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Drosophila cyclin E interacts with components of the Brahma complex

Anthony M.Brumby[1], Claudia B.Zraly[2], Julie A.Horsfield[3,4], Julie Secombe[3,5], Robert Saint[3], Andrew K.Dingwall[2,6] and Helena Richardson[1,6]

[1]Peter MacCallum Cancer Institute, Locked bag 1, A'Beckett Street, Melbourne, Victoria 8006, [3]Department of Molecular Biosciences, University of Adelaide, Adelaide, South Australia 5005, Australia and [2]Department of Biology, Syracuse University, Syracuse, NY 13244-1270, USA

[4]Present address: Department of Molecular Medicine, School of Medicine, University of Auckland, Auckland, New Zealand
[5]Present address: Fred Hutchinson Cancer Research Center, Seattle, WA, USA

[6]Corresponding authors
e-mail: h.Richardson@pmci.unimelb.edu.au or akdingwa@mailbox.syr.edu

Cyclin E–Cdk2 is essential for S phase entry. To identify genes interacting with *cyclin E*, we carried out a genetic screen using a hypomorphic mutation of *Drosophila cyclin E* (*DmcycE^{JP}*), which gives rise to adults with a rough eye phenotype. Amongst the dominant suppressors of *DmcycE^{JP}*, we identified *brahma* (*brm*) and *moira* (*mor*), which encode conserved core components of the *Drosophila* Brm complex that is highly related to the SWI–SNF ATP-dependent chromatin remodeling complex. Mutations in genes encoding other Brm complex components, including *snr1* (*BAP45*), *osa* and deficiencies that remove *BAP60* and *BAP111* can also suppress the *DmcycE^{JP}* eye phenotype. We show that Brm complex mutants suppress the *DmcycE^{JP}* phenotype by increasing S phases without affecting DmcycE protein levels and that DmcycE physically interacts with Brm and Snr1 *in vivo*. These data suggest that the Brm complex inhibits S phase entry by acting downstream of DmcycE protein accumulation. The Brm complex also physically interacts weakly with *Drosophila* retinoblastoma (Rbf1), but no genetic interactions were detected, suggesting that the Brm complex and Rbf1 act largely independently to mediate G_1 arrest.

Keywords: Brahma/cell cycle/cyclin E/retinoblastoma/S phase

Introduction

The coordination of cell proliferation and differentiation is fundamental for development of multicellular organisms. The G_1 to S phase transition is a critical point in the cell cycle where a cell makes the decision to proliferate or differentiate. Entry into S phase is driven by G_1 cyclin–Cdk protein kinases (reviewed by Ekholm and Reed, 2000). In multicellular organisms, there are three classes of G_1 cyclins, cyclin D, E and A, that are rate limiting and essential for the G_1 to S phase progression. Cyclin E and cyclin A form a complex with Cdk2, while cyclin D forms a complex with Cdk4 or Cdk6. The activity of these cyclin complexes is regulated by the binding of the p21^{CIP1} and p16^{INK4a} families of inhibitor proteins (reviewed by Sherr and Roberts, 1995). The only essential target of the cyclin D–Cdk4(6) protein kinase is the retinoblastoma (Rb) tumor suppressor protein (reviewed by Dyson, 1998; Harbour and Dean, 2000). Rb in its unphosphorylated form binds to the E2F/DP transcription factor, forming an inactive complex at S phase gene promoters. Phosphorylation of Rb by cyclin D–Cdk4(6) is important to inactivate Rb and allow S phase gene transcription. Cyclin E–Cdk2 and cyclin A–Cdk2 are also required for further phosphorylation and complete inactivation of Rb. However, cyclin E–Cdk2 and cyclin A–Cdk2 have other essential roles in promoting entry into S phase, possibly by phosphorylating proteins involved in the initiation of DNA replication (reviewed by Ekholm and Reed, 2000).

The regulation of entry into S phase is similar between *Drosophila* and mammalian cells. Fly homologs of many of the essential mammalian proteins have been identified and characterized, including cyclin E, cyclin D, cyclin A, Cdk2, Cdk4(6), p21 (Dacapo), E2F, DP and Rb (reviewed by Edgar and Lehner, 1996). In *Drosophila*, cyclin E (DmcycE) appears to be the most important cyclin in the G_1 to S phase transition. *DmcycE* is expressed in proliferating cells and is down-regulated as cells exit into G_1 phase (Richardson *et al*., 1993, 1995). Furthermore, mutant and overexpression studies have shown that *Drosophila DmcycE* is both sufficient and rate limiting for the G_1 to S phase transition (Knoblich *et al*., 1994; Richardson *et al*., 1995).

Two E2F (E2F1 and E2F2), one DP and two Rb (Rbf1 and Rbf2) homologs are present in flies (reviewed by Dyson, 1998; Harbour and Dean, 2000). As in mammalian cells, E2F/DP regulates the S phase genes, such as *PCNA* and *RNR2*, and induces cells into S phase. Mutant and ectopic expression analyses have shown that Rbf1 abrogates E2F1/DP function during *Drosophila* development (Du *et al*., 1996a; Du and Dyson, 1999; Du, 2000). In *Drosophila,* DmcycE–Cdk2 is also able to phosphorylate Rbf1, thereby leading to inactivation of Rbf1 and entry into S phase. However, in many tissues in the embryo, except nervous system cells, *DmcycE* transcription is also regulated by E2F1/DP (Duronio and O'Farrell, 1995; Sauer *et al*., 1995; Jones *et al*., 2000). Thus, in many tissues, DmcycE and E2F1/DP work in a positive feedback loop to induce entry into S phase by abrogating Rbf1. In addition, other positive and negative cell cycle regulators are likely to play an important role in potentiating the G_1 to

S phase transition, including growth factors and cell–cell communication signaling pathways.

In order to identify novel G_1/S phase regulators in *Drosophila*, we have taken advantage of a hypomorphic *DmcycE* mutation (*DmcycE^{JP}*), which exhibits defects in both eye and wing development. The *DmcycE^{JP}* mutant displays a rough eye phenotype due to a reduction in S phases during eye development and exhibits wing notching and shortening of the L5 wing vein (Secombe *et al*., 1998). We carried out a genetic modifier screen of X-ray and ethyl methanesulfonate (EMS) mutagenized flies to isolate dominant suppressors and enhancers of the *DmcycE^{JP}* rough eye phenotype (to be reported in detail elsewhere). By genetic analysis, we have identified two of the suppressors to be the *brahma* (*brm*) and *moira* (*mor*) genes, which are members of the SWI–SNF group of chromatin remodeling, general transcriptional regulatory genes (Tamkun *et al*., 1992; Crosby *et al*., 1999; reviewed by Tamkun, 1995). *brm* and *mor* alleles were isolated originally as dominant suppressors of a *Polycomb* (*Pc*) mutant that resulted in aberrant expression of homeotic genes (Kennison and Tamkun, 1988). Due to their positive regulatory affect on homeotic gene expression, *brm* and *mor* have been classed in the *trithorax* group (trx-G) of genes (Kennison and Tamkun, 1992), generally thought to act as global transcriptional activators. Both Brm and Mor are components of the fly counterpart of the yeast SWI–SNF complex that utilizes the energy of ATP hydrolysis to remodel chromatin, thereby overcoming the repressive effects of chromatin structure on transcription. The SWI–SNF complex, originally identified in yeast, is a large (~2 MDa) multisubunit complex composed of 8–11 stably associated proteins (reviewed in Kingston and Narlikar, 1999; Peterson and Workman, 2000). Several of the core subunits are highly conserved among metazoan SWI–SNF counterparts, known as the Brm complex in *Drosophila* (Dingwall *et al*., 1995; Papoulas *et al*., 1998) and the hBrm and Brg1 complexes in mammals (Wang *et al*., 1996). The biochemical properties of the purified yeast and mammalian SWI–SNF complexes have been examined in detail (Peterson and Workman, 2000); however, the biological roles of the complex in metazoan development are not well understood. The *Drosophila* Brm complex has been purified and shown to contain homologs of several yeast SWI–SNF proteins, including Brm (SWI2/SNF2), BAP155 (Mor/SWI3), BAP45 (Snr1/SNF5) and BAP60 (SWP73/RSC6), as well as novel proteins BAP111 (a HMG-like protein), BAP74 (Hsp70 cognate 4), BAP55 (actin-related protein) and BAP47 (actin) (Papoulas *et al*., 1998). A potential SWI1 homolog was not identified among the purified Brm complex components; although the trx-G gene *osa/eyelid* encodes a protein with limited homology to SWI1, *osa* mutants strongly interact genetically with Brm complex genes and Osa may be a component of some Brm complexes (Collins *et al*., 1999). Other purified Brm complexes appear to contain Osa, as well as several additional unidentified proteins, but not BAP74 (Kal *et al*., 2000). Therefore, similarly to mammalian SWI–SNF complex counterparts (hBrm and Brg1 complexes; Wang *et al*., 1996), the composition of the Brm complex may be heterogeneous, varying in different developmental contexts. In addition to *brm*, *mor* and *osa*, specific mutations have been described in the Brm complex gene *snr1*, which shows genetic interactions with *brm* (Dingwall *et al*., 1995; Triesman *et al*., 1997; Vazquez *et al*., 1999), and *Hsc70-4* (Mollaaghababa *et al*., 2001).

