

# Role for a *Drosophila* Myb-containing protein complex in site-specific DNA replication

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There is considerable interest in the developmental, temporal and tissue-specific patterns of DNA replication in metazoans<sup>1,2</sup>. Site-specific DNA replication at the chorion loci in *Drosophila* follicle cells leads to extensive gene amplification, and the organization of the *cis*-acting DNA elements that regulate this process may provide a model for how such regulation is achieved<sup>3</sup>. Two elements important for amplification of the third chromosome chorion gene cluster, *ACE3* and *Ori-β*, are directly bound by Orc<sup>4-6</sup> (origin recognition complex), and two-dimensional gel analysis has revealed that the primary origin used is *Ori-β* (refs 7-9). Here we show that the *Drosophila* homologue of the Myb (Myeloblastosis) oncoprotein family is tightly associated with four additional proteins, and that the complex binds site-specifically to these regulatory DNA elements. *Drosophila* Myb is required *in trans* for gene amplification, showing that a Myb protein is directly involved in DNA replication. A *Drosophila* Myb binding site, as well as the binding site for another Myb complex member (p120), is necessary *in cis* for replication of reporter transgenes. Chromatin immunoprecipitation experiments localize both proteins to the chorion loci *in vivo*. These data provide evidence that specific protein complexes bound to replication enhancer elements work together with the general replication machinery for site-specific origin utilization during replication.

To identify proteins that bind to either *ACE3* or *Ori-β*, we fractionated *Drosophila* tissue culture nuclear extracts as outlined in Fig. 1. DNase I protection was used to assay site-specific *ACE3*- and *Ori-β*-binding proteins, and to follow their purification. The final glycerol gradient fractions contained five polypeptides (Fig. 1a) that co-eluted with binding activity for both DNAs (Fig. 1b, c) in multiple independent fractionation schemes from either Schneider L2 or Kc cell lines (data not shown). Utilizing peptide sequences from proteolysed purified protein, database searches identified *Drosophila* Myb (p85) and Caf1 p55 proteins, as well as three new *Drosophila* proteins (p40, p120, p130; Berkeley *Drosophila* Genome Project CG15119, CG6061, CG3480).

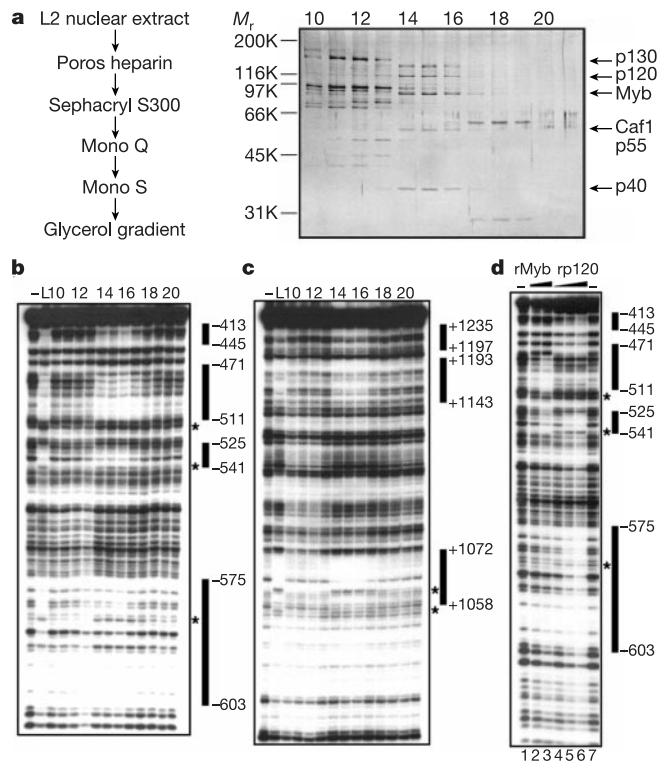
*Drosophila* Myb recognizes a highly conserved DNA sequence<sup>10,11</sup>, but the specific binding properties of the glycerol gradient fractions might be more complex than that of Myb alone. We therefore tested whether any of the other proteins in our footprinting fractions might interact site-specifically with DNA. Each protein was produced individually and purified to homogeneity as either a (His)<sub>6</sub>- or Flag-tagged protein using the baculovirus system. Only recombinant (r) Myb and rp120 bound to *ACE3* (Fig. 1d, lanes 2-6 and data not shown). rMyb protected nucleotides -471 to -511, and at higher concentrations protected -525 to -541, whereas rp120 protected -413 to -445, -525 to -541, and -575 to -603. However, the protection from the glycerol gradient fractions was more complex than the simple sums of the protections observed for these two purified proteins (Fig. 1d, compare lanes 3 and 6 to Fig. 1b, fractions 14-16). Moreover, rMyb on its own did not bind to *Ori-β*,

whereas p120 did (data not shown).

