Table 1. Progeny from Mating-I, Cy/Pm x Pm/C-1367

	Cy/Pm	Pm	Cy
	86	106	0
	40	41	0
	22	40	1
	113	129	1
	82	84	0
	112	112	0
	125	126	0
	104	109	0
	67	87	1
	153	143	2
	142	150	0
	116	125	0
	132	136	0
	71	83	2
	101	115	1
Total	1466	1586	8

Table 2. Progeny from Mating II, Pm/C-1367 x Pm/C-1367.

	Pm	+
	59	2
	60	0
	52	1
	70	0
	71	0
	72	1
	134	0
	141	1
	111	3
	100	3
	155	0
	138	1
	146	0
	163	2
Total	1472	14

Mating-I in Figure 1 was repeated and the number of progenies in generation 3 is given in Table 1. As clearly shown in Table 1, one of second chromosomes sampled from a natural population in Szentendre, Hungary, was found to be lethal in combination with the second chromosome balancers. This lethal chromosome and lethal gene were tentatively named C-1367 and *l-Cy*, respectively. It should be noted that it was impossible to detect *l-Cy*, if *Cy* males were singly mated with *Cy/Pm* females in generation 2. The result clearly shows that C-1367 is carrying a recessive lethal gene, although it is not complete. *Pm* males in generation 3 of Mating-I were also mated with *Pm* females repeatedly. The result shown in Table 2 indicates the same conclusion, that *Cy*/*l-Cy* is substantially lethal and the viability of Cy*/l-Cy* is less than 0.005 of *Pm* /*l-Cy* heterozygote. Several wild type phenol-types appeared in the progenies in this mating, because *Pm* chromosome is not complete as a balancer. These results indicate that C-1367 has a lethal allele, *l-Cy*, and this lethal allele is concealed in the second chromosome balancers in the heterozygous condition, and that *l-Cy* is completely linked with *Cy*.

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Ovary phenotype and expression of *bab1* and *bab2* paralogs in the larval ovary of two mutants of the *bab* locus in *Drosophila melanogaster*.

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Introduction

The *bric-à-brac* locus (*bab*, Godt and Laski, 1995) is composed of two evolutionarily related genes, *bab1* and *bab2* (Couderc *et al.*, 2002), that play important roles in various developmental processes and sex-specific differentiation (Sahut-Barnola, *et al.*, 1995; Godt, *et al.*, 1993; Barmina and Kopp, 2007; Randsholt and Santamaria, 2008; Kopp *et al.*, 2000; Williams, *et al.*, 2008). In

particular, *bab* function is necessary for organogenesis of the *D. melanogaster* ovary (Godt and Laski, 1995), involving both *bab1* and *bab2* paralogs (Couderc *et al.*, 2002). However, *bab1* and *bab2* have different expression patterns in the larval ovary, since *bab2* is expressed in all somatic cells whereas *bab1* is restricted to the terminal filament (TF) cells that form the tip of the ovariole (Couderc *et al.*, 2002).

In an attempt to assign specific functions to each paralog, *bab* alleles were classified according to associated defects in legs as well as in ovarian morphogenesis and fertility, in correlation with expression levels of *bab1* and *bab2* gene products (Couderc *et al.*, 2002). Two mutants of the *bab* locus, $bab1^{P}$ and $bab2^{E1}$, were shown to affect specifically *bab1* and *bab2*, respectively (Couderc *et al.*, 2002), and to display a strong phenotype in the ovary. The $bab1^{P}$ allele corresponds to a *P*[lacZ] insertion in the first intron of *bab1*, whereas $bab2^{E1}$ is an EMS-induced mutation. Available antibodies allow the detection of BAB1 and BAB2 by tissue immunostaining, and only BAB2 on Western blot. In $bab1^{P}$ mutants, no BAB1 protein was detected and BAB2 protein levels were unaffected. In $bab2^{E1}$ mutants, BAB2 was undetectable by tissue immunostaining and almost absent on Western blot, and BAB1 levels were unaffected (Couderc *et al.*, 2002).

The recessive ovary phenotype of $bab1^{P}$ and $bab2^{E1}$ mutants is characterized by a reduction of the size of the ovary (Couderc *et al.*, 2002), which is stronger in $bab2^{E1}$ than in $bab1^{P}$ mutants. This result suggested that the two paralogs are necessary for organogenesis of the ovary. Results of rescue experiments of *bab* mutants using transgenes expressing either *bab1* or *bab2* cDNAs suggested that the two paralogs play redundant functions, *bab2* being, however, more important than *bab1* in ovary morphogenesis (Bardot *et al.*, 2002).

