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Original Article

Functional analysis of *Drosophila* DNA polymerase ε p58 subunit

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Abstract: DNA polymerase ε (polε) plays a central role in DNA replication in eukaryotic cells, and has been suggested to the main synthetic polymerase on the leading strand. It is a hetero-tetrameric enzyme, comprising a large catalytic subunit (the A subunit ~250 kDa), a B subunit of ~60 kDa in most species (~80 kDa in budding yeast) and two smaller subunits (each ~20 kDa). In *Drosophila*, two subunits of polε (dpolε) have been identified. One is the 255 kDa catalytic subunit (dpolεp255), and the other is the 58 kDa subunit (dpolεp58). The functions of the B subunit have been mainly studied in budding yeast and mammalian cell culture, few studies have been performed in the context of an intact multicellular organism and therefore its functions in this context remain poorly understood. To address this we examined the in vivo role of dpolεp58 in *Drosophila*. A homozygous dpolεp58 mutant is pupal lethal, and the imaginal discs are less developed in the third instar larvae. In the eye discs of this mutant S phases, as measured by BrdU incorporation assays, were significantly reduced. In addition staining with an anti-phospho histone H3 (PH3) antibody, (a marker of M phase), was increased in the posterior region of eye discs, where usually cells stop replicating and start differentiation. These results indicate that dpolεp58 is essential for *Drosophila* development and plays an important role in progression of S phase in mitotic cell cycles. We also observed that the size of nuclei in salivary gland cells were decreased in dpolεp58 mutant, indicating that dpolεp58 also plays a role in endoreplication. Furthermore we detect a putative functional interaction between dpolε and ORC2 in discs suggesting that polε plays a role in the initiation of DNA replication in *Drosophila*.

Keywords: DNA polymerase ε B subunit, *Drosophila melanogaster*

Introduction

In eukaryotic cells, three DNA polymerases (pol), polα, polδ and polε are essential for replicating nuclear DNA. Polα is responsible for primase activity at the initiation of DNA replication and during Okazaki fragments synthesis, where it synthesizes about 10 nucleotides of primer RNA followed by 20-30 nucleotides of DNA. After primer synthesis, polα dissociates and is replaced by polδ and/or polε to perform the bulk of the DNA elongation [1-3].

DNA polymerase ε was first isolated from yeast in 1970 [4]. In budding yeast, polε is composed of four subunits comprising the catalytic subunit Pol2, the middle subunit Dpb2 and two small subunits Dpb3 and Dpb4 [5-8]. The subunit structure of this heterotetrameric enzyme appears to be evolutionarily conserved in all eukaryotes [9, 10]. Pol2 has DNA polymerase and exonuclease domains at the N-terminus and ATPase and/or ATP-binding domains and two Zn finger domains at the C-terminus. Although it has been suggested that polymerase epsilon is responsible for leading strand synthesis [11], genetic studies in yeast demonstrated that the N-terminus of pol2 is dispensable for cell viability [12-14], whereas the non-catalytic C-terminal domain is essential for cell viability [12-15]. The C-terminal domain has multiple sites that interact with DNA [16] and in mammalian cells, shows associations with

other polε, PCNA [17] and MDM2 (an E3 ubiquitin ligase activating p53 degradation) [18]. Pol2 also genetically and physically interacts with Dpb11, which is needed for chromosomal DNA replication and the S-phase checkpoint in *Saccharomyces cerevisiae* [19-21]. Recruitment of pol2 and pol1 (polα) to origins depends on Dbp11 [21].

