doi:10.1016/j.jmb.2005.07.009 J. Mol. Biol. (2005) 352, 329–337

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Drosophila BAP60 is an Essential Component of the Brahma Complex, Required for Gene Activation and Repression

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The SWI/SNF-like chromatin remodeling complex of *Drosophila*, the Brahma complex, contains four subunits (Brahma, BAP155/Moira, SNR1 and BAP60) conserved from yeast to humans. A reconstituted human complex lacking the BAP60 homolog shows full remodeling activity, suggesting that BAP60 is not essential for the core function. We generated *Drosophila* mutants and found that BAP60 carries a vital function and participates in complex-mediated transcriptional activation and repression. BAP60 binds DNA and shows genetic and physical interactions with the sex-determining transcription factors encoded by *sisterless A* and *scute*. The results support the conclusion that BAP60 participates in site-specific recruitment of the Brahma complex in *Drosophila*.

Keywords: gene regulation; chromatin remodeling; position effect variegation; DNA binding; sex determination

Introduction

Transcriptional regulation in eukaryotic cells occurs on DNA compacted in nucleosomes and involves ATP-dependent chromatin remodeling processes by a number of different protein complexes.[1] The best-characterized chromatin remodeling complex, the SWI/SNF complex first identified in yeast, has counterparts in flies (Brahma complex) and mammals (BRG1/hBRM complexes). SWI/SNF-like complexes take part in a diverse array of cellular functions, including the activation and repression of gene transcription, the control of cell cycle progression and the chromosomal integration of retroviral DNA.[2–8] They are recruited to specific target genes through the association with sequence-specific transcription factors.[9–12]

In *Drosophila*, the name-giving component of the SWI/SNF-like Brahma complex, the gene *brahma* (*brm*), was identified in a genetic screen for regulators of homeotic gene expression.[13] In addition to Brahma (BRM), which corresponds to the ATP-hydrolysing subunit SWI2/SNF2 of yeast,[2] three subunits, termed BAP155/Moira, SNR1 and BAP60, are conserved in SWI/SNF and SWI/SNF-like complexes from yeast to mammals.[14] A minimal complex composed of BRM, BAP155/Moira and SNR1 can achieve full chromatin remodeling activity *in vitro*,[15] indicating that, despite its evolutionary conservation from yeast to man, BAP60 is not required for the basic chromatin remodeling function. Instead, it may play a role in the recruitment of the complex to specific promotors. Its mammalian homolog BAF60a was found to associate with the AP1 heterodimer cJun/cFos,[16] as well as with nuclear receptors,[17,18] and a second mammalian homolog, BAF60c, was shown to interact with various transcription factors.[19,20]

Here, we report that BAP60 is a functional component of the Brahma complex and participates in both repressive and activating transcriptional activities. BAP60 interacts also with the transcription factors encoded by *sisterless A* and *scute* in the context of X-chromosomal dosage compensation. The results imply that BAP60 is involved in the recruitment and site-specific anchoring of the Brahma complex at specific promoter sites and participates in X-chromosomal dosage compensation in *Drosophila*.

Results

Generation and characterization of a *Bap60* mutant

To assess the *in vivo* requirement for BAP60, we

Abbreviation used: GST, glutathione-*S*-transferase.

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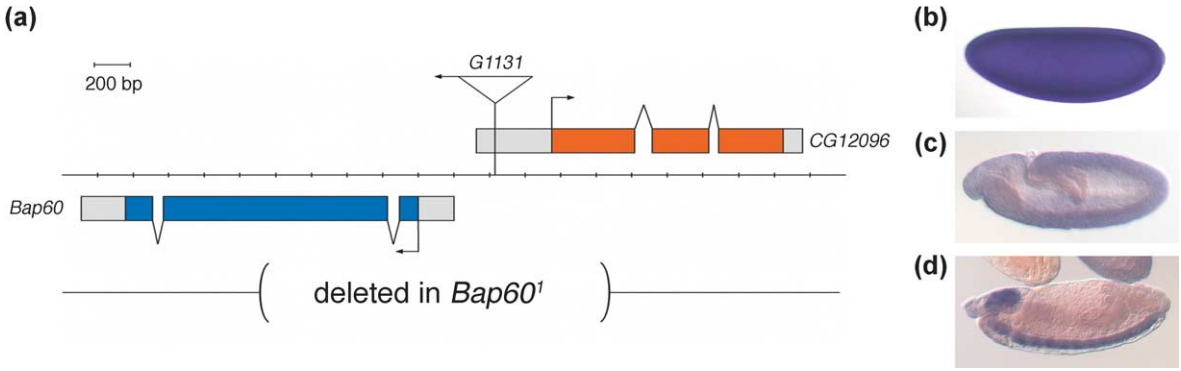

