

Development 137, 3167–3176 (2010) doi:10.1242/dev.050575
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Drosophila Ataxin 2-binding protein 1 marks an intermediate step in the molecular differentiation of female germline cysts

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SUMMARY

In the *Drosophila* ovary, extrinsic signaling from the niche and intrinsic translational control machinery regulate the balance between germline stem cell maintenance and the differentiation of their daughters. However, the molecules that promote the continued stepwise development of ovarian germ cells after their exit from the niche remain largely unknown. Here, we report that the early development of germline cysts depends on the *Drosophila* homolog of the human ataxin 2-binding protein 1 (*A2BP1*) gene. *Drosophila* *A2BP1* protein expression is first observed in the cytoplasm of 4-, 8- and 16-cell cysts, bridging the expression of the early differentiation factor Bam with late markers such as Orb, Rbp9 and Bruno encoded by *arrest*. The expression of *A2BP1* is lost in *bam*, *sans-fille* (*snf*) and *mei-P26* mutants, but is still present in other mutants such as *rbp9* and *arrest*. *A2BP1* alleles of varying strength produce mutant phenotypes that include germline counting defects and cystic tumors. Phenotypic analysis reveals that strong *A2BP1* alleles disrupt the transition from mitosis to meiosis. These mutant cells continue to express high levels of mitotic cyclins and fail to express markers of terminal differentiation. Biochemical analysis reveals that *A2BP1* isoforms bind to each other and associate with Bruno, a known translational repressor protein. These data show that *A2BP1* promotes the molecular differentiation of ovarian germline cysts.

KEY WORDS: Ataxin 2-binding protein 1, *Drosophila*, Oogenesis, Ovarian tumor

INTRODUCTION

The development of naïve cells towards a terminally differentiated fate often proceeds through a number of distinct steps. Complex transcriptional and post-transcriptional hierarchies regulate transitions between these stages so that differentiation occurs in a linear and tissue-specific manner. *Drosophila* ovarian germline cyst development has served as a useful platform for studying how diverse mechanisms coordinate to establish specific cell fates, particularly with regards to stem cells and their differentiating progeny.

Ovarian cyst development begins in the germarium with the asymmetric division of a germline stem cell (GSC) (for a review, see Wong et al., 2005). This division results in one of the daughters being displaced away from the cap cell niche. This cell, called the cystoblast, proceeds through four incomplete mitotic divisions to form an interconnected 16-cell cyst. Within this cyst, one cell becomes the oocyte, whereas the remaining cells become supportive nurse cells. Once encapsulated by follicle cells, the cyst buds off of the germarium to become an egg chamber.

Several molecular and morphological markers highlight changes within differentiating germline cysts. One widely used marker has been the fusome, a germline-specific organelle that has many properties of the endoplasmic reticulum (Lighthouse et al., 2008; Snapp et al., 2004). The fusome plays roles in regulating the mitotic cell cycle within germline cysts and in oocyte specification (Lin and

Spradling, 1995; Lin et al., 1994). Within GSCs, the fusome appears small and round. During the incomplete mitotic divisions, the fusome becomes branched and runs through the ring canals of the interconnected cells within the germline cyst (de Cuevas and Spradling, 1998). The degree of fusome branching reflects the number of cells within a developing cyst and has served as a primary marker for evaluating germ cell differentiation within the germarium.

Expression of *bam* is both necessary and sufficient for the early differentiation of germline cysts. Cytoplasmic Bam is first observed within cystoblasts and persists through eight-cell cysts. Loss of *bam* results in a tumorous phenotype in which all germline cells remain as single cells in a pre-cystoblast state (McKearin and Ohlstein, 1995; McKearin and Spradling, 1990). The biochemical function of Bam remains unclear but recent findings show at least one of its roles is to repress *nanos* translation (Li et al., 2009).

Mutations in a number of other genes, including *mei-P26*, *sex-lethal* (*sxl*), *sans fille* (*snf*), *arrest* and *rbp9* block the terminal differentiation of 16-cell cysts and often result in the formation of cystic tumors that can be easily distinguished from *bam* mutant tumors based on the presence of branched fusomes (Chau et al., 2009; Kim-Ha et al., 1999; Nagengast et al., 2003; Neumuller et al., 2008; Page et al., 2000; Parisi et al., 2001; Pauli et al., 1993; Schupbach, 1985). These cystic tumors contain a range of single, 2-, 4-, 8- and 16-cell cysts. However, these mutants are not all arrested at equivalent stages. For example, mutations in *mei-P26*, *sxl* and *snf*, a splicing factor needed for the germline expression of *sxl*, all result in the formation of large tumors and ‘pseudo-egg chambers’ that contain many undifferentiated germ cells (Chau et al., 2009; Nagengast et al., 2003; Neumuller et al., 2008; Page et al., 2000). Significantly, a recent study shows that loss of *snf* prevents germ cells from adopting a committed differentiated fate (Chau et al., 2009). *snf* mutant germ cells carry abnormal fusomes and exhibit expanded expression of early markers such as Piwi and Pumilio

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(Chau et al., 2009). Similarly, *mei-P26* mutant cells do not progress beyond the earliest steps of differentiation (Neumuller et al., 2008; Page et al., 2000). By contrast, *arrest* and *rbp9* mutants form smaller tumors and do not produce large pseudo-egg chambers (Kim-Ha et al., 1999; Parisi et al., 2001). The *arrest* gene encodes the translational repressor protein Bruno and functions during the later stages of cyst development as germ cells transition from a mitotic to meiotic cell cycle (Parisi et al., 2001; Sugimura and Lilly, 2006). Bruno binds *cyclin A* and *sxl* mRNAs, and represses their translation (Sugimura and Lilly, 2006; Wang and Lin, 2007). The *rbp9* gene encodes the *Drosophila* homolog of the RNA-binding protein Hu (Kim-Ha et al., 1999; Lee et al., 2000). The expression of Rbp9 increases dramatically within 16-cell cysts and phenotypic analysis suggests the protein functions during the later steps of cyst differentiation (Kim-Ha et al., 1999; Lee et al., 2000).

Here, we provide evidence that the *Drosophila* homolog of mammalian ataxin 2-binding protein 1 (*A2BP1*) functions during the intermediate stages of germline cyst development. Cytoplasmic *A2BP1* protein is expressed in a novel pattern within four-, eight- and 16-cell cysts, bridging the expression of Bam and the expression of late markers associated with terminal cyst differentiation. Disruption of *A2BP1* results in cell counting defects and tumor formation. *A2BP1* physically and genetically interacts with other factors that function before the onset of meiosis. Together, these data extend our understanding of the molecular changes that occur during germline cyst differentiation.

MATERIALS AND METHODS

Fly stocks

Fly stocks were maintained on standard cornmeal molasses agar at 25°C unless otherwise noted. *w¹¹¹⁸* was used as a control in all our experiments. Other lines used include a *histoneGFP* transgene inserted on 3L (gift from J. Duffy), *enc^{d4}* (gift from T. Schupbach), *bam^{Δ86}* (McKearin and Ohlstein, 1995), *rbp9²⁶⁹⁰*, *rbp9^{Δ1}* (Kim-Ha et al., 1999) (gifts from J. Kim-Ha), *snf^{Δ48}* (Chau et al., 2009; Nagengast et al., 2003) (gift from H. Salz), *mei-P26^{mf51}* (Page et al., 2000) (gift from R. S. Hawley), *A2BP1^{KG06463}* (Bellen et al., 2004), *Df(3L)ED4457* (Ryder et al., 2004) (obtained from Bloomington Stock Center; BL#9355) and *A2BP1^{e03440}*, *A2BP1^{f02600}*, *A2BP1^{f01889}* (Thibault et al., 2004) (obtained from Harvard Stock Center). In addition to the *Df(3L)ED4457* deficiency, we generated another small molecularly defined deficiency using FRT/FLP mediated recombination in combination with two insertions surrounding the *A2BP1* region: *d05504* and *f00028* (Parks et al., 2004). Recombination between these elements resulted in the deletion of 124,285 bp that uncovered ten genes. The *A2BP1* alleles behaved in an identical manner when placed over either deletion.