Aiming to characterize further the possible differences between *bab1* and *bab2* functions, we compared the structure of the ovary in $bab1^{P}$ and $bab2^{E1}$ mutants and found that the reduced ovaries in these two mutants are in fact very different structurally. In parallel, we quantified the levels of BAB1 and BAB2 proteins in $bab1^{P}$ and $bab2^{E1}$ mutant larval ovaries by immunofluorescent confocal microscopy and showed that both alleles affect the expression of both paralogs.

Results and Discussion

The ovary phenotype of $bab1^{P}$ and $bab2^{E1}$ mutants was compared to that of bab^{AR07}, a deficiency that covers both *bab1* and *bab2*. As previously described (Couderc *et al.*, 2002), the size of the ovary was reduced in all three mutants (Figure 1B-D) compared to that of the wild-type Canton-S control flies (Figure 1A). However, the structure of the ovary differed significantly between the three mutants. In wild-type Canton-S flies, ovarioles are characterized by the presence of a germarium at their distal tip (Figure 1A', arrowheads). In both $bab1^{P}$ and bab^{AR07} ovaries, the whole structure of the ovary was affected. In $bab1^{P}$ mutants, germaria and ovarioles were in most cases indistinguishable, though egg chambers at various stages of oogenesis and even mature eggs were observed in rare cases (Figure 1B'). In bab^{AR07} mutants, ovarioles were not distinguishable (Figure 1D'). In addition, mature eggs were never observed, although disorganized egg chambers appeared to be present (Figure 1D' and inset). In contrast, $bab2^{E1}$ mutant ovaries contained about 20 ovarioles (Figure 1C'), as is observed in Canton-S flies. However, $bab2^{E1}$ mutant ovarioles appeared to contain only early stages of oogenesis (before stage 9) and mature eggs were rarely observed (Figure 1C'). These results clearly differentiate the phenotype of $bab1^{P}$ and $bab2^{E1}$ mutant ovaries: both are reduced in size, however likely through different mechanisms, since $bab1^{P}$ mutant ovaries lack ovarioles, whereas $bab2^{E1}$ mutant ovaries contain a wild-type number of ovarioles in which oogenesis is blocked early. If the previous characterization of these two *bab* mutations is taken into

account, the results of the present phenotypical analysis would lead us to conclude that *bab1* is required for the formation of ovarioles and *bab2* for oogenesis.

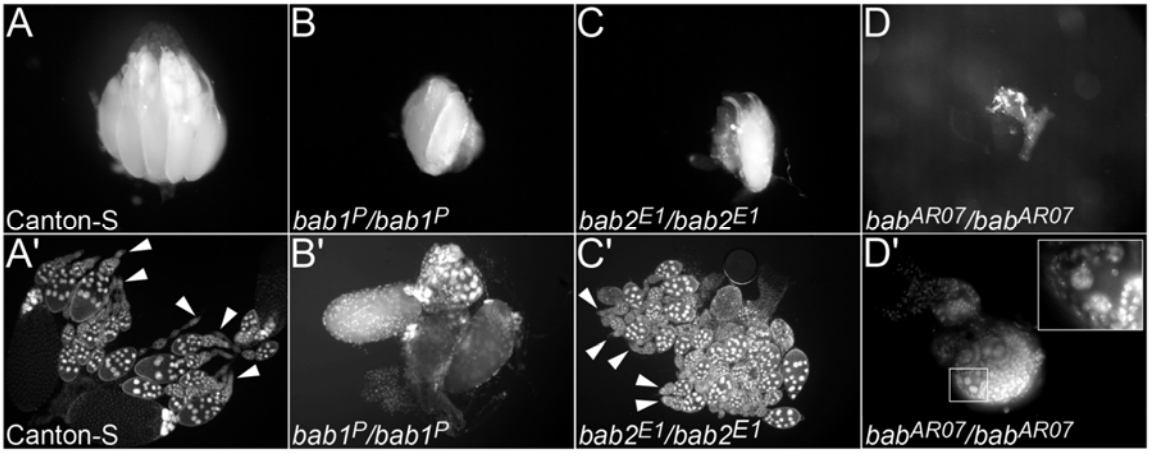

Figure 1**.** Morphology of whole adult ovaries visualized using bright field microscopy (A-D) and ovariole structure revealed by DAPI staining and epifluorecence microscopy (A'-D') of Canton-S (A, A'), *bab1*^{P} homozygotes (B, B'), *bab2*^{E1} homozygotes (C, C'), and *bab*^{AR07} homozygotes (D, D') characterized by a layer of nuclei embedding (inset).

