Ataxin 2-binding protein 1 is a context-specific positive regulator of Notch signaling during neurogenesis in *Drosophila melanogaster*

Jay Prakash Shukla[1],*, Girish Deshpande[1,2] and L. S. Shashidhara[1],*

ABSTRACT

The role of the Notch pathway during the lateral inhibition that underlies binary cell fate choice is extensively studied, but the context specificity that generates diverse outcomes is less well understood. In the peripheral nervous system of *Drosophila melanogaster*, differential Notch signaling between cells of the proneural cluster orchestrates sensory organ specification. Here we report functional analysis of *Drosophila* Ataxin 2-binding protein 1 (A2BP1) during this process. Its human ortholog is linked to type 2 spinocerebellar ataxia and other complex neuronal disorders. Downregulation of *Drosophila* A2BP1 in the proneural cluster increases adult sensory bristle number, whereas its overexpression results in loss of bristles. We show that A2BP1 regulates sensory organ specification by potentiating Notch signaling. Supporting its direct involvement, biochemical analysis shows that A2BP1 is part of the Suppressor of Hairless [Su(H)] complex in the presence and absence of Notch. However, in the absence of Notch signaling, the A2BP1 interacting fraction of Su(H) does not associate with the repressor proteins Groucho and CtBP. We propose a model explaining the requirement of A2BP1 as a positive regulator of context-specific Notch activity.

KEY WORDS: Neurogenesis, Notch, S2 cells, Sensory organ precursor, Su(H)

INTRODUCTION

Cell fate specification during the development of multicellular organisms is dependent upon regulatory influences that are both cell-autonomous and non-cell-autonomous. The cell-autonomous pathway(s) relies on intrinsic factors, whereas the non-cell-autonomous mode of determination utilizes either short- or long-range signaling that in turn is mediated either by secreted ligands or cell-cell communication between neighbors (Perrimon et al., 2012; Schweisguth, 2015). In addition to different combinations of signaling pathways, the outcome of each pathway is thought to be fine-tuned by context-specific effectors to enable the generation of cellular diversity within a multicellular organism (Perrimon et al., 2012).

Lateral inhibition constitutes an important mode of regulation that is contingent upon communication between neighboring cells (Heitzler and Simpson, 1991; Simpson, 1990). In a canonical scenario involving lateral inhibition, within an equipotent field of cells, a stochastic determinative event first confers a distinct identity on a randomly selected cell. The determined cell subsequently initiates an inhibitory signal that prevents its neighbors from acquiring the same identity (Simpson, 1990). The respective identities are ultimately fixed using autocrine as well as paracrine feed-forward regulation precluding the cells from acquiring identical fates (Artavanis-Tsakonas et al., 1995). Lateral inhibition is thus a key mechanism that is deployed to specify diverse cell types during embryonic differentiation and adult tissue homeostasis (Artavanis-Tsakonas et al., 1999; Cabrera, 1990; Heitzler and Simpson, 1991).

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, the specification of thoracic bristles, which are part of the peripheral nervous system (PNS), utilizes lateral inhibition as a strategy (Heitzler and Simpson, 1991; Jan and Jan, 1994). These bristles arise from sensory organ precursors (SOPs) and subsequently divide and form a complete sensory organ comprising shaft, socket, sheath, neuron and glia (Hartenstein and Posakony, 1989; Reddy and Rodrigues, 1999). SOPs are specified from a group of equipotent cells belonging to a proneural cluster (PNC) (Skeath and Carroll, 1991). Initially, cells within the PNC express equivalent levels of proneural proteins such as Achaete (Ac) and Scute (Sc) (Gómez-Skarmeta et al., 2003). SOP fate is conferred by a relatively modest and stochastic increase in Ac and Sc levels in a random subset of cells (Cubas et al., 1991; Skeath and Carroll, 1991). As a result of this increase, SOP precursors express higher levels of Delta ligand, which in turn activates Notch signaling in the neighboring cells. 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 (Heitzler and Simpson, 1991).

Canonical Notch signaling, for example during neurogenesis or wing patterning in *Drosophila*, requires activation of Notch (N) receptor by one of the ligands of the Delta/Serrate/Lag-2 (DSL) family at the cell surface, typically presented by the neighboring cells (Bray, 2006). This receptor-ligand interaction results in the release of N intracellular domain (N^{ICD}), which is translocated into the nucleus where it interacts with a transcription factor(s) of the CBF1/Suppressor of Hairless/LAG-1 (CSL) family and Mastermind (Mam) to regulate the transcription of downstream target genes (Bray, 2006; de Celis et al., 1996; Kim et al., 1996; Lecourtois and Schweisguth, 1995; Neumann and Cohen, 1996). N negatively regulates sensory organ specification by regulating the *Enhancer of split* gene complex [*E(spl)-C*] (Culí and Modolell, 1998; Heitzler et al., 1996; Lecourtois and Schweisguth, 1995) and positively regulates patterning of the wing epithelium along the dorsoventral (D/V) axis by regulating *wingless* (*wg*), *cut* (*ct*) and *vestigial* (*vg*) (de

[1]Indian Institute of Science Education and Research Pune, Dr Homi Bhabha Road, Pune, Maharashtra 411008, India. [2]Department of Molecular Biology, Princeton University, Princeton, NJ 08540, USA.

*Authors for correspondence (shuklajp@students.iiserpune.ac.in; ls.shashidhara@iiserpune.ac.in)

G.D., 0000-0002-5200-7090; L.S.S., 0000-0001-9306-1432

Celis et al., 1996; Kim et al., 1996; Neumann and Cohen, 1996). Notably, N regulates target genes either in an instructive or in a permissive manner (Furriols and Bray, 2001). For example, *wg* activation needs the permissive function, whereas *ct* and *E(spl)m8* [*E(spl)m8-HLH* – FlyBase] both require an instructive role (Janody and Treisman, 2011). In a permissive mode, the presence of N receptor is sufficient to neutralize the repressors, thereby facilitating activation of downstream targets. By contrast, N requires different sets of additional co-factors to regulate target gene expression when operating in an instructive capacity (Furriols and Bray, 2001; 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. Here we report that the *Drosophila* ortholog of Ataxin 2-binding protein 1 (A2BP1; also known as Rbfox1) is a potential regulator of the Notch signaling pathway in the context of sensory organ specification.

A2BP1 was first reported in human as a nuclear RNA-binding protein that interacts with ataxin 2 in a yeast two-hybrid system and was subsequently linked to type 2 spinocerebellar ataxia (SCA2) (Shibata et al., 2000). It acts as a splicing regulator for a number of genes involved in nerve conduction (Lee et al., 2009; O'Brien et al., 2012; Underwood et al., 2005). Several mutations mapped to the *A2BP1* locus have been linked to complex neuronal disorders (Bhalla et al., 2004; Martin et al., 2007; Sebat et al., 2007). However, the precise role of A2BP1 during development and homeostasis of the nervous system is not known. We have previously shown that *Drosophila* A2BP1 contributes to the Hedgehog (Hh) signaling pathway. Specifically, it interacts with the transcription factor Cubitus interruptus (Ci) to regulate Hh target genes during vein-intervein specification (Usha and Shashidhara, 2010). Although the involvement of A2BP1 during nervous system development has not been analyzed, it has been reported that downregulation of *A2BP1* in the early fly embryo leads to a reduction in neuronal cell number (Koizumi et al., 2007).

During our functional analysis of A2BP1, we noticed that compromising its activity in a nervous system-specific manner gave rise to the tufted phenotype, i.e. supernumerary bristles in adult flies. Here we show that A2BP1 functions as a negative regulator of sensory organ specification by modulating Notch signaling. Our genetic and biochemical data indicate that A2BP1 achieves this via physical association with Su(H), a crucial regulator of the Notch pathway. Based on these data we propose a model incorporating context-specific regulation, as engineered by A2BP1, that calibrates the outcome of the Notch pathway during SOP specification.

RESULTS
A2BP1 is expressed in SOPs
Immunohistochemical analysis performed on different *Drosophila* tissues has revealed that A2BP1 is a nuclear protein that is expressed in the developing embryo (Koizumi et al., 2007) and imaginal discs (Usha and Shashidhara, 2010; Tastan et al., 2010). Although A2BP1 protein is broadly distributed across different tissues and developmental stages, it does not appear to be ubiquitous. For instance, in wing imaginal discs, A2BP1 is detected throughout the entire wing pouch and myoblasts of the notum region, but even within the wing pouch the protein is absent from the D/V boundary (Bajpai et al., 2004; Usha and Shashidhara, 2010).

To understand in more detail the role of A2BP1 in the development of the nervous system, we examined its expression patterns in the developing CNS and PNS. In late stage embryos (stage 14), A2BP1 is expressed in the chordotonal organs of the PNS, as indicated by colocalization with the neuronal marker Futsch

(22c10) (Fig. S1A). Furthermore, A2BP1 colocalizes with another neuronal marker, Neurotactin (Nrt) (Fig. S1B), in the ventral nerve cord (VNC). As reported previously (Usha and Shashidhara, 2010), A2BP1 is expressed throughout the entire notum of the wing imaginal disc, while myoblasts show the strongest expression. SOPs, the progenitors of adult thoracic sensory bristles, are specified in the notum region of the wing disc. *E(spl)m8* expression marks PNCs and Senseless (Sens) expression marks the SOP fate. We detected A2BP1 expression in PNCs, where it overlaps with E(spl)m8 (Fig. 1B,C). Moreover, A2BP1 is also present in partially specified SOPs that are marked with both E(spl)m8 and low-level Sens expression (Fig. 1E). Together, these observations suggest that A2BP1 is expressed in the neuronal precursor cells of both the CNS and PNS. In this report, however, we have restricted our functional analysis of A2BP1 to the context of adult thoracic sensory organ specification in the PNS.

A2BP1 regulates external sensory organ development
External sensory organs are composed of sensory bristles and campaniform sensilla. They develop from neuroepithelium, where a single SOP is selected from clustered progenitors (Heitzler and Simpson, 1991; Jan and Jan, 1994). SOP specification thus provides an ideal developmental context in which to elucidate mechanisms underlying cell fate determination, including lateral inhibition (Heitzler and Simpson, 1991). To examine whether the expression of A2BP1 is relevant for neuronal specification, we investigated whether a neuronal phenotype is associated with A2BP1. We manipulated A2BP1 levels/activity using the UAS-GAL4 system and employed several GAL4 drivers with spatially and temporally differing expression patterns. For downregulation of *A2BP1*, we used a transgenic RNAi method (Usha and Shashidhara, 2010), which knocks down A2BP1 to near complete levels (Fig. S2C). Downregulation of *A2BP1* using RNAi with *sca*-GAL4, which is expressed in PNCs, showed an increase in thoracic bristle number (Fig. 1G,L). These extra bristles were always specified in close proximity to the extant bristles and not at a distant, ectopic location. Identical phenotypes were consistently observed with a number of early third instar GAL4 drivers expressing in the notum (Fig. S3B-E,J). Next, we examined the effect of overexpression of A2BP1. While overexpression of A2BP1 showed embryonic or early larval lethality with most of the nota expressing GAL4 drivers, a few escapers with *sca*-GAL4 showed loss of the majority of macrochaetae and microchaetae and had a bald appearance (Fig. 1H,L). Taken together, these observations suggest that A2BP1 is a negative regulator of sensory bristle development.