For clonal analysis, a *hsFLP* (X); *histoneGFP* [FRT]79D stock was crossed to *A2BP1^{e03440}* [FRT]79D flies. Adult females were subjected to a heat shock at 37°C for 1 hour, three times per day for 2 days.

Immunohistochemistry and in situ hybridization

Ovaries were dissected in Grace's Medium. Tissue was fixed for 10 minutes with gentle rocking in 4% formaldehyde (EM grade) in PBS. After fixation, ovaries were washed four times in PBT (PBS + 0.5% BSA + 0.3% Triton-X 100) at RT for 10 minutes. Primary antibodies were incubated overnight at 4°C. Ovaries were then washed four times with PBT for 10 minutes, incubated for five hours with secondary antibodies. Ovaries were then washed and mounted in VectaShield Mounting medium with DAPI (Vector Laboratories).

The following antibodies were used (dilutions noted in parentheses): rabbit anti-GFP (1:1000) (Molecular Probes), mouse monoclonal anti-Orb 6H4 (1:10), anti-Sxl (1:10), anti-Cyclin A (1:10), anti-Cyclin B (1:20) and anti-BamC A7 (1:10) (Developmental Studies Hybridoma Bank, Iowa); rabbit polyclonal anti-Spectrin (1:1000) (Byers et al., 1987) (a gift from Ron Dubreuil); rabbit anti-Nanos (a gift from Akira Nakamura); rabbit polyclonal anti-C(3)G (1:3000) (Hong et al., 2003) and anti-Bruno (1:5000) (gifts from Mary Lilly); goat anti-Vasa (1:200) (Santa Cruz

Biotechnology); and rabbit polyclonal anti-Rbp9 (1:5000) (a gift from J. Kim-Ha). Fluorescence-conjugated secondary antibodies were used at a 1:200 dilution. Actin was labeled with rhodamine-conjugated Phalloidin (Molecular Probes) at 1:100 and DNA with DAPI.

In situ hybridization was carried out using standard procedures using an *A2BP1* antisense probe containing sequences within the third common exon.

Antibody generation

Sequence corresponding to residues 84-186AA of *A2BP1*-PE was cloned into PROEX (Invitrogen) and expressed as a 6xHistidine fusion in *Escherichia coli*. Recombinant protein was purified using Ni²⁺ resin (Qiagen) under denaturing conditions. The purified protein was used to generate polyclonal guinea pig antisera (Covance).

Genomic rescue construct

The CH321-94L16 P[acman] clone (Venken et al., 2009) was obtained from the CHORI BAC/PAC Resource Center. CH321-94L16 contains the entire *A2BP1* locus plus 10 kb of upstream sequence. We replaced the nested *CG6527* and most of the large second intron of *A2BP1* with a 3×P3-RFP/KAN cassette using recombineering techniques (Venken et al., 2006). This cassette was flanked with 50 bp homology arms using the following primers: 5'-CAATATGATATGACTGGGGGGGATACAATCA-CAATATTGTGTTCCCTAAGGCGCGCCACCCCTTATACTTC-3' and 5'-TACTTTTCATTTCACATACTTGGGCTTCCCACTTCCCGTACCT-CATCATGCCACCCCTTTGCTGCTGCG-3'. The resulting PCR product was introduced into DY380 cells carrying the CH321-94L16 clone. Potential recombinants were selected on chloramphenicol and kanamycin, and correct targeting of the cassette was sequence verified. The construct was then introduced into the VK00037 landing site (Venken et al., 2006) on the second chromosome using phi-C31 integrase (Rainbow Transgenics).

Immunoprecipitation and Yeast 2-Hybrid

Tissue lysates were made by homogenizing ovaries from ~100 females in lysis buffer [25 mM HEPES, 50 mM KCl, 1 mM Mg(OAc)₂, 0.01% Triton-X-100, 1 mM DTT (added fresh) and protease inhibitor (Roche)]. For S2 cells experiments, plasmids were transfected using Effectene Transfection reagent (Qiagen) and analyzed 24 hours later. Cells were lysed in Resin Binding Buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.1% Triton-X 100) supplemented with protease inhibitors. Lysates were spun at maximum speed for 15 minutes at 4°C and the supernatant was precleared with 40 µl of Protein G beads. Antibodies were incubated with the cleared supernatant at 4°C for 2 hours. Protein A Agarose beads (Invitrogen) were added and incubated overnight at 4°C. After 14 hours, the beads were washed four times in lysis buffer for 20 minutes and resuspended in an equal volume of protein loading buffer. SDS-polyacrylamide gel electrophoresis and transfer onto nitrocellulose (Hybond ECL, AP Biotech) were carried out according to manufacturer's instruction (BIO-RAD).

For IP experiments with RNase A treatment, 100 µg/ml of RNase A was boiled and added to the ovarian extracts and incubated for 30 minutes. For each RNase A-treated sample, a parallel sample was used with 40 U of an RNase Inhibitor (Protector RNase Inhibitor, Roche).

Westerns were performed using standard procedures with the following antibodies: anti-A2BP1 (1:5000), anti-Bruno (1:5000), anti-HA (1:5000) (Roche) and anti-FLAG (1:10,000) (Sigma). Amido Black staining of membranes served as a loading control. Different *A2BP1* isoforms were subcloned into pEG202 or pJG4.5 destination vectors using LR clonase (Invitrogen). To test interactions in yeast, standard LexA-based two-hybrid techniques were used.

RESULTS

A2BP1 is expressed in developing germline cysts

The initial differentiation of *Drosophila* ovarian germ cells depends on the expression of the *bam* gene. Bam represses *nanos* translation through elements within the 3'UTR of *nanos* mRNA (Li et al., 2009). Once Bam levels decrease, Nanos expression re-emerges in 16-cell cysts. However, during the course of our studies, we