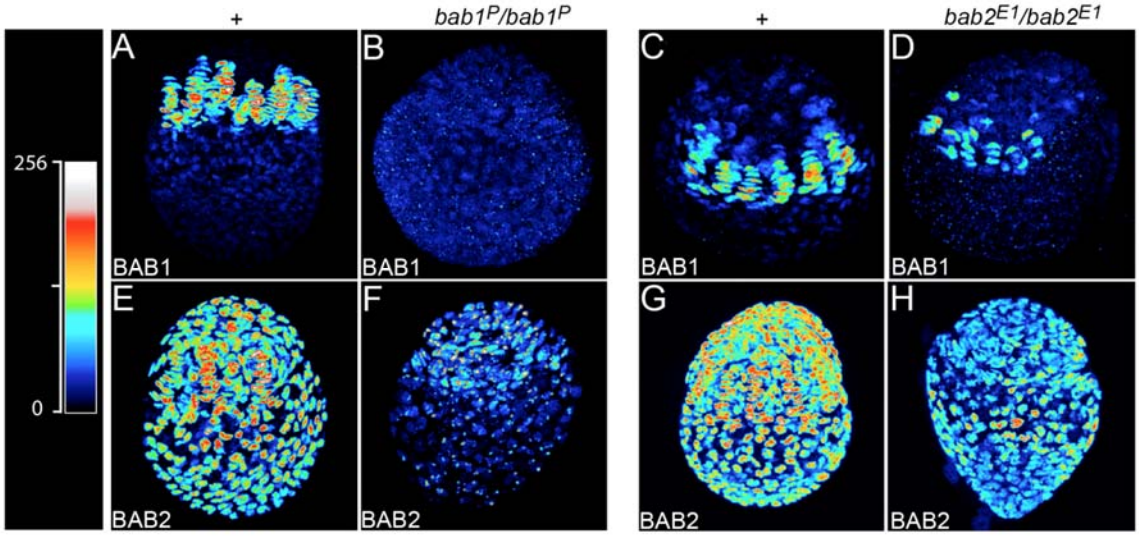

Figure 2**.** Expression of *bab1* and *bab2* in wandering third instar larval ovaries. Comparison of BAB1 and BAB2 protein levels between control (A, E, C, G) and *bab1*^{P} (B, F) or *bab2*^{E1} (D, H) homozygotes as measured by immunostaining and confocal analysis followed by quantification using the lookup table shown on the left of the panel. BAB1 positive cells in A, C and D correspond to TF cells.

The latter conclusion is, however, challenged by the results of the present analysis of *bab1* and *bab2* expression in the *bab1*^{P} and *bab2*^{E1} mutant ovaries. BAB1 and BAB2 protein levels were measured by immunofluorescent confocal microscopy in homozygous *bab1*^{P} and *bab2*^{E1} mutant

larval ovaries. As expected from previous work (Couderc *et al.*, 2002), BAB1 was undetectable in *bab1*^{P} mutant ovaries (Figure 2B, compare to wild-type in 2A). BAB2 was reduced in *bab2*^{E1} mutant ovaries (Figure 2H, compare to wild-type in 2G), whereas it was undetectable in Couderc *et al.* (2002). This difference is not surprising, since low levels of BAB2 had been detected on Western blot and since fluorescent immuno-detection with confocal microscopy is more sensitive than classical enzymatic immunostaining as was previously used. However, BAB2 levels were also strongly reduced in *bab1*^{P} (Figure 2F, compare to wild-type in 2E), and BAB1 levels were reduced in *bab2*^{E1} mutant ovaries (Figure 2D, compare to wild-type in 2C). These results suggest that the *P*-element insertion in *bab1*^{P} as well as the EMS-induced mutation in *bab2*^{E1} affect the expression of both *bab* paralogs in the larval ovary. Therefore, it is impossible to conclude on the specific implication of each *bab* paralog in ovarian morphogenesis and oogenesis based solely on the analysis of the phenotype of these mutants.

The *bab1* and *bab2* genes may interact in *trans*, regulating each other at the transcriptional level. In fact, it was shown that the *bab2* gene product binds to both *bab1* and *bab2* sequences (Lours *et al.*, 2003). If binding of BAB2 on *bab1* gene sequences normally activates transcription, the reduction of BAB2 in *bab2*^{E1} ovaries may lead to a reduction of *bab1* transcription and, consequently, to a reduction of BAB1 protein levels in the ovary, as observed in our data (Figure 2D). Since the BAB1 protein is restricted to TF cells, whereas *bab2* is expressed in all somatic cells of the ovary, direct transcriptional control of *bab2* by the BAB1 protein is unlikely to occur outside TF cells. However, BAB2 protein levels are strongly reduced in all somatic cells in *bab1*^{P} ovaries. A non autonomous and thus indirect regulation of *bab2* by *bab1*, involving long-distance signaling between *bab1*-expressing cells and other somatic cells in the ovary, cannot be excluded.