Next, we investigated whether only sensory bristles are sensitive to manipulation in A2BP1 levels/activity or whether it has a more general function in the PNS. We examined the effect of manipulation of A2BP1 expression on another class of external sensory organs, the campaniform sensilla. Bristle and campaniform sensilla share a developmental path up to a point of SOP selection and division. They also have similar cell composition in mature sensory organs, except that the external hair-like structures in the bristles are replaced by cup-shaped papilla (Cole and Palka, 1982; Palka et al., 1986). Downregulation of *A2BP1* using *sca*-GAL4 resulted in an increase in campaniform sensilla proximal to the L3 vein and proximal and distal twin sensilla of the adult wing margin (p-TSM, d-TSM) (Fig. S4D,H). To our surprise, we also observed campaniform sensillum to bristle transformation at both L3 vein sensillum and p-TSM, d-TSM positions (Fig. S4E,G), where the dome-shaped papilla were morphologically converted to trichoid-like structures, i.e. the bristles. This suggests that A2BP1 might
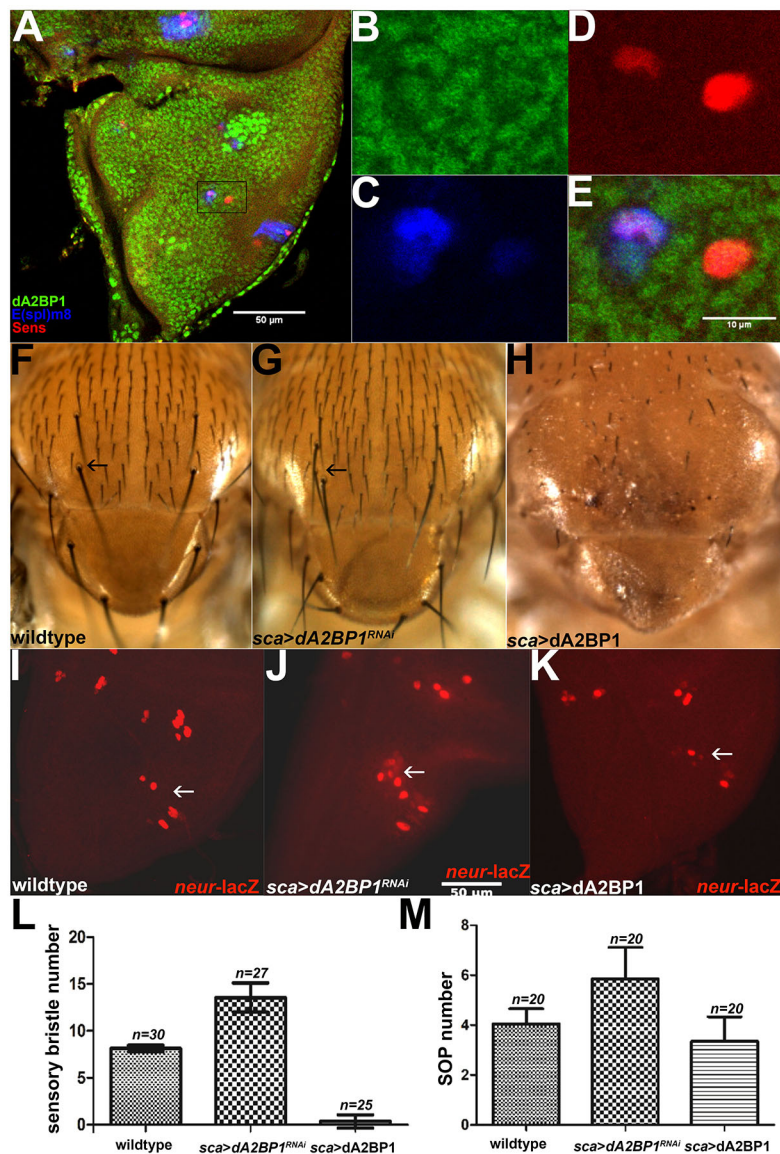
Fig. 1. A2BP1 is expressed in sensory precursor cells of the wing imaginal discs and negatively regulates bristle development. (A-E) Wild-type third instar larval imaginal wing disc stained for A2BP1 (green), *E(spl)m8-lacZ* (blue) and Sens (red). Boxed region in A is shown at higher magnification in single channel (B-D) and merge (E). A2BP1 expression overlaps with that of *E(spl)m8* and Sens. (F-H) Adult thorax of wild-type (F), UAS-*A2BP1^RNAi*; *sca*-GAL4 (G) and *sca*-GAL4/UAS-A2BP1 (H) flies. Downregulation of *A2BP1* leads to an increase in bristle number (G; representative bristles are marked with black arrows), whereas *A2BP1* overexpression results in complete loss of macrochaetae and substantial loss of microchaetae. (I-K) Third instar larval wing imaginal discs of wild-type (I), UAS-*A2BP1^RNAi*; *sca*-GAL4 (J) and *sca*-GAL4/UAS-A2BP1 (K) flies stained for *neur-lacZ*, which marks mature SOPs. Downregulation of *A2BP1* leads to an increase in SOP number, whereas *A2BP1* overexpression results in a reduction in SOP number (representative SOPs are marked with white arrows). (L,M) Quantitative analysis of changes in bristle number (DC+SC; L) and SOP number (M) in the indicated genetic backgrounds. All genotype combinations were significantly different from the wild type at *P*<0.05 (for details see Table S1); error bars indicate s.d.

have a general role during organ specification in the PNS. However, owing to the ease of analysis, here we have largely focused on the development of the sensory bristles of the adult thorax.

The increase in sensory bristle number upon reduction in *A2BP1* can be explained by one of two distinct mechanisms: (1) an increase in SOP number at the initial stages of specification in PNCs; or (2) a fate switch between daughter cells during asymmetric division (Schweisguth, 2015). To distinguish these possibilities, we downregulated *A2BP1* with different GAL4 drivers expressed at early and late stages of bristle development. *c253*-GAL4, which is expressed much more weakly at late stages of SOP specification (compared with *sca*-GAL4) showed a very mild phenotype (Fig. S3F). Downregulation of *A2BP1* during SOP division using *prospero*-GAL4 and *numb*-GAL4 (which are expressed only after SOP specification) did not result in any bristle phenotype (Fig. S3G, H). This suggests that A2BP1 is required during early events leading to SOP specification and not during later stages. This is supported by the fact that A2BP1 levels are much lower at early pupal stages (e.g. 4 h after puparium formation) as compared with third instar larval discs (Fig. S5).

To further confirm that the sensory bristle phenotype is due to a change in SOP number, we examined SOP number during A2BP1 manipulation. *neuralized* (*neur*) expression marks SOP cells in late third instar wing imaginal discs (Huang et al., 1991). Consistent with the adult phenotype, i.e. increased dorsocentral (DC), scutellar (SC) and anterior postalar (aPA) bristles, there was an increase in SOP number at equivalent locations of the wing disc (Fig. 1J,M). Conversely, A2BP1 overexpression resulted in decreased numbers of SOPs (Fig. 1K,M). Together, these results suggest that A2BP1 specifically regulates SOP specification to regulate bristle development.

A2BP1 is upstream of Senseless during bristle development

To understand the precise role of A2BP1, we performed epistasis experiments with several known regulators of the SOP specification pathway. SOP specification is a tightly regulated process as it involves coordination among various signaling pathways that are interconnected via complex molecular mechanisms. Sens occupies the most downstream effector position, as the activity of different signaling pathways converges on Sens (Acar et al., 2006; Frankfort and Mardon, 2004). Sens marks the SOPs and its expression is necessary and sufficient to induce SOP and sensory bristle

formation even in the absence of other proneural genes (Nolo et al., 2000).

We examined genetic interactions between *A2BP1* and *sens* using the null alleles *sens⁵⁸* and *sensᴱ²*. In heterozygous backgrounds, *sens* mutations do not display any sensory bristle phenotype. However, these mutations suppressed the supernumerary bristle phenotype observed with downregulation of *A2BP1* (Fig. S6C,D). Conversely, Sens overexpression, which results in an increase in bristle number (Nolo et al., 2000), suppressed the phenotype (loss of sensory bristles) caused by the overexpression of A2BP1 (Fig. S6F). Together, these results suggest that A2BP1 is upstream of Sens. To confirm this we examined Sens expression after downregulating A2BP1 levels. Downregulation of *A2BP1* led to an increase in the total number of Sens-positive cells in PNCs (marked by *neur* expression) (Fig. 2B). Taken together, these results confirm that A2BP1 regulates SOP specification and acts upstream of Sens.

### A2BP1 determines SOP number by regulating Ac expression
Notch signaling negatively regulates bristle development by repressing proneural genes of the *achaete-scute* (*ac-sc*) complex. For acquisition of SOP fate, PNC cells must express higher levels of *ac-sc* complex genes (Skeath and Carroll, 1991). Expression of Ac

and Sc proteins leads to Sens expression, which in turn activates Ac expression by a feed-forward loop, ultimately resulting in the SOP fate (Acar et al., 2006). The Notch pathway restricts SOP number by inhibiting the expression of Ac and Sc in neighboring cells (Heitzler et al., 1996; Heitzler and Simpson, 1991).

We examined whether A2BP1 regulates SOP number via alteration in Ac expression levels. Downregulation of *A2BP1* led to an increase in Ac levels in PNCs without affecting PNC size (Fig. 2D), suggesting that more than the normal number of PNC cells are crossing the threshold of Ac expression. This also corresponds to an increase in the number of Sens-expressing cells (Fig. 2B). Conversely, overexpression of A2BP1 resulted in a drastic reduction in Ac expression (Fig. 2E). These results suggest that, like N, A2BP1 also restricts Ac expression to limit SOP specification and, ultimately, the total number of bristles.

A2BP1 regulates *E(spl)* transcription to regulate Ac expression
As a proneural gene, *ac* is very tightly regulated both at the level of transcription and at the protein level. Notch signaling-based, E(spl)-C-mediated transcriptional regulation is crucial for Ac and Sc expression (Culí and Modolell, 1998; Heitzler et al., 1996; Lecourtois and Schweisguth, 1995). E(spl) acts as an effector of
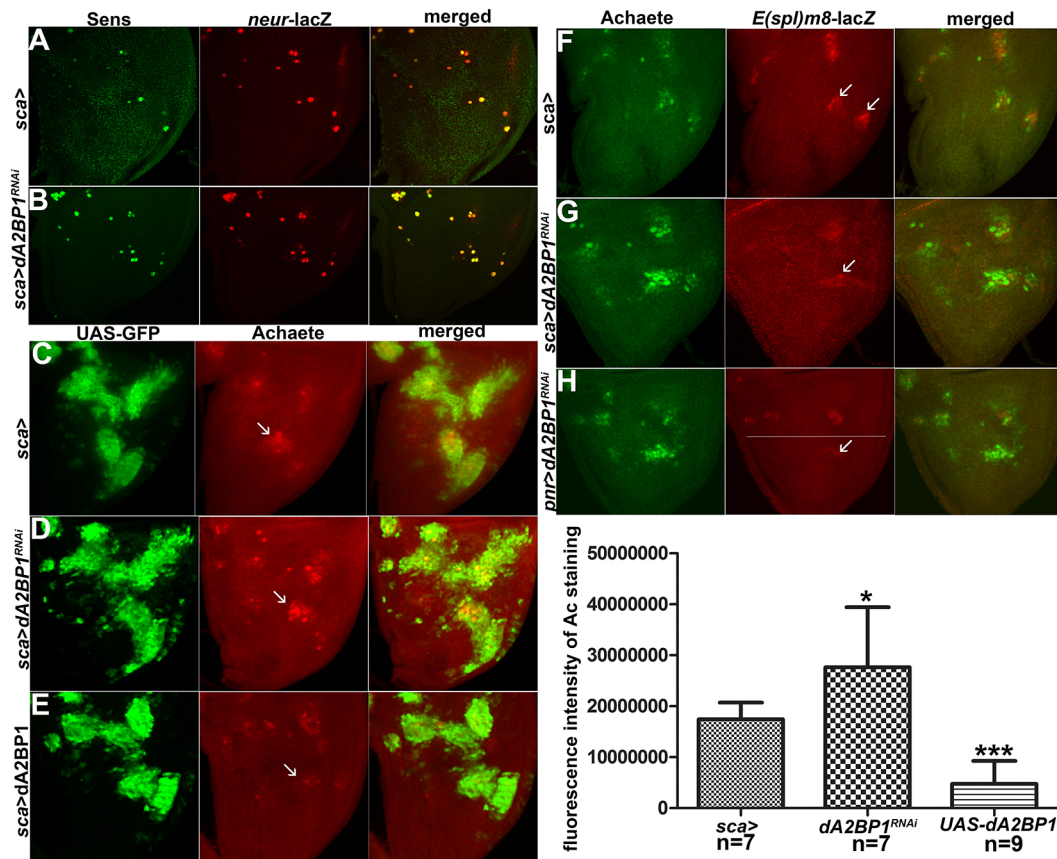
Fig. 2. A2BP1 regulates the expression of both the effector (Ac) and mediator [E(spl)m8] of the Notch pathway. (A,B) Third instar larval wing imaginal discs of *sca*-GAL4; *neur-lacZ* (A) and UAS-*A2BP1ᴿᴺᴬⁱ*; *sca*-GAL4; *neur-lacZ* (B) stained for β-galactosidase (red) and Sens (green). Increased number of cells expressing both *neur* and Sens suggest an increase in SOPs due to downregulation of *A2BP1*. (C-E) Third instar larval wing imaginal discs of *sca*-GAL4; UAS-GFP (C), UAS-*A2BP1ᴿᴺᴬⁱ*; *sca*-GAL4; UAS-GFP (D) and *sca*-GAL4/ UAS-A2BP1; UAS-GFP (E) stained for GFP [marks proneural cluster (PNC)] and Ac. Expression levels and the number of Ac-expressing cells are increased (quantitative analysis of Ac staining is shown to the right) in the background of *A2BP1* downregulation and are reduced when A2BP1 is overexpressed (representative PNCs are marked with arrows). See Table S1 for *P*-values; error bars indicate s.d. (F-H) Third instar larval wing imaginal discs of wild type (F), UAS-*A2BP1ᴿᴺᴬⁱ*; *sca*-GAL4/*E(spl)m8-lacZ* (G) and UAS-*A2BP1ᴿᴺᴬⁱ*; *E(spl)m8-lacZ/+*; *pnr*-GAL4/+ (H) stained for Ac (green) and *E(spl)m8-lacZ* (red). Increase in Ac staining in the background of downregulation of *A2BP1* is associated with a decrease in *E(spl)m8* expression. *pnr*-GAL4 is expressed in the dorsalmost notum (below the line in H). Representative PNCs are marked with white arrows. Note the decreased levels of *E(spl)m8* in the *pnr*-GAL4-expressing domain compared with more ventral regions.

Notch signaling. Most of the E(spl)-C group proteins directly regulate *ac* expression by binding to its promoter (Jennings et al., 1999). As *ac* is a direct target of E(spl)-C, we next asked whether A2BP1 regulates Ac and Sc via E(spl).

Owing to its role as a negative regulator of SOP specification, overexpression of E(spl)m8 suppressed sensory bristle formation (Fig. S6J). We downregulated *A2BP1* in the background of overexpression of E(spl)m8. Although downregulation of *A2BP1* results in increased bristle number, it did not rescue the loss of sensory bristle phenotype caused by the overexpression of E(spl)m8 (Fig. S6K). This suggests that A2BP1 is upstream of *E(spl)-C*.