Figure 1. (a) Representation of the organization of the *Bap60* gene locus based on the mapping of respective cDNAs from the Berkeley Drosophila Genome Project (BDGP; http://www.fruitfly.org) and on release 2 of the *Drosophila* genome sequence. Location and orientation of the EP element *G1131* in the 5′ UTR of *CG12096* and the genomic area deleted in $Bap60^1$ are indicated. (b)–(d) Distribution of the *Bap60* mRNA during embryogenesis. The transcript pattern was determined by *in situ* hybridization of antisense RNA probes to whole-mount preparations. The pattern of *Bap60* expression is similar to those of *brm* and *mor*, and to the protein expression pattern of SNR1. *Bap60* mRNA is a maternal transcript that is distributed uniformly at the blastoderm stage. Zygotic transcripts become predominant at the extended germ band stage. After germ band retraction, *Bap60* expression is restricted to the ventral nerve chord and the brain; thus, as has been suggested for *snr1*, the expression of *Bap60* may be linked to the occurance of cell division.[22]

generated a *Bap60* loss-of-function mutant by imprecise excision of the EP element *G1131*.[21] This element is inserted into the X-chromosomal DNA, approximately 240 bp upstream of the *Bap60* transcription start (Figure 1(a)). Neither the EP element insertion nor Gal4-driven ubiquitous overexpression of *Bap60* interferes with normal development or viability.[21] Remobilization of the EP element resulted in a 2 kb deletion that removed the N-terminal portion of the *Bap60* coding region ($Bap60^1$) (Figure 1(a)). About two-thirds of $Bap60^1$ hemizygous male embryos die prior to hatching, the remaining male individuals hatch from the egg but die during early larval stages. As observed for null mutations of the Brahma complex subunits *brm*, *snr1* and *osa*,[2,22,23] no morphologically distinct cutical defect was evident. In addition, no abnormality of internal structures such as the muscle pattern or the nervous system could be observed. To show that loss of *Bap60* activity is the cause of the lethal phenotype, we performed rescue experiments with a transgene expressing *Bap60* cDNA under the control of the constitutively active *Act5C* promotor in $Bap60^1$ mutant individuals (see Materials and Methods). Hemizygous $Bap60^1$ mutant males bearing the *Act5c-Bap60* transgene developed into normal-looking, fertile adults, indicating that the mutant effect is due to the lack of *Bap60* activity.

In addition to the similar mutant phenotype, *Bap60* shows an embryonic expression profile equivalent to that observed for *brm*, *moira* (*mor*) and *snr1* (Figure 1(b)–(d)),[22,24,25] including maternal expression. To explore a possible requirement for maternal *Bap60* activity, we generated homozygous $Bap60^1$ germline clones using the FLP-DFS technique.[26] Eggs resulting from such germline clones were partially deformed or deflated, and no larvae hatched from these eggs. This implies that BAP60 carries an essential function during oogenesis, as had been reported earlier for mutations of the Brahma complex subunits BRM, SNR1 and MOR.[27–29] The similar expression patterns as well as the essential functions of BAP60 and other core subunits of the Brahma complex suggest that BAP60 participates in one or several functions of the BRM complex *in vivo*.

BAP60 participates in BRM complex activity

Biochemical studies revealed that BAP60 is a component of the Brahma complex. In order to provide a functional link between BAP60 and the Brahma complex, we examined possible genetic interactions of *Bap60* with genes encoding subunits of the complex. Mutations in *brm* cause a loss of humeral bristles.[28] Transheterozygous $Bap60^1$, brm^2 and $Bap60^1$, mor^1 individuals show an increased frequency of this humeral bristle phenotype (Table 1), implying that the genes act in the same genetic pathway. In order to show that BAP60 is involved directly in the Brahma complex-dependent regulation of gene activity, we took advantage of the finding that *brm* acts as a suppressor of Nasobemia, an allele of *Antennapedia* ($Antp^{NS}$), that causes the homeotic transformation of antenna to leg.[13] The Nasobemia transformation is due to the ectopic activation of the *Antp* P2 promoter in the eye-antennal disc.[30] The same homeotic transformation is observed with the $Antp^{73b}$ allele, but in this case the effect is due to a chromosomal inversion in which the *Antp* transcript is placed under the control of a promotor that is specifically active in the eye-antennal imaginal disc.[31,32] Suppression of the Nasobemia phenotype was shown for mutations of *brm*, *osa*, *mor* and *snr1*, whereas the same mutations fail to suppress the phenotype

Table 1. *Bap60* interacts genetically with *brm* and participates in the transcriptional activation of *Antp*

Genotype	Control[a,b]	$Bap60^1$ [b]	Phenotype scored
$Bap60^1/+;; brm^2/+$	20/292 (7)	44/321 (14)	Loss of humeral bristles
$Bap60^1/+;; brm^2 trx^{E2}/+$	6/128 (5)	52/351 (15)	Loss of humeral bristles
$Bap60^1/+;; trx^{E2}/+$	Not determined	0/162 (0)	Loss of humeral bristles
$Bap60^1/+;; mor^1/+$	8/281 (3)	25/357 (7)	Loss of humeral bristles
$Bap60^1/+;; Antp^{NS}/+$	362/363 (100)	157/171 (92)	Transformation of antenna to leg
$Bap60^1/+;; Antp^{73b}/+$	135/135 (100)	104/104 (100)	Transformation of antenna to leg

