxin-2 in a yeast two hybrid screen. The A2BP1 interacts with C-terminus of the Ataxin-2 independent of its poly Q expansion. It is normally a nuclear protein, but in SCA2, it is deposited in the cytoplasm of neuronal cells along with the Ataxin-2 (Shibata et al., 2000). A2BP1 has an RNA binding domain and it regulates RNA splicing of neuronal and muscle specific genes including NMDA receptor and Calcium ATPase (Lee et al., 2009; Underwood et al., 2005). Mutations in *A2BP1* either by gene disruption due to translocation or single nucleotide polymorphism (SNP) have been implicated in several human diseases including autism, epilepsy and mental retardation (Bhalla et al., 2004; Martin et al., 2007).

dA2BP1, *Drosophila* homologue of A2BP1, is a component of Hedgehog (Hh) signaling pathway. Specifically, it interacts with the transcription factor Cubitus interruptus (Ci) to regulate Hh-target genes (Usha and Shashidhara, 2010). Down-regulation of dA2BP1 in early fly embryo results in reduction in the neuronal cell number (Koizumi et al., 2007). However, detailed functional studies on A2BP1 during nervous system

development have not been carried out. *Drosophila* is a good model system to learn function of any gene, specifically in the context of development. Here, we have employed peripheral nervous system (PNS) of *Drosophila* to understand the role of dA2BP1 during nervous system development. In spite of reasonable work on poly Q expansion in Ataxin2 and its role in the disease, we still do not understand the molecular basis of SCA2 pathogenicity completely. Understanding the cellular and developmental role of dA2BP1 may provide some insights into the cellular pathogenicity of SCA2. In this context, we have undertaken a study to examine the precise function of dA2BP1 during neuronal development.

Thoracic sensory bristles are a part of peripheral nervous system and a good model system to understand cell differentiation during nervous system development. It originates from the epidermis of 3rd instar wing imaginal disc. During sensory organ development, first a small subset of cells form proneural cluster (PNC) by expressing proneural proteins, Achaete/Scute. In the next step, a single sensory organ precursor (SOP) emerges from the PNC. The SOP cell then divides asymmetrically to give rise to a complete bristle composed of socket, shaft, sheath, neuron and glia (Reddy and Rodrigues, 1999).

SOP selection is a crucial event to regulate sensory organ number for precise sensory input. Notch signaling, which has been studied in detail for its function in lateral inhibition and cell-fate specification during development, is implicated in SOP specification. Notch is a trans-membrane receptor activated by other trans-membrane ligands Delta and Serrate presented by the neighboring cells (Heitzler and Simpson, 1991; Kunisch et al., 1994). After activation, the Notch receptor undergoes several proteolytic cleavages to release its intracellular domain (NICD), which further translocates to the nucleus to act as transcriptional factor. In the nucleus, Notch signaling activates *Enhancer of split* group of genes, which in turn negatively regulate proneural genes *achaete/scute* complex to inhibit neuronal fate in those cells (Culí and Modolell, 1998; Heitzler et al., 1996a; Lecourtois and Schweisguth, 1995). However, nuclear entry of Notch and its context-specific cofactors involved in activation of its targets are not well understood.

Both Notch and Su(H) has Poly-Q domain. Dynamics of dA2BP1 interactions with these proteins may change when there is poly-Q expansion of Ataxin-2 leading to tissue homeostasis. Thus, our study suggests a new avenue to study molecular basis of SCA2.

Specific Objectives

1. To elucidate the expression of dA2BP1 during the development of nervous system in *Drosophila*
2. To examine neuronal phenotypes associated with loss- and gain-of-dA2BP1.
3. To identify of pathways that are directly regulated by dA2BP1 during neuronal specification.
4. To study the precise mechanism by which dA2BP1 regulates neuronal development.

Results-

dA2BP1 regulates external sensory organ development-

External sensory organs i.e., sensory bristles and campaniform sensilla are present in multiple locations on the thorax and thoracic organs, particularly on the dorsal thorax and the wing. Down regulation of *dA2BP1* leads to increase in thoracic macrochaetae (sensory bristles) and campaniform sensilla number on the wing. It also transforms campaniform sensilla into sensory bristle. Over expression of dA2BP1 in PNC leads to loss of majority of macrochaetae and microchaetae. These results suggests that dA2BP1 negatively regulates sensory bristle formation.

dA2BP1 shows genetic interaction with Notch pathways-

Although Sensory organ development involves various signaling pathway, Notch plays an important role during Sensory organ precursor (SOP) specification and division. Its role in negative regulation of SOP specification helps in limiting sensory bristle number and their spacing on the thorax. Reduction in Notch signaling results in increase in the number of sensory bristles. We have observed that down regulation of *dA2BP1* in the background of lower Notch signaling further enhances sensory bristle number phenotype. Notch gain of function mutants show reduction in bristle number; which we could rescue

by down regulating *dA2BP1*. This observation was supported by using both classical loss-of function and gain-of-function alleles and transgenic lines; Notch dominant negative (NDN) and Notch intracellular domain (NICD), a constitutively form of Notch. Our results suggest that dA2BP1 is a positive regulator of Notch signaling and functions down-stream of events leading to internalization of Notch receptor, but may function at par with Notch in the nucleus.

dA2BP1 regulates *Enhancer of Split* to regulate Achaete expression and SOP number-

Increase or decrease in sensory bristle number described above could be an effect of either increase in sensory organ precursor (SOP) number at the initial stages of bristle development or a fate switch between daughter cells later. We used *neur*-lacZ (*A101*) to mark SOP during dA2BP1 manipulation. Down-regulation of *dA2BP1* (which showed increase in sensory bristle number) showed increase in SOP number, whereas dA2BP1 over-expression (showed loss of sensory bristles) resulted in decreased number of SOP. These results show that dA2BP1 may function at initial stages of sensory organ development. As dA2BP1 manipulation affects the SOP number, next we examined the Achaete levels. SOP specification largely depends on Achaete expression. Down regulation of *dA2BP1* in PNC leads to increase in Achaete level and conversely, over-expression of dA2BP1 resulted in reduction in Achaete expression. These results suggest that dA2BP1 regulates the number of cells that cross the threshold levels Ac and thereby, may modulate SOP fate.

Notch signaling regulates Ac/Sc expression by regulating proteins encoded by the *E(spl)* complex. As dA2BP1 shows genetic interaction with Notch, next we examined *E(spl)m8* and *E(spl)mβ* expression during dA2BP1 manipulation. *dA2BP1* down-regulation in PNC using *sca*-GAL4 showed reduction in *E(spl)m8*-lacZ expression. Its down regulation in wing pouch using *dpp*-GAL4 resulted in reduction in E(spl)mβ expression, suggesting that dA2BP1 positively regulates *E(spl)* complex genes. As these are context-specific mediators of Notch singling, our results suggests that dA2BP1 may interact with Notch in its multiple functions.

dA2BP1 is part of Su(H) complex both in the presence and the absence of Notch-

In the absence of Notch signaling, its target genes are repressed by repressor complex which includes Suppressor of Hairless (Su(H)), Hairless (H), Groucho (Gro) and C-terminal binding protein (dCtBP) (Barolo et al., 2002; Nagel et al., 2005). During active Notch signaling, activated Notch intracellular domain (NICD) and Mastermind (Mam) bind to the Su(H) complex. This binding facilitates removal of the repressor proteins, H, Gro and dCtBP from the complex, and thereby, activate target genes (Helms et al., 1999; Le Gall and Giniger, 2004; Lecourtois and Schweisguth, 1995). Because dA2BP1 is positive regulator of Notch signaling and upstream of E(spl), the direct target of Notch, next we examined, whether dA2BP1 is a part of Su(H) complex. We performed IP with anti-dA2BP1 antibodies on extract of S2 cells, which does not express functional Notch. We detected Su(H) in the co-immunoprecipitate (co-IP), suggesting that dA2BP1 is an interactive partner of Su(H). To understand if the dA2BP1 and Su(H) interaction is continued after addition of the Notch to the complex, we performed co-IP on S2-N (Notch activated) cell extract using dA2BP1 antibody. We detected both Su(H) and Notch in the co-IP, suggesting that dA2BP1 and Su(H) interaction persists even after addition of Notch to the complex and thus it may act as a transcription co-factor with Notch and Su(H). However, we did not observe other components of the repressor complex such as Gro and dCtBP in the co-IP suggesting that dA2BP1 might be part of Notch- Su(H) activator complex only.

Loss of function of dA2BP1 suppresses loss of function phenotype associated with Hairless during SOP formation-

Next we examine whether loss of function of dA2BP1 suppresses phenotype associated with loss of function of Hairless (H). H is a part of suppressor complex and acts as an antagonist of Notch signaling. Loss of H leads to elevated Notch target expression and results in phenotypes equivalent to Notch gain of function even in absence of Notch. These phenotypes could be categorized into either complete loss of sensory bristles or transformation of shaft cell to socket cell (Bang et al., 1991; Bang et al., 1995). H heterozygous flies show either complete loss of sensory bristle or shaft to socket

conversion. Expression of *dA2BP1*^{RNAi} in H mutant could partially rescue the former effect of H, i.e., complete loss of sensory bristles, but there was no retransformation of socket cells to shaft cells. Together these results suggests that dA2BP1 regulates Notch signaling during SOP specification.

In summary, the results obtained in this study suggest that dA2BP1 is a positive regulator of Notch signaling during peripheral nervous system development. It is likely that dA2BP1 competes with Hairless to bind Su(H). However, it remains to be investigated, if dA2BP1 binds directly to Notch or only when Notch is bound to Su(H). This has an implication in our understanding of how intracellular domain of Notch is regulated from the time it is generated at the membrane to its translocation to the chromatin in the nucleus, wherever Su(H) is already present.

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1 Chapter-1: Introduction

Development and tissue homeostasis share some common intrinsic and extrinsic mechanisms. These mechanisms include cell signaling and differentiation. They are controlled by various genetic and epigenetic factors (Beerman and Rossi, 2015; Gatchel et al., 2008; Hooper et al., 2008; Zhou et al., 2010). Both events depend on stem cell differentiation and self-renewal. Malfunction of tissue homeostasis where stem cell pools could lose renewal capacity or differentiation lead to compromised tissue homeostasis and repair (Biteau et al., 2011). Thus, developmental defects, which could be result of defective cell signaling can trigger early aging and pathological disorder including, cancer and onset of progressive neurodegenerative diseases such as Huntington, Alzheimer's, Parkinson's and various types of ataxia (Gatchel et al., 2008; Hooper et al., 2008; Oh et al., 2014; Xu et al., 2008b).

Triplet repeat expansions lead to variety of genetic diseases and nervous system is a frequently affected tissue. Spinocerebellar ataxia 2 (SCA2) is one such disease, which leads to severe loss of function of cerebellum which co-ordinates muscle movement. Degeneration of the pontine nuclei and inferior olive with extensive loss of Purkinje cells is observed as in other ataxia. Degeneration of the Substantia nigra is the characteristic feature which separates it from other SCA diseases (Fig. 1.1)(Taroni and DiDonato, 2004). This is attributed to CAG repeat (resulting poly-Q domains) in a protein named Ataxin-2. A Poly Q domain of 22-23 repeats exists in the wildtype Ataxin-2. Whenever this crosses 34 repeats, it results in SCA2 (Lorenzetti et al., 1997), while intermediate poly Q length modifies the risk of other neurodegenerative disease such as amyotrophic lateral sclerosis (Elden et al., 2010). Normally, Ataxin-2 is distributed in the nervous system and after Poly Q expansion, it accumulates in Purkinje cells and its aggregation increases with age. Ataxin-2 binding protein (A2BP1) was identified as an interactive partner of Ataxin-2 in a yeast two hybrid screen (Shibata et al., 2000). It interacts with C-terminus of the Ataxin-2 independent of its poly Q expansion. A2BP1 is predominantly a nuclear protein, but it is deposited in the cytoplasm of neuronal cells along with the Ataxin-2 in SCA2 (Shibata et al., 2000). Unlike various other types of Spinocerebellar ataxia, SCA2 does not form intra-nuclear aggregates or nuclear inclusions (Huynh et al., 2000).

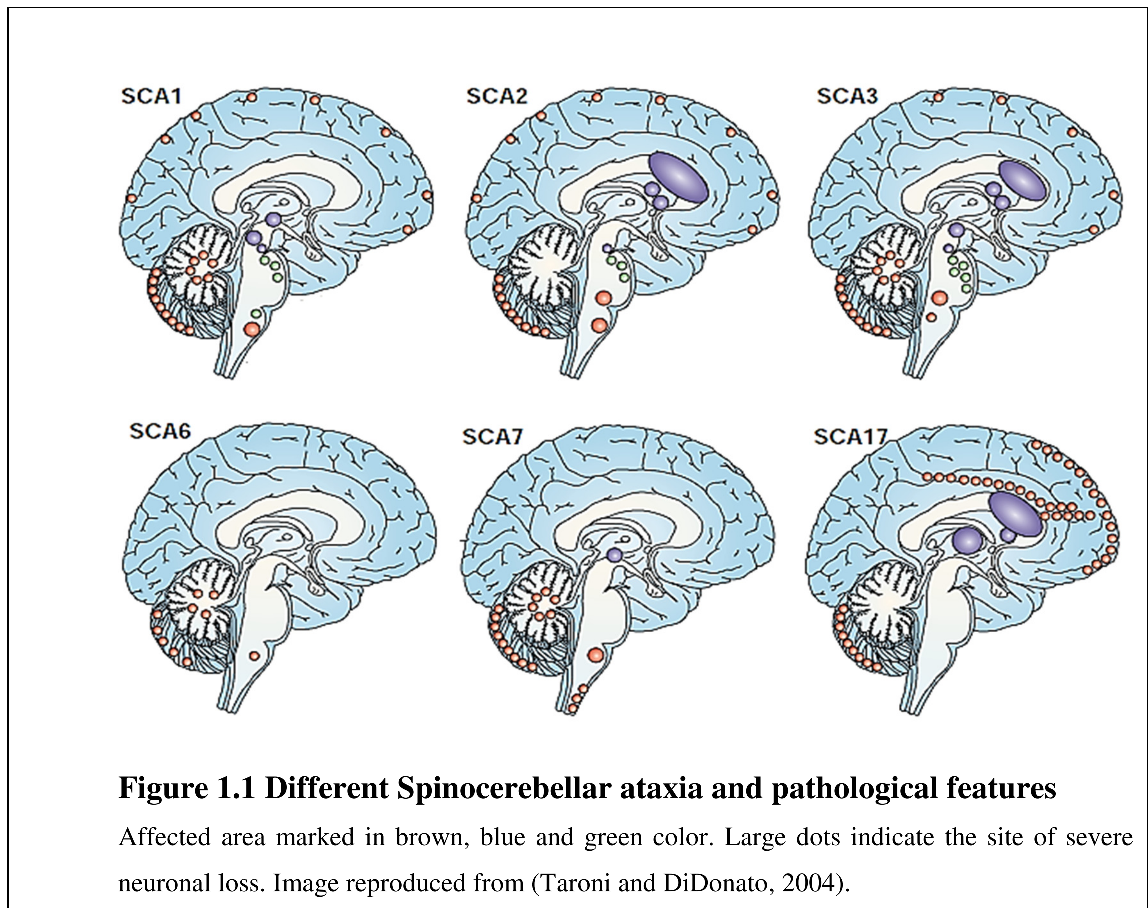


Figure 1.1 Different Spinocerebellar ataxia and pathological features

Affected area marked in brown, blue and green color. Large dots indicate the site of severe neuronal loss. Image reproduced from (Taroni and DiDonato, 2004).

Mutations in *A2BP1* is directly linked to neuronal diseases. Loss of *A2BP1* either by point mutation, translocation or deletion results in wide range of neuronal disorders including mental retardation, epilepsy, schizophrenia and autism (Bhalla et al., 2004; Lal et al., 2015; Martin et al., 2007; Sebat et al., 2007; Xu et al., 2008a). A2BP1 knock out mouse shows severe susceptibility toward epileptic seizure and abnormal brain development (Gehman et al., 2011).

A2BP1 is a RNA-binding nuclear protein and regulates alternative splicing of tissue-specific exons. It is widely associated with RNA metabolism including RNA splicing, polyadenylation, localization, export, translation and degradation (Kinzy et al., 2008). In mammals, there are 3 homologues of A2BP1 viz. Fox1 (A2BP1), Fox2 (RBM9) and Fox3 (NeuN). Fox 1 or A2BP1 is expressed in post-mitotic neurons, heart and skeletal

muscle cells; Fox2 expresses during developing embryo, stem cells as well as in mature neurons and muscle cells, while Fox3 is expressed only in neurons

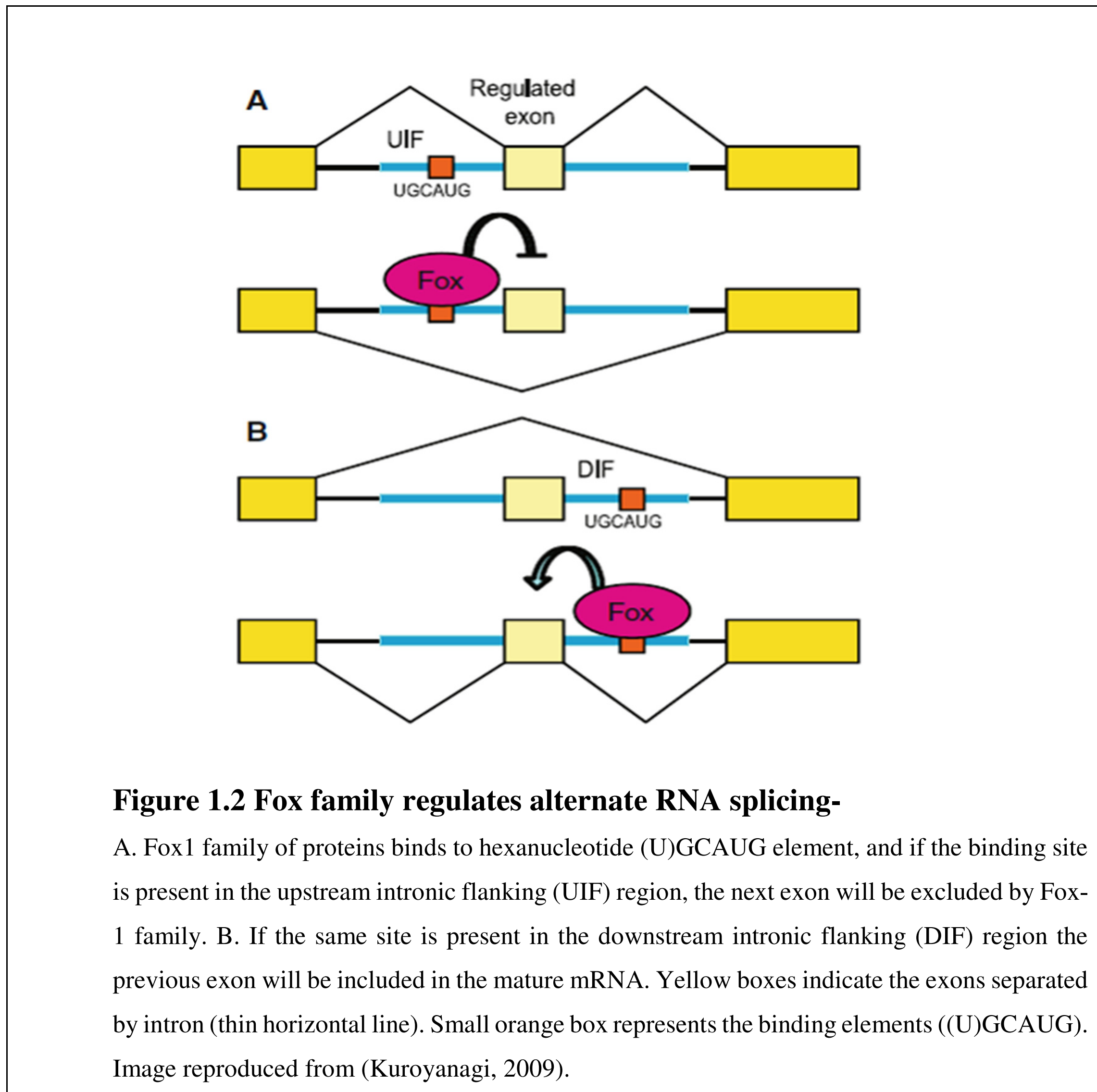


Figure 1.2 Fox family regulates alternate RNA splicing-

A. Fox1 family of proteins binds to hexanucleotide (U)GCAUG element, and if the binding site is present in the upstream intronic flanking (UIF) region, the next exon will be excluded by Fox-1 family. B. If the same site is present in the downstream intronic flanking (DIF) region the previous exon will be included in the mature mRNA. Yellow boxes indicate the exons separated by intron (thin horizontal line). Small orange box represents the binding elements ((U)GCAUG). Image reproduced from (Kuroyanagi, 2009).

(Kim et al., 2009; Ponthier et al., 2006; Underwood et al., 2005). All 3 mammalian homologues have conserved RNA binding domain (RBD, also called RNA recognition motif, RRM) and they all bind to a hexa-nucleotide UGCAUG motif present in hetero-nuclear RNA (pre-mRNA) to regulate RNA splicing (Jin et al., 2003). Whether exons are included or excluded in mature mRNA is dependent on the location of the binding element. If motif is present upstream of the intronic region, it will exclude the exon present before this intron, whereas if the motif is present downstream of the intronic region, it will include

the exon present after this intron (Fig. 1.2) (Kuroyanagi, 2009). Cell culture based study shows that A2BP1 regulates splicing and transcription of its targets during neuronal development (Fogel et al., 2012).

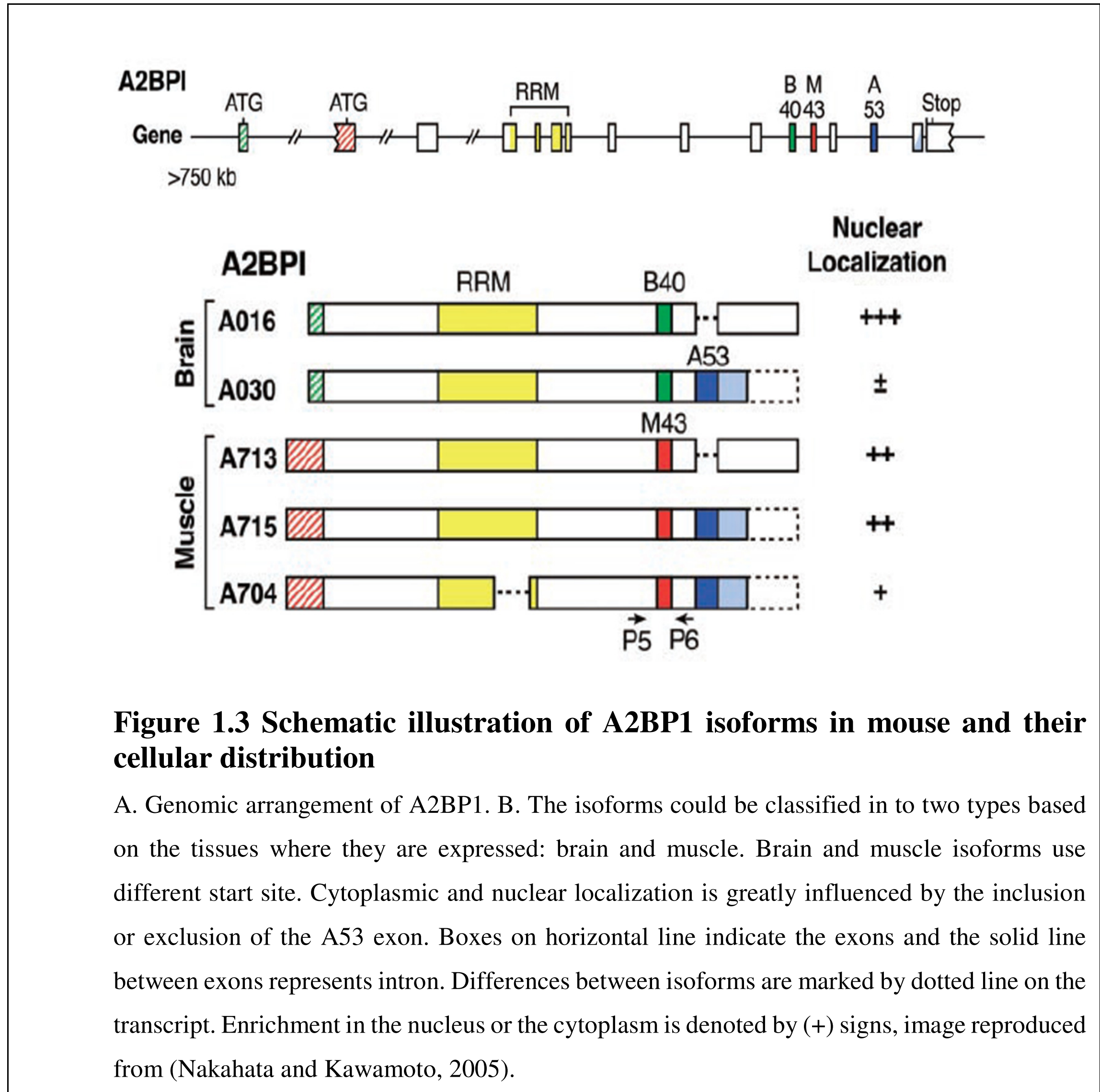


Figure 1.3 Schematic illustration of A2BP1 isoforms in mouse and their cellular distribution

A. Genomic arrangement of A2BP1. B. The isoforms could be classified in to two types based on the tissues where they are expressed: brain and muscle. Brain and muscle isoforms use different start site. Cytoplasmic and nuclear localization is greatly influenced by the inclusion or exclusion of the A53 exon. Boxes on horizontal line indicate the exons and the solid line between exons represents intron. Differences between isoforms are marked by dotted line on the transcript. Enrichment in the nucleus or the cytoplasm is denoted by (+) signs, image reproduced from (Nakahata and Kawamoto, 2005).

A2BP1 regulates mRNA splicing of several genes that are important for synaptic function including NMDA receptor 1, Calcium ATPase and Sodium channel SCN8A (Lee et al., 2009; O'Brien et al., 2012; Underwood et al., 2005). It along with Fox2 regulates mRNA splicing of Mef 2 during muscle development (Runfola et al., 2015; Zhang et al., 2008). Different isoforms of A2BP1 are expressed in muscles and neurons to carry out

tissue specific RNA splicing. In neuronal cells, isoform A016 is localized to the nuclei whereas, A030 is majorly present in the cytoplasm. Muscle specific isoforms i.e., A713 and A715 are both localized to the cytoplasm (Fig. 1.3) (Nakahata and Kawamoto, 2005).

The RRM itself is highly conserved from *C. elegans* to humans (Fig 1.4A and E) (Kuroyanagi, 2009). Feminizing locus on X (Fox1) is closest homologue of A2BP1 in *C. elegans*. Fox1 acts as sex determination gene where complementation of male (XO) embryo with *fox1* genes results in development into a hermaphrodite (XX) adult suggesting that Fox1 alone is enough and sufficient for sex determination in *C. elegans* (Hodgkin et al., 1994). In *Drosophila*, the closest homologue of *fox1* is *CG32062* (*dA2BP1*), whose RBD shows 90 percent identity with human A2BP1 (Fig. 1.4E) (Tastan et al., 2010; Usha and Shashidhara, 2010). Comparison of tertiary structure of human A2BP1 and dA2BP1 shows that RNA binding region is highly conserved (Fig.1.4C and D). *dA2BP1* is located on Chromosome 3L and maps to 67E4-67E5. It expands over a 112 kb region and has a 50 kb long second intron (Fig.1.5A). EST database suggests that there are eight different dA2BP1 isoforms. All isoforms contain the 92 amino acid RRM (Fig.1.5B and C) (Tastan et al., 2010; Usha and Shashidhara, 2010).

dA2BP1 was first discovered as a target of the Hox protein Ultrabithorax (Ubx)(Bajpai et al., 2004). Ubx specifies the development of haltere in the third thoracic segment. Loss of Ubx in 3rd thoracic segment results in haltere to wing transformations, while gain of function of Ubx in 2nd thoracic segment leads to wing-to-haltere transformations (Lewis, 1978). dA2BP1 was discovered as a differentially expressed gene between wing and haltere. In wing imaginal disc, it is expressed in both anterior and posterior compartments. On the contrary, in the haltere disc it is expressed only in the posterior compartment. Loss of *Ubx* in haltere results in the activation of dA2BP1 in the anterior compartment. Similarly in *Contrabithorax* (Cbx^{Hm}, gain of function allele of *Ubx*), dA2BP1 expression is repressed in the anterior compartment of the wing imaginal disc suggesting that Ubx negatively regulates dA2BP1 (Bajpai et al., 2004). dA2BP1 regulates Hedgehog (Hh) signaling pathway during wing development (Usha and Shashidhara, 2010).