Several recent studies have provided strong connections between metazoan SWI–SNF complexes and regulation of the cell cycle. In yeast, the SWI–SNF complex is not essential for viability, and whole genome analyses of *swi/snf* mutants have shown roles in activation and repression of transcription (Holstege *et al*., 1998; Sudarsanam and Winston, 2000; Sudarsanam *et al*., 2000). A screen for modifiers of E2F1/DP function in *Drosophila* identified new alleles of *brm* and *mor* as enhancers of the rough eye phenotype associated with ectopic expression of E2F1 and DP in the developing *Drosophila* eye imaginal disc (Staehling-Hampton *et al*., 1999). In support of this, mammalian homologs of Brm and Mor (hBrm/Brg1 and BAF55, respectively) have been recently reported to be present in cyclin E complexes and to be phosphorylated by cyclin E–Cdk2 (Shanahan *et al*., 1999). Significantly, human homologs of Brm (hBrm and Brg1) inhibit entry into S phase and achieve this at least in part by cooperation with the tumor suppressor, Rb (Dunaief *et al*., 1994; Muchardt *et al*., 1998; Reyes *et al*., 1998; Shanahan *et al*., 1999). Furthermore, Rb can bind to Brg1 and hBrm (Dunaief *et al*., 1994; Strober *et al*., 1996; Trouche *et al*., 1997), and the ability of Rb to induce G_1 arrest has been shown to depend upon hBrm and Brg1 (Strobeck *et al*., 2000a,b; Zhang *et al*., 2000). However, the precise mechanism by which the mammalian Brm complexes cooperate with Rb to achieve G_1 arrest is unclear. The recent identification of two Rb–Brg1 (hBrm) complexes, one of which also includes a histone deacetylase (Hdac), has revealed more complexity (Zhang *et al*., 2000). Hdac is required for gene repression by removing the acetyl groups from histones and binds to and cooperates with Rb in repression of E2F-dependent gene transcription (reviewed by Harbour and Dean, 2000; Zhang and Dean, 2001). The Hdac–Rb–Brg1(hBrm) complex appears to be important to repress *cyclin E* transcription, while the Rb–Brg1(hBrm) complex is involved in repression of the *cyclin A* and *cdc2* genes (Zhang *et al*., 2000). The Hdac-associated co-repressor protein Sin3a has also been detected in hBrm and one of two different Brg1 complexes (Sif *et al*., 2001), although the exact biological role it plays in the specific function of these complexes is not clear. The importance of the Brm complex in cell cycle regulation is reinforced by the identification of truncating mutations of the *hSNF5/INI1/SMARCB1* gene, a human homolog of *snr1*, in pediatric rhabdosarcomas and other tumors (Versteege *et al*., 1998; Sevenet *et al*., 1999).

Here, we have investigated the role of the Brm complex in the G_1 to S phase transition in flies. We isolated *brm* and *mor* alleles in a screen for dominant suppressors of a *DmcycE* hypomorphic allele. We show that other Brm complex genes also interact genetically with *DmcycE*. Although the most obvious manner in which the Brm complex mediates negative regulation of S phase is via transcriptional regulation of *DmcycE* or E2F1/DP target genes, neither we nor others have observed any transcriptional effects. Consistent with this, we find that Brm and Snr1 physically interact with DmcycE *in vivo*. In addition

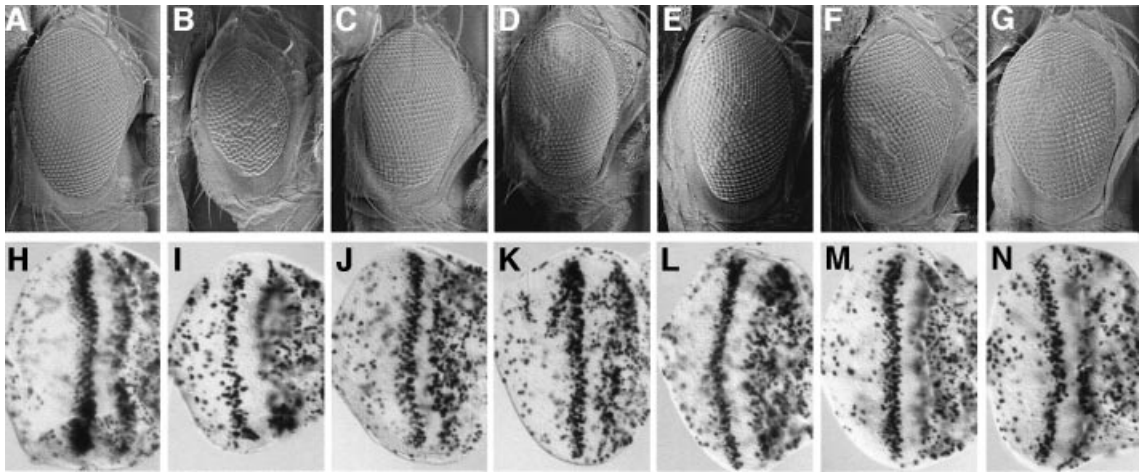

Fig. 1. *brm* and *mor* dominantly suppress the *DmcycE^{JP}* rough eye phenotype by increasing S phases. (A–G) Scanning electron micrographs of adult eyes. (**A**) Wild-type (*w^{1118}*); (**B**) *DmcycE^{JP}*; (**C**) *DmcycE^{JP}; brm^{25S14}*/+; (**D**) *DmcycE^{JP}; brm^{2}*/+; (**E**) *DmcycE^{JP}*; *mor^{35S1}*/+; (**F**) *DmcycE^{JP}; mor^{1}*/+; and (**G**) *brm^{K804R}; DmcycE^{JP}*. (H–N) Third instar larval eye imaginal discs labelled with BrdU. (**H**) Wild-type (*w^{1118}*); (**I**) *DmcycE^{JP}*; (**J**) *DmcycE^{JP}; brm^{25S14}*/+; (**K**) *DmcycE^{JP}; brm^{2}*/+; (**L**) *DmcycE^{JP}; mor^{35S1}*/+; (**M**) *DmcycE^{JP}; mor^{1}*/+; and (**N**) *brm^{K804R}; DmcycE^{JP}*. Adult eyes and larval imaginal discs are orientated anterior to the right in this and all subsequent figures.

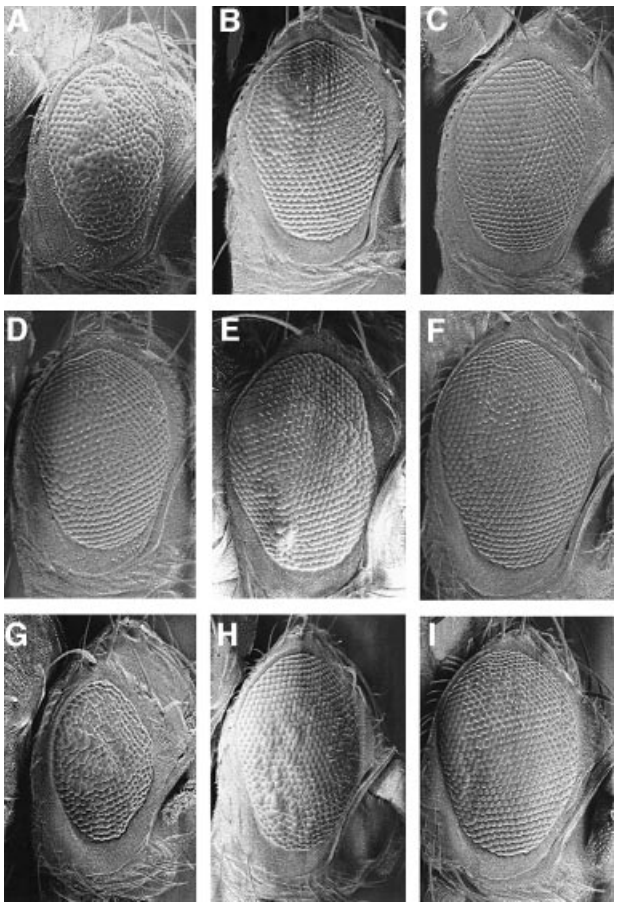

Fig. 2. Dominant suppression of *DmcycE^{JP}* rough eye phenotype by SWI–SNF genes. Scanning electron micrographs of *DmcycE^{JP}* adult eyes. (A–F) In the background of *w/+; b DmcycE^{JP} bw/DmcycE^{JP}*. (**A**) +; (**B**) *snr1^{01319}*/+; (**C**) *snr1^{R3}*/+; (**D**) *osa^{00090}*/+; (**E**) *osa^{S3263b}*/+; (**F**) *Df(BAP111)/+*. (G–I) In the background of *w*; *DmcycE^{JP}*, which is slightly more extreme than *w/+; b DmcycE^{JP} bw/DmcycE^{JP}*. (**G**) +; (**H**) *P[w^{+}; UAS$_{GAL}$hsp70-snr1-cdel.3] snr1^{R3}/P[w^{+}; 69B-GAL4]*. (**I**) *P[w^{+}; UAS$_{GAL}$hsp70-snr1-cdel.3] snr1^{R3}/P[w^{+}; Act5C-GAL4]*.

we show that Rbf1 and Brm complex proteins weakly associate *in vivo*; however, we have observed no genetic interactions between *brm* or *mor* and *rbf1*. This suggests that the Brm complex and Rbf1 function largely independently in negatively regulating the G_1 to S phase transition.