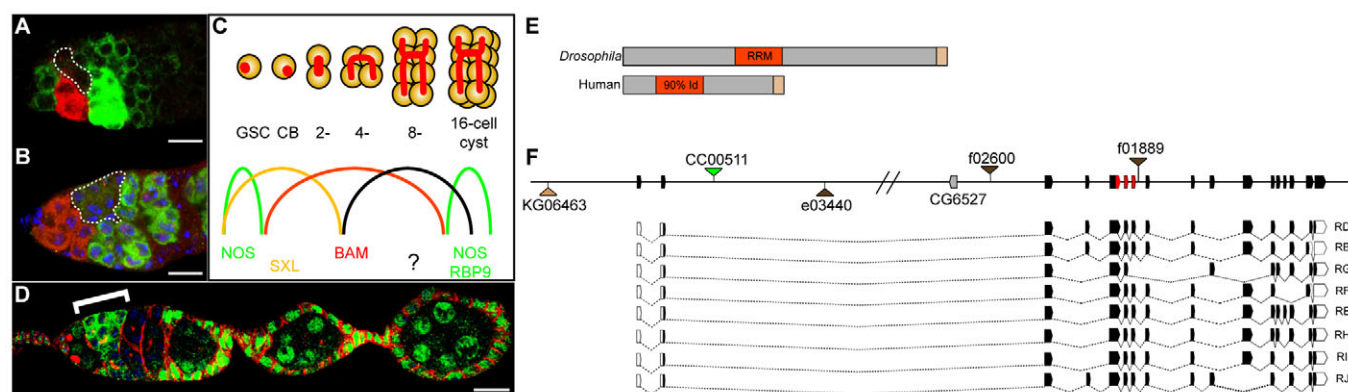


Fig. 1. The *CC00511* protein trap reports the expression of A2BP1. (A) A gerarium stained for endogenous Nanos (green) and Bam (red). A cyst containing low levels of Nanos and Bam is outlined. (B) A gerarium stained for Sxl (red) and Bruno (green). (C) Summary of the known expression patterns of available markers. The black line and question mark signify a hypothetical protein that bridges the expression of early and late markers during cyst differentiation. (D) *A2BP1^{CC00511}* protein trap line stained for GFP (green), 1B1 (red) and DAPI (blue). GFP expression is detected in the cytoplasm of early multicellular germline cysts (bracket). (E) The RRM domains (red) of *Drosophila* A2BP1 and human A2BP1 are 90% identical. (F) Structure of the *A2BP1* gene and transposon insertions in the region. Scale bars: 20 μ m.

occasionally noticed a delay in when high levels of Nanos expression reappeared after Bam antibody staining had decreased below the levels of detection (Fig. 1A). In addition, we observed a similar gap in when cysts express high levels of cytoplasmic Sxl and Bruno, two factors previously described as having mutually exclusive expression patterns (Fig. 1B) (Wang and Lin, 2007). Based on these results, we hypothesized that unidentified factors might function in four- and eight-cell cysts (Fig. 1C). To identify such genes, we screened a previously described protein trap collection (Buszczak et al., 2007). These efforts led to the identification of *CC00511*. GFP expression within *CC00511* ovaries was first observed in the cytoplasm of early multicellular germline cysts (Fig. 1D). In region 3 of the gerarium, the GFP expression shifted from the cytoplasm to the nuclei of the germline cells where it remained for the rest of oogenesis. GFP was also observed in the nuclei of follicle cells beginning in region 2b of the gerarium.

Previous molecular analysis showed the *CC00511* P-element was inserted in an intron of the *CG32062* gene downstream of the translational start site (Fig. 1F) (Buszczak et al., 2007). RT-PCR confirmed the GFP sequence from the protein trap was spliced in frame to the first exon downstream of the element's insertion site. The *CG32062* locus covers ~79 kb and contains many splice variants (Fig. 1F). Five of these isoforms have been previously annotated (FlyBase). Sequencing several other available cDNA clones revealed at least three additional splice variants (Fig. 1F). The molecular function of *CG32062* remains uncharacterized but a recent large-scale RNAi screen implicated this gene in *Drosophila* nervous system development (Koizumi et al., 2007). A second nested gene, *CG6527*, which encodes a major sperm protein (FlyBase), lies in the reverse orientation within the large second intron of *CG32062*.

Motif searches revealed the *CG32062* protein contains a RNA Recognition Motif (RRM) (marked in red; Fig. 1E,F). Sequence alignments showed this RRM domain is 90% (77/85 amino acids) identical to the RRM domain found in human ataxin 2-binding protein 1 (A2BP1). This protein is also referred to as Fox1 based on its homology to the *C. elegans* gene product Feminizing locus On X. Aligning the entire amino acid sequence of *CG32062* against human A2BP1 revealed another short region of homology (55% identical; 22/40 residues) at the C termini of both proteins.

Performing BLAST analysis of human A2BP1 sequence against the *Drosophila* genome showed *CG32062* to be the closest homolog of *A2BP1*. Because of these similarities, we will refer to *CG32062* as *A2BP1* hereafter.

Human A2BP1 was first identified based on its association with human ataxin 2, a protein implicated in spinocerebellar ataxia type 2 (Shibata et al., 2000). Recent studies suggest A2BP1 may also contribute to several other human diseases, including cancer, autism and osteoarthritis (Cheung et al., 2008; Huang et al., 2009; Martin et al., 2007; Zhai et al., 2009). Previous molecular analysis of mammalian A2BP1 has focused on its role in alternative splicing (Auweter et al., 2006; Fukumura et al., 2009; Jin et al., 2003; Kuroyanagi et al., 2007; Lee et al., 2009; Minovitsky et al., 2005; Nakahata and Kawamoto, 2005; Underwood et al., 2005; Zhang et al., 2008; Zhou et al., 2007; Zhou and Lou, 2008). However, the localization of A2BP1 is not always restricted to the nucleus. In mammals, *A2BP1* itself undergoes alternative splicing and several of the resulting isoforms localize to the cytoplasm, leaving open the possibility that the protein carries out additional functions (Nakahata and Kawamoto, 2005). Given the dynamic subcellular localization patterns of A2BP1 during oogenesis, we considered the possibility that *Drosophila* A2BP1 protein also has multiple functions relating to RNA metabolism.

A2BP1 protein is expressed in a novel pattern during early cyst development

We raised a polyclonal antibody against a region of the endogenous A2BP1 protein common to all annotated isoforms. Staining *A2BP1^{CC00511}* heterozygotes with this A2BP1 antibody showed the GFP protein trap displayed the same expression pattern and subcellular localization as the endogenous protein (see Fig. S1A in the supplementary material). In situ hybridization suggested the expression of endogenous *A2BP1* within the gerarium was regulated at the level of transcription (see Fig. S1B in the supplementary material).

To further define the spatial and temporal expression pattern of A2BP1, we compared its protein expression to a set of molecular markers that define different developmental stages during early cyst development (Fig. 2). The first two markers we examined were Nanos and Bam (Fig. 2A). Nanos is expressed in all GSCs