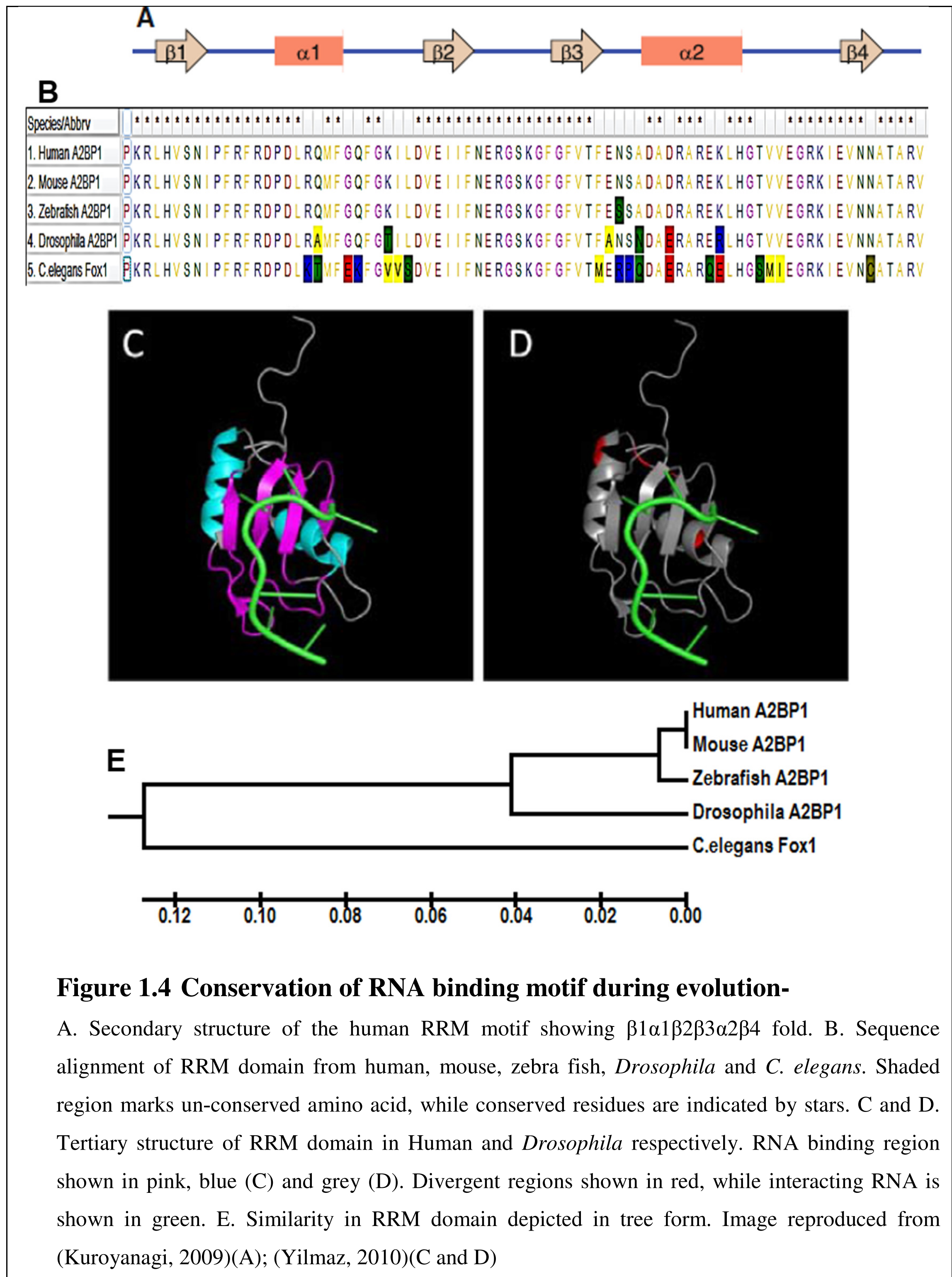
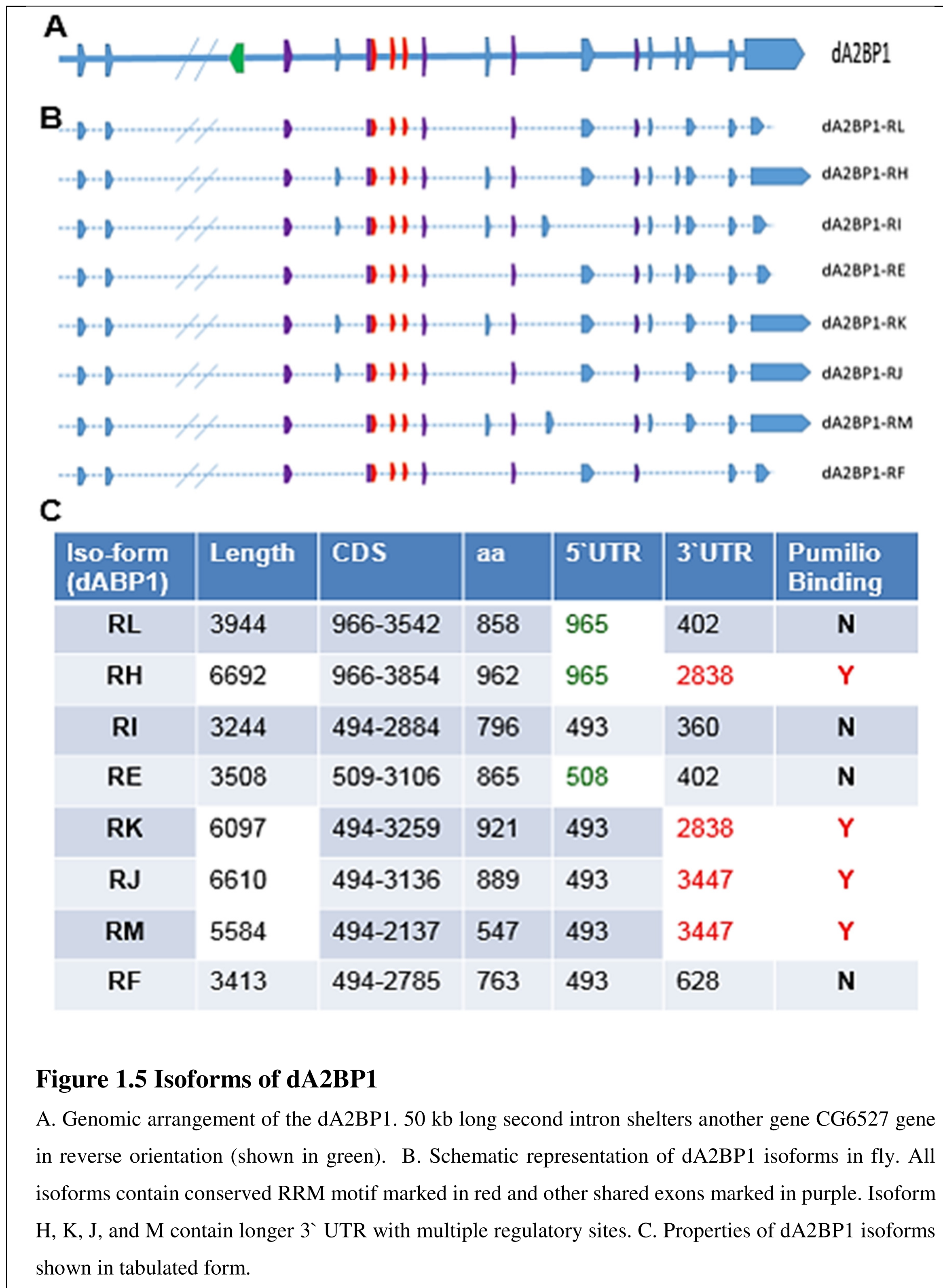


Figure 1.4 Conservation of RNA binding motif during evolution-

A. Secondary structure of the human RRM motif showing β1α1β2β3α2β4 fold. B. Sequence alignment of RRM domain from human, mouse, zebra fish, *Drosophila* and *C. elegans*. Shaded region marks un-conserved amino acid, while conserved residues are indicated by stars. C and D. Tertiary structure of RRM domain in Human and *Drosophila* respectively. RNA binding region shown in pink, blue (C) and grey (D). Divergent regions shown in red, while interacting RNA is shown in green. E. Similarity in RRM domain depicted in tree form. Image reproduced from (Kuroyanagi, 2009)(A); (Yilmaz, 2010)(C and D)



Iso-form (dABP1)	Length	CDS	aa	5`UTR	3`UTR	Pumilio Binding
RL	3944	966-3542	858	965	402	N
RH	6692	966-3854	962	965	2838	Y
RI	3244	494-2884	796	493	360	N
RE	3508	509-3106	865	508	402	N
RK	6097	494-3259	921	493	2838	Y
RJ	6610	494-3136	889	493	3447	Y
RM	5584	494-2137	547	493	3447	Y
RF	3413	494-2785	763	493	628	N

Figure 1.5 Isoforms of dA2BP1

A. Genomic arrangement of the dA2BP1. 50 kb long second intron shelters another gene CG6527 gene in reverse orientation (shown in green). B. Schematic representation of dA2BP1 isoforms in fly. All isoforms contain conserved RRM motif marked in red and other shared exons marked in purple. Isoform H, K, J, and M contain longer 3` UTR with multiple regulatory sites. C. Properties of dA2BP1 isoforms shown in tabulated form.

Loss of dA2BP1 in the wing pouch results in loss of intervein region between L3 and L4, the area regulated by Hh signaling via Knot expression. Cubitus interruptus (Ci), a zinc finger protein, acts as effector of Hh signaling. In the absence of Hh signaling, Ci undergoes posttranslational modification and proteolytic cleavage. The cleaved N-terminal Ci75 goes to the nucleus and acts as a repressor for target genes. Hh signaling prevents post-translational modification and proteolytic cleavage of Ci155, full length Ci, which acts as a transcription factor for expression of *knot, dpp* and other Hh-responsive genes. dA2BP1 physically interacts with Ci and acts as its cofactor during the *knot* regulation (Usha and Shashidhara, 2010). dA2BP1 is predominantly a nuclear protein but is also present in the cytoplasm during intermediate stage of ovarian cyst development (Tastan et al., 2010; Usha and Shashidhara, 2010). It interacts with another RNA-binding protein Bruno to regulate germline cyst differentiation and loss of dA2BP1 function results in tumor formation (Tastan et al., 2010). Loss of dA2BP1 during embryonic development leads to severe neuronal loss and affects axon guidance (Fig. 1.6C and D) (Koizumi et al., 2007).

As described above A2BP1 and its homologue regulates RNA splicing and nervous system development in vertebrates. Role of dA2BP1, the only fly homologue of human A2BP1, in the nervous system has not been explored. *Drosophila* is a good model system to learn about gene function specifically in the context of development. Here, we have examined the role of dA2BP1 during nervous system development of *Drosophila*, specifically in the context of peripheral nervous system (PNS). In spite of reasonable work on poly Q expansion in Ataxin2 and its role in diseases, we still do not understand SCA2 pathogenicity completely. Unlike other types of Spinocerebellar ataxia, SCA2 pathogenicity does not depend on intra-nuclear aggregate or nuclear inclusions formation. Toxicity through aggregate formation may have little effect on pathogenicity (Huynh et al., 2000). Understanding the cellular and developmental role of dA2BP1 may provide some insights into the cellular pathogenicity of SCA2.

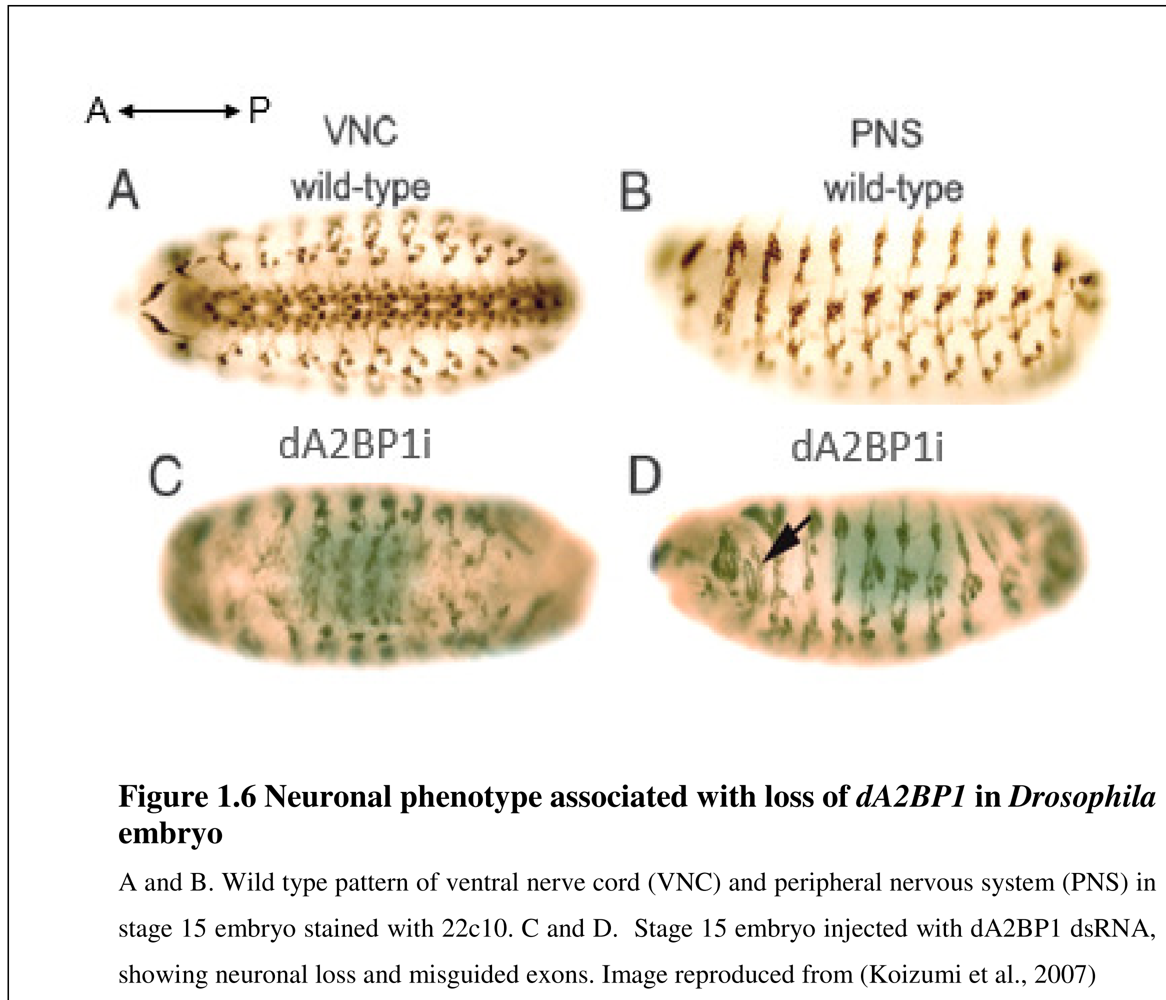


Figure 1.6 Neuronal phenotype associated with loss of *dA2BP1* in *Drosophila* embryo

A and B. Wild type pattern of ventral nerve cord (VNC) and peripheral nervous system (PNS) in stage 15 embryo stained with 22c10. C and D. Stage 15 embryo injected with dA2BP1 dsRNA, showing neuronal loss and misguided exons. Image reproduced from (Koizumi et al., 2007)

1.2 Bristle development and Notch signaling an overview:

In particular, in a developing nervous system, determination of distinct cell types that are physically proximal and share a common progenitor cell has been shown to depend upon lateral inhibition. For instance, specification of thoracic bristles, which are part of the peripheral nervous system (PNS), utilizes lateral inhibition as a strategy. These bristles arise from sensory organ precursors (SOPs) and subsequently divide and form complete sense organ comprised of shaft, socket, sheath, neuron and glia (Reddy and Rodrigues, 1999). SOPs are specified from a group of equipotent cells belonging to a pro-neural cluster (PNC). Initially, cells within the PNC cluster express equivalent levels of pro-neural proteins such as Achaete and Scute (Ac/Sc). SOP fate is conferred upon by a relatively modest and stochastic increase in Ac/Sc levels in a random subset of cells. As a

result of this increase, the SOP precursors express more levels of Delta ligand which in turn activates Notch signaling in the neighboring cells. Activation of Ac/Sc and differential Notch/Delta signaling activates Senseless (Sens) and Neuralized (Neur) expression in SOP cells to confirm the SOP fate. Activation of the Notch pathway inhibits SOP specification limiting the total number of SOPs (Heitzler and Simpson, 1991; Kunisch et al., 1994). Supporting the pivotal role of Notch signaling in this specification event, loss of Notch mitigates the lateral inhibition and leads to supernumerary bristles, also known as tufting.

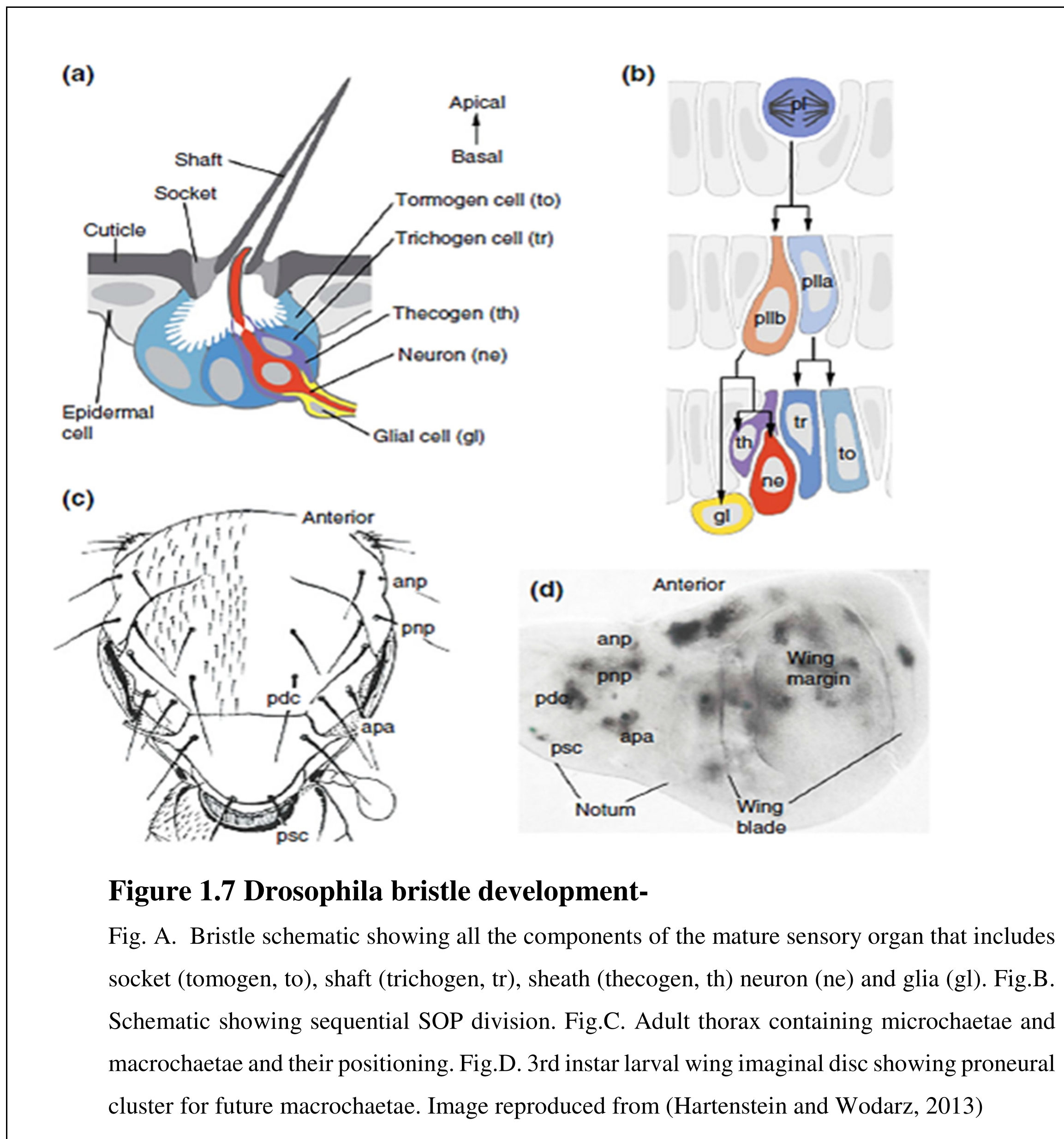


Figure 1.7 Drosophila bristle development-

Fig. A. Bristle schematic showing all the components of the mature sensory organ that includes socket (tomogen, to), shaft (trichogen, tr), sheath (thecogen, th) neuron (ne) and glia (gl). Fig.B. Schematic showing sequential SOP division. Fig.C. Adult thorax containing microchaetae and macrochaetae and their positioning. Fig.D. 3rd instar larval wing imaginal disc showing proneural cluster for future macrochaetae. Image reproduced from (Hartenstein and Wodarz, 2013)

As in the case of most signaling pathways, depending on the context, Notch signaling also follows canonical and non-canonical modes of signal transduction. Canonical signaling requires activation of Notch receptor by one of the ligands of the Delta/Serrate/Lag-2 family (DSL) family at the cell surface typically presented by the neighboring cells. This receptor ligand interaction results in release of Notch intracellular domain (NICD), which is translocated into the nucleus where it interacts with the transcription factor(s) of CBF1/Suppressor of Hairless/LAG-1 (CSL) family and Mastermind (Mam) to regulate transcription of the down-stream target genes such as *Enhancer of split E(spl)-Complex*, *wingless* (*wg*), *cut* and *Vestigial* (vg)(Guruharsha et al., 2012). Notch regulates sensory organ development by regulating *Enhancer of split* group of genes (Culí and Modolell, 1998; Heitzler et al., 1996a; Lecourtois and Schweisguth, 1995). Notably, it regulates target genes either in an instructive or in a permissive manner. For example, *wg* activation needs permissive function whereas *cut* and *E(spl)m8* both require instructive role (Janody and Treisman, 2011). In a permissive mode, presence of Notch receptor is sufficient to neutralize the repressors thereby facilitating the activation of the downstream targets. By contrast, Notch requires different sets of additional co-factors to regulate target gene expression while participating in an instructive capacity(Janody and Treisman, 2011). Interestingly however, although context specific co-factors have been conjectured on a number of occasions their molecular identity has remained elusive.

Detailed introduction on various aspects of structure and organization of PNS, signaling pathways involved in PNS development, etc. relevant to this study are provided in subsequent chapters.

Thesis has been divided in following section.

1. A2BP1 introduction and overview of bristle development and Notch signaling.
2. Materials and method
3. Peripheral nervous system of drosophila and neuronal phenotype associated with dA2BP1.
4. dA2BP1 genetic interaction with signaling pathways.
5. Molecular role of dA2BP1 in bristle development.
6. Conclusion and future prospects.

1.3 Specific Objectives:

1) To elucidate the expression of dA2BP1 during the development of nervous system in *Drosophila*
2) To examine neuronal phenotypes associated with loss- and gain-of-dA2BP1.
3) To identify of pathways that are directly regulated by dA2BP1 during neuronal specification.
4) To study the precise mechanism by which dA2BP1 regulates neuronal development.

2 Chapter 2: Materials and Methods

2.1 Fly Stocks-

2.1.1 Various alleles:

w^{1118} and Canton-S strains of *Drosophila melanogaster* were used as control.
$A2BP1^{EY01049}$, (BL.15489); $A2BP1^{PL00487}$, (BL.19522); $A2BP1^{MB03305}$,(BL.24263); $A2BP1^{MI07729}$, (BL.43760); $A2BP1^{MI09134}$, (BL.54491); $A2BP1^{MI01918}$, (BL.44669); $A2BP1^{MI04255}$,(BL.37417); $A2BP^{MI09677}$, (BL.54505); $A2BP1^{c03982}$,(Harvard Stock Center); $A2BP1^{e03440}$, (Harvard Stock Center); $A2BP1^{f01889}$, (Harvard Stock Center); $A2BP1^{KG06463}$,(Harvard Stock Center); $A2BP1^{C00511}$, (Buszczak et al.; 2007), N^{55e11}(loss of function allele of Notch; BL 28813), N^{Ax16} (Notch gain of function allele; BL 52014), $Delta^{7}$ (hypomorphic allele of Delta; BL 485), $Sens^{58}$ (loss of function allele of Senseless, BL 5312), $Sense^{E2}$ (Senseless loss of function allele, BL 5311), H^{1} (loss of function allele of Hairless; BL 515), H^{2} (Hairless loss of function allele; BL 517), H^{3} (loss of function allele of Hairless; BL 518),

2.1.2 GAL4 drivers:

dpp-GAL4$^{40.6}$ (Morimura et al., 1996), *c253*-GAL4 (BL 6980), *pnr*-GAL4(Calleja et al., 1996), *sca*-GAL4 (BL 6479), *omb*-GAL4 (Calleja et al., 1996), *ap*-GAL4(Calleja et al., 1996), *iroqous*-GAL4, *c765*-GAL4, *EM46*-GAL4 (*notal*-GAL4 Kind gift from Juan Modollel)

2.1.3 UAS lines for over-expression:

UAS-dA2BP1 (Usha and Shashidhara, 2010), UAS-Notch^{DN} (dominant negative Notch; (Rebay et al., 1993), UAS-Notch^{intra} (constitutively active Notch; (Rebay et al., 1993), UAS-E(spl)m8 (BL 26827), (Freeman, 1996), UAS-EGFR^{DN} (EGFR dominant negative, (Golembo et al., 1996), UAS-Senseless (BL 39681), UAS-nuclear lacZ (Brand and Perrimon, 1993).

2.1.4 **UAS lines for the induction of RNAi:**

UAS-*A2BP1*^{RNAi} TRiP line.JF02600, (BL.27286); UAS-*A2BP1*^{RNAi} TRiP line.HMS00478, (BL.32476); UAS-*A2BP1*^{RNAiKK} (line: 109100) VDRC (Vienna, Austria); UAS-*A2BP1*^{RNAi} (NIG collection) and UAS- d*A2BP1*^{RNAi} (Usha and Shashidhara, 2010).

2.1.5 **LacZ reporter lines:**

Enhancer of split m8 (E(spl))-lacZ, *E(spl)mβ*-lacZ (kind gift from Sarah Bray), *neuralized*-lacZ (A101; BL 4369),

2.2 Fly work-

Unless stated otherwise, all experiments were carried out at 25°C on standard fly medium containing corn-floor, sugar, yeast, agar and malt (corn-floor 75g, sugar 80g, yeast 24g, agar 10g and, malt 60g per 1000ml) Propionic acid (5ml/l), ortho-phosphoric acid (1ml/l) and p-methyl benzoate (5ml of 5% solution/l) were added before pouring the media into vials and bottles.

2.2.1 **Larval dissection and immunohistochemistry:**

The larvae were collected in a glass cavity block and washed and dissected in PBS. To access wing and other imaginal discs, the larvae were cut open at the posterior 2/3 part of the larvae. The remaining 1/3rd was turned inside out with the help of a pair of needles. The exposed imaginal discs along with inverted larval cuticle were fixed in 4% paraformaldehyde (made in PBS, pH 7.4) for 20 minutes. After fixation, the larval tissue was washed 3 times with PBS (5 minutes each) and processed for immunohistochemistry.

The fixed and washed larval tissue was blocked for an hour with blocking solution (PBS + 0.1% TritonX-100 (Sigma) + 0.5% BSA (Sigma)). The tissue was further incubated with the primary antibodies (diluted in blocking solution) either 3 hours at room temperature or overnight at 4°C on a rotating plate. After incubation, the primary antibodies were removed and tissue was washed 4 times with PBT (PBS + 0.1% TritonX-100 (Sigma), 10 minutes each. The tissue was then incubated with secondary antibodies (diluted in blocking solution) for 2 hour at room temperature or overnight at 4°C on a rotating plate. As the secondary antibodies were conjugated with fluorophore, further processing was done under dim light or in dark conditions. After incubation, the secondary antibodies were

removed and tissue was washed 4 times with PBT, 10 minutes each. Finally, the tissue was washed with PBS to remove the detergent. The desired imaginal discs were detached and mounted in mounting medium (PBS + 80% glycerol, Sigma) on a glass slide and covered with a cover slip. Excess mounting medium was removed with the help of tissue paper and the cover slip was sealed with nail polish.

2.2.2 **Embryo collection:**

Embryos were collected on agar plate with yeast paste. The agar plate consists of agar, 6.25gms; sugar, 7.50gms; dissolved in 250 ml water and boiled. Anti-fungal reagents including propionic acid (5ml/l), ortho-phosphoric acid (1 ml/l) and p-methyl benzoate (5ml of 5% solution/l) were added when solution was cooled below 55°C. The media was poured in petri-plates, cooled overnight and then smeared with yeast paste on the corner. The plates were set with embryo collection cages. Flies with desired genotype were added to these cages. The first 48 hour collection was discarded as the egg laying is normally poor in the start. The plates were regularly changed and desired stage embryos were collected and aged. The embryos were collected on an 111 µm mesh sieve and washed thoroughly with running tap water. The embryos were further dechorionated in 50% sodium-hypochlorite solution for 2-3 minutes and rinsed with water several times to remove the sodium-hypochlorite. Finally embryos were dried on tissue paper and fixed in 1:1 heptane and 4% paraformaldehyde (made in PBS, pH 7.4) for 20 minutes. After fixation, embryos were washed 3 times with PBS (5 minutes each) and devitellinized in 1:1 methanol and heptane. The devitellinized embryo was either preceded for blocking and immunohistochemistry or serially dehydrated and stored in 100% methanol for future use. Immunohistochemistry protocol used was common for embryo and imaginal disc staining.

2.2.3 **Primary antibodies for immunohistochemistry:**

The following antibodies were used in this study:

Rabbit polyclonal anti-dA2BP1(Usha and Shashidhara, 2010)1:150), mouse anti-Futsch 22c10 (1:10), (DSHB); mouse monoclonal anti-Neurotactin BP106 (1:200), (DSHB); mouse monoclonal anti-Achaete (1:10), (DSHB); mouse monoclonal anti-Notch^{intra} C17.9C6, (1:200), (DSHB); mouse monoclonal anti-Wingless (1:200; (Brook and Cohen,

1996); mouse monoclonal anti-Cut 2B10 (1:50), DSHB); guineapig polyclonal anti-Senseless (1: 1000; Kind gift from Hugo Bellen), goat polyclonal anti-Su(H) (1:1000; SantacruZ), goat polyclonal anti-CtBP (1:1000; SantacruZ, sc-26610), rabbit polyclonal anti-Groucho (1:1000; kind gift from Girish Ratnaparkhi), mouse monoclonal anti-β galactosidase 41-1ea (1:800), chicken polyclonal anti-β galactosidase (Abcam 1:1000), rabbit polyclonal anti-GFP (Invitrogen 1:2000) and chicken polyclonal anti-GFP (1:500; Invitrogen).

2.2.4 **Secondary antibodies for immunohistochemistry:**

Following secondary antibodies from Invitrogen were used: goat anti-mouse Alexafluor 488 (1:1000), goat anti-mouse Alexafluor 568 (1:1000), goat anti-rabbit Alexafluor 488 (1:1000), goat anti-rabbit Alexafluor 568 (1:1000), goat anti-gunea pig Alexafluor 488 (1:1000), goat anti-guinea pig Alexafluor 633 (1:1000) and goat anti-chicken Alexafluor 633 (1:1000).

Fly wing processing and mounting:

The adult flies of desired phenotype were collected in 50% ethanol and serially dehydrated with 70%, 90% and 100% ethanol: 10 min each. Finally the flies were transferred to clove oil for overnight clearing and mounted on glass slide in DPX mounting solution. The slides were dried overnight before microscopy.

2.3 Microscopy-

The adult fly phenotype was recorded using either Leica S8APO with LAS EZ software or scanning electron microscope Zeiss EVO LS10 with Smart SEM software. Fluorescent images were taken by either Zeiss imager Z1 with Axiovision 4.8 software or confocal using Zeiss LSM 710 with ZEN 2010 software. Images were processed with Adobe Photoshop (version CS6) and Image J. All images processing comply with standard ethical practices.

2.4 Statistical tests-

To understand the role of dA2BP1 in fly nervous system, we scored sensory organ precursory (SOP) cells of wing discs and adult dorso-central (DC) and scutellar (SC)

macrochaete in various genetic backgrounds. We analyzed our results using student's t-test and error bar represents standard deviation (SD).

2.5 S2 Cell Culture and Transfection-

S2 cells were cultured at 23°C in Schneider's *Drosophila* media (GIBCO) supplemented with 10% FBS, 50 units/ml penicillin and streptomycin. S2 cells do not express Notch due to a mutation at the 5' UTR of *Notch* gene. We made S2-Notch stable cell line by transfecting pMT-Notch (DGRC#1022). 2 µg of pMT-Notch was mixed with 0.1 µg of pico-hygro and transfected using transfection reagent (Mirus Bio , MIR 5400A). Stable cell lines were selected for hygromycin (Invitrogen Cat# 10687-010) resistance (150µg/ml) for one month and continuously maintained in the presence of hygromycin (150µg/ml). Whenever required, Notch expression was induced by adding 600 µM CuSO4 for 20-24 hours. Cleavage of the Notch receptor was activated by treating Notch expressing S2 cells with 1 µM EDTA in PBS for 30 minutes (Krejci and Bray 2007).

2.6 dsRNA Production-

cDNA of A2BP1 and Su(H) were obtained from Gold collection DGRC. We used pre-designed primers against A2BP1 (primer reference: DGRC# DRSC25775) and Su(H) (primer reference: DGRC# HFA03445) to amplify templates for in-vitro transcription. T7 polymerase binding site –taatacgactcactataggg- was included at 5' end of the primers. In-vitro transcription was carried out using a MEGASCRIPT T7 transcription kit (Ambion) according to the manufacturer's protocol. After the transcription, RNA was precipitated overnight with Lithium Chloride and Ethanol (110µl water+1/10 V 4M LiCl (13.5µl) + 2.5V 100% Ethanol (370µl)) at -20^{0}C. After incubation, the precipitate was pelleted down by centrifugation @12000 RPM, 4^{0}C for 15 minutes. The pellet was washed with 70% ethanol by centrifugation @12000 RPM, 4^{0}C for 15 minutes. Supernatant was removed and pellet was air dried and re-suspended in DEPC-treated water. RNA was annealed at 65°C for 12 min and slowly cooled to room temperature and stored at -20^{0}C (Clemens et al., 2000). RNA quality was checked on Agarose gel to insure annealing and quantitated on Nonodrop. For dsRNA treatment, cells were seeded at 40% cell density in 6 well plate, supplemented with 30 µg dsRNA in 400 µl of serum free medium for 90 minutes; followed

by addition of 800 µl cell culture medium. After 3 days, cells were split and supplemented with 10 µg of dsRNA and cultured for 12 h before CuSO4 induction.