[a] Controls are the same genotypes without the *Bap60* mutation.
[b] The values in parentheses are percentages.

caused by the $Antp^{73b}$ mutation. The different interactions of the Brama complex mutations with the two *Antp* alleles were taken as an argument that the Brahma complex is specifically required to activate *Antp* expression *via* its natural P2 promotor.[2,23,27,29] $Bap60^1$ causes a slight but significant suppression of the $Antp^{NS}$-dependent transformation, whereas transformations caused by $Antp^{73b}$ remain unaffected (Table 1). These results indicate that BAP60 participates in the BRM-dependent activation of *Antp* involving the P2 promotor. As several subunits of the yeast and metazoan Brahma complexes are known to interact directly with transcription factors,[9,17,33,34] it is interesting to speculate that the Brahma complex is recruited to the Antp P2 promotor by the interaction of one of its subunits with the known activators of the P2 promotor, namely Fushi tarazu and Hunchback.[35]

An assay based on the phenomenon of position effect variegation also revealed a repressive effect of the BAP60 protein. This assay takes advantage of an inversion chromosome, $In(1)w^{m4h}$, that places the *white* gene in the vicinity of centromeric heterochromatin. The juxtaposition of the *white* gene and heterochromatin represses *white* in a substantial number of eye ommatidia (Figure 2(a)).[36] Using $In(1)w^{m4h}$ in combination with the $Bap60^1$ mutant, we found that transheterozygous individuals had normal eye-colour, indicating that *white* repression is completely relieved in the absence of one functional copy of the *Bap60* gene (Figure 2(b)). The same effect had been observed with mutations of *snr1*, which codes for a BRM complex core component.[27] The strength of the Su(var) effect of $Bap60^1$, and the fact that $In(1)w^{m4h}$ in combination with chromosomes in which the $Bap60^1$ mutant was generated had no such effect,[21] make it unlikely that the observed effect is caused by modifiers in the genetic background of the $Bap60^1$ flies. Heterochromatin-dependent gene repression by components of the BRM complex is also consistent with the finding that the human BRM homolog BRG1 interacts with HP1α, a chromatin component necessary for heterochromatin formation.[37] It should be noted, however, that suppressors of the phenomenon of position effect variegation do not necessarily have to localize to heterochromatic regions, as they can theoretically act by repressing the activity of transcriptional activators in euchromatic regions. In fact, *Drosophila* BRM was shown to localize almost exclusively to areas of active transcription.[38]

Collectively, our results indicate that BAP60 is a functional component of the BRM complex. To address possible functions of BAP60 within the complex, we investigated two activities BAP60 had been implicated with in previous studies: DNA binding and the interaction with transcription factors.

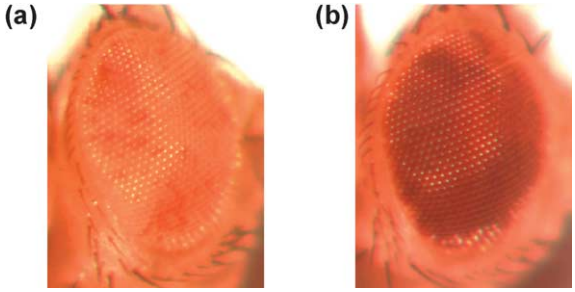

Figure 2. $Bap60^1$ acts as a suppressor of heterochromatin-promoted position effect variegation. (a) Eye of a female with the inversion chromosome $In(1)w^{m4h}$ in a *white* background. The eye shows variegated *white* expression. (b) Eye of a female that carries both $In(1)w^{m4h}$ and $Bap60^1$ in a *white* background. The presence of the $Bap60^1$ mutation restores normal expression of the *white* gene, thus suppressing the variegated phenotype of the $In(1)w^{m4h}$ inversion.

BAP60 has non-essential DNA binding properties

Studies on the yeast SWI/SNF complex implied that the BAP60 homolog SWP73 has an ability to bind DNA.[39] However, neither SWP73 nor BAP60 contain a known DNA binding motif. To address possible DNA binding by BAP60, we performed an *in vitro* co-immunoprecipitation experiment with unspecific DNA using a glutathione-*S*-transferase (GST)-BAP60 fusion protein (see Materials and Methods). Figure 3 shows that the GST-BAP60 fusion protein was able to retain DNA fragments (Figure 3(a)), that the amino acid interval 116–204 of BAP60 ("$BAP60^{116–204}$") was sufficient for the binding of DNA (Figure 3(b)) and that alanine replacements of three conserved basic amino acids in $BAP60^{116–204}$ resulted in a loss of DNA binding (Figure 3(c) and (d)). These findings confirm that

