The PBAP remodeling complex is required for histone H3.3 replacement at chromatin boundaries and for boundary functions

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SUMMARY

Establishment and maintenance of epigenetic memories are essential for development. Replacement of canonical histone H3 by its variant H3.3 has been implicated in cellular memory. *Drosophila* sequence-specific DNA-binding protein GAGA factor and a chromatin factor FACT direct H3.3 replacement in conjunction with H3.3-specific chaperone HIRA at chromatin boundaries to counteract the spreading of silent chromatin. However, little is known about which ATP-driven chromatin remodeling factor is responsible for the H3.3 replacement at chromatin boundaries. Here, we report that GAGA factor associates with the Polybromo-associated Brm (PBAP) remodeling complex, which consists of many Trithorax group proteins, and recruits this complex to chromatin boundaries *d1* (which is downstream of *w*), the *Fab-7* DNase-hypersensitive site (HS) 1 of *Abd-B* and the *bxd* region of *Ubx*. *Trl*-encoding GAGA factor, *brm* and *polybromo/bap180* mutations compromise the H3.3 replacement and boundary functions in a synergistic manner. Furthermore, Polybromo is necessary for generation of the DNase HS at *d1*, and HIRA functions to restore the alteration. Taken together, we propose that FACT and PBAP complexes are recruited to chromatin boundaries in a GAGA factor-dependent manner, and are needed for H3.3 replacement to execute boundary functions. Our results provide new insight into the function of the *trithorax* group during development.

KEY WORDS: PBAP complex, Histone H3.3, Chromatin boundary, FACT, *trithorax* group, *Drosophila*

INTRODUCTION

DNA is wrapped around a histone octamer to form the basic unit of chromatin structure. During embryogenesis, dynamic changes of chromatin structure and chromatin modification occur after fertilization; subsequently, the epigenetic information is inherited through many rounds of the cell cycle. Thus, chromatin is essential for the determination of cell identity. Two strategies are used to modulate a chromatin environment: the covalent modification of histone tails and energy-dependent chromatin remodeling. The acetylation, methylation or phosphorylation of histone tails can have profound effects on chromatin structure and transcription (Jenuwein and Allis, 2001). Chromatin remodeling reactions are catalyzed by large protein complexes that use the energy of ATP hydrolysis to alter the structure or positioning of nucleosomes (Becker and Hörz, 2002; Clapier and Cairns, 2009). In addition to these events, histone variants play important roles in modulating chromatin structure (Henikoff and Ahmad, 2005; Kamakaka and Biggins, 2005; Sarma and Reinberg, 2005).

One histone variant, H3.3, has been studied extensively. Although histone H3.3 differs by only four amino acids from canonical histone H3.1, they differ in their mechanisms of chromatin deposition. Histone H3.1 is assembled into chromatin during DNA replication, whereas deposition of histone H3.3 occurs throughout the cell cycle (Ahmad and Henikoff, 2002).

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Furthermore, the chaperone complex for H3.1 contains CAF-1, p150, p60 and p48, whereas the complex for H3.3 contains HIRA (Tagami et al., 2004). Biochemical analysis revealed that H3.3 is associated with active histone modifications such as H3 Lys 4 methylation (McKittrick et al., 2004). Moreover, a nucleosome containing H3.3 exhibits instability (Jin and Felsenfeld, 2007). Therefore, histone H3.3 replacement can change pre-existing epigenetic states.

Chromatin boundaries are transcriptional regulatory elements that modulate interaction between enhancer and promoter, and protect gene expression from positive or negative effects of the adjacent chromatin (Bell et al., 2001; Maeda and Karch, 2007). Establishment and maintenance of chromatin boundaries play a crucial role in development. For example, the *Fab-7* boundary governs space-specific expression of *Abd-B* by ensuring the autonomous activities of the *iab-6* and *iab-7* cis-regulatory regions (Mihaly et al., 1997). Previously, we have shown that GAGA factor interacts with FACT, induces chromatin remodeling and directs H3.3 replacement through an interaction with HIRA at chromatin boundaries *d1* and *Fab-7* to counteract the spreading of silent chromatin (Shimojima et al., 2003; Nakayama et al., 2007). Furthermore, H3.3 replacement peaks at multiple boundaries in the *Bithorax* complex, including *Mcp*, *Fab-7* and *Fab-8* (Mito et al., 2007). Thus, histone H3.3 replacement can influence chromatin boundary functions.

Although FACT displaces a histone H2A-H2B pair from a nucleosome (Belotserkovskaya et al., 2003), it alone cannot accomplish the H3.3 replacement that accompanies extensive chromatin remodeling, as revealed by DNase hypersensitivity (Nakayama et al., 2007). Furthermore, chromatin remodeling factors Chd1 and ATRX contribute to H3.3 deposition on the male pronucleus after fertilization and on pericentric or telomeric chromatin, respectively (Konev et al., 2007; Drané et al., 2010;

Goldberg et al., 2010). Therefore, we assume that an ATP-dependent chromatin remodeling factor is necessary for the replacement at chromatin boundaries. Among the ATP-driven remodelers, a Swi/Snf-type complex is thought to be most suitable for the replacement because it has a unique activity to transfer a histone octamer from one piece of DNA to another (Lorch et al., 1999; Workman, 2006). There are two subclasses of the Swi/Snf-type complex that consist of many Trithorax group proteins in *Drosophila* (Mohrmann et al., 2004). One is the Brm-associated proteins (BAP) complex, which contains Osa in addition to core subunits that are shared by the two complexes: Brm, Mor/Bap155, Bap111, Bap60, Bap55, Actin, and Snr1. The other is PBAP complex, which harbors Polybromo/Bap180 and Bap170 in place of Osa. These two complexes correspond to mammalian Brg1- or Brm-associated factors (BAF), and Polybromo-associated BAF (PBAF) complexes, respectively. However, involvement of these complexes in H3.3 replacement is not clear in either *Drosophila* or mammals.

In this study, we isolated GAGA factor-associated proteins to identify an ATP-dependent chromatin remodeling factor responsible for the H3.3 replacement at chromatin boundaries. We found association of the GAGA factor with PBAP complex and GAGA factor-dependent recruitment of the PBAP complex to chromatin boundary regions. Furthermore, our data indicate that PBAP complex is required for the H3.3 replacement at chromatin boundaries and for boundary functions.