2.7 RNA isolation and quantification-

Total RNA was isolated using Trizol reagent (Invitrogen). Cells or tissue sample were collected in 1.5 ml microfuge tube and mixed with 5 volume of Trizol and homogenized at 4^0C by mixing vigorously. After complete homogenization, the sample was added with 1/5 volume of chloroform and mixed by inverting the tube 5 times. The sample was incubated further for 5 min at RT and centrifuged @12000 RPM, 4^0C for 15 minutes. Aqueous layer was harvested without disturbing the interphase. The harvest was mixed with equal volume of 100 % isopropanol and precipitated overnight at −20 °C. The precipitate was then pelleted down by centrifugation @12000 RPM, 4^0C for 15 minutes. The pellet was washed with 70% ethanol by centrifugation @12000 RPM, 4^0C for 15 minutes. Supernatant was removed and the pellet was air dried and re-suspended in DEPC-treated water. RNA quality was checked on agarose gel electrophoresis. The RNA was quantified by spectrophotometry (Nanodrop) and 2 µg of RNA was reverse transcribed to cDNA using superscript kit, Invitrogen and oligo dT primers. To check gene expression, real time PCR was performed with KAPA syber fast qPCR kit and mRNA was normalized against rp49. Details of primer are mentioned in Table 1.

2.8 Notch expression and activation in S2 cell

To understand precise mechanism by which dA2BP1 regulates targets of Notch pathway, we examined physical interactions between dA2BP1, Notch and Su(H) by immuno-precipitation experiments. For this, we used S2 cells. As S2 cells do not have functional Notch, we expressed it under an inducible expression system.

2.8.1 Experimental procedure

S2 cell were transfected with pMT-Notch pico-hygro and stable cell line was made after a month-long selection for hygromycin resistance. Notch expression was induced with varying amount of $CuSO_4$ for 20-24 hours and Notch receptor cleavage was induced with

1 μM EDTA in PBS for 30 minutes. For detail, please refer materials and method (2.5, 2.6 and 2.7)

Notch encodes a 300 kDa single pass transmembrane protein. Notch activation by proteolytic or EDTA induced cleavage generates a 120 kDa cytoplasmic domain (NICD). Monoclonal antibody C17.9C6 (DSHB) generated against NICD recognizes both full length Notch and NICD. We observed Notch expression after $CuSO_4$ induction and expression varies with varying amount of CuSO4. We also observed proteolytically cleaved form NICD, which was induced by EDTA (Fig. 2.1).

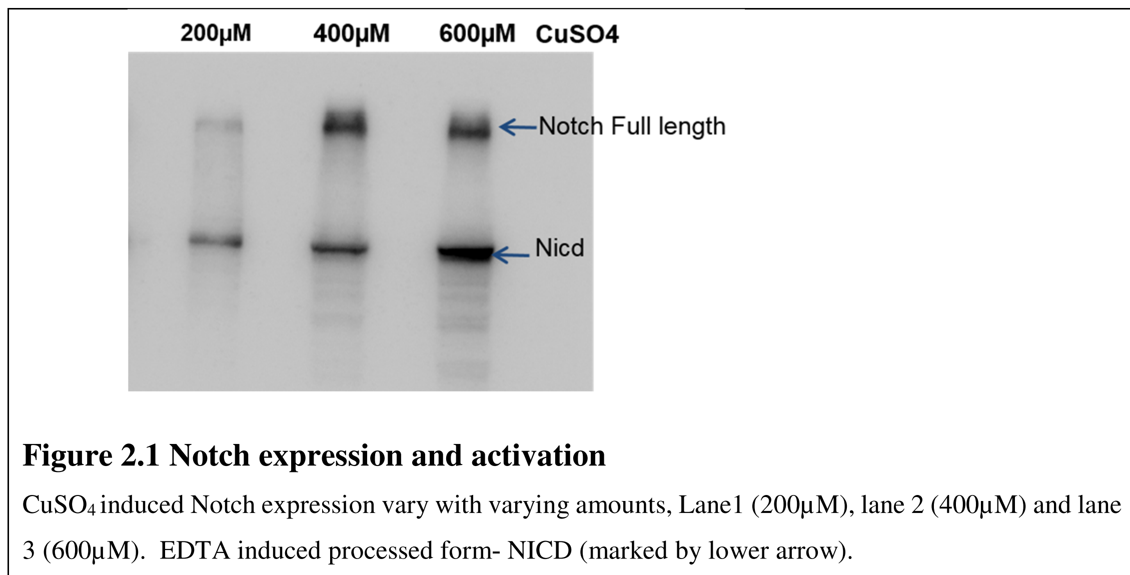


Figure 2.1 Notch expression and activation

$CuSO_4$ induced Notch expression vary with varying amounts, Lane1 (200μM), lane 2 (400μM) and lane 3 (600μM). EDTA induced processed form- NICD (marked by lower arrow).

2.8.2 Activation of Notch targets after EDTA induction-

To validate functionality of expressed Notch, we examined levels of its target expression in both EDTA induced and un-induced backgrounds. E (spl)-C is a direct target of Notch. E (spl)-C houses 11 genes, and among these, 7 genes - viz., mδ, mγ, mβ, m3, m5, m7 and m8 - encode for helix loop helix proteins. We quantified mγ, mβ, m3, m7 and m8 expression using real time PCR and mRNA expression was normalized against rp49. We observed that after EDTA induction, all of Notch targets except m8 showed increase in

expression. Other studies have also observed same response during EDTA based activation (Krejci et al.; 2007).

Table 1. Primer list used in Real time PCR

Gene Name	**Primer Name**	**Primer sequence**
RP49	RP49F	AGT ATCTGATGCCCAACATCG
	RP49R	TTCCGACCAGGTTACAAGAAC
E(spl)m3	DMM3F	AACAGCAACAACACCAGCAG
	DMM3R	GGACTCCTGCGAGCTAACC
E(spl)mβ	DMMBF	CTACGTTCATGCTGCCAATG
	DMMBR	ATTCAGAGGGTGGTGGAGTG
E(spl)mγ	DMMGF	GTCAATGAGGTCTCCCGTTC
	DMMGR	GGTCAACAGGGAATGGCTGG
E(spl)m7	DMM7F	AGTGGATGTGGCTTTTGGAACC
	DMM7R	GACGATACTGAGTGGAGTGTTGACG
E(spl)m8	DMM8F	ATGAACAAGTGCCTGGACAACC
	DMM8R	CTTCCTGAGCCACCTTCTTTGG

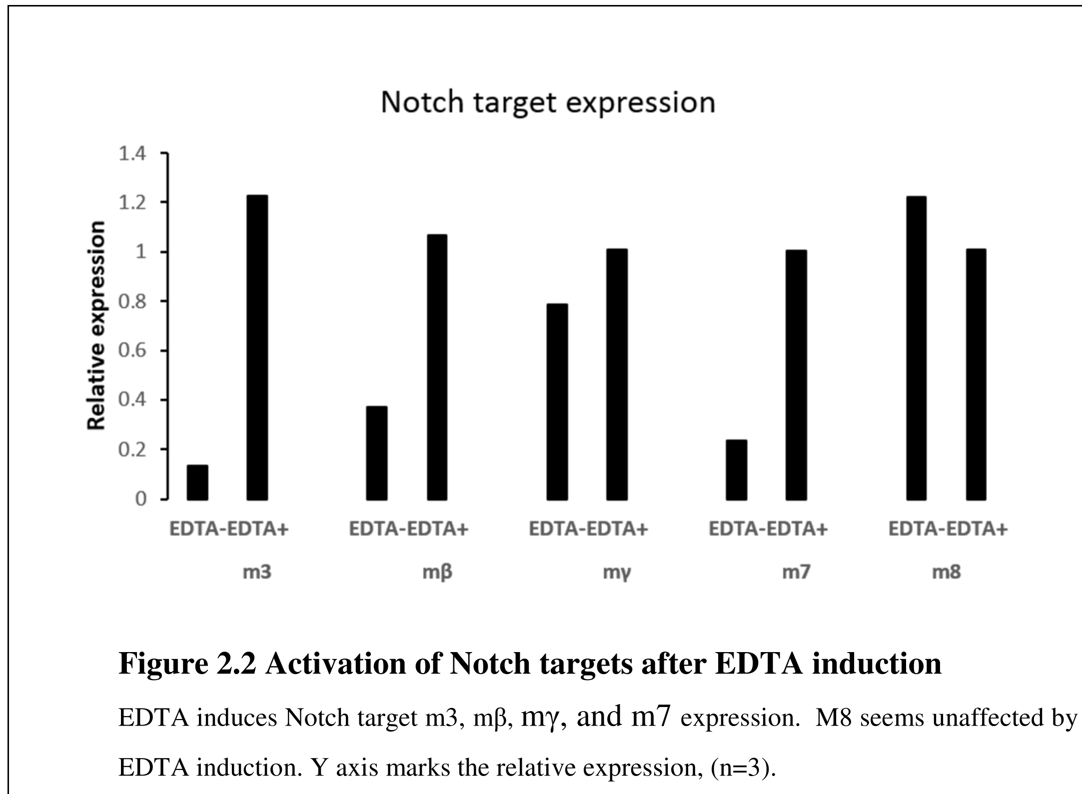


Figure 2.2 Activation of Notch targets after EDTA induction

EDTA induces Notch target m3, mβ, mγ, and m7 expression. M8 seems unaffected by EDTA induction. Y axis marks the relative expression, (n=3).

S2 cells expressing functional Notch were then used for immune-precipitation experiments.

2.9 Immunoprecipitation (IP) and Western blot hybridization-

Cells with 70 to 80% confluency were collected by centrifugation at 1800 rpm and washed with buffer 1 containing 50 mM Tris-HCl and 2 mM EDTA. Cells in buffer 1 were mixed with equal volume of high salt lysis buffer containing 600 mM NaCl, 50 mM Tris-HCl, 2 mM EDTA, 1% triton X100 and lysed for 30 min at 4^0C on a rotator. After lysis, the salt concentration was adjusted to 100mM for further IP by adding buffer1. The cell lysate was centrifuged at 12000 rpm for 20 minutes at 4^0C to remove cell debris and pre-cleared with Protein A magnetic beads for 1-2 hours before proceeding for IP. 10% of total cell lysate was retained as input. The diluted cell lysate (1500µl) was divided into equal halves, one was incubated with 3 µg of either rabbit polyclonal anti-A2BP1or rabbit polyclonal anti-Groucho or Goat anti-Su(H) (Santacruz) antibodies and the other half was incubated with equal amount of Rabbit IgG- (Bethyl) or Goat IgG (Santacruz) or Rat IgG (Santacruz),

respectively as control. The mixture was rotated for 8-16 hours at 4^0C and further incubated with Protein A magnetic beads (Ademtech) for 3 hours. The beads were washed 4 times with 150 mM NaCl, 50 mM Tris-HCl, 2 mM EDTA, 0.1% triton X and eluted in 50 µl wash buffer and 15 µl of 5X SDS loading buffer.

Protein sample was resolved by running 10% SDS PAGE and blotted on PVDF membrane (Millipore). The blot was blocked with 5% fat free milk in TBST buffer (20 mM Tris, pH 7.6; 150 mM NaCl; 1% Tween 20) and incubated with primary antibodies (diluted in blocking solution) either 3 hours at room temperature or overnight at 4^oC under rotating condition. After incubation, the primary antibodies were removed and the blot was washed 4 times with TBST, 10 minutes each. The blot was further incubated with secondary antibodies conjugated with HRP (diluted in blocking solution) for 1 hour at room temperature under rotating condition. After incubation, the secondary antibodies were removed and blot was washed 4 times with TBST, 10 minutes each. Finally, the blot was exposed with HRP substrate (Millipore) and imaged by CCD camera LAS4000. The images were processed with Image J or Fuzifilm software.

Following secondary antibodies were used in Western blot hybridization (obtained from Jackson laboratories): goat anti-rabbit-HRP (1:10000), goat anti-mouse-HRP (1:10000), rabbit anti-goat-HRP (1:10000), goat anti-Rat-HRP (Abcam 1:5000).

3 Chapter-3: Expression and function of dA2BP1 during sensory organ development in *Drosophila*

3.1 Introduction-

***Drosophila* Peripheral Nervous System –**The nervous system is largely divided into two parts: the central nervous system (CNS) and the peripheral nervous system (PNS) on the basis of the distribution of the neurons and their polarity. PNS collect sensory information from distant body parts, which will be finally integrated and analyzed in the CNS and the feedback response is initiated by motor neurons at the neuromuscular junctions. *Drosophila* CNS originates from the progenitor cells known as neuroblasts. Neuroblast specification and proliferation takes place during embryogenesis and neurons generated in this period form the larval and adult nervous system, while PNS originates from the epidermis during the course of development.

PNS consists of 4 types of sensory transducers: external sensory organs (es), chordotonal organs (ch), multiple dendritic (md) neurons and photoreceptors (Fig. 3.1) (Jan and Jan, 1994). Based on dendrite structure of the neurons, these sensory organs can be divided into two types. Type I sensory organs that include chordotonal organ and external sensory organ, contain a single dendrite and are associated with accessory cells. Type 2 sensory organs contain a neuron with multiple dendrites and they lack accessory cells. These include the multi dendritic sensory neurons.

3.1.1 **Multidendritic neurons:**

Multidendritic (Md) neurons can be found in embryo, larvae and adult. They appears like cluster of 5 cells just beneath the epidermal layer. They are also connected with trachea and muscle. Md neurons can be further divided into 3 types, tracheal dendrite neurons (td), bipolar dendrite neurons (bd) and dendritic arbarisation (da) (Bodmer and Jan, 1987). Depending upon the arborisation pattern, da neurons can be further classified into 4 types: Class I to Class IV (Grueber et al., 2002; Sugimura et al., 2003). Class I da neurons are very simple comb like small dendrites, which will get bigger in terms of their territory size and branching as we move from class I to class IV (Fig. 3.2A).

3.1.2 **Photoreceptors:**

Fly eye is composed of approximately 800 hexagonal structures called ommatidia (Fig. 3.2B) and each ommatidium is composed of eight photoreceptors, four cone cells, two primary pigment cells and 12 supporting inter-ommatidial cells. Photoreceptors are neuronal cells and originate from eye imaginal disc during development. The apical region of the photoreceptor cell develops microvillus structure called rhabdomere, which contains the photosensitive molecule, opine, that changes its conformation during light perception. Photoreceptor specification also requires Atonal expression (Jan and Jan, 1994, 1995).

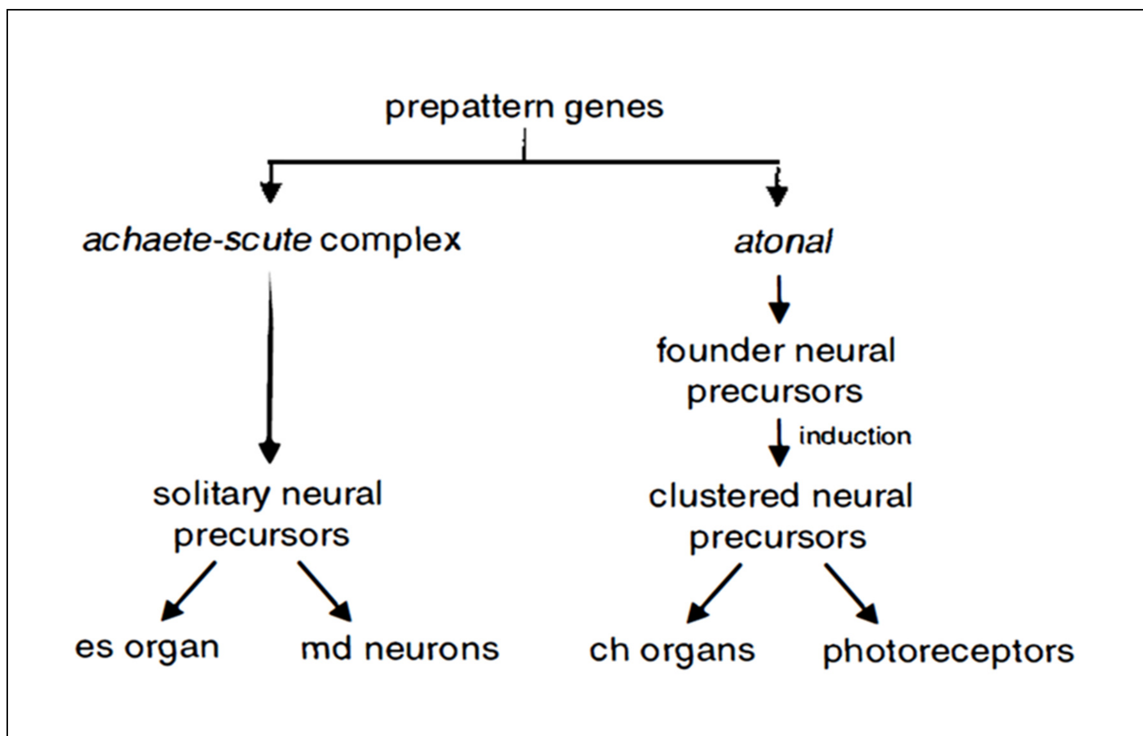


Figure 3.1 Drosophila PNS classification on the basis of proneural protein requirement-

Achaete/Scute guides sensory organ development by solitary sensory organ selection from clustered progenitors, whereas Atonal regulates clustered sensory organ development Image reproduced from (Jan and Jan, 1994).

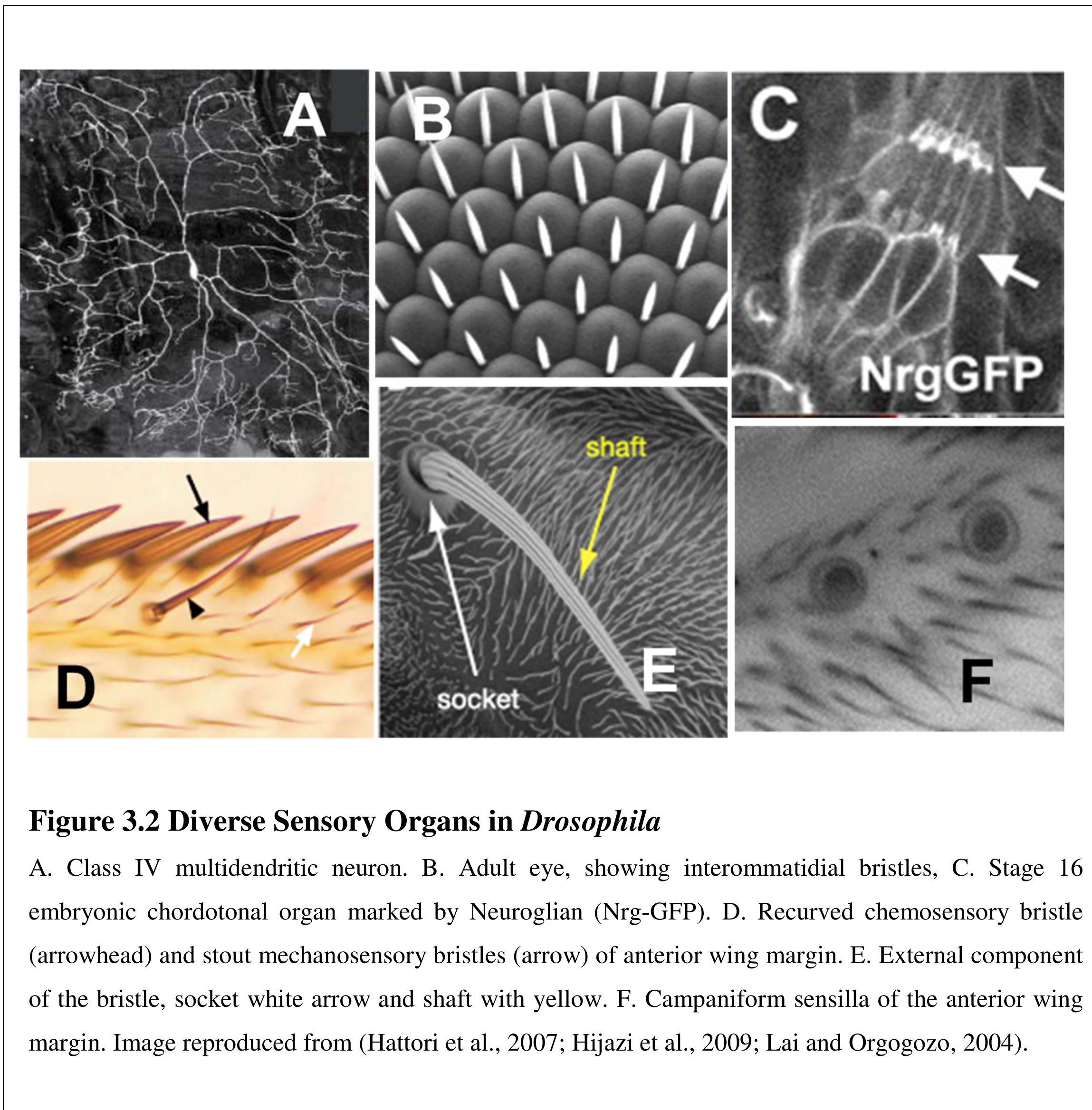


Figure 3.2 Diverse Sensory Organs in *Drosophila*

A. Class IV multidendritic neuron. B. Adult eye, showing interommatidial bristles, C. Stage 16 embryonic chordotonal organ marked by Neuroglian (Nrg-GFP). D. Recurved chemosensory bristle (arrowhead) and stout mechanosensory bristles (arrow) of anterior wing margin. E. External component of the bristle, socket white arrow and shaft with yellow. F. Campaniform sensilla of the anterior wing margin. Image reproduced from (Hattori et al., 2007; Hijazi et al., 2009; Lai and Orgogozo, 2004).

3.1.3 **Chordotonal organs:**

Chordotonal (ch) organs lie in sub-epidermal layer of the embryo and larvae (Fig. 3.2C). They function as stretch receptors and help in larval locomotion. In the adult fly, they are located near the hinge of wing and haltere and leg joints. They are also present in thoracic and abdominal segments. They form Johnston's organ of adult antenna by combining several Ch organs. Ch organ formation requires expression of proneural protein called

Atonal (Jarman et al., 1993; Jarman et al., 1995). Homeo-box protein Cut represses ch fate (Bodmer et al., 1987).

3.1.4 **External sensory organs:**

External sensory (es) organs are present on the adult body surface and they provide information about external environment (Lees and Waddington, 1942). The es organs are located in the epidermis. They contain 1 to 5 neurons and four support cells. Neuronal cell-body is located just beneath the epidermis, while dendrites penetrate the epidermis to reach the cuticle apically and the axon migrates basally to the CNS. Based on the signal perception, es organ can be of two types, mechanosensory and chemosensory (Hartenstein and Posakony, 1989). The mechanosensory es organs contain single bipolar neurons whereas chemosensory have additional four neurons which come from additional division of neuronal cell. Paired box protein (Pox neuro) regulates chemosensory organ development (Awasaki and Kimura, 1997). Loss of function of *pox neuro* directs the neurons towards mechanosensory cell fate (Dambly-Chaudière et al., 1992). Based on the external appearance and cell composition external sensory organs can be classified into 3 sub-types: wing margin bristle, campaniform sensillum and thoracic bristle.

3.1.5 **Wing margin bristle:**

Wing margin bristles are present only on anterior part of the wing. These bristles are arranged in three rows: dorsal surface of the wing contains two rows and ventral surface contains one. The first dorsal row consists of sparsely arranged recurved chemosensory bristles, while second row contains densely arranged mechanosensory stout bristles (Fig. 3.2D). The ventral row recurved chemosensory bristles are spaced by four non-sensory epithelial hairs. The triple row pattern follows until the end of L2 vein after that two dorsal rows combine and form a single row and continues until L3. The posterior wing margin contains only non-sensory epithelial hairs (Hartenstein and Posakony, 1989).

3.1.6 **Thoracic bristles:**

Drosophila has two kinds of thoracic bristles: macrochaetae and microchaetae. As the names suggest, macrochaetae are bigger in size, they have a definite number and place on thorax. Microchaetae are smaller in size and very well aligned all over the thorax except

scutellum (Fig. 3.2E). There are 11 stereotypically arranged macrochaetae per hemi-notum. These can be named differently based on their position on the thorax i.e, anterior dorsocentral (aDC), posterior dorsocentral (pDC), anterior scutellar (aSC), posterior scutellar (pSC), posterior postalar (pPA), anterior postalar (aPA), posterior supraalar (pSA), anterior supraalar (aSA), posterior notopleural (pNP), anterior notopleural (aNP), and presutural (PS). Except macrochaetae, other types of bristles show variation in number and position from fly to fly and between male and female. Both microchaetae and macrochaetae show common mode of development, cellular composition and cell lineages. Sensory organ precursors for microchaetae and macrochaetae appear at different time points of fly development (Ghysen et al., 1993). The macrochaetae that originate from proneural cluster are marked by Achaete/Scute expression but can be regulated by a different set of genes (Cubas et al., 1991). Macrochaetae sensory organ precursor (SOP) appears during late third instar larval stages and its division starts in early pupal stages giving it much needed time to secrete more cuticle for bigger bristles, whereas microchaete SOP selection and division starts in pupal stages resulting in smaller bristle (Fig1.7).

3.1.7 **Campaniform Sensillum:**

Campaniform sensilla appear like flattened oval discs present on both larvae and adult body surface (Fig. 3.2F) (Cole and Palka, 1982). They function as flex receptors embedded in the exoskeleton. In larvae, they are present only on the abdominal body segment (A1 to A7) and in the adult fly, all over the body surface (Higashijima et al., 1992). Campaniform sensilla present on leg, wing and haltere have been explored in detail (Cole and Palka, 1982). A single campaniform sensillum is composed of 5 different cell type including socket, papila, glia, sheath and neuron. Homeo-box protein BarH1 and BarH2 expression decides the campaniform sensillum fate over trochoid bristle (Higashijima et al., 1992).

As discussed in Chapter 1, a major objective of this study is to examine the function of dA2BP1 during normal development of the *Drosophila* nervous system. We examined its expression patterns by antibody staining against dA2BP1 and function by RNAi-mediated knock-down of dA2BP1 in different parts/components of the nervous system. We then identified an appropriate component of the nervous system for studying the mechanism of dA2BP1 function in more detail.

3.2 Results-

3.2.1 **dA2BP1 is expressed in the nervous system of *Drosophila*:**

dA2BP1 is expressed in the developing embryo (Koizumi et al., 2007), most of the imaginal discs (Usha and Shashidhara, 2010) and also in the developing ovarian cyst (Tastan et al., 2010). In wing imaginal discs, dA2BP1 expresses in the entire wing pouch except the dorso-ventral (DV) boundary. dA2BP1 is also strongly expressed in myoblasts of the wing disc, located in the notal region (Bajpai et al., 2004; Usha and Shashidhara, 2010). dA2BP1 is majorly a nuclear protein. However, cytoplasmic localization is also reported (Tastan et al., 2010). Although dA2BP1 is expressd ubiquitously in most of the imaginal discs, before understanding its role in the nervous system development, we first examined its expression in the nervous system. In early stages of embryo (stage 14), dA2BP1 is expressed in the chordotonal organs, as indicated by its co-localization with the neuronal marker Futsch (22c10, Fig3.3A). Based on the co-localization of dA2BP1 and Neurotactin (BP106, Fig3.3B), we observed that dA2BP1 is expressed in neuroblasts and post-mitotic neurons of the ventral nerve chord (VNC).

We also examined its expression in proneural cluster (PNC) and sensory organ precursors (SOP) of the wing imaginal disc. The notum region of the wing imaginal disc forms fly thorax. SOP develops from PNC and differentiates into complete sensory organs (which are also called bristles). *Enhancer of split m8 (E(spl) m8)*-LacZ expression marks PNCs and Senseless (Sense) expression marks the SOP fate. dA2BP1 staining was observed in PNCs and upcoming SOPs as shown by co-localization with the E(spl) m8 and Sense (Fig.3.3C). These observations suggest that dA2BP1 is expressed in the neuronal precursor cells of both CNS and PNS.

3.2.2 **dA2BP1 regulates external sensory organ development:**

To understand role of the dA2BP1 in the nervous system, we examined neuronal phenotype in various available mutants of *dA2BP1*. Various attempts have been made in the past in the lab to generate loss of function mutants either by EMS mutagenesis or by P-element insertion. These attempts failed to generate loss-of-function mutations in the coding region of *dA2BP1*.

Table.2- dA2BP1 mutation and phenotype

Mutant	Location	Collection	Remark
A2BP1^{EY01049}	1st Intron	P(EPgy2)/ (Bellen et al., 2004)	10% homozygous flies, No wing and sensory bristle phenotype
A2BP ^{PL00487}	2nd Intron	PiggyBac/ (Häcker et al., 2003)	No wing and sensory bristle phenotype
A2BP1^{MB03305}	9th intron	Mi(ET1)/ (Bellen et al., 2004; Jordan et al., 2012)	No homozygous flies, No wing and sensory bristle phenotype
A2BP1^{MI07729}	2nd Intron	Mi(MIC)/(Nagarkar-Jaiswal et al., 2015)	No homozygous flies, No wing and sensory bristle phenotype
A2BP1^{MI09134}	2nd Intron	Mi(MIC)/(Nagarkar-Jaiswal et al., 2015)	6% homozygous flies, No wing and sensory bristle phenotype
A2BP1^{MI01918}	5`UTR	Mi(MIC)/(Nagarkar-Jaiswal et al., 2015)	Most homozygous flies, No wing and sensory bristle phenotype
A2BP1^{MI04255}	7th intron	Mi(MIC)/(Nagarkar-Jaiswal et al., 2015)	No wing and sensory bristle phenotype
A2BP^{MI09677}	2nd Intron	Mi(MIC)/(Nagarkar-Jaiswal et al., 2015)	No homozygous flies, No wing and sensory bristle phenotype
A2BP1^{c03982}	2nd Intron	PiggyBac / (Thibault et al., 2004),	No homozygous flies, No wing and sensory bristle phenotype
A2BP1^{e03440}	2nd Intron	PiggyBac / (Thibault et al., 2004),	No homozygous flies, No wing and sensory bristle phenotype
A2BP1^{f01889}	7th intron	PiggyBac / (Thibault et al., 2004),	No homozygous flies, No wing and sensory bristle phenotype
A2BP1^{KG06463}	5`UTR	P element/ (Bellen et al., 2004),	2% flies homozygous, No wing and sensory bristle phenotype
A2BP1^{C00511}	2nd Intron	P element/ (Buszczak et al., 2007)	2% male flies homozygous, No wing and sensory bristle phenotype
UAS-*A2BP1*^{RNAi} TRiP line.JF02600	3rd Chr.	-	No wing and sensory bristle phenotype
UAS-*A2BP1*^{RNAi} TRiP line.HMS00478	3rd Chr.	-	No wing and sensory bristle phenotype
UAS-*A2BP1*^{RNAiKK} (line: 109100) VDRC	2nd Chr.	-	No wing and sensory bristle phenotype
UAS-*A2BP1*^{RNAi} (NIG collection)	3rd Chr.		Increase in sensory bristle
UAS- *A2BP1*^{RNAi}	X Chr.	(Usha and Shashidhara, 2010)	Increase in sensory bristle
UAS-dA2BP1	2nd Chr.	(Usha and Shashidhara, 2010)	Loss of sensory bristle

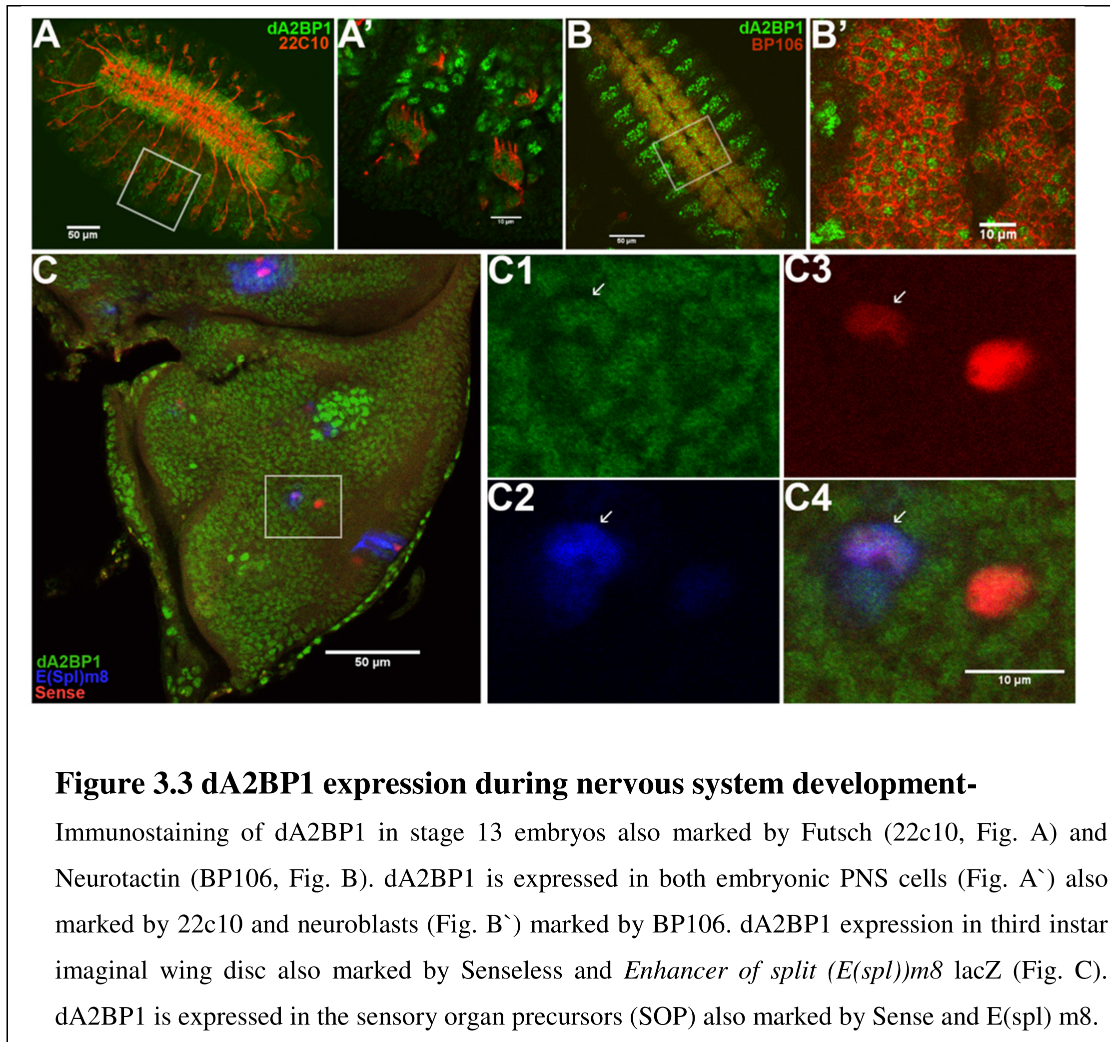


Figure 3.3 dA2BP1 expression during nervous system development- Immunostaining of dA2BP1 in stage 13 embryos also marked by Futsch (22c10, Fig. A) and Neurotactin (BP106, Fig. B). dA2BP1 is expressed in both embryonic PNS cells (Fig. A`) also marked by 22c10 and neuroblasts (Fig. B`) marked by BP106. dA2BP1 expression in third instar imaginal wing disc also marked by Senseless and *Enhancer of split (E(spl))m8* lacZ (Fig. C). dA2BP1 is expressed in the sensory organ precursors (SOP) also marked by Sense and E(spl) m8.