The polε second largest subunit (polε B subunit), Dpb2 is also required for viability in yeast [5]. Studies in yeast suggest that Dpb2 is essential for chromosomal replication and required for the stability of enzyme activity [22]. Dpb2 binds to the C-terminal domain of the catalytic subunit [23] and is phosphorylated by Cdc28 at the late G1 phase of cell cycle [24]. Dpb2 has also been shown to interact with GINS, and helps DNA polymerases bind to pre-RC [25]. The GINS complex associates with origins during S phase in a pre-replication complex (pre-RC) and CDK-dependent manner, and interacts with the Mcm2-7 complex and Cdc45 to form the CMG complex, thought to be responsible for the replicative DNA helicase activity in eukaryotes [26-28]. In *Xenopus*, budding yeast and fission yeast GINS is required for Cdc45 and polymerase loading [25, 29-31]. In budding yeast, the amino-terminal domain of dpb2 associates with the B domain of Psf1, subunit of GINS. This interaction is essential for assembly of the CMG helicase during the initiation of chromosome replication, and also serves subsequently to integrate polε into the replisome at DNA replication forks [32]. In mouse cultured cells, the polε B subunit has been reported to interact with SAP18 to recruit the transcriptional co-repressor Sin3 [33]. The deacetylase activity associated with SIN3 has been reported to be involved in controlling the timing of replication [34].

In *Drosophila*, two subunits of DNA polymerase ε (dpolε) have been identified [35]. One is the 255 kDa catalytic subunit (dpolεp255) and the other is the 58 kDa subunit (dpolεp58). The N-terminus of dpolεp255 carries the well-conserved six DNA polymerase subdomains and five 3'→5' exonuclease motifs as observed with Polε in other species. Biochemical analysis of dpolεp255 suggests that the catalytic subunit synthesizes DNA progressively in the presence of both Mn^{2+} and Mg^{2+} ions [36]. Genetic studies in *Drosophila* demonstrated that dpolεp255 is required for the G1-S transition and/or S-phase progression [37] of the mitotic cycle as well as for endocycle progression [37, 38]. In the mitotic cell cycle, the catalytic N-terminal region of dpolεp255 is dispensable, but in endocyles it is crucial and its defect cannot be complemented by other DNA polymerases [38]. As *Drosophila* DNA polymerase α (dpolα), *Drosophila* DNA polymerase δ (dpolδ) and dpolε show similar expression patterns during development, it is suggested that dpolε, in combination with dpolα and dpolδ, participates in DNA replication. So far there is no report on the function of dpolεp58.

Since most of the previous studies on the polε B subunit were carried out in yeast and cultured mammalian cells, the functions of this subunit in intact multi-cellular organisms are poorly understood. We have therefore conducted an *in vivo* assessment of dpolεp58 in *Drosophila*, a well-defined multi-cellular model organism. In this paper, we examine the roles of dpolεp58 during *Drosophila* development using *dpolεp58* mutant fly lines. We demonstrate that dpolεp58 plays an essential role in the G1–S transition and/or S-phase progression of the mitotic cycle as well as in endocycle progression, as is the case with polεp255. Moreover our results suggest a previously undocumented interaction between dpolεp58 and ORC2. We discuss a possible role for this interaction in initiation of DNA replication.

Materials and methods

Fly stocks

Fly stocks were cultured at 25°C on standard food. The Canton S strain was used as a wild-type strain. *DNA polymerase ε subunit p58* (*dpolεp58*) gene mutant strain (*Drosophila* Genetic Resource Center, stock number 142095) was obtained from *Drosophila* Genetic Resource Center in Kyoto. All RNAi lines used in this study were obtained from the Vienna *Drosophila* RNAi Center in Vienna. Gal4 strains such as Actin5C-GAL4 and MS1096-GAL4 were obtained from Bloomington *Drosophila* stock center in Indiana. GMR-GAL4 (strain 16) was described earlier [39]. To express GAL4, flies were cultivated at 28°C.

Plasmid construction

To generate pUAST-dpolεp58, the dpolεp58 cDNA fragment (1,578 base pair) was amplified

from the EST clone (Clone ID: LD25702, Open Biosystems, Huntsvill, AL, USA) by PCR using KOD plus (TOYOBO, OSAKA, Japan) with the primer oligonucleotides 5'-*EcoR*I-dpolεp58 (5'-CGGAATTCATGGATGTGGATTTACTGCC) and 3'-dpolεp58-XhoI (5'-ATACTCGAGCTACTCCAATTCGTCCGGTA). The PCR product was digested with EcoRI and XhoI, and then inserted into EcoRI and XhoI sites of pUAST [40, 41].