MATERIALS AND METHODS
Antibodies
For generation of antibodies against Brm, Polybromo or Bap60, we amplified cDNA encoding a polypeptide corresponding to residues 1 to 500 of Polybromo, residues 1301 to 1630 of Brm, or full length Bap60, followed by subcloning it into the pET21 expression vector. Recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) pLysS at 25°C for 3 hours and were purified with Ni-NTA (Qiagen), followed by immunization of rabbits. Mouse anti-*Drosophila* Osa monoclonal antibody (Concentrate) was purchased from Developmental Studies Hybridoma Bank (DSHB, Iowa University, IA, USA). The purified anti-HIRA antibodies PG (Bonnefoy et al., 2007) were a gift from B. Loppin (Universite Lyon, Lyon, France). The anti-SSRP1, SPT16 and GAGA factor antibodies have been described previously (Shimojima et al., 2003; Nakayama et al., 2007).

Isolation of FLAG-GAGA factor associated proteins
All procedures were performed at 4°C. *Drosophila* embryos (125 g, 0-24 hours after egg laying) were collected from the fly line expressing FLAG-GAGA factor and dechorionated. Nuclear extract (NE, 100 mg protein) was prepared from the embryos, and FLAG-GAGA factor and its associated proteins were purified using 10 ml settled volume of FLAG M2 beads (Sigma-Aldrich) as described previously (Shimojima et al., 2003). To identify FLAG-GAGA factor-associated proteins, the eluted proteins were resolved by SDS-PAGE and transferred to a PVDF membrane (Roche Diagnostics) by a Trans-Blot Semi-Dry Apparatus (Bio-Rad, CA, USA). The membrane was subsequently stained with Coomassie Brilliant Blue, and protein in each band was subjected to MALDI-TOF mass analysis after digestion with lysylendopeptidase. For Superose 6 gel filtration, the pooled eluate was concentrated up to 500 μl with Amicon Ultra-15 (MWCO1000, Millipore). The concentrated proteins were loaded on a column of Superose 6 10/32 (GE Healthcare) and eluted with buffer F [20 mM HEPES Na$^+$ (pH 7.9), 50 mM NaCl, 0.2 mM EGTA, 10% (v/v) glycerol, 0.5 mM DTT, 0.01% (v/v) NP-40 and 0.5 mM PMSF]. Each 500 μl fraction was collected and proteins in each fraction were analyzed by western blotting.

Immunoprecipitation
NE containing 500 μg proteins from 0- to 24-hour-old *Drosophila* embryos of the *yw* line, the transgenic line expressing HIRA-FLAG or third instar larvae homozygous for *bap180Δ86* was pre-cleared by a 60-minute rotation with 10 μl of pre-immune rabbit IgG pre-adsorbed to 25 μl settled volume of Protein G agarose (Millipore) followed by centrifugation at 10,000 rpm (8000 *g*) for 5 minutes. The pre-cleared NE was incubated with 10 μl of rabbit antibodies against GAGA factor, Polybromo, Brm, SSRP1, SPT16, HIRA or anti-mouse monoclonal antibody against Osa or FLAG M2 pre-adsorbed to 25-μl settled volume of Protein G agarose in 500 μl of a binding buffer [20 mM HEPES Na$^+$ (pH 7.9), 50 mM (Fig. 1A,B) or 150 mM NaCl (Fig. 1C,D), 3 mM MgCl$_2$, 10% (v/v) glycerol, 0.2 mM EDTA, 1/1000 volume of Protease inhibitor cocktail, 500 μg/ml BSA, 0.5 mM DTT, and 0.1% (v/v) NP-40] for 2 hours. The beads were washed five times with a wash buffer [the binding buffer + 0.4% (v/v) NP-40] by rotating tubes slowly for 5 minutes. Twenty-five μl of 2×SDS sample buffer was added to the beads and eluted proteins were heat-denatured at 95°C for 5 minutes and then were centrifuged at 3000 rpm (700 *g*) for 5 minutes. Proteins in the supernatant were loaded on a 6.5% SDS polyacrylamide gel. Whenever positive results were obtained by co-immunoprecipitation, we confirmed that the association was not mediated through nucleic acids by repeating the experiments in the presence of ethidium bromide.

Western blotting
Proteins were resolved on a 6.5% or 8% SDS polyacrylamide gel and were transferred to a PVDF membrane (Millipore) in a transfer buffer [48 mM Tris base, 390 mM glycine, 20% (v/v) methanol, 0.1% SDS] for 180 minutes at 35 V. The membrane was blocked with 5% skim milk in TBST [20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.1% (v/v) Tween 20] for 30 minutes at room temperature. The membrane was incubated with each primary antibody (1:2000) in 1% skim milk in TBST for 1-2 hours at room temperature or at 4°C overnight. The membrane was washed three times with TBST for 5 minutes at room temperature. The membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) in 1% skim milk in TBST for 1 hour at room temperature. Signals were detected with Supersignal+ (Pierce) and a LAS-4000 Mini Imaging System (FUJIFILM, Tokyo, Japan).