We then examined whether A2BP1 regulates the expression of *E(spl)m8*, a member of the *E(spl)-C*. *E(spl)m8-lacZ* expression was reduced in PNCs (Fig. 2G) when *A2BP1* was downregulated using *sca*-GAL4, which is consistent with the derepression of Ac expression in those clusters and a subsequent increase in the number of Sens-expressing cells. We confirmed this result by engineering spatially restricted expression of A2BP1 within the disc using the *pnr*-GAL4 driver. This driver is expressed only in DC and SC PNCs, whereas PNCs at other positions remain unaffected and thereby serve as an excellent control within the same disc. We observed reduction in *E(spl)m8-lacZ* expression only in DC and SC PNCs (Fig. 2H).

A2BP1 interacts with Notch

Notch signaling is one of the best-studied pathways involved in cell fate specification and cell differentiation (Artavanis-Tsakonas et al., 1999). Both SOP selection and subsequent division to form a complete sensory organ comprising different cell types require Notch signaling. Reduction in Notch signaling weakens lateral inhibition, which results in an increase in sensory bristle number (Heitzler and Simpson, 1991). Based on the genetic interactions and regulation of components of the N pathway, we investigated whether A2BP1 interacts with *N* itself to regulate bristle development. First, we tested genetic interactions between the two genes. N^{55e11} is a null allele that shows a mild increase in bristle number in the heterozygous background (Fig. 3B) (Brennan et al., 1997). Downregulation of *A2BP1* in this background further enhanced the phenotype (Fig. 3D,G). Interestingly, apart from bristle enhancement at DC, SC and aPA positions, the increase in bristle number also extended to other positions (Fig. 3D'). This suggests that loss of function of *A2BP1* enhances the *N* phenotype at most bristle positions. *Abruptex*[16] is a gain-of-function mutation of *N* (N^{Ax16}) and, in the heterozygous condition, it shows the loss of bristle number phenotype (Fig. 3E) (Go and Artavanis-Tsakonas, 1998; Heitzler and Simpson, 1993). We observed suppression of the *N* gain-of-function phenotype displayed by N^{Ax16} when *A2BP1* was downregulated in this genetic background (Fig. 3F,G).

N functions as a transcriptional activator of *E(spl)-C* in combination with Su(H) protein (Bray, 2006). Our results suggest that A2BP1 acts upstream of *E(spl)-C*. Suppression of the N^{Ax16} phenotype by downregulation of *A2BP1* (Fig. 3F) suggests that A2BP1 is downstream of N. Activation of N depends on its binding to an extracellular ligand and subsequent processing of the receptor by enzymatic cleavage to release N^{ICD} (Bray, 2006). Overexpression of the dominant-negative form of N (N^{DN}, which lacks the intracellular domain) with *c253*-GAL4 weakens Notch signaling (by sequestering the ligand) and results in an increase in sensory bristle number per PNC (Fig. 3I) (Rebay et al., 1993). Downregulation of *A2BP1* in this background showed a dramatic enhancement of this phenotype (Fig. 3J). Although this resembled the tufted phenotype (giving an impression of multiple shafts emanating from a single socket), we also observed additional

socketed bristles (Fig. 3J'), suggesting that this phenotype is likely to arise from aberrant SOP specification. Furthermore, larval wing discs of this genetic background showed a significant increase in Sens expression, with Sens-expressing cells in clusters (Fig. S7D), reminiscent of the tufted phenotype in the adult. Consistent with this and the conclusion that the role of A2BP1 is restricted to early stages of SOP specification, A2BP1 and N^{DN} did not show any interactions during SOP division (Fig. S8D).

Overexpression of A2BP1 in the N^{DN} background resulted in complete loss of macrochaetae, similar to the phenotype caused by overexpression of A2BP1 in a wild-type background (Fig. 3L). Since overexpression of A2BP1 could mitigate the N^{DN} phenotype (and that of N^{Ax16}), this suggests that A2BP1 functions downstream of N. As A2BP1 is upstream of *E(spl)-C*, which is a direct target of N, this suggests the possibility that A2BP1 functions at parity with N.

Overexpression of N^{ICD} (which lacks the extracellular and membrane-spanning domains and hence functions as a constitutively active form of N) causes strong gain-of-function phenotypes. As N^{ICD} expression at early stages is lethal, we expressed it in late SOPs to examine its interaction with A2BP1. N^{ICD} overexpression results in loss of bristles (Fig. 3M) (Mumm and Kopan, 2000; Struhl et al., 1993). This phenotype was unaffected by downregulation of *A2BP1* (Fig. 3N), suggesting that A2BP1 might not interact with N during late stages of neurogenesis and, in particular, during SOP division.

We also studied interactions between A2BP1 and Sanpodo (Spdo). Spdo regulates SOP division in a Numb-dependent manner. It binds to N receptor in Numb-positive daughter cells and promotes its internalization to reduce signaling via N. However, in the absence of Numb, Spdo binds to γ-Secretase and facilitates Notch signaling (Schweisguth, 2015; Upadhyay et al., 2013). Overexpression of Spdo in PNCs increases the number of sensory organs, indicating loss of N signaling. Downregulation of *A2BP1* further enhanced this phenotype (Fig. S9), which suggests that A2BP1 is an integral component of the N pathway.

A2BP1 is part of the Su(H) complex in the presence and absence of Notch

As the above experiments indicated that A2BP1 might function as an integral component of the N pathway, we next examined A2BP1 and N interactions at the molecular level, employing biochemical techniques. During Notch signaling, N^{ICD} and Su(H) act as transcription factors for downstream targets. In the absence of signal, Su(H) binds to the enhancer regions of *E(spl)-C* genes along with a repressor complex consisting of Hairless (H), C-terminal binding protein (CtBP) and Groucho (Gro) (Bray, 2006; Nagel et al., 2005). During active Notch signaling, activated N^{ICD} and Mam bind to Su(H) and this binding facilitates removal of the repressor proteins (Bray, 2006).

To examine whether A2BP1 is a constituent of the activator and/or repressor complex, we performed immunoprecipitation with anti-A2BP1 antibodies on extracts of S2 cells, which do not express functional N due to a mutation in the endogenous gene. We detected Su(H) in the immunoprecipitate, suggesting that it interacts with A2BP1 (Fig. 4B). However, we could not detect the co-repressors Gro and CtBP in the immunoprecipitate (Fig. 4E,F,H). We tried reverse immunoprecipitation using anti-Su(H) antibodies, but could not co-precipitate A2BP1, which might be due to lower abundance of A2BP1 in the immunoprecipitate (Fig. 4D). This further suggests that only a small fraction of Su(H), perhaps that free from repressor complex, is associated with A2BP1. Next, we examined whether the A2BP1-Su(H) interaction is sustained in the presence of activated N. As S2 cells do not express N, we made a stable cell line by transfecting pMT-N full length into S2 cells and developed an inducible system for Notch
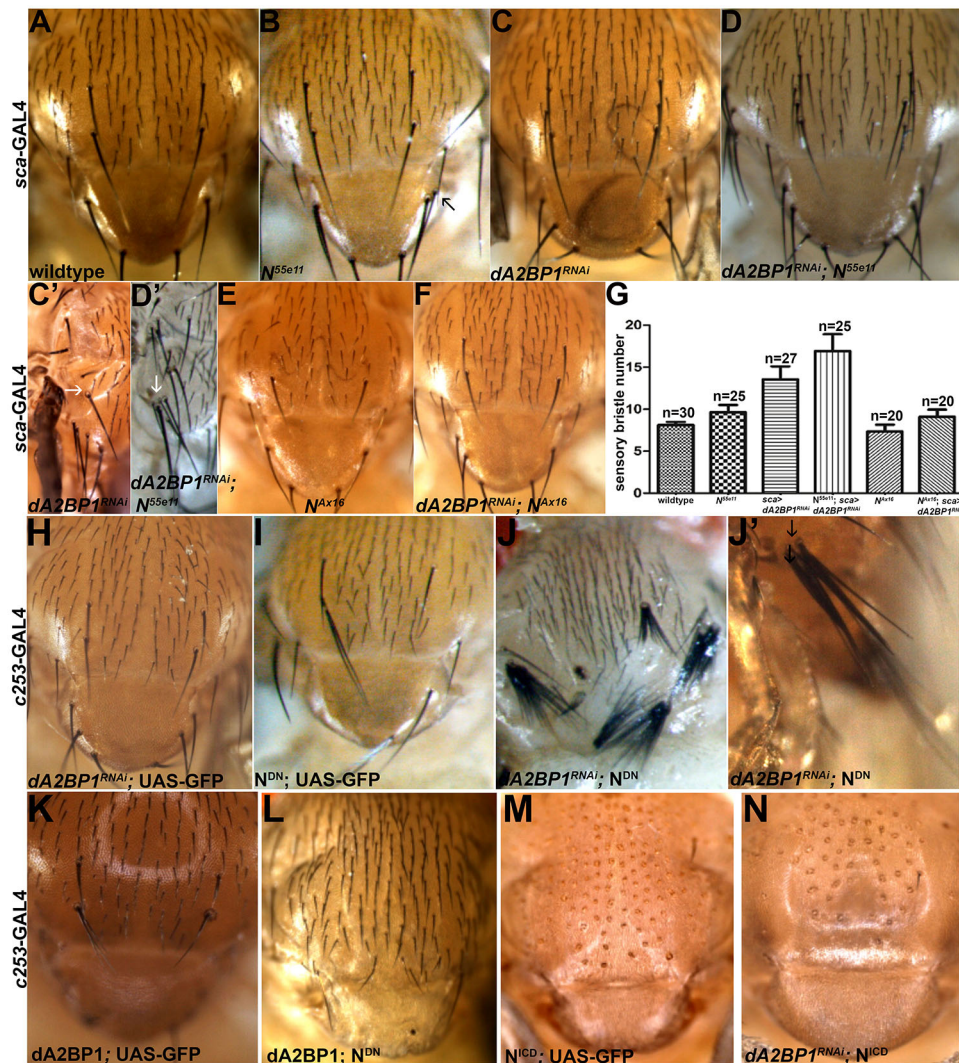
Fig. 3. A2BP1 may function at parity with Notch. (A-D′) Adult thorax of wild-type (A), N^{55e11}/+; *sca*-GAL4/+ (B), UAS-*A2BP1*^{RNAi}/+; *sca*-GAL4 (C) and N^{55e11}/ UAS-*A2BP1*^{RNAi}; *sca*-GAL4/+ (D) flies. Loss of both *A2BP1* and *N* (in the heterozygous N^{55e11} background) results in an enhanced supernumerary bristle phenotype compared with the loss of either of the two genes individually. (C′,D′) The lateral bristle positions of C and D, respectively. Combined loss of *A2BP1* and *N* leads to an increase in sensory bristle number at these positions too (arrows in C′,D′), which is not seen when either gene is downregulated individually. (E,F) Adult thorax of N^{Ax16}/+; *sca*-GAL4 (E) and N^{Ax16}/UAS-*A2BP1*^{RNAi}; *sca*-GAL4 (F) flies. The gain of *N* (in the heterozygous N^{Ax16} background) leads to a decrease in sensory bristle number. The phenotype is rescued when *A2BP1* is downregulated in this background. (G) Quantitative analysis of changes in bristle number (DC+SC) in the indicated genetic backgrounds. All comparisons (enhancement or suppression) are significant at $P<0.0001$ (for details see Table S1); error bars indicate s.d. (H-L) Adult thorax of UAS-*A2BP1*^{RNAi}; *c253*-GAL4; UAS-GFP (H), *c253*-GAL4/UAS-N^{DN}; UAS-GFP (I), UAS-*A2BP1*^{RNAi}; *c253*-GAL4/ UAS-N^{DN} (J), *c253*-GAL4/UAS-A2BP1; UAS-GFP (K) and *c253*-GAL4/UAS-N^{DN} (L) flies. Strong genetic interactions between *N* and *A2BP1* is evident from the extreme tufted phenotypes in flies expressing N^{DN} in the background of loss of function for *A2BP1* (J). The tufted phenotype is caused by an increase in the number of the entire sensory organs and not just a change in the specification of subtypes to bristles, as shown in the higher magnification image in J′. Overexpression of A2BP1 is able to suppress the N^{DN} phenotype (L), suggesting that A2BP1 is downstream of N. (M,N) Adult thorax of *c253*-GAL4/UAS-N^{ICD}; UAS-GFP (M) and UAS-*A2BP1*^{RNAi}; *c253*-GAL4/UAS-N^{ICD} (N). Overexpression of N^{ICD} results in complete loss of both macrochaetae and microchaetae (M). Downregulation of *A2BP1* in the N^{ICD} background has no effect on the N^{ICD}-induced phenotype (N).

signaling (Krejci and Bray, 2007). We detected both Su(H) (Fig. 4J) and N (Fig. 4K) in the immunoprecipitate, suggesting that A2BP1 is part of the Su(H)-N transcription complex both in the absence and presence of N and might function as a transcriptional co-factor to regulate the expression of *E(spl)-C*.

Loss of function of *A2BP1* suppresses the loss-of-function phenotype associated with H

Reduction in *Su(H)* function suppresses loss-of-function phenotypes associated with *H* [hence the name Su(H)] (Ashburner, 1982). Subsequently, it was discovered that H is a component of the repressor complex that negatively regulates targets of Notch

signaling. Loss of *H* function enhances the expression of targets of N and, thereby, results in N gain-of-function phenotypes. These phenotypes could be categorized as either the complete loss of sensory bristles, where SOP development stalls at the specification stage (Fig. 5C), or socket cell duplication, where the shaft cell is transformed into a socket cell during SOP division (Bang et al., 1991; Bang et al., 1995). Thus, similar to N, H functions during both SOP initiation and SOP division.