All five proteins co-immunoprecipitated together when any of the five antibodies were used, whereas a control antibody failed to immunoprecipitate any of the proteins (Fig. 2a). This association was not mediated through DNA, because ethidium bromide did not disrupt the interactions (Fig. 2b, compare lanes 2-4 with lanes 5-7). We obtained identical results using either 0-12-h embryo or ovary nuclear extracts (data not shown). Immunoblotting of fractions derived from the first two columns in our purification revealed that Myb co-fractionated only with the four other complex members (data not shown). No indication of free or other Myb forms was found. We therefore conclude that most of the Myb in these extracts is in a tight complex with the four additional proteins.

Antibodies against each of the five proteins individually super-shifted the nucleo-protein complex formed on *ACE3* DNA (Fig. 2c, compare lane 2 with lanes 3-7), whereas the control antibody did not (Fig. 2c, lane 8). Furthermore, when all of the recombinant proteins were co-expressed using baculovirus, the entire complex was isolated using nickel-chelate or Flag-tag affinity chromatography when only one subunit was tagged (data not shown).

As the *Drosophila* Myb complex bound to both *ACE3* and *Ori-β* *in vitro*, we tested whether the Myb complex directly interacted with Orc. Immunoprecipitation from Kc cell nuclear extracts showed that anti-Orc1 or anti-Orc2 antibodies co-immunoprecipitated



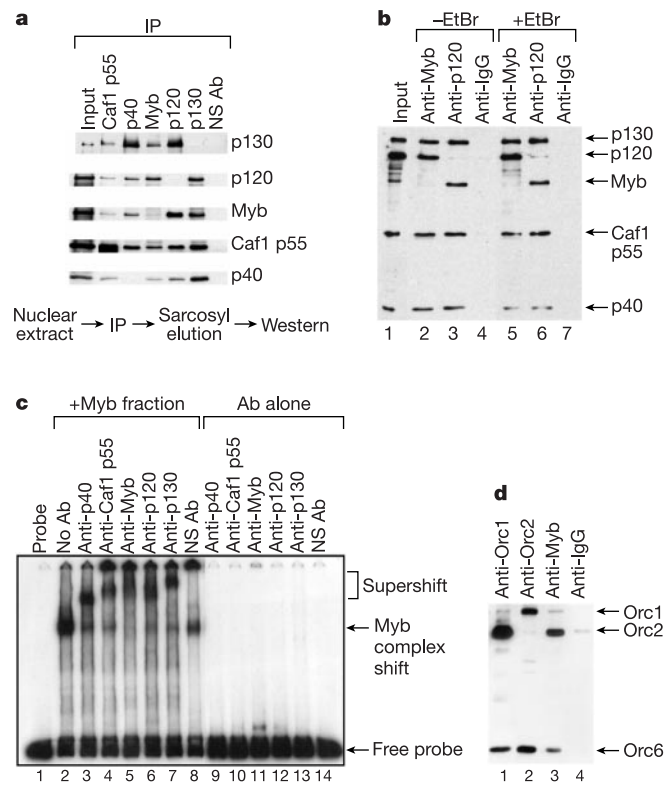
**Figure 1** Purification of proteins binding to *ACE3* and *Ori-β*. **a**, Silver-stained gel showing the proteins from glycerol gradient fractions isolated using the scheme shown. The fraction numbers from the gradient (top), the five proteins that co-fractionated with the site-specific DNA-binding activity (right), and markers of relative molecular mass ( $M_r$ ) (left) are indicated. **b**, DNase I protection by glycerol gradient fractions indicated on the top using a 5'-end-labelled *ACE3* DNA fragment. Minus sign indicates no protein; L shows glycerol gradient load from mono S peak. Black bars denote the regions of the probe that were protected from DNase I digestion; numbering on the right indicates the location of the sites within the 5'-untranslated region (5'-UTR) of the s18 chorion gene of *ACE3* (ref. 26). Asterisks denote DNase I hypersensitive sites. **c**, Same as **b** except that a 5'-end-labelled *Ori-β* fragment was used. **d**, Same as **b** except: lanes 1, 7, no protein; lanes 2-3, increasing amounts of recombinant (His)<sub>6</sub>-Myb; lanes 4-6, increasing amounts of recombinant Flag-p120.

Orc1, 2 and 6 (Fig. 2d, lanes 1 and 2). Immunoprecipitations with anti-Myb antibodies, but not control IgG, co-immunoprecipitated Orc (Fig. 2d, lanes 3 and 4). Reciprocal experiments showed that anti-Orc2 antibodies co-immunoprecipitated the Myb complex (data not shown).