Chromatin immunoprecipitation (ChIP)
ChIP assays using larval nuclei were performed as follows. Approximately 3500 third instar larvae homozygous for *Trl^{13C}* were collected and stored at −80°C. The frozen larvae were thawed and homogenized in 5 ml of the buffer A [10 mM HEPES Na$^+$ (pH 7.9), 10 mM KCl, 3 mM MgCl$_2$, 0.2 mM EGTA, 1 M sucrose, 0.5 mM DTT, 0.1% (v/v) NP-40, 0.5 mM PMSF and 1/1000 volume of Protease inhibitor cocktail (Sigma-Aldrich, P8340)]. The homogenate was filtered through two layers of 63-μm nylon mesh and was centrifuged at 1400 rpm (180 *g*) for 90 seconds to remove debris. The supernatant was centrifuged at 6000 rpm (3200 *g*) for 10 minutes. The pelleted nuclei were suspended with 5 ml of buffer G [20 mM HEPES Na$^+$(pH 7.9), 10 mM KCl, 0.2 mM EGTA, 10% (v/v) glycerol, 0.5 mM DTT, 0.1% (v/v) NP-40, 0.5 mM PMSF and 1/1000 volume of Protease inhibitor cocktail]. The suspended nuclei were transferred into a 15-ml tube and equal volume of buffer H [20 mM HEPES Na$^+$(pH 7.9), 10 mM KCl, 0.2 mM EGTA, 10% (v/v) glycerol, 0.1% (v/v) NP-40, 0.5 mM PMSF, 1/1000 volume of Protease inhibitor cocktail and 2% formaldehyde] was added. Crosslinking was carried out by rotating the tube for 15 minutes at 4°C. To stop the crosslink, one-ninth the volume of 1.25 M glycine was added and the tube was rotated for another 5 minutes. The tube was centrifuged at 3500 rpm (1600 *g*) for 10 minutes. Sonicated chromatin was prepared from the pelleted nuclei and subjected to ChIP as described previously (Nakayama et al., 2007). After phenol/chloroform extraction followed by ethanol precipitation, the DNA was dissolved in 100 μl of TE. Quantitative (q) PCR using 2-10 μl of the DNA was performed with a Light Cycler 2.0 (Roche Diagnostics, Mannheim, Germany). The experiments were repeated at least three times. For ChIP assays of *Trl$^+$* (wild type) or *polybromo* mutant samples, ~800 third instar larvae of wild type or *bap180Δ86* (Carrera et al., 2008) homozygote were collected and processed as above. ChIP assays using embryos were carried out as described previously (Nakayama et al., 2007), except that amounts of DNA were quantified with qPCR as above.
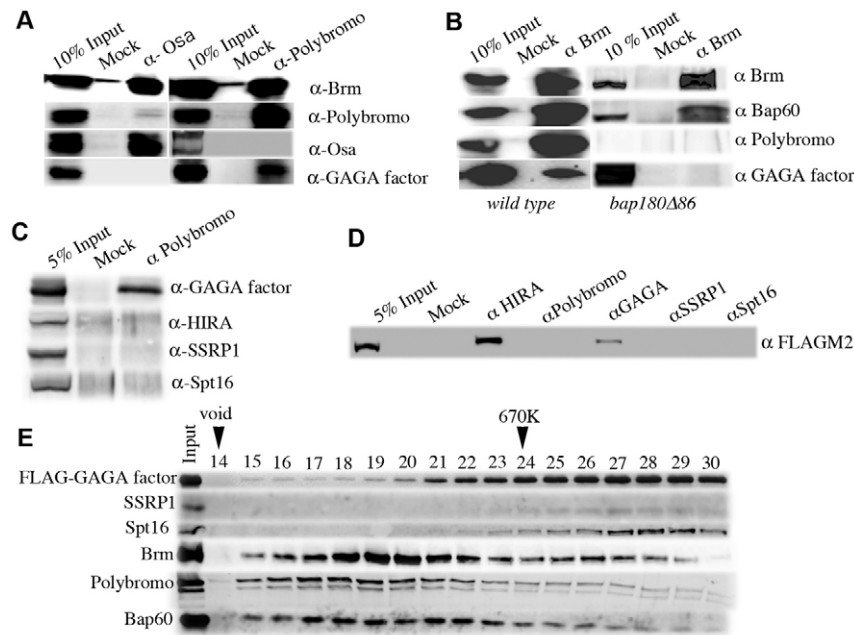
Fig. 1. PBAP complex is associated with GAGA factor. (**A**) Co-immunoprecipitation analyses using nuclear extracts (NEs) from *yw* embryos with anti-Osa antibody (left panel) or with anti-Polybromo antibodies (right panel). Eluted proteins were immunoblotted with antibodies against Brm, Polybromo, Osa or GAGA factor. (**B**) Co-immunoprecipitation analyses using NEs from *yw* embryos (left panel) or *bap180Δ86* homozygous larvae (right panel) with anti-Brm antibodies. Eluted proteins were immunoblotted with antibodies against Brm, Bap60, Polybromo or GAGA factor. (**C**) Co-immunoprecipitation analyses using NE from *yw* embryos with anti-Polybromo antibodies. Eluted proteins were immunoblotted with antibodies against GAGA factor, HIRA, SSRP1 or Spt16. (**D**) Co-immunoprecipitation analyses of HIRA-FLAG. Proteins in NEs from a fly line expressing HIRA-FLAG were immunoprecipitated with antibodies against HIRA, Polybromo, GAGA factor, SSRP1 or Spt16, and analyzed by immunoblotting using anti-FLAG antibody M2. (**E**) Gel filtration profile. Immunoaffinity-purified FLAG-GAGA factor-associated proteins were fractionated by Superose 6 gel filtration and proteins in each fraction were analyzed by immunoblots using antibodies against FLAG, FACT subunits, Brm, Polybromo or Bap60.

Reverse transcription (RT)-qPCR

Total RNAs were prepared from third instar larvae using Sepasol (Nakalai Tesque). cDNAs were synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) in a 20 μl-reaction mixture according to the manufacturer's protocol. Subsequently, 20 μl of sterilized water were added to the reaction mixtures. The real-time qPCR were conducted using the Light Cycler 2.0 (Roche Diagnostics, Mannheim, Germany). Reactions were carried out using each 2 μl of the cDNA as a template in a 20-μl reaction mixture. The amount of starting cDNA was normalized to that of β-tubulin. The experiments were repeated at least three times. Primer sequences used for qPCR are shown in supplementary material Table S1.

Position effect variegation

For position effect variegation (PEV) assays, w^{m4} or w^{m4}; $Trl^{R85}/TM6B$ females were crossed with wild-type or a mutant males, and male progenies with a desired genotype were used for quantification of eye pigment levels. Twenty adult male flies eclosed within 24 hours were collected and processed as described previously (Nakayama et al., 2007). More than eight independent samples were measured.

Abdominal segment (A) 6 to A5 transformation

yw, yw; $Trl^{R85}/TM6B$ or *yw;Δspt16/TM3* females were crossed with wild-type or a mutant males, and male progenies with a desired genotype were used for detection of ectopic bristles on A6.

*Spe*I accessibility assay

The assays were carried out as described (Jack and Eggert, 1990). Nuclei were isolated from third instar larvae and ~5 μg DNA was treated with 10 units of *Spe*I (Takara, Kyoto) at 37°C for 30 minutes. Ten independent incubations were made for each line.

Transgenic lines and other fly lines

Full-length Polybromo cDNA was obtained from National Institute of Genetics. The cDNA was subcloned into pET21 and a FLAG-encoding sequence was introduced to the N-terminal region. The full-length cDNA of FLAG-Polybromo was digested with *Mlu*I and *Not*I, and inserted into a germline transform vector pCasperHS83_EGFP (Nakayama et al., 2007). Transgenic flies were generated by *Drosophila* Embryo Injection Services (Best Gene). We used a line termed *yw; P[bap180⁺]*, in which the transgene was inserted into the second chromosome. We also established fly lines such as *P[hsp83-H3.3-FLAG,EGFP];bap180Δ86/TM3*, *P[hsp83-H3-FLAG,EGFP];bap180Δ86/TM3*, *P[hsp83-H3.3-FLAG,EGFP]; brm²/TM3*, *P[hsp83-H3-FLAG,EGFP];brm²/TM3*, *P[hsp83-H3.3-FLAG,EGFP];Trl¹³C/bap180Δ86*, *P[hsp83-H3-FLAG,EGFP];Trl¹³C/bap180Δ86*, *P[hsp83-H3.3-FLAG,EGFP];Trl¹³C/brm²*, *P[hsp83-H3-FLAG,EGFP];Trl¹³C/brm²*, w^{m4}*;Trl^{R85}/TM6B*, *P[bap180⁺];bap180Δ86*, *P[hsp83-H3-FLAG,EGFP];osa²/TM3*, *P[hsp83-H3.3-FLAG,EGFP];osa²/TM3*, *ssm/FM7;P[hsp83-H3-FLAG,EGFP]*, *ssm/FM7;P[hsp83-H3.3-FLAG,EGFP]*, *ssm/FM7GFP* and *ssm/FM7GFP;bap180Δ86/TM6B* through appropriate crosses. *bap180Δ86/TM6B* and *Trl^{R85}/TM6B* were gifts from Drs J. Treisman (New York University School of Medicine, NY, USA) and C. Wu (National Institutes of Health, MD, USA), respectively. *brm²/TM6B* and *osa²/TM6B* were obtained from the Bloomington Stock Center (Indiana University, IN, USA). Other fly lines have been described previously (Shimojima et al., 2003; Nakayama et al., 2007).