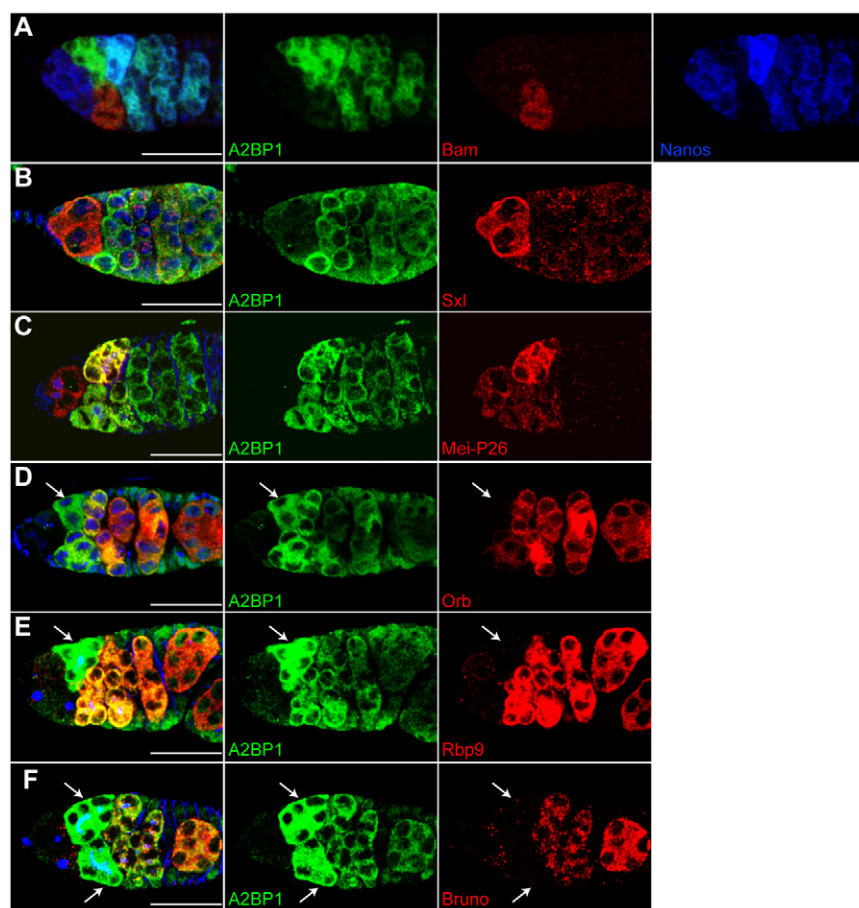


Fig. 2. A2BP1 is expressed in a novel pattern in the gerarium. (A) Gerarium stained for A2BP1 (green), Bam (red) and Nanos (blue).

A2BP1 is expressed in intermediate cysts that express low levels of Bam and Nanos.

(B) Gerarium stained for A2BP1 (green), Sxl (red) and DNA (blue). (C) Gerarium stained for A2BP1 (green), Mei-P26 (red) and 1B1 (blue).

(D-F) A2BP1 expression precedes high levels of expression of other differentiation markers such as Orb, Rbp9 and Bruno. (D) Gerarium stained for A2BP1 (green), Orb (red) and DNA (blue).

(E) Gerarium stained for A2BP1 (green), Rbp9 (red) and 1B1 (blue). (F) Gerarium stained for A2BP1 (green), Bruno (red) and 1B1 (blue).

Scale bars: 20 μ m. Arrows point to cysts that express high levels of A2BP1 and low levels of the indicated differentiation markers.

but largely repressed in dividing cysts (Wang and Lin, 2004). Nanos reappears at high levels in newly formed 16-cell cysts. By contrast, Bam expression is first observed in cystoblasts and persists up until the eight-cell cyst stage. Staining for these two proteins and A2BP1 revealed that A2BP1 exhibits a novel expression pattern. A2BP1 was not expressed in the earliest Bam-positive cells but Bam and A2BP1 expression overlapped in four-cell and eight-cell cysts. As A2BP1 levels increased in eight-cell cysts, Bam expression decreased. A2BP1 expression peaked in cysts after Bam expression was no longer observed and as Nanos expression began to re-emerge.

Next, we compared A2BP1 expression with Sxl and Mei-P26, two markers that label GSCs and early cysts (Fig. 2B,C). Others have shown Sxl is cytoplasmic in GSCs, the cystoblast and two-cell cysts (Bopp et al., 1993; Chau et al., 2009). In later cysts, Sxl cytoplasmic expression levels decrease and the protein moves into the nucleus. Double labeling with Sxl and A2BP1 antibodies revealed that cytoplasmic Sxl did not overlap with A2BP1 in the gerarium (Fig. 2B). Like Sxl, Mei-P26 protein is first detected in GSCs (Liu et al., 2009; Neumuller et al., 2008). Mei-P26 expression continues to increase during cyst development, peaking in 16-cell cysts. Cytoplasmic A2BP1 expression overlapped with Mei-P26 expression and persisted in cysts even after Mei-P26 levels had fallen below the levels of detection (Fig. 2C).

Finally, we compared the expression of A2BP1 with Orb, Rbp9 and Bruno, the translational repressor encoded by the *arrest* gene (Fig. 2D-F). All three of these proteins exhibit very low levels of expression in GSCs and early cysts. The expression of these proteins dramatically increases in 16-cell cysts. We found A2BP1

expression preceded the highest levels of expression of Orb, Rbp9 and Bruno. Together, these data suggest A2BP1 exhibits a novel expression pattern that bridges the expression of the early differentiation marker Bam and the expression of late markers such as Orb, Rbp9 and Bruno.

A2BP1 germline expression depends on *bam*, *mei-P26* and *snf*, but not on *rbp9* or *arrest*

Although changes in fusome morphology reflect cell division (de Cuevas and Spradling, 1998), we reasoned that A2BP1 protein expression might provide a new molecular marker for evaluating germline cysts as they progress through differentiation. To test this idea, we examined A2BP1 antibody staining in ovaries from a variety of female sterile mutants. We also co-stained these samples with the 1B1 monoclonal antibody that labels fusomes and cell membranes. We first looked at A2BP1 expression in *bam* mutants that contain only single stem cell-like cells. As expected, given their early block in differentiation, *bam* mutant germline cells do not express high levels of A2BP1 (Fig. 3B). Next, we examined A2BP1 expression in *snf*, *mei-P26*, *rbp9* and *arrest* mutant ovaries, all of which contain cystic germline tumors of varying severity. We did not observe high levels of cytoplasmic A2BP1 staining in the germline cells of *mei-P26* and *snf* mutant ovaries, despite the presence of multicellular cysts (Fig. 3C,D). By contrast, germline cells from *rbp9* and *arrest* mutants both expressed readily detectable levels of A2BP1 at the appropriate stages (Fig. 3E,F). These data show, consistent with previous findings, that different ovarian tumor mutants arrest cyst development at distinct steps.

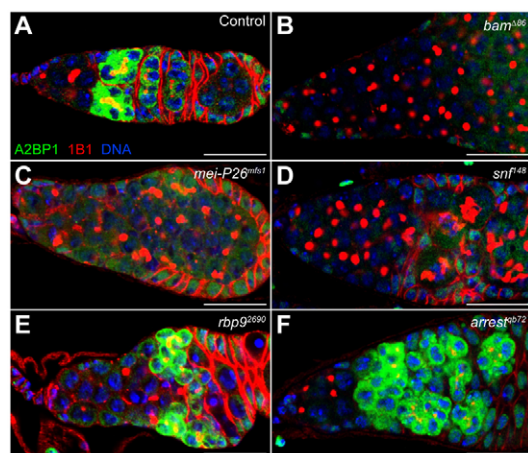


Fig. 3. A2BP1 expression shows that different female sterile mutations block cyst differentiation at discrete steps. (A) Control, (B) *bam*^{Δ86}, (C) *mei-p26*^{mf51}, (D) *snf*^{l48}, (E) *Rbp9*²⁶⁹⁰ and (F) *arrest*^{qb72} ovaries stained for A2BP1 (green), 1B1 (red) and DNA (blue). A2BP1 is not readily detected in *bam*^{Δ86}, *mei-p26*^{mf51} or *snf*^{l48} mutant ovaries but is expressed at normal levels in *Rbp9*²⁶⁹⁰ and *arrest*^{qb72} mutant ovaries. Scale bars: 20 μm.