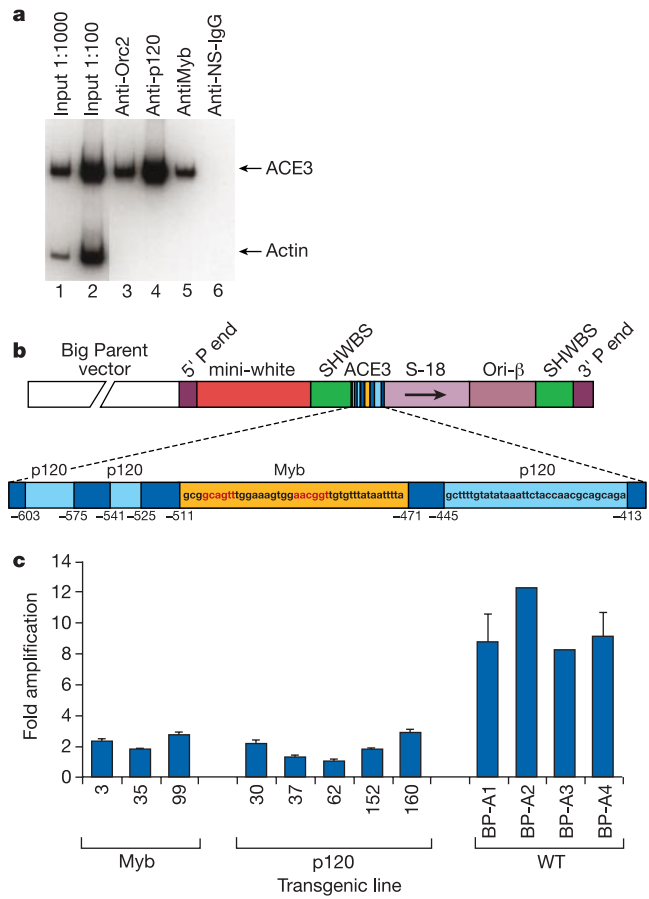
We performed chromatin immunoprecipitation assays on whole ovaries dissected from females that were aged to maximize the number of stage-10 egg chambers<sup>4</sup> as a first step in exploring the role of the *Drosophila* Myb complex *in vivo*. We found that antibodies against Myb, Orc2 and p120 specifically precipitated *ACE3*-containing chromatin but did not precipitate the *actin* locus (Fig. 3a, lanes 3–5). Neither *ACE3* nor *actin* were precipitated with control IgG (Fig. 3a, lane 6). Thus, Myb, p120 and Orc are bound to *ACE3* in ovaries enriched for egg chambers undergoing chorion gene amplification. E2F-containing complexes<sup>5</sup> can bind to Orc and are associated with *ACE3* in ovaries, but the location of this E2F *cis*-element is unknown. The interactions between the Myb complex, Orc and E2F proteins in sculpting the properties of the *ACE3* element will be critical to understanding how this element functions as a replication enhancer.

Small P element transgenes containing *ACE3* and *Ori-β* amplify efficiently at ectopic genomic sites only when both elements are

present<sup>9</sup>. We used a minimal replication reporter to assess the role of protein binding sites within *ACE3* to minimize the complications of redundant *cis*-elements. We used ‘suppressor of hairy wing binding sites’ (*SHWBS*) to insulate the transgenes from chromosomal position effects (Fig. 3b). Such reporters allow us to investigate, at various chromosomal positions, if the binding sites in *ACE3* for Myb and p120 are important *cis*-acting elements for amplification. We constructed transgenes that contained deletions of each of the binding sites identified by DNase I protection (Fig. 1b and Fig. 3b) and generated several transgenic lines for each deletion. Mutations abolished DNA binding of both recombinant Myb and the entire complex to the regulatory sequences in electrophoretic mobility shift assays (EMSA) experiments (Supplementary Information). Deletion of either the Myb (–471 to –511) or one of the p120 binding sites (–413 to –445) resulted in severely reduced amplification in stage-13 egg chambers (Fig. 3c). Deletion of the other two p120 binding sites resulted in reduced levels of amplification, but not as severe as the sites shown in Fig. 3c (data not shown).