EN403 line was generated in the lab by GAL4 insertion in the 2nd intron of *dA2BP1*. These flies show high levels of lethality in homozygous conditions (Bajpai et al., 2004). *R27.1*, generated by EMS mutagenesis, shows homozygous lethality during first instar larval stage, however, mitotic clones show variety of low penetrance phenotypes including L3 vein thickening, loss of wing marginal bristles, and small eye (Ph.D thesis, Bajpai, R. 2002; Ph.D thesis, Usha, N. 2007). As part of this study, additional mutants generated by the fly community subsequent to the studies of Bajpai (2002) and Usha (2007) were examined for any neuronal phenotypes (Table 2). Most of these mutations have not been characterized in classical way. However, based on the mutation map, we analyzed that almost all the mutations map to intronic regions and majority of them to the 2nd intron. Similar to *R27.1*, majority of these mutants are homozygous lethal (Table 2). *CC00551* is a GFP trap line

generated by Buszczak et al., 2007 and characterized by Tastan et al., 2010. *CC00551* is one such example, wherein homozygous progeny shows high degree of lethality and the escapers are sterile. While these findings indicate the indispensable role of dA2BP1 in fly development, it did not specifically confirm its role during nervous system development.

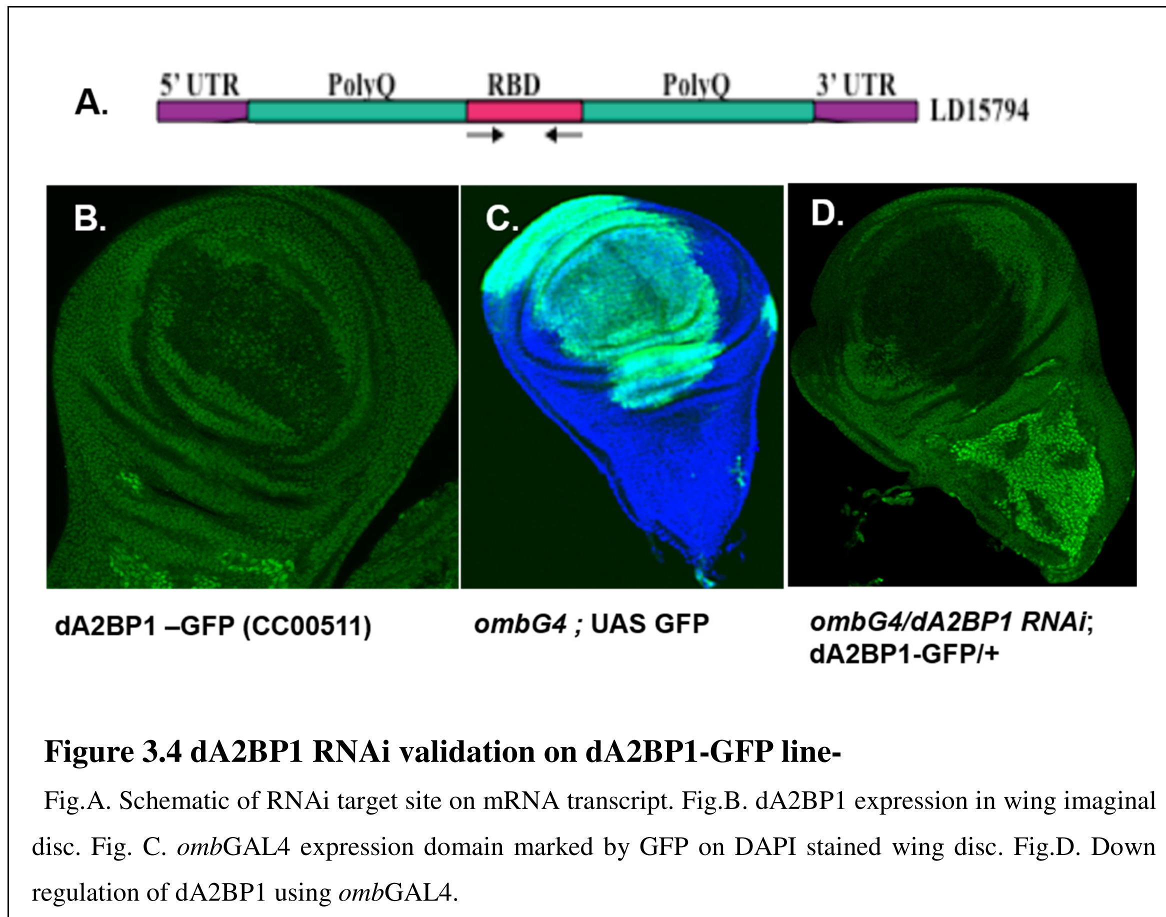
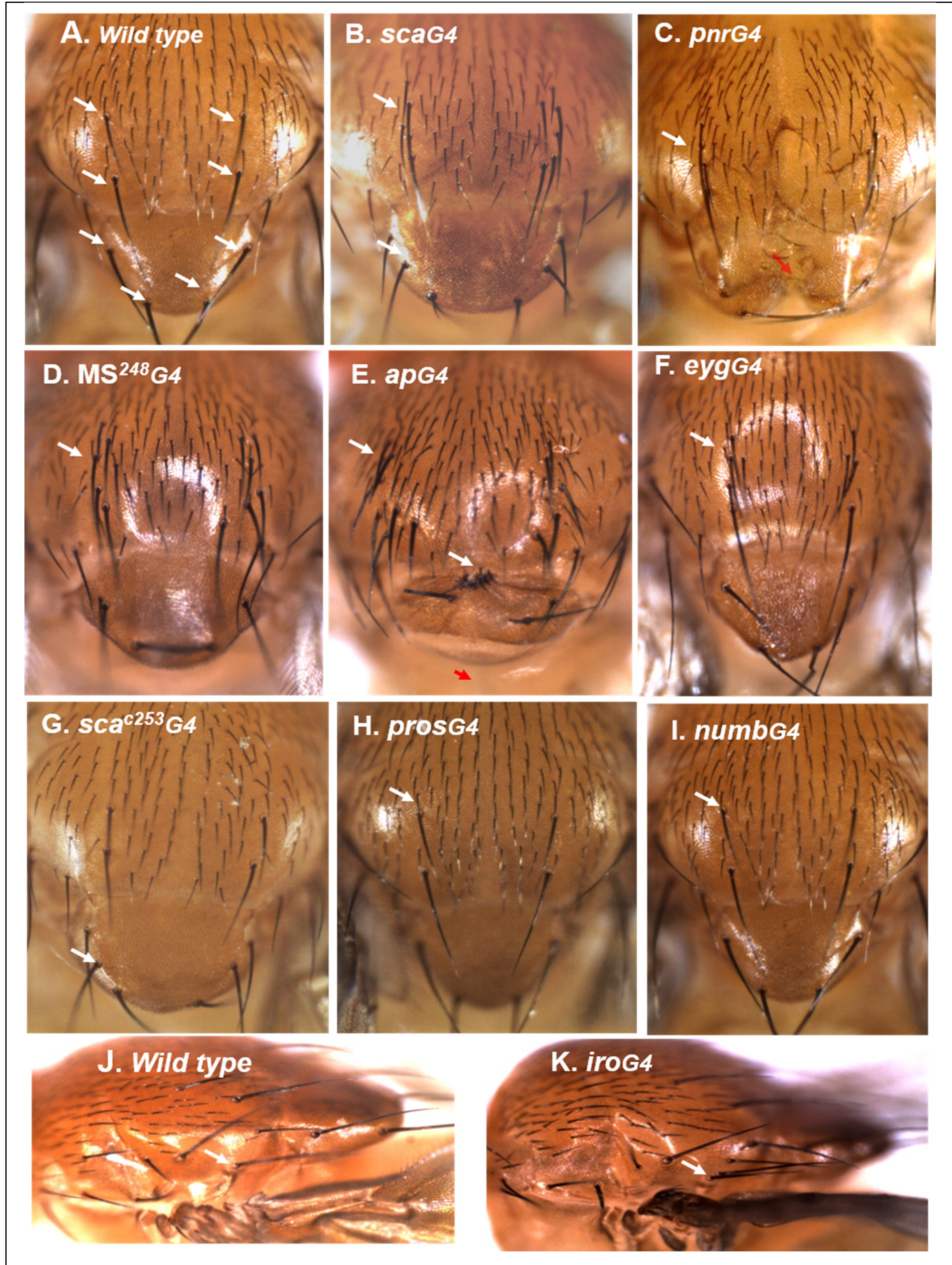


Figure 3.4 dA2BP1 RNAi validation on dA2BP1-GFP line-

Fig.A. Schematic of RNAi target site on mRNA transcript. Fig.B. dA2BP1 expression in wing imaginal disc. Fig. C. *omb*GAL4 expression domain marked by GFP on DAPI stained wing disc. Fig.D. Down regulation of dA2BP1 using *omb*GAL4.

To achieve spatial and temporal specificity in the down regulation of *dA2BP1* function, we used RNAi-based approach coupled with UAS-GAL4 system (Brand and Perrimon, 1993). We used transgenic RNAi lines from various resources, namely Bloomington TRiP, VDRC, NIG RNAi collection and RNAi lines generated in the lab (Usha and Shashidhara, 2010). We used *scabrous*-GAL4 (*sca*-GAL4), which is expressed in both developing CNS and PNS, to down regulate *dA2BP1*. We did not observe any phenotype with either Bloomington TRiP or VDRC RNAi lines. Only down regulation of *dA2BP1* using NIG RNAi collection and the RNAi line generated in our lab showed increase in the thoracic bristle number (Fig 5.6B). This difference in phenotype could be


A. Wild type
B. scaG4
C. pnrG4
D. MS248G4
E. apG4
F. eygG4
G. scac253G4
H. prosG4
I. numbG4
J. Wild type
K. iroG4

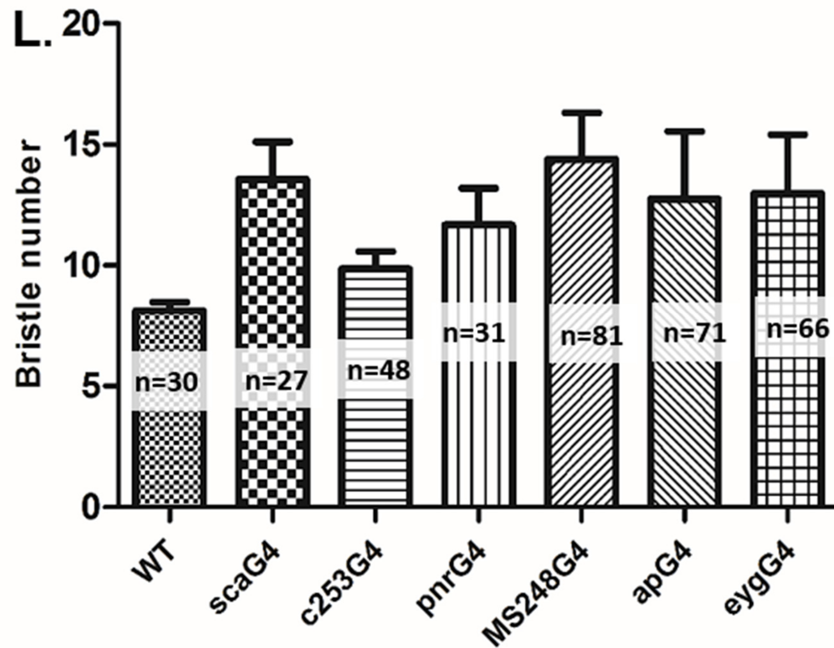


Figure 3.5 dA2BP1 loss of function causes increase in the number of sensory bristles on the *Drosophila* thorax:

A. Wild-type thorax; aDC, pDC, aSC and pSC bristles are marked with white arrows. B-G Down regulation of dA2BP1 using *sca*-GAL4, *pnr*-AL4, MS^{248}-GAL4, *apterus*-GAL4, *EM461(eyg)*-GAL4 and sca^{253}-GAL4. Note that the extra macrochaetae (white arrows) arise near the extant ones and not at an ectopic location. H-I Down regulation of dA2BP1 with *prospero*-GAL4 (H) and *numb*-GAL4 (I) do not cause generation of extra bristles. K. Down regulation of dA2BP1 with *iroquos*-GAL4 results in bristle formation at lateral position (aPA) only (white arrow). L. Quantitative analysis of bristle number in different genetic backgrounds. Error bars represent the S.D. The increase in bristle numbers is significant in all genetic backgrounds with $p<0.001$.

attributed to either strength of the RNAi line and/or if not all isoforms of dA2BP1 are strongly downregulated. The RNAi line generated in our lab has been shown to target all isoforms and shows complete knock down at the protein level (Fig.3.4).

There are 11 stereotypically arranged macrochaetae per hemi-notum, the positions that were affected include, dorsocentral (DC), scutellar (SC), and postalar (pSA) without

affecting the relative location of the sensory organs. To confirm this phenotype, we down regulated *dA2BP1* with other notum-specific GAL4 drivers including, *apterous, pannier, iroquos, eyegone (EM461), MS*^{248}*, apteorus*-GAL4*,* which are expressed in dorsal compartment of the disc. They all showed significant increase in bristle number and compressed scutellar region (Fig 3.5E). *pannier*-GAL4*,* which is expressed only in the dorsocentral and scutellar region, showed increase in bristle only at DC and SC position along with additional bifurcation of the mesothorax phenotype (Fig 3.5C). *iroquos*-GAL4*,* which is expressed only at lateral region of the thorax showed increase in bristle number only at postalar position (Fig 3.5K). *MS*^{248}-GAL4 has a wider expression domain and downregulation of dA2BP1 using this GAL4 resulted in increase in bristle number at both DC and SC positions (Fig 3.5D). However, down-regulation with *prospero*-GAL4*,* and *numb*-GAL4 drivers*,* which express at later stages of bristle development, did not show any bristle phenotype (Fig 3.5H and I). This indicated that dA2BP1 has a role in early stages of bristle development, perhaps in cell fate specification.

3.2.3 **Down regulation of dA2BP1 leads to increase in Campaniform sensilla and transformation to bristle:**

Campaniform sensilla are also external sensory organs, but different from bristles. They share common developmental mechanism and only differ in terminal cell division, which gives rise to external flattened oval disc like structures rather than a hair like structure that we see in bristles. The campaniform sensilla that are present on the wing and haltere have been studied in detail for their structure and function (Cole and Palka, 1982). Wing blade contain campaniform sensilla at various positions, including proximal and distal twin sensilla of the margin (p-TSM and d-TSM), anterior and posterior cross-vein sensilla (ACV, PCV), and three campaniform sensilla along the L3 vein (Fig 3.6A). Down regulation of dA2BP1 by using *sca*-GAL4 resulted in increase in campaniform sensillum number at p-TSM, d-TSM, and L3 vein sensillum (Fig 3.6D and J). We also observed duplication of ACV sensillum, but less frequently (Fig 3.6I). Interestingly, we observed campaniform sensillum to bristle transformation most often at p-TSM, d-TSM position (Fig 3.6L) and less frequently of ACV and L3 vein sensillum (Fig 3.6K and E). Campaniform sensillum at L3 showed higher rate of bristle transformation with *dpp*-GAL4

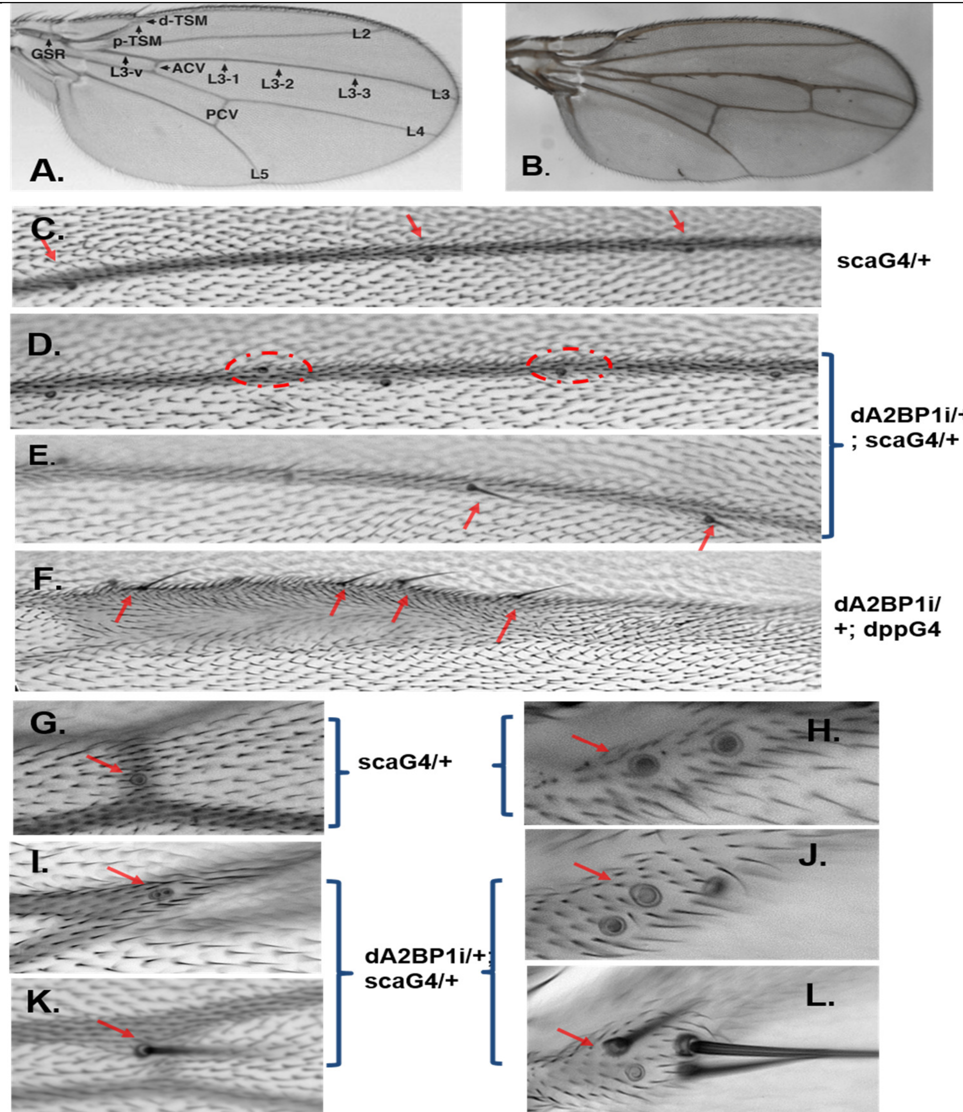


Figure 3.6 Down regulation of dA2BP1 causes increase in campaniform sensilla number and its transformation to bristle type:

A. Wild type wing showing the L1-L5 veins and position of campaniform sensilla. B. dA2BP1 down regulation with *sca*-GAL4 results in thickening of L3 and formation of extra cross vein between L3 and L4. C. Magnified view of three L3 vein campaniform sensilla marked by arrow in wild type wing. D-E. Down regulation of dA2BP1 results in increase in number of campaniform sensilla marked by dotted circles (in D) and transformation into bristle (E; marked by arrow). F. With *dpp*-GAL4 showing stronger phenotype. G. Magnified view of the ACV (red arrow). I, K. Down regulation of dA2BP1 results in duplication of ACV marked by red arrow (I) and transformation into bristle (K; marked by arrow). Magnified views of the Wild type (Fig. H) and dA2BP1 down regulated (J and L) p-TSM and d-TSM of the margin. Note the duplication of the p-TSM and d-TSM sensillum in J and transformation into bristle in L.

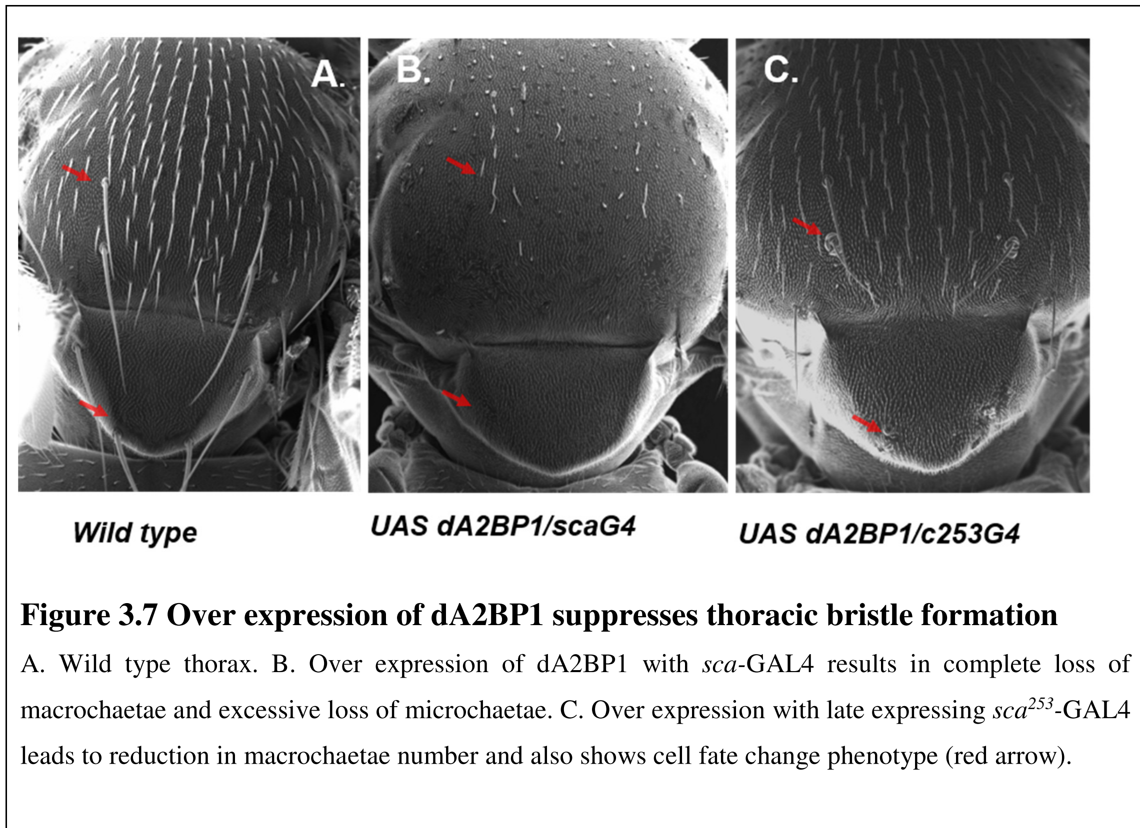


Figure 3.7 Over expression of dA2BP1 suppresses thoracic bristle formation

A. Wild type thorax. B. Over expression of dA2BP1 with *sca*-GAL4 results in complete loss of macrochaetae and excessive loss of microchaetae. C. Over expression with late expressing *sca*^{253}-GAL4 leads to reduction in macrochaetae number and also shows cell fate change phenotype (red arrow).

(Fig 3.6F). These results suggest that normally dA2BP1 may promote the fate of campaniform sensillum by suppressing bristle formation.

3.2.4 Over-expression of dA2BP1 results in complete loss of bristle:

To understand the increase in bristle number caused by the down regulation of dA2BP1 and to reconfirm its role during sensory organ development, we also took a gain of function approach. Over-expression of dA2BP1 showed embryonic and larval lethality with most of the GAL4 drivers. Over-expression of dA2BP1 with PNS specific GAL4, *sca*-GAL4 also showed lethality but at a lower rate. There were fewer eclosions of these progeny. The emerged flies showed loss of majority of macrochaetae and microchaetae and had a bald appearance (Fig.3.7B). dA2BP1 over-expression with *sca*^{c253}-GAL4, which is expressed during later stage of SOP specification and division, showed mild reduction in bristle number and in addition it also caused duplication of socket or shaft at the bristle position (Fig. 3.7C). As over-expression of dA2BP1 resulted in reduction in bristle number, it is

probable that dA2BP1 negatively regulates bristle formation. The observed socket or shaft duplication phenotype suggested that dA2BP1 can influence cell fate decisions.

3.3 Discussion.

On the basis of requirement of proneural transcription factor during development, we can classify PNS broadly in to two types. The one which are dependent on Achaete/Scute and the other that are dependent on Atonal (Jan and Jan, 1994). In this study, we show that dA2BP1 is expressed in both Atonal-based chordotonal organs (Fig.3.3A) and in Achaete/Scute based SOPs (Fig3.3C). To understand the role of dA2BP1, if any, and the mechanism of its function in PNS development, here we have focused only on SOP development. Both loss and gain-of-function studies suggest that dA2BP1 regulates Achaete/Scute based PNS development. As down regulation of dA2BP1 increased the number of sensory bristles and its gain-of-function results in complete loss of bristles, dA2BP1 is likely a negative regulator of bristle development.

Bristle and campaniform sensillum share similar cell composition except that the shaft seen in bristles is replaced by papilla in campaniform sensillum. Down regulation of dA2BP1 shows increase in the campaniform sensillum number but it also transforms it to the bristles. It is likely that dA2BP1 regulates the number of sensory organs by conferring sensilla fate and suppressing bristle-fate.

3.3.1 dA2BP mediates early events during sensory organ development:

There are 11 stereotypically arranged macrochaetae per hemi-notum. SOPs for different macrochaetae arise at different time point from different PNCs. The first SOP appears almost 30 hours before puparium formation and the process completes 3 hours before pupariation (Held Jr et al., 2005; Huang et al., 1991). Down regulation of dA2BP1 resulted in phenotypes at the levels of pSC, pDC and, aPA. SOPs specification for these positions starts very early and completes before 24 hours of puparium formation, suggesting that dA2BP1 might be regulating early events during bristle development.

Wide range of early GAL4 including both PNC specific and epithelial specific (including PNC) gave us similar phenotype. We also observe that, extra bristles arise near the extant ones and not at an ectopic place suggesting that dA2BP1 does not regulate bristle development beyond the PNC. We did not observe increase in bristle number with late

GAL4 drivers, which express during SOP division, suggesting that dA2BP1 function is limited to SOP specification. As the phenotype is PNC specific and regulation is limited till SOP specification, we speculate that dA2BP1 might be regulating lateral inhibition to regulate bristle development.

In the next chapter, we examine the developmental pathway in which dA2BP1 participates during the development of sensory bristles.

4 Chapter-4: dA2BP1 interacts with Notch pathway during sensory organ development

4.1 Introduction-

4.1.1 Notch Signaling:

Notch is named after a century old phenotype in *Notch* (N) locus, fly wing notching (Dexter, 1914). It is one of the most studied singling pathway, both in flies and in vertebrates including human. Notch signaling has been extensively explored with respect to its role in cell fate specification. It regulates early patterning events, wherever one cell amongst a group of cells needs to function differently necessitating during complex tissue organization during metazoan development. Many developmental mechanisms and signaling pathways are well conserved during evolution. In the context of Notch signaling, the conservation is very high both at the molecular level as well as the networks level. Notch signaling is known to regulate blastomere determination in *C. elegans* (Priess, 2005). In flies, it is required for embryonic segmental patterning, sensory organ specification, leg joint specification, photoreceptor development and dorso-ventral patterning during wing development (Bishop et al., 1999; Heitzler and Simpson, 1991; Wiellette and McGinnis, 1999). Notch signaling has multiple roles during human development including cardiomyocyte differentiation, astrocyte differentiation, inner ear hair formation and endocrine cell differentiation during insulin secretion (Andersson et al., 2011). It regulates crypt and goblet cell specification during intestine development. It has been widely studied for its role during immune system development. It not only maintains hematopoietic stem cell pool but also decides both B cell and T cell differentiation. As Notch signaling regulates differentiation of major cell types in human, malfunction of Notch signaling has been reported in many cancer types including T cell acute lymphoblastic leukemia (Andersson et al., 2011). It is also linked to several genetic diseases, where gene network related to Notch pathway is mutated. Mutation in the ligand *Jag1* and the receptor *Notch2* leads to Alagille syndrome, *Lunatic Fringe* mutants develops spondylocostal dysostosis, mutations in receptor *Notch3* results in CADASIL syndrome and in *Notch1* causes aortic valve disease (Andersson et al., 2011; Fortini, 2009).

