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METHODS AND TECHNICAL ADVANCES

Novel mutants of the *aubergine* gene

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ABSTRACT

Aubergine is an RNA-binding protein of the Piwi clade, functioning in germline in the piRNA pathway that silences transposons and repetitive sequences. Several mutations of this gene exist, but they mostly result in truncated proteins or correspond to mutations that also affect neighboring genes. We have generated complete *aubergine* knock-out mutants that do not disrupt the neighboring genes. These novel mutants are characterized by PCR and sequencing. Their nature is confirmed by female sterility and by the presence of crystals in testes, common to the *aubergine* loss of function mutations. These mutants provide novel and more appropriate tools for the study of the piRNA pathway that controls genome stability.

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Aubergine; argonaute; crystal-stellate; knockout mutant; mutagenesis; piRNA; sterility

Introduction

The Aubergine (Aub) protein belongs to the Piwi clade (Piwi, Aubergine, Argonaute 3) of the Argonaute family of proteins. The main role of this protein clade is to prevent transposon movement in germline cells.[1] Interacting with the RISC complex too,[2] these proteins have been shown to bind Piwi interacting RNAs (piRNAs) and to silence transposable elements,[1] in a Dicer independent way,[3] as reviewed in Siomi et al.[4] piRNAs are 24–30 nucleotide long non-coding RNAs active in germline cells.[5,6] They are utilized to ensure genetic stability in animal gonads, as seen in *Drosophila*,[7] rat,[8] and zebrafish.[9] In this process, the Aub protein also interacts with dFmr1, the *Drosophila* ortholog of the Fragile X Mental Retardation Protein, which is already known as a translational regulator.[10] Besides piRNA maturation, Aub is involved in other RNA-related mechanisms including *nanos* mRNA localization[11] and Poly A tail shortening complex localization,[12] epigenetic regulation such as Polycomb group response element clustering,[13] and chromosome condensation during mitosis.[14] As expected, Aub is highly expressed in ovaries and testes,[15] where it is involved in the piRNA pathway occurring in the germline. It is also expressed in the embryo, where it has a role in pole cell formation. Finally, recent studies call for a role of Aub in the nervous system.[10] The human ortholog of Piwi clade, HIWI was shown to have a similar role[16] and to be expressed in undifferentiated cells.[17] These data highlight the importance of the Piwi clade of proteins in the stability of the genome and in development.

Functional analyses tightly rely on the availability of mutations that disrupt gene activity. The *BSC213* line serves as an *aub* mutant;[18] however this deficiency comprises several genes including *nos, porin, dpr2, SCAR* and *piwi*, in addition to *aub*. Flies that are homozygous for this deficiency are lethal before the 3rd instar larval stage. Other alleles have also been routinely used: aub^{QC42}, aub^{HN2},[19] aub^{N11},[20] and aub^{K86},[21] all generated by ethyl methanesulfonate (EMS) mutagenesis. aub^{HN2} and aub^{K86} carries nonsense mutations and codes for a truncated, supposedly non-functional proteins.[20] aub^{N11} contains a frameshift mutation[20] (Fig. 1A). The molecular lesion of aub^{QC42} is unknown; it is considered a strong hypomorph EMS-induced *aub* allele,[19] confers female sterility, when homozygous.[22] At our growing conditions aub^{QC42} is not completely vital, only few escapers survive; as a consequence we and other groups routinely used the

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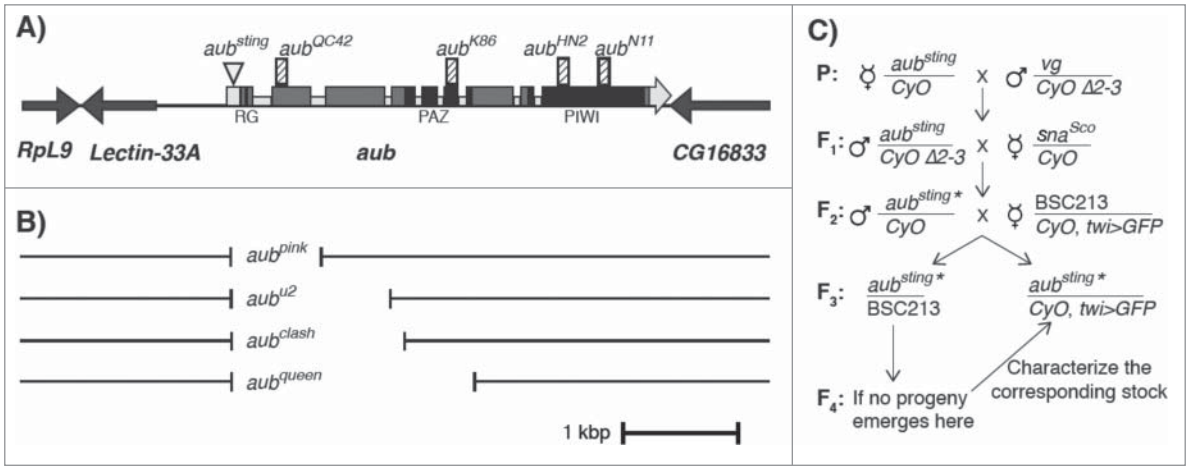