**Figure 2** A Myb complex in *Drosophila*. **a**, Proteins from extracts were immunoprecipitated (IP) with a given antibody (top), the precipitates washed with RIPA buffer containing 300 mM KCl, and half of the 0.4% sarcosyl eluates were analysed using a single immunoblot successively probed with antibodies indicated (right): ‘input’ indicates 1/50 total extract. **b**, Same as in **a** except that the IPs were either performed with (lanes 5–7) or without (lanes 2–4) ethidium bromide (EtBr) in the extracts before precipitation. The immunoblot was performed with the mixture of antibodies. **c**, EMSA with mono S peak fraction and radiolabelled *ACE3* DNA (lane 2). Supershifts caused by given antibodies are indicated. NS, nonspecific control (affinity-purified anti-BPV-E2 polyclonal sera). Affinity-purified antibodies alone do not interact with DNA (lanes 9–13). **d**, IPs performed as in **a** in the presence of EtBr using the antibodies indicated in the top of the figure. Half of the sarcosyl eluate was simultaneously blotted for the presence of Orc1, 2 and 6 as indicated on the right. Lane 4, non-specific rabbit IgG.



**Figure 3** *Drosophila* Myb and p120 associate with *ACE3*, and binding sites are essential for amplification *in vivo*. **a**, *ACE3*- and *actin*-specific PCR following chromatin immunoprecipitation from whole ovaries using affinity-purified: anti-Orc2 (lane 3), anti-p120 (lane 4), anti-Myb (lane 5) or rabbit IgG (lane 6). PCR products from tenfold serial dilutions of the input DNA are shown in lanes 1 and 2. **b**, The P-element vector with insulator elements (*SHWBS*) surrounding the amplification unit. The *ACE3* region is expanded, showing the three p120 sites (pale blue) and the two Myb sites (yellow box with consensus sites in red) inferred from the DNase I protection experiment. Three amplification reporters were used to create transgenic lines: wild type (WT), a reporter with base pairs –511 to –471 (Myb sites), or base pairs –445 to –413 (the most 3’ p120 site) deleted. **c**, Each blue bar (with strain no. given) represents data from an independent transgenic fly line; for each such line, at least two independent experiments were measured and each had side-by-side duplicates (mean  $\pm$  s.d.).

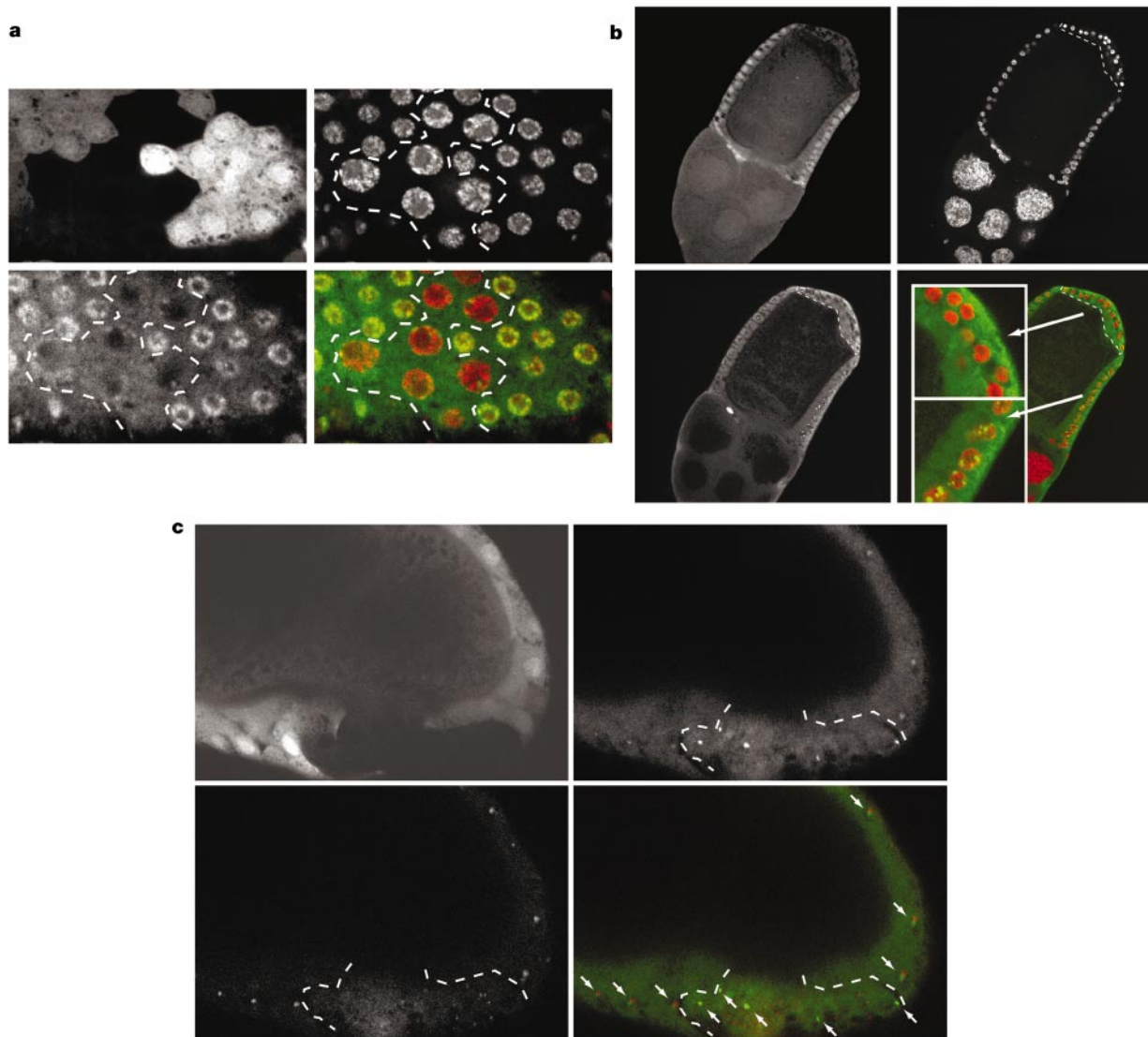
The mature somatic follicle cells that surround the developing oocyte derive from a series of mitotic cell divisions followed by genome-wide endocycles<sup>3</sup>. At stage 10B, global DNA replication shuts down and the chorion loci on the X and third chromosomes (and two additional unidentified loci) begin locus-specific amplification. Amplification can be visualized by the incorporation of bromodeoxyuridine (BrdU) at four sub-nuclear foci using immunofluorescence microscopy, where the two largest foci represent incorporation on the X and third chromosomes<sup>12</sup>.