Results

brm and mor alleles suppress the DmcycE^{JP} rough eye phenotype by increasing S phases

Based on deficiency mapping and complementation tests, two alleles of *brahma* (an X-ray-induced allele, *25S14* and an EMS-induced allele, *E6S8*) and one allele of *moira* (an X-ray-induced allele, *35S1*) were isolated in a *DmcycE^{JP}* modifier screen (to be published elsewhere). These alleles, as well as previously isolated alleles of *brahma* (*brm^{2}*) and *moira* (*mor^{1}*), suppressed the *DmcycE^{JP}* rough eye phenotype (Figure 1C–F compared with B; data not shown). In addition, when Brm function was reduced in a *DmcycE^{JP}* background by using the *brm* dominant-negative transgene, *brm^{K804R}* (Papoulas *et al*., 1998), suppression of the *DmcycE^{JP}* rough eye phenotype was also observed (Figure 1G). To test whether the suppression of the *DmcycE^{JP}* rough eye phenotype by *brm* and *mor* was due to an increase in S phases, we carried out bromodeoxyuridine (BrdU) labeling of *DmcycE^{JP}; brm/+* and *DmcycE^{JP}; mor/+* eye imaginal discs from third instar larvae. Halving the dosage of *brm* or *mor*, using either alleles obtained in our genetic screen or previously isolated alleles, as well as the *brm* dominant-negative transgene, resulted in a dramatic increase in the number of S phases relative to *DmcycE^{JP}* eye discs (Figure 1J–N compared with I). No significant effects on adult eye phenotype or entry into S phase were observed with mutant alleles of *brm* or *mor* alone (results not shown), indicating that *brm* and *mor* alleles only increase S phases in the sensitized *DmcycE^{JP}* background. These results

show that *brm* and *mor* interact genetically with *DmcycE* and are consistent with a role for Brm and Mor in negatively regulating entry into S phase.

Other Brm complex genes interact genetically with cyclin E

Since Brm and Mor are conserved core components of the fly SWI–SNF chromatin remodeling complex (Brm complex), we wished to test genes encoding other proteins present in this complex for a genetic interaction with *DmcycE*. The *Drosophila* Brm complex has been shown to consist of Brm, Mor/BAP155, BAP111 (HMG domain protein), BAP74 (Hsp70 cognate Hsc4), BAP60 (SWP73/RSC6 homolog), BAP55 (actin-related protein), BAP47 (actin 5C or 42A) and Snr1–BAP45 (Papoulas *et al*., 1998). The Osa/Eyelid protein, related to yeast SWI1, has also been shown to exist in some, but not all, Brm complexes (Collins *et al*., 1999; Kal *et al*., 2000). Using loss-of-function alleles of *Hsc70-4* and *snr1*, and deficiencies of *BAP111* and *BAP60*, we examined whether halving the dosage of these genes modified the rough eye phenotype of $DmcycE^{JP}$ (Figure 2; Table I). *BAP55* could not be tested since no deficiencies covering this gene exist and, due to the uncertainty of the identity of *BAP47*, deficiencies of *Actin5C* or *Actin42A* were not investigated. Our results showed that decreasing the dosage of *snr1* using several different alleles resulted in moderate suppression of the $DmcycE^{JP}$ eye phenotype (Figure 2B and C compared with A). However, strong suppression was observed when a C-terminal deletion of *snr1* that acts as a dominant-negative (*snr1-cdel.3*) was ectopically expressed in the eye using the *GAL4/UAS* binary system (Brand and Perrimon, 1993) in $DmcycE^{JP}$ flies heterozygous for $snr1^{R3}$ (Figure 2H and I compared with G). Expression of *snr1-cdel.3* via the *Act5C-GAL4* driver, which is expressed ubiquitously at high levels, resulted in better suppression than the *GawB[69B]-GAL4* driver,

Table I. Interaction of $DmcycE^{JP}$ with Brm complex genes

Brm complex gene	Allele	Suppression of $DmcycE^{JP}$
mor (*moira*)	*1* (hypomorph)	+++
(*SWI3* homolog) 89B1	*35S1* (X-ray)	+++
brm (*brahma*)	*2* (amorph)	++
(*SWI2* homolog) 72A3	*25S14* (X-ray)	++
	E6S8 (EMS)	++
Snr1	*01319* (*P* allele)	++
(*SNF5-related 1*) 83A5-6	*R3* (lethal recessive)	++
	Act5C-snr1-cdel.3, $snr1^{R3}$	+++
osa (*eyelid*)	*00090* (*P* allele)	++
(*SWI1* homolog) 90C1-2	*krycheck*	++
	s3263b (*P* allele)	++
Hsc70-4 (*Hsp70 cognate 4*)	*03550* (*P* allele)	+
(*Bap74*) 88E8-9	*L3929* (*P* allele)	–
BAP111 (HMG-like)	*Df(1)lz-90b24*	+++
8C9-13	*Df(1)M38-c5*	+++
BAP60 (*RSC6/SWP73*) 11D5-10	*Df(1)c246*	+++
E(brm)25D-26B	*Df(2L)cl-h3*	+++
E(brm)64E1-65C	*Df(3L)ZN47*	+++

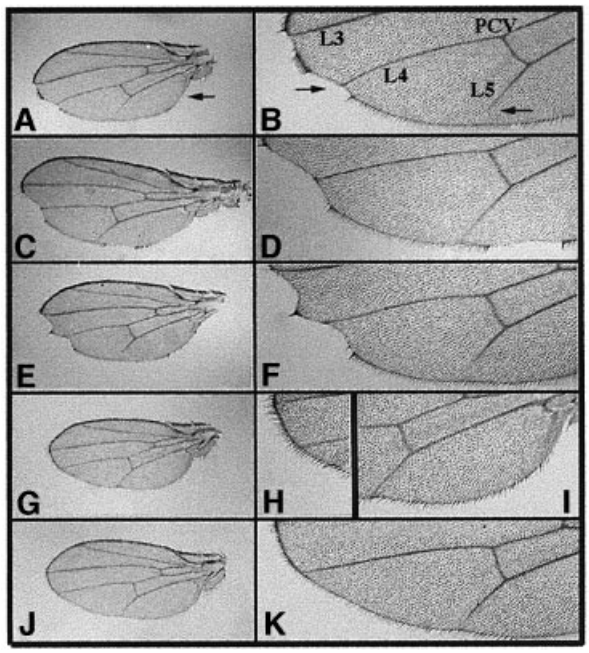

Fig. 3. Suppression of $DmcycE^{JP}$ wing phenotypes by mutations in *brm* and by ectopic expression of a *snr1* deletion transgene. Wings were dissected from flies homozygous for the $DmcycE^{JP}$ mutation on the second chromosome and either wild-type or heterozygous for various mutations and/or transgenes carried on the third chromosome. Wings shown in (A), (C), (E), (G) and (J) are at the same magnification, as are the magnified views of the same wings shown in (B), (D), (F), (H), (I) and (K). (**A** and **B**) Flies homozygous for $DmcycE^{JP}$ alone shown as a whole wing view (A) or at increased magnification (B). Note the notching at the posterior/distal wing margin, the missing hairs along the posterior/proximal wing blade and shortening of the fifth longitudinal vein (L5), indicated by arrows in (A) and (B). Also indicated are the positions of the L3 and L4 longitudinal veins and the posterior cross-vein (PCV). (C–K) $DmcycE^{JP}$ containing heterozygous mutations and/or transgenes on the third chromosome. (**C** and **D**) *w; DmcycE^{JP}; P[w$^+$; Act5C-GAL4]/TM3*. Note the phenotypes similar to those observed in (A) and (B). (**E** and **F**) *w; DmcycE^{JP}, P[w$^+$; UAS$_{GAL}$hsp70-snr1-cdel.3], snr1^{R3}/TM3*. (**G–I**) *w; DmcycE^{JP}, P[w$^+$; UAS$_{GAL}$hsp70-snr1-cdel.3], snr1^{R3}/P[w$^+$; Act5C-GAL4]*. Note the suppression of both the wing margin and L5 defects in flies that ubiquitously overexpress the *snr1-cdel.3* truncated transgene with a heterozygous $snr1^{R3}$ mutation. Shown in (H) and (I) are magnified views of the wing margin (shown in G) between the L3 and L4 veins. (**J** and **K**) *w; DmcycE^{JP}; brm^2/TM3*. Note the suppression of the wing defects similar to those observed with overexpression of the *snr1-cdel.3* truncation transgene shown above.

Table II. Suppression of $DmcycE^{JP}$ wing defects by *snr1* and *brm*

Genotype	No. of wings examined	Notching			% complete L5
		None	Mild	Severe	
$DmcycE^{JP}$; *X-GAL4/TM3*	192	4%	23%	73%	50%
$DmcycE^{JP}$; *GawB[69B]-GAL4/UAS-snr1-cdel.3*, $snr1^{R3}$	72	55%	28%	17%	87%
$DmcycE^{JP}$; *Act5C-GAL4/UAS-snr1-cdel.3*, $snr1^{R3}$	32	91%	9%	0%	100%
$DmcycE^{JP}$; brm^2/*TM3*	56	82%	18%	0%	100%