Figure 1. Genomic organization of the *aubergine* region. (A) Layout of *RpL9, Lectin-33A, aub* and *CG16833* genes, arrows indicate their orientation. The aub^{sting} insertional mutation and nonsense mutations aub^{QC42} (Y94, aub^{K86} (Q377), aub^{HN2} (Q622) and frameshift mutation aub^{N11} (G741) are indicated as a triangle and as vertical striped bars on the top of the panel. Light gray boxes represent the 5′, 3′ UTR (big boxes) and the introns (small boxes), dark gray boxes represent the exons; line represents the intergenic regions. Sequences corresponding to RG, PAZ and PIWI domains of *aub* are indicated by black boxes on the exons. (B) 5′ and 3′ breakpoints of the novel *aub* mutants. The graph is in-scale. The scale bar is 1 kg base-pair (kbp). aub^{pink} and aub^{U2} are sequenced using a promoter forward primer (Prom. Fw2 AAATGTGTCCGGAGATTTACAA, see Fig. 2) and the reverse primer in exon 4 (E4 Rv: CCATAATTGCATGCGGAAAT, see Fig. 2). aub^{clash} is sequenced using the forward primer (Prom. Fw GAATTCAACGATGCCTTTTCA see Fig. 2) and the reverse primer in exon 7 (E7 Rv: CAGCTCGATGTTCCAGGACT, see Fig. 2). aub^{queen} is sequenced using the forward primer Prom. Fw, and the reverse primer in exon 9 (E9 Rv GTGGAGGGGGATCACTACCT, see Fig. 2). (C) Crossing scheme for *aub* mobilization. Only the 2nd chromosome is represented. *Δ2–3* indicates the transposase containing line. *vg* and sna^{Sco} are selectable phenotypic markers. *CyO* represents the balancer second chromosome and *CyO, twi>GFP* the fluorescent labeled (*twist-Gal4, UAS-GFP*) balancer chromosome. *BSC213* indicates the deficiency line eliminating *aub* and many other neighboring genes. aub^{sting*} represents a potential *aub* mutant upon P element excision.

aub^{HN2}/aub^{QC42} transheterozygotes, in order to analyze *aub* "loss of function."[3,23-25]

For the above reasons, we decided to produce novel and null *aub* alleles by mobilizing the P element transposon present in the *P{lacW}* aub^{sting} allele, also known as aub^{sting}. P-element mutagenesis is a convenient method to obtain knock-out mutants. Here we report the phenotypic characterization of a novel *aub* allele that completely eliminates the expression of the Aub protein and leaves the adjacent genes unaffected. This provides a novel and efficient tool to study the role of the Aub protein in genome stability, sterility and neuronal plasticity.

Results

Molecular characterization of the aub^{QC42} *allele*

The Aub protein contains typical domains as reviewed by Höck.[26] The arginine-glycine rich domain (a.k.a. RG domain, aminoacids 11–17) is necessary to interact with Tudor proteins;[27,28] the PAZ domain (Piwi-Argonaut-Zwille) located on the N terminal half (aminoacids 280–413) serves as a docking site for 3′ end of small RNA;[29] and the PIWI domain, located on the C-terminal half (amino acids 554–852) of the protein having a function similar to RNase H[30] (Fig. 1A).

First, we characterized the molecular lesion contained in the aub^{QC42} flies. This mutation was isolated in a screen for EMS-induced female sterility.[19] FlyBase describes this allele as a point mutation, but no description of the aub^{QC42} lesion has been so far reported. We amplified the *aub* cDNA, obtained from RNA extracted from heterozygous animals, with different primer pairs (see Materials and Methods) and sequenced the amplicons. We found a putative point mutation at the 393rd base of cDNA in some fragments, leading to a premature stop at codon 94 (Fig. 1A). In order to confirm the aub^{QC42} molecular lesion, we cloned and sequenced genomic fragments and the analysis of two independent cDNA clones confirmed the lesion.