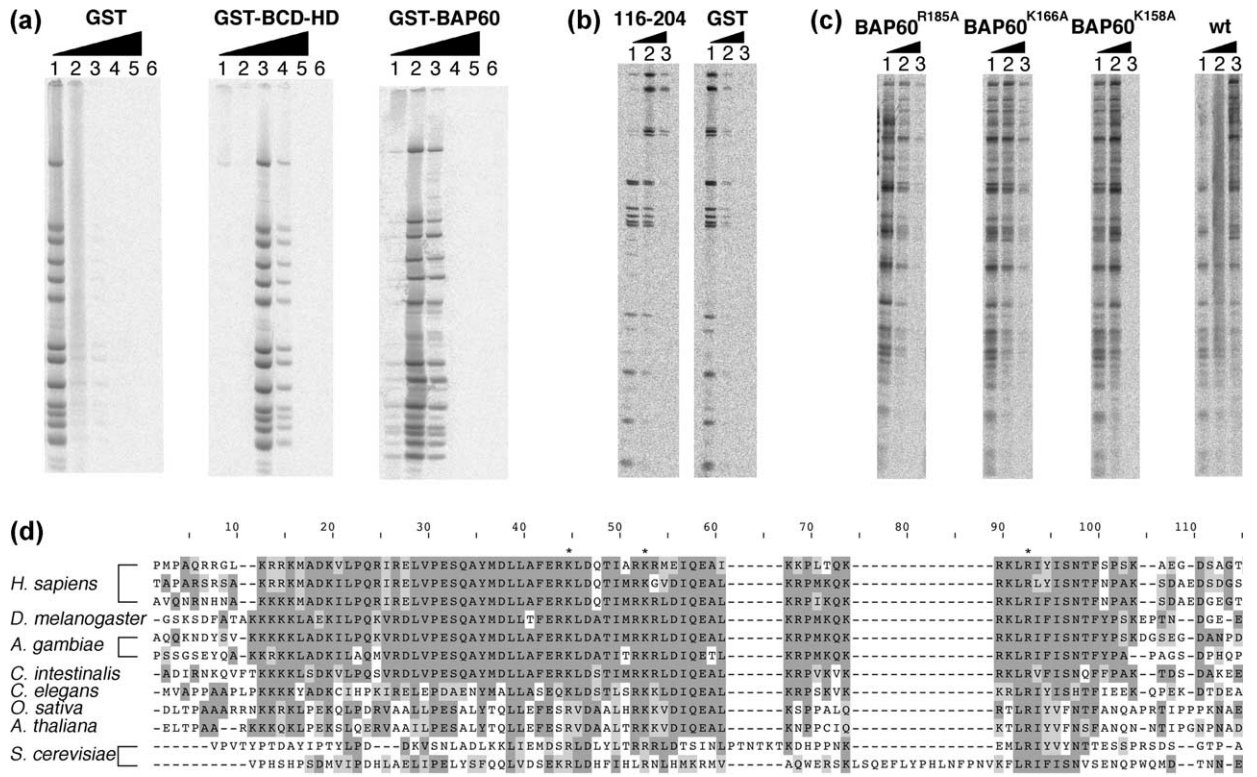

Figure 3. (a)–(c) DNA co-immunoprecipitation experiments. Labeled fragments of plasmid DNA were purified with Sepharose beads that carried the indicated GST fusion proteins. Lanes 1 to 5 show the DNA fragments that were eluted after washing with buffers containing 100 mM NaCl (lane 1), 200 mM NaCl (lane 2), 500 mM NaCl (lane 3), 750 mM NaCl (lane 4) or 1 M NaCl (lane 5); lane 6: beads boiled in 1% SDS, 200 mM NaCl after the final elution step. (a) BAP60 shows DNA binding activity. GST-BAP60 is able to retain DNA up to elevated concentrations of salt, while in the case of GST alone, the DNA fragments are eluted with the binding buffer. The *bicoid* homeodomain (BCD-HD) served as a positive control for DNA binding. (b) Amino acid residues 116 to 204 of the BAP60 protein are sufficient to cause retention of DNA. (c) Three conserved basic amino acid residues within the BAP60-DBD were mutated to alanine. Note that instead of retaining DNA fragments in buffers containing up to 500 mM NaCl, the DNA now elutes mainly under low-salt conditions. All three mutations therefore impair DNA binding by the full-length BAP60 protein. (d) Alignment of the BAP60 DNA binding domain with homologous sequences from BAP60 orthologs. The following sequences are shown (from top to bottom): *Homo sapiens* BAF60b, BAF60c and BAF60a; *Drosophila melanogaster* BAP60; *Anopheles gambiae* ENSANGP00000021019 and ENSANGP00000020546; *Ciona intestinalis* AK112677.1 (mRNA identification number); *Caenorhabditis elegans* NP_491329.2; *Oryza sativa* CAD40740.2; *Arabidopsis thaliana* AAL38282.1; *Saccharomyces cerevisiae* RSC6 and SWP73. All sequences were retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov). Alignments for the non-yeast sequences were generated using Clustal_W 1.81.[52] The two sequences from yeast were aligned with the BAP60 sequence based on PSI-BLAST[53] results and were then incorporated manually into the overall alignment. Asterisks (*) mark conserved basic residues that we mutated to alanine for our *in vitro* and *in vivo* experiments.