As Orc2 also localizes to these foci, we stained ovaries with either anti-Myb or anti-Orc2 antibodies. We found that Myb is diffusely nuclear and not localized to the distinct sub-nuclear foci that contain Orc2 (compare Fig. 4a, lower left panel with Fig. 4c, upper right panel). Identical results were observed with the four other complex members (data not shown).

*Drosophila* Myb has been suggested to have a general role in S phase in several different tissues<sup>13</sup>. However, a direct role for

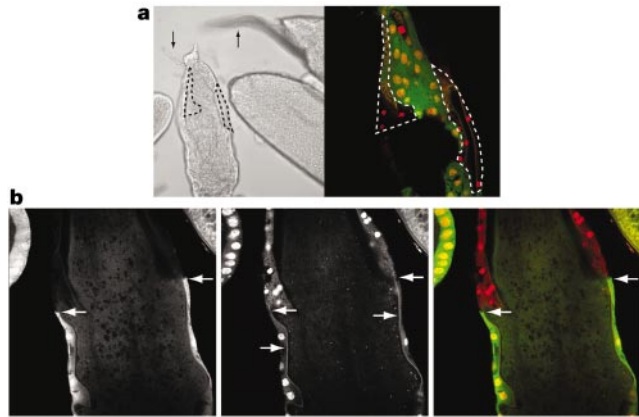
*Drosophila* Myb in replication at a specific location has not been demonstrated. To test the need for *Drosophila* Myb in *trans* for replication at the chorion loci, we used a directed mosaic system to generate green fluorescent protein (GFP)-negative, homozygous *Drosophila* Myb mutant clones in a heterozygous ovary<sup>14,15</sup>. Figure 4a shows follicle cells from one such mutant clone in a stage-10 egg chamber that was stained with anti-Myb antibodies (lower left panel) and with propidium iodide for DNA (PI, upper right panel). There was negligible Myb protein expression in the GFP-negative clone (Fig. 4a, lower right panel).

In contrast to the surrounding normal cells, no subnuclear foci of BrdU incorporation were detected in *Drosophila* Myb mutant clones of stage-10 egg chambers (Fig. 4b). Double-labelling with anti-Orc2 antibodies and BrdU revealed that in *Myb* mutant cells, Orc2 was present at the sub-nuclear foci even though these cells lacked BrdU foci (Fig. 4c). Similar foci of Cdt1/Dup immunostaining were also present in *Myb* mutant cells (data not shown). We obtained



**Figure 4** *Drosophila* Myb is required for chorion gene amplification. Stage-10 mosaic egg chambers from heterozygous *Myb* females (MH30 in **a** and **c**; MH107 in **b**). GFP-negative, *Myb* mutant mitotic clones (upper left in all three figures) are indicated with dotted lines. **a**, Propidium iodide (PI, upper right and red in lower right) and anti-Myb (lower left and green in lower right), showing an absence of Myb staining in the mutant clone. **b**, PI (upper right and red in lower right) and BrdU incorporation (lower left and green in lower right), showing the absence of subnuclear foci of BrdU incorporation in the *Myb* mutant clone (enlarged panels, lower right). **c**, Anti-Orc2 (upper right and green in lower right) and BrdU incorporation (lower left and red in lower right, downward-pointing arrows), showing normal Orc2 foci despite the absence of BrdU foci in the *Myb* mutant clone (upward-pointing arrows).

left and green in lower right), showing the absence of subnuclear foci of BrdU incorporation in the *Myb* mutant clone (enlarged panels, lower right). **c**, Anti-Orc2 (upper right and green in lower right) and BrdU incorporation (lower left and red in lower right, downward-pointing arrows), showing normal Orc2 foci despite the absence of BrdU foci in the *Myb* mutant clone (upward-pointing arrows).