P element mediated mutagenesis

The fact that aub^{QC42}, the allele coding for the smallest truncated Aub protein available in the community still codes for the first 93 amino acids that contain the arginine-glycine rich domain prompted us to perform

a mutagenesis in order to produce a null allele. aub^{sting} carries a *P{lacW}* element inserted 58 base upstream to the translational start site of the *aub-RA* transcript[15] (Fig. 1A). This allele behaves as an *aub* gain of function in somatic cells, due to its ectopic expression in these cells, and as a loss of function in the germline.[10] *P{lacW}* is an engineered[31] P element that carries two inverted repeats at the termini, a *white*$^{+}$plus; (w^{+}) wild type gene leading to red eye as a phenotypic marker of the insertion[32] and no longer contains the gene coding for the transposase. Strains that had lost the w^{+} marker were selected as potential precise/imprecise excisions (Fig. 1C).

aub^{sting}, the P{lacW} element inserted in the first exon of *aub* was mobilized to create knockout mutants by imprecise excision in this experiment.

The putative *aub* mutants were then selected based upon female sterility and subsequently characterized by analytical PCR using primers matching to different regions (Fig. 2). The exact breakpoints were determined by sequencing (Fig. 1B). In total, 103 crosses were set up at F2 with single *w* males. 44 lines carry internal deletions (where just part of the P element is deleted); 49 lines represent precise excisions (the entire P element is excised out, without disrupting the surrounding sequence), 10 carry imprecise excisions (where the insertion flanking area is disrupted). The interesting lines were isogenized upon crossing with a multiple balancer (Bloomington Stock Center #3703) (Fig. 1C).

The 10 imprecise excisions represent independent events and carry different mutations. The largest deletion, named aub^{queen}, removes 2144 bp (2047 bp after the AUG codon). The border sequences are 5′–ATAACTCACATCCCTGGGCG–3′ on the 5′ UTR and 5′–GGACTCCGCCTTGGTGGAGA–3′ in exon 7. The second largest deletion, aub^{clash} removes 1452 bp (1355 bp after the AUG). The border sequences are 5′–ATAACTCACATCCCTGGGCG–3′ on the 5′ UTR and 5′–CACAAGGTTATGCGAACTGA–3′ in exon 4. The aub^{U2} allele lacks 1345 bp total (1248 bp after the AUG), from 5′ UTR until mid-intron 3 whereas aub^{pink} lacks 759 bp total (662 bp after the AUG), from 5′ UTR until mid-intron 2. Unlike the 3 other female sterile alleles, aub^{pink} is only partially sterile. aub^{btls}, an internal deletion, contains 2 kb of the P element present in the promoter region of *aub*. Although this mutant could not be sequenced, it might serve as a proper *aub* mutant as female sterility suggests.

We decided to further characterize the aub^{clash} allele. The deletion spans from 11001465 to position 11000010 of the AE014134.6 genomic clone, which carries the *aubergine* gene. The 1452 bp deletion eliminates the AUG of the Aub protein and no possible protein can be produced from the rearranged region, which we also verified by Western Blot on protein extract from mutant testes (Fig. 2D). Thus, aub^{clash} can be considered a null *aubergine* allele.

Phenotypic characterization of the aub^{clash} null allele

In order to analyze the new *aub* allele we assessed several phenotypes that are typical of the *aub* mutation: 1) sterility,[19] 2) the presence of crystals made of the Stellate protein in the mutant testes, 3) the presence of morphological abnormalities and variation in the adult, 4) the potential to rescue the crystal phenotype observed in testes that lack the dFmr1 protein.[10,15,19]

We compared the sterility of aub^{clash} males and females in comparison with that of aub^{HN2}/aub^{QC42} transheterozygous, aub^{sting} homozygous and control animals. The graph in Figure 3A,B shows that aub^{clash} mutants females are completely sterile as are the aub^{HN2}/aub^{QC42} transheterozygous females. aub^{clash} mutant males are partially fertile (16% compared to wild type) and this phenotype is more severe than that of aub^{HN2}/aub^{QC42} transheterozygous animals, whose fertility is 84% of that of wild type animals.

As the other *aub* mutants, aub^{clash} testes display Stellate-made crystalline aggregates in their spermatocytes[10,15] (Fig. 3C–E) and are enlarged[10] (Fig. 3J–L).