BAP60 is able to bind DNA. The BAP60$^{116–204}$ region is conserved in all known BAP60 homologs (Figure 3(d)), but is not observed in any other type of protein (as determined by PSI-BLAST analysis). However, attempts to determine a DNA binding motif for BAP60$^{116–204}$ by SELEX failed to reveal a distinct DNA binding motif (data not shown), indicating that BAP60 has non-sequence-specific DNA binding properties.

We next asked whether the non-specific DNA binding ability of BAP60 is essential for its *in vivo* function. Full-length BAP60 containing the three amino acid replacements that cause the protein to lose its *in vitro* DNA binding properties can fully rescue *Bap60*^{1} mutant individuals when expressed from a transgene under the control of the *Act5C* promotor. Thus, the *in vitro* DNA binding properties of the BAP60$^{116–204}$ region are not essential for the function of the protein *in vivo*. These results suggest that BAP60 does not participate directly in the selection of specific DNA target sites, but may instead enhance the affinity of the Brahma complex once bound to DNA, for example after recruitment *via* sequence-specific transcription factors.

Bap60 interacts genetically with sex-determining genes *sisterless A* and *scute*

Mammalian BAP60 homologs have been shown to interact with sequence-specific transcription factors.[16–20] Furthermore, a previous study had shown a function of the human BAP60 homolog BAF60c in heart development. In addition, it was demonstrated by cell culture experiments that

Table 2. Maternal heterozygosity for *Bap60*^{1} reduces the viability of female offspring heterozygous for mutant alleles of *sisA* or *sc*

Genotype[a]	Maternal genotype	Zygotic genotype	Sex	Viability (%)	No. progeny
A	*Bap60*/+	*Bap60/sisA sisC*	F	24	64
		+/*sisA sisC*	F	19	52
		+/*Y*	M	100[b]	269
B	*Bap60*/+	*Bap60* or +/*sisA sisC*	F	44	87
		+/*Y*	M	100[b]	99
C	+/+	+/*sisA sisC*	F	98	166
		+/*Y*	M	100	170
D	*Bap60*/+	*Bap60* or +/*sisA*	F	52	57
		+/*Y*	M	100[b]	55
E	+/+	+/*sisA*	F	105	132
		+/*Y*	M	100	126
F	*Bap60; Act5c-Bap60*$^{+}$	*Bap60/sisA sisC; Act5c-Bap60*$^{+}$/+	F	96	55
		Bap60/Y; Act5c-Bap60^{+}/+	M	100	57
G	*Bap60*/+	*Bap60* or +/*Df(sc)*	F	52	295
		+/*Y*	M	100[b]	283
H	+/+	+/*Df(sc)*	F	82	166
		+/*Y*	M	100[c]	101
I	*Bap60*/+	*Bap60* or +/*sc*	F	61	75
		+/*Y*	M	100[b]	61

[a] Genotypes of crosses: A: *y w Bap60*^{1}/*FM7c* ff × mm *y v sisA*^{1}*os*^{sisC1}*B/Y*, B: *y w Bap60*^{1}/*w*^{1118} ff × mm *y v sisA*^{1}*os*^{sisC1}*B/Y* (females siblings to cross C), C: *FM7c/w*^{1118} ff × mm *y v sisA*^{1}*os*^{sisC1}*B/Y* (females siblings to cross B), D: *y w Bap60*^{1}/*w*^{1118} ff × mm *y cm ct sisA*^{1}/*Y* (females siblings to cross E), E: *FM7c/w*^{1118} ff × mm *y cm ct sisA*^{1}/*Y* (females siblings to cross D), F: *y w Bap60*^{1}*; Act5c-Bap60*$^{+}$ ff × mm *y v sisA*^{1} *os*^{sisC1} *B/Y*, G: *y w Bap60*^{1}/*w*^{1118} ff × mm *Df(1)sc*^{19}*,y sc/Y-Dp(1:Y)y*$^{+}$*ASC*$^{+}$ (females siblings to cross H), H: *FM7c/w*^{1118} ff × mm *Df(1) sc*^{19}*,y sc/Y-Dp(1:Y)y*$^{+}$*ASC*$^{+}$ (females siblings to cross G), I: *y w Bap60*^{1}/*w*^{1118} ff × mm *In(1)sc*$^{10\text{-}1}$/*Y-Dp(1:Y)y*$^{+}$*ASC*$^{+}$

[b] *Bap60*^{1}/*Y* males do not survive. Reference is #+/*Y* males ×2.