A2BP1 mutants display defects in early germline differentiation

In order to determine whether A2BP1 functioned during cyst differentiation, we sought to characterize *A2BP1* mutant phenotypes. Previous transposon screens yielded a number of inserts in and around the *A2BP1* locus (Fig. 1F). These included both *P*-elements (*A2BP1*^{CC00511}, *A2BP1*^{KG06463}) and *piggyBac* elements (*A2BP1*^{e03440}, *A2BP1*^{f01889}, *A2BP1*^{f02600}) (Bellen et al., 2004; Buszczak et al., 2007; Thibault et al., 2004). We crossed these alleles to molecularly defined deficiencies that uncover the region and to each other (see Materials and methods). This complementation analysis revealed that some of these insertions resulted in a female sterile phenotype (see Table S1 in the supplementary material). To further characterize how *A2BP1* mutations disrupt normal oogenesis, we stained ovaries with anti-Vasa antibodies to specifically label germline cells and the 1B1 monoclonal antibody. This analysis revealed that both fertile and sterile *A2BP1* mutants exhibited a range of related phenotypes (see Table S1 in the supplementary material; Fig. 4). Hemizygous and transheterozygous *A2BP1*^{CC00511} and *A2BP1*^{f02600} females were fertile but a large percentage of their egg chambers contained 31 nurse cells and one oocyte (Fig. 4B,C; see Fig. S2 in the supplementary material). In addition to cell counting defects, *A2BP1*^{f02600} mutant ovaries often appeared slightly tumorous and their egg chambers contained fusome remnants (Fig. 4C). Counting the number of ring canals within *A2BP1*^{CC00511} oocytes revealed that the extra nurse cells within these mutants arose from an extra round of mitosis prior to the egg chambers pinching off of the germarium (see Fig. S2 in the supplementary material). The same phenotype has been observed in *encore* mutants (Hawkins et al., 1996; Hawkins et al., 1997; Van Buskirk et al., 2000). The *encore* gene encodes a conserved protein that negatively regulates Bam protein expression during late cyst development. Interestingly, we observed an expansion of Bam expression in *A2BP1*^{CC00511} mutants (see Fig. S2D in the supplementary material). We also found that a *bam* null allele dominantly suppressed the extra nurse cell phenotype of *A2BP1*^{CC00511} mutant cysts (see Fig. S2E in the

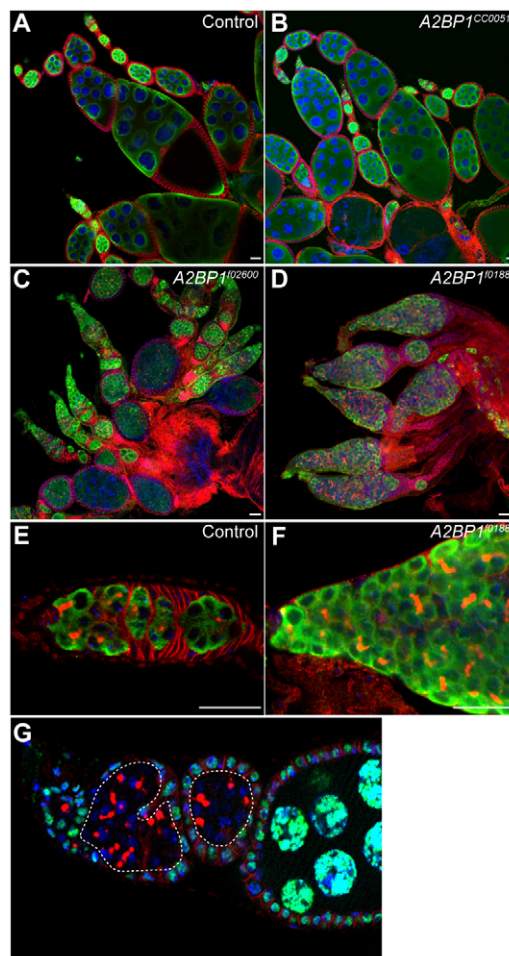


Fig. 4. A2BP1 mutations disrupt normal germline cyst development. (A,E) Control, (B) *A2BP1*^{CC00511}, (C) *A2BP1*^{f02600} and (D,F) *A2BP1*^{f01889} hemizygous ovaries stained for VASA (green), 1B1 (red) and DNA (blue). The *A2BP1*^{CC00511} mutation results in the formation of egg chambers with extra nurse cells. The *A2BP1*^{f02600} allele displays both germ cell counting defects and a mildly tumorous phenotype. *A2BP1*^{f01889} mutants exhibit a pronounced germline cystic tumor phenotype. (G) Negatively marked germline clones of the *A2BP1*^{e03440} mutation stained for GFP (green), 1B1 (red) and DAPI (blue). Mutant germline cells (outlined with broken line) do not differentiate and form pseudo-egg chambers. Scale bars: 20 μm.

supplementary material). Moreover, the *encore*^{q4} allele strongly enhanced the *A2BP1*^{CC00511} phenotype so that fusomes persisted in egg chambers well beyond the time when the organelle would normally be degraded (see Fig. S2F-H in the supplementary material). These findings suggested *A2BP1* was required for the final maturation of 16-cell germline cysts within the germarium.

Stronger hypomorphic alleles of *A2BP1* exhibited more severe phenotypes that further implicated *A2BP1* as a crucial regulator of germline cyst development. Females homozygous and hemizygous for *A2BP1*^{KG06463}, *A2BP1*^{f01889} and *A2BP1*^{e03440} were sterile. Ovaries from these females were small and did not contain maturing egg chambers. Anti-Vasa and 1B1 staining revealed strong *A2BP1* mutants displayed a tumorous phenotype (Fig. 4D,F). Germaria from these females had a large number of multicellular germline cysts, reminiscent of cystic tumors caused by mutations in *mei-P26* and *snf* (Fig. 4D) (Chau et al., 2009; Page

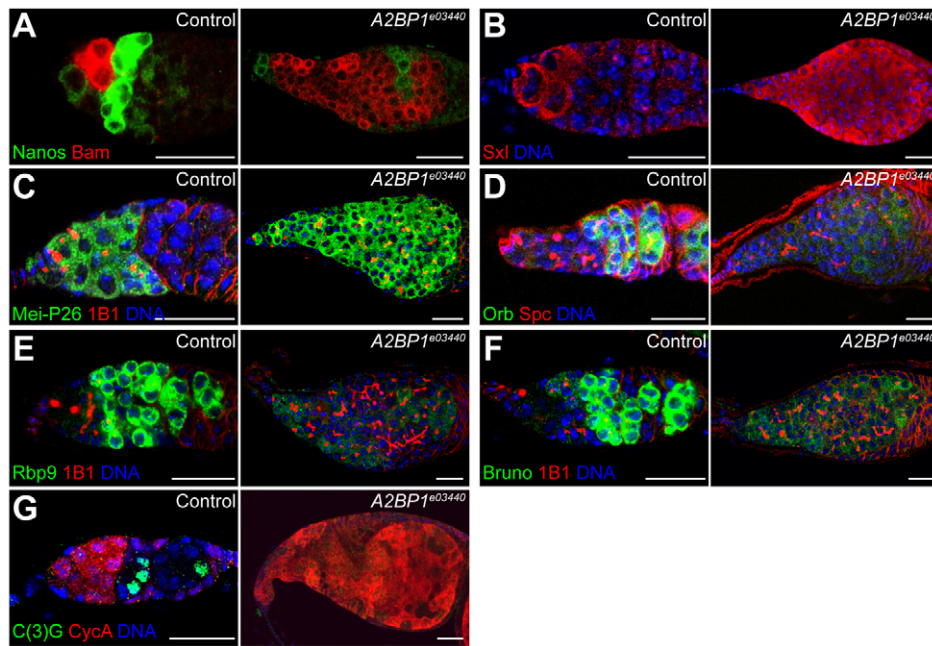


Fig. 5. Disruption of *A2BP1* blocks intermediate cyst differentiation.