It has been previously shown that the reduced levels of the Hsp83 and SpindleE proteins, 2 other components of the piRNA pathway, generate phenotypic variation by transposon-mediated mutagenesis (at 0.9% and 12% frequency, respectively, see[33]). This includes the lack of bristles on the notum (Sco-like phenotype), a dark notum, notched or abnormally everted wings. We hence tested the possibility that the reduction of Aub levels has a similar effect and analyzed aub^{clash} homozygous as well as aub^{HN2}/aub^{QC42} transheterozygous adults. Morphological phenotypes are indeed present in a significant fraction of the mutant animals: aub^{clash} homozygotes exhibit a 7.6% frequency

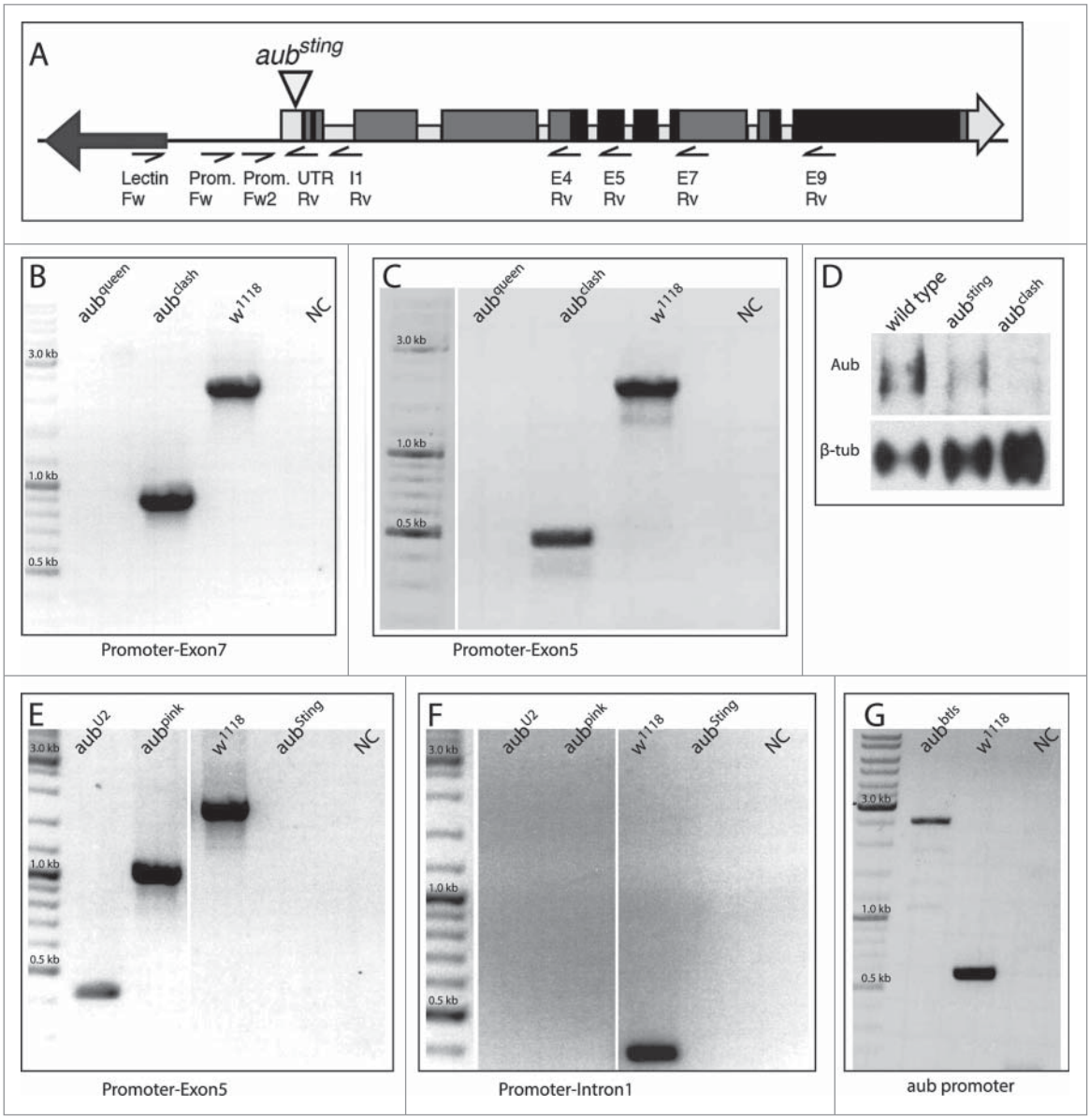

Figure 2. Analytical PCR for *aub* mutants. (A) Map of the primers used to characterize the *aub* mutants. (B, C) Characterization of aub^{queen} and aub^{clash}. (B) PCR using primers from the *aub* promoter (Prom. Fw) and from exon 7 (E7 Rv). w^{1118} is expected to show a band at 2367 bp. aub^{queen} is not expected to show a PCR product, since exon 7 is mostly deleted; and aub^{clash} is expected to show a band at 915 bp (due to a deletion of 1452 bp). (C) PCR using primers from the *aub* promoter (Prom. Fw) and from exon 5 (E5 Rv, AATGGCGTCGATTGAAAGTC). w^{1118} is expected to show a band at 1903 bp. aub^{queen} is not expected to give a band, since exon 5 is entirely deleted; and aub^{clash} is expected to show a band at 451 bp. (D, E) Characterization of aub^{U2} and aub^{pink}. (D) Western Blot on protein extracts from adult testes of the indicated genotypes. Tubulin was used as a loading control. (E) PCR using primers from the *aub* promoter (Prom. Fw2) and from exon 5 (E5 Rv). w^{1118} is expected to show a band at 1757 bp. aub^{U2} is expected to show a band at 412 bp (due to a deletion of 1345 bp), aub^{pink} is expected to show a band at 998 bp (due to a deletion of 759 bp). aub^{Sting} is not expected to produce a product using these PCR conditions, since the P element is too large (>10 kb). (F) PCR using primers from *aub* promoter (Prom. Fw2) and intron 1 (I1 Rv, CAAGGCCAAGCTAATTTTGGA). w^{1118} is expected to show a band at 333 bp. aub^{U2} and aub^{pink} are not expected to display a PCR product since intron 1 is entirely deleted in both. aub^{Sting} is not expected to display a product in these PCR conditions, due to the large size of the P element. (G) Characterization of aub^{btls}. PCR using primers from the *Lectin-33A* promoter (Lectin Fw, TAAACGCTCGGCAGAGAACT) and from *aub* 5′ UTR (UTR Rv, GTTAGACGCCCAGGGATGT). w^{1118} is expected to display a band at 575 bp. aub^{btls} displays a 2.5 kb band due to the presence of P element sequences. For all panels, w^{1118} is used as wild type control; NC stands for negative control, that is, a PCR reaction with no DNA. The DNA ladder used is Thermo-Fisher GeneRuler™ High Range SM0331.