**Figure 5** Absence of *Drosophila Myb* results in thin chorion and abnormal dorsal appendages. **a**, Left panel, differential interference contrast image of late-stage egg chambers from heterozygous *Myb* mutant, *AlkB*<sup>+</sup> females. Arrow on left: abnormal dorsal appendage of the mosaic egg chamber; GFP-negative *Myb* mutant patches are outlined in black. Arrow on right: normal dorsal appendage of wild-type egg chamber. Right panel, mosaic egg chamber stained with PI (red) to visualize nuclei. The GFP (green)-negative *Myb* mutant patches are outlined in white. **b**, Egg chamber described in **a** showing GFP (left panel and green in right panel) and PI (centre panel and red in right panel). The chorion underlying the follicle cells in the GFP-positive, *Myb*-containing patch (right-pointing arrows) disappears at the border of the GFP-negative *Myb*-mutant patch (left-pointing arrows).

identical results with both of our previously described *Myb* mutants<sup>13</sup>: MH107 removes the promoter and 5'-untranslated region of *Myb* and the adjacent *AlkB* gene, and MH30 removes *AlkB*, *Myb* and a gene 3' of *Myb*. We also found that all of the MH107 phenotypes were complemented with a transgene that contained *Myb* without *AlkB*, but not with a transgene that contained *AlkB* without *Myb*<sup>13</sup> (data not shown).

Together, these data demonstrate that in the absence of *Drosophila Myb*, members of the pre-replication-complex (RC) are present at the sub-nuclear foci but are unable to initiate detectable replication. One prediction from these results is that in late-stage mosaic egg chambers with *Myb* clones, patches of thin, fragile eggshell and thin dorsal appendages should result from reduced chorion gene expression. Figure 5a shows a mosaic egg chamber in which GFP-negative, *Myb* mutant patches in the regions that are normally responsible for dorsal appendage formation<sup>16</sup> resulted in greatly reduced appendages (Supplementary Information). Note that as egg chambers age, the *Myb* mutant nuclei appear more compact. Using laser light scattering following propidium iodide staining, we found an absence of the underlying chorion beneath the follicle cells in the mutant patch (Fig. 5b, middle panel).

Studies of *Myb* family members have largely focused on their functions as transcription factors, though important targets for gene activation that might explain the role of these proteins in the cell cycle remain unclear. We show here that *Drosophila Myb* has a direct role in DNA replication. Our biochemical experiments imply that this protein functions in tight complex with four other proteins. Recent studies suggest that in a variety of tissues, but not all, *Drosophila Myb* seems to be important for S-phase progression<sup>13,17</sup>. Our findings support a role for *Drosophila Myb* in tissue-specific and temporally defined DNA replication, much as enhancer proteins define site-specific transcription.

The genetic studies and, in particular, the mosaic analyses indicate that *Drosophila Myb* probably functions at a late step in the replication process, as *Orc2* and *Cdt1* were both localized at discrete foci within *Drosophila Myb* mutant stage-10B follicle cells. We infer that *Orc* and other general replication proteins were localized at *ACE3*. However, we do not know when in the develop-

mental process *Orc* appears at *ACE3* with regard to *Drosophila Myb* loss. Thus, *Drosophila Myb* perdurance after genomic deletion could certainly complicate any conclusions about the role of *Drosophila Myb* in establishing a pre-RC at the amplification foci. In any case, *Myb* family members interact with both acetylases and deacetylases<sup>18</sup>; thus, it is intriguing to consider the potential roles of this modification in either early or late steps of DNA replication initiation. □

**Methods**

**Myb complex purification and recombinant protein expression**

Schneider L2 or Kc cell nuclear extracts were prepared as described<sup>19</sup>. Details of the fractionation protocol will be provided upon request. Fractions containing the DNA-binding activity were loaded onto a 15–35% glycerol gradient, and peptides sequenced from the purified *Drosophila Myb* complex members<sup>20</sup> were used to identify the corresponding proteins from database searches. Complementary DNA clones were obtained from Research Genetics, sequenced, and following polymerase chain reaction (PCR) amplification, cloned into either pFastBacHTa (Gibco) to generate (His)<sub>6</sub>-tagged proteins or pFastBac1 (Gibco) to generate untagged versions. Flag-tagged p120 was generated in pFastBac1 following PCR amplification of the 5' end with oligonucleotides encoding the Flag epitope. Recombinant proteins were purified from infected HI-5 nuclear extracts using standard methods.