[c] *FM7c* males were not counted. Reference is # *w*^{1118}/*Y* males ×2.

BAF60c interacts physically with transcription factors involved in heart development.[20] However, the effects of such interactions at the level of an entire organism have not yet been demonstrated. We therefore set out to establish the relevance of interactions between *Drosophila* BAP60 and transcription factors at the organismal level by using a genetic approach combined with *in vivo* and *in vitro* binding studies of BAP60 and the genetically identified transcription factors.

A first hint towards an interaction of BAP60 with transcription factors came from an unrelated screen for modifiers of the sex-determining genes *sisterless A* (*sisA*) and *scute* (*sc*), which encode transcription factors.[40–42] We found that when present maternally, the two overlapping deficiencies *Df(1)JA26* and *Df(1)N12*, which uncover *Bap60*, cause female-specific lethality in heterozygous *sisA* or *sc* mutant offspring (data not shown). We next tested whether a reduced amount of maternal BAP60 is responsible for the observed lethality of heterozygous mutant *sisA* and *sc* females by asking whether the absence of one maternal copy of *Bap60* causes a deficiency-like gender-specific effect.

Table 2 shows the results of corresponding genetic interaction studies. Since the X-chromosomal *Bap60*^{1} allele causes lethality in hemizygous males, heterozygous *Bap60*^{1} females produce male and female offspring in a 1:2 ratio, indicating that decreased maternal *Bap60* activity does not affect the normal male/female ratio. However, when in addition the zygotic wild-type gene dose of either *sisA* or *sc* is reduced to one copy, a specific reduction of female offspring is observed (Table 2).

BAP60 interacts with SISA and SC both *in vivo* and *in vitro*

The fact that *sisA* and *sc* encode transcription factors suggests that the genetic interactions observed with *Bap60* could be provided by direct physical interactions between the corresponding proteins and BAP60. We tested this possibility by performing co-immunoprecipitation experiments using epitope-tagged proteins expressed in *Drosophila* S2 cells. Figure 4(a) shows that both SISA and SC are co-precipitated upon immunoprecipitation with antibodies directed against the FLAG epitope of FLAG-tagged BAP60. Control experiments showed that SISA and SC were not precipitated in the absence of the FLAG-BAP60 protein. The result indicates that SISA and SC are associated with BAP60 in cell lysates. However, this association could be of indirect nature, for example *via* a different subunit of the Brahma complex. In order to test this possibility, we performed GST-pulldown experiments using purified GST-BAP60 fusion protein and *in vitro* translated SISA and SC proteins. The results of these experiments show that the association of BAP60 with each of the two transcription factors is direct, at least *in vitro* (Figure 4(b)).

SXL expression is not visibly affected

The specific loss of female offspring observed in the genetic interactions between *Bap60* and the transcription factors *sisA* and *sc* is reminiscent of that seen when *Sex-lethal* (*Sxl*) function is impaired.

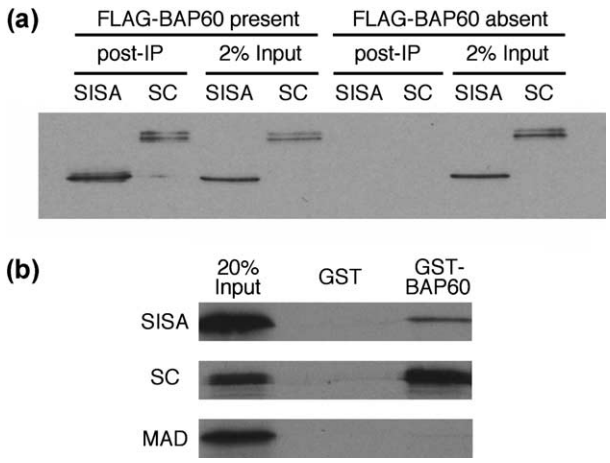

Figure 4. (a) SISA and SC co-immunoprecipitate with BAP60 in cell culture. FLAG epitope-tagged BAP60 and Myc epitope-tagged SISA or SC were co-expressed in *Drosophila* S2 cells. Immunoprecipitations were directed against the FLAG epitope tag. The presence of SISA and SC in immunoprecipitates was determined after Western blotting by immunostaining against the Myc epitope tag. The ability of SISA and SC to co-precipitate depends on co-transfection with the expression vector for FLAG-BAP60. (b) GST-pulldown experiments. *In vitro* translated SISA, SC and MAD were incubated with beads loaded with GST or GST-BAP60. After five wash steps, the beads were loaded onto an SDS/polyacrylamide gel. Target proteins were visualized by autoradiography. BAP60 interacts physically with both SISA and SC. BAP60 does not interact with the transcription factor MAD,[54] which was included as a control.