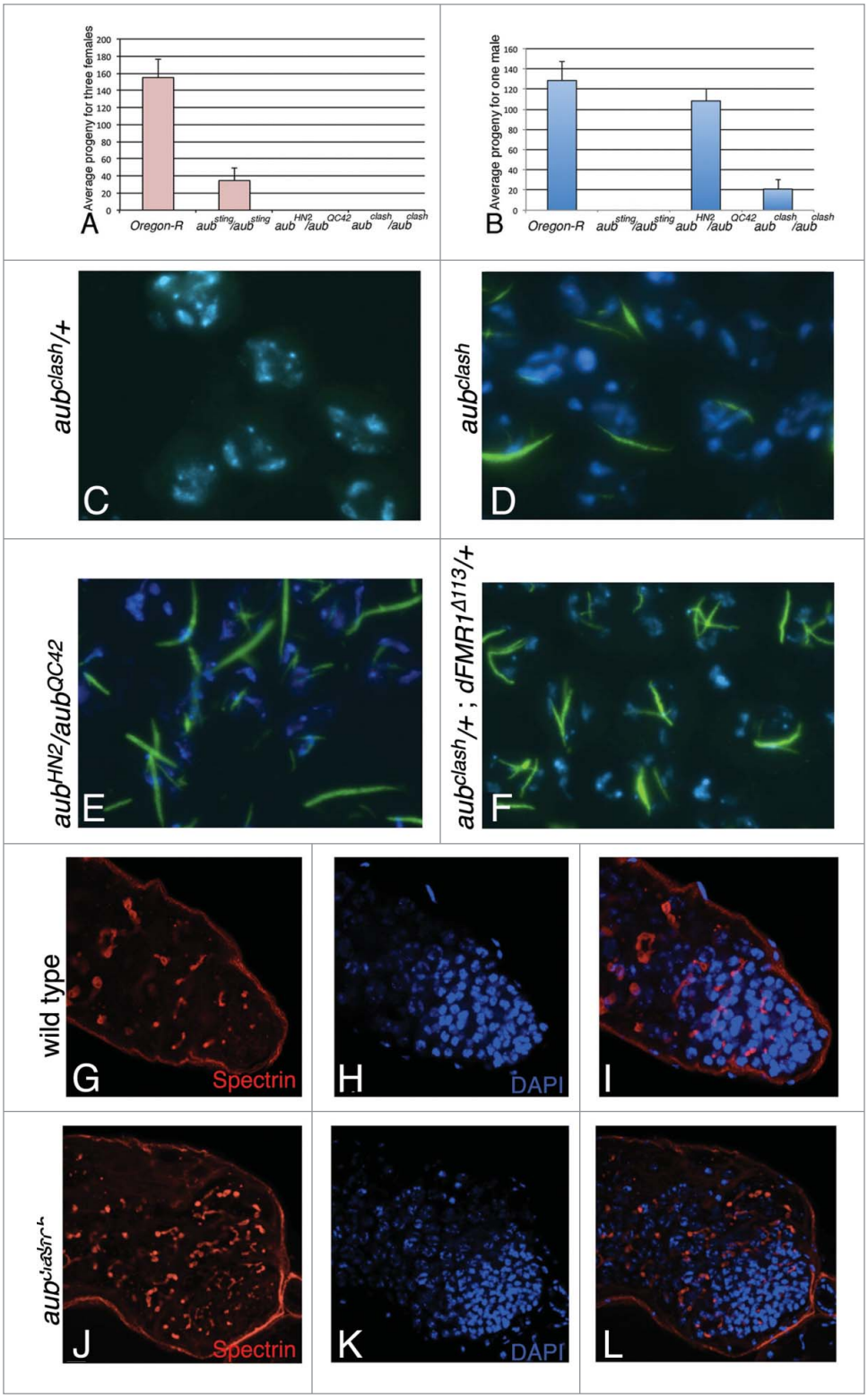

Figure 3. Phenotypic characterization of the *aub*^{clash} allele (A) Female fertility in the mentioned genotypes. (B) Male fertility in the mentioned genotypes. Results represent mean ± s.d., n = 3. (C-F) Crystal phenotype of adult testes analyzed by immunolabeling: anti-Stellate labeling is in green, DAPI in blue in (C) *aub*^{clash} /+. (D) *aub*^{clash}/*aub*^{clash}. (E) *aub*^{HN2}/*aub*^{QC42}. (F) *aub*^{clash}/+; *dFmr1*$^{\delta 113}$ /+. (G-I) Confocal projections (3 sections) from a wild type testis labeled with anti-α-Spectrin antibody (in red, G) DAPI (in blue, H) and merge (I). (J-L) Confocal projections (3 sections) from an *aub*^{clash} testis labeled with anti-α-Spectrin antibody, which recognizes the fusome, a germline-specific organelle (in red, J), DAPI (in blue, K) and merge (L).

(91/1197) of phenotypic variants, the aub^{HN2}/aub^{QC42} transheterozygotes show a 1.2% frequency (10/800); both frequencies are higher than the one exhibited by the aub^{clash} heterozygotes 0.08% (3/3510).

Finally, the presence of one aub^{sting} allele rescues the "crystal" phenotype of animals mutant for dFmr1, which has been recently defined as a component of the piRNA pathway. This is due to overexpression of the Aub protein in the somatic compartment of aub^{sting} testes. aub^{HN2} or aub^{QC42} alleles, which are considered as loss of function mutations, do not rescue the *dFmr1*-mediated phenotype.[10] We set up the appropriate crosses and found that $aub^{clash}/+$; $dFmr1^{\Delta 113}/+$ individuals behave like the aub^{HN2} or aub^{QC42} alleles, that is there, is no rescue of the *dFmr1*-mediated "crystal" phenotype, as expected from a loss of function *aub* allele[10] (Fig. 3F).