RESULTS
Isolation of GAGA factor-associated proteins

GAGA factor induces chromatin remodeling at chromatin boundaries (Nakayama et al., 2007) and, hence, we presumed that an ATP-dependent chromatin remodeler would be recruited to the boundaries through association with GAGA factor. When we isolated FLAG-GAGA factor-associated proteins in the previous study, the anti-FLAG immunoaffinity purification was conducted in the presence of 420 mM NaCl. Under this condition, only FACT

Table 1. MALDI-TOF mass spectrometric analyses of GAGA factor-associated proteins

Symbol	Locus tag	Accession number	Full length (amino acids)	Total peptides	Mascot score	Total number of amino acid residues	Coverage (%)
Nurf301	CG8274	AAL16644.1	2669	25	122	298	13.1
Kismet	CG3696	AAF51530.1	2151	10	35	103	5.7
dMi-2	CG8103	AAF49099.2	1982	26	152	285	13.3
Polybromo	CG11375	AAF56339.1	1654	48	290	670	41.0
Tho2	CG31671	AAF51302.2	1641	46	256	527	33.0
Brm	CG5942	AAA19661	1638	57	233	662	29.9
Mor	CG18740	NP_524373.1	1209	24	148	410	23.2
Spt16	CG1828	NP_476610.2	1083	27	161	332	24.7
ISWI	CG4119	AAM11261.1	1027	12	84	120	11.6
SSRP1	CG4817	AAF47064.1	723	19	84	191	26.6
Rpd3	CG7471	AAF47924.1	521	7	64	98	22.5
Bap60	CG4303	AAF48235.1	515	13	123	137	24.3
Bap55	CG6546	NP_611209.1	425	13	165	184	39.3
Thoc6	CG5632	XM_003804691.1	350	6	93	62	17.7
Actin	CG4027	AAF46098.1	376	7	108	96	25.5
Snr1	CG1064	AAC77830.1	370	5	77	69	18.6

was co-eluted with FLAG-GAGA factor (Shimojima et al., 2003). In the present study, we decreased the concentration of NaCl to 50 mM throughout sample application, washing and elution to isolate proteins that are weakly associated with GAGA factor. MALDI-TOF mass spectrometric analyses revealed that FLAG-GAGA factor was co-eluted with numerous proteins, such as Mi-2, Brm and Kismet, and the previously described GAGA factor-associated proteins Nurf 301, ISWI (Xiao et al., 2001) and FACT (Shimojima et al., 2003) (Table 1). Interestingly, the GAGA factor-associated proteins also included multiple core subunits of Brm complex, such as Mor, Bap60, Bap55, Snr1 and Actin, in addition to Brm. Therefore, we focused here on the Brm complex as a candidate ATP-dependent remodeler that is responsible for H3.3 replacement at chromatin boundaries.

MALDI-TOF mass spectrometry showed that GAGA factor was co-eluted with Polybromo but not with Osa (Table 1), suggesting that GAGA factor associates with the PBAP complex but not with the BAP complex. To confirm this, we performed co-immunoprecipitation with antibodies against Polybromo or Osa, followed by immunoblotting with antibodies against GAGA factor, Brm, Polybromo or Osa. We found that GAGA factor and Brm but not Osa were co-precipitated with Polybromo when nuclear extract (NE) from *yw* embryos was immunoprecipitated with anti-Polybromo antibodies (Fig. 1A, right panel). By contrast, neither GAGA factor nor Polybromo was co-precipitated with Osa when we used anti-Osa antibody (Fig. 1A, left panel). Reciprocal immunoprecipitation showed that Bap60, Polybromo and GAGA factor were co-precipitated with anti-Brm antibodies (Fig. 1B, left panel). Taken together, these data demonstrate that GAGA factor associates with the PBAP complex.

We then carried out the same set of immunoprecipitation with anti-Brm antibodies as above using NE from larvae homozygous for a *polybromo* mutation *bap180Δ86*. Polybromo protein was not detectable in both input and immunoprecipitated lanes (Fig. 1B, right panel), confirming that the allele is protein null (Carrera et al., 2008). Although co-immunoprecipitation of Bap60 with Brm suggests the presence of the Brm complex, GAGA factor was not co-precipitated with Brm. These results indicate that Polybromo is necessary for the association of GAGA factor with the PBAP complex.

Previously, we have shown that GAGA factor is associated with FACT (Shimojima et al., 2003) and HIRA (Nakayama et al., 2007). To examine the relationship among GAGA factor, FACT, HIRA

and PBAP, we extended the co-immunoprecipitation experiments. When proteins in NE from *yw* embryos were immunoprecipitated with anti-Polybromo antibodies, FACT components SSRP1 and Spt16, and HIRA were not co-precipitated (Fig. 1C). Furthermore, using NE from embryos of the transgenic line expressing HIRA-FLAG (Loppin et al., 2005), we observed that HIRA-FLAG was co-precipitated with GAGA factor as reported previously (Nakayama et al., 2007), but not with Polybromo, SSRP1 and Spt16 (Fig. 1D). From these results, although GAGA factor is associated with FACT, PBAP and HIRA, it is most likely that GAGA factor-FACT, GAGA factor-PBAP and GAGA factor-HIRA are distinct complexes.