To generate pGEX-dpolpε58, the cDNA fragment was cut out from the plasmid pUAST-dpolεp58 and subcloned into EcoRI and XhoI sites of pGEX-6P-1 (GE healthcare). All plasmids were propagated in *Escherichia coli* (*E. coli*) XL-1 Blue, isolated by standard procedures [40] and further purified using a Qiagen plasmid Midi kit (Qiagen, Valencia, CA, USA). DNA sequencing was carried out with a BigDye TM kit and a 310 NT Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Chemically synthesized oligonucleotides were used as sequencing primers.

Expression of GST fusion proteins and purification of dpolεp58 protein

The full-length dpolεp58 cDNA was cloned into pGEX6p-1 as described above. Expression of GST-dpolεp58 fusion proteins in *E. coli* BL21 was carried out as described elsewhere [40]. Lysates of cells were prepared by sonication in 0.3% Triton X-100 in PBS (PBST) containing 1 mM phenylmethylsulfonyl fluoride, and 1 mM each of pepstatin and leupeptin. Lysates were cleared by centrifugation at 12,000×g for 30 min at 4°C. The pellet was dissolved in PBS-T containing 4M urea, stored for 30 min at 4°C, and cleared by centrifugation at 7,500×g for 40 min at 4°C. The pellet was dissolved in PBS-T containing 8M Urea, stored for 40 min at 4°C, and cleared by centrifugation at 12,000×g for 30 min at 4°C. The supernatant was replaced sequentially with 150 ml PBS-T containing 8M Urea, 7M Urea, 6M Urea, 5M Urea, 4M Urea, 3M Urea, 2M Urea and 1M Urea, PBS-T per 1 h by dialysis.

Antibodies

The purified dpolεp58 protein was used to elicit monoclonal antibody production in rat. The antibodies were purified by E-Z-SEP (Pharmacia Biotech) following procedures described. The purified antibodies were resuspended with PBS (pH7.4). The following dilutions for the antibodies were used for Western blot analyses. The rat-monoclonal anti-dpolεp58 antibody (1:5,000 dilution), the rabbit-polyclonal anti-dpolεp255 antibody [42], the rabbit-polyclonal anti-Cdc45 antibody [43] at 1:2,000 dilution, the rabbit-polyclonal anti-dOrc2 antibody [44] at 1:2,000 dilution, the rabbit-polyclonal anti-dMcm2 antibody [45] at 1:2,000 dilution, the rabbit-polyclonal anti-dMcm3 antibody [45] at 1:2,000 dilution, the rabbit-polyclonal anti-dMcm6 [45] antibody at 1:2,000 dilution, the rabbit-polyclonal anti-dMcm7 [46] antibody at 1:2,000 dilution were used for Western immunoblot analysis.

Western immunoblot analysis

Third instar larvae frozen in liquid nitrogen were homogenized in a solution containing 50 mM Tris-HCl (pH6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol blue, 1.3% β-mercaptoethanol, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.15 μM aprotinin, 1 μM E-64 protease inhibitor, 1 μM leupeptin hemisulfate monohydrate, 0.5 mM disodium dihydrogen ethylenediaminetetraacetate dehydrate. Homogenates were centrifuged at 15,000×g for 15 min, and the extracts were collected. These extracts were electrophoretically separated on 6.0% or 10% polyacrylamide gels containing 0.1% SDS and then transferred to Polyvinylidene difluoride (PVDF) membrane (BIO-RAD) in a solution containing 25 mM Tris (hydroxymethyl) aminomethane, 192 mM glycine and 20% methanol at 4°C for 2 or 4 h. The blotted membranes were blocked with a blocking buffer (Blocking One (Nacalai Tesque, Kyoto, Japan)) at 25°C for 1 h and then incubated with the primary antibody at 4°C for 16 h. After washing with TBS containing 0.05% Tween20, the blots were incubated with a horseradish peroxidase-conjugated anti-rat IgG (GE healthcare) at 1:10,000 dilution or anti-rabbit IgG (GE healthcare) at 1:20,000 dilution at 25°C for 1 h. Detection was performed with Chemi-Lumi One Super (Nacalai Tesque) and images were analyzed with Lumivision Pro HSII image analyzer (Aisin Seiki). For normalization using anti-α-tubulin antibody (Sigma), membrane was washed with TBS containing 0.05% Tween20 and first antibody was stripped with Stripping Solution (Wako Pure Chemical Industries, Osaka, Japan) at 25°C for 15 min. After washing the membrane with TBS containing 0.05% Tween20, anti-α-tubulin antibody was incubated at 1:10,000 dilution in TBS containing 0.05% Tween20, the blots were then