Discussion

Aub belongs to the Argonaute family of proteins, which are necessary for keeping germline genomic integrity. In addition, recent data also suggest a role for Aub in tumorigenesis,[34-37] stem cell identity renewal [17] and in the nervous system.[38] Characterizing the role and the genetic interactions of *aub* in the different tissues is essential. Knock-out mutants and targeted overexpression are indispensible for such characterization. Some alleles generated by EMS mutagenesis (aub^{HN2}, aub^{QC42} etc.) have been used as loss of function, however, they contain premature stop codons so that truncated proteins or alternative transcripts from a downstream translation start site might be expressed. The fact that these alleles may not be nulls is in line with the finding that homozygous aub^{clash} males are much less fertile than the transheterozygous aub^{HN2}/aub^{QC42} males. Other alleles (e.g. $aub^{sting\text{-}3a}$) represent large deletions that completely eliminate Aub expression, but adjacent genes are disrupted as well. RNA interference has been used to affect the activity of Aub, however, this condition represents a knockdown and in addition we cannot exclude off target effects, due to the high sequence similarity among the members of the Argonaute family of proteins.

We have generated clean mutants of *aub*, where the neighboring genes are not affected and we have characterized at least one null mutation, aub^{clash} by molecular and phenotypic means. This allele will provide the scientific community with an efficient and specific tool to study the role of Aub in RNA biology.