(A) Control and *A2BP1*^{e03440} hemizygous ovaries stained for Nanos (green) and Bam (red). (B) Control and *A2BP1*^{e03440} hemizygous ovaries stained for Sxl (red) and DNA (blue). (C) Control and *A2BP1*^{e03440} hemizygous ovaries stained for Mei-P26 (green), 1B1 (red) and DNA (blue). (D) Control and *A2BP1*^{e03440} hemizygous ovaries stained for Orb (green), Spectrin (Spc) (red) and DNA (blue). (E) Control and *A2BP1*^{e03440} hemizygous ovaries stained for Rbp9 (green), 1B1 (red) and DNA (blue). (F) Control and *A2BP1*^{e03440} hemizygous ovaries stained for Bruno (green), 1B1 (red) and DNA (blue). (G) Control and *A2BP1*^{e03440} hemizygous ovaries stained for C(3)G (green), Cyclin-A (CycA) (red) and DNA (blue). Scale bars: 20 μm.

et al., 2000). Follicle cells often enveloped these undifferentiated germ cells to form pseudo-egg chambers. A genomic construct containing the *A2BP1* locus without the nested *CG6527* gene rescued the tumor phenotypes of both *A2BP1*^{e03440} and *A2BP1*^{KG06463} mutants, verifying that the observed phenotypes resulted from disruption of *A2BP1*.

To determine whether *A2BP1* functions in the germline or in the surrounding somatic cells, we employed FRT-mediated mitotic recombination using the *A2BP1*^{e03440} allele. This clonal analysis showed germ cells homozygous for the *A2BP1*^{e03440} allele failed to differentiate properly and became tumorous (Fig. 4G). By contrast, egg chambers in which the entire follicle cell layer was homozygous for the *A2BP1*^{e03440} allele appeared normal through the early stages of oogenesis (data not shown). These results indicate that *A2BP1* functions cell-autonomously within the germline during early cyst development.

Strong *A2BP1* mutants arrest before the mitotic to meiotic transition

To characterize the molecular function of *A2BP1*, we tested whether a strong hypomorphic allele of *A2BP1* affected the expression of early and late markers within the germline. First, we compared Nanos and Bam staining in wild-type and *A2BP1*^{e03440} hemizygous ovaries. This analysis showed disruption of *A2BP1* resulted in a dramatic expansion of Bam expression and a subsequent delay in late Nanos expression (Fig. 5A). The early expression of Nanos within GSCs remained unaffected, suggesting that *A2BP1* mutations do not cause a GSC phenotype (see Fig. S3 in the supplementary material). Previous results have shown that Bam helps repress the translation of *nanos* mRNA (Li et al., 2009). We found expression of Nanos and Bam remained mutually exclusive from one another in *A2BP1* mutant cysts. These data indicated that *A2BP1* does not directly participate in the negative regulation of Nanos (see Fig. S3 in the supplementary material) and suggested that *A2BP1* functions downstream of the initial steps of cyst differentiation.

In wild-type ovaries, cytoplasmic Sxl and *A2BP1* were expressed in mutually exclusive patterns (Fig. 2B). Given this relationship, we tested whether disruption of *A2BP1* results in an

expansion of cytoplasmic Sxl expression (Fig. 5B). Normally cytoplasmic Sxl expression is limited to the GSCs, cystoblasts and early multicellular cysts in the anterior region of the ovarium. However, *A2BP1*^{e03440} hemizygous mutants displayed cytoplasmic Sxl expression in germ cells throughout the ovarium, including in eight-cell and 16-cell cysts. Likewise, the expression of Mei-P26 appeared greatly expanded in *A2BP1*^{e03440} mutants (Fig. 5C). The expanded expression of both these markers indicated that *A2BP1* mutants were unable to properly regulate gene expression during cyst development.

To further examine how mutations in *A2BP1* affected gene expression during cyst development, we examined the expression of three late markers in control and *A2BP1*^{e03440} mutant ovaries: Orb (Fig. 5D), Rbp9 (Fig. 5E) and Bruno (Fig. 5F). Normally, early developing cysts express low levels of Orb, Rbp9 and Bruno. The expression of all three genes increases in 16-cell cysts. By contrast, *A2BP1*^{e03440} mutant ovaries displayed severe reductions in Orb, Rbp9 and Bruno expression, even though 16-cell cysts were present in the samples. These findings support the conclusion that *A2BP1* promotes the expression of proteins needed for the final steps of cyst development.

A2BP1 mutant cysts appeared to remain mitotically active beyond the point when the meiotic program should normally be initiated. To test formally whether *A2BP1* mutant germline cells displayed defects in the mitotic to meiotic transition, we stained control and mutant ovaries for C(3)G and Cyclin A (Fig. 5G). C(3)G is a component of the synaptonemal complex (Page and Hawley, 2001) and in wild-type samples accumulates at high levels after Cyclin A levels drop and cells have exited the mitotic cell cycle (Sugimura and Lilly, 2006). Hemizygous *A2BP1*^{e03440} mutants displayed defects in Cyclin A and C(3)G staining (Fig. 5G). Cyclin A expression persisted throughout the ovarium and robust C(3)G accumulation was not observed. Together, these experiments indicate that *A2BP1* functions to promote changes in gene expression within four-, eight-cell and 16-cell cysts. The misregulation of these gene expression programs within *A2BP1* mutants appears to block the normal transition from a mitotic cell cycle to a meiotic cell cycle.