Identification of GAGA factor-PBAP complex
To further confirm association of GAGA factor with PBAP complex, we analyzed the affinity-purified FLAG-GAGA factor-associated proteins by gel filtration through Superose 6 (Fig. 1E). The eluted fractions were resolved by SDS-PAGE and immunoblots were probed with antibodies against Brm, Polybromo, Bap60, SSRP1, Spt16 or FLAG. Components of PBAP, i.e. Brm, Polybromo and Bap60, were co-eluted mainly in fraction numbers 17-21. This represents the PBAP complex with a molecular mass of ~2 MDa (Mohrmann et al., 2004). A small but significant proportion of FLAG-GAGA factor was co-eluted with the PBAP complex. By contrast, the profile of FACT (SSRP1 and Spt16) did not overlap with the peak of the PBAP complex. Rather, FACT was co-eluted with the majority of FLAG-GAGA factor in fraction numbers 27-29, which correspond to the molecular mass of the FLAG-GAGA factor-FACT complex. These data indicate that GAGA factor forms a complex with PBAP remodeler and support the notion that the GAGA factor-PBAP complex and the GAGA factor-FACT complex are distinct from each other.

GAGA factor recruits PBAP complex and HIRA to chromatin boundaries
If PBAP complex functions at chromatin boundaries, it should be detected at boundaries. To examine occupancy of PBAP complex at chromatin boundaries, we carried out ChIP assays using chromatin fragments prepared from third instar larvae of wild type. GAGA factor, a FACT subunit SSRP1 and subunits of PBAP (Polybromo, Brm and Bap60) but not Osa were detected at three

chromatin boundaries: *d1*, *Fab-7* HS1 and the *bxd* region (Fig. 2A,B, wild type). These results indicate that GAGA factor, the PBAP complex and FACT are present at the chromatin boundaries. We also analyzed occupancies of HIRA at the chromatin boundaries. Confirming our previous study (Nakayama et al., 2007), HIRA was detected at *d1* (Fig. 2B). We also found occupancies of HIRA at *Fab-7* and *bxd* (Fig. 2B).

GAGA factor is expected to recruit these complexes to the chromatin boundaries, as all these boundaries carry a cluster of GAGA factor-binding sites. To test the possibility, we searched for a GAGA factor-deficient condition. RNAi-mediated knockdown of GAGA factor was not efficient because of the self-regulation of *Trl*-encoding GAGA factor (Bernués et al., 2007). Therefore, we employed a hypomorphic allele, Trl^{13C}, in which a P-element is inserted into the first intron of *Trl* (Farkas et al., 1994). Although bulk of Trl^{13C} homozygotes died during embryogenesis, rare escapers grew until third instar larvae. We raised the $Trl^{13C}/TM6B$ line in thousands of vials and collected the escapers without the *Tb* phenotype of *TM6B* for ChIP assays. We found that the levels of occupancy of GAGA factor at the three boundaries were markedly reduced in Trl^{13C} compared with those in wild type (Fig. 2A, Trl^{13C}). The levels of SSRP1, Polybromo, Brm, Bap60 and HIRA at the boundaries were also severely reduced in a similar manner to that of GAGA factor (Fig. 2A,B, Trl^{13C}). This is not due to reduction in the expression levels of SSRP1, Polybromo, Brm, Bap60 and HIRA in Trl^{13C}, as RT-qPCR showed that the expression levels of SSRP1, Polybromo, Brm, Bap60 and HIRA mRNAs were not affected in Trl^{13C} (Fig. 2C). Taken together, these data demonstrate GAGA factor-dependent recruitment of FACT, PBAP complex and HIRA to the chromatin boundaries.

Conversely, it is possible that the PBAP remodeler could facilitate the recruitment of GAGA factor, FACT and HIRA to the boundaries. To test the possibility, we collected third instar larvae that are homozygous for the *polybromo* mutation *bap180Δ86* and carried out ChIP assays. As shown in Fig. 2A,B, the occupancies of GAGA factor, FACT and HIRA were barely affected by the *polybromo* mutation, although the levels of Polybromo, Brm and Bap60 were severely reduced in the mutant. These results suggest that PBAP remodeler is not required for the recruitment of GAGA factor, FACT or HIRA to the boundaries.

PBAP complex and HIRA function in H3.3 replacement at chromatin boundaries

We next investigated whether PBAP complex and HIRA are involved in the H3.3 replacement at chromatin boundaries. To this end, we first established a fly line expressing histone H3.3-FLAG or H3-FLAG, which is heterozygous for *brm²*, *bap180Δ86*, *osa²* or *hira* mutation *ssm* (Loppin et al., 2005), or trans-heterozygous for Trl^{13C} and *brm²*, or for Trl^{13C} and *bap180Δ86*. The expression level of the H3.3-FLAG or H3-FLAG was ~1% the level of endogenous histone H3 (Nakayama et al., 2007). The low expression level would not disturb fly physiology but allow us to use the FLAG-tagged histone as a tracer. We then collected embryos 10-22 hours after egg laying and performed ChIP assays using a FLAG M2 monoclonal antibody. The heterozygous population consisted of embryos heterozygous for the mutation (50%), homozygous for the mutation (25%) and homozygous for a balancer or for the other mutation in the case of trans-heterozygote (25%), but we call the population simply heterozygote or trans-heterozygote here. We found that the ratio of H3.3 to H3 in Trl^{13C}, *bap180Δ86*, *brm²* or *ssm* heterozygote was significantly reduced at *d1*, *Fab-7* HS1 and *bxd* regions compared
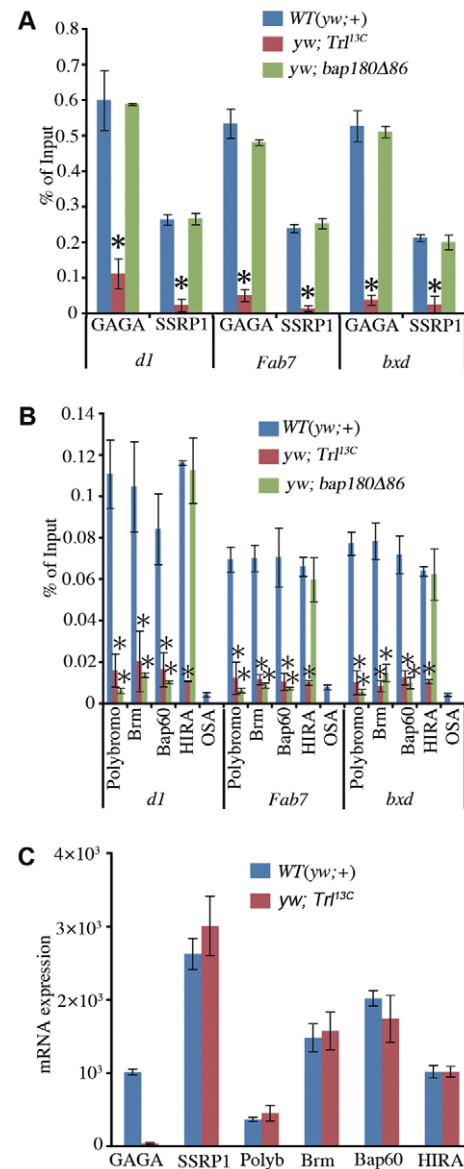
Fig. 2. GAGA factor recruits PBAP complex and HIRA to chromatin boundaries. (A,B) Occupancies of GAGA factor, FACT, PBAP complex and HIRA at chromatin boundaries *d1*, *Fab-7* HS1 and *bxd*. ChIP assays were carried out with antibodies against GAGA factor, SSRP1, Polybromo, Brm, Bap60, HIRA and Osa using larval nuclei from wild type, Trl^{13C} or *bap180Δ86*. **(C)** Expression levels of GAGA factor, SSRP1, Polybromo, Brm, Bap60 and HIRA mRNAs in wild-type or Trl^{13C} larvae. Data were normalized to β-tubulin mRNA. Experiments were repeated three times. Error bars represent s.d. Asterisks indicate statistical significance according to Welch's *t*-test: *$P < 0.005$.