Female-specific lethality is caused by a lack of SXL-dependent repression of X-chromosomal dosage compensation in females.[40,43] Since the *Sxl* gene is a common target gene of the transcription factors SISA and SC,[40–42,44,45] we reasoned that the combination of maternally reduced BAP60 activity and partial loss of either *sisA* or *sc* activities may directly affect *Sxl* expression in early embryos. We tested this possibility by immunostainings on whole-mount embryos using anti-SXL antibodies, asking whether the pattern and/or amount of SXL protein is visibly affected in embryos with reduced activities of *Bap60* and *sisA*, or *Bap60* and *sc.* Although the female offspring among such embryos are clearly affected, as revealed by the genetic interaction studies (see above), neither the amount nor the patterns of SXL expression were found to be visibly altered in such embryos (data not shown). This observation implies that BAP60-dependent SISA and SC activities, which specifically affect the female offspring, do not affect *Sxl* transcription *per se* but rather the efficiency of X-chromosomal dosage compensation at a different, and as yet unidentified level.

Discussion

Our results provide the first evidence that BAP60 carries a vital function and participates in several aspects of BRM complex function at the organism level. They also complement and confirm previous tissue culture studies with vertebrate BAP60 homologs by showing that BAP60 has DNA binding properties and is able to associate with transcription factors. Our studies demonstrate that the transcription factors SISA and SC interact with BAP60 by genetic means and that they are able to associate directly with BAP60. However, BAP60 is not directly required for *Sx*l expression but functions in a process referred to as *Sxl*-dependent suppression of X-chromosomal dosage compensation in females.[40,43] Genetic interaction studies suggest that the physical interactions of BAP60 with SISA and SC, observed both *in vitro* and in cell culture, are indeed relevant for the survival of the female offspring. On the basis of these observations, it was surprising to find no detectable change of SXL expression in embryos affected by the genetic interactions. This finding formally suggests that the interaction of BAP60 with SISA and SC might not be relevant for the regulation of SXL expression *per se*, but rather for the regulation of other target genes that act downstream of, in parallel with, or independent of *Sxl*.

Our results also provide evidence for a conserved domain that provides BAP60 with non-sequence-specific DNA binding properties *in vitro*. The ability of BAP60 to bind DNA may explain an earlier finding showing that the presence of BAF60a increases the *in vitro* affinity of the cJun/cFos heterodimer for its AP-1 binding site.[16] The stabilization of BRM complex/DNA association by unspecific DNA binding *via* BAP60 could be of importance for fixing the complex at specific promotors. Notably, our finding that BAP60 binds in a functional manner to sequence-specific transcription factors in *Drosophila* supports the proposal that mammalian BAP60 homologs mediate interactions between the Brahma complex and sequence-specific transcription factors.[16–20] BAP60-dependent recruitment of the BRM complex *via* transcription factors as different as the AP1 heterodimer cJun/cFos[16] and nuclear receptors[17,18]in mammals, as well as SISA and SC in the fly, exemplifies the multiple regulatory processes BAP60 is involved in. These interactions help to explain why a minimal BRM complex can achieve full *in vitro* chromatin remodeling activity in the absence of BAP60.[15]

Materials and Methods

Drosophila stocks and mutant analysis

Bap60 mutants were generated by crossing the EP element line *G1131*[21] to flies containing a transposase-expressing transgene. Remobilization of the EP element resulted in the generation of a deletion that was identified by virtue of the lethality it causes in male flies. The exact position and extent of the deletion was determined by

PCR amplification of the region of the original EP element insertion site, followed by sequencing of the amplified DNA. To generate *Bap60* germline clones, we employed the FLP-DFS technique.[26] For this purpose, the *Bap60*^{1} mutation was recombined onto the *y, w, v, P[mini w*$^{+}$*]; FRT]14A-B* chromosome and resulting female flies were crossed to *ovo*^{D1}, *P[mini w*$^{+}$*]; FRT]14A-B/Y; hs-FLP38/hs-FLP38* males. Larvae from such a cross were heat-shocked for 1 hour at 39 °C on the third and fourth day. *In situ* hybridization of whole-mount embryo preparations was performed with digoxigenin (Dig)-labeled antisense RNA. Probes were detected using an alkaline phosphatase-coupled anti-Dig antibody with NBT/BCIP staining. The EST used for rescue transgene construction, probe generation and recombinant protein expression was LD09078 (BDGP†). For the generation of a rescue construct, *Bap60* was brought under the control of the *Actin 5c* (*Act5c*) promotor[46] in a pCaSpeR4 background.[47] Fly stocks of the mutants *brm*^{2}, *mor*^{1}, *brm*^{2}*trx*^{E2}, *osa*^{2}, *Antp*^{NS}, *Antp*^{73b}, of the deficiencies *Df(1)JA26* and *Df(1)N12* and of the *In(1)w*^{m4h} chromosome were obtained from the Bloomington stock center ‡. The *snr1*^{R3} mutant[27] was provided by A. K. Dingwall. All strains listed in Table 2 are as described elsewhere.[40,41,48]