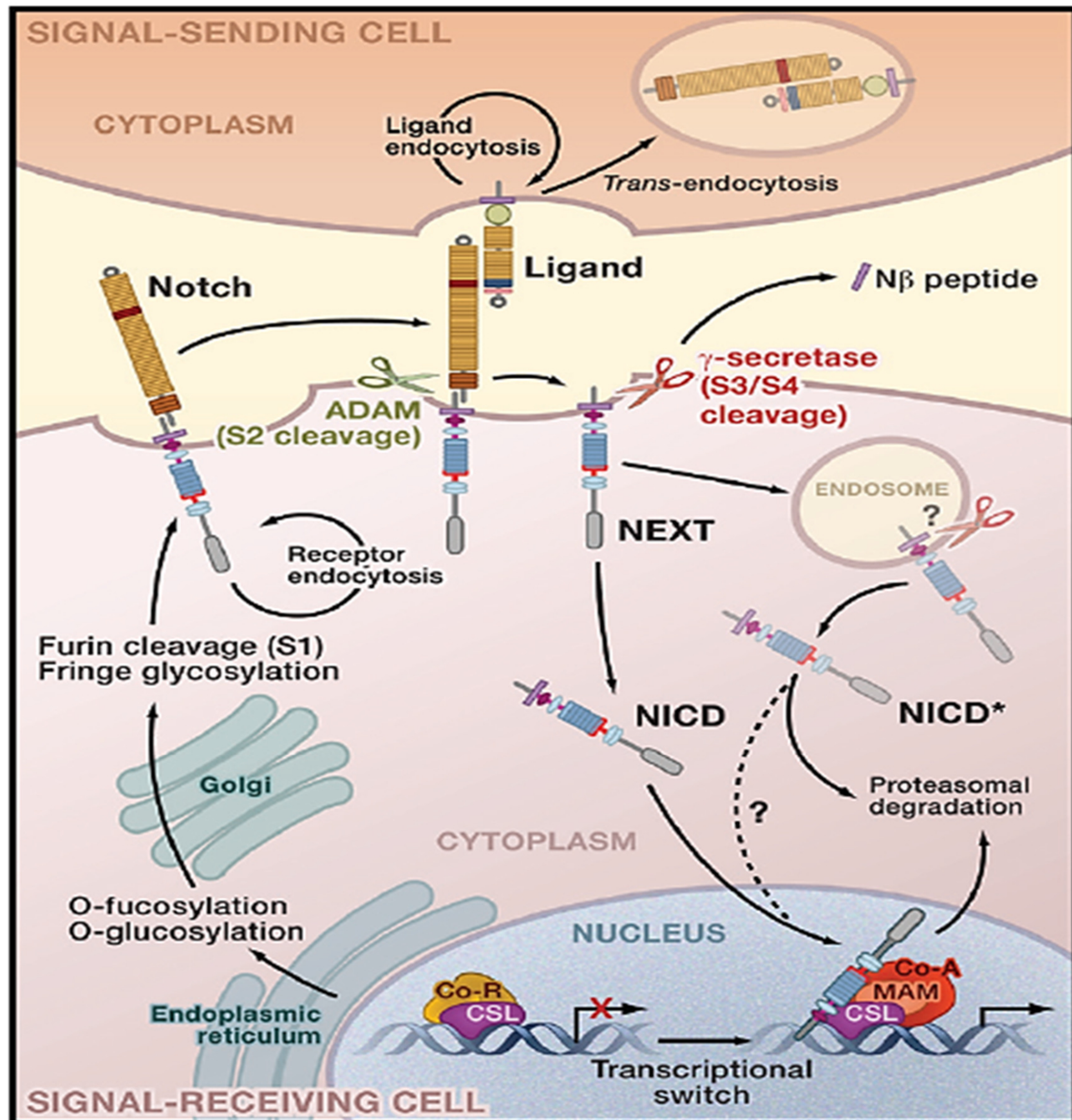


Figure 4.1 An overview of Notch signaling.

Notch receptor is synthesized and modified in ER. It is then glycosylated in Golgi and transported to plasma membrane. Notch extra cellular domain interacts with the ligand and triggers receptor cleavage by ADAM metalloproteases. Subsequent cleavage near the trans-membrane domain by γ-secretase enzymes releases the intra cellular domain (NICD). NICD moves to the nucleus and acts as transcriptional activator along with Su(H) (a CSL family transcription factor) and Mastermind. Image reproduced from (Kopan and Ilagan, 2009)

Notch signaling is classified as two types: canonical and non-canonical. Canonical signaling requires activation of Notch receptor by ligand of Delta/Serrate/Lag-2 (DSL) family at the cell surface and release of Notch intracellular domain (NICD). NICD further moves to the nucleus and interacts with transcription factor of CBF1/Suppressor of Hairless/LAG-1 (CSL) family to transcribe down-stream target genes including *E(spl)-Complex*. Activation of Notch signaling without involving DSL ligand is classified as non-canonical.

4.1.2 **The Notch Receptor:**

Notch is a single pass transmembrane receptor present on the plasma membrane of signaling cell. It is a glycoprotein and it undergoes post translational modification in both Endoplasmic reticulum (ER) and Golgi before being transported to the plasma membrane as a Notch receptor. The post translational modification, which includes proteolytic cleavage and glycosylation, is essential for its function (Moloney et al., 2000). After polypeptide synthesis in the ER, Notch extracellular domain undergoes o-linked glucosylation on serine residue by glucosyltransferase Rumi (Acar et al., 2008). O-fucosyltransferase starts the new site for glycosylation by addition of fucose on Notch extracellular domain in trans-Golgi, which is further extended by Fringe, another glycosyltransferase (Fig.4.1) (Fortini, 2009; Moloney et al., 2000; Okajima et al., 2005). Fringe mediated glycosylation increases receptor sensitivity towards ligand Delta when compared to the other ligand Serrate.

Proteolytic cleavages are also equally critical events during Notch signaling. During its first proteolytic cleavage in the Golgi, Notch gets cleaved near the trans-membrane domain by furine like convertase into two parts: Notch N-terminal extracellular and Notch C-Terminal intracellular subunits (Kopan and Turner, 1996). These two fragments form disulfide linkages and are exported to the plasma membrane as Notch receptor. Second round of proteolytic cleavage is initiated after ligand interaction. When Notch extracellular receptor interacts with DSL ligand, it undergoes conformational change and exposes second proteolytic site to membrane bound metalloprotease Kuzbanian (Kuz; (Lieber et al., 2002)). After second cleavage, the extracellular part of the receptor is

endocytosed along with DSL ligand by the signal sending cell. The remaining cytoplasmic tail is embedded in the membrane endocytosed by the signal receiving cell. Final proteolytic cleavage by γ-secretases in late endosome or on plasma membrane releases Notch intracellular domain (NICD) (Struhl and Greenwald, 1999). After activation NICD moves to the nucleus and acts as a transcription factor (Fig.4.1) (Kidd et al., 1998). NICD has a very short half-life and it has been observed that it undergoes multiple ubiquitination and phosphorylation and is finally degraded by the proteasome. Other post-translational modifications such as hydroxylation and acetylation have been observed for mammalian NICD but have not yet been explored in *Drosophila*.

4.1.3 **Activation of Notch by its Ligands:**

Notch receptor can be activated by two different ligands Delta and Serrate. Together they can be classified as Delta/Serrate/Lag-2 family (DSL) proteins. Unlike many of the traditional secretory ligands, they are membrane bound and activate paracrine signaling. These two ligands have different expression patterns and differ in function. They both interact with Notch at the same region i.e., between 11-12 EGF like repeats. Ectopic expression of Serrate can compensate for Delta function to a certain extent (Gu et al., 1995). However, Delta shows greater affinity for glycosylated Notch receptor. Serrate plays leading role during embryonic segmentation, whereas Notch mediated lateral inhibition during SOP selection is more Delta-dependent. Delta interacts with Notch via EGF like domain at the N terminus and activates downstream signaling in signal receiving cell. However, in the signal sending cell which expresses Delta, the Delta receptor along with Notch extra-cellular domain are endocytosed by the cell (Fig.4.1). After endocytosis, Delta/Serrate undergoes mono-ubiqitination by RING finger E3 ligase Mind bomb and Neuralized and finally undergoes proteasomal degradation (Lai et al., 2005).

4.1.4. Downstream of Notch internalization:

After ligand binding and protealytic cleavage, the activated NICD moves to the nucleus to further activate downstream targets. Normally, Notch targets are repressed by a suppressor complex, which includes, Suppressor of Hairless (Su(H)), Hairless (H), C-terminal binding protein (dCtBP) and Groucho (Gro). Su(H) is a DNA binding protein that acts as both

activator and repressor (Bailey and Posakony, 1995; Brou et al., 1994). In the absence of Notch signaling, Hairless interacts with Su(H) and acts as an adapter to recruit Gro and CtBP. Both Gro and CtBP are long range and short range chromatin modifiers respectively (Barolo et al., 2002; Nagel et al., 2005). They binds on targets and further recruit histone deacetylase (HDAC) for further chromatin silencing. Together this complex represses Su(H)-dependent transcription of Notch targets. During active Notch signaling, NICD bind to Su(H) and facilitates removal of Hairless from the repressor complex. Devoid from repressor factors, Notch and Su(H) complex further recruits other transcription factors including Mastermind (Mam) to transcribe the target genes (Helms et al., 1999; Le Gall and Giniger, 2004; Lecourtois and Schweisguth, 1995).

4.2 Results-

4.2.1 **Genetic interaction between dA2BP1 and N signaling during sensory bristle development:**

Notch signaling is known for its role during cell fate specification and cell differentiation. What has fascinated researchers most is its role in sensory organ specification that require Notch-Delta mediated lateral inhibition. Reduction in Notch signaling weakens lateral inhibition, which results in increase in the number of sensory bristles (Beerman and Rossi, 2015). To further explore if dA2BP1 functions in Notch pathway, we down-regulated dA2BP1 in the heterozygous background of Notch null mutant, N^{55e11} (Fig.4.2B). Heterozygous *Notch* mutants show mild increase in sensory bristle number and this was enhanced when dA2BP1 was also down-regulated (Fig. 4.2D). Interestingly, apart from bristle enhancement at DC, SC and aPA positions, (Fig.4.2C`, dA2BP1 loss of function) increase in bristle number extends to other positions too (Fig.4.2D`, dA2BP1 loss of function in combination with N^{55e11}). *Abruptex*^{16} (Ax^{16}) is a gain of function mutation of Notch (Hartley et al., 1987; Kelley et al., 1987). These mutants also affect SOP specification, where flies develops less bristles in compare to wild type (Go and Artavanis-Tsakonas, 1998; Heitzler and Simpson, 1993). We observed suppression of this phenotype by the down-regulation of dA2BP1 (Fig.4.2E and F).

As down-regulation of dA2BP1 is capable of reducing Notch signaling, we further extended our genetic interaction studies at the level of distinct phases of Notch signaling. Over-expression of Notch dominant negative (NDN) weakens Notch signaling and results in increase in sensory bristle number per PNC (Fig.4.3C) (Rebay et al., 1993). Down-regulation of dA2BP1 in the background of over-expression of NDN showed dramatic enhancement of this phenotype (Fig.4.3D). Conversely, over-expression of dA2BP1 in NDN background resulted in complete loss of macrochaetae, similar to the phenotype caused by the over-expression of dA2BP1 (Fig.4.3F). To confirm this interaction further, we down-regulated dA2BP1 in the background of constitutively active form of Notch. Over-expression of Notch^{intra} (NICD) in wild type SOP results in multiple socket phenotype (Fig.4.3G) (Mumm and Kopan, 2000; Struhl et al., 1993).

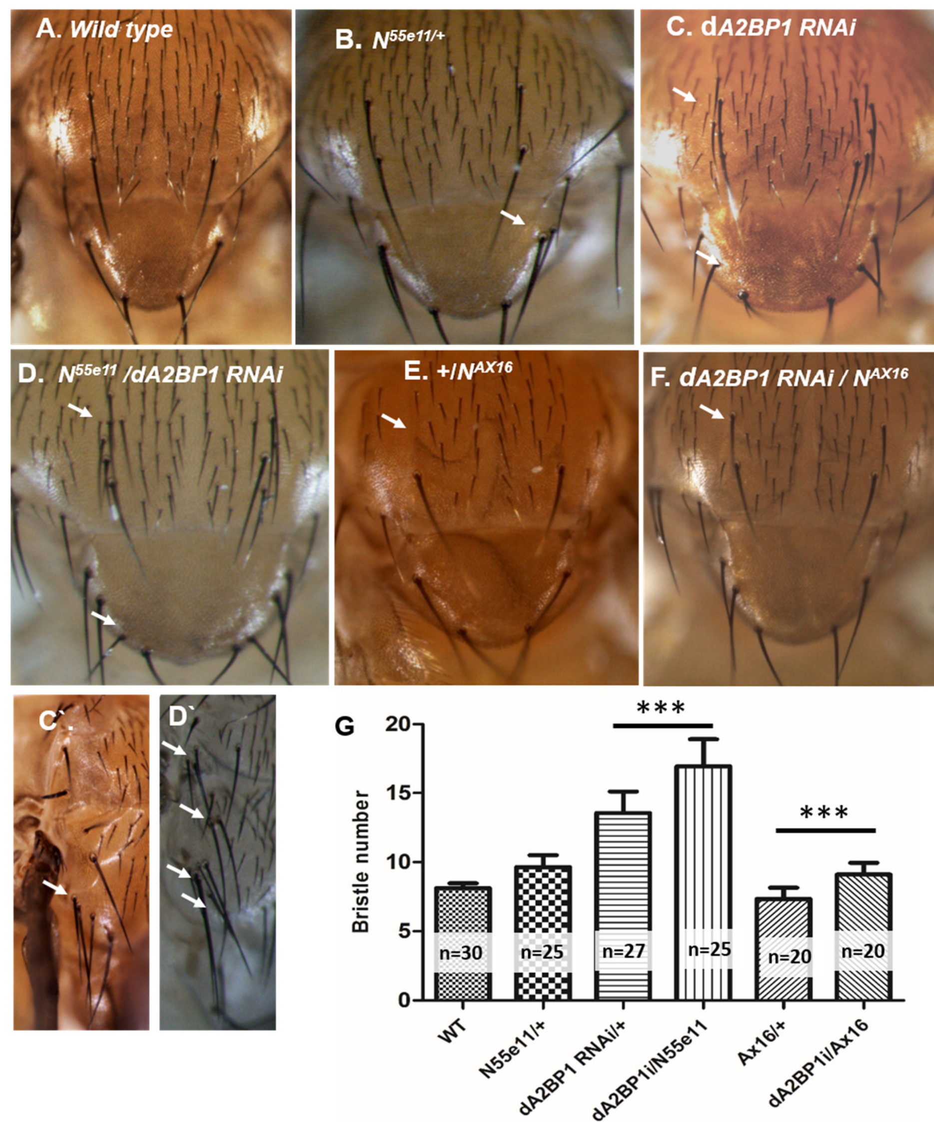


Figure 4.2 Genetic interactions between dA2BP1 and Notch

A. Wild type thorax. B. Loss of *Notch* (N^{55e11}) leads to increase in sensory bristle number (red arrow). C. dA2BP1 down regulation also cause increase in sensory bristle number. D. Loss of dA2BP1 in N^{55e11} background further enhances this phenotype at DC and SC position. C`. lateral bristles in dA2BP1i. D` show increase in lateral bristle number in addition to DC and SC. Combination of loss of dA2BP1 and loss of N leads to increase in sensory bristle number at these positions. E. Gain of Notch (in gain of function mutation N^{AX16}) leads to decrease in sensory bristle number. F. Down regulation of dA2BP1 in this background recues sensory bristle number. G. Sensory bristle number quantification in different genetic backgrounds. Error bars represent the S.D. The Changes in sensory bristle numbers are significant in all genetic backgrounds with $p<0.001$ when compared to their respective controls.

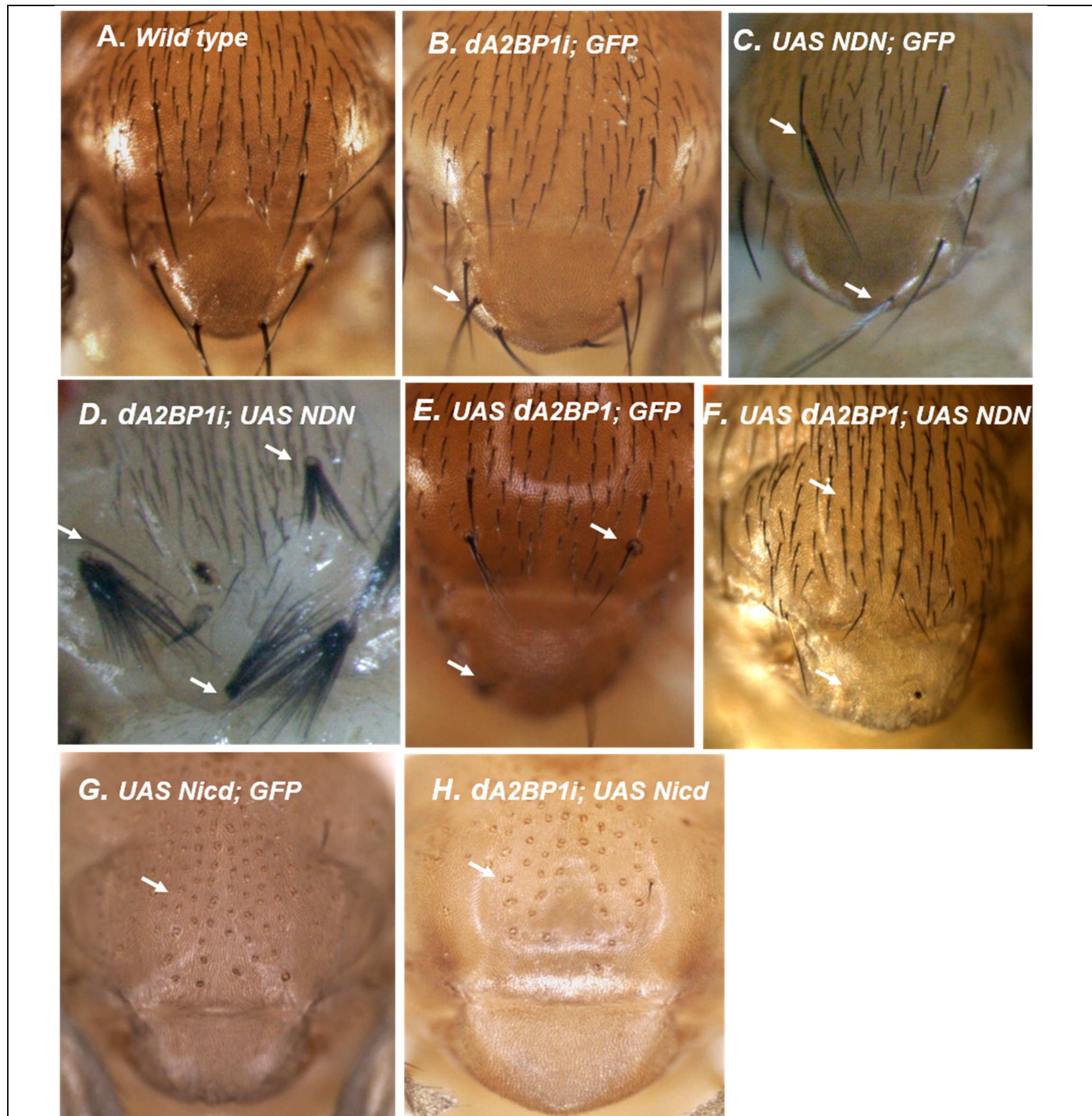
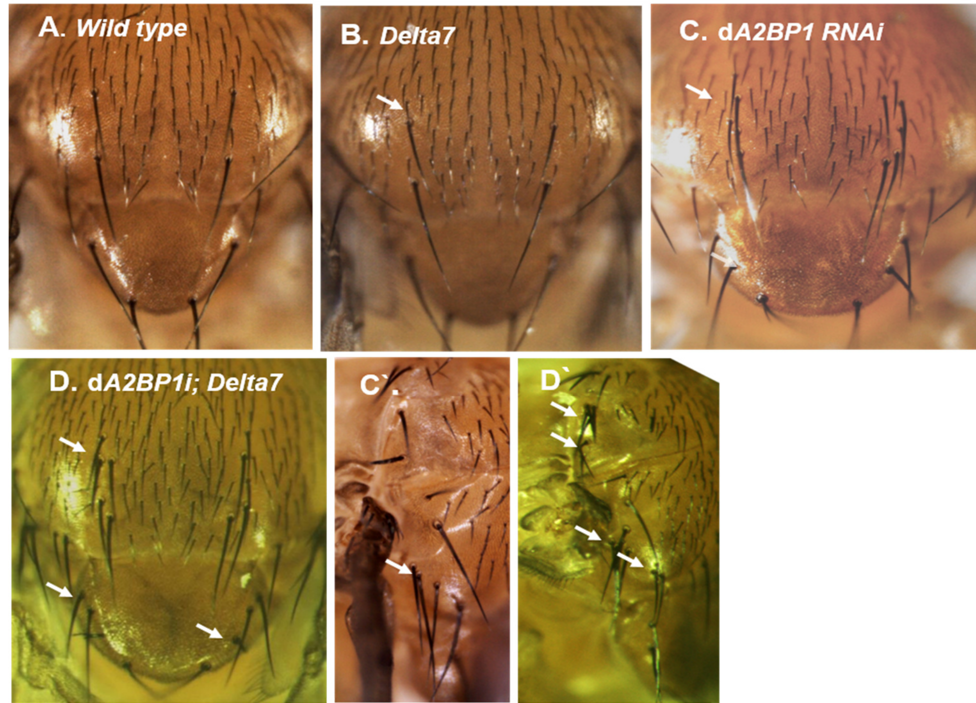


Figure 4.3 dA2BP1 may function upstream of (or at par with) Notch

A. Wild type thorax. B. Down regulation of *dA2BP1* with *c253*-GAL4 results in mild increase in sensory bristle number (white arrow). C. Over-expression NDN with *c253*-GAL4 leads to increase in sensory bristle number per PNC (white arrow). D. Loss of dA2BP1 in NDN background further enhances the NDN phenotype. Note, the multiple sensory bristles emerging from a single PNC. E. Over-expression of dA2BP1 with *c253*-GAL4 results in mild reduction in sensory bristle number. F. Over expression of dA2BP1 in NDN background leads to complete loss of macrochaetae. G. Over expression NICD results in multi-socket phenotype. H. Loss of dA2BP1 shows no change in NICD over expressed phenotype.



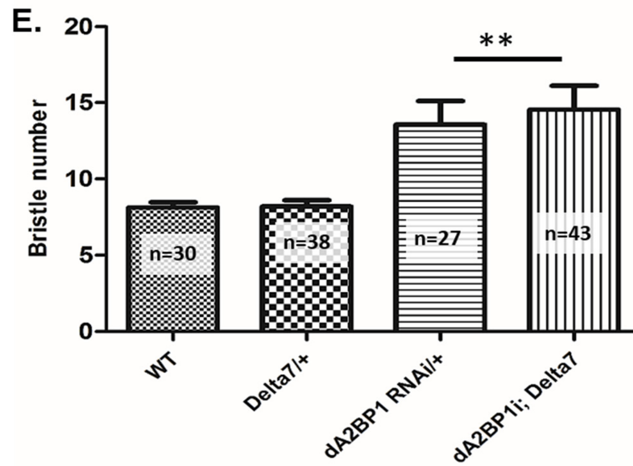


Figure 4.4 dA2BP1 shows genetic interaction with Delta-

A. Wild type thorax. B. Hypomorphic allele *Delta* (*Dl*^{7}) does not show any sensory bristle phenotype. C. Phenotypes induced by RNAi against *dA2BP1*. D. dA2BP1 phenotype is enhanced by *Dl*^{7}. C` and D` show the lateral sensory bristle positions. Combination of both dA2BP1 down regulation and *D*^{7} leads to increase in sensory bristle number at these positions. E. Sensory bristle number quantification in different genetic backgrounds. Error bars represent the S.D. Changes in sensory bristle number are significant with $p<0.05$

This phenotype was unaffected by the down-regulation of dA2BP1 (Fig.4.3H), suggesting that dA2BP1 may function upstream or at par with N.

Notch plays two important roles during sensory bristle development, one during SOP specification and the other during asymmetric SOP division. Our observation suggests that dA2BP1 function is limited to SOP specification.

4.2.2 dA2BP1 shows genetic interaction with Delta:

Prompted by the dA2BP1 epistatic relation with N, we examined its interaction with the Delta (Dl); the ligand of N. Dl^{7} is hypomorphic allele of Dl. In heterozygous background, it causes thickening of 2nd wing vein and also shows small delta phenotype near posterior cross-vein (PCV) (van de Hoef et al., 2013). Dl^{7} does not show sensory bristle phenotype in heterozygous background (Fig.4.4B). Down regulation of dA2BP1 in this background resulted in enhanced sensory bristle phenotype of dA2BP1 (Fig.4.4D). Furthermore, similar to N allele, N^{55e11} (Fig.4.2D`), we observed increase in sensory bristle number at lateral positions (Fig.4.4D`).

4.2.3 Genetic interactions between dA2BP1 and EGFR pathway:

Egfr/Ras pathway is one of the important pathways, which regulate various aspects of cell fate specification, differentiation, proliferation, migration and survival (Domínguez et al., 1998; Perrimon and Perkins, 1997). In fly, EGFR regulates embryonic patterning, wing development, photoreceptor specification, dorsal follicle cell fate specification etc. Notch and EGFR signaling is best known for their antagonistic function during photoreceptor specification (Rohrbaugh et al., 2002). We, therefore, studied genetic interactions between dA2BP1 and EGFR. EGFR pathway acts as a positive regulator of sensory organ development (Culí et al., 2001). Over expression of EGFR in PNC leads to a mild increase in sensory bristle number (Fig.4.6 B). Down-regulation of dA2BP1 in the background resulted in further increase in sensory bristle number (Fig4.6 D), suggesting that dA2BP1 and EGFR have opposite roles during sensory bristle development. Over expression of dominant negative form of EGFR (EGFRDN) causes decrease in EGFR signaling and thus reduction in sensory bristle number (Fig4.6 E). We down regulated dA2BP1 in EGFRDN

background. Down-regulation of the dA2BP1 showed suppression of EGFRDN-induced phenotype (Fig4.6 F). This further confirmed the inverse relationship between dA2BP1 and EGFR.

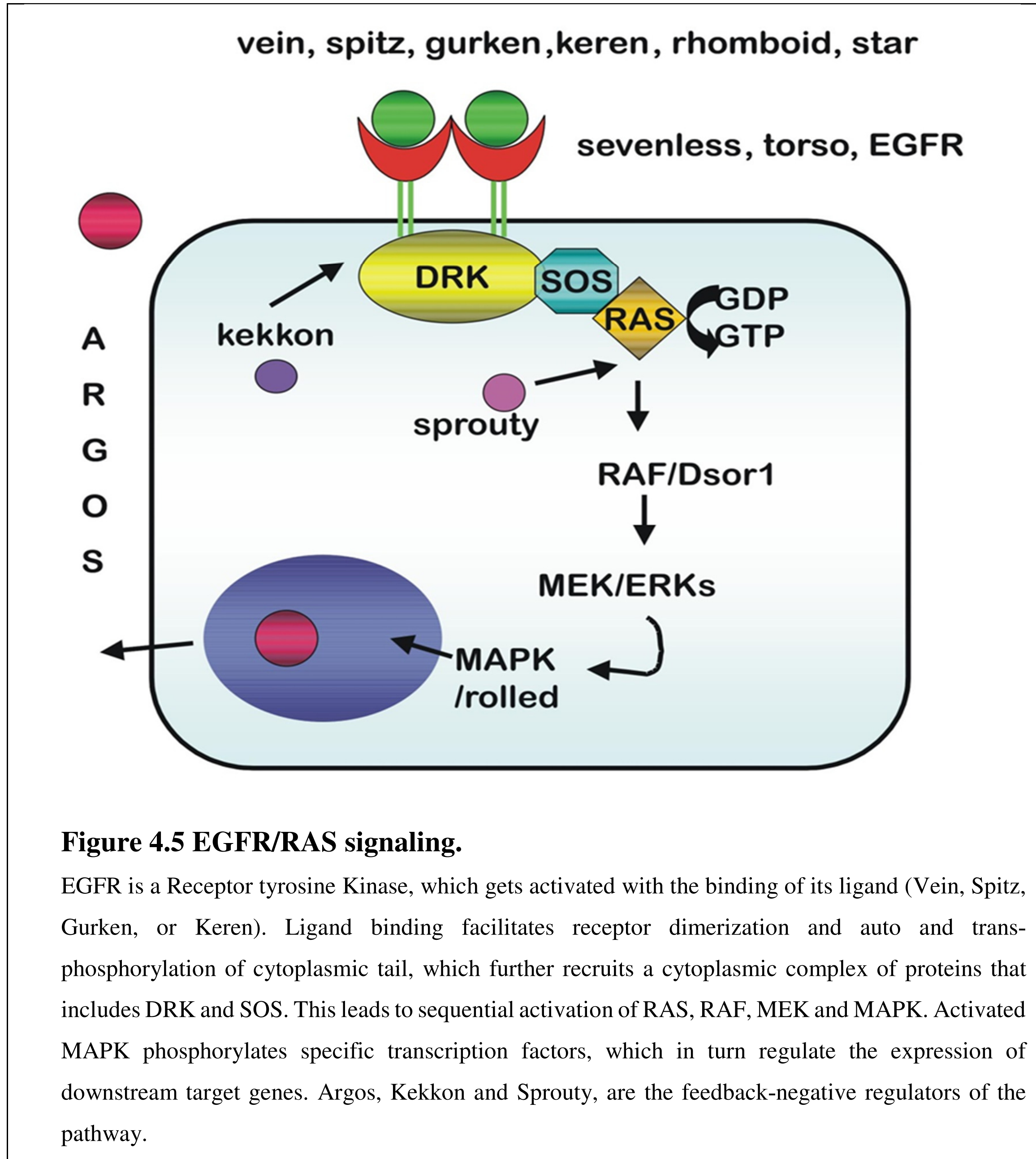


Figure 4.5 EGFR/RAS signaling.

EGFR is a Receptor tyrosine Kinase, which gets activated with the binding of its ligand (Vein, Spitz, Gurken, or Keren). Ligand binding facilitates receptor dimerization and auto and trans-phosphorylation of cytoplasmic tail, which further recruits a cytoplasmic complex of proteins that includes DRK and SOS. This leads to sequential activation of RAS, RAF, MEK and MAPK. Activated MAPK phosphorylates specific transcription factors, which in turn regulate the expression of downstream target genes. Argos, Kekkon and Sprouty, are the feedback-negative regulators of the pathway.