As our results suggest that A2BP1 is part of the Su(H)-N complex, which is downstream of H, we were prompted to examine whether loss of A2BP1 could also suppress the phenotypes associated with loss of *H*. We used three dominant null alleles:
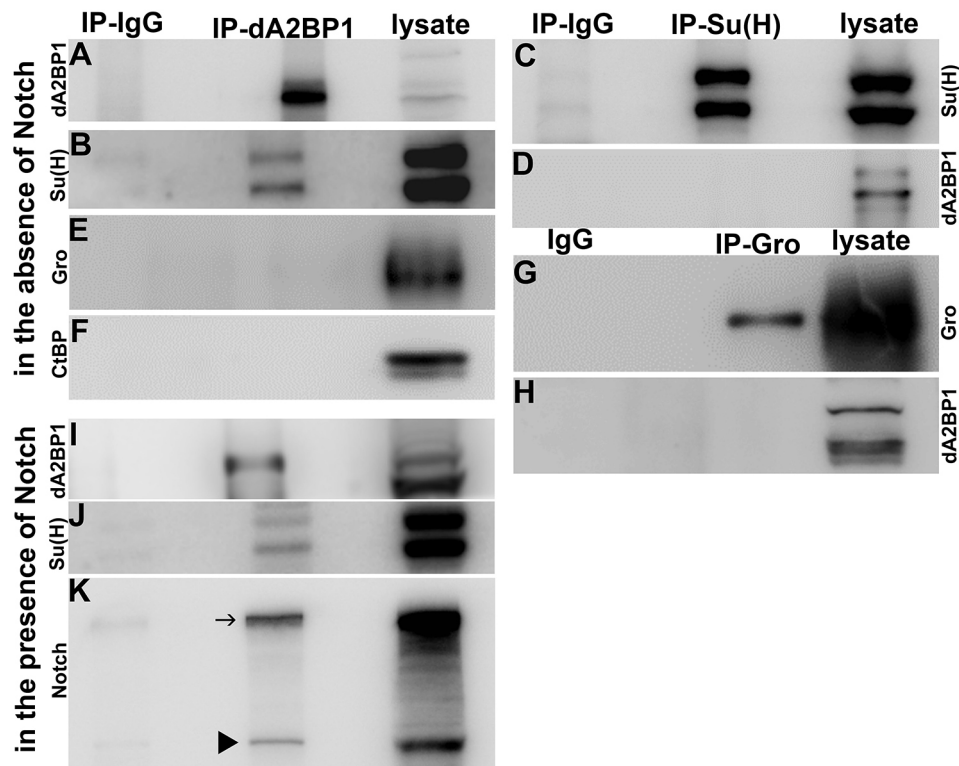
Fig. 4. A2BP1 is part of the Su(H) complex in the presence and absence of Notch. (A-H) Western blot to detect proteins present in immunoprecipitates from S2 cell (in which *N* is not functional) extracts. Su(H) co-precipitates with A2BP1, suggesting that the two are part of the same protein complex. However, only a small fraction of Su(H) is co-immunoprecipitated with A2BP1 (compare with input lane in B). This suggests that not all cellular Su(H) is in a complex with A2BP1, which is confirmed by the observation that A2BP1 is not detected in the immunoprecipitate obtained using anti-Su(H) antibodies (D). Gro and CtBP, which form the repressor complex with Su(H), are not co-precipitated with A2BP1 (E,F). The absence of A2BP1 and Gro interactions is confirmed by the reverse immunoprecipitation (H). (I-K) Western blot to detect proteins present in immunoprecipitates from S2 cell extracts. Both Su(H) and N are co-precipitated (J,K), suggesting that A2BP1 is part of the Su(H)-activator complex. (K) The arrow marks full-length N protein, and the arrowhead marks N[ICD]. IgG, from the corresponding host, was used as negative control in all immunoprecipitation experiments. Lane 'lysate' represents a fraction of the whole lysate, from the same batch used for corresponding immunoprecipitation experiments. The amount of total lysate loaded on the gel varied from 5% (B,C,J), 10% (A,D-G) and 15% (H,I,K) of the total amount used for immunoprecipitation.

H[1], *H[2]* and *H[3]*. All show loss of one or more sensory bristles. *A2BP1* loss of function in an *H* heterozygous background resulted in sockets in the place of bristles (Fig. 5D,E), suggesting the rescue of SOP specification but not of events during SOP division. This confirmed our previous observations that A2BP1 functions only during the specification of SOPs.

Taken together, our results suggest that A2BP1 is a context-specific co-factor of the Su(H)-N complex.

DISCUSSION

N receptor and one or more of its ligands are ubiquitously expressed throughout the development of invertebrates and vertebrates. Notch pathway activity is nonetheless tightly regulated in a spatiotemporal manner and the outcomes of this signaling pathway are diverse and vary in a context-dependent fashion. Although it is likely that post-translational modifications of N, such as glycosylation, ubiquitylation and phosphorylation, play an important role in the spatiotemporal regulation of its activity, it is not well understood how this well conserved and canonical pathway is employed in such versatile ways (Fortini, 2009). This is especially confounding with the specific involvement of Notch signaling during nervous system development, as Notch pathway is deployed recurrently to ultimately confer unique cellular and functional identities. Several models have thus depended on the presence of context-

specific regulators to achieve the multipurpose signaling that leads to the generation of cellular and functional diversity.

E(spl)-C is an important effector of the Notch pathway and it is directly regulated by the Su(H)-N complex. Although N and Su(H) regulate both SOP specification and SOP division, the role of E(Spl)m8 is limited to SOP specification (Nagel et al., 2000). This specificity makes it an ideal candidate to explore the context-dependent co-factors that either potentiate or dampen Notch signaling. Our data illustrate this point convincingly as they demonstrate that A2BP1 functions as a context-specific factor of the N- and Su(H)-based protein assembly to regulate *E(Spl)-C* expression, which in turn regulates SOP specification. Interestingly, this function is restricted to the specification phase alone. Our genetic studies with *H* corroborate this selectivity (Fig. 5). Our data are thus consistent with the conclusion that A2BP1 activity regulates SOP specification. SOP division, by contrast, appears to be unaltered. One potential reason for this functional distinction could relate to the differential expression of A2BP1. Supporting this possibility, A2BP1 levels seem to be reduced during SOP division in early pupal wing discs as compared with L3 wing discs (Fig. S5).

It would be interesting to explore if A2BP1 and N interactions are restricted to epidermal cells of the PNCs or whether their interaction also extends to other epithelial contexts. Preliminary observations suggest that expression of *E(spl)mβ* [*E(spl)mβ-HLH* – FlyBase], a target of N signaling in the wing pouch, is dependent on A2BP1
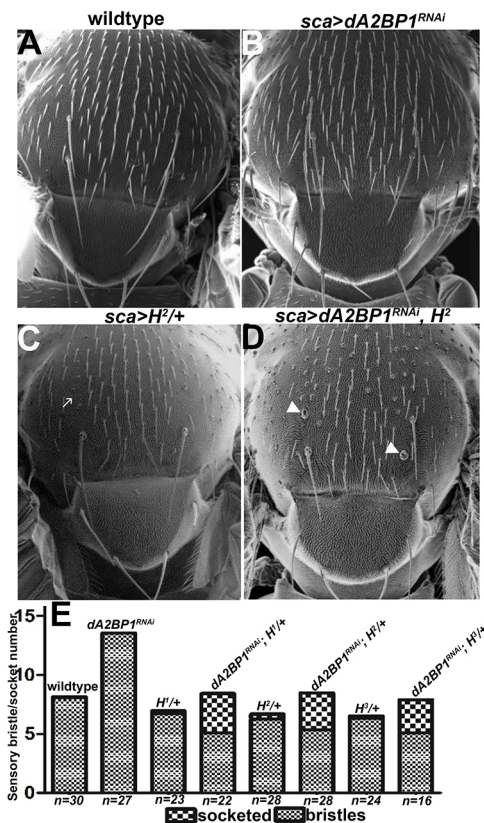
Fig. 5. A2BP1 is also a suppressor of H. (A-D) Adult thorax of *sca*-GAL4 (A), UAS-*A2BP1*^{RNAi}; *sca*-GAL4 (B), *sca*-GAL4; H^2/+ (C) and UAS-*A2BP1*^{RNAi}; *sca*-GAL4; H^2/+ (D) flies. Loss of *A2BP1* results in a supernumerary bristle phenotype (B), whereas *H* heterozygosity causes loss of sensory bristles (C; arrow). Removal of *A2BP1* in an H^2/+ background resulted in the appearance of socketed sensory organs (arrowheads in D) in the place of bristles. This suggests that, although the loss of *A2BP1* suppresses the H^2/+ phenotype, it does so only at the level of specification of the sensory organ. (E) Quantitative analysis of changes in bristle and socket numbers (DC+SC) in the indicated genetic backgrounds. Suppression of the H^2/+ phenotype is significant at *P*<0.0001 (for details see Table S1).

(Fig. S10B). During wing development, Notch signaling determines the D/V organizer by regulating the expression of *wg*, *ct* and *vg*. Previously, we have shown that A2BP1 is not expressed at the D/V boundary (Bajpai et al., 2004) and that downregulation of *A2BP1* has little effect on Wg, Ct and Vg expression (data not shown). However, overexpression of A2BP1 can induce ectopic Wg, consistent with aberrant activation of N signaling (Fig. S11B). We therefore conclude that A2BP1 is not essential for N-dependent *wg*, *ct* and *vg* expression, although it can affect their expression if overexpressed.

Role of A2BP1 in the Su(H)-N complex

Genetic interactions between *A2BP1* and *H* suggest that A2BP1 might function in conjunction with Su(H). Supporting this conclusion, immunoprecipitation experiments showed that these two proteins are indeed part of the same complex. Moreover, as A2BP1 and Su(H) are able to form a complex even in the absence of N, their interaction seems to be independent of Notch signaling. Reports suggest that N^{ICD} and Su(H) physically interact *in vitro* (Kelly et al., 2007). As A2BP1 interacts with Su(H) both in the absence and presence of N and both are positive regulators of the Notch pathway, we propose that A2BP1 is likely to prime Su(H)-N

interactions by setting aside a fraction of Su(H) from the repressor complex (Figs 5 and 6). As lateral inhibition is thought to depend on a stochastic distinction between 'equivalent' cells, a molecular priming event of this nature could serve as a key component of SOP determination. It is tempting to speculate that the presence of Ac and Sc renders all cells competent to acquire the SOP fate, whereas the complex between A2BP1 and Su(H) allows the cells to be in a 'poised' state to take over the epithelial fate. This priming complex would also ensure that as soon as lateral inhibition is activated epithelial fate is enforced. It is also possible that, although lower in absolute amounts, A2BP1 has a higher affinity for Su(H) than for components of the repressor complex and helps to present Su(H) to N^{ICD} (Fig. 6). Future analysis will involve detailed molecular interaction studies among these three proteins to elucidate the mode of action of the protein complexes.

Our data have established a unique function for A2BP1 in regulating cell fate specification, although it is likely to participate in transcriptional regulation [we have monitored transcriptional regulation using *lacZ* reporter genes of E(spl); Fig. 2G,H; Fig. S10B]. This is unanticipated because A2BP1 is a known splicing factor. It is, however, possible that the two molecular roles are coupled, a point that requires further experimental exploration.

Interestingly, A2BP1 has emerged as a significant player in the regulation of cell fate specification and cell division in both invertebrates and vertebrates. For instance, in *C. elegans* the A2BP1 ortholog acts as a dose-sensitive sex determinant (Hodgkin et al., 1994). In vertebrates, loss of *A2BP1* leads to tumorigenesis, including glioma and neuroblastoma (Hu et al., 2013). In *Drosophila*, loss of *A2BP1* during oogenesis leads to the tumorigenic phenotype of the egg chamber (Tastan et al., 2010). These diverse phenotypes that result from loss of A2BP1 in different organisms need to be viewed in the light of our observations that place A2BP1 as a context-specific regulator of the Notch pathway. It will be interesting to identify and characterize additional context-specific regulators, as it is likely that such regulators are crucial in deciding the end result of a signaling event. Our earlier studies had shown that A2BP1 modulates Hh signaling. Taken together, our observations raise the possibility that context-specific regulators not only fine-tune an individual signaling pathway but are also likely to play a crucial role in coordinating outcomes of multiple signaling events. Although such players have been postulated, so far their identity has remained elusive. More importantly, understanding their precise involvement in developmental and morphogenetic contexts is likely to open up new and unexpected avenues of investigation.

A2BP1 in the context of SCA2

Expansions of polyglutamine (polyQ) tracks and neurological diseases such as SCA2 have a causal relationship. After polyQ expansion, proteins such as ataxin 2 acquire new potential to interact with other proteins and small molecules to form aggregates and, thereby, hamper many crucial metabolic activities of the cell, ultimately causing disease (Broude and Cantor, 2003; Dueñas et al., 2006). *Drosophila* A2BP1 has two polyQ domains and it regulates the Notch pathway, which itself has a polyQ domain. Su(H) is also a transcription factor with polyQ domains. N^{ICD} undergoes polymerization and causes aggregate formation under high calcium conditions (Kelly et al., 2007). Because A2BP1 is known to interact with polyQ-expanded ataxin 2 and N is also implicated in SCA (Tong et al., 2011), the results of this study might help understand the crucial roles of polyQ domains in general and specifically in the context of SCA2.