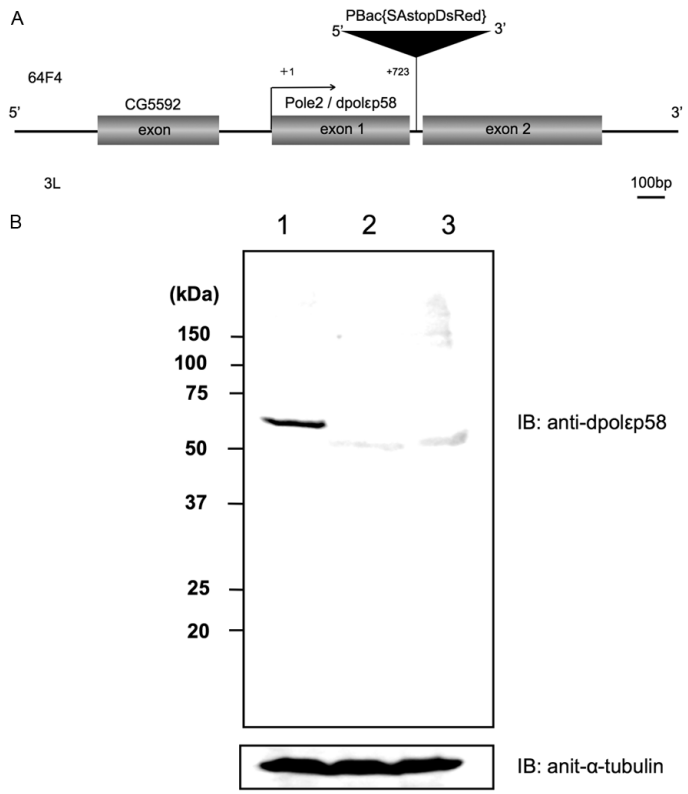

Figure 1. PBac{SAstopDsRed}LL07627 is a dpolεp58 null allele. (A) Schematic structure of the genomic region encoding Pole2/dpolε p58. The top horizontal line indicates the portion of the 64F4 region that includes *dpolεp58* gene and the *CG5592* gene. The inverted triangle indicates the insertion site of the PiggyBac transposon, PBac{SAstopDsRed}LL07627. The major transcription initiation site is designated as +1. Scale bar indicates 100 bp (B) Western blot with anti-dpolεp58 antibody and extracts from three lines: lane 1: Canton S, lane 2: *yw; +; PBac{SAstopDsRed}LL07627*, lane 3: *yw; +; PBac{SAstopDsRed}LL07627/Df(3L)Exel6107*. There is a typo on the western blot should be anti.

incubated with a horseradish peroxidase-labeled anti-mouse IgG (GE healthcare) at 1:10,000 dilution and detected as above.

Measurement of sizes of various larval tissues

Third instar larvae were dissected in PBS using a SMZ645 (Nikon) microscope, Images of samples were taken from CAMEDIA C-3030 (OLYMPUS) of the microscopy, SZX12 (OLYMPUS). The size of each tissue was measured by Image J.