Table III. Interaction of *DmcycE^{JP}* with trx-G and Pc-G genes

Trithorax gene	Allele	Effect on *DmcycE^{JP}*		
		Level of suppression	No effect	Level of enhancement
Mod(mdg4) modifier of Mdg4 93D7	*L3101* (*P* allele)	+++		
	03852 (*P* allele)	+++		
Trl (*Trithorax-like*) 70F1-2	*13C* (hypomorph)	++		
	R85 (hypomorph)	+		
	R67 (lethal recessive)	+		
	62 (lethal recessive)	+		
skd (*skuld, S(Pc), S(Sev^{act})*) 3-51	*2* (hypomorph)	++		
kto (*kohtalo*) 76B1-D5	*1* (hypomorph)	+		
dev/btl (*devenir/breathless*/FGFR) 70D2	*1* (hypomorph)	+		
	00208 (*P* allele)	+		
Hth (*Homothorax*) 86C	*5E04* (lethal recessive)	+		
trx (*trithorax*) 88B3	*1* (hypomorph)	++ (when homozygous)		
	*E2 (*amorph)	+		
	00347 (semi-lethal, *P* allele)		No effect	
urd (*urdur*) 87F12-15	*2* (hypomorph)	+		
lawC (*leg arista wing complex*)	*EF520* (loss of function)		No effect	
sls (*sallimus, S(Pc)*) 62C1-3	*1* (recessive lethal)			+
ash1 (*absent, small or homeotic1*) 76B9	*B1* (hypomorph)			+
	22 (amorph)			+
ash2 (*absent, small or homeotic2*) 96A17	*1* (amorph)			+
	18 (recessive lethal)			+
lid (*little imaginal discs/E(ash1)*)	*1* (lethal recessive)			+
(RBP2 homolog) 26A-B	*2* (lethal recessive)			+
kis (*kismet*) 21B7	*1* (loss of function)		No effect	
	07812 (*P* allele)		No effect	
Polycomb gene				
Pc (*Polycomb*) 78C9-78D	*7* (EMS allele)	++		
	1 (amorph)	+		
	4 (loss of function)	+		
	2 (antimorph)		No effect	
	6 (EMS allele)		No effect	
Scr (*Sex combs reduced*) 84A5-B1	*1* (loss of function)			+
Scm (*Sex combs on midleg*) 85E1-10	*D1* (loss of function)		No effect	
ph-d (*polyhomeotic distal*) 2D1-5	*503* (amorph)		No effect	
Pcl (*Polycomblike*) 55B	*11* (amorph)		No effect	
E(Pc) (*Enhancer of Polycomb*) 47F13-17	*1* (lethal recessive)			+
Psc (*Posterior sex combs*) 49E1	*1* (hypomorph)		No effect	
Asx (*Additional sex combs*) 51A2	*1* (gain of function)		No effect	

which is expressed at low levels in the eye imaginal disc (Figure 2G–I). Dominant suppression of the *DmcycE^{JP}* eye phenotype was also observed with *osa* (Figure 2D and E compared with A) and with one of the two *Hsc70-4* alleles tested (Table I). The difference in interaction of the two *Hsc70-4* alleles with *DmcycE^{JP}* may reflect different allele strengths, specific functions affected by one allele but not the other, or genetic background affects. However, Hsc70-4 may not be a core component of the Brm complex since it is not always present in purified Brm complexes, nor have genetic interactions been detected between *brm* and *Hsc70-4* (Kal *et al*., 2000; Mollaaghababa *et al*., 2001). Strong suppression was also observed with two deficiencies that removed *BAP111* (Figure 2F compared with A; Table I) and *BAP60/RSC6* (Table I). We also tested two deficiencies that were identified as enhancers of the *brm* dominant-negative allele, *brm^{K804R}* (Papoulas *et al*., 1998). Both of these deficiencies, *E(brm)25D-26B* and *E(brm)64E1-65C*, showed strong suppression of the *DmcycE^{JP}* rough eye phenotype (Table I). Thus, all *Drosophila* Brm complex genes and *brm*-interacting genes tested interact genetically with *DmcycE*.

brm and snr1 alleles suppress the DmcycE^{JP} wing defects

To explore further the genetic interaction between Brm complex genes and *DmcycE*, we examined another phenotype of *DmcycE^{JP}*, that of wing notching and L5 wing vein truncation (Secombe *et al*., 1998; Table II; Figure 3). The control *DmcycE^{JP}* flies show mild notching at the wing blade periphery at a penetrance of ~96%, and only 50% of wings show a complete L5 vein (Table II; Figure 3A–F). Halving the dosage of *brm* strongly suppressed the wing blade notching and the truncated L5 wing vein phenotypes of *DmcycE^{JP}* flies (Figure 3J and K). In addition, ectopic expression of the dominant-negative *snr1-cdel.3* transgene using either *Act5C-GAL4* or *GawB[69B]-GAL4*, in combination with reducing the

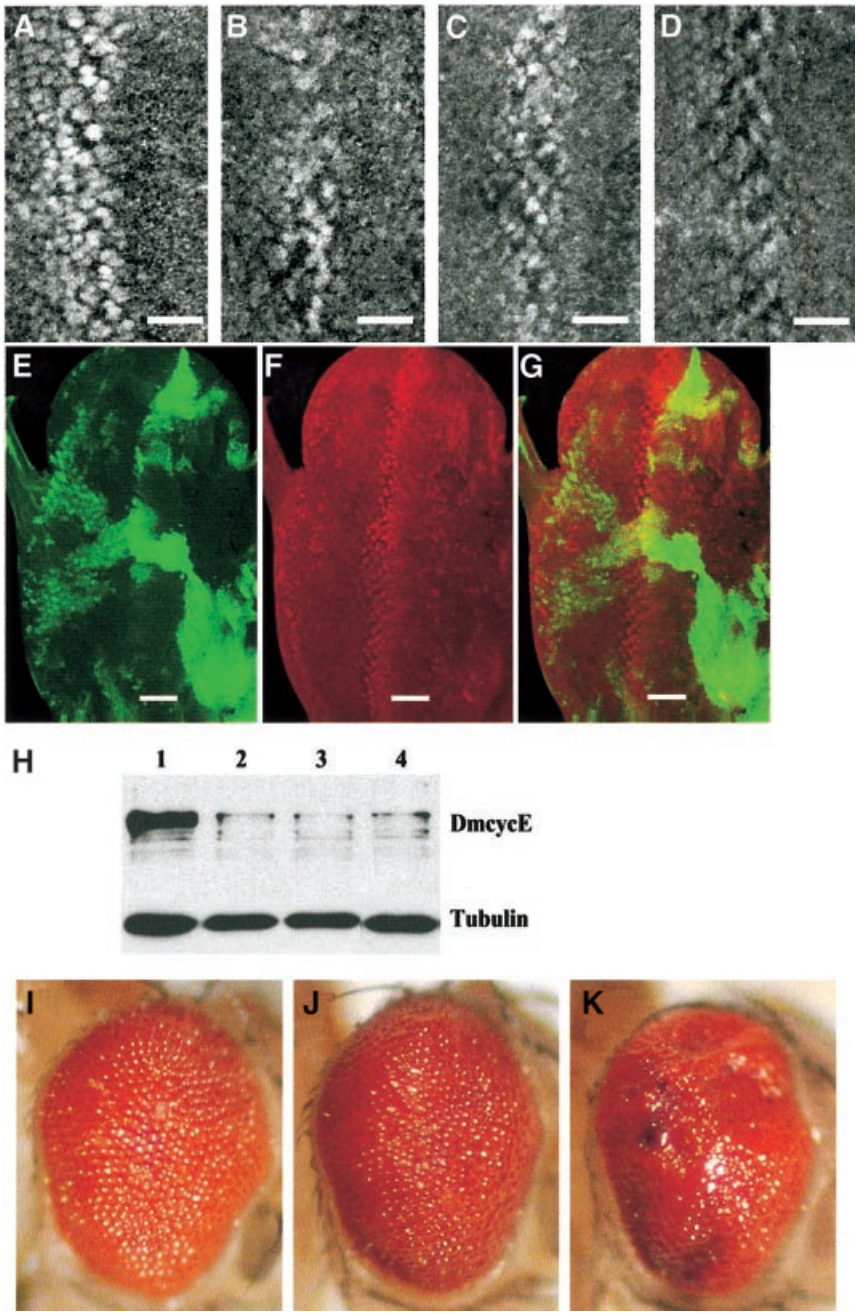

Fig. 4. The Brm complex does not affect DmcycE protein levels and functions genetically downstream of *DmcycE* transcription. DmcycE antibody staining of larval eye imaginal discs from (**A**) wild-type; (**B**) $DmcycE^{JP}$; (**C**) $DmcycE^{JP}$; brm^{25S14}/+; and (**D**) $DmcycE^{JP}$; mor^{1}/+. (E–G) DmcycE antibody staining and GFP fluorescence from an *ey-FLP, UAS-GFP; Tb-GAL4 FRT(82B) GAL80/FRT(82B) UAS-*brm^{K804R} eye disc. (**E**) GFP (green) marks the clones expressing *UAS-*brm^{K804R}. (**F**) DmcycE antibody staining. (**G**) Merge. Note that in clones expressing *UAS-*brm^{K804R} distant from the normal band of cyclin E staining, cyclin E is not expressed ectopically. The bar indicates the position of the morphogenetic furrow. (**H**) Western analysis of DmcycE protein (upper panel) or tubulin (lower panel) in eye imaginal discs from wild-type (lane 1); $DmcycE^{JP}$ (lane 2); $DmcycE^{JP}$; brm^{25S14}/+ (lane 3); and $DmcycE^{JP}$; mor^{1}/+ (lane 4). Since $DmcycE^{JP}$ affects the eye imaginal disc but not the antennal disc, the antennal disc was removed from the eye disc before protein was prepared. Quantitation of band intensities from the DmcycE immunoblot normalized to tubulin showed that the level of DmcycE in $DmcycE^{JP}$ eye discs was not increased by halving the dosage of *brm* or *mor*. (I–K) Adult eyes from (**I**) *GMR-GAL4, UAS-DmcycE/+; GMR-p35/+*; (**J**) *GMR-GAL4, UAS-DmcycE/+; GMR-p35/*brm^{25S14}; and (**K**) *GMR-GAL4, UAS-DmcycE/+; GMR-p35/*mor^{35S1}.

dosage of the endogenous *snr1* gene, resulted in the suppression of both the wing notching and the truncated wing vein phenotypes (Table II; Figure 3G–I; data not shown). Expression of the dominant-negative *snr1* transgene via the stronger *Act5C-GAL4* driver resulted in a greater suppression of both of these phenotypes (Table II). Thus, *brm* and *snr1* show a dosage-sensitive suppression of $DmcycE^{JP}$ wing and eye phenotypes.