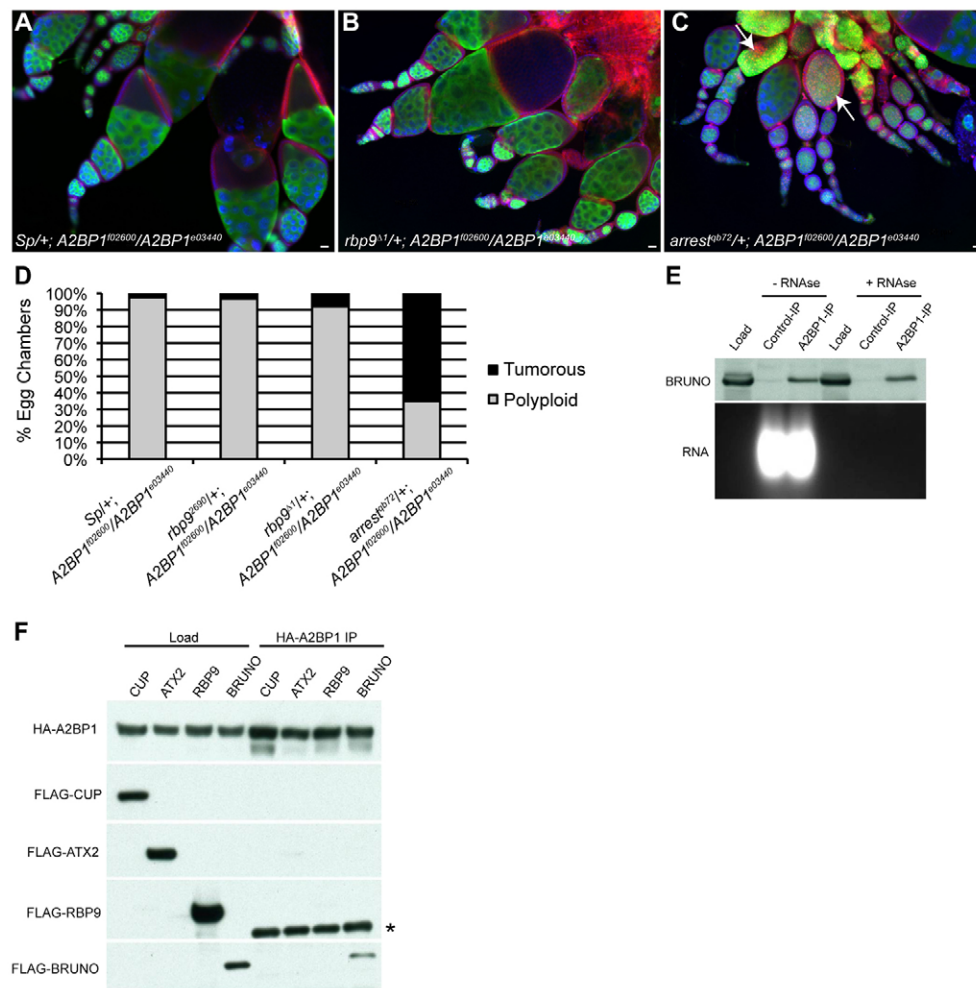


Fig. 6. A2BP1 interacts with Bruno but not with Rbp9. (A) *Sp/+; A2BP1^{f02600}/A2BP1^{e03440}*, (B) *rbp9^{Δ1}/+; A2BP1^{f02600}/A2BP1^{e03440}* and (C) *arrest^{qb72}/+; A2BP1^{f02600}/A2BP1^{e03440}* ovaries stained for Vasa (green), 1B1 (red) and DAPI (blue). A null mutation in *rbp9* does not enhance the weak *A2BP1^{f02600}/A2BP1^{e03440}* phenotype. However, one copy of the *arrest^{qb72}* mutation modifies the weak *A2BP1* phenotype so that tumorous pseudo-egg chambers (arrows) are now observed. (D) Graph showing quantification of the phenotype. (E) (Top panel) Western blot of an A2BP1 immunoprecipitation probed for Bruno. (Bottom panel) Gel showing RNA depletion in the RNAse treated extracts. A2BP1 and Bruno physically interact even in the absence of RNA. (F) Western blot of immunoprecipitations using extracts from S2 cells expressing FLAG-tagged proteins and HA-tagged A2BP1. Scale bars: 20 μ m.

A2BP1 isoforms associate with each other

We considered the possibility that different A2BP1 isoforms might form a functional complex together. To test this hypothesis, we performed a series of genetic and biochemical assays. Interestingly, our phenotypic analysis showed that the *A2BP1^{KG06463}* allele was fertile when placed over either of the other strong sterile alleles (*A2BP1^{f01889}* or *A2BP1^{e03440}*) but not when placed over a small molecularly defined deficiency (see Table S1 in the supplementary material). However, careful examination revealed that germline cysts from *A2BP1^{KG06463}/A2BP1^{f01889}* and *A2BP1^{KG06463}/A2BP1^{e03440}* ovaries displayed counting defects at high penetrance and a mildly tumorous phenotype at low penetrance (see Fig. S4A,B in the supplementary material). Western blot analysis showed that these different mutations disrupt the expression of different A2BP1 isoforms, thus providing a possible explanation for their partial complementation (see Fig. S4C in the supplementary material). We tested for interactions between different isoforms using both yeast 2-hybrid and co-

immunoprecipitation assays. The yeast 2-hybrid experiments revealed an interaction between A2BP1-PI and A2BP1-PE (see Fig. S4D in the supplementary material). Furthermore, co-immunoprecipitation experiments using epitope-tagged versions of different A2BP1 isoforms also indicated various A2BP1 isoforms associated with themselves and with each other (see Fig. S4E in the supplementary material; data not shown).

A2BP1 interacts with Bruno but not Rbp9

To further investigate the developmental function of A2BP1, we tested for genetic interactions between *A2BP1* and several other mutants. We focused our efforts on *rbp9* and *arrest*, two genes that function during the late stages of cyst development within the germarium. We reasoned that if *A2BP1* worked together with *rbp9* or *arrest*, mutations in these genes would enhance weak *A2BP1* mutant phenotypes. To test this, we crossed single copies of *rbp9* and *arrest* mutations into the *A2BP1^{f02600}/A2BP1^{e03440}* background. *A2BP1^{f02600}/A2BP1^{e03440}* transheterozygotes were weakly fertile

but nearly all cyst cells from these mutants underwent an extra mitotic division so that the resulting egg chambers contained 32 germline cells. Of note, the presence of germline tumors and pseudo-egg chambers was extremely rare in this background (Fig. 6D). Interestingly, the *rbp9^{Δ1}* and *rbp9²⁶⁹⁰* alleles did not modify this weak *A2BP1* mutant phenotype (Fig. 6B,D). These flies continued to lay eggs and there was little evidence of increased tumor formation (Fig. 6D). By contrast, the *arrest^{qb72}* mutation dramatically enhanced the phenotype of *A2BP1^{l02600}/A2BP1^{e03440}* transheterozygotes (Fig. 6C). These flies were sterile and displayed a tumor phenotype similar to the strongest *A2BP1* mutants. Moreover, we observed a marked increase in the number of pseudo egg chambers within these samples (Fig. 6D). These genetic interactions suggested a functional link between *A2BP1* and *arrest*.

The *arrest* gene encodes the translational repressor Bruno. Previous studies have shown that Bruno protein binds to the 3'UTR of *sxl*, *Cyclin A* and other target mRNAs and represses their translation in the posterior region of the germarium (Sugimura and Lilly, 2006; Wang and Lin, 2007). Given the cytoplasmic localization of A2BP1 during cyst development and its genetic interaction with *arrest*, we considered the possibility that A2BP1 might physically associate with Bruno. To test this, we made extracts from whole ovaries and conducted immunoprecipitation experiments using anti-A2BP1 antibodies (Fig. 6E; data not shown). In parallel, we treated separate extracts with RNase A to test whether any observed interactions were dependent on the presence of RNA. We probed the resulting immunoprecipitation pellets for the presence of Bruno. These experiments revealed that A2BP1 associated with Bruno even in the absence of RNA.

Previous work has demonstrated interactions between mammalian A2BP1 and ataxin 2 (Atx2), and between *Drosophila* Bruno and Cup (Nakamura et al., 2004; Shibata et al., 2000). To test whether *Drosophila* A2BP1 interacted with these proteins, we cloned sequences corresponding to *Drosophila* cup and *Atx2* into FLAG-tagged S2 cell expression vectors. We also placed the open reading frame of *rbp9* into this vector. A2BP1-RE sequence was cloned into a separate S2 cell expression vector that carried a Hemagglutinin (HA) tag. We expressed these proteins in S2 cells and performed immunoprecipitations on the resulting extracts using HA resin (Fig. 6F). The pellets were probed for the presence of FLAG-tagged proteins. These experiments confirmed that A2BP1 and Bruno physically associated with each other. However, we did not detect interactions between A2BP1 and FLAG-tagged Cup, Atx2 or Rbp9.

DISCUSSION

Here, we report the *Drosophila* homolog of human A2BP1 promotes the differentiation of germline cells into 16-cell cysts. A2BP1 is expressed in a novel pattern during early cyst development and mutations in *A2BP1* disrupt early oogenesis, resulting in the formation of cystic tumors. Our findings suggest that *A2BP1* helps regulate changes in gene expression programs during the intermediate steps of germline cyst development.