Immunostaining of imaginal discs

Third instar larvae were dissected in PBS. The discs were fixed in 4% paraformaldehyde in PBS at 25°C for 20 min or 45 min on ice (only anti-cyclinE antibody) and washed with 0.3% Triton X-100 in PBS (PBST) and blocked in PBS containing 0.1% Triton X-100 and 10% goat serum at 25°C for 30 min. The samples were incubated with rabbit anti-phospho histone H3 (PH3) antibody (Cell Signaling) at 1:200 dilution at 4°C for 16 h. After extensive washing with PBST, the samples were incubated with anti-rabbit or anti-rat IgG conjugated with Alexa FluorTM 488 (Molecular Probes) at 1:400 dilution at 4°C for 16 h. DNA was stained with propidium iodide or 4',6-diamidino-2-phenylindole (DAPI). After extensive washing with PBST and PBS, samples were mounted in Floroguard Antifade Reagent (Bio-Rad Laboratories). Preparations were examined with florescence microscopy (BX-50, Olympus) equipped with cooled CCD camera (ORCA-ER, Hamamatsu Photo).

5-bromo-2'-deoxyuridine (BrdU) labeling

For labeling wing imaginal discs with BrdU, third instar larvae were dissected in PBS. The imaginal discs were resuspended in Grace's insect medium and then incubated in the presence of 75 μg/ml BrdU (Roche) at 25°C for 1 h. The samples were fixed in Carnoy's fixative (ethanol: acetic acide: chloroform, 6:1:3) at 25°C for 20 min, and further fixed in 80% ethanol/ 50 mM glycine buffer (pH2.0) at -20°C for 16 h. BrdU incorporation was visualized using the 5-bromo-2'-deoxyuridine Labelling and Detection Kit I (Roche).

Data analysis

The relative area of tissue and the relative number, size in salivary gland nuclei were quantified using Image J software and statically analyzed by Welch's t-test or student's t-test.

Results and discussion

PBac{SAstopDsRed}LL07627 is a dpolεp58 null allele

To clarify the cellular function of the Polε subunit B in a whole organism context we have analysed the consequences of depleting this