Interaction of $DmcycE^{JP}$ with trithorax and Polycomb group genes and global transcriptional regulators

To determine whether the genetic interaction observed between $DmcycE^{JP}$ and several Brm complex genes was specific or reflected a more global effect on transcription regulation, we also examined a number of trx-G and Pc-G genes for whether they could dominantly modify the

DmcycE^{JP} eye phenotype. As shown in Table III, most trx-G or Pc-G gene mutants did not show any strong dominant interactions with *DmcycE^{JP}*, although subtle effects were observed with several.

Among the trx-G genes tested that did affect the *DmcycE^{JP}* phenotype, the most striking dominant suppression was observed with *mod(Mdg4)*, which encodes a BTB/POZ domain transcription factor (Read *et al*., 2000). Moderate suppression was observed upon halving the dosage of *skuld*, which was isolated as a suppressor of *Pc* (Kennison and Tamkun, 1988). Although the product encoded by *skuld* is not known, its genetic map position (3–51) does not correlate with any known gene encoding a Brm complex subunit (Papoulas *et al*., 1998). Moderate suppression was also observed with one mutant allele of *Trithorax-like* (*Trl^{13C}*), while other alleles showed slight suppression (Table III). However, it is likely that a homozygous viable mutant obtained as a dominant suppressor of *DmcycE^{JP}* in our genetic screen (*65S19*) is an allele of *Trl*, since when crossed to known *Trl* alleles it gives a *Trl* abdominal transformation phenotype (data not shown). The *Trl* gene encodes the GAGA factor, which is a BTB/POZ domain transcription factor that regulates the expression of many genes in collaboration with the NURF chromatin remodeling complex (Farkas *et al*., 1994; Tsukiyama and Wu, 1995). A hypomorphic allele of *trithorax* (*trx^{1}*) suppressed the *DmcycE^{JP}* eye phenotype when homozygous, but stronger alleles of *trithorax* did not show dominant suppression. In contrast, some mutant alleles of other trx-G genes (*sallimus*, *ash1*, *ash2* and *lid*) resulted in slight dominant enhancement of the *DmcycE^{JP}* rough eye phenotype (Table III).

Many of the Pc-G genes are thought to function in opposition to trx-G genes in transcriptional regulation of specific targets. Of the Pc-G genes tested, very few showed any significant interactions with *DmcycE^{JP}* (Table III). Thus, although some dominant genetic interactions were observed with trx-G or Pc-G genes and *DmcycE^{JP}*, most of these were subtle compared with interactions observed with Brm complex genes. This suggests that while global transcriptional regulation may be loosely linked to the G_1 to S phase transition, the Brm complex plays a more important role. The strong suppression of *DmcycE^{JP}* observed with *mod(Mdg4)* also suggests that *mod(Mdg4)* may play an important role in *DmcycE* regulation and entry into S phase.

We also examined whether other global transcriptional regulators dominantly interacted with *DmcycE^{JP}*. Hdacs are important in changing gene expression states (reviewed by Kouzarides, 1999). Sin3a interacts biochemically and genetically with Hdacs and is thought to tether Hdacs to transcription repressor proteins such as Mad–Max and Rb, which are important in the G_1 to S phase transition. In *Xenopus* oocytes, a SWI2 family member, Mi-2, forms a complex that includes the Hdac Rpd3, the deacetylase-associated protein Sin3a and the Rb-associated protein RpAp46/48 (Wade *et al*., 1998). Among these genes, specific mutations exist in flies for *rpd3*, one of the four known Hdacs that act to enhance gene silencing at heterochromatic regions (de Rubertis *et al*., 1996), and for *sin3a* (Pennetta and Pauli, 1998). No effect on the *DmcycE^{JP}* eye phenotype was observed when we halved the dosage of *rpd3* (data not shown), suggesting that Rpd3 is not rate limiting for DmcycE function or is redundant with other Hdacs. Surprisingly, mild enhancement of the *DmcycE^{JP}* eye phenotype was observed when the dosage of *sin3a* was decreased using homozygous viable *P* element alleles (not shown). This enhancement was increased when the dosage of *sin3a* was decreased further using a deficiency of *sin3a*. However, since this deficiency also removes other genes including *ISWI* (*nurf-140/chrac*), another *SWI2* family nucleosome remodeling gene (Deuring *et al*., 2000), it is possible that this enhancement is due to halving the dosage of *ISWI* or to other genes as well as *sin3a*. This genetic interaction was opposite to what was expected, given that Sin3a is required for Hdac-mediated repression. The mechanism by which this occurs requires further investigation.

brm and *mor* do not function to suppress *DmcycE^{JP}* by increasing DmcycE levels

The *DmcycE^{JP}* mutation is a hypomorph that exhibits decreased DmcycE protein levels (Secombe *et al*., 1998). To explore whether *brm* and *mor* suppression of the *DmcycE^{JP}* eye phenotype was due to effects on *DmcycE* expression, we examined DmcycE protein levels in eye discs by immunostaining and western blot analyses (Figure 4). DmcycE antibody staining of eye imaginal discs from *DmcycE^{JP}; brm/+* and *DmcycE^{JP}; mor/+* larvae revealed that there was no significant increase in DmcycE protein levels relative to *DmcycE^{JP}* alone (compare Figure 4B with C and D; data not shown). To explore this further, we used the *eyeless-FLP*; *UAS-GFP, Tb-GAL4, FRT, Tb-GAL80* system (Lee and Luo, 1999) to generate clones of cells overexpressing the dominant-negative *brm* transgene (*UAS-brm^{K804R}*) within otherwise wild-type eye imaginal discs (Figure 4E–G). In this system, clones expressing *UAS-brm^{K804R}* are marked by green fluorescent protein (GFP) (Figure 4E). If Brm acts to repress *DmcycE* transcription, we would expect that in *UAS-brm^{K804R}*-expressing clones *DmcycE* should be ectopically expressed. By DmcycE antibody staining, no ectopic expression of *DmcycE* was observed in *UAS-brm^{K804R}*-expressing clones (Figure 4F and G). Furthermore, we examined DmcycE protein levels in *DmcycE^{JP}; brm/+* and *DmcycE^{JP}; mor/+* third instar larval eye imaginal discs by western analysis (Figure 4H). No significant increase in DmcycE protein level was observed when *brm* or *mor* dosage was halved relative to *DmcycE^{JP}* eye discs (Figure 4H). Thus, the Brm complex appears to function downstream of DmcycE protein accumulation to inhibit S phase entry. Consistent with these data, *brm* and *mor* mutants dominantly enhanced the rough eye phenotype produced by the ectopic expression of *DmcycE* (Figure 4I–K). In these flies, *DmcycE* is produced independently of its normal transcriptional regulation using the *GMR-GAL4* driver, and apoptosis is inhibited by *GMR*-driven expression of the caspase inhibitor p35 (*GMR-p35*), leading to an overgrown and rough eye phenotype (Figure 4I). Only genes that act downstream of *DmcycE* transcription are expected to show modification of this rough eye phenotype. Halving the dosage of either *brm* or *mor* enhanced this rough eye phenotype and increased the number of S phase cells (Figure 4J and K; data not shown). Thus, genetically, the Brm complex functions downstream of *DmcycE* transcription.

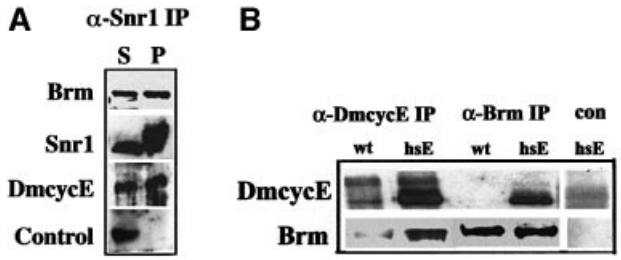

Fig. 5. Brm and Snr1 form a complex with cyclin E. (**A**) Snr1 and Brm form a complex with DmcycE in embryos. Native wild-type embryo extracts (500 μg) were incubated with affinity-purified Snr1 rabbit antibodies and precipitated with protein G–Sepharose beads. The presence of Brm, Snr1, DmcycE and a control nuclear protein was examined in the supernatant (S) and in the pelleted material eluted from the Sepharose beads (P) by immunoblotting. The supernatant tracks represent one-tenth of the immunoprecipitated tracks. (**B**) Brm forms a complex with DmcycE in larval brains/discs. Larval extracts were prepared from w^{1118} or a line transgenic for *hsp70-DmcycE* and incubated with an anti-DmcycE (8B10) or anti-Brm antibodies and precipitated with protein A–Sepharose beads. Pelleted proteins were examined for the presence of Brm (lower panel) and DmcycE (upper panel) by immunoblotting. The control immunoprecipitation was carried out using DmcycE pre-immune serum.