Materials and methods

Drosophila strains

The *aub* gene is on the 2nd chromosome. The aub^{sting} allele has been described in.[15] w^{1118}; aub^{QC42} cn^1 bw^1/*CyO, P{sevRas1.V12}FK1* is ordered from Bloomington Stock Center, number 4968. The transposase carrying line is a gift from P. Heitzler (Strasbourg), with the genotype w^{1118} ; *cn,* vg^{μ}*, bw, sp/CyO, H[*w^{+}*, Δ2–3] Ho 2.1* on the 2nd chromosome. The fluorescent balancer abbreviated as *CyO, twi>GFP* is *CyO, twist-Gal4, UAS-GFP* on the 2nd chromosome. The multiple balancer line, with Bloomington Stock Center number 3703, is $w^{1118}/Dp(1;Y)y^{+}$; *CyO/*nub^1 b^1 sna^{Sco} lt^1 stw^3; *MKRS/TM6B,* Tb^1, bearing mutations on the 1st, 2nd and 3rd chromosomes. $dFmr1^{\Delta 113}$ is a null allele.[10]

Mutagenesis

aub^{sting} flies can be recognized by orange eyes, while all the balancers and deficiency chromosomes are in a white eye background. The mobilization started by crossing aub^{sting}/*CyO* virgins (orange eyes) with *vg/CyO Δ2–3* males that carry the transposase. Bringing the P element and transposase enzyme together allows P element mobilization in aub^{sting}/*CyO Δ2-3* flies of the F_1. aub^{sting}/*CyO Δ2–3* males were mated with virgin females carrying the balancer. In the F_2, which likely contains mutants, the sna^{Sco} negative and *CyO* flies with white eyes were collected, since the white eye indicates that the P element had been excised. Individual aub^{sting*} (*aub* mutant candidate) males were crossed with 6 virgins of the following genotype *BSC213/CyO, twi>GFP* (*aub* deficiency over the fluorescent balancer line); 103 such crosses were set. In the F_3, an aub^{sting*}/*CyO, twi>GFP* line was established as a stable stock from each cross, which also allowed the identification of homozygous mutant candidates. From the same vials, *CyO* negative flies were also kept to establish an aub^{sting*}/*BSC213* line to test for sterility at the same time (Fig. 1C). The sterile strains were selected as primary candidates for imprecise excision. The matching stocks were characterized by *CyO* negative selection from their corresponding aub^{sting*}/*CyO, twi>GFP* adults. Deletion size and location were calculated on the bases of *aub* transcript-RA (FBtr0080165, release r6.09).

DNA analyses

The DNA isolation buffer contains 50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, 1%

SDS. DNA was extracted from 5 anesthetized adults from each genotype, in presence of 1 mg/ml proteinase K. The solution was centrifuged at 15000 g for 10 min; the supernatant was transferred to clean 1.5 ml tubes. NaCl to final concentration 0.24 M and half volume isopropanol was added. The DNA was pelleted with centrifuge at 13000 g, 4 C°, for 10 minutes. The pellets were washed with 70% ethanol and air-dried. DNA was resuspended in 100 μl ddH_2O.

Total RNA extraction, cDNA production and cloning

Total RNA was extracted from 30 mg of male or female gonadal tissues using the RNAqueos-4 PCR Kit (AMBION) reagent, following the manufacturer's protocol as described previously.[39] Samples were incubated with DNase I RNase free (AMBION) (2U DNase up to 2 μg RNA) at 37°C for 30 min (100 μl). DNase-treated RNA was precipitated at −80°C overnight and after centrifugation (10,000 g for 15 min) it was dissolved in 50 μl of nuclease-free water. The RNA concentration and purity were determined photometrically. 5 μg of total RNA were used as a template for oligonucleotide dT-primed reverse transcription using SuperScriptIII RNaseH-reverse transcriptase (Invitrogen), according to manufacturer's instructions.

For the cDNA preparation the M-MLV Reverse Transcriptase kit (Invitrogen) was used following the manufacturer's protocol.