**Antibody purification and immunoprecipitations**

PCR fragments for *Drosophila Myb*, p40 (CG15119), p120 (CG6061) and p130 (CG3480) were cloned into pVCH6<sup>21</sup> and proteins purified from *Escherichia coli*. Polyclonal rabbit antibodies were affinity purified<sup>22</sup> using cyanogen bromide (CNBR)-sepharose (Amersham) coupled to maltose binding protein (MBP)-*Myb*, (His)<sub>6</sub>-p40, glutathione S-transferase (GST)-p120 or (His)<sub>6</sub>-p130 and dialysed into PBS. Anti-Caf1 p55 antibodies were affinity purified from serum provided by J. Kadonaga. For IP and chromatin immunoprecipitation experiments, antibodies were isolated from sera using Gamma-bind plus beads (Amersham) and immunoprecipitations performed as described using affinity-purified rabbit IgG (Sigma) as a control<sup>23</sup>. Where indicated, 50 µg ml<sup>-1</sup> ethidium bromide was included in the extracts before IP.

**Biochemical assays**

DNase I protection experiments were performed as described<sup>24</sup>, except that the probes were derived from the third chromosome chorion locus<sup>4</sup>. For EMSA, binding reactions were set up on ice and contained 0.1 M KCl, 0.2 mg ml<sup>-1</sup> bovine serum albumin (BSA), 5 mM MgCl<sub>2</sub>, 2 µg ml<sup>-1</sup> poly [d(G-C)][d(G-C)], and ~1 fmol of end-labelled *ACE3* probe. Reactions were incubated for 15 min at room temperature, affinity-purified antibodies were added, and further incubated for 15 min. Samples were electrophoresed for 5 h at 4.7 V cm<sup>-1</sup> in 4.2% (60:1 acrylamide:bisacrylamide) gels containing 0.5X TGE buffer (12.5 mM Tris, pH 8.3; 95 mM glycine; 0.5 mM EDTA), 0.02% ethyl phenyl—polyethylene glycol (NP-40; Shell), and 3% glycerol.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitations were performed as described<sup>4</sup> with the following modifications: whole ovaries were used instead of dissected stage-10 egg chambers, and immunoprecipitations were performed with 5 µl of Gamma-bind plus antibody beads. Fifteen cycles of PCR using *ACE3* and *actin* primers<sup>25</sup> and Southern blot hybridization with <sup>32</sup>P-labelled *ACE3* and *actin* probes were used to detect the amplified products.

**Amplification assay**

PCR products derived from oligonucleotides containing a deletion of the *Drosophila Myb* or p120 binding sites were used to replace the non-mutated *ACE3* fragment from the Big Parent vector<sup>9</sup> and these constructs were transformed into *Drosophila*. The wild-type Big Parent lines were as described<sup>9</sup>. Amplification of the *ACE3* deletion transgenes was assayed<sup>9</sup> with the following modification: the hybridization probe for the loading control was derived from a fragment of the *CG1115* single copy gene. For each independent transgenic line, at least four assays were performed, and the data presented as the mean ± s.d.

**Genetics**

The MH30 and MH107 alleles of *Drosophila Myb* were previously described<sup>13</sup>. Each allele was recombined with an X chromosome containing an FRT site at 18E (P{w<sup>+</sup>mW.hs = FRT(w<sup>hs</sup>)9-2; Bloomington Stock Center). MH107, FRT18E was further recombined with an X chromosome P element containing a 9.8-kb *XbaI-BamHI Drosophila* genomic DNA fragment from 13F that contains the entire *G-beta* and *AlkB* genes, but only the 5' half of the *Drosophila Myb* gene<sup>13</sup>. Flies carrying y<sup>1</sup>, w<sup>1118</sup>, P{w<sup>+</sup>mC,Ubi-GFP.nls}, P{w<sup>+</sup>mW.hs.FRT(w<sup>hs</sup>)9-2} or P{w<sup>+</sup>mW.hs = en2.4-GAL4}e22c P{w<sup>+</sup>mC = UAS-FLP1.D}JD1 chromosomes were kindly provided by J. Duffy. Ovaries were dissected from adult female flies with genotypes: *Drosophila Myb* mutant, FRT18E/Ubi-GFP, FRT18E; e22c-GAL4, UAS-FLP. For testing the *Myb* mutation in the presence of wild-type *AlkB*, the *AlkB* + transgene was also present on the MH107, FRT18E chromosome. For testing the *AlkB* mutation in the presence of wild type *Myb*, we used homozygous MH107 females with a third chromosome P element containing a 6.6-kb *MluI-BamHI-MluI-BamHI* genomic DNA fragment containing all of *Myb* but only a small 3' fragment of *AlkB*<sup>13</sup>.