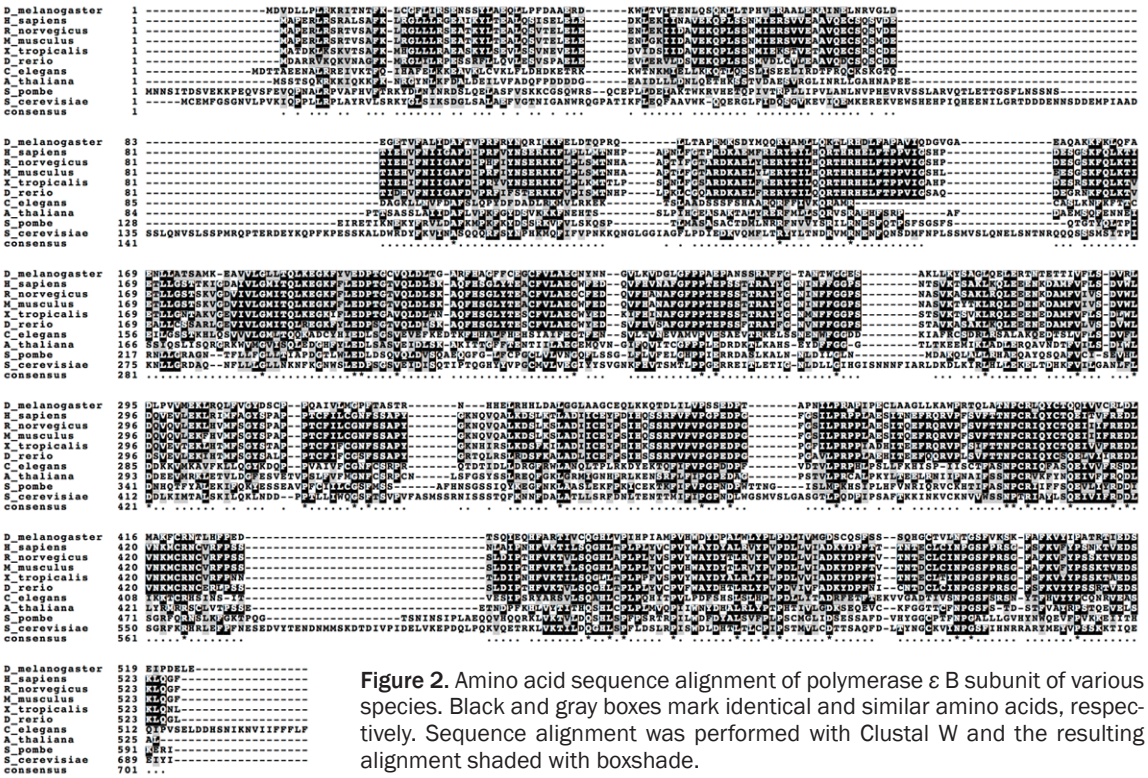

Figure 2. Amino acid sequence alignment of polymerase ε B subunit of various species. Black and gray boxes mark identical and similar amino acids, respectively. Sequence alignment was performed with Clustal W and the resulting alignment shaded with boxshade.

Table 1. Amino acid sequence comparison of *Drosophila* pol ε B subunit with other species

Species	Identity (%)	Similarity (%)
H. sapiens	42.3	74.1
R. norvegicus	41.1	73.3
M. musculus	41.1	72.4
X. tropicalis	41.3	74.6
D. rerio	38.3	71.6
C. elegans	29.4	61.1
A. thaliana	31.0	60.9
S. pomb	29.3	62.7
S. cerevisiae	25.5	55.6

subunit on the development of *Drosophila melanogaster*. Depletion of the polymerase was achieved using the PiggyBac transposon insertion line, PBac{SAstopDsRed}LL07627. In this line a transposon is inserted in the *dpolεp58* intron between exon 1 and exon 2 (**Figure 1A**). To show the level of dpolεp58 protein expression in flies we first produced rat-monoclonal antibodies against the dpolεp58 protein. To check the specificity of the antibody we performed Western blot analysis with third instar larval extracts from Canton S (**Figure 1B**, lane 1), the homozygous mutants (*yw; +; PBac{SAstopDsRed}LL07627*) (**Figure 1B**, lane 2) and the heterozygous alleles with chromosome deficiency, Df(3L)Exel6107, which lacks a genomic region including the entire *dpolεp58* gene locus (*yw; +; PBac{SAstopDsRed}LL07627/Df(3L)Exel6107*) (**Figure 1B**, lane 3). A 58 kDa band was detected with the monoclonal antibody in the third instar larval extracts from Canton S (**Figure 1B**, lane 1). This is consistent with the predicted size of the protein from the amino acid composition (525 aa, 58,759 Da). By contrast, the 58 kDa band was not detectable in the homozygous or hemizygous mutant lines (**Figure 1B**, lanes 2 and 3). Normalization using anti-α-tubulin antibody confirmed that the level of α-tubulin protein was almost the same in all examined larval extracts. These results indicate that the monoclonal antibodies specifically recognize polεp58 protein. They also show that PBac{SAstopDsRed}LL07627 is truly a *dpolεp58* null mutant and could be used for functional analysis of dpolεp58.

DNA polymerase ε p58 subunit plays a role in development of larval tissues

The polymerase ε p58 subunit is well conserved across species (**Figure 2** and **Table 1**), suggest-