DEVELOPMENT
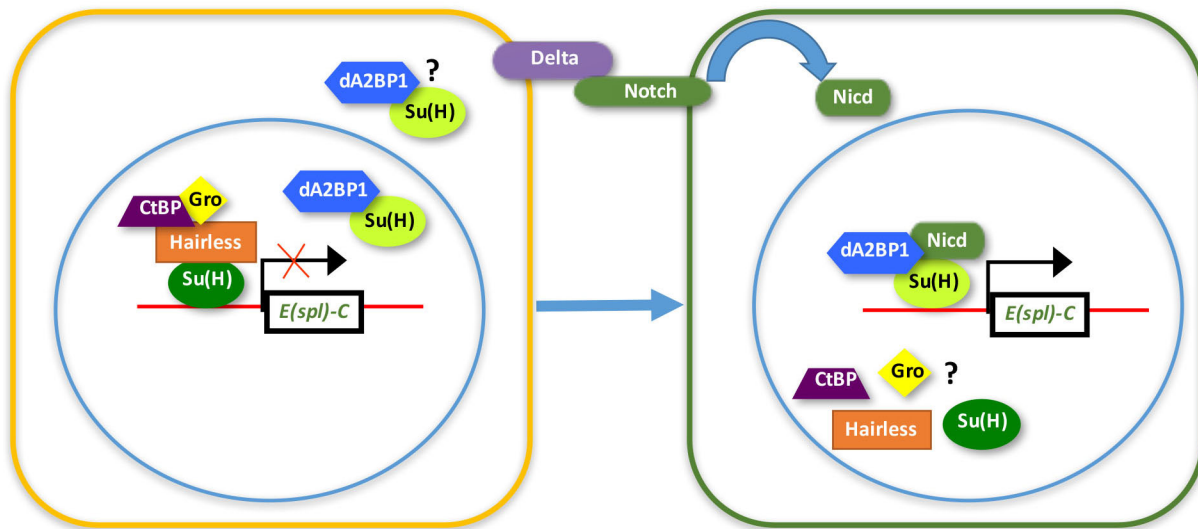
Fig. 6. Model for A2BP1 function in the Notch pathway. In the absence of Notch signaling, Su(H), Gro, CtBP and H form a repressor complex and transcriptionally downregulate the targets of N, such as the *E(spl)-C* genes. In the presence of activated N (N^{ICD}), this repressor complex is dissociated and the Su(H)-N complex activates target genes. Our results suggest that A2BP1 acts as a positive regulator of Notch signaling. It is part of the Su(H)-N complex and activates *E(spl)-C*. Our biochemical analysis indicates that, irrespective of Notch signaling, A2BP1 is complexed with Su(H). Interestingly, however, A2BP1 is not part of the repressor complex as it is not detected with either CtBP or Gro. Thus, the fraction of Su(H) complexed with A2BP1 is likely to be different from that which participates in the repressor complex. This indicates that A2BP1 might be involved in setting aside a small fraction of Su(H) to potentiate lateral inhibition. In the presence of N^{ICD}, increased levels of Su(H) would be available upon dissociation from the repressor complex to strengthen the process further.

MATERIALS AND METHODS

Fly stocks
Unless stated otherwise, experiments were carried out at 25°C and *w^{1118}* and Canton-S strains of *Drosophila melanogaster* were used as control.

The following fly stocks were used. UAS lines: UAS-A2BP1 (Usha and Shashidhara, 2010), UAS-*A2BP1^{RNAi}* (Usha and Shashidhara, 2010), UAS-N^{DN} (Rebay et al., 1993), UAS-N^{ICD} (Rebay et al., 1993), UAS-E(spl)m8 (Bloomington Stock Center, BL 26827), UAS-Senseless (Bloomington Stock Center, BL 39681), UAS-Spdo (Bloomington Stock Center, BL 9934). GAL4 drivers: *dpp*-GAL4$^{440.6}$ (Morimura et al., 1996), *pnr*-GAL4 (Calleja et al., 1996), two different versions of *sca*-GAL4 namely *sca*-GAL4 (BL 6479) and *c253*-GAL4 (BL 6980), *ap*-GAL4 (Calleja et al., 1996), *iroqous*-GAL4, *EM461*-GAL4 (*notal*-GAL4, kind gift from Prof. Juan Modollel, Centro de Biología Molecular Severo Ochoa, Madrid, Spain), *pros*-GAL4 (Prof. C. Doe, University of Oregon, USA), *numb*-GAL4 (kind gift from Prof. Stephen Cohen, University of Copenhagen, Denmark). Mutant alleles: *A2BP1^{C00511}* (Buszczak et al., 2007), *N^{55e11}* (BL 28813), *N^{Ax16}* (BL 52014), *sens^{58}* (BL 5312), *sens^{E2}* (BL 5311), *H^1* (BL 515), *H^2* (BL 517) and *H^3* (BL 518). Reporter transgenes: *E(spl)m8-lacZ*, *E(spl)mβ-lacZ* (kind gifts from Prof. Sarah Bray, University of Cambridge, UK), *neur-lacZ* (A101; BL 4369) and UAS-GFP (Chalfie et al., 1994).

Immunohistochemistry
Immunohistochemical analysis was performed on embryo and imaginal discs as described previously (Patel et al., 1989). Primary antibodies used in immunohistochemistry were: rabbit polyclonal anti-A2BP1 (1:150) (Usha and Shashidhara, 2010), mouse anti-Futsch 22c10 (1:10; DSHB), mouse monoclonal anti-Neurotactin (1:20; DSHB, BP106), mouse monoclonal anti-Achaete (1:10; DSHB), mouse anti-Wingless (1:200; DSHB), guinea pig polyclonal anti-Senseless (1: 1000; kind gift from Hugo Bellen, Baylor College of Medicine, Houston, USA), mouse monoclonal anti-β-galactosidase (1:800; DSHB, 41-1ea), chicken polyclonal anti-β-galactosidase (1:1000; Abcam, ab9361), rabbit polyclonal anti-GFP (1:2000; Invitrogen, A-6455) and chicken polyclonal anti-GFP (1:500; Invitrogen, A-1262). The following secondary antibodies (all Invitrogen, 1:1000) were used: goat anti-mouse Alexa Fluor 488, goat anti-mouse Alexa Fluor 568, goat anti-rabbit Alexa Fluor 488, goat anti-rabbit Alexa Fluor 568, goat anti-guinea pig Alexa Fluor 488, goat anti-guinea pig Alexa Fluor 633 and goat anti-chicken Alexa Fluor 633.

Fly processing and mounting
Adult flies of the desired genotype were collected in 50% ethanol and serially dehydrated with 70%, 90% and 100% ethanol for 10 min each step. Finally, the flies were transferred to clove oil for overnight clearing and mounted on a glass slide in DPX mounting solution (MERCK Chemicals, 61803502501730). The slides were dried overnight before microscopy.

Microscopy
Adult fly phenotypes were recorded using either a Leica S8APO with LAS EZ software or a Zeiss EVO LS10 scanning electron microscope with Smart SEM software. Fluorescent images were taken by either a Zeiss imager Z1 with Axiovision 4.8 software or using a Zeiss LSM 710 confocal with ZEN 2010 software. Fluorescence intensity was quantified using Imaris 7 software (Bitplane). Images were processed with Adobe Photoshop (CS6) and ImageJ (NIH). All image processing complied with standard ethical practices.

Statistical tests
We scored SOPs of wing discs and adult DC and SC macrochaetae in various genetic backgrounds and analyzed the results using Student's *t*-test. Details of all statistical tests are provided in Table S1.

S2 cell culture and transfection
S2 cells were cultured at 23°C in Schneider's *Drosophila* medium (Gibco) supplemented with 10% FBS, 50 units/ml penicillin and streptomycin. S2 cells do not express *N* owing to a mutation at the 5′ UTR of the endogenous gene (which we reauthenticated). We made an S2-N stable cell line by transfecting pMT-N (DGRC#1022): 2 μg pMT-N was mixed with 0.1 μg pCoHygro (Invitrogen, K413001) and transfected using TransIT-2020 transfection reagent (Mirus Bio, MIR 5400A). Stable cell lines were selected for hygromycin (Invitrogen, 10687-010) resistance (150 μg/ml) for 1 month and continuously maintained in the presence of hygromycin (150 μg/ml). Whenever required, N expression was induced by adding 600 μM CuSO$_4$ for 20-24 h. Cleavage of N receptor was activated by treating N-expressing S2 cells with 1 μM EDTA in PBS for 30 min (Krejci and Bray, 2007).

Immunoprecipitation (IP) and western blot
IP and western blot were carried using standard protocols; for details see the supplementary Materials and Methods. IP was carried out with rabbit

polyclonal anti-A2BP1, rabbit polyclonal anti-Groucho (kind gift from Girish Ratnaparkhi, IISER, Pune, India) or goat anti-Su(H) (Santa Cruz, sc-15813) antibodies. Rabbit IgG (Bethyl) or goat IgG (Santa Cruz) were used as control.

Western blots were probed with rabbit polyclonal anti-A2BP1 (1:2500), goat polyclonal anti-Su(H) (1:1000), mouse monoclonal anti-N^{ICD} (1:1000; DSHB, C17.9C6), goat polyclonal anti-CtBP (1:1000; Santa Cruz, sc-26610) or rabbit polyclonal anti-Groucho (1:1000). Secondary antibodies used in western blots (Jackson Laboratories, 1:10,000, unless stated otherwise) were: goat anti-rabbit-HRP, goat anti-mouse-HRP, rabbit anti-goat-HRP and goat anti-rat-HRP (1:5000; Abcam).

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Supplementary material

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 buffer 1. All IP buffers were supplemented with protease inhibitor cocktail tablets (Roche, 11873580001). 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-dA2BP1 or rabbit polyclonal anti-Groucho or Goat anti-Su(H) (Santacruz) antibody 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.

The 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 4ºC 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 the blot was washed 4 times with TBST, 10 minutes each. Finally, the blot was exposed with HRP substrate (Millipore) and imaged by CCD camera LAS 4000. The images were processed with Image J or Fujifilm software.

Table S1. Statistics of comparison of sensory bristle numbers between wildtype and various experimental genotypes. Significant differences are represented as *** for *p*-value < 0.001, ** for *p*-value < 0.01, * for *p*-value < 0.05. Figure numbers of corresponding figures/graphs are indicated in the table. NS = Not Significant.

Genotypes	*p*-value	Significance
Figure 1L		
Wildtype (n=30) *vs* *sca*G4> *dA2BP1^{RNAi}* (n=27)	5.64E-17	***
Wildtype (n=30) *vs* *sca*G4> UAS dA2BP1 (n=25)	2.41E-34	***
Figure 1M		
Wildtype (n=20) vs scaG4> dA2BP1RNAi (n=20)	4.19E-06	***
Wildtype (n=20) vs scaG4> UAS dA2BP1 (n=20)	0.01100	*
Figure 2		
*sca*G4>UAS GFP (n=7) *vs* *sca*G4> *dA2BP1^{RNAi}* (n=7)	0.0484	*
*sca*G4>UAS GFP (n=7) *vs* *sca*G4> UAS dA2BP1 (n=9)	2.07E-05	***
Figure 3G		
Wildtype (n=30) *vs* N^{55e11}/+ (n=25)	6.58E-11	***
Wildtype (n=30) *vs* *sca*G4> *dA2BP1^{RNAi}* (n=27)	5.64E-17	***
*sca*G4> *dA2BP1^{RNAi}* (n=27) *vs* *sca*G4> *dA2BP1^{RNAi}* / N^{55e11}(n=25)	1.19E-08	***
N^{55e11}/+ (n=25) *vs* *sca*G4> *dA2BP1^{RNAi}* / N^{55e11} (n=25)	1.15E-21	***
Wildtype (n=30) *vs* N^{Ax16}/+ (n=20)	4.13E-05	***
*sca*G4> *dA2BP1^{RNAi}* (n=27)*vs* *sca*G4> *dA2BP1^{RNAi}* / N^{Ax16} (n=20)	4.24E-15	***
N^{Ax16}/+ (n=20) *vs* *sca*G4> *dA2BP1^{RNAi}* / N^{Ax16} (n=20)	7.44E-08	***
Figure 5E		
Wildtype (n=30) *vs* *sca*G4> *dA2BP1^{RNAi}* (n=27)	5.64E-17	***
Wildtype (n=30) *vs* *sca*G4/+; H^1/+ (n=23)	1.78E-10	***
*sca*G4/+; H^1/+ (n=23) *vs* *dA2BP1^{RNAi}* /+; *sca*G4/+; H^1/+ (n=22)	3.60E-07	***
Wildtype (n=30) *vs* *sca*G4/+; H^2/+ (n=28)	1.63E-13	***
*sca*G4/+; H^2/+ (n=28) *vs* *dA2BP1^{RNAi}* /+; *sca*G4/+; H^2/+ (n=28)	1.56E-12	***
Wildtype (n=30) *vs* *sca*G4/+; H^3/+ (n=24)	7.19E-13	***
*sca*G4/+; H^3/+ (n=24) *vs* *dA2BP1^{RNAi}* /+; *sca*G4/+; H^3/+ (n=16)	2.82E-06	***
Figure S3K		
Wildtype (n=30) *vs* *sca*G4> *dA2BP1^{RNAi}* (n=27)	5.64E-17	***
Wildtype (n=30) *vs* *c253*G4> *dA2BP1^{RNAi}* (n=48)	9.11E-20	***
Wildtype (n=30) *vs* *pnr*G4> *dA2BP1^{RNAi}* (n=31)	3.09E-18	***