**Antibody staining and BrdU labelling of ovaries**

Ovaries were fixed and processed as described<sup>25</sup> except that all antibody incubations were performed in PBS with 0.1% Triton X-100 (PBT) and 1% BSA. BrdU labelling was performed as described<sup>12</sup> with the following modifications (kindly provided by B. Calvi): ovaries were washed 2 × 15 min in PBS + 0.6% Triton X-100, 2 × 15 min in DNase I buffer (66 mM Tris pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM fresh 2-mercaptoethanol), then incubated in 25 units DNase I (Worthington)/0.5 ml DNase buffer at 37 °C for 30 min, washed 3 × 1 min then 1 × 30 min in PBT, blocked in PBT with 5% normal goat serum for at least 1 h, and stained with anti-BrdU monoclonal (Becton-Dickinson), affinity-purified anti-Myb polyclonal, and/or anti-Orc2 polyclonal<sup>4</sup> antibodies followed by fluorophore-conjugated secondary antibodies (Molecular Probes). Ovaries were mounted in Vectashield with or without propidium iodide (Vector Laboratories) and visualized with a Nikon PCM confocal microscope.

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**Supplementary Information** accompanies the paper on Nature's website (<http://www.nature.com/nature>).

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**The RNA processing exosome is linked to elongating RNA polymerase II in *Drosophila***

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The RNA polymerase II elongation complex contains several factors that facilitate transcription elongation and catalyse the processing of precursor messenger RNAs (pre-mRNAs)<sup>1–3</sup>. The conserved elongation factor Spt6 is recruited rapidly and robustly to sites of active transcription<sup>4,5</sup>. Here we show that *Drosophila* Spt6 (dSpt6) co-purifies with the exosome, a complex of 3' to 5' exonucleases that is implicated in the processing of structural RNA and in the degradation of improperly processed pre-mRNA<sup>6–10</sup>. Immunoprecipitation assays of *Drosophila* nuclear extracts show that the exosome also associates with the elongation factor dSpt5 and RNA polymerase II. *In vivo*, exosome subunits colocalize with dSpt6 at transcriptionally active loci on polytene chromosomes during normal development and are strongly recruited to heat-shock loci on gene induction. At higher resolution, chromatin immunoprecipitation analysis shows that the exosome is recruited to transcriptionally active units of heat-shock genes. These data provide a physical basis for the hypothesis that exosome-mediated pre-mRNA surveillance accompanies transcription elongation.

The production of full-length mRNA transcripts requires the coordinated effort of many factors, including the general elongation protein Spt6, which is essential and conserved. In yeast, Spt6 interacts genetically and biochemically with transcription elongation factors that regulate the processivity of RNA polymerase II (Pol II)<sup>11</sup>. Spt6 may also modulate chromatin structure during transcription, through direct interactions with histone H3 (ref. 12). dSpt6 is recruited to sites of active transcription and colocalizes with the elongating form of Pol II (refs 4, 5). On the basis of these observations, we considered that dSpt6 might associate functionally with the transcriptional elongation machinery.

We took a biochemical approach to identify the factors with which dSpt6 associates. Full-length dSpt6 was cloned, tagged with an epitope and expressed in a stable *Drosophila* cell line (Fig. 1a). Nuclear extracts containing dSpt6-Flag-His<sub>6</sub> (dSpt6FH) were prepared and subjected to Flag affinity chromatography followed by glycerol gradient sedimentation (Fig. 1b). Several polypeptides co-purified with the tagged dSpt6 (Fig. 1c) and were identified unequivocally. We excised bands from the gel and identified the proteins by a combination of peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and mass spectrometric sequencing using NanoES triple-quadrupole tandem MS (MS/MS).

We identified nine bands with approximate stoichiometry to dSpt6 that represent homologues of the subunits of the yeast and human exosome (Table 1). An additional band corresponded to untagged dSpt6, consistent with the self-association of Spt6 previously deduced from a large-scale analysis of *Saccharomyces cerevisiae* protein complexes<sup>13</sup>. This large-scale analysis failed to detect an interaction between Spt6 and the exosome, which is not unexpected, as our observations suggested that only fractions of the *Drosophila* Spt6 and exosome are associated physically (Fig. 1e, h, i). Nonetheless, this physical association could withstand