with wild type (Fig. 3). Moreover the ratio of H3.3 to H3 was further reduced in $Trl^{13C}/brm²$ or $Trl^{13C}/bap180Δ86$ trans-heterozygote compared with Trl^{13C}, *brm²* or *bap180 Δ86* single heterozygote. As a negative control, *osa²* heterozygote did not affect the H3.3/H3 ratio at the boundaries. Furthermore, at control sites such as *iab-6* and *d6*, the H3.3/H3 ratios in the mutants were almost the same as that in wild type. These data indicate that PBAP complex and HIRA are required for the H3.3 replacement at the chromatin boundaries.
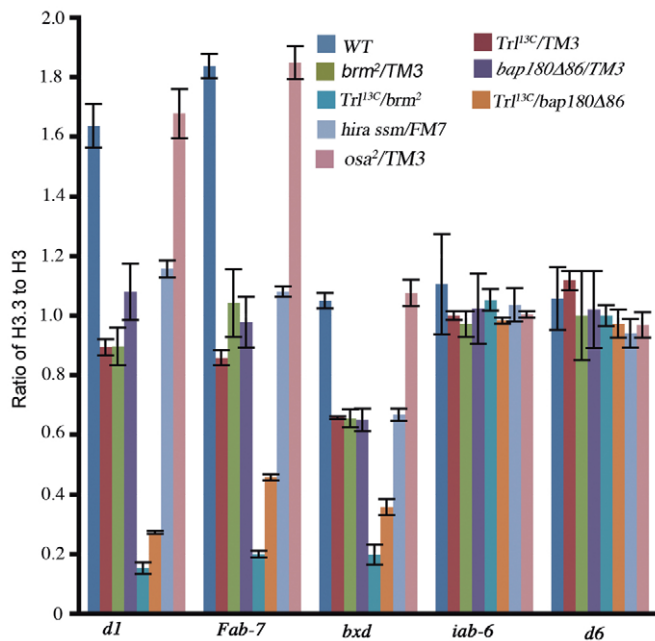
Fig. 3. PBAP complex and HIRA are required for H3.3 replacement at chromatin boundaries. ChIP assays were carried out with the anti-FLAG M2 antibody using embryos from wild type or from each mutant line harboring either *P[EGFP, hsp83-H3.3-FLAG]* or *P[EGFP, hsp83-H3-FLAG]* transgene. Shown is the ratio of H3.3 to H3 in each line. The experiments were repeated at least four times. Data are mean±s.d. Differences between wild type and each heterozygote (except *osa²* heterozygote) or between *Trl¹³C* heterozygote and each trans-heterozygote are significant (*P*<0.001; Welch's *t*-test).

Fig. 4. *Spe*I accessibility assays. The assays were carried out in wild type and indicated mutants with qPCR levels relative to undigested control (%). A *Spe*I recognition sequence is present immediately before $(GA)_8$ at *d1*. A control *Spe*I site was chosen arbitrarily ~11 kb downstream of *d1*. The *Spe*I incubations were repeated ten times in each line. Data are mean±s.d. Differences between wild type and *bap180Δ86* or *ssm* are significant (*P*<0.001; Welch's *t*-test).

Polybromo is responsible for alteration of chromatin structure and HIRA functions to restore this alteration

Dynamic histone H3.3 replacement at a chromatin boundary would generate a DNase HS. Indeed, we have shown that GAGA factor directs formation of a DNase HS at the *d1* boundary (Nakayama et al., 2007). To test whether PBAP complex is involved in the alteration of chromatin structure, we carried out *Spe*I accessibility assays. The assay relies on inhibition of qPCR encompassing an *Spe*I site upon *Spe*I-mediated cleavage of DNA (Fig. 4, naked DNA). When nuclei isolated from wild-type larvae were treated with *Spe*I, the qPCR levels at *d1* was ~50% of undigested control, whereas the level at a control *Spe*I site was ~90% of undigested control (Fig. 4). These results confirm our previous conclusion that *d1* is a DNase HS. By contrast, the *Spe*I accessibility at *d1* was severely compromised in nuclei from *bap180Δ86* homozygous larvae. Expressing Polybromo/Bap180 from a transgene restored the accessibility in *bap180Δ86*. These results demonstrate that Polybromo is responsible for the formation of DNase HS at *d1*.

As HIRA is another key player in the H3.3 replacement, we also analyzed the *Spe*I accessibility in nuclei from *hira ssm* larvae. Interestingly, the accessibility at *d1* in *ssm* larvae was even higher than that in wild type, while the control *Spe*I site was barely accessible. These results suggest that the *Spe*I accessibility at *d1* in wild type is under equilibrium between Polybromo-dependent enhancement and HIRA-dependent reduction. Furthermore, this *ssm* phenotype was suppressed by the homozygous *bap180Δ86* allele: the accessibility in *ssm; bap180Δ86* double mutant was
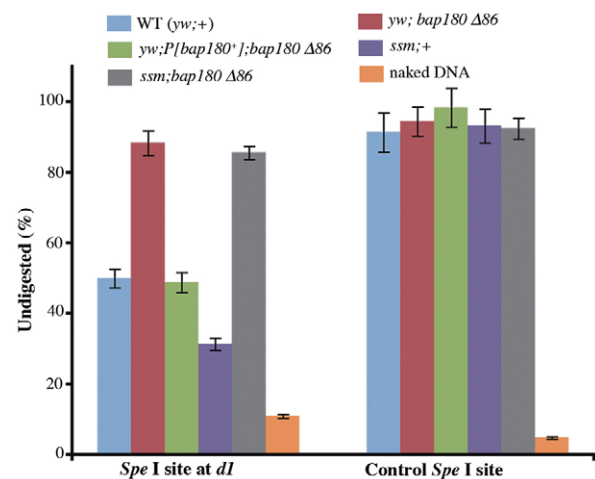
almost the same level as that in the *bap180Δ86* single mutant. These data suggest that HIRA restores the Polybromo-induced alteration of chromatin structure and that this HIRA activity is dependent on the Polybromo function.