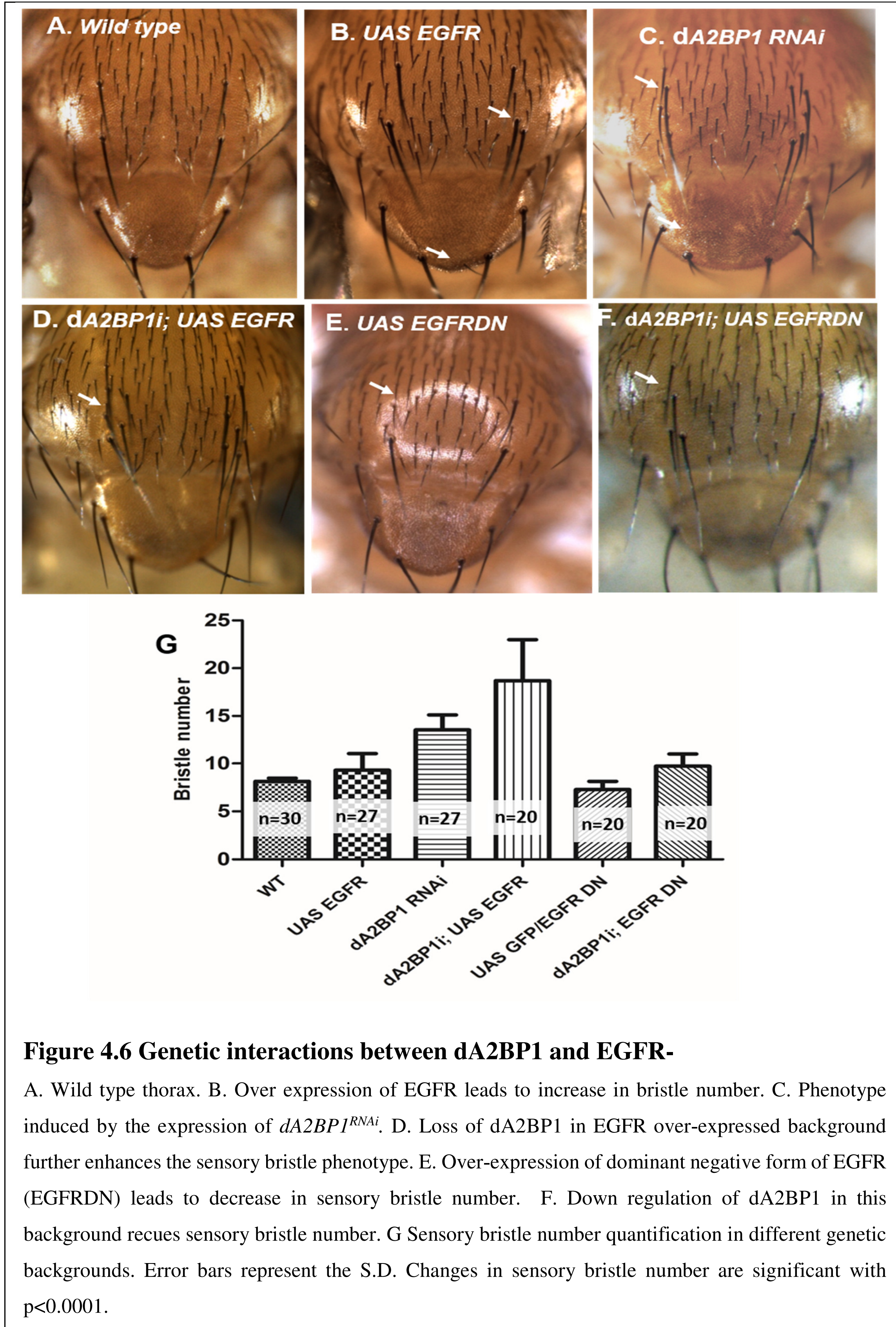
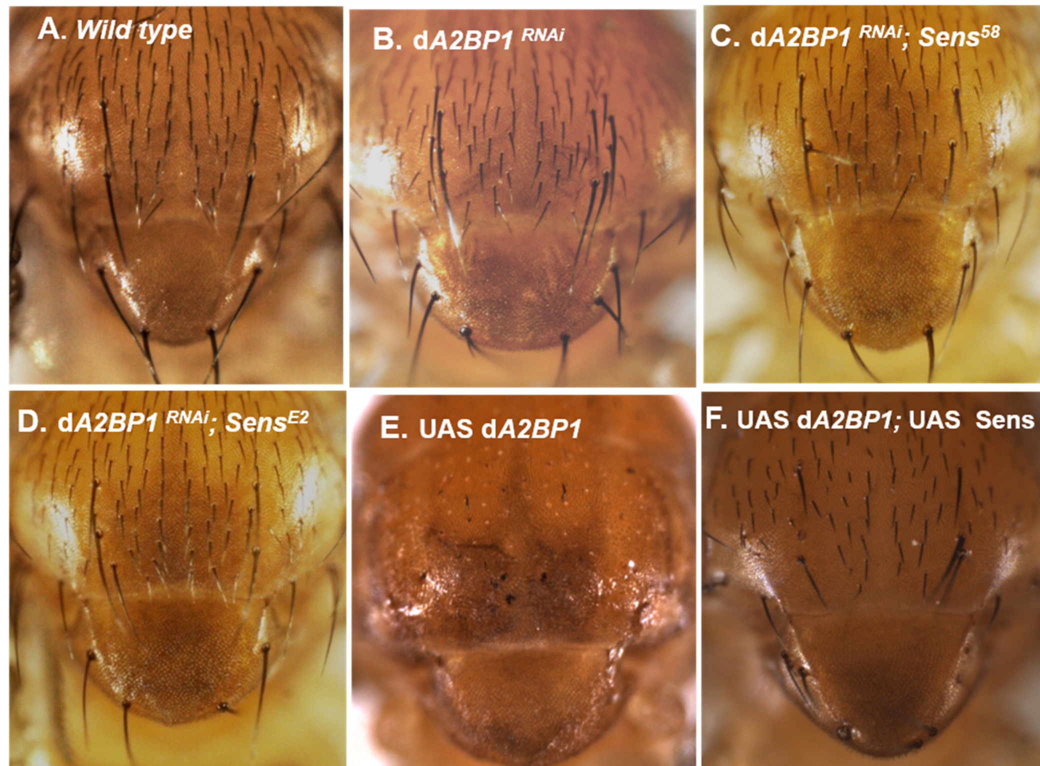


Figure 4.6 Genetic interactions between dA2BP1 and EGFR-

A. Wild type thorax. B. Over expression of EGFR leads to increase in bristle number. C. Phenotype induced by the expression of *dA2BP1*^{RNAi}. D. Loss of dA2BP1 in EGFR over-expressed background further enhances the sensory bristle phenotype. E. Over-expression of dominant negative form of EGFR (EGFRDN) leads to decrease in sensory bristle number. F. Down regulation of dA2BP1 in this background recues sensory bristle number. G Sensory bristle number quantification in different genetic backgrounds. Error bars represent the S.D. Changes in sensory bristle number are significant with $p<0.0001$.



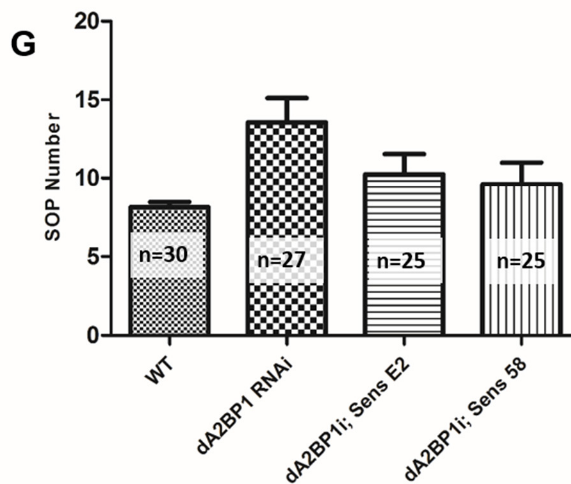


Figure 4.7 Genetic interaction between dA2BP1 and Senseless.

A. Wild type thorax. B. Phenotype induced by the down regulation of dA2BP1. C-D. Loss of single copy of Sens (*sens*^{58} and *sens*^{E2}), while does not show sensory bristle phenotype, it can suppress the phenotype induced by the down regulation of dA2BP1. E. Over expression of dA2BP1 suppresses sensory bristle formation. F. Over expression of Sens rescues sensory bristle phenotype caused by dA2BP1 over expression. G. Sensory bristle number quantification in different genetic backgrounds. Error bars represent the S.D. Changes in sensory bristle number are significant with $p<0.0001$.

4.2.4 **dA2BP1 is upstream of Senseless:**

EGFR and Notch signaling together regulate sensory bristle development by defining the number of SOPs. Genetic interactions with both EGFR and Notch pathways confirms that dA2BP1 is functional during SOP specification. The zinc finger protein Senseless (Sens) is a downstream effector of Notch signaling (Acar et al., 2006; Jafar-Nejad et al., 2003). Its expression is down regulated by N singling. Sens expression is enough and sufficient to induce SOP and sensory bristle even in the absence of other proneural genes (Nolo et al., 2000). $sens^{58}$ and $sens^{E2}$ are null alleles for *sens*. In heterozygous background, they do not display any sensory bristle phenotype. However, they suppressed supernumerary bristle phenotype observed with down regulation of dA2BP1. Conversely, Sens over-expression suppressed phenotype (loss of sensory bristles) caused by the over-expression of dA2BP1 (Fig.4.7F). This suggests that Sense is downstream of dA2BP1.

4.3 Discussion-

4.3.1 **dA2BP1 shows synergy with Notch function:**

Notch pathway has been explored for its role during sensory bristle development. It is one of the best known pathways for differential regulation of gene expression. Sensory bristle phenotype caused by the manipulation of dA2BP1 might be the result of its role in Notch pathway. Loss of both Notch and dA2BP1 results in synergistic effect. Moreover, Notch gain of function could be rescued by lowering dA2BP1 suggesting that dA2BP1 is positive regulator of Notch. This observation is further strengthened by dA2BP1 manipulation in the backgrounds of specific alterations of N function. Over expression of Notch extra cellular domain (NECD or NDN) hampers Notch signaling (dominant negative effect) and leads to increase in sensory bristle number per PNC. Down regulation of dA2BP1 in this background further enhanced this phenotype: with dramatic appearance of tufts of sensory bristles. In contrast, over-expression dA2BP1, which suppresses sensory bristle formation, suppressed dominant negative effect of NECD. This suggests that dA2BP1 is downstream to Notch in the pathway.

Gain of function of Notch results in two types of phenotypes: complete loss of sensory bristles and appearance of multi-socketed sensory organs (caused by the alteration in the cell fate during SOP division). Over-expression of full length Notch results in complete bald phenotype, whereas over expression of Notch intracellular domain (NICD; gain of function) induces multi-socket phenotype. Effect of over-expression of Notch (N^{AX16}) was rescued when dA2BP1 was down regulated. However, RNAi-mediated knockdown of dA2BP1 did not rescue the multi-socket phenotype associated with the over-expression of constitutively active form of Notch (NICD). These observation suggest role of dA2BP1 during sensory bristle development is limited to SOP formation. This is supported by the earlier observation that down regulation of dA2BP1 with late GAL4 drivers (those that express post-SOP specification) shows no phenotype.

4.3.2 **Genetic interaction with EGFR:**

EGFR promotes sensory bristle formation by positively regulating Achaete expression (Culí et al., 2001). Notch and EGFR signaling pathways are best known for their antagonistic relationship during photoreceptor development. Here, we have observed that

dA2BP1 also has similar interactions with EGFR. Loss of function of dA2BP1, which causes supernumerary sensory bristles, is further enhanced by increased levels of EGFR. We have also shown that the loss of sensory bristle formation by a dominant negative form of EGFR is rescued when dA2BP1 is down regulated. Notch and EGFR pathways regulate Achaete expression during sensory bristle development. At this point, we cannot discriminate whether dA2BP1 is regulating Notch or EGFR pathway. Observations from Culi et al.; 2001; suggests that Notch signaling negatively regulates *vein/rhomboid* to regulate EGFR signaling, but they have not seen any effect of EGFR on E(spl)m8 expression. Thus, to understand the role of dA2BP1 in the context of specific pathway, we have to establish its genetic interactions with E(spl)m8. This is discussed in the next chapter.

4.3.3 **Senseless (Sens) interaction confirms that dA2BP1 has upstream role in the pathway:**

During sensory organ specification, Sens is downstream effector of Notch signaling (Fig.5.2). Sens and Achaete/Scute regulate each other's expression, while they both are regulated by E(spl)-C (Acar et al., 2006; Jafar-Nejad et al., 2003). We have observed that loss of function of Sens suppresses the phenotype (extra sensory bristles) caused by the loss of dA2BP1, suggesting that the dA2BP1 function is dependent on normal function of Sens. Over-expression of senseless can bypass Achaete and promotes bristle formation. We have observed that gain of function of Sens suppresses loss of sensory bristles observed when dA2BP1 is over expressed. This suggests that Sens is downstream to dA2BP1. Taken together these observation suggests that dA2BP1 might be regulating Achaete directly or via E(spl)-C.

5 Chapter-5: dA2BP1 regulates lateral inhibition by regulating Notch pathway

5.1 Introduction:

Sensory bristle development is a highly regulated phenomenon. Various signaling pathways not only control their number but also their position on the thorax. Sensory organ specification and development starts during larval stages. During development, the signaling molecules initially set up the platform by deciding a cluster of progenitor cells and then lateral inhibition specifies the SOPs by using regional cues. SOPs for sensory bristles of wing and T2 thorax are specified in the wing imaginal disc of third instar larvae (Cubas et al., 1991). Later during pupal stages, asymmetric divisions of a SOP form a complete sensory organ composed of socket, shaft, sheath, neuron and glia (Reddy and Rodrigues, 1999; Walt and Tobler, 1978).

5.1.1 **Positioning of proneural cluster:**

In the past years, several pathways have been discovered to play a role in the formation of the progenitor cluster. Certain early wing patterning events such as anterior/posterior patterning have a very significant role in sensory bristle positioning. SOPs for sensory bristles appear only at the anterior region of wing imaginal disc. Hedgehog (Hh) signaling positively regulates sensory bristle development. Over expression of either Hh or its signaling components, Patched (Ptc) or Cubitus interruptus (Ci) result in ectopic SOPs and sensory bristle development (Mullor et al., 1997). Hh pathway regulates homeo-box containing Iro-C complex to regulate lateral bristle development (Leyns et al., 1996). Decapentaplegic (Dpp), a transforming growth factor-β (TGFβ) family member, is a target of Hh signaling that fine tunes the positioning of the proneural clusters (Fig.5.1). It regulates sensory bristle development independent of Hh signaling (Phillips and Whittle, 1993). Dpp and Wingless (Wg, a Wnt family member) are long range signaling molecules. They widely affect sensory bristle development, while their role in dorsocentral (DC) bristle positioning has been extensively explored. Both Dpp and Wg are positive regulators of dorsocentral enhancer of *achaete/scute* (*ac/sc*) (Fig.5.1) (Tomoyasu et al., 1998). Dpp

regulates Ac/Sc by regulating Pannier (Pnr), a GATA transcription factor (García-García et al., 1999; Heitzler et al., 1996b). Pnr and its antagonist U shaped (Ush) decide the positioning of the DC bristles. Expression of Ush overlaps with the Pnr expression in mesonotum area. It represses Pnr-dependent transcription of *ac* and thus shifts DC bristles positioning away from the center of the thorax (Sato and Saigo, 2000). Pnr further represses *Iroquois Complex* (*Iro-C*) and limits it to the lateral position to define the medial notum (Fig.5.1) (Sato et al., 1999; Tomoyasu et al., 1998). The other pathway is the EGFR signaling pathway, which regulates Ac/Sc by regulating the MAP kinase signaling pathway (Culí et al., 2001). Over expression of EGF antagonist Argos prevents bristle formation (Fig.4.5). Negative regulators of Ac/Sc play very important role in bristle positioning: they limit Ac/Sc activity in such a way that neural cluster confines to a small, very tightly regulated boundary. Hairy (h) forms a heterodimer with Groucho (Gro), and the complex acts as a repressor for Ac/Sc (Ohsako et al., 1994; Van Doren et al., 1994), while Extamacrochaetae (Emc) a helix loop helix protein forms heterodimer with Ac/Sc and sequesters it from Da/Ac interaction (Fig.5.1)(Ellis et al., 1990; Garrell and Modolell, 1990; Van Doren et al., 1991). Stripe (Sr) an early growth response like factor prevents SOP selection by regulating Ac/Sc (Usui et al., 2008).

5.1.2 **SOP selection and lateral inhibition:**

Each proneural cluster, which consists of an average 20 to 30 cells, selects a single SOP (except DC and SC, which select two SOP each) and among them rest acquire an epidermal cell fate. Proneural clusters differ from each other in terms of shape, size and onset timing (Cubas et al., 1991; Skeath and Carroll, 1992). Cells within proneural clusters are arrested in G2 phase of cell cycle to provide equal opportunity for SOP fate (Kimura et al., 1997; Usui and Kimura, 1992). The SOP fate requires high expression of the proneural protein Ac/Sc. At the beginning, cells within progenitor cluster are equivalent in Ac/Sc expression and as time progresses, for unknown reasons or perhaps due to stochasticity, some cells start expressing more Ac/Sc. The cell that expresses more Ac/Sc, transcribes more *delta* to activate Notch signaling in neighboring cells (Heitzler and Simpson, 1991; Kunisch et al., 1994). Activation of Notch further represses *ac/sc* by transcribing *Enhancer of split complex (E(spl)-C)* genes. E(spl) binds *ac/sc* enhancer and negatively regulates its

expression (Fig.5.3)(Bailey and Posakony, 1995; Culí and Modolell, 1998; Lecourtois and Schweisguth, 1995).

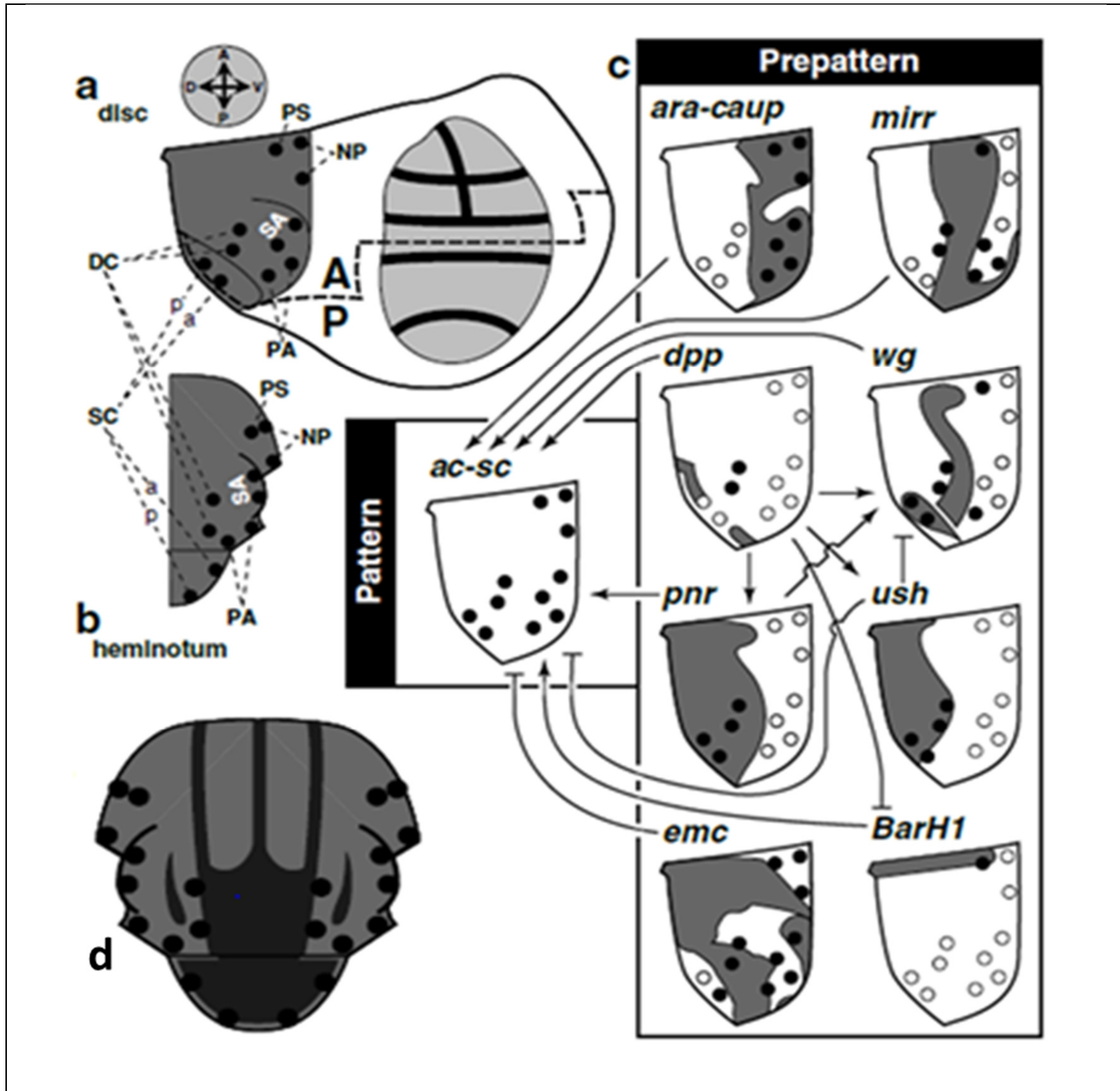


Figure 5.1 Positioning of proneural clusters-

A-B. Schematic representation of wing imaginal disc, marking wing pouch (light shading) and notum (dark shading). Dots in B mark the SOP positions, which form sensory bristles in the adult thorax. C. Expression pattern of different genes (shaded) around the proneural cluster and their final imprint on Achaete/Scute expression. Sharp arrowhead marks the activation, whereas blunt arrow marks the inhibition. Araucan-caupolican, Mirror, Pannier, Dpp, Wg and BarH1 act as activators, whereas Extramacrochaetae and U-shaped act as repressors. D Complete thorax with marked sensory bristle position and pigmentation. Image reproduced from (Held Jr et al., 2005).

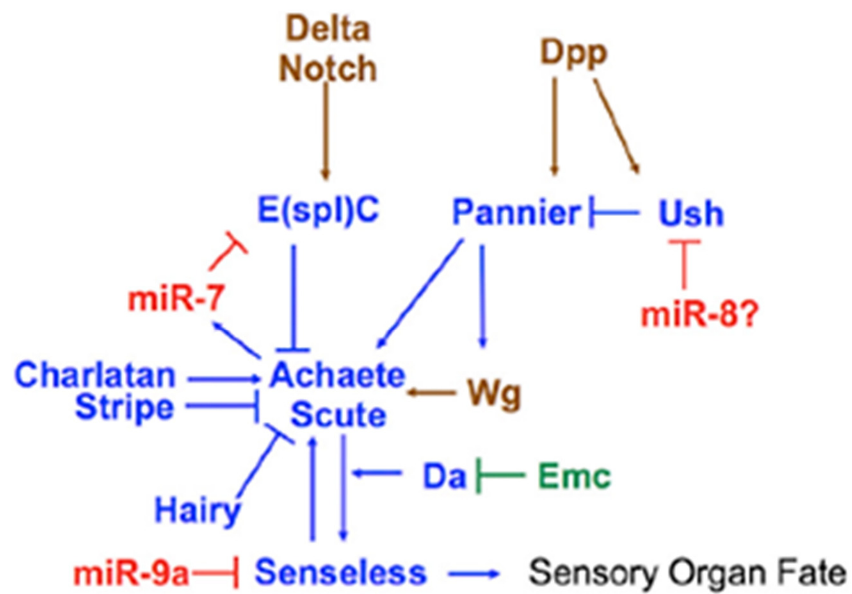


Figure 5.2 Regulation of Achaete/Scute –

Achaete/Scute regulation is complex. This figure shows only the simplified outline of Achaete/Scute regulation. Factors which regulate transcription are in blue, protein-protein interactors are in green and miRNAs based regulation depicted in red. Image reproduced from (Cassidy et al., 2013).

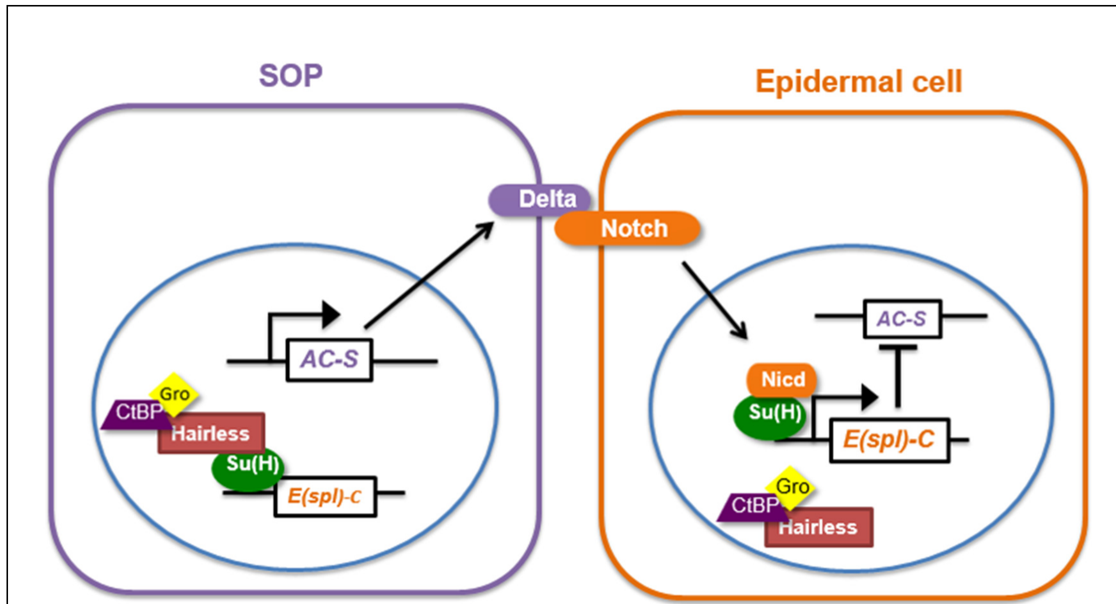


Figure 5.3 SOP Selection and Lateral inhibition-

High Achaete/Scute (AC-S) expressing cells express more Delta on their surface to activate Notch signaling in neighboring cells. Activation of Notch signaling represses AC-S expression by via E(spl)-C, thus less expression of Delta those cells, which take up epidermal fate..

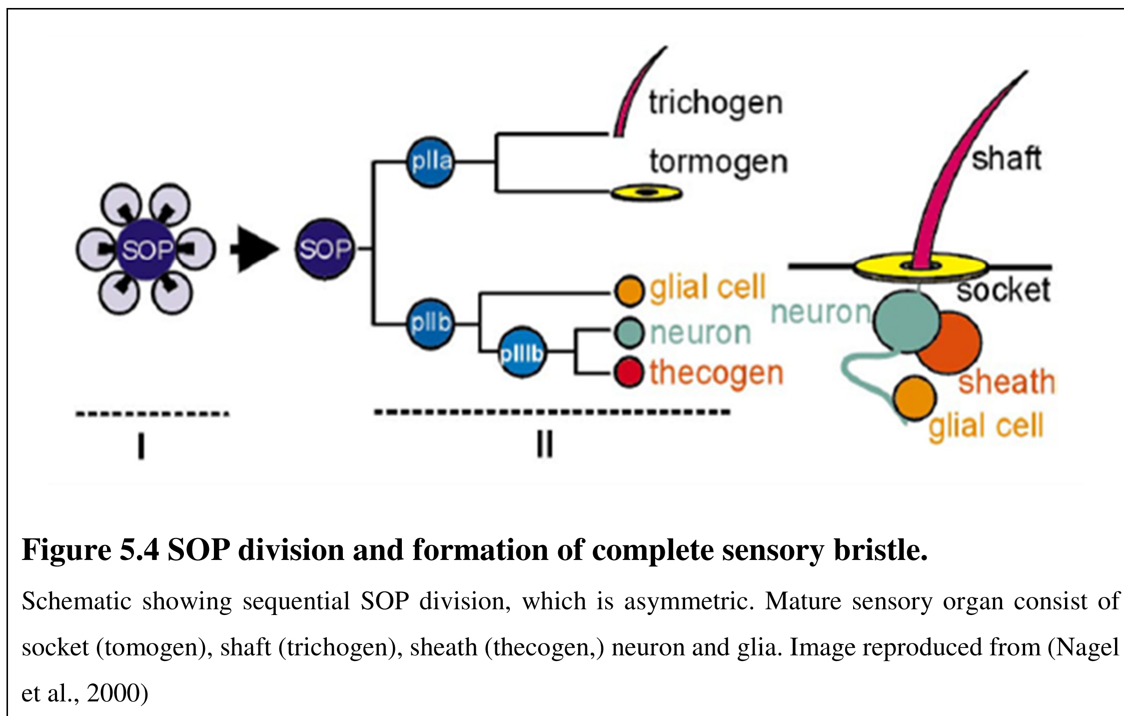


Figure 5.4 SOP division and formation of complete sensory bristle.

Schematic showing sequential SOP division, which is asymmetric. Mature sensory organ consist of socket (tomogen), shaft (trichogen), sheath (thecogen,) neuron and glia. Image reproduced from (Nagel et al., 2000)

5.1.3 **SOP division:**

SOP division starts during early pupal stages. First division forms two daughter cells pIIa and pIIb (Fig.5.4). This cell division is asymmetric in terms of Neuralized and Numb distribution. pIIb cell, which later forms inner cells, glia, neuron and sheath, inherits Numb and Neuralized from pI to establish differential Notch signaling (Le Borgne et al., 2005; Schweisguth, 2004). Numb and Sanpodo inhibit Notch receptor recycling and thus makes pIIb express fewer number of Notch receptors, while Neuralized promotes ubiqitination and degradation of Delta (Couturier et al., 2013). Degradation of Delta has positive feedback on its expression and finally more Delta expression on the surface (Lai et al., 2005). pIIa cell division will form two outer cells: shaft (hair like, tricogen) and socket (tomogen). Socket cell undergoes endo-replication and secretes cuticle to form dendritic cap. Shaft cell undergoes endo-replication and secretes cuticle to form bristle (Pierce et al., 2004).

As discussed in the previous chapter, we have observed that dA2BP1 negatively regulates sensory bristle formation. In this chapter, its precise role in SOP specification and differentiation is examined.

5.2 Results-

5.2.1 **dA2BP1 regulates SOP number to regulate sensory bristle number:**

Gain or loss of sensory bristle number could be an effect of either increase in SOP number at the initial stages of development or a fate switch between daughter cells during asymmetric division. As our previous results suggest that downregulation of the dA2BP1 during SOP division has no phenotype, we speculated that dA2BP1 might be regulating the SOP specification rather than SOP division. SOP specification for thoracic macrochaetae starts during early 3rd instar wing imaginal disc and SOPs from different PNC arise asynchronously at the notal area of wing imaginal disc. The first SOP appears almost 30 hours before puparium formation and all SOP specification completes before 3 hours of pupariation (Held Jr et al., 2005). Neuralized (Neur) expression marks the SOP cells in late 3rd instar wing imaginal disc (Huang et al., 1991). We used *neur*-lacZ (*A101*) to mark SOP during dA2BP1 manipulation. Since the sensory bristle phenotype is limited to dorsocentral (DC) and scutellar (SC) and anterior postalar (aPA), we observed change in SOP number at these locations (Fig.5.5A). dA2BP1 down-regulation showed increase in SOP number (Fig.5.5B) and dA2BP1 over-expression resulted in decreased number of SOPs (Fig.5.5C).

5.2.2 **dA2BP1 regulates SOP number by regulating Achaete expression:**

Achaete/scute complex acts as a proneural gene for nervous system development (Cubas et al., 1991). *Ac/Sc-Complex* consists of basic helix loop helix proteins clustered together and acts as a transcription factor for downstream targets such as *delta* (Fig.5.3) (Hing et al., 1994). Most of these genes shows partial functional redundancy and are regulated by similar set of transcription factors, together they are required for their downstream events. *Ac/Sc-Complex* expands over 100kb near the tip of the X chromosome and is the genomic locus for *achaete, scute, lethal of scute* and *asense* (García-Bellido and de Celis, 2009; Ghysen and Dambly-Chaudiere, 1988). Although the complex houses 4 genes, *achaete* has been explored extensively for its transcriptional regulation and protein-protein interactions. Major regulatory regions on *achaete* enhancer are E box, CAGGT (positively regulated by

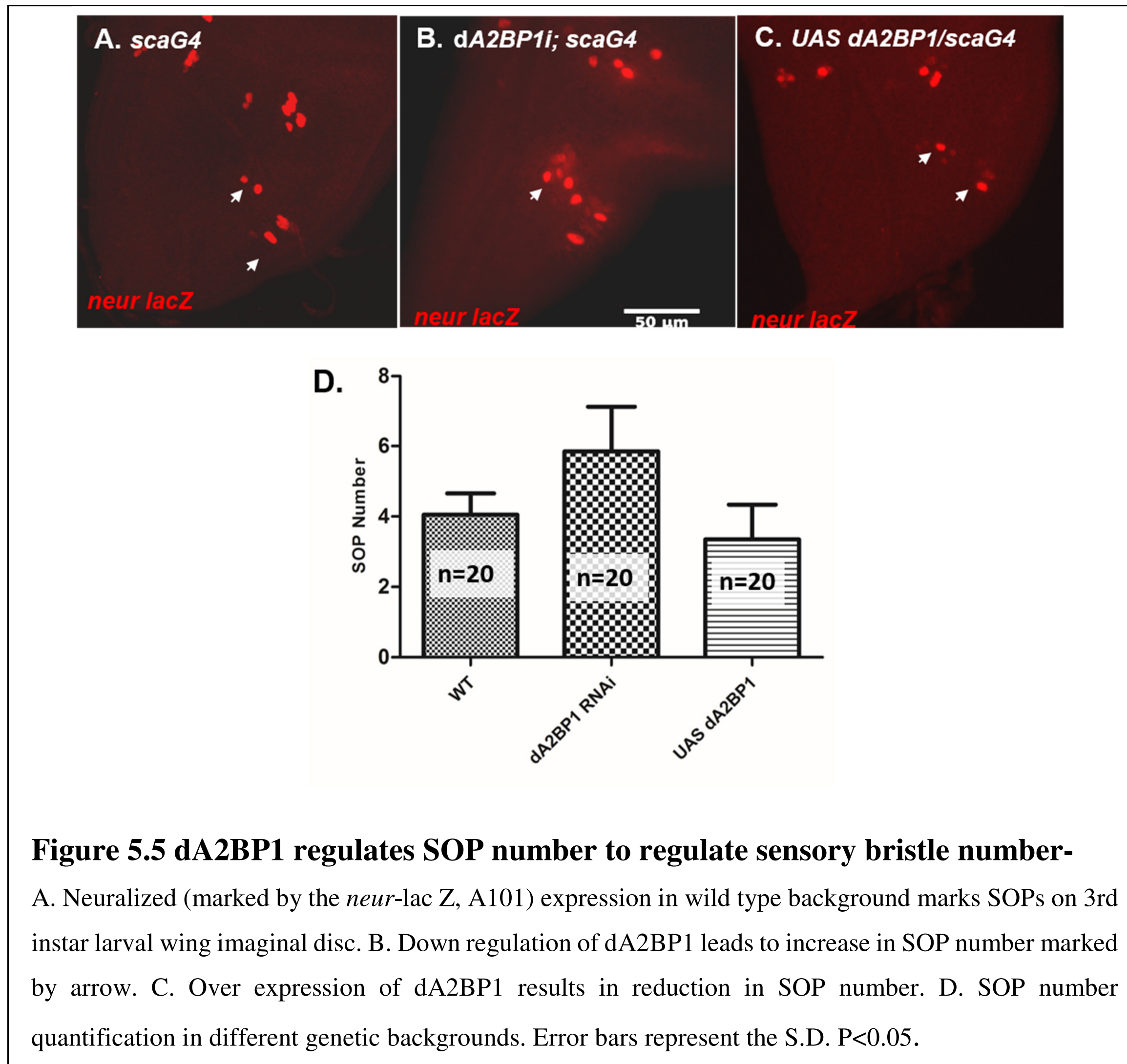


Figure 5.5 dA2BP1 regulates SOP number to regulate sensory bristle number- A. Neuralized (marked by the *neur*-lac Z, A101) expression in wild type background marks SOPs on 3rd instar larval wing imaginal disc. B. Down regulation of dA2BP1 leads to increase in SOP number marked by arrow. C. Over expression of dA2BP1 results in reduction in SOP number. D. SOP number quantification in different genetic backgrounds. Error bars represent the S.D. P<0.05.