Wildtype (n=30) ***vs*** *MS*[248]*G4> dA2BP1*[RNAi] (n=81)	7.95E-34	***
Wildtype (n=30) ***vs*** *ap*G4> *dA2BP1*[RNAi] (n=71)	3.80E-14	***
Wildtype (n=30) ***vs*** *eyg*G4> *dA2BP1*[RNAi] (n=66)	3.38E-18	***
Figure S6G		
Wildtype (n=30) *vs scaG4> dA2BP1*[RNAi] *(n=27)*	5.64E-17	***
scaG4> dA2BP1[RNAi] *(n=27) vs scaG4> dA2BP1*[RNAi]*; sens*[E2]*/+ (n=25)*	4.79E-11	***
scaG4> dA2BP1[RNAi] *(n=27) vs scaG4> dA2BP1*[RNAi]*; sens*[58]*/+ (n=25)*	4.34E-13	***
Figure S8E		
*pros*G4> UAS GFP (n=20) ***vs*** *pros*G4> *dA2BP1*[RNAi] (n=20)	0.15164	NS
*pros*G4> UAS GFP (n=20) ***vs*** *pros*G4/N[DN] (n=20)	0.02707	*
*pros*G4> *dA2BP1*[RNAi] (n=20) ***vs*** *dA2BP1*[RNAi]; *pros*G4/N[DN] (n=20)	0.43192	NS
*pros*G4/N[DN] (n=20) ***vs*** *dA2BP1*[RNAi]; *pros*G4/N[DN] (n=20)	0.81928	NS

Supplemental Figures
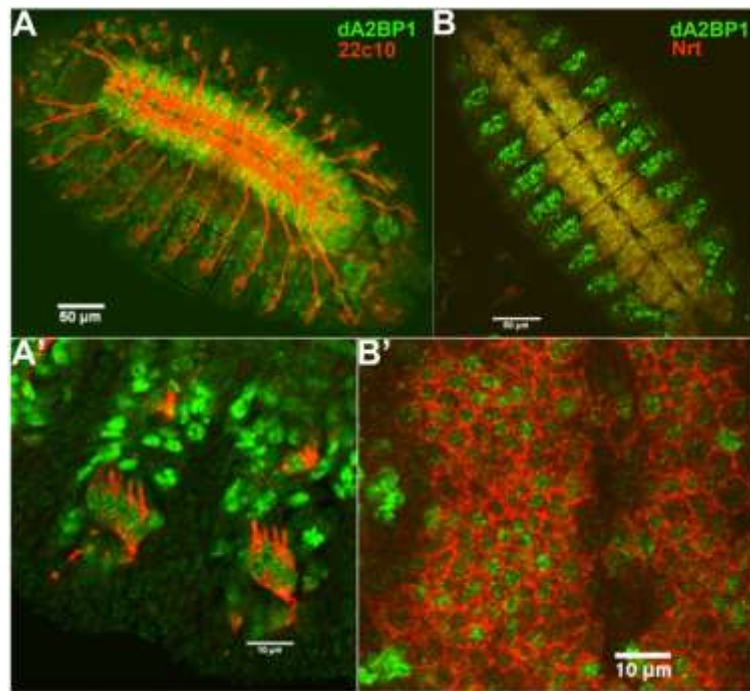
Figure S1. dA2BP1 is expressed in developing central and peripheral nervous systems of *Drosophila* embryos.

(A, B) Stage 14 embryos stained for dA2BP1 (green) and Futsch (22c10, red, A) or Neurotactin (Nrt, red, B). A' and B' show higher magnification images of A and B, respectively. Futsch marks peripheral nervous system, while Nrt marks the neuronal population of VNC.
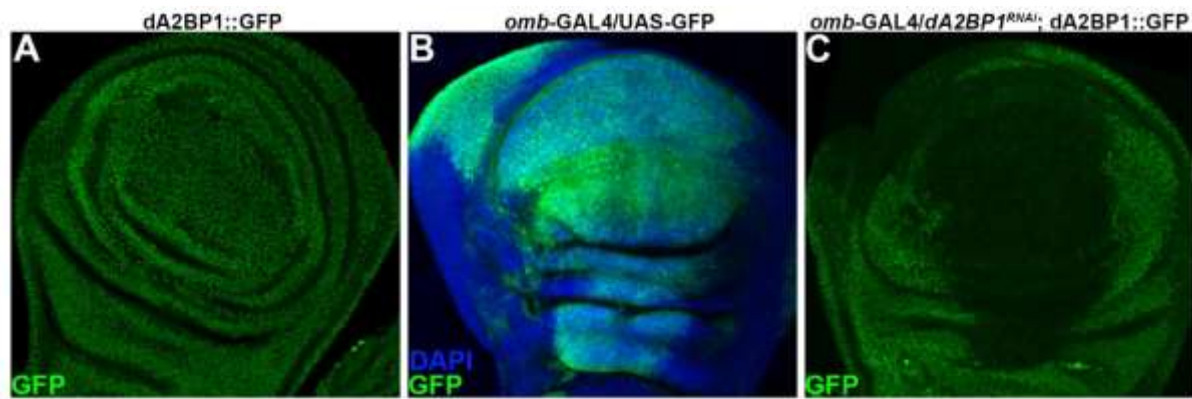
Figure S2. RNAi-mediated downregulation of dA2BP1 expression.

(A) dA2BP1::GFP wing disc of 3rd instar larvae showing wild-type expression pattern of dA2BP1. (B) *omb*-GAL4/UAS-GFP wing disc showing expression pattern of the omb-GAL4 driver in the 3rd instar larval stage. (C) *omb*-GAL4/UAS-*dA2BP1RNAi*; dA2BP1::GFP wing disc showing down-regulation of *dA2BP1* in the entire region where RNAi is induced by the *omb*-GAL4 driver confirming the efficiency of the RNAi transgene.
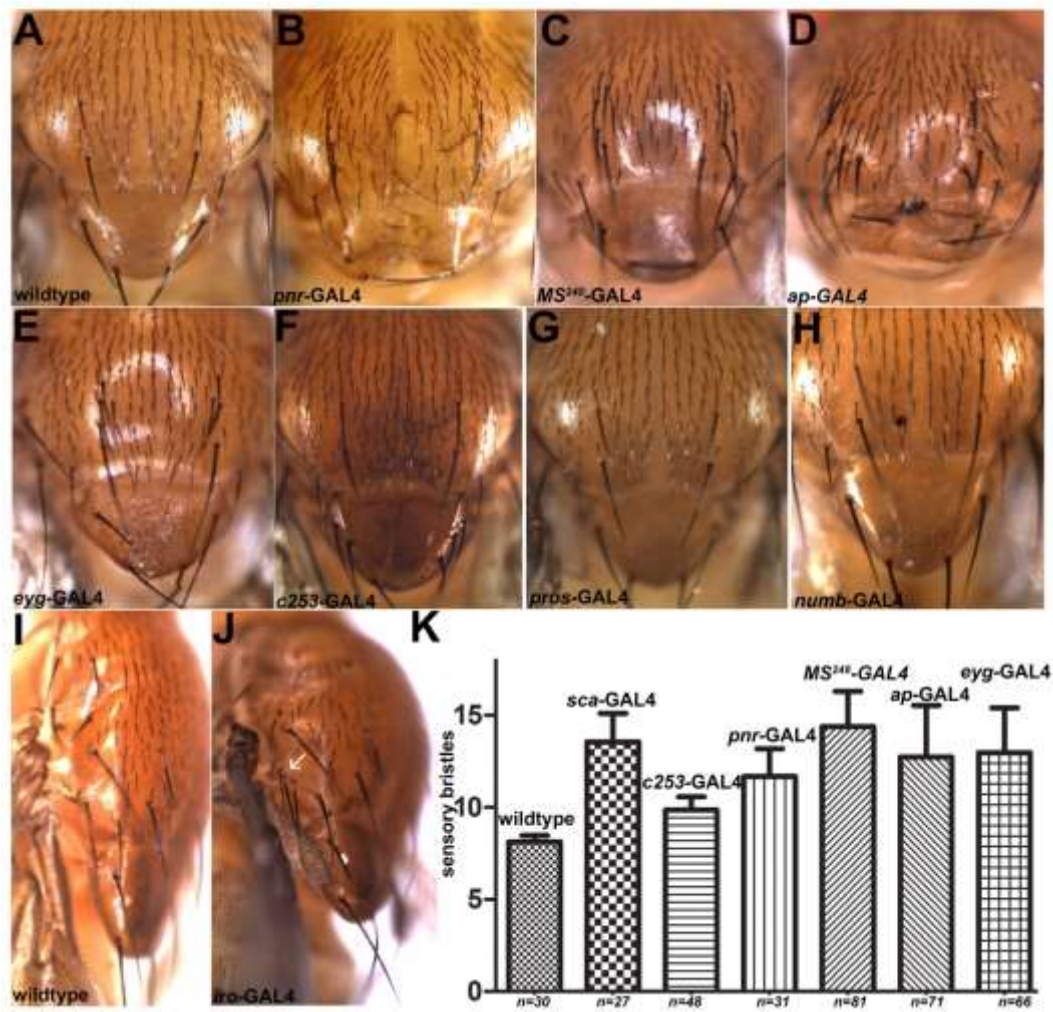
Figure S3. dA2BP1 loss of function causes increase in the number of sensory bristles on the *Drosophila* thorax.

(A-J) Adult thorax of *wildtype* (A, I), UAS-*dA2BP1^RNAi^; pnr*-GAL4 (B), UAS-*dA2BP1^RNAi^; MS^24B^*-GAL4 (C), UAS-*dA2BP1^RNAi^; ap*-GAL4 (D), UAS-*dA2BP1^RNAi^; eyg*-GAL4 (E), UAS-*dA2BP1^RNAi^; c253*-GAL4 (F), UAS-*dA2BP1^RNAi^; pros*-GAL4 (G), UAS-*dA2BP1^RNAi^; numb*-GAL4 (H) and UAS-*dA2BP1^RNAi^; iro*-GAL4 flies (J). Arrow marks the increase in bristle at *iro*-GAL4 expressing region (J). Except with *numb*-GAL4 and *pros*-GAL4, *dA2BP1^RNAi^*–mediated downregulation of dA2BP1 causes increase in bristle number. (L) Statistical analysis of changes in bristle number (DC + SC) in different genetic backgrounds. Error bars represent standard deviation. All genotypic combinations were significantly different than wild type at *p* <0.0001.
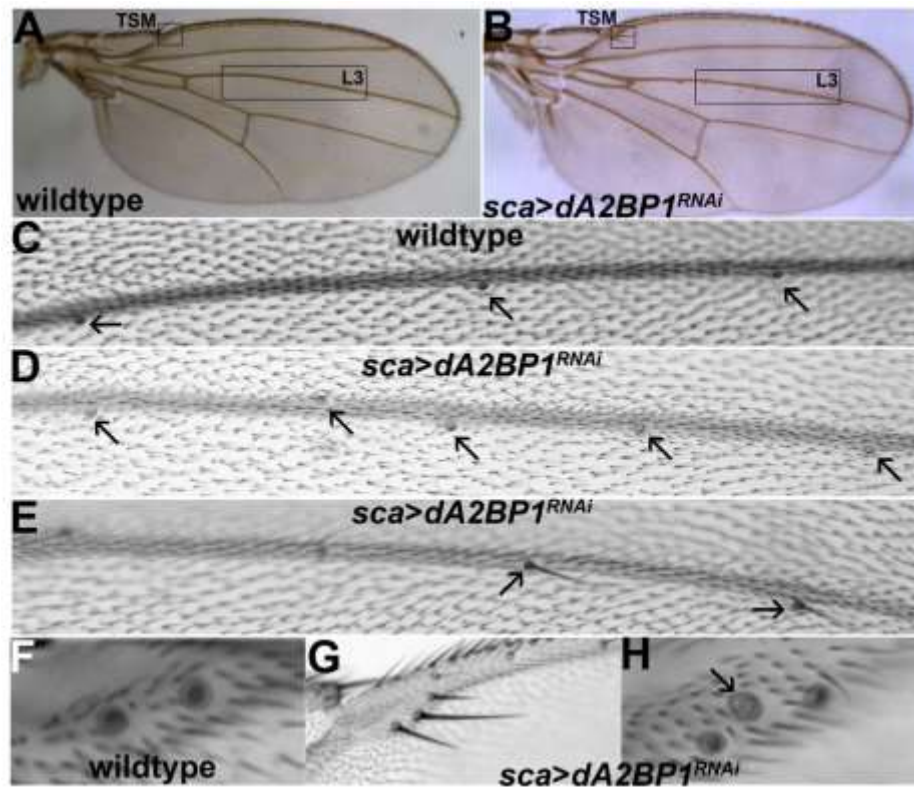
Figure S4. Downregulation of *dA2BP1* causes increase in campaniform sensilla number and its transformation to bristle type.