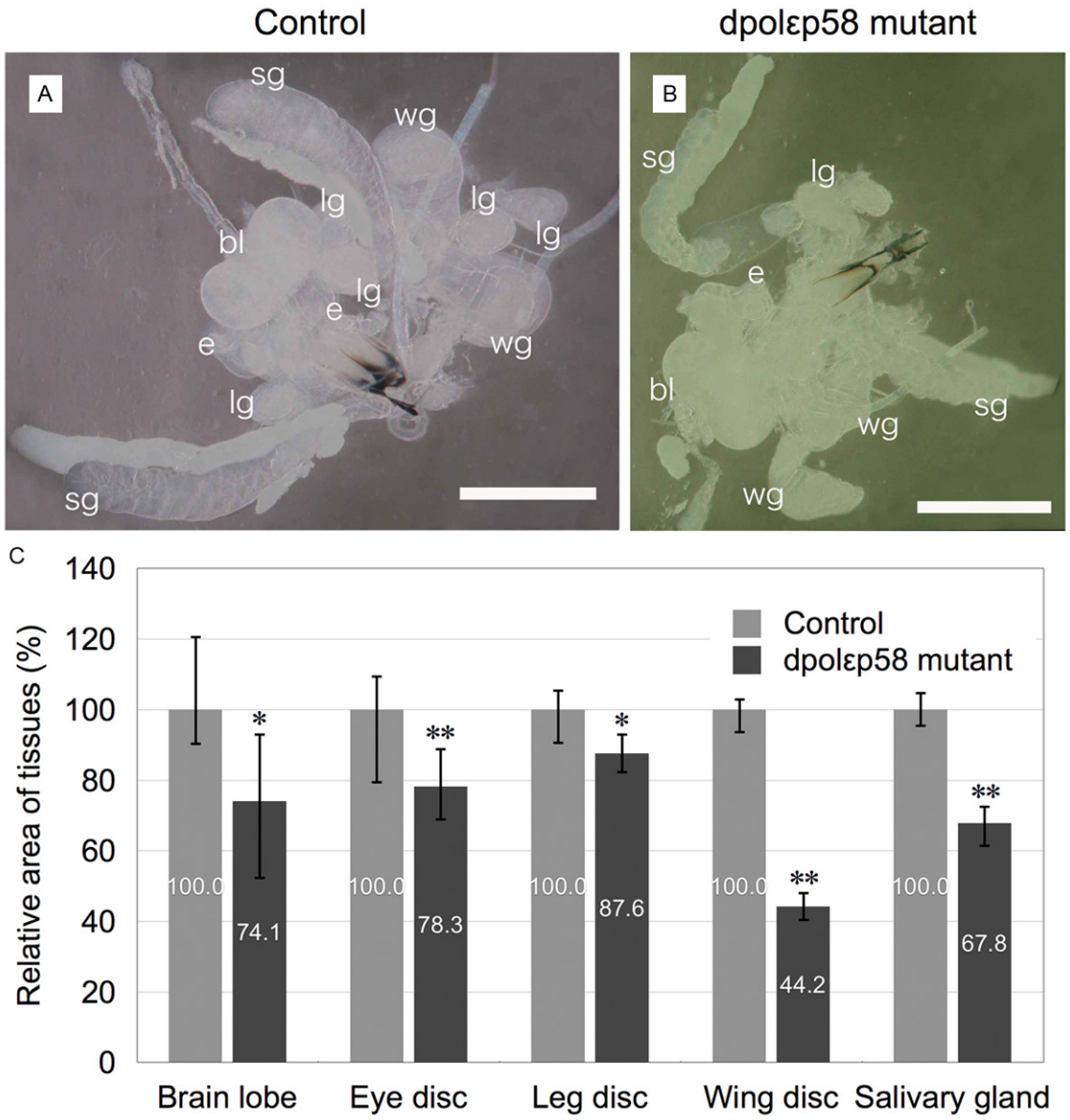

Figure 3. The DNA polymerase ε p58 subunit plays a role in development of larval brain lobes, imaginal discs and salivary glands. A and B: Tissues from third instar larvae. A: Canton S, B: *yw; +; PBac{SAstopDsRed}/LL07627*. sg; salivary gland, bl; brain lobe, e; eye disc, lg: leg disc, wg; wing disc. Scale bars show 500 μm. C: Average size of imaginal discs in wild type and dpolεp58 mutant flies