Ac/Sc or Ac/Da); S box, AATC (positively regulated by Senseless); N box, CACG/AAG (negatively regulated by E(spl)) (Acar et al., 2006; Culí and Modolell, 1998; Van Doren et al., 1992). Being a basic helix loop helix protein, Ac forms a homodimer and can also bind to other helix loop helix proteins to form heterodimers. It can form heterodimers with Scute, and Daughterless (Da) and together this complex serves as an activator for target genes (Cabrera and Alonso, 1991). Alternatively, it can form heterodimers with Extramacrochaetae (Emc), which lacks basic domain to bind DNA and sequesters it from Da and Scute interaction. Thus, Emc negatively regulates Ac function (Van Doren et al., 1992). During SOP specification, all cells in PNC have equal opportunity to acquire SOP

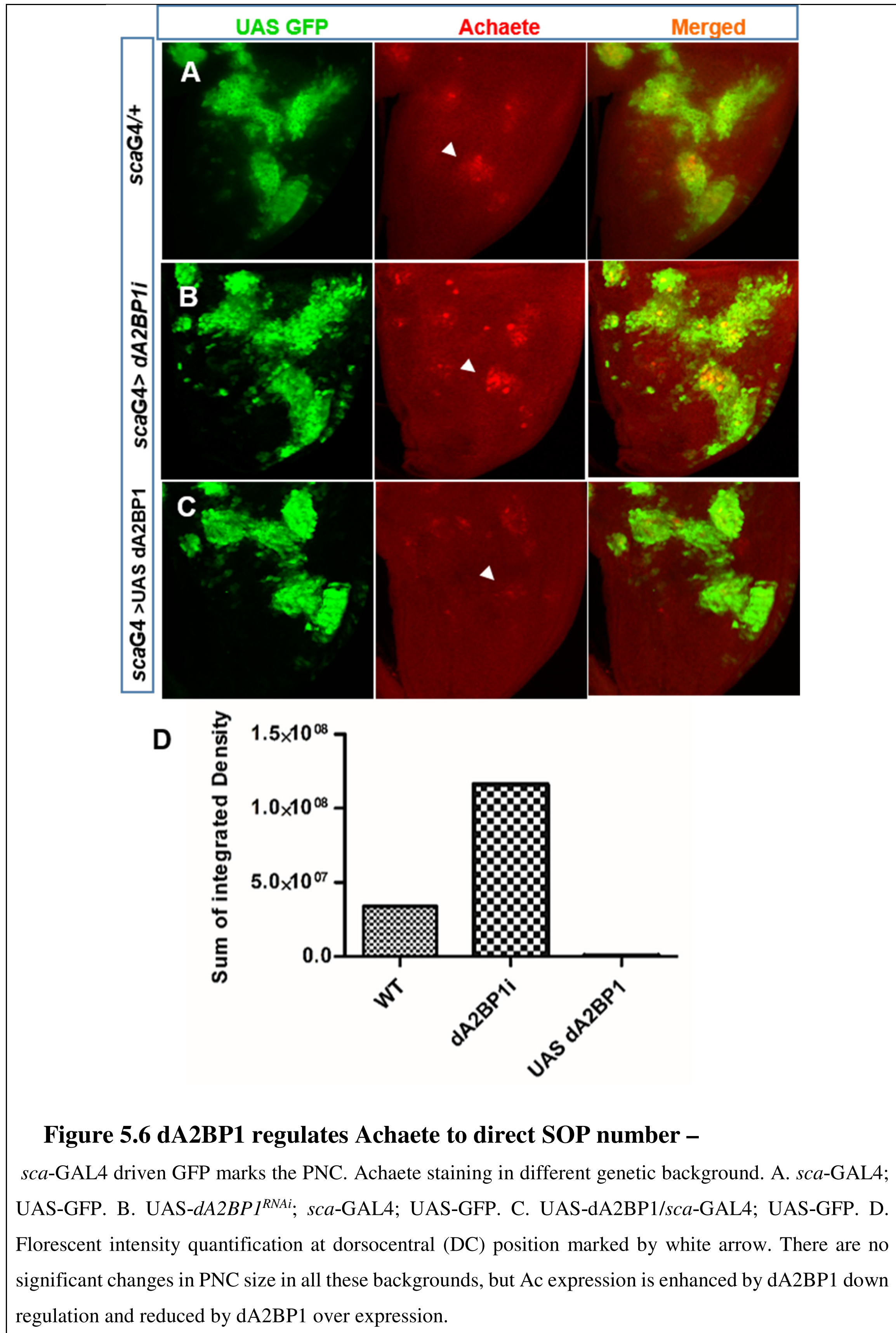


Figure 5.6 dA2BP1 regulates Achaete to direct SOP number –

sca-GAL4 driven GFP marks the PNC. Achaete staining in different genetic background. A. *sca*-GAL4; UAS-GFP. B. UAS-*dA2BP1*^{RNAi}; *sca*-GAL4; UAS-GFP. C. UAS-dA2BP1/*sca*-GAL4; UAS-GFP. D. Florescent intensity quantification at dorsocentral (DC) position marked by white arrow. There are no significant changes in PNC size in all these backgrounds, but Ac expression is enhanced by dA2BP1 down regulation and reduced by dA2BP1 over expression.

fate initially but later on due to lateral inhibition, only one cell secures the SOP fate by reaching the threshold Ac/Sc expression.

As dA2BP1 manipulation affects the SOP number, to explore whether dA2BP1 regulates *achaete* expression to bring change in SOP number, we examined the Achaete levels. Down regulation of dA2BP1 lead to increase in Ac levels in PNC without affecting PNC size (Fig.5.6B). Conversely, over-expression of dA2BP1 resulted in reduction in Ac expression (Fig.5.6C). These results suggest that dA2BP1 regulates the number of cells that cross the threshold Ac levels and thereby, may modulate SOP fate.

5.2.3 **dA2BP1 regulates *Enhancer of split* (*E(spl)*) expression to regulate Achaete expression:**

Being a proneural gene, Ac is very tightly regulated both at the level of transcription and at the level of protein-protein interactions. Although there are many transcriptional regulators for Ac/Sc, Notch signaling based, E(spl)-C mediated regulation is critical for Ac/Sc expression (Fig5.2 and 5.3) (Culí and Modolell, 1998; Heitzler et al., 1996a; Lecourtois and Schweisguth, 1995). *E(spl)-C* expands over 50 kb on right arm of 3rd chromosome. Among these, 7 genes viz., mδ, mγ, mβ, m3, m5, m7 and m8 encode for helix loop helix proteins and can partially substitute for each other's function (Jennings et al., 1999). Rest four members mα, m2, m4, and m6 are a part of Bearded family proteins (Lai et al., 2000). Being a neurogenic factor, E(spl) complex is very tightly regulated, its enhancer region has binding site for Suppressor of Hairless Su(H), basic helix loop helix dimer (E box), and N box (Castro et al., 2005). E(spl) proteins form a dimer and bind to N box (CACnAG) (Jennings et al., 1999). The C-terminal tail of E(spl) proteins contains WRPD domain, which recruits Groucho and thus this complex acts as a transcriptional repressor (Paroush et al., 1994). E(spl) complex mediated Notch signaling is limited to SOP specification, further Notch signaling during asymmetric SOP division is independent of E(spl) (Nagel et al., 2000).

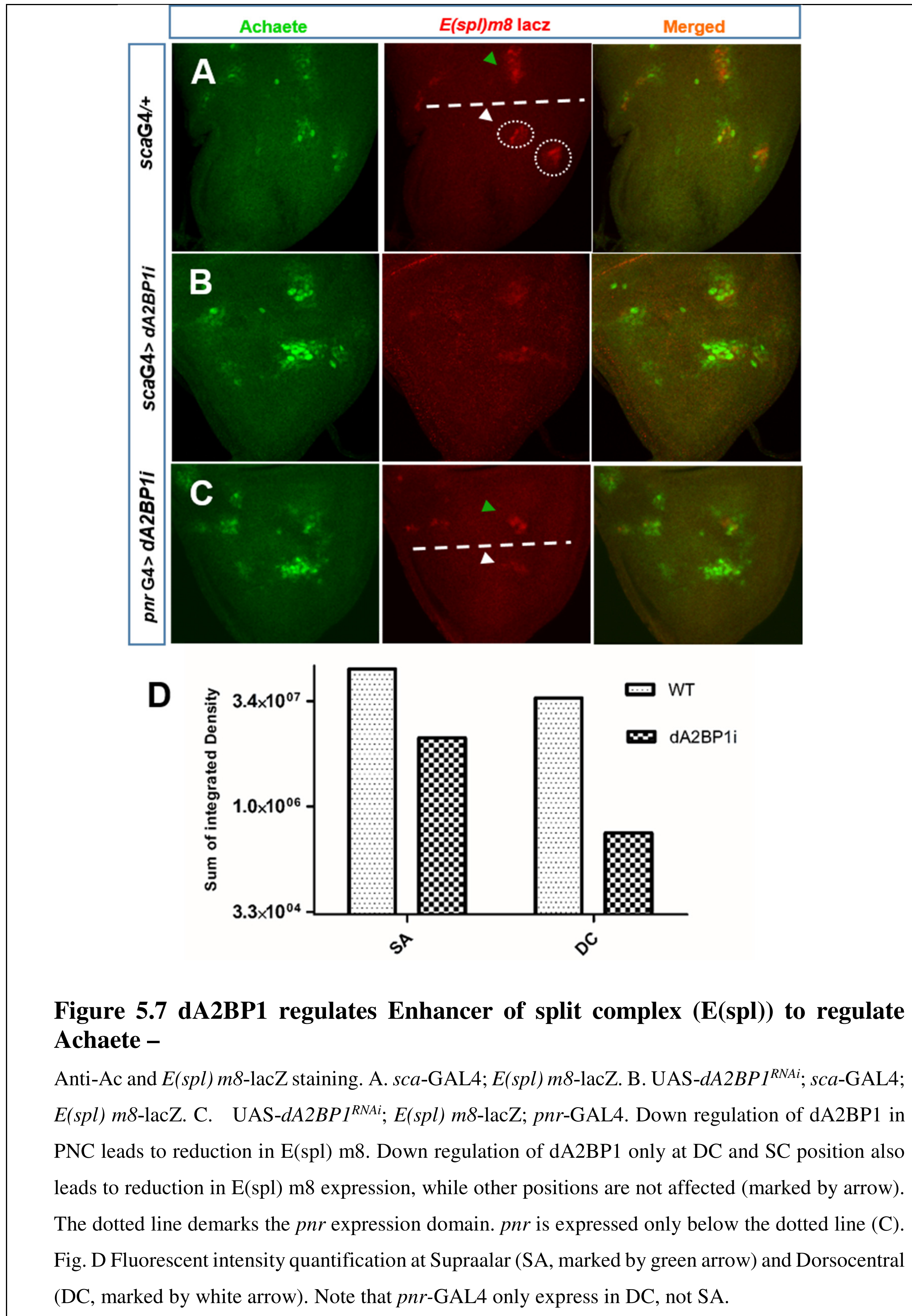


Figure 5.7 dA2BP1 regulates Enhancer of split complex (E(spl)) to regulate Achaete –

Anti-Ac and *E(spl) m8*-lacZ staining. A. *sca*-GAL4; *E(spl) m8*-lacZ. B. UAS-*dA2BP1*^{RNAi}; *sca*-GAL4; *E(spl) m8*-lacZ. C. UAS-*dA2BP1*^{RNAi}; *E(spl) m8*-lacZ; *pnr*-GAL4. Down regulation of dA2BP1 in PNC leads to reduction in E(spl) m8. Down regulation of dA2BP1 only at DC and SC position also leads to reduction in E(spl) m8 expression, while other positions are not affected (marked by arrow). The dotted line demarks the *pnr* expression domain. *pnr* is expressed only below the dotted line (C). Fig. D Fluorescent intensity quantification at Supraalar (SA, marked by green arrow) and Dorsocentral (DC, marked by white arrow). Note that *pnr*-GAL4 only express in DC, not SA.

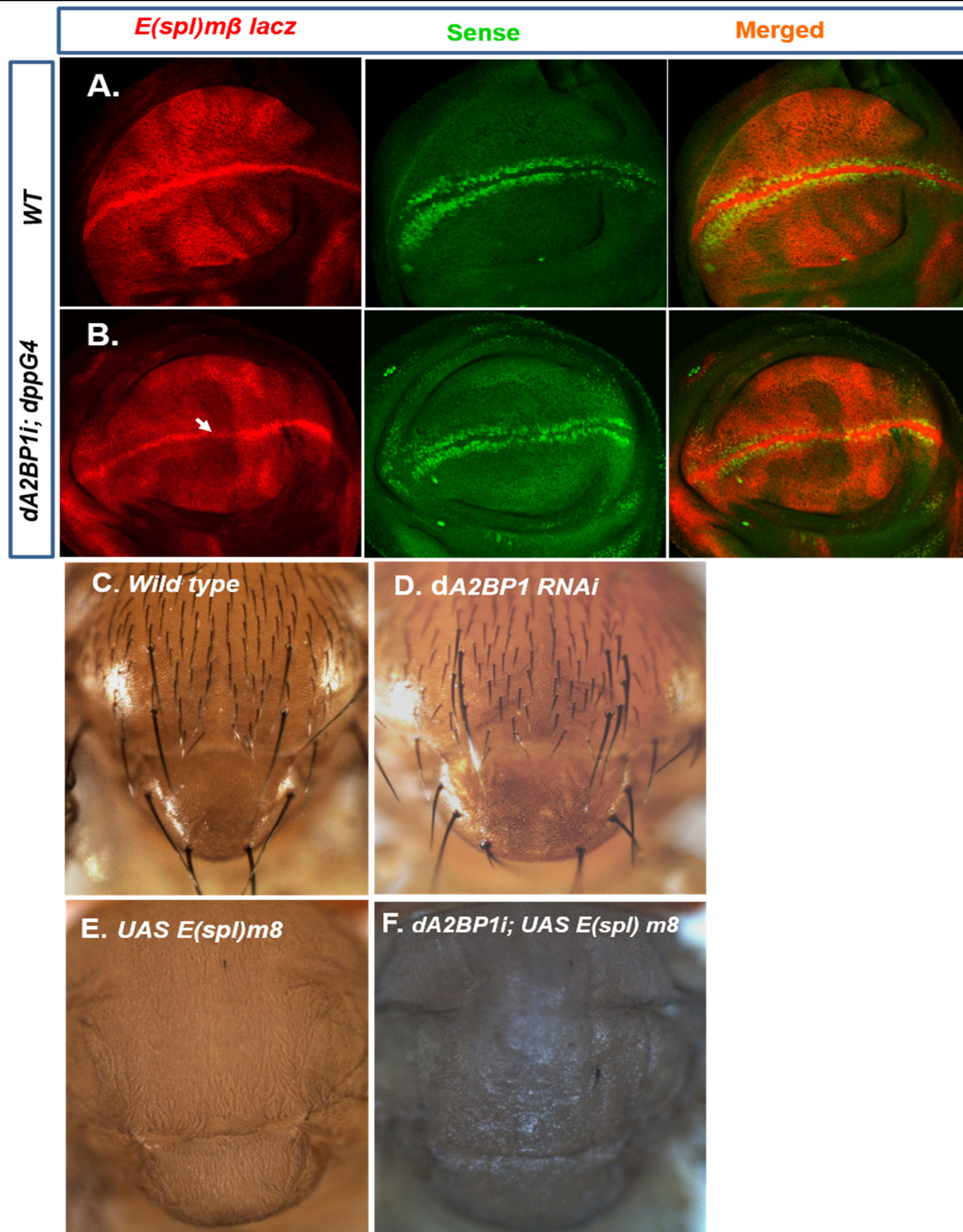


Figure 5.8 dA2BP1 is upstream of E(spl)

A. E(spl)mβ and Sense expression in the wild type wing disc. B. dpp-GAL4; *E(spl)mβ*-lacZ; UAS-*dA2BP1*^{RNAi}. Down regulation of dA2BP1 at AP boundary results in loss of E(spl)mβ expression (white arrow). C. Wild type thorax; D. *sca*-GAL4; UAS-*dA2BP1*^{RNAi} E. *sca*-GAL4; UAS-E(spl)m8; F. *sca*-GAL4; UAS-*dA2BP1*^{RNAi}; UAS-E(spl)m8. Over expression of E(spl) m8 represses sensory bristle formation. Down regulation of dA2BP1, which normally results in increased number of sensory bristles; could not rescue the phenotypes caused by the over-expression of E(spl) m8.

As dA2BP1 shows genetic interaction with Notch and regulates Achaete expression, we looked at *E(spl)m8* and *E(spl)mβ* expression. dA2BP1 down-regulation in PNC using *sca*-GAL4 not only showed reduction in *E(spl)m8*-lacZ expression as seen by reduced fluorescence intensity, but also showed much lower expression in a broader domain which may be leading to up-regulation of Ac in these domains (Fig.5.7B).

We then extended our experiments to investigate another member of *E(spl)* complex viz. *E(spl)mβ* which is expressed in the entire wing pouch. Down-regulation of dA2BP1 in few cells abutting the A/P boundary using *dpp*-GAL4 leads to reduction of *E(spl)mβ*-lacZ expression (Fig.5.8B). However, *E(spl)mβ* expression at the D/V boundary was not affected since dA2BP1 is not expressed at the D/V boundary. This clearly indicates that dA2BP1 impinges on Notch signaling via regulation of *E(spl)-C* genes. Over-expression of E(spl)m8 represses SOP specification and thus suppresses sensory bristle formation (Fig.5.8E). To confirm that the role of dA2BP1 is upstream to *E(spl)-C*, we down regulated dA2BP1 in E(spl)m8 over-expressed background. dA2BP1 down regulation did not rescue sensory bristle phenotype (Fig.5.8F). This confirmed the role of dA2BP1 upstream of *E(spl)-C* in the Notch pathway.

5.2.4 **Over-expression of dA2BP1 results in the elevated levels Notch accumulation and its activity:**

Notch signals via two pathways: canonical and non-canonical (Guruharsha et al., 2012). In both modes, various proteins modulate Notch function via posttranslational modification either to stabilize active Notch towards its role in transcription or for protein degradation to attenuate signaling (Couturier et al., 2012; Mukherjee et al., 2005). We have already shown that down-regulation of dA2BP1 significantly reduced *E(spl)m8* and *E(spl)mβ* expression indicating its requirement for Notch signaling. Epistatic relation between A2BP1 and *E(spl)-C* shows that dA2BP1 is upstream to *E(spl)-C,* which is a direct target of Notch.

Next we examined the precise role of dA2BP1 in Notch signaling. Notch expresses uniformly in the wing pouch. Over-expression of dA2BP1 using *dpp*-GAL4 resulted in elevated levels of Notch in GAL4-expression regions (Fig.5.9B), suggesting that dA2BP1 is a positive regulator of Notch. However, we failed to locate NICD in the nucleus using

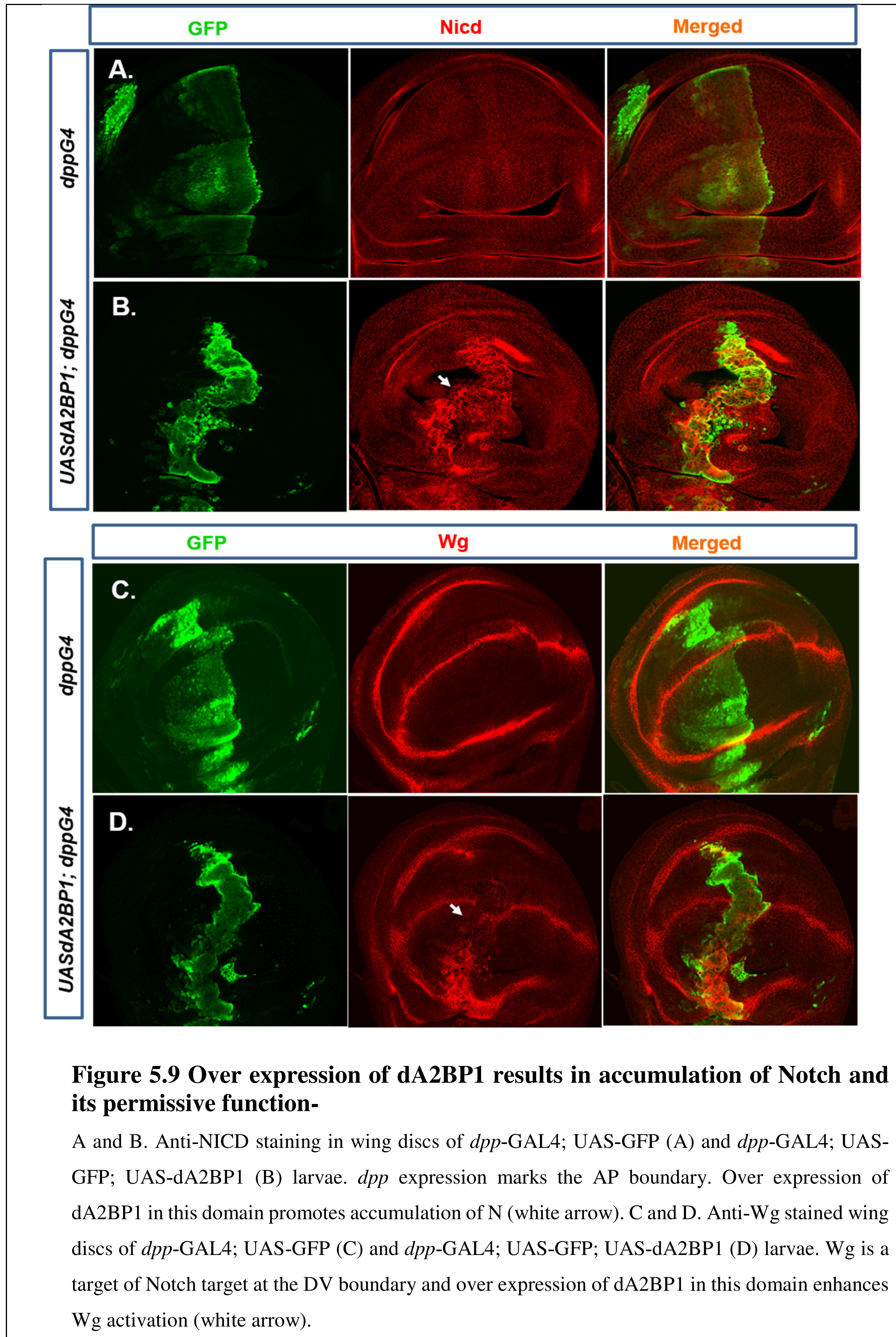


Figure 5.9 Over expression of dA2BP1 results in accumulation of Notch and its permissive function-

A and B. Anti-NICD staining in wing discs of *dpp*-GAL4; UAS-GFP (A) and *dpp*-GAL4; UAS-GFP; UAS-dA2BP1 (B) larvae. *dpp* expression marks the AP boundary. Over expression of dA2BP1 in this domain promotes accumulation of N (white arrow). C and D. Anti-Wg stained wing discs of *dpp*-GAL4; UAS-GFP (C) and *dpp*-GAL4; UAS-GFP; UAS-dA2BP1 (D) larvae. Wg is a target of Notch target at the DV boundary and over expression of dA2BP1 in this domain enhances Wg activation (white arrow).

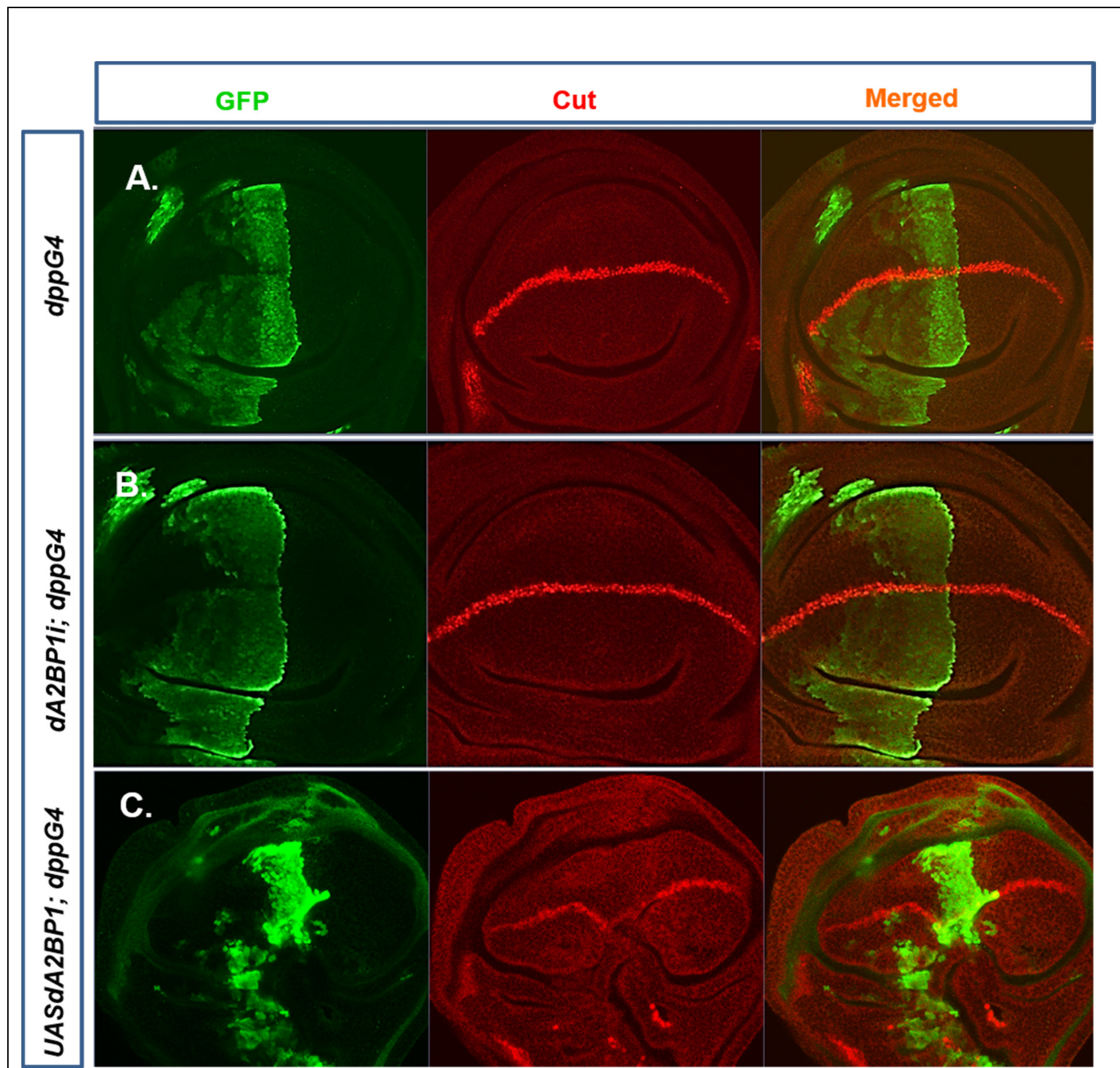


Figure 5.10 dA2BP1 is not part of the instructive function of Notch.
A, B and C. Anti-Cut antibody staining of wing discs of *dpp*-GAL4; UAS-GFP (A); *dpp*-GAL4; UAS-GFP; UAS-*dA2BP1*^{RNAi} (B) and *dpp*-GAL4; UAS-GFP; UAS-dA2BP1 (C) larvae. dA2BP1 does not express in the DV boundary, expression of *dA2BP1*^{RNAi}, as expected, does not affect Cut expression (B). Over-expression of dA2BP1, which causes increased accumulation of Notch (Fig. 5.9B) does not affect levels or pattern of Cut expression (C).

available antibody. The role of Notch for regulation of downstream targets could be instructive or permissive (Bray and Furriols, 2001; Janody and Treisman, 2011). While Notch activity in removal of repressors is considered as permissive function, its interactions

with different sets of additional co-factors upregulating target expression is termed instructive function. Wg activation needs permissive Notch function while *cut* and *E(spl)m8* both require instructive role (Janody and Treisman, 2011). To test whether dA2BP1-stabilized Notch is functionally active towards its permissive role or instructive role, we examined Wg levels after elevated Notch accumulation. Over-expression of dA2BP1 using *dpp*-GAL4 enhanced Wg levels in the D/V boundary (Fig.5.9D). However, over-expression of dA2BP1 was not sufficient to activate Cut expression (Fig.5.10C). This suggested that while dA2BP1 is a positive regulator of Notch signaling, it is not necessary for all functions of Notch.

5.2.5 **dA2BP1 is a part of Su(H) complex both in the presence and absence of Notch:**

During Notch signaling, NICD acts as a transcription factor for downstream targets. In the absence of Notch signaling, promoter and enhancer regions of the *E(spl)-Complex* genes are occupied by a repressor complex, which silences targets of Notch at the chromatin level (Fig 5.3). DNA binding protein Suppressor of Hairless (Su(H)) acts as a repressor in the absence of Notch signal (Bailey and Posakony, 1995; Brou et al., 1994). In the repressor complex, the Su(H) physically interacts with Hairless (H) which further recruits other proteins including Gro and C-terminal binding protein (dCtBP) (Barolo et al., 2002; Nagel et al., 2005). Gro further recruits histone deacetylase (HDAC), which results in chromatin silencing (Fig 5.3). During active Notch signaling, activated NICD and Mastermind (Mam) bind to the Su(H) complex and this binding facilitates removal of the repressor proteins, H, Gro and dCtBP from the complex, and thereby, activate target genes (Helms et al., 1999; Le Gall and Giniger, 2004; Lecourtois and Schweisguth, 1995).

To understand molecular role of dA2BP1 in the complex towards stabilizing Notch, we performed immunoprecipitation (IP) experiment for dA2BP1 on the lysate isolated from S2 cells. As S2 cell do not express Notch, we made a stable cell line by transfecting pMT-Notch full length in S2 cells and developed an inducible system for Notch signaling (Krejci and Bray, 2007).