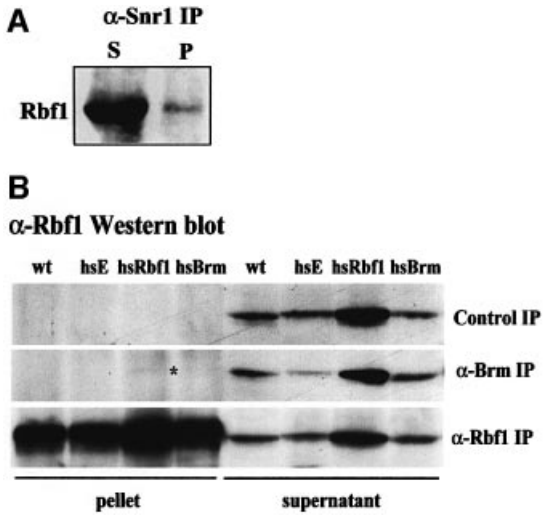

Fig. 6. Brm and Snr1 physically interact weakly with Rbf1. (**A**) Snr1 and Rbf1 physically interact weakly in embryos. Embryo extracts were incubated with affinity-purified anti-Snr1 antibodies and precipitated with protein G–Sepharose beads. The presence or Rbf1 was examined in the supernatant (S) and in the pelleted material eluted from the beads (P) by immunoblotting. (**B**) Rbf1 and Brm weakly physically interact in larval brains/discs. Extracts prepared from w^{1118} or heat-shocked *hsp70-DmcycE* (hsE), *hsp70-rbf1* (hsRbf1) or *hsp70-GAL4, UAS-brm*^{K804R} (hsBrm) larvae were immunoprecipitated with anti-Rbf1 or anti-Brm antibodies. Pelleted proteins and the supernatants were examined for the presence of Rbf1 by immunoblotting. Rbf1 was detected weakly in Brm immunoprecipitates in the hsRbf1 track (*). The control immunoprecipitation was carried out using protein A–Sepharose beads alone. (A and B) The supernatant tracks represent one-tenth of the immunoprecipitated tracks. Quantitation of band intensities showed that only a small fraction of total Rbf1 is co-immunoprecipitated with Snr1 or with Brm.

Furthermore, the study of Staehling-Hampton *et al*. (1999) showed that the E2F target genes, *rnr2* and *dhfr*, were also not affected by *brm* or *mor* mutants. Taken together, these results suggest that the Brm complex has a role, independent of DmcycE protein accumulation and E2F-dependent gene transcription, in mediating negative regulation of S phase.

DmcycE physically associates with the Brm complex in vivo

The genetic interactions we have observed with *DmcycE* and the Brm complex genes is consistent with the observation in mammalian cells that cyclin E–Cdk2 can form a complex with the Brahma homolog Brg1 and the Moira homolog BAP155 (Shanahan *et al*., 1999). To confirm that a biochemical interaction was also occurring between these proteins in *Drosophila*, we initially examined embryonic extracts, which have higher levels of DmcycE protein than larval tissues (Figure 5A). Embryonic extract immunoprecipitated with an anti-Snr1 antibody co-precipitated Brm and DmcycE, but not a control nuclear transcription factor. Comparison of the precipitated versus supernatant protein fractions revealed that a significant portion of DmcycE in the embryonic extract was co-precipitated efficiently with Snr1, along with Brm. We have also observed co-precipitation of Brm complex with the Cdk2 protein (data not shown; C.Zraly and A.Dingwall, in preparation). Co-precipitation of Cdk2 and DmcycE is expected, since Cdk2 is the sole catalytic partner of DmcycE in *Drosophila* (Sauer *et al*., 1995).

As the $DmcycE^{JP}$ mutant phenotype result from decreased S phases during larval imaginal disc development (Secombe *et al*., 1998), we examined whether DmcycE was stably associated with the Brm complex in larval tissues. When anti-DmcycE was used for the immunoprecipitation, Brm was readily detectable by immunoblotting (Figure 5B). However, in Brm immunoprecipitates, DmcycE was not detected (Figure 5B), probably because most cells in these extracts are arrested in G_1 and do not contain DmcycE. To increase the level of DmcycE, we ectopically expressed DmcycE in larvae by using the *hsp70-DmcycE* transgene (Richardson *et al*., 1995). This resulted in a dramatic increase in DmcycE and in the ability of DmcycE and Brm to be co-immunoprecipitated (Figure 5B, hsE tracks). Thus, Brm and DmcycE physically associate in both larval and embryonic tissues.

Rbf1 physically associates with the Brm complex in vivo

In mammalian cells, Brm and Brg1 can bind to Rb (Dunaief *et al*., 1994; Strober *et al*., 1996; Trouche *et al*., 1997). To determine whether *Drosophila* Rbf1 could also form stable associations with the Brm complex *in vivo*, we carried out co-immunoprecipitation experiments from both embryo and larval tissue extracts. As shown above in Figure 5, both Brm and DmcycE are present in anti-Snr1 immunoprecipitates from wild-type embryonic extracts. Similarly, we observed co-precipitation of Rbf1 with Snr1, although only a small portion of total Rbf1 was associated with Snr1 [Figure 6A, compare supernatant (S) with pellet (P) lanes]. We next examined associations in larval tissues where the relative amount of Brm complex and Rbf1 is much reduced compared with embryos. In anti-Brm immunoprecipitates, Rbf1 was not detectable in wild-type larval extracts, but could be weakly detected when Rbf1 levels were increased after heat shock induction of an *hsp70-rbf1* transgene in larvae (Figure 6B, compare supernatant with pellet lanes). Conversely, in anti-Rbf1

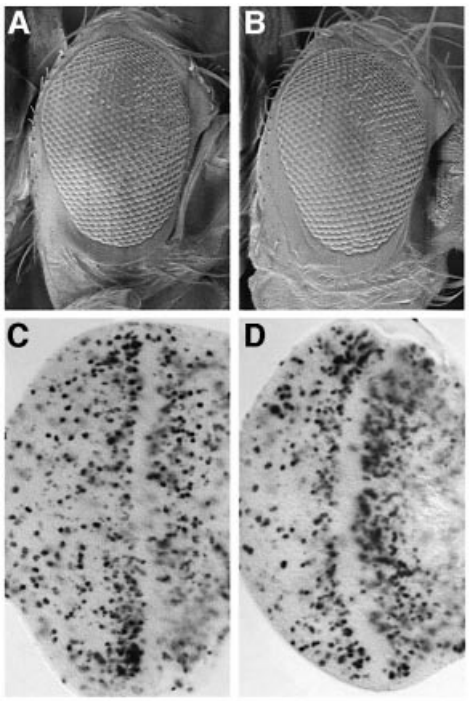

Fig. 7. *brm* or *mor* do not interact genetically with *rbf1*. Scanning electron micrographs of adult eyes from (**A**) $rbf1^{120a}/rbf1^{11}$ and (**B**) $rbf1^{120a}/rbf1^{11}$; brm^{25S14}, +/+, mor^{35S1}. (C and D) BrdU labeling of eye imaginal discs from (**C**) $rbf1^{120a}/rbf1^{11}$ and (**D**) $rbf1^{120a}/rbf1^{11}$; brm^{25S14}, +/+, mor^{35S1}.

immunoprecipitates, low levels of Brm were detected by immunoblotting in heat-shocked *hsp70-rbf1* larval extracts (results not shown). In contrast, E2F1 was readily detected in Rbf1 immunoprecipitates (data not shown). These results show that Rbf1 physically associates with Brm complex proteins in *Drosophila*, albeit at relatively low levels compared with DmcycE and Brm or Snr1.

brm or mor do not interact genetically with rbf1

In mammalian cells, co-transfection of Brm and Rb cooperates to mediate G_1 arrest (reviewed by Muchardt and Yaniv, 2001) and it is possible that a similar cooperation occurs in *Drosophila*. To obtain evidence for a functional interaction between the Brm complex and Rbf1, we first looked for genetic interactions between *rbf1* and *brm* or *mor* alleles. Using the $rbf1^{11}$ null allele, we examined transheterozygous combinations of $rbf1^{11}$ and *brm* or *mor* mutants for adult phenotypes, including eye or bristle patterning effects. In this assay, we did not observe any significant specific defects (data not shown). To compromise the function of *rbf1* further, we made use of a female sterile allele of *rbf1*, $rbf1^{120a}$ (Du, 2000; Bosco *et al*., 2001). Transheterozygous $rbf1^{11}/rbf1^{120a}$ females are viable but sterile due to defects in the endoreplication cycles of the follicle cells that surround the egg chamber (Bosco *et al*., 2001). During oogenesis, there are two phases of endoreplication in the follicle cells (Royzman *et al*., 1999). Until stage 10A, genomic endoreplication occurs asynchronously, while at stage 10B these cycles switch to synchronous amplification of discrete foci including the *chorion* genes. In *rbf1* female sterile mutants, there are defects both in the switch from endoreplication to *chorion* gene amplification and in over-replication of the *chorion* gene foci (Bosco *et al*., 2001). We examined follicle cell S phases by BrdU labeling of $rbf1^{11}/rbf1^{120a}$ females, also heterozygous for *brm*, *mor* or both. Under these circumstances, we failed to observe any significant enhancement of the replication defects of $rbf1^{11}/rbf1^{120a}$ follicle cells (data not shown). Furthermore, we investigated whether halving the dosage of *brm* or *mor* could enhance the S phase defect of $rbf1^{11}/rbf1^{120a}$ in eye imaginal discs. $rbf1^{11}/rbf1^{120a}$ eye discs have a severe disruption in the post-morphogenetic furrow S phase band, but have excessive S phases in the posterior region (Figure 7C compared with Figure 1H). Despite this disruption, the adult eye phenotype of $rbf1^{11}/rbf1^{120a}$ flies is only mildly disorganized (Figure 7A compared with Figure 1A). Halving the dosage of *brm*, *mor* or both did not significantly increase the number of S phases in the posterior region of the eye disc, nor was the $rbf1^{11}/rbf1^{120a}$ adult eye phenotype affected (Figure 7B and D; data not shown). In addition, we examined if we could detect interactions between *rbf1* and *brm* by using the rough eye phenotype generated by overexpression of the *brm* dominant-negative allele in the developing eye (*GMR-GAL4*; $UAS\text{-}brm^{K804R}$; data not shown). Reducing or increasing the dosage of *rbf1* in this background did not significantly affect the *GMR-GAL4*; $UAS\text{-}brm^{K804R}$ eye phenotype or affect S phases (data not shown).