PCR amplification was conducted using specific pair of primers and the Platinum Taq Polymerase (Invitrogen) for fragment up to 1000 bp. To amplify fragment longer than 1000 bp, the Expand Long Template PCR System kit (Roche) was used. All the primers used for the amplification are reported below. The product of single PCR was then purified using QIAquick PCR Purification Kit (Qiagen) following the manufacturer's protocol. It was directly used for sequencing or used for the cloning in the TA-vector (Strata Clone PCR Cloning Kit (Stratagene) and the StrataClone™ SoloPack® CompetentCells, following the manufacturer's protocol. We used this vector for the cloning of the cDNA amplified fragments and for the genomic fragments as well. Primers used in the PCR reactions:

1F5′*sting* upper 5′ CTGAACGGCATTTGTGACGA 3′
1F*sting* upper 5′ CGTGGTCGAGGAAGAAAGCC 3′
2U*sting* upper 5′ GAGGCAATGGTGGTGGTGGT 3′
3U*sting* upper 5′ CGTGCTGGCGAAAACATTGA 3′
6U*sting* upper 5′ CGGGAATGACGGACGCTATG 3′
8U*sting* upper 5′ CGCAACGGCACTTACTCCCA 3′
3L*sting* lower 5′ GGTTCCGTCAAAGATGTAGC 3′
5L*sting* lower 5′ ATGCGATAGGTTTTGTTATT 3′
9L*sting* lower 5′ TGCTGTCGAGGCGCGATAAC 3′
9L-2*sting* lower 5′ TGCGATGCCCAGTAAAGTAG 3′

Immunofluorescence of Stellate-made crystals

Testes were dissected in Ringer's modified solution (182 mM KCl, 46 mM NaCl, 3 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5), fixed in methanol, washed in PBST (1x PBS, 1% Triton X-100, 0.5% acetic acid) for 15 min, washed in 1X PBS for 5 min 3 times and incubated with the polyclonal mouse anti-Stellate antibody (1:100).[40] Samples were washed in 1X PBS for 5 min 3 times, incubated 2 h with 1:100 FITC-conjugated anti-mouse-IgG antibody (Jackson) and examined by epifluorescence microscopy (Nikon-Optiphot 2); DAPI was used at 100 ng/ml for nuclear labeling.

Antibody production and Western blot analyses

The anti-Aub antibody was made in rabbit against the C-terminal peptide (847–866) also used by Brennecke.[1] The antibody was affinity purified according to the sulfolink coupling gel protocol (Pierce #20401) using the peptide employed to immunize the animals. 10 ml of centrifuged sera were washed with 20 ml of PBS and eluted with 5 ml of 0.1M Glycine pH2.8. 0.5 ml fractions were collected and neutralized with 25 μl of Tris pH 9.5 1M. They were then were quantified by Bradford assay.

Adult testes from 10 flies (control and mutant) were dissected in cold PBS buffer and lysed in 30 μl Laemli Sample Buffer 2X (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), with the addition of the protease inhibitor (Roche), using small pestle, on ice, and then the samples were boiled (8 min, 80°C). Proteins were transferred from 8% SDS-polyacrylamide gels to the membrane using constant amperage (200mA), for 1 h, in transfer buffer and the membrane was subsequently rinsed in TBS1X/0.1% Tween 20 buffer (TBST). To avoid non-specific binding, the membrane was placed in a 5 % milk solution (in TBST) and incubated for 1 h at room temperature (RT) with slow shaking. The filter was then incubated with the affinity purified rabbit anti-Aubergine diluted (1:500) and mouse monoclonal anti−β−tubulin (Millipore) diluted (1:4.000) in

TBST at 4°C overnight. Upon incubation, the membrane was rinsed 3 x with TBST and washed 3 x for 10 min at RT with shaking. Secondary antibody conjugated to horseradish peroxidase (HRP) 1:5000 (Jackson Immunoresearch), was added and incubated for 1h at RT. Colorimetric analysis was performed with the detection system for the HRP, as described by the company.

Male and female fertility testing

One young male was mated to 3 control virgin females, and 3 virgin females were mated with 3 control males. 10 individual males and 30 females were tested for each genotype. After 4 days the crosses were transferred to a fresh vial. The parental flies were removed from the last vial after an additional 4 days. The number of the adult progeny from each vial was counted.

Immunofluorescence of the gonads and confocal microscopy

Drosophila gonads were dissected in Ringer's solution and fixed in 4% paraformaldehyde for 20 min, washed in PBT (1X PBS with 0.5% Triton X-100), blocked in 5% NGS for 1 h and incubated with rabbit mouse anti-α-Spectrin (DSHB) antibody at 4°C overnight. Samples were washed in PBST and then incubated for 2 h with the secondary antibodies against mouse IgG, conjugated to Cy3 dyes (1:500, Jackson). All the samples were examined and captured using a laser-scanning confocal microscope (Zeiss LSM 700 on Axio imager M2).

Abbreviations and Acronyms

aub	aubergine
AGO	Argonaute
bp	base-pair
FMRP	Fragile X Mental Retardation Protein
GFP	Green Fluorescent Protein
piRNA	Piwi interacting RNA
RISC	RNA-induced silencing complex
UTR	untranslated region

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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