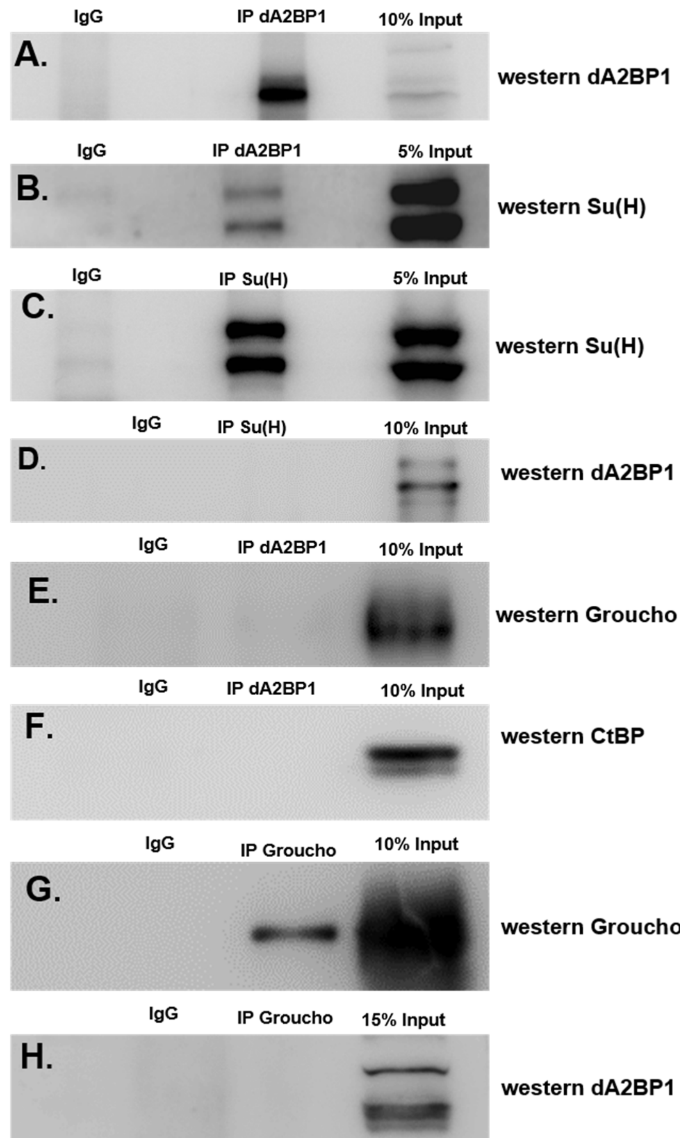


Figure 5.11 dA2BP1 is part of Su(H) complex in the absence of Notch – (A–F) Western blot hybridization to detect proteins present in immunoprecipitates from S2 cell (in which Notch is mutated and not functional) extracts isolated with the help of different antibodies as shown on the image. Western blots were probed with antibodies against dA2BP1 (A,D,H), Su(H) (B and C), Gro (E and G) and CtBP (F). IgG was used for control IP. Control IPs were performed with antisera generated from the same host. Input indicates the lysate amount used for IP. Note, dA2BP1 is part of a complex with Su (H) when it is not complexed with Gro or CtBP indicating that dA2BP1 binds to Su (H) only after Notch signaling removes Gro and CtBP from the complex.

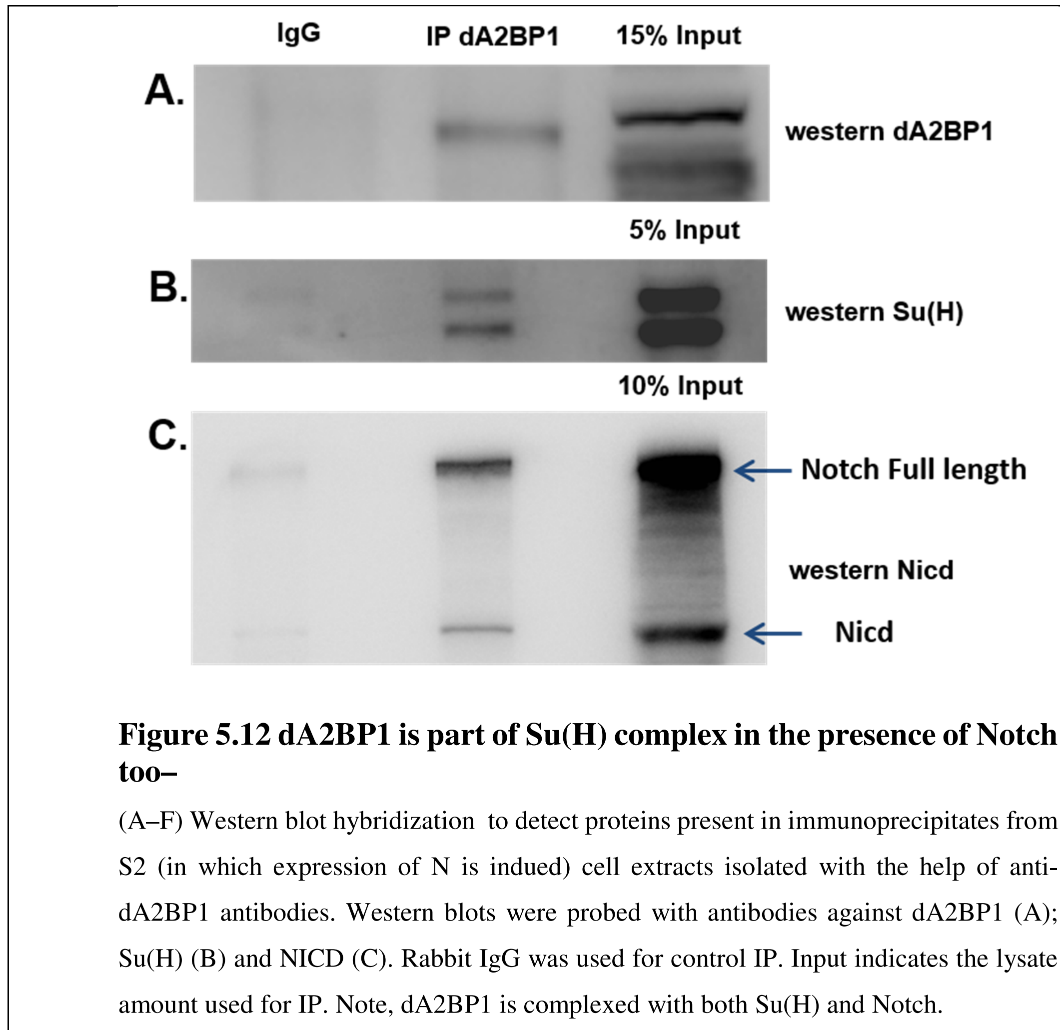


Figure 5.12 dA2BP1 is part of Su(H) complex in the presence of Notch too–

(A–F) Western blot hybridization to detect proteins present in immunoprecipitates from S2 (in which expression of N is indued) cell extracts isolated with the help of anti-dA2BP1 antibodies. Western blots were probed with antibodies against dA2BP1 (A); Su(H) (B) and NICD (C). Rabbit IgG was used for control IP. Input indicates the lysate amount used for IP. Note, dA2BP1 is complexed with both Su(H) and Notch.

dA2BP1 has 8 different known isoforms. Polyclonal antibodies generated against isoform dA2BP1-RE recognizes almost all isoforms on Western blot hybridization, but it strongly precipitates 100 kDa isoform in immunoprecipitation (IP) experiments (Fig. 5.11A) (Usha and Shashidhara, 2010). We performed IP with anti-dA2BP1 antibodies on extracts of S2 cells, which does not express functional Notch. We detected Su(H) in the immunoprecipitate, suggesting that it is as an interactive partner of dA2BP1 (Fig.5.11B). However, reverse IP using anti-Su(H) antibodies could not co-precipitate dA2BP1, which may be due to lower abundance of the latter (Fig.5.11D). We also examined if other components of the repressor complex such as Gro (Fig.5.11E) and CtBP (Fig.5.11F) are also part of dA2BP1-Su(H) complex. We, however, did not observe these repressor complex components in the immunoprecipitates. We carried out reverse IP with anti-Gro

antibodies and did not detect dA2BP1 in the immunoprecipitate (Fig.5.10H), suggesting that the dA2BP1 is not a part of the repressor complex. It also suggests that dA2BP1 is part of Su(H) and Notch complex only after the Notch signaling is activated and has replaced Gro and CtBP.

We then carried out IP with anti-dA2BP1 antibodies in Notch activated S2 cells (S2-N cells) to understand if the dA2BP1-Su(H) interaction is continued after addition of the Notch to the complex. We detected both Su(H) (Fig.5.12B) and Notch (Fig.5.12C) in the immunoprecipitate, suggesting that dA2BP1 is part of the Su(H)-Notch transcription complex and acts as transcription co-factor for the Notch signaling.

5.2.6 **Loss of function dA2BP1 suppresses loss of function phenotype associated with Hairless for SOP formation:**

During sensory bristle development, Notch function is required during both SOP initiation and SOP division. Similar to Notch, Hairless also acts at both levels. Hairless is a negative regulator of Notch signaling: loss of the Hairless function enhances the expression of targets of Notch and thereby, resulting in gain of Notch phenotype. These phenotypes could be categorized in to either complete loss of sensory bristles, where SOP development stalls at the specification stage (Fig.5.13C, white arrow) or results in shaft cell to socket cell conversion during SOP division (Fig.5.13C, white arrow head) (Bang et al., 1991; Bang et al., 1995). Reduction in Su(H) function suppresses loss of function phenotypes associated with Hairless (and hence the name Su(H)) (Ashburner, 1982).

As our results suggest that dA2BP1 is part of Su(H) complex, both in the presence and absence of Notch, we speculated that Su(H) and dA2BP1 function together to regulate SOP specification. To understand this mechanism, we down regulated dA2BP1 in the background of loss of function alleles of Hairless. We used 3 different null (and dominant) alleles of *Hairless i.e.,* H^1, H^2*and,* H^3. The heterozygous flies show either complete loss of sensory bristle or shaft to socket conversion. We observed, albeit partially, rescue of only one phenotype: loss of sensory bristles (Fig.5.13D). Presence of sockets in the place of shafts was not affected (Fig.5.13D, white arrowhead), further confirming observations mentioned in the previous sections that dA2BP1 functions only during the specification of SOPs.

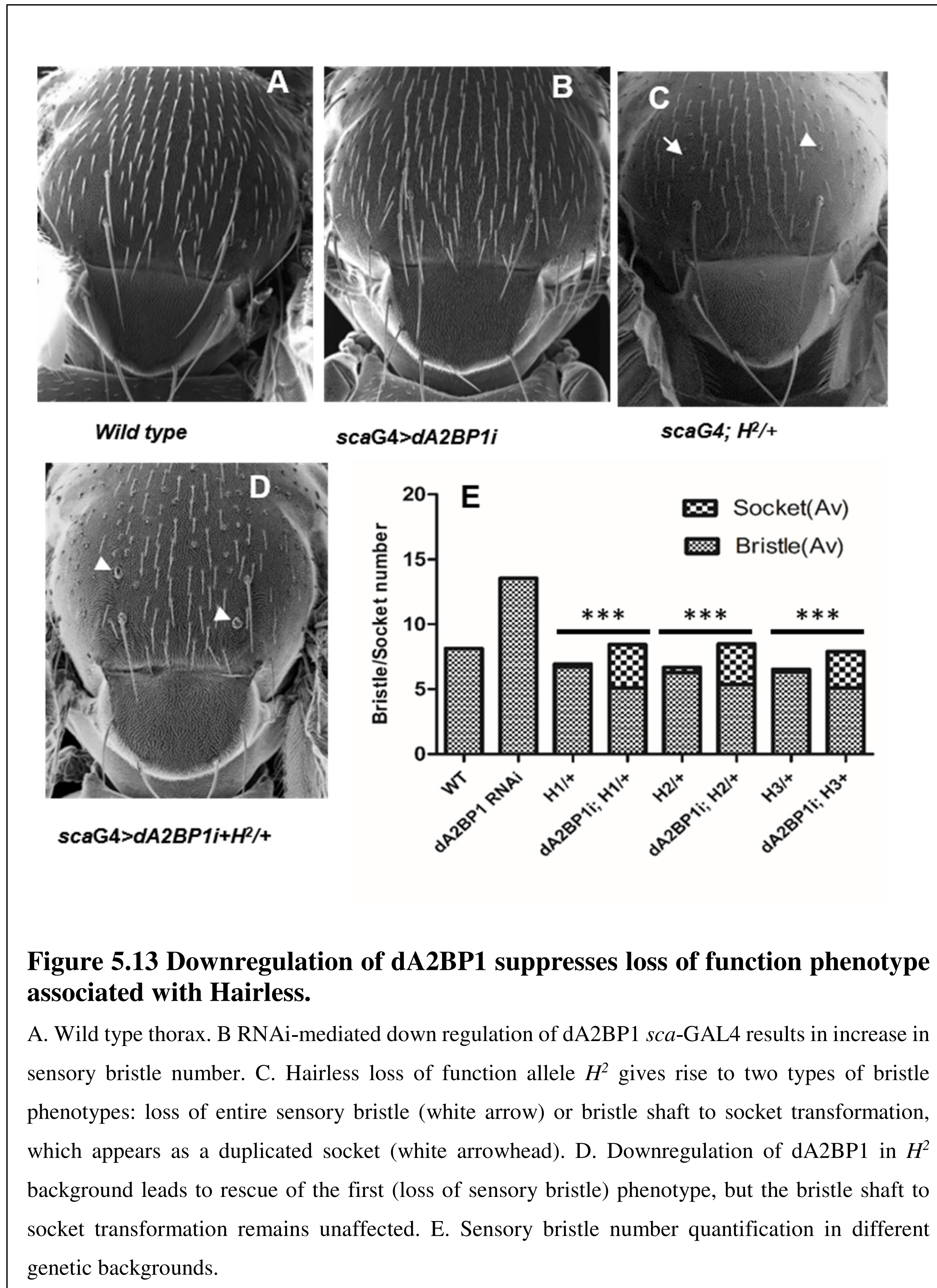


Figure 5.13 Downregulation of dA2BP1 suppresses loss of function phenotype associated with Hairless.

A. Wild type thorax. B RNAi-mediated down regulation of dA2BP1 *sca*-GAL4 results in increase in sensory bristle number. C. Hairless loss of function allele H^2 gives rise to two types of bristle phenotypes: loss of entire sensory bristle (white arrow) or bristle shaft to socket transformation, which appears as a duplicated socket (white arrowhead). D. Downregulation of dA2BP1 in H^2 background leads to rescue of the first (loss of sensory bristle) phenotype, but the bristle shaft to socket transformation remains unaffected. E. Sensory bristle number quantification in different genetic backgrounds.

5.3 Discussion-

Unlike CNS, PNS develops from epithelial cells, where every cell from proneural cluster has equal opportunity to develop into sensory organ. As it is important to have precise number of sensory organ, this program is very tightly regulated. Notch signaling, which plays an important role during SOP selection, has been extensively studied in this context. Down regulation of dA2BP1 leads to increase in sensory bristle number, also shows increase in SOP suggesting that dA2BP1 regulates sensory bristle number by regulating SOP number. Increase in SOP number per PNC is largely due to enhanced Ac expression. We observed increased Ac expression when dA2BP1 is down regulated; suggesting that dA2BP1 indeed regulates SOP specification. Over expression of dA2BP1 suppresses the development of sensory bristle number completely, although reduction in SOP number is not proportional to the sensory bristle number. It is possible that while over expression of dA2BP1 leads to drastic reduction in Ac expression, it might still allow SOP selection based on the differential Ac expression amongst PNC (Culí and Modolell, 1998; Held Jr et al., 2005).

Achaete expression is largely restricted by repressors including Emc, Stripe and Hairy (Usui et al., 2008). These factors limit the size and position of the PNC and proneural gene expression during SOP selection. No significant change was observed in PNC size in either loss or gain of function background of dA2BP1. In addition to the regulators of PNC, E(spl)-C regulates Ac within PNC. Down regulation of dA2BP1 suppresses E(spl)m8 expression. It is possible that this reduction in inhibitory loop results in some activation of Ac expression.

As dA2BP1 is positive regulator of Notch signaling, we speculated stabilization/accumulation of Notch in the gain of function background of dA2BP1. Over expression of dA2BP1 not only enhanced Notch levels (Fig.5.9B), but also enhanced its permissive role towards Wg activation (Fig.5.9D). However, its over-expression was not sufficient to enhance the instructive role of Notch towards the activation of Cut expression (Fig.5.10C). To enhance Notch accumulation and function, overexpressed dABP1 could act either by elevating Notch expression or by stabilization of Notch. While we did observe increased levels of Notch caused by the over-expression of dA2BP1, we do not know if this caused by transcriptional regulation or by stabilization. As during Notch signaling

Notch activity is largely dependent on protein modifications, we predict this is due to stabilization of N post-its activation. Our IP data also suggests that dA2BP1 is part of the Su(H) complex in the presence of Notch (Fig.5.12) and may play a role in stabilizing activated form of Notch.

Su(H) functions as both repressor and activator of its targets. In the presence of Notch, it activates the target gene expression, while in the absence of Notch, it represses the same targets. Our observation suggests that dA2BP1 is part of the the Su(H) complex both in the presence and absence of activated Notch. As loss- and gain-of-function phenotypes suggest that dA2BP1 is a positive regulator of Notch signaling, it is likely that dA2BP1-Su(H) interaction is relevant only during Notch signaling. This is further supported by that fact that loss of dA2BP1 can suppress the phenotypes associated with loss of Hairless, which is an antagonist of Su(H). It is likely that dA2BP1 competes with Hairless to bind Su(H). However, it remains to be investigated, if dA2BP1 binds to Notch even in the absence of Su(H) or only when Notch is in the form bound to Su(H). This has an implication in our understanding of how intracellular domain of Notch is regulated from the time it is generated at the membrane to its translocation to the chromatin in the nucleus, wherever Su(H) is already present.

5.4 dA2BP1 mode of action in Notch signaling

This study conclusively shows that dA2BP1 is involved in regulation of Notch signaling. It is upstream of Senseless, Achaete and *E(spl)-C*. We also show that dA2BP1 specifically regulates *E(spl)-C* transcription as down regulation of dA2BP1 leads to reduction in *E(spl)m8*-LacZ and *E(spl)mβ*-LacZ expression. Thus, dA2BP1 is a positive regulator of Notch pathway. Our results further show that dA2BP1 over expression elevates Notch accumulation which lead us to speculate that it might be stabilizing Notch by protein-protein interaction. By biochemical studies, we show that dA2BP1 is part of Notch-Su(H) complex during target activation. We also observe that dA2BP1 interacting Su(H) pool is devoid of Gro and CtBP; suggesting that dA2BP1 is not part of repressor complex before Notch activation. Putting it together, dA2BP1 activates Notch pathway by interacting with Notch-Su(H) activator complex. It can be speculated that dA2BP1 activates the Su(H)

complex by relieving the repressor proteins. However, this interaction requires presence of the Notch. Furthermore, this interaction ultimately helps cell fate determination by lateral inhibition. Then again, this speculation needs further testing.

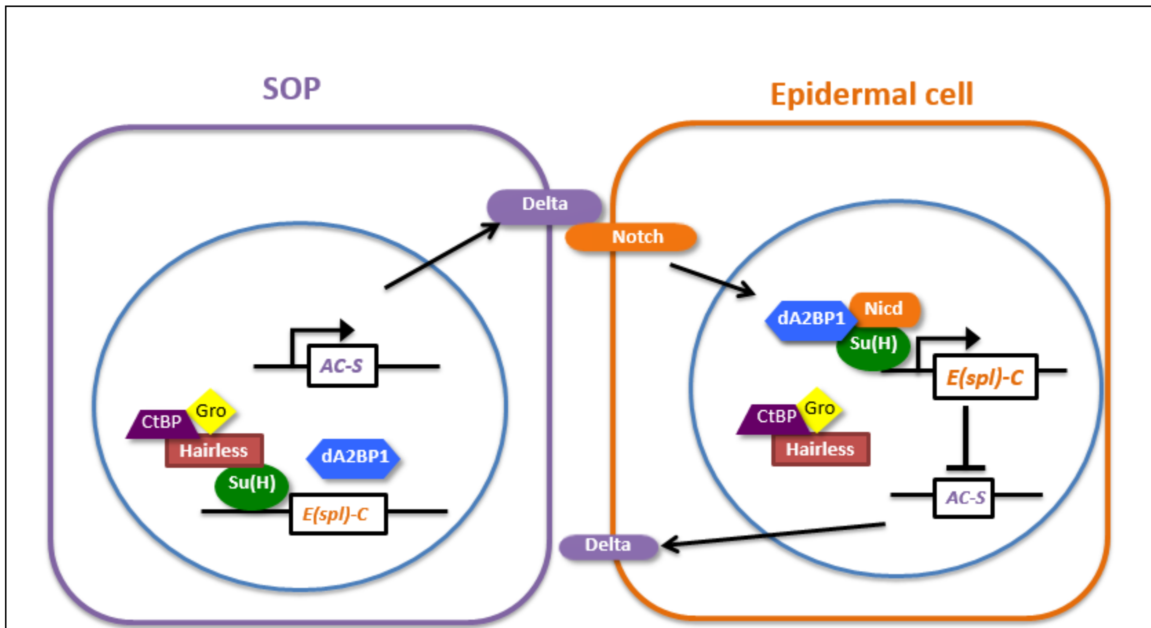


Figure 5.14 Mechanism of Notch regulation by dA2BP1

dA2BP1 is expressed in PNC cells including SOPs. During SOP specification, the future SOP cell expresses high Achaete/Scute (AC-S) to express more Delta on their surface to activate Notch signaling in neighboring cells. dA2BP1 is part of the Notch-Su(H) complex in Notch active cells. Activation of Notch signaling represses AC-S via E(spl)-C expression leading to lowered Delta expression in these neighboring epidermal cells, which take up epidermal fate.

6 Chapter-6: Conclusion and Future directions

Salient observations of this study are:

1. dA2BP1 is expressed in the developing nervous system, specifically, in the sensory organ precursors (SOPs).
2. Loss of dA2BP1 results in supernumerary sensory bristles on the dorsal thorax and gain of dA2BP1 causes baldness i.e. complete loss of both microchaete and macrochaete.
3. dA2BP1 genetically interacts with Notch pathway. It is positioned upstream or at par with Notch itself.
4. dA2BP1 regulates only the SOP specification at early stages of development. It has no role during asymmetric cell division of SOPs.
5. dA2BP1 physically interacts with Notch and Su(H). The interactions with the latter appears to be Notch-independent.
6. It appears that dA2BP1 is required for the transition of Su(H) as a repressor of targets of Notch to their activator.

6.1.1 **dA2BP1 isoforms in *Drosophila* and differential regulation:**

Here we have shown that dA2BP1 is widely expressed in the nervous system, and its expression in the developing embryos appears to be more specific to the nervous system than in other cells. The antibodies used here detect all isoforms and we are not sure, which of them are specific, if any, to the nervous system. In vertebrate A2BP1 expresses in different isoforms and their distribution varies not only from tissue to tissue, but also in different cellular compartments (Fig. 1.3). In *Drosophila*, dA2BP1 has 8 isoforms and their differential regulation is not known. Two isoforms RL and RH use different start site and thus have slight difference at the N- terminus. The other most striking feature is the length of 3'UTR amongst these isoforms (Fig 1.5). Isoform RH, RJ, RK, and RM have additional 2.4kb to 2.8kb longer 3'UTR (Fly Base). Longer 3'UTR has been correlated with transcript stability, translation efficiency and subcellular localization. Recent advances in transcript analysis show that mRNAs with longer 3'UTR are specific to the neuronal tissue (Gerber et al., 2006). Our analysis shows that dA2BP1 isoforms with longer transcript do have Pumilio (Pum) binding motif. Pum is well known translation repressor

and may regulate dA2BP1 expression (Hilgers et al., 2011). Micro-RNA (miR) based translational regulation is also a widely employed mechanism for differential gene expression. Prediction made by Burgler and Macdonald (2005) suggests that dA2BP1 is a target of miR-12 and miR-280 (Burgler and Macdonald, 2005). Thus, molecular tools are available to study the regulation of dA2BP1 expression in specific tissues, particularly in the nervous system. Regulation of its expression may well correlate its specific role in the neuronal development.

6.1.2 **dA2BP1 is an important factor for cell fate specification:**

A2BP1 has emerged as a significant player that regulates cell specification and division in both invertebrates and vertebrates. In *C. elegans* it acts as sex determinant factor. Its heterozygous background results in male, while in double the normal levels, the worm is hermaphrodite (Hodgkin et al., 1994). In vertebrates, A2BP1 is important for maintaining the specificity of the cell: loss of the A2BP1 leads to tumorogenesis including glioma and neuroblastoma (Hu et al., 2013). In *Drosophila* too, loss of dA2BP1 during oogenesis leads to tumorogenenic phenotype of the egg chamber (Tastan et al., 2010). We have shown here that loss of dA2BP1 during SOP specification leads to increase in sensory bristle number. However, the increase in the SOP number is due to change in cell fate early during development, not due to mere increase in cell division. This study has further established a role for dA2BP1 in regulating Notch signaling during cell fate specification. However, this role of dA2BP1 is at the level of transcriptional regulation and not as a splicing factor. It is, however, possible that the two molecular roles are coupled, which requires additional studies.

6.1.3 **dA2BP1-Notch interaction is specific to the transcriptional regulation of *E(spl)-C*:**

dA2BP1 genetically interacts with Notch. We have observed that down regulation of dA2BP1 leads to reduction in Notch reporters, *E(spl)m8*-LacZ and *E(spl)mβ*-LacZ expression. As these reporters are specific to promotor readout, we conclude that dA2BP1 regulates *E(spl)-C* at the transcriptional level. The functional implications of this interaction are similar to the role of Enhancer of the split (E(spl) in the Notch pathway (Jennings et al.; 1995). Although Notch and Su(H) regulates both SOP specification and

SOP division, role of E(Spl) is limited to SOP specification (Nagel et al., 2000). Similarly, we observed that dA2BP1 down regulation affects only bristle number, and not cell fate.

During wing development, Notch signaling determines D/V organizer by regulating the expression of Wg, Ct and Vg. Amongst the targets, Ct expression is mediated by E(spl)-C (Ligoxygakis et al., 1999). While over-expression of dA2BP1 affected Wg expression, it did not affect Ct expression, further suggesting that dA2BP1-dependent regulation of Notch pathway is limited and specific to regulating the expression of *E(spl)-C*.

To further confirm the dA2BP1 role in Notch target activation, we tried down-regulating dA2BP1 using dsRNA in S2-N cells and quantify Notch-dependent transcription activation using real time PCR. However, we failed to knockdown dA2BP1 sufficiently to see the change. S2 cell based assay will also be helpful to investigate precise nature of Notch-dA2BP1 interactions. Different domains of dA2BP1 including RRM may be expressed in S2-N cells and quantification of Notch targets could pinpoint the specific domain of dA2BP1 required for Notch signaling.

6.1.4 **Role of dA2BP1 in Su(H) complex:**

Genetic interactions between dA2BP1 and Hairless suggests that dA2BP1 may function at par with Su(H). Immunoprecipitation experiments confirm that the two are part of the same protein complex. As dA2BP1 and Su(H) are able to form a complex even in the absence of Notch, their interaction is not dependent on Notch signaling. Observation from Kelly et al., (2007) suggests that NICD and Su(H) physically interact in vitro (Kelly et al., 2007). As signaling events inside a cell are complex and context-dependent, we do not have sufficient information on the dynamics of Notch-Su(H) interactions inside a cell. As dA2BP1 interacts with Su(H) both in the absence and the presence of Notch and they both are positive regulators of Notch; we speculate that dA2BP1 might stabilize and strengthen Notch-Su(H) interactions on chromatin. It is also possible that function of Su(H) is to stabilize dA2BP1 and Notch interactions, which help in regulating the targets of Notch both at the transcriptional level and at the level of intron splicing. Detailed molecular interaction studies amongst these three proteins are needed to further elucidate the mechanism of function of dA2BP1 and Su(H).

6.1.5 **Notch-Su(H) paradox:**

Binding of NICD to Su(H) complex has been long debated. One school advocates that NICD binds to Su(H) repressor complex at the chromatin and turns the complex as activator by removing repressor proteins such as H, Gro and dCtBP from the complex (Castro et al., 2005; Koelzer and Klein, 2006). However, another school of thought speculate that NICD and Su(H) complex is formed before they bind to DNA (Krejci and Bray, 2007). dA2BP1 is the first transcription factor to be reported as the one which interacts with Su(H) even in the absence of Notch. This interaction continues even after activation of Notch in those cells. We have also observed that dA2BP1 immunoprecipitated pool of Su(H) is devoid of known repressors (Gro, H and CtBP) that interact with the former. Comparison of genomic sites that are bound by dA2BP1 on the chromatin in the presence and absence of Notch may throw some light on the existing debate on Notch and Su(H) interactions and also to understand precise role of dA2BP1 in this phenomenon.

6.1.6 **dA2BP1 as chromatin regulator:**

We have observed two transcription-related activities of dA2BP1, it acts as transcriptional co-factor in two well characterized signaling pathways: Hedgehog and Notch. We have also observed that in both pathways, in the absence of the signal, the mediators (Ci and Su(H), respectively) act as transcriptional repressors for the same set of genes for which they act as activators in the presence of the signal. dA2BP1 interacts with both repressor Ci^{75} and activator Ci^{155} forms of Ci (Usha and Shashidhara, 2010). Here we have shown that it interacts with Su(H) in the presence as well as the absence of Notch. Loss of dA2BP1 in both cases leads to reduction in the expression of downstream target genes. Repressor to activator transition needs major chromatin demodulation and we speculate a significant role for dA2BP1 in this phenomenon. Transformation of campaniform sensillum into sensory bristle is a rare phenotype. Very few genes have been reported to regulate this phenomenon and confer the identity of the campaniform sensillum. Loss of homeobox genes, BarH1 and BarH2; DNA binding activity of transcription factor SMAD; and loss of chromatin regulator Brahma leads to this transformation (Elfring et al., 1998; Higashijima et al., 1992; Takaesu et al., 2005). Thus, dA2BP1-mediated regulation of this phenomenon suggests a chromatin-related role.

6.1.7 **RNA binding role of the dA2BP1:**

In the vertebrate system, A2BP1 is widely known for its role in tissue specific RNA splicing. It has RRM motif, which binds to hexa-nucleotide sequence –UGCAUG- to regulate RNA splicing (Underwood et al., 2005). The fly homologue, dA2BP1 shows more than 90% similarity to the mammalian A2BP1 at the level of RRM. It is possible that dA2BP1 also has RNA binding role. Bruno is an RNA binding protein, known to regulate translation of target proteins by regulating RNA splicing (Park et al., 2004). dA2BP1 physically interacts with Bruno and regulates its targets such as *Cyc A* and *Cyc B* (Tastan et al., 2010). However, Usha and Shashidhara (2010) and this study demonstrate dA2BP1 role in transcriptional regulation of target genes. Usha and Shashidhara (2010) have also demonstrated DNA binding activity of dA2BP1. Many proteins such as Bicoid and Modulo play dual role and binds to both DNA and RNA to regulate targets during fly development (Lasko, 2000; Li et al., 2008; Mikhaylova et al., 2006). It is likely that dA2BP1 is playing dual role during nervous system development in *Drosophila*.

6.1.8 **A2BP1 vis-à-vis Poly Q expansion:**

Expansion of poly glutamine (Poly Q) tracks and neurological diseases have causal relationship. After poly Q expansion, proteins such as Ataxin-2 acquires new potential to interact with other proteins and small molecules to form aggregates and, thereby, hamper many crucial metabolic activities of the cell and finally cause diseases (Broude and Cantor, 2003; Dueñas et al., 2006). However, we know very little about the general poly Q role of the protein in normal cells. dA2BP1 has two poly Q domains and it regulates Notch pathway, which is full of poly Q domains. Su(H) is also a transcription factor with Poly Q domain repressed. Its regulators too are proteins with Poly Q domains, Notch and Mam. Notch intracellular domain undergoes polymerization and causes aggregate formation under high Calcium conditions (Kelly et al., 2007). Now we have included a fourth transcription factor to this list, dA2BP1. Because A2BP1 is known to interact with poly Q expanded Ataxin2, results of this study may help understand critical roles of poly Q-domains in general and specifically in the context of SCA2.

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