Since we only observed a physical interaction between Brm and Rbf1 in larval tissues when Rbf1 was overexpressed, we wished to determine if under these conditions a genetic interaction between Brm and Rbf1 could be observed. Overexpression of *rbf1* in the eye, using four copies of a *GMR-rbf1* transgene, results in a mild rough eye phenotype, while two copies of *GMR-rbf* have no significant effect (Du *et al*., 1996a). We wished to determine whether we could enhance this phenotype by co-expression of Brm complex components. Flies overexpressing *brm* or *mor* are not available; however, *UAS-osa* transgenic flies have been described (Collins *et al*., 1999). We observed that overexpression of wild-type *osa* via the *GMR* driver resulted in a mild rough eye phenotype (data not shown). However, co-expression of *rbf1* and *osa* did not result in an enhanced rough eye phenotype or in a decrease in S phase cells in eye imaginal discs relative to *GMR-osa* or *GMR-rbf1* alone (results not shown). In summary, using several different genetic assays, we were unable to detect any significant functional interactions between *rbf1* and Brm complex genes.

Discussion

In this study, we have presented evidence that *brm*, *mor* and *snr1* interact genetically with *DmcycE*. This interaction is specific to the Brm complex genes, rather than being a general feature of the Trithorax group (trx-G) of global transcriptional activators, since very few trx-G genes other than Brm complex genes strongly suppressed the $DmcycE^{JP}$ eye phenotype. This is consistent with the finding that Ash1 and Ash2, at least, are present in high molecular weight complexes distinct from the Brm complex (Papoulas *et al*., 1998). We also showed that Brm and Snr1 form complexes with DmcycE in embryo and larval brain/disc extracts. Although we have not

examined all components of the Brm complex, the dominant genetic interactions that we have observed between *DmcycE*, and *mor* and *osa* alleles and deficiencies removing *BAP60* and *BAP111* suggest that these gene products may also be present in a complex with DmcycE, along with Brm and Snr1.

The $DmcycE^{JP}$ suppression we observed occurred by reducing the dosage of Brm complex genes, as well as by reducing Brm complex function with the dominant-negative brm^{K804R} transgene. The brm^{K804R} mutation abolishes Brm function by blocking ATP binding, but does not affect the assembly or stability of the complex (Elfring *et al*., 1998). This suggests that an ATP-dependent function is required for the Brm complex to negatively regulate S phase entry.

A role for the Brm complex downstream of DmcycE

The genetic interactions with *DmcycE* or *E2F1/DP* and Brm complex genes initially were thought to be due most probably to effects on *DmcycE* transcription or E2F/DP-dependent transcription, given the role of the Brm complex in transcriptional regulation (Tamkun, 1995). Surprisingly, the results of this study suggest that the Brm complex functions downstream of DmcycE transcription and protein accumulation. (i) No significant effect on DmcycE protein levels in $DmcycE^{JP}$ eye discs was observed when the dosage of *brm* or *mor* was halved. (ii) The rough eye phenotype due to overexpression of *DmcycE* from the *GMR* driver was enhanced by halving the dosage of *brm* and *mor*, indicating that Brm and Mor act to inhibit S phase entry downstream of *DmcycE* transcription. (iii) DmcycE forms a complex with Brm and Snr1. Taken together, these data provide strong evidence that the Brm complex does not inhibit the G_1 to S phase transition by acting to down-regulate *DmcycE* transcription.

It is also likely that the Brm complex does not act to down-regulate E2F1/DP-dependent gene transcription, since no effect was observed for at least two E2F1/DP targets in *brm* mutants (Staehling-Hampton *et al*., 1999). Thus, mutations in Brm complex genes suppress the $DmcycE^{JP}$ mutant phenotypes by allowing progression into S phase without increasing either DmcycE protein levels or the expression of E2F1/DP-dependent genes. This suggests that one function of the *Drosophila* Brm complex is to restrict entry into S phase by inhibiting DmcycE–Cdk2 activity or by acting downstream of DmcycE–Cdk2 function. A function for Brm downstream of DmcycE–Cdk2 is consistent with reports that mammalian cyclin E can bind to and phosphorylate components of the Brm complex and thereby inactivate it (Shanahan *et al*., 1999). Thus the Brm complex may be acting as a curb to S phase entry that needs to be overcome by phosphorylation and inactivation by cyclin E–Cdk2.

Brm and Rbf1

Consistent with studies in cultured mammalian cells (Dunaief *et al*., 1994; Trouche *et al*., 1997; Zhang *et al*., 2000), we observed that the Rbf1 protein was present in complexes with Brm or Snr1 in larval and embryonic extracts. However, in embryos, only a small portion of total cellular Rbf1 was present in Snr1 immunoprecipitates, in contrast to a significant fraction of the cellular DmcycE, suggesting that most Brm complexes do not contain Rbf1. Our observation that *Drosophila* Rbf1 and Brm form a complex *in vivo* is consistent with studies in mammalian cells showing that hBrm and/or Brg1 can bind to and cooperate with Rb in transcriptional repression, and that hBrm and Brg1 are required for Rb-induced G_1 arrest (reviewed by Muchardt and Yaniv, 2001; Zhang and Dean, 2001). However, in *Drosophila*, we were unable to obtain clear evidence for cooperation of *brm* or *mor* with *rbf1* in S phase entry. It is possible that the phenotypes we were examining were not sensitive enough for S phase effects to be observed. However, the lack of a strong effect of Brm complex mutants on the *rbf1* mutant S phase phenotype, when strong genetic interactions were observed with Brm complex genes and *DmcycE*, suggests that Rbf1 and Brm primarily function independently in negatively regulating S phase entry. Therefore, the suppression of the S phase defect of $DmcycE^{JP}$ by Brm complex mutants may not involve *rbf1*. Independent roles for Brm and Rb are also likely in mammalian cells since Rb knockout mice have a different mutant phenotype from that of Brg1 or Brm knockouts (reviewed by Muchardt and Yaniv, 2001).

In mammalian cells, Rb can form a complex containing both Brg1 and Hdac1, which is required to repress *DmcycE* transcription (Dahiya *et al*., 2000; Zhang *et al*., 2000) and may also have a role at replication origins (Lai *et al*., 2001). However, reducing the dose of the *Drosophila* Hdac gene, *rpd3*, did not suppress the $DmcycE^{JP}$ rough eye phenotype. It is possible that no interaction was observed for *rpd3* and *DmcycE*, because there are a least three other Hdacs in flies that may perform overlapping functions with *rpd3*. However, mutations in *sin3a*, which encodes a Hdac-interacting protein, enhanced the $DmcycE^{JP}$ rough eye phenotype, suggesting that Sin3a functions in opposition to Brm in regulating DmcycE or S phase entry. Further studies using specific mutations in other *Drosophila* Hdacs, and Hdac-interacting proteins are required to analyze further their role in the G_1 to S phase transition.

How does the Brm complex mediate negative regulation of the G_1 to S phase transition?

Our results, along with those of Staehling-Hampton *et al*. (1999), suggest that the Brm complex is playing a role independent of *DmcycE* transcription and E2F/DP-dependent transcription in negatively regulating the G_1 to S phase transition. One way in which this may occur is by transcriptional regulation of other critical G_1/S phase genes. For example, there is evidence that in *Drosophila*, the Brm complex is important in negatively regulating Armadillo-dTCF target genes in the Wingless signaling pathway (Collins and Treisman, 2000). Although as yet there have been no studies showing directly that G_1/S phase-inducing genes are targets of the Wingless signaling pathway in *Drosophila*, this is possible based on studies in mammalian cells (reviewed by Nollet *et al*., 1999). Furthermore, the Wingless pathway clearly has a role in cell proliferation in some *Drosophila* tissues (e.g. Neumann and Cohen, 1996). Whether this is the mechanism by which the Brm complex mediates negative regulation of cell cycle entry requires further investigation.

Another way in which the Brm complex may function is by restricting or regulating access to chromosomal origins of replication. Several studies have shown that ATP-dependent chromatin remodeling is important for modulating the initiation of chromosomal DNA replication (Hu *et al*., 1999; Li, 1999; Lipford and Bell, 2001). Our data are consistent with the view that the Brm complex may play a role in this process, possibly functioning to restrict entry into S phase by acting directly to remodel nucleosomes at replication origins. In this scenario, DmcycE–Cdk2 may then act to phosphorylate and inactivate the Brm complex, allowing assembly or function of the pre-replication complex and replication origin firing. Indeed, cyclin E–Cdk2 has been shown recently to be recruited by the Cdc6 pre-replication complex protein to replication origins at the G_1 to S phase transition (Furstenthal *et al*., 2001).

Intriguingly, recent studies have shown that the E2F/DP complex also acts directly at replication origins. In the amplification of the *chorion* gene clusters during the ovarian follicle cell endoreplicative cycles, it has been shown that E2F1/DP is important in localizing the origin of replication complex specifically to the *chorion* gene origins and activating replication, and that Rbf1 is important in limiting DNA replication (Austin *et al*., 1999; Royzman *et al*., 1999; Bosco *et al*., 2001). This mechanism is not limited to these specialized cycles, since transcription-independent roles for E2F1 in inducing S phase have also been documented in the eye imaginal disc (Du, 2000). Taken together, these studies suggest that the E2F1/DP–Rbf1 complex plays a non-transcriptional role in S phase by acting directly at DNA replication origins (Bosco *et al*., 2001). In mammalian cells, a similar non-transcriptional role for Rb in DNA replication inhibition has been demonstrated (Knudsen *et al*., 1998), possibly through its functional association with the pre-replication complex protein Mcm7 (Sterner *et al*., 1998) and its localization to replication foci (Kennedy *et al*., 2000).