A2BP1 marks a distinct step in the molecular differentiation of germline cysts

Past studies have sought to characterize the mechanisms that control *bam* expression in germline stem cells and cystoblasts. These efforts led to the understanding that *dpp* signaling from the cap cells initiates a phosphorylation cascade that results in the transcriptional repression of *bam* in stem cells through a well-defined element within its promoter (Chen and McKearin, 2003a; Chen and McKearin, 2003b; Song et al., 2004; Xie and

Spradling, 1998). Once a stem cell daughter leaves the niche, this repression subsides resulting in active *bam* transcription. The expression of Bam continues up until the eight-cell cyst stage whereupon it is again repressed (McKearin and Ohlstein, 1995). Given these findings and the lack of two-, four- and eight-cell cyst specific markers, the prevailing view has been that all Bam-expressing cysts are roughly equivalent on a molecular level. Subsequently, fusome branching has served as a widely used marker to track the progress of cyst differentiation. However, the expression of A2BP1 now shows that the number of mitotic divisions undertaken by a cyst does not necessarily reflect the underlying molecular state of these cells. In addition to undergoing successive rounds of incomplete mitotic divisions, cystoblasts, and two-, four- and eight-cell cysts also exhibit distinct changes in their gene expression programs. For example, the cystoblast expresses both cytoplasmic Sxl protein and Bam. In two-cell cysts, Sxl expression begins to recede while Bam levels increase. In four-cell cysts, cytoplasmic Sxl protein is no longer detectable, Bam expression continues and A2BP1 protein expression is induced. In eight-cell cysts, Bam expression begins to decrease while A2BP1 expression continues to increase. Finally, in 16-cell cysts, Bam is absent, A2BP1 is present and the expression of other proteins such as Nanos, Orb, Rbp9 and Bruno is upregulated. These markers probably reflect much broader changes in gene expression during cyst development.

In situ hybridization suggests that the regulation of *A2BP1* expression occurs at the level of transcription (see Fig. S1 in the supplementary material). However, given the complexity of the *A2BP1* locus, we cannot completely rule out the possibility that alternative splicing and translational regulation also restrict *A2BP1* expression. Examining A2BP1 protein expression in various mutant backgrounds has allowed us to further subdivide cystic tumors. Consistent with previous phenotypic characterization, A2BP1 expression shows that *mei-P26*, *snf*, *rbp9* and *arrest* tumors are arrested at different stages of cyst differentiation. Loss of *mei-P26* and *snf* blocks the molecular differentiation of cysts prior to the induction of A2BP1 expression. The absence of A2BP1 expression within *mei-P26* and *snf* mutant germline cysts indicates that mitotic divisions can continue in the absence of molecular differentiation. These findings suggest *mei-P26*, *snf* and, by inference, *sxl* help drive cyst development to a point defined by A2BP1. In turn, A2BP1 promotes cyst progression towards a terminally differentiated state marked by reduced levels of Bam and Sxl and increased levels of late markers such as Rbp9 and Orb.

Progressive germline differentiation depends on the ability to repress earlier programs

What is the developmental function of *A2BP1*? A number of mutations that result in germline tumors have been isolated. Some of these mutations disrupt genes involved in the regulation of sex-specific splicing and germline sexual identity. However, the issue of why defects in sexual identity result in tumorous phenotypes remains largely unresolved (Casper and Van Doren, 2009). Other mutations such as *mei-P26* and *arrest* do not have a clear role in establishing sexual identity, suggesting that disruption of other molecular pathways can also block cyst differentiation. Our data, together with previous findings, suggests cysts must turn off earlier programs of gene expression to move to the next stage of differentiation. For example, Bam expression must be repressed in 16-cell cysts. In *encore* and *A2BP1* mutants, the expansion of Bam expression results extra mitotic divisions and a subsequent delay in meiosis (Hawkins et

al., 1996; Van Buskirk et al., 2000). Significantly, *bam* null mutations can suppress both *encore* and weak *A2BP1* mutant phenotypes (Hawkins et al., 1996). Similarly, expansion of cytoplasmic Sxl and Cyclin A in *arrest* and *A2BP1* mutants correlates with an inability to enter meiosis.

The molecular function of A2BP1

A2BP1 contains a highly conserved RNA recognition motif. The mammalian homolog of A2BP1 was first identified based on its association with ataxin 2. We find little evidence that A2BP1 physically or genetically interacts with *Drosophila* Ataxin 2 in S2 cell extracts or during early cyst development (Fig. 6; data not shown). However, this conclusion is based on negative data and the findings that *A2BP1* functions during nervous system development (Koizumi et al., 2007) leaves open the possibility that these two proteins may interact in different contexts.

Our studies suggest a functional link between A2BP1 and Bruno. Interestingly, high levels of A2BP1 expression precede high levels of Bruno expression. However, there are detectable levels of Bruno within four- and eight-cell cysts. These low levels of Bruno expression may be sufficient to coordinate with A2BP1 to promote cyst development. A2BP1 may also have additional functions that do not require interaction with Bruno. Differences between *A2BP1* and *arrest* mutant phenotypes may reflect these separate functions. However, it is important to note that the *Drosophila* genome encodes three highly related Bruno-like proteins (FlyBase). Genetic redundancy between these genes may mask other functions during early cyst development. Regardless, the observed genetic and physical interactions between A2BP1 and Bruno support a model in which these two proteins cooperate to regulate germline cyst differentiation.

Mammalian A2BP1 binds to UGCAUG RNA elements within introns and regulates the alternative splicing of specific messages (Jin et al., 2003; Lee et al., 2009; Underwood et al., 2005). Given the RRM domain in A2BP1 is 90% identical to its mammalian homologs, A2BP1 may also bind to similar elements. The original study that defined the A2BP1 RNA binding sites in vitro showed that the protein associated with GCAUG sites with a slight bias for UGCAUG and AGCAUG sequences (Jin et al., 2003). Further work showed A2BP1 bound preferentially to UGCAUG sites in vivo (Underwood et al., 2005). The cytoplasmic localization of A2BP1 protein in four-, eight- and 16-cell cysts and its association with Bruno suggests it may participate in translational repression. Examination of annotated sequences (FlyBase) reveals that a small number of *Drosophila* transcripts contain multiple UGCAUG and AGCAUG sites within their 3'UTRs. For example, *sxl*-RH 3'UTR has five AGCAUG sites but none of the preferred UGCAUG sites. The functional significance of these elements remains uncertain. Interestingly, Bruno has also been implicated in alternative splicing (Park et al., 2004). Therefore, identifying in vivo mRNA targets of A2BP1 will help clarify its molecular functions and further enhance our understanding of the complex genetic hierarchies that control germline cyst development.

Acknowledgements

We thank T. Schüpbach, H. Salz, R. S. Hawley, J. Knoblich, P. Lasko, H. Lin, M. Lilly, A. Nakamura, J. Kim-Ha, R. Dubreuil, the Developmental Hybridoma Bank, the Bloomington Stock Center and the Harvard Stock Center for reagents. We thank Dhananjay Chaturvedi, Robin Hiesinger and Nevine Shalaby for comments on the manuscript. This work was supported by the E.E.

and Greer Garson Fogelson Endowment (UTSW Medical Center) to M.B. and by the National Institutes of Health grant GM045820 to D.M. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.050575/-DC1>

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