(A-B) Wildtype (A) and UAS-*dA2BP1^RNAi^; sca*-GAL4 (B) wing blades marking the campaniform sensilla position; the L3 vein campaniform sensillum and the twin sensilla of the margin (TSM). Regions marked as L3 in A and B are shown at higher magnification in C and D, respectively. Please note 2 additional L3 vein campaniform sensilla (D). We also occasionally observed sensory bristles on L3 (E; image was taken from a different wing blade than shown in B). (F-G) A section of wild-type (F) and UAS-*dA2BP1^RNAi^; sca*-GAL4 (G) wing blades showing twin sensilla of the margin at higher magnification. Note sensory bristles in the wing margin in the place of socketed sensory organs (G). In some wing blades, we also observed 3 sensilla in the place of normal two (H; image was taken from a different wing blade than shown in B).
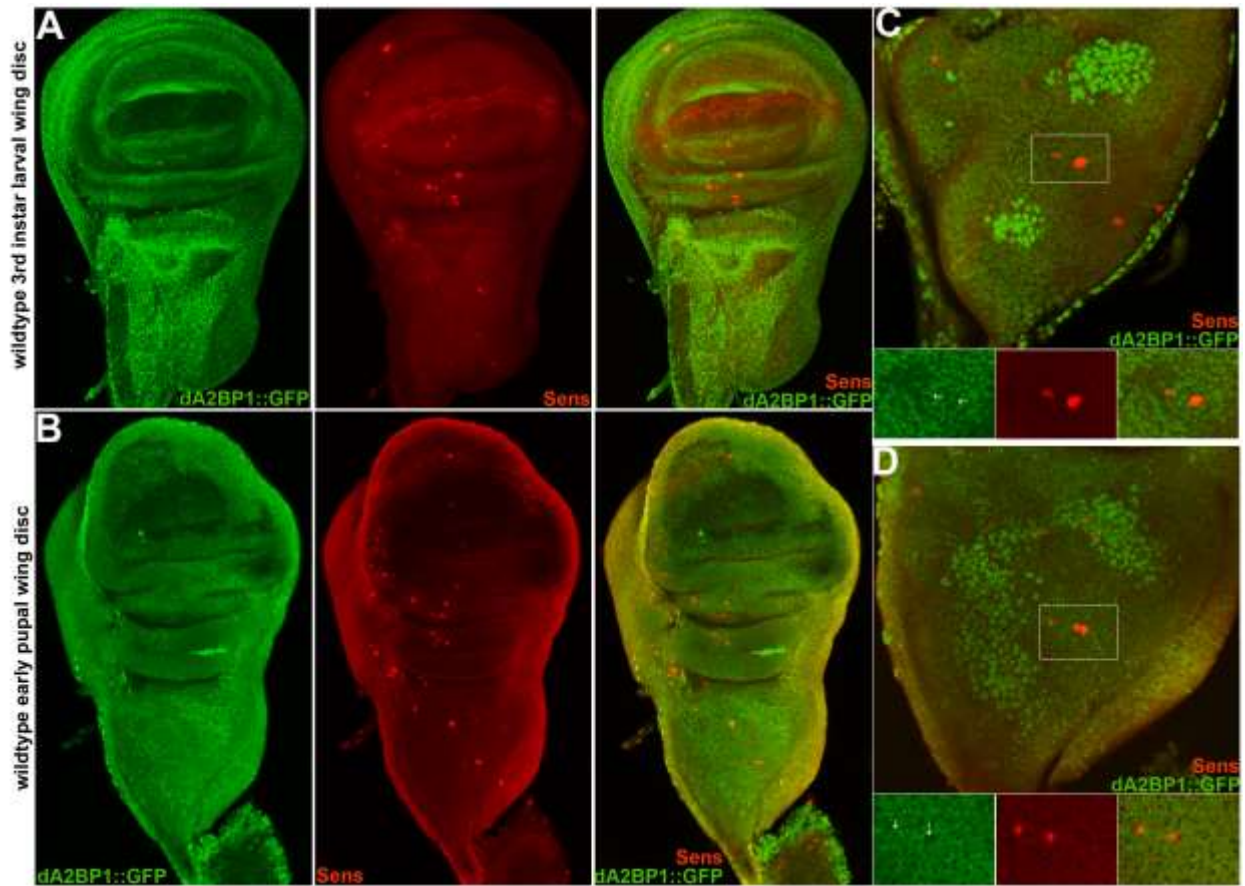
Figure S5. Reduced levels of dA2BP1 expression in the SOPs of pupal wing disc compared to larval stages.

(A) 3rd instar larval wing disc of dA2BP1::GFP showing the expression pattern of dA2BP1 and Sens. dA2BP1 expression is strong in most parts of the wing disc including SOPs, as marked by Sens. (B) Wing disc of dA2BP1::GFP at 4h after puparium formation showing the expression pattern of dA2BP1 and Sens. The discs have started everting as evidenced by wing margin expression pattern of Sens. Relative to larval stages, dA2BP1 expression is much lower in most parts of the wing disc including SOPs, as marked by Sens. Discs in A and B are at 20X magnification. (C-D) 3rd instar larval (C) and early pupal (D) discs at higher magnification (40X). Insets in both C and D show corresponding boxed region for better clarity of dA2BP1 expression in SOPs. In larval discs, all SOPs show dA2BP1 expression (arrows in C). At pupal stages, we did observe expression of dA2BP1, specifically in dividing SOPs (dotted arrow in D). Even at this stage, a SOP, which has not yet started dividing, still expresses dA2BP1 (arrow in D).
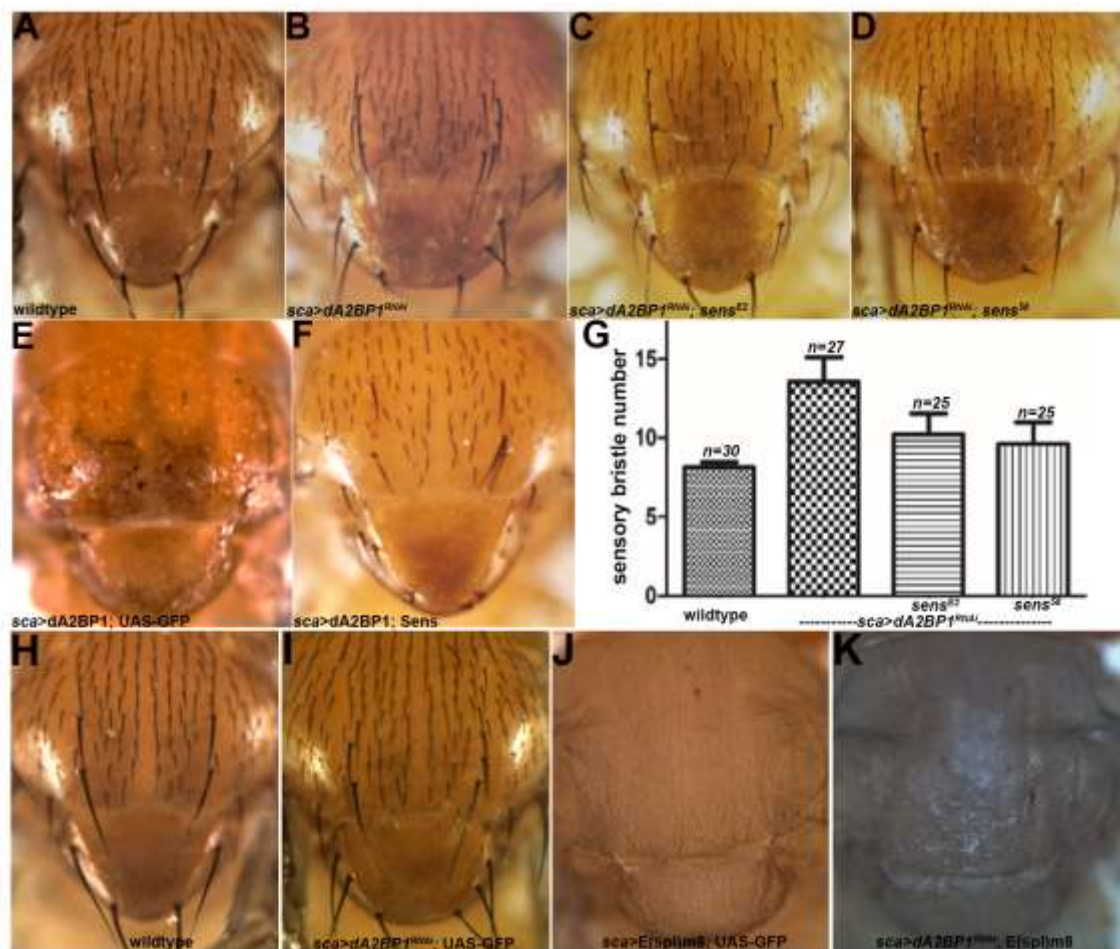
Figure S6. dA2BP1 is upstream of Senseless and E(spl)m8 in the Notch pathway

(A-D) Adult thorax of wildtype (A), UAS-*dA2BP1^{RNAi}; sca*-GAL4 (B), UAS-*dA2BP1^{RNAi}; sca*-GAL4; *sens^{E2}*/+ (C) and UAS-*dA2BP1^{RNAi}; sca*-GAL4; *sens^{58}*/+ (D) flies. *sens* heterozygous flies do not have any sensory bristle phenotype. *dA2BP1^{RNAi}*–induced supernumerary phenotype, however, is suppressed by heterozygous *sens* alleles. (E-F) Adult thorax of *sca*-GAL4/UAS-dA2BP1; UAS-GFP (E) and UAS-Sens; *sca*-GAL4/UAS-dA2BP1 (F). Over-expression of dA2BP1 results in loss of sensory bristle on the adult thorax (E). This phenotype is suppressed by the over-expression of Sens (F). (G) Statistical analysis of changes in bristle number (DC + SC) in different genetic backgrounds. Error bars represent standard deviation. Suppression of *dA2BP1^{RNAi}*–induced supernumerary phenotype is significant at p<0.0001. Numbers of adult thoraces examined are indicated against each genotype in the bar diagram.

(H-K) adult thorax of wildtype (H) UAS-*dA2BP1^{RNAi}; sca*-GAL4; UAS-GFP (I), *sca*-GAL4; UAS-E(spl)m8/UAS-GFP (J) and UAS-*dA2BP1^{RNAi}; sca*-GAL4; UAS-E(spl)m8 (K) flies.