Crucial role of PBAP complex in boundary functions

In the w^{m4} line, where *w* is juxtaposed with pericentric heterochromatin, counteraction of the heterochromatin spread at the *d1* boundary maintains *w* expression (Nakayama et al., 2007). Therefore, the boundary function at *d1* can be assessed by position effect variegation (PEV). To test whether PBAP complex is involved in the boundary function at *d1*, we analyzed PEV in wild type and various mutants under the w^{m4} background (Fig. 5). Trl^{R85} is a deletion allele that is obtained by excision of the P-element in Trl^{13C}. Its deletion removes extensive regions of the *Trl* transcription unit and, hence, it is presumed to be a null allele (Farkas et al., 1994). As reported previously (Farkas et al., 1994), PEV was enhanced (i.e. eye pigmentation was reduced) in Trl^{R85}/+. brm^2/+ also enhanced PEV. Similarly, PEV was enhanced in *bap180Δ86*/+ and the enhancement was restored by expressing Polybromo/Bap180 from the transgene. Moreover, in Trl^{R85} and brm^2 or Trl^{R85} and *bap180Δ86* trans-heterozygotes, PEV was enhanced more than in each single heterozygote. As a control, osa^2/+ did not affect PEV. These data indicate that the PBAP complex plays a crucial role in the boundary function at *d1* together with GAGA factor.

Fab-7 is the boundary that insulates between *iab-6* and *iab-7* enhancers of *Abd-B* (Mihaly et al., 1997). Defects in the *Fab-7* boundary function results in a homeotic transformation of the male A6 to A5, as revealed by formation of extra bristles on male A6. Previously, we have shown that defects in *Trl* and *spt16* encoding a FACT subunit cause the A6 to A5 transformation and genetic interaction between *Trl* and *spt16* on the phenotype (Shimojima et al., 2003). To examine whether PBAP complex is involved in the
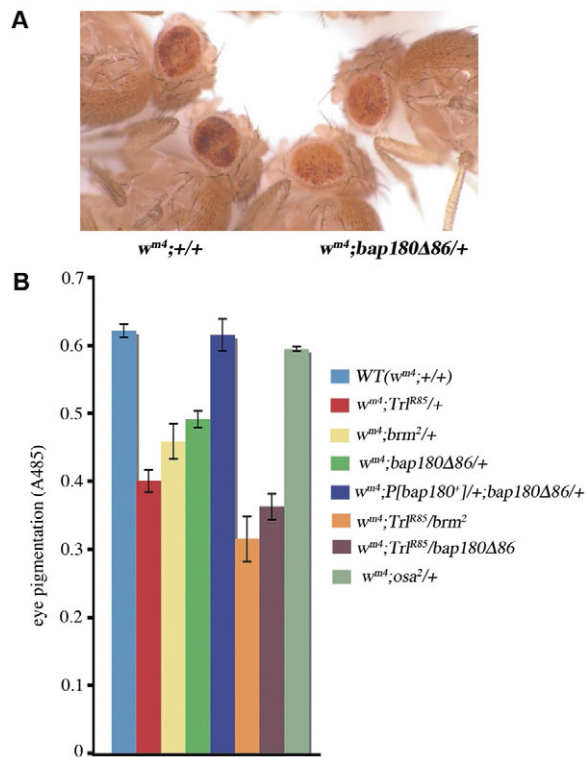
Fig. 5. Importance of PBAP complex to counteract heterochromatin spreading at *d1* boundary. (**A**) A typical example of position effect variegation (PEV), demonstrating enhancement in *bap180Δ86/+* (right two flies) compared with *+/+* (left two flies). (**B**) PEV assays were carried out by measuring eye pigmentation in male flies of indicated genotypes under the *w^m4* background. The experiments were repeated at least eight times. Data are mean±s.d. Differences between wild type and each heterozygote, except *osa²/+*, or between *Trl^R85/+* and each trans-heterozygote are significant (*P<0.001*; Welch's *t*-test).

Table 2. The PBAP complex is required for boundary function at *Fab-7*

Genotype	A6 to A5 transformation/ total number	Percentage
Wild type	0/504	0
Trl^R85/+	19/472	4
brm²/+	18/452	4
bap180Δ86/+	27/706	4
P[bap180⁺]/+;bap180Δ86/+	0/807	0
bap180Δ86	104/260	40
P[bap⁺];bap180Δ86	0/511	0
Δspt16/+	21/461	5
Trl^R85/brm²	36/313	12
Trl^R85/bap180Δ86	33/275	12
Δspt16/bap180Δ86	45/305	15
osa²/+	0/516	0

Frequencies of the A6 to A5 transformation were measured in males of indicated genotypes. Differences between wild type and each single heterozygote, except *osa²/+*, or between *Trl^R85/+* or *Δspt16/+* and each transheterozygote were significant (*P<0.001*; Fisher's exact test).

boundary function at *Fab-7*, we analyzed frequencies of the A6 to A5 transformation in wild type and various mutants (Table 2). We were unable to find the A6 to A5 transformation in wild type and *osa²/+*. However, we observed the transformation in *Trl^R85/+*, *brm²/+* and *Δspt16/+*, although the frequency was low. A similar frequency of the transformation was detected in *bap180Δ86/+*, and this phenotype was rescued by expressing Polybromo/Bap180 from the transgene. In the *bap180Δ86* homozygote, we observed a marked increase in the frequency of this transformation compared with the *bap180Δ86* heterozygote and this phenotype was rescued by expressing Polybromo/Bap180 from two doses of the transgene. Moreover, in *Trl^R85* and *brm²*, *Trl^R85* and *bap180Δ86* or *Δspt16* and *bap180Δ86* trans-heterozygotes, the frequency of this transformation was higher than in each single heterozygote. These data indicate that PBAP complex functions at the *Fab-7* boundary together with GAGA factor and FACT.

DISCUSSION

In the previous study, we have shown that the GAGA factor-FACT complex directs the H3.3 replacement at chromatin boundaries in conjunction with the histone chaperone HIRA (Nakayama et al., 2007). The present study demonstrates that PBAP remodeling complex is recruited to the boundaries in a GAGA factor-dependent manner and contributes to the H3.3 replacement at the boundaries.