Alternatively, *cis*-regulatory effects between *bab1* and *bab2* may also be responsible for the effect of the *bab1*^{P} mutation on *bab2* expression or for that of *bab2*^{E1} on *bab1* expression. It was shown that at least one *cis*-regulatory element (CREs) located in *bab1* sequences is shared between the two *bab* paralogs for the control of *bab1* and *bab2* expression in the abdomen (Williams *et al.*, 2008). This CRE, or other yet unidentified CREs, may also co-regulate the two paralogs in the ovary. The *bab1*^{P} allele contains a *P*-element inserted in the first intron of *bab1*. This insertion may disrupt the function of an unidentified ovary-specific CRE, thereby also affecting the expression of *bab2* in the ovary. This *cis*-regulatory effect may not occur in other tissues, since BAB2 protein levels were unaffected in larval imaginal discs and brains as detected by Western blotting (Couderc *et al.*, 2002). Since the *bab2*^{E1} allele has not been characterized at the sequence level, *cis*-regulatory effects as well as alteration of *bab1* sequences in this mutation, leading to *bab1* down-regulation in TF cells, may also explain the low level of *bab1* expression in *bab2*^{E1} mutant ovaries.

Taken together, our results show that the presence of reduced ovaries in *bab* mutants may be due to different mechanisms depending on the mutation and that, due to possible *cis*- and *trans*-regulatory effects, specific functions of *bab1* and *bab2* cannot be assigned using the two strongest *bab* locus alleles previously characterized as affecting only one of the two paralogs. Other approaches such as RNAi silencing of the individual *bab* paralogs will be necessary to address these questions.

Material and Methods

Fly stocks and analysis of adult ovaries

Flies were grown on standard corn-agar medium under uncrowded conditions at 25°C. *bab* mutant stocks were kind gifts from J-L. Couderc (*bab2*^{E1}), D. Godt (*bab1*^{P}), and M. Boube (*bab*^{AR07}), and *hh-lacZ* from A.-M. Pret. Female flies were dissected 24 to 48 hours after eclosion in phosphate-

buffered saline 0.66× (PBS tablets, Sigma). Ovaries were fixed in PBS 1× 3.7% formaldehyde during 30 min at room temperature and stored at 4°C. General morphology of the ovary was observed under a stereoscopic microscope (Leica MZFL III). Ovaries were labeled in DAPI (5 µg/ml in PBS) and observed under epifluorescence (Leica DMRD).

Immuno-fluorescence and imaging

bab mutant stocks were balanced over *TM6Tb*. Female wandering third instar larvae of the Tb+ phenotype were dissected in PBS and whole fat bodies to which ovaries are attached were collected, fixed in PBS containing 2% BSA (Sigma A2058), 3.7% formaldehyde (Sigma), 1% Triton during 30 min at room temperature, washed in PBS 0.3% Triton (PBT), and blocked in PBT, BSA 1% (PBTA). Fixed larval ovaries were incubated in PBTA with the appropriate combination (see Figure 2 legend) of rabbit anti-BAB1 (1 :1000, (Williams *et al.*, 2008)), rat anti-BAB2 (1 :1000, (Couderc *et al.*, 2002)), and mouse anti ß-galactosidase (DSHB) overnight at 4°C. Ovaries were incubated during 2 hours at room temperature with the appropriate Alexa 488- and Alexa 568-coupled secondary antibodies (1:500 in PBTA, Molecular Probes). Ovaries were mounted in Citifluor (AF1, Biovalley, FR) and directly observed under an inverted confocal microscope NIKON TE2000-U. Fixation and immuno-fluorescence of ovaries of mutant and control genotypes were performed in the same tube, thus allowing direct comparison of signal levels between genotypes. Control and mutant ovaries were genotyped using *lacZ* reporters. Control ovaries were Canton-S for $bab1^{P}$, and *hh-lacZ* for $bab2^{E1}$. Genotypes were identified after imaging: $bab1^{P}$ carries a *lacZ* reporter that is expressed in TF cells, allowing to positively discriminate the $bab1^{P}$ homozygotes from Canton-S controls; $bab2^{E1}$ mutants were identified by the absence of anti-betaGalactosidase immunostaining, and *hh-lacZ* controls by the presence of anti-betaGalactosidase immunostaining in TF cells (data not shown). The bab^{AR07} deficiency that covers both *bab1* and *bab2* was used as a control for the specificity of BAB1 and BAB2 signals in a separate experiment (data not shown). Confocal images were analyzed using ImageJ (NIH) and Photoshop CS2 (Adobe) softwares, using identical settings for all samples of the same experimental series.

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Mutants in *D. simulans* and *D. sechellia.*

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Here we report the isolation of nine new spontaneous mutants in *D. simulans* identified this year, as well as notes on mutants described in Sousa-Neves *et al*. (2009). We also report the genetic