DNA co-immunoprecipitation analysis

GST fusion proteins for the *in vitro* DNA binding assay were generated by cloning the respective coding sequences into the plasmid pGEX-4T-3 (Amersham Biosciences) and by subsequent expression of the fusion protein in BL21(DE3) cells (Invitrogen). The GST fusion proteins were purified from the bacterial lysates by binding to GSH-Sepharose beads (Amersham Biosciences) and were then dialyzed against DNA binding buffer (see below). Restriction enzyme-digested plasmid DNA (~1 μg) was end-labeled with [^{33}P]ATP using phage T4 polynucleotide kinase (Fermentas). Protein A-Sepharose beads (Calbiochem) were incubated with polyclonal anti-GST antiserum (provided by U. Schmidt-Ott) in binding buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100. Up to 100 ng of labeled restriction fragments were incubated with 300 ng of GST fusion protein in 50 μl reactions in binding buffer for 30 minutes at room temperature. After addition of 50 μl of a 50% anti-GST-Sepharose bead suspension (see above) the reaction was further incubated for 30 minutes at 4 °C with rotation. The beads were then washed with binding buffer followed by washes (500 μl each) with increasing concentrations of NaCl. During each washing step, the beads were rotated for 5 minutes. The supernatants of each step were precipitated in ethanol and applied to a 4% (w/v) polyacrylamide gel. DNA fragments were detected by autoradiography.

Analysis of the BAP60 DNA-binding domain

To search for occurences of the domain outside the family of BAP60 homologs, a PSI-BLAST search with the BAP60 domain sequence was done using the default settings at the NCBI web site§ with three iterations of the search algorithm. The SELEX-based search for binding motifs of the BAP60 DNA binding domain was performed essentially as described.[49] Binding and wash buffer contained 20 mM Tris (pH 8.0), 40 mM KCl, 0.5 M EDTA, 5% glycerol, 20 μg/ml of bovine serum albumin, 2 μg/ml of poly(dIdC), 0.5 mM DTT. Approximately 70 ng of the GST fusion protein of the BAP60 DNA-binding domain were employed in each round. The search for motifs in the sequences obtained from the SELEX experiment after seven to nine rounds was done with the help of the MEME algorithm‖.[50] The Stratagene QuikChange Kit was used to achieve site-directed mutagenesis of conserved amino acids within the DNA binding domain.

Co-immunoprecipitation and GST-pulldown experiments

For co-immunoprecipitation experiments, *Drosophila* S2 cells were transiently co-transfected with pBS-based plasmids encoding SISA or SC fused at their amino termini to ten copies of the Myc epitope tag (pBS-Ubip-10myc-sisA, pBS-Ubip-10myc-sc) and with a plasmid encoding BAP60 fused at its amino terminus to three copies of the FLAG epitope tag (pBS-Ubip-3Flag-Bap60). Expression of the epitope-tagged proteins was achieved by virtue of the constitutively active ubiquitin promotor.[51] The cells (three wells of a six-well plate) were transfected using the Effectene reagent (Qiagen) and lyzed three days post-transfection in 500 μl of lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol) supplemented with Complete Protease Inhibitor tablets (Roche). After centrifugation (10 min, 20,000 ***g***) of the lysate, the supernatant was incubated overnight with 10 μl of ANTI-FLAG M2 Affinity Gel (Sigma) with rotation at 4 °C. The beads were washed four times with 500 μl of lysis buffer and were then boiled in 10 μl of 2× SDS sample buffer and loaded onto a Laemmli SDS/polyacrylamide gel. After electrophoresis, SISA and SC were visualized by Western blotting and immunostaining using an antibody directed against the Myc epitope tag (provided by A. Herzig).

For the GST-pulldown experiments, GST-BAP60 or GST alone (5 μg) was bound to 10 μl of GSH-Sepharose beads (Amersham Biosciences) and incubated for 1 hour at 4 °C with 5 μl of *in vitro* translated [^{35}S]methionine-labeled target protein (TNT Quick Coupled Transcription/Translation System, Promega) in 500 μl of pulldown buffer (50 mM Hepes–KOH (pH 7.9), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5 mM DTT, 1% Triton X-100). After five washes (500 μl each) with pulldown buffer, the beads were boiled in 10 μl of 2× SDS sample buffer and loaded onto a Laemmli SDS/polyacrylamide gel. After electrophoresis, target proteins were visualized by autoradiography.

Acknowledgements

We thank our colleagues in the laboratory for critical discussions and support. We especially thank U. Schäfer for providing the EP-element line *G1131*, and U. Jahns-Meyer, I. Plischke and G. Dowe for technical assistance.

† http://www.fruitfly.org
‡ http://flystocks.bio.indiana.edu
§ http://www.ncbi.nlm.nih.gov
‖ http://meme.sdsc.edu

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Edited by M. Yaniv

(Received 9 April 2005; received in revised form 27 June 2005; accepted 11 July 2005)
Available online 25 July 2005