Our study also revealed that Polybromo is responsible for the generation of a DNase HS and that HIRA functions to restore the altered state. Taken together, we propose the following model for the H3.3 replacement at a chromatin boundary (Fig. 6). GAGA factor recruits FACT and PBAP to boundaries carrying multiple GAGA factor-binding sites. FACT displaces a H2A-H2B pair from a nucleosome with the displaced H2A-H2B being anchored by FACT. This facilitates access of PBAP to a H3-H4 tetramer because the tetramer is sandwiched between two H2A-H2B dimers in a nucleosome core. Then PBAP would displace H3-H4. The resulting nucleosome-free region would be a DNase HS and become a target of the HIRA-ASF1 chaperone complex (Tagami et al., 2004) for deposition of H3.3-H4, as proposed by Ray-Gallet et al. (Ray-Gallet et al., 2011). The deposition of H3.3-H4 would mask the DNase HS. Finally, FACT deposits the tethered H2A-H2B pair to reconstitute the nucleosome. The process would be repeated continuously to achieve boundary function. As FACT, Polybromo-containing Brm complex, HIRA and ASF1 are conserved among species from *Drosophila* to human, a similar mechanism may also operate in mammals.

What is the role of H3.3 replacement in boundary function? It has been reported that H3.3 is associated with active histone modifications such as Lys 4 methylation but tends to avoid Lys 9 methylation (McKittrick et al., 2004). Indeed, we have detected a peak of H3K4 methylation and a dip of H3K9 methylation at the *d1* boundary (Nakayama et al., 2007). Therefore, the continuous H3.3 replacement at chromatin boundaries can prevent the spreading of specific histone modifications. Alternatively, the intrinsic instability of a nucleosome containing H3.3 (Jin and Felsenfeld, 2007) and the transient disruption of nucleosome structure during H3.3 replacement would generate a nucleosome-free region. This nucleosome gap can serve as a barrier against the spreading of chromatin with specific epigenetic memories.

Before isolation of GAGA factor-associated proteins, we anticipated the presence of a huge complex containing GAGA factor, FACT and a chromatin remodeling factor. However, our data suggest the presence of two distinct complexes: the GAGA factor-FACT complex and the GAGA factor-PBAP complex. This means that at least two GAGA factor-binding sites, one for the GAGA factor-FACT complex and the other for the GAGA factor-PBAP complex, are necessary within a boundary to allow
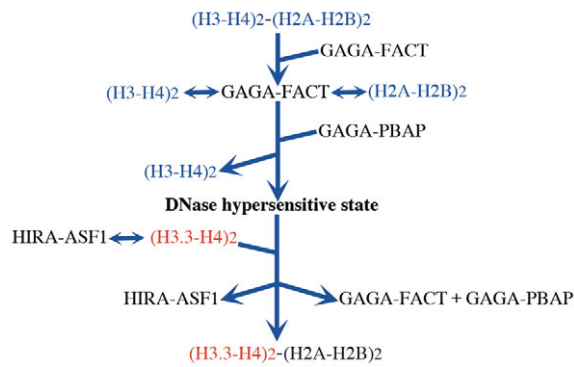
Fig. 6. Model for H3.3 replacement at the chromatin boundary.
GAGA factor recruits FACT and PBAP to a chromatin boundary
harboring a cluster of GAGA factor-binding sites. FACT interacts with a
nucleosome and displaces a H2A-H2B pair while anchoring the
displaced pair. This would facilitate PBAP-mediated displacement of an
H3-H4 tetramer. The histone-displaced region of DNA would be a
DNase HS. Then HIRA-ASF1 complex would deposit H3.3-H4 on the
region to restore from the DNase-hypersensitive state. Finally, FACT
deposits the tethered H2A-H2B pair to reconstitute the nucleosome.

concerted actions of FACT and PBAP for the H3.3 replacement.
This may preclude the H3.3 replacement to occur at a single
GAGA factor-binding site that is present frequently within the
genome. In good agreement with the idea, chromatin boundaries
harbor a cluster of GAGA factor-binding sites that are occupied
with the factors. For example, ChIP assays using anti-GAGA factor
antibodies showed a prominent signal at *d1* consisting of $(GA)_8$
compared with other single GAGA factor-binding sites around *w*
(Nakayama et al., 2007). The present study revealed that the level
of GAGA factor at *Fab-7* HS1 or *bxd*, which harbor multiple
closely spaced GAGA factor-binding sites, was comparable with
that at *d1* (Fig. 2A,B, wild type). Because multiple GAGA factor-
binding sites are present in other boundaries [such as *Fab-8* and
Mcp in the *Bithorax* complex (Busturia et al., 2001; Moon et al.,
2005), regulatory elements of the α1-tubulin gene (O'Donnell et
al., 1994), and the SF1 insulator of the *Antennapedia* complex
(Belozerov et al., 2003)], the FACT- and PBAP-dependent H3.3
replacement may also occur at these boundaries. Multiple closely
spaced GAGA factor-binding sites are presumably an indicator of
the chromatin boundary.

The present study demonstrates that the PBAP complex but not
the BAP complex is required for the boundary functions. These two
subtypes of Swi/Snf remodeling factor possess overlapping, distinct
and counteracting functions (Collins and Treisman, 2000; Moshkin
et al., 2007; Carrera et al., 2008). As for the distinct functions,
while the BAP complex controls the cell cycle and Wingless target
genes (Collins and Treisman, 2000; Moshkin et al., 2007), the
PBAP complex regulates metamorphosis and the immune response
(Carrera et al., 2008). Whether the H3.3 replacement-mediated
boundary function is involved in the regulation of metamorphosis
or the immune response remains to be clarified in future studies.

Recent articles have reported involvement of ATP-dependent
remodeling factors in deposition of H3.3 into chromatin. First, it
has been shown that Chd1 is required for the deposition of H3.3
into decondensing sperm chromatin after fertilization (Konev et al.,
2007). Others have demonstrated contribution of ATRX to the
deposition of H3.3 at pericentric chromatin and telomeres (Drané
et al., 2010; Goldberg et al., 2010). We show here that PBAP is

required for H3.3 replacement at the chromatin boundaries. These
data support the idea that distinct ATP-dependent remodelers work
for H3.3 deposition at specific locations within the genome
(Goldberg et al., 2010). Interestingly, null mutations in *Drosophila
polybromo/bap180*, *chd1* and *hira* exhibit the same phenotype, i.e.
viable but female sterile (Bonnefoy et al., 2007; Konev et al., 2007;
Carrera et al., 2008), suggesting that the H3.3 deposition at the
specific genomic locations is essential for early embryogenesis.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
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