Given the data for a role for Rb–E2F/DP directly at replication origins and the evidence that chromatin remodeling is important in replication initiation, it is possible that Brm and Rbf1 may both have a role at replication origins to prevent premature origin firing in G_1. However, the failure to detect a genetic interaction between *brm* complex genes and *rbf1* suggests that they also have other important roles, independent of each other, in the G_1 to S phase transition.

In summary, our results have shown that mutations in genes encoding components of the Brm chromatin remodeling complex can dominantly suppress a *DmcycE* hypomorphic allele by increasing the number of S phase cells without affecting cyclin E protein levels. Consistent with this view, DmcycE physically interacts with Brm and Snr1. Although a complex was also observed between the Brm complex and Rbf1, no genetic interactions were detected between Brm complex genes and *rbf1*, suggesting that Rbf1 and Brm function largely independently in negatively regulating the G_1 to S phase transition. Taken together, these data suggest that the Brm complex negatively regulates entry into S phase, possibly in partial collaboration with Rbf1, and that this negative regulation can be abrogated by the action of cyclin E at the G_1 to S phase transition.

Materials and methods

Fly strains and genetic manipulations

To examine genetic interactions between $DmcycE^{JP}$ and trx-G or Pc-G genes, stocks were generated that contained $DmcycE^{JP}$ (either heterozygous over CyO or homozygous) together with the test allele over a balancer chromosome. A stock of $DmcycE^{JP}$ isogenic for the second and third chromosomes was used for all crosses. Marked second chromosome $DmcycE^{JP}$ stocks (*dp cl b* $DmcycE^{JP}$ *cn* or *b* $DmcycE^{JP}$ *cn bw*) were used to generate recombinants of $DmcycE^{JP}$ and second chromosome genes. These stocks were outcrossed to $DmcycE^{JP}$, and at least 50 progeny heterozygous for the test allele were scored for modification of the $DmcycE^{JP}$ rough eye phenotype. Mutant alleles of Brm complex, trx-G, Pc-G and Hdac complex genes (as listed in Table I) were tested for interaction with $DmcycE^{JP}$ at 25°C. Deficiencies used to examine the effect of halving the dosage of several Brm complex genes and the Brm-interacting genes identified by Papoulas *et al*. (1998) were: *E(*brm^{K804R}*)25D-26B, Df(2L)cl-h3*; *E(*brm^{K804R}*)64E1-65C, Df(3L)ZN47*; *BAP111, Df(1)lz-90b24*; *Df(1)M38-c5*; and *BAP60, Df(1)C246*. Flies showing a modified eye phenotype were analyzed further by scanning electron microscopy as previously described (Secombe *et al*., 1998). The *GMR-GAL4, UAS-DmcycE; GMR-p35* stock was generated by recombination of second chromosome lines of *GMR-GAL4* and *UAS-DmcycE* (containing a genomic *DmcycE* transgene under control of UAS_{GAL4}, obtained from C.Lehner) followed by crosses to obtain the stock with *GMR-p35* (third chromosome). Flies co-expressing *rbf1* and *osa* via the *GMR* driver were generated by first making a recombinant of *GMR-GAL4* with *UAS-osa* (second chromosome) and the crossing to *GMR-rbf1* flies. To analyze genetic interaction with the *brm* dominant-negative allele, a *GMR-GAL4*; *UAS-*brm^{K804R} stock was generated, and crosses were carried out with *GMR-rbf1* or $rbf1^{11}$ flies. To generate brm^{K804R}-overexpressing clones in the eye, *ey-FLP UAS-GFP; +; Tb-GAL4 FRT(82B) Tb-GAL80* flies were crossed to *FRT(82B)*, *UAS-*brm^{K804R} flies and larval progeny eye imaginal discs were dissected. Sources for fly stocks were: brm^{K804R} transgenic flies, J.Tamkun; *UAS-osa* and *ey-FLP, UAS-GFP; Tb-GAL4 FRT(82B), Tb-GAL80* flies, J.Treisman; *lid* alleles, A.Shearn; and *Trl* alleles, T.Greenberg. *osa* alleles isolated as enhancers of *GMR-E2F1, DP* were obtained from N.Dyson. All other fly stocks were obtained from the Bloomington stock center.

The *UAS-snr1-cdel.3* deletion transgene was constructed by first placing a 108 amino acid C-terminal deletion of the Snr1 open reading frame under the control of the *GAL4(UAS)* and minimal *hsp70* promoter in *pUAST* (Brand and Perrimon, 1993). An insertion on the third chromosome was obtained following introduction by *P*-element transformation (C.Zraly and A.Dingwall, unpublished). The *UAS-snr1-cdel.3,* $snr1^{R3}$ strain used in these studies *(w; P[*w^+*; UAS*$_{GAL}$*hsp70-snr1-cdel.3], snr1*^{R3}*/TM6B)* was generated by recombining the *UAS-snr1-cdel.3* transgene with the $snr1^{R3}$ mutation (Dingwall *et al*., 1995). To induce expression of the transgene in a homozygous $DmcycE^{JP}$ background, *w; DmcycE*^{JP}*/CyO; UAS*$_{GAL}$*hsp70-snr1-cdel.3,* $snr1^{R3}$*/TM3* flies were crossed to either *w*; $DmcycE^{JP}$*/CyO; GawB[69B]-GAL4* or *w;* $DmcycE^{JP}$*/CyO; Act5C-GAL4/TM3* flies. Progeny of the genotype *w;* $cycE^{JP}$*/*$cycE^{JP}$*; P[*w^+*, UAS*$_{GAL}$*hsp70-snr1-cdel.3], snr1*^{R3}*/P[*w^+*, Act5C-GAL4]* and progeny homozygous for $DmcycE^{JP}$ and heterozygous for either *UAS-snr1-cdel.3,* $snr1^{R3}$ or the *GAL4* insertion were scored for suppression of the eye and wing defects. No obvious differences in suppression frequency or extent were correlated with the parental source of the GAL4 protein. Wings were dissected and mounted in DPX mountant (Fluka) for microscopic examination and photography.

Preparation of native extracts and immunoprecipitation assays

Native protein extracts from Oregon R embryos were prepared as described in Dingwall *et al*. (1995). For each immunoprecipitation experiment, ~500 μg of native protein extract from Oregon R embryos was pre-cleared with protein G–Sepharose beads (Pharmacia), and then incubated with Snr1 affinity-purified antibody (Dingwall *et al*., 1995). Protein complexes were precipitated using protein G–Sepharose beads, and bound and unbound proteins were fractionated on a 10% SDS–polyacrylamide gel and analyzed by western blotting.

Protein extracts were prepared from dissected third larval instar head tissues following heat shock induction of w^{1118}*, hsp70-DmcycE* or *hsp70-*

rbf1 larvae. Equilibrated protein A–Sepharose CL-4B was incubated with anti-DmcycE monoclonal antibody (8B10), anti-Rbf1 monoclonal (gift of W.Du and N.Dyson; Du *et al*., 1996a) or rabbit polyclonal anti-Brm antiserum (gift of C.Muchardt and M.Yaniv) for 6 h at 4°C. By western analysis of immunoprecipitated pellets and supernatants, we have shown that the anti-Brm and anti-DmcycE monoclonal (8B10) antibodies can immunoprecipitate their respective proteins efficiently and specifically (data not shown). Protein A–Sepharose-cleared protein extract was added to the antibody-bound Sepharose and incubation continued overnight. After extensive washing of the Sepharose beads, bound proteins were fractionated on a 7.5% SDS–polyacrylamide gel and analyzed by western blotting. Other antibodies used for immunoprecipitation–western analysis included anti-DmcycE polyclonal antibody raised in rats (Crack *et al*., 2002), and anti-dE2F1 (Du *et al*., 1996b) and control antiserum directed against a nuclear transcription factor that does not physically interact with Snr1 (Zraly *et al*., 2002).

Comparison of DmcycE protein levels in *DmcycE^{JP}; brm*/+ and *DmcycE^{JP}; mor*/+ eye discs relative to *DmcycE^{JP}* and wild-type was performed by dissecting 20 pairs of eye discs from each sample away from the antennal disc portion. The dissected tissue was homogenized in protein sample buffer before loading onto a 10% SDS–polyacrylamide gel. Western blot analyses were performed using the anti-DmcycE 8B10 monoclonal antibody followed by detection with a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Jackson Immunochemicals) and enhanced chemiluminescence (ECL) detection. As a loading control, the blots were also incubated with an anti-β-tubulin antibody (E7; Developmental Studies Hybridoma Bank). The DmcycE and tubulin detection signals were measured by scanning densitometry of X-ray films to quantify the relative level of DmcycE protein in each sample.

Immunohistochemistry and BrdU labeling

DmcycE antibody staining of larval eye imaginal discs was carried out as described previously (Secombe *et al*., 1998) using the anti-DmcycE antibody raised in rats, followed by detection using indirect immunofluorescence with anti-rat biotin and streptavidin–rhodamine. BrdU labeling of eye discs and ovaries was carried out as described previously (Secombe *et al*., 1998; Royzman *et al*., 1999).

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