Loss of dA2BP1 in super-numerary bristle phenotype, while over-expression of E(spl)m8 results in complete loss of both macrochaetae and microchaetae (J). The phenotype caused by the over-expression of E(spl)m8 is dominant to that of loss of function of dA2BP1 (K) suggesting that the E(spl)m8 is downstream of dA2BP1.
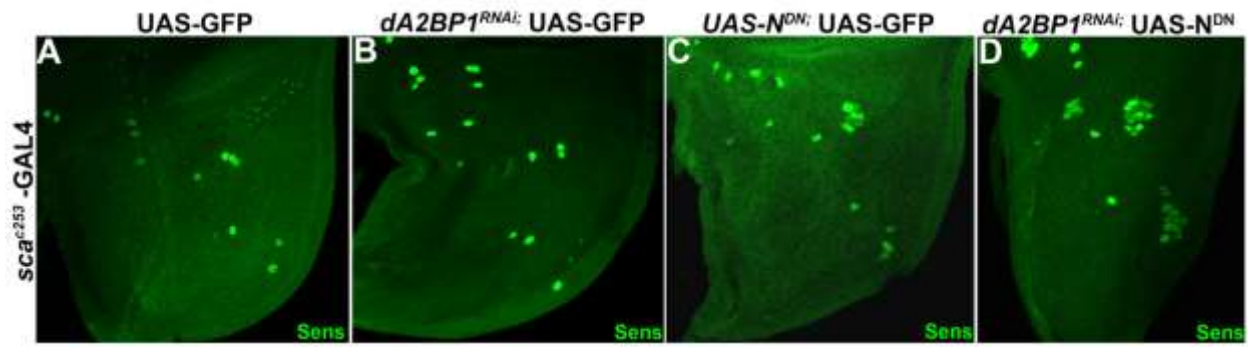
Figure S7. Increase in sensory bristles due to downregulation of both *dA2BP1* and N-is due to increase in the number of SOPs at the larval stages.
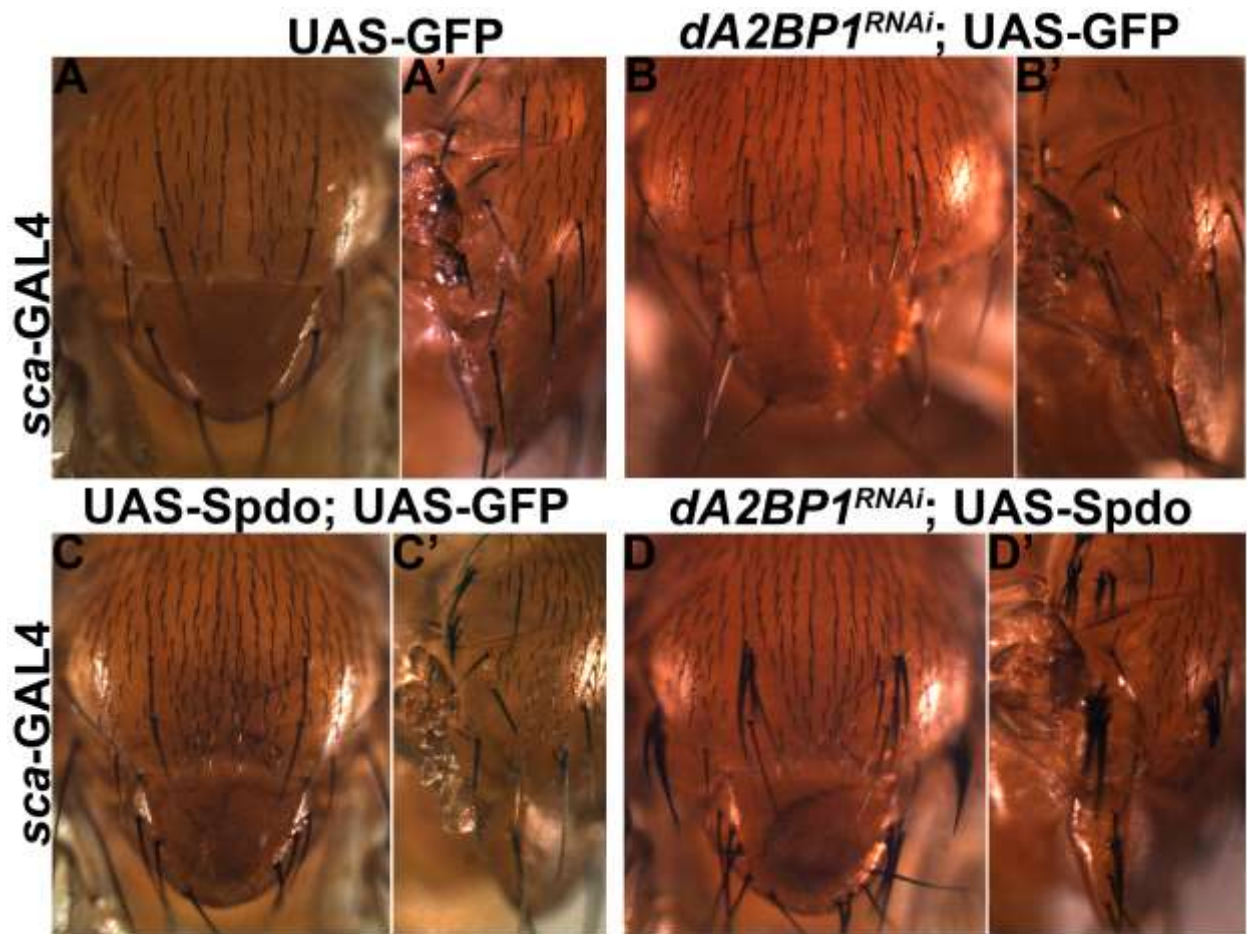
(A-D) *sca^{c253}*-GAL4; UAS-GFP (A), UAS-*dA2BP1^{RNAi}*; *sca^{c253}*-GAL4; UAS-GFP (B), UAS-N^{DN}/*sca^{c253}*-GAL4; UAS-GFP (C) and UAS-*dA2BP1^{RNAi}*; UAS-N^{DN}/*sca^{c253}*-GAL4 (D) wing imaginal discs stained for Sens expression. Note increase in the number of Sens-expressing cells. The large clusters of Sens-expressing cells in D corresponds to tufted bristles phenotypes seen in the adult thorax of the same genotype (Fig. 4G). This suggests increase in the number of SOPs in this genotypic combination rather than the just increase in external bristle cells of the sensory organs.
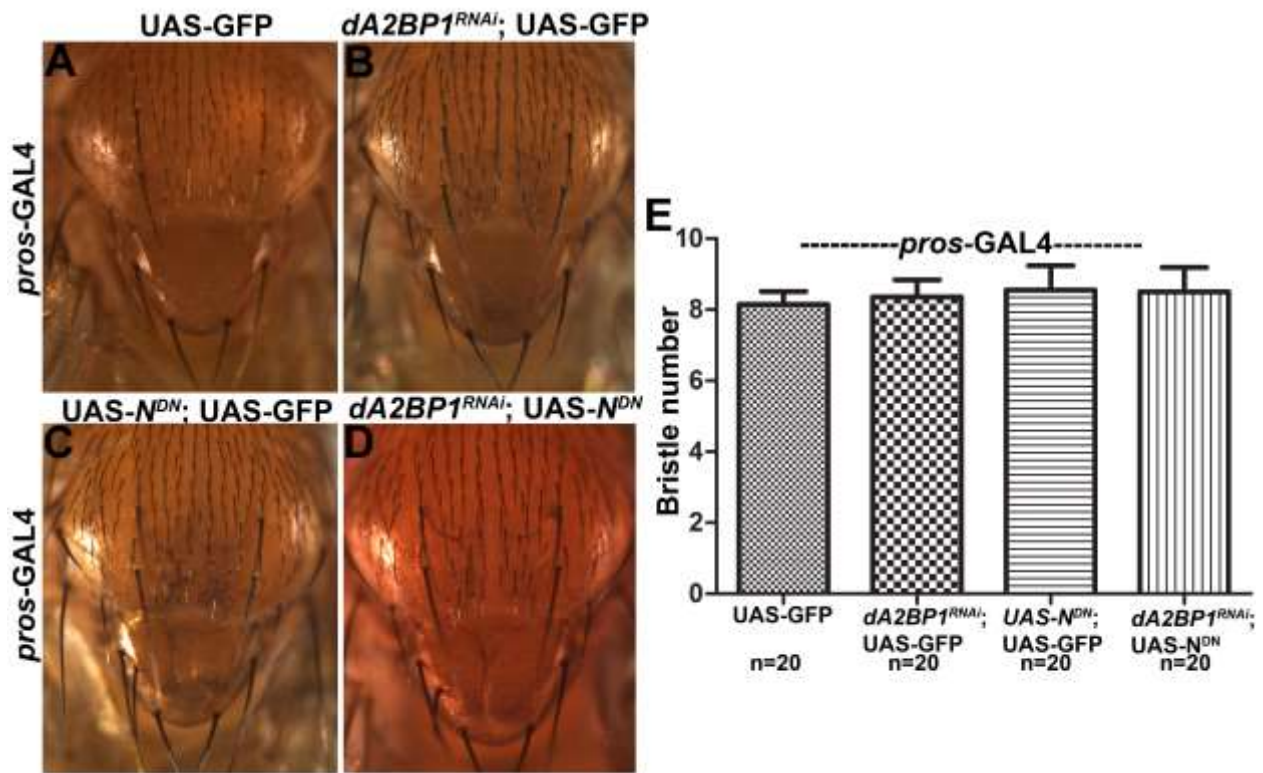
**Figure S8. dA2BP1 has no role after the specification of SOPs.**

(A-D) Images of adult thorax of *pros*-GAL4; UAS-GFP (A), UAS-*dA2BP1^RNAi*; *pros*-GAL4; UAS-GFP (B), UAS-N^DN/*pros*-GAL4; UAS-GFP (C) and UAS-*dA2BP1^RNAi*; UAS-N^DN/ *pros*-GAL4 (D). There is mild increase in the number or pattern of sensory bristles when N pathway is downregulated in late stages of SOP specification using *pros*-GAL4 driver. However, this phenotype is unaffected when *dA2BP1* is downregulated in this background.

Figure S9. Loss of dA2BP1 enhances the phenotype caused by the over-expression of Sanpodo, a negative regulator of Notch pathway.

(A-D) Adult thorax of *sca*-GAL4; UAS-GFP (A), UAS-*dA2BP1^{RNAi}*; *sca*-GAL4; UAS-GFP (B), UAS-Spdo/*sca*-GAL4; UAS-GFP (C) and UAS-*dA2BP1^{RNAi}*; UAS-Spdo/*sca*-GAL4 (D). Note increase in the number of sensory bristles in both B and C, which is significantly further enhanced in D. The number of full sensory organs is increased rather than just bristles, as shown in D' at higher magnification.
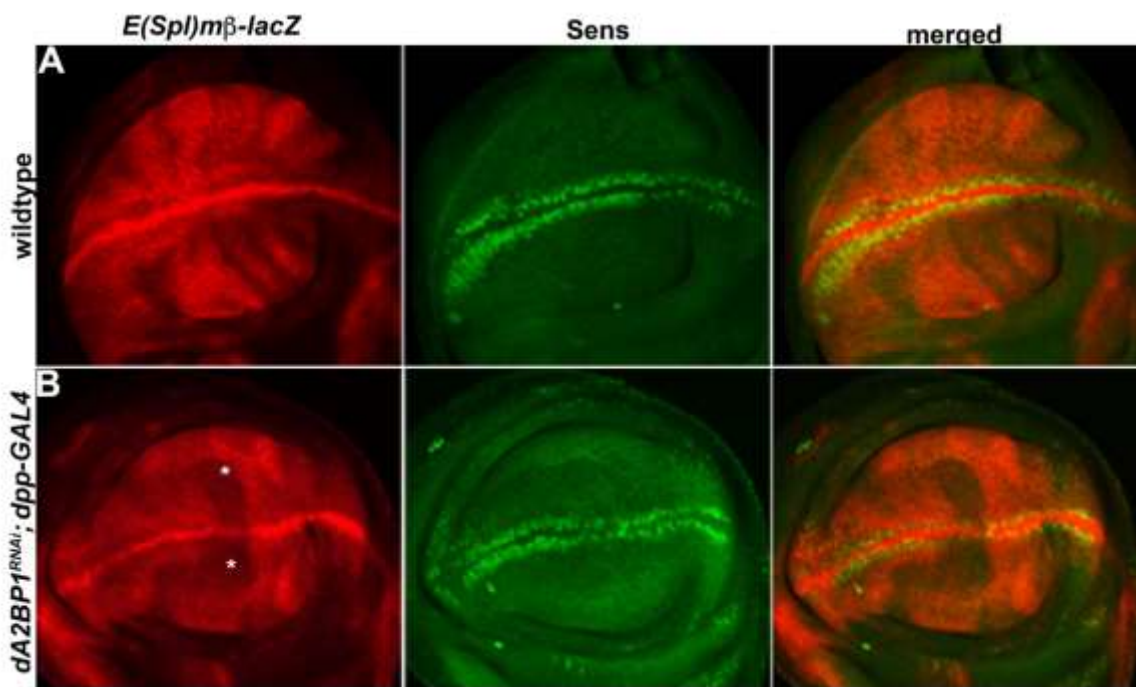
Figure S10. dA2BP1 regulates the expression of *E(spl)mβ* in wing epithelium

(A-B) *E(spl)mβ* –lacZ (A) and UAS-*dA2BP1^RNAi^; E(spl)mβ –lacZ; dpp*-GAL4; (B) wing discs stained for lacZ and Sens expression. Downregulation of *dA2BP1* at the A/P boundary results in loss of *E(spl)mβ* expression in non-DV cells (white asterisks). Expression of *E(spl)mβ* in the D/V boundary, wherein dA2BP1 is not expressed (Bajpai et al., 2004) is unaffected.
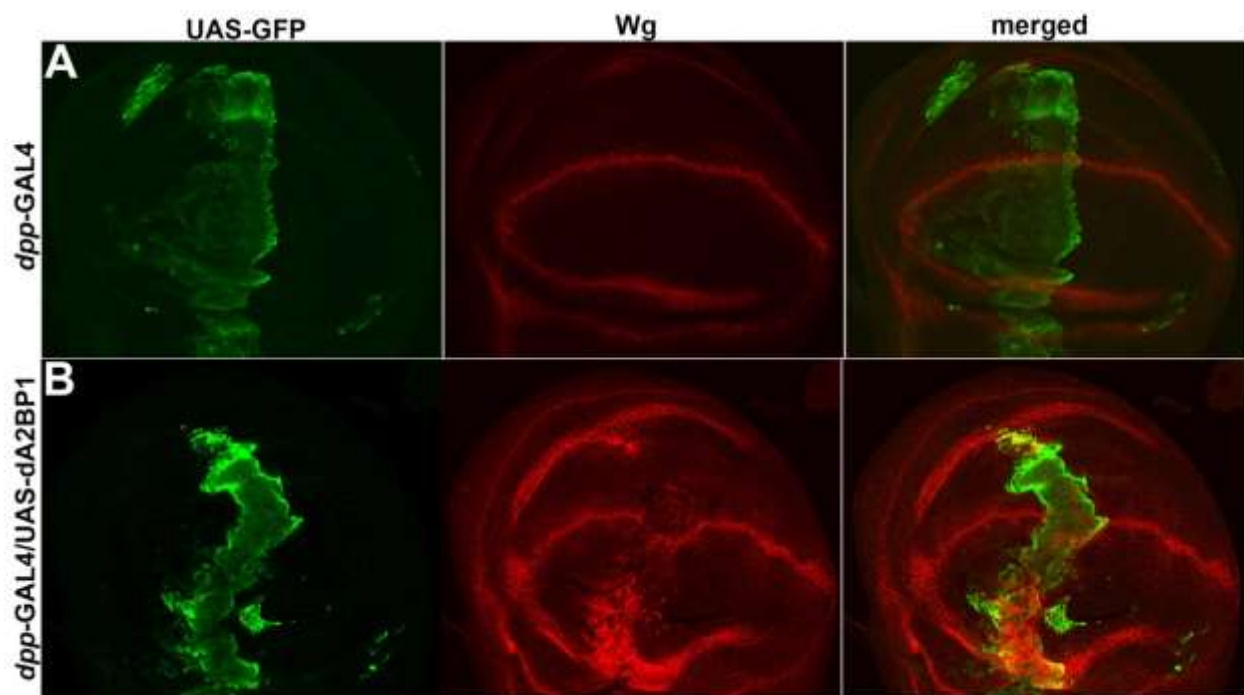
Figure S11. Ectopic expression of dA2BP1 activates Wg expression in wing epithelium
(A-B) *dpp*-GAL4/UAS-GFP (A) and UAS-dA2BP1; *dpp*-GAL4/UAS-GFP (B) wing discs stained for GFP and Wg expression. Upregulation of dA2BP1 at the A/P boundary results in the activation of Wg expression along the boundary, an indication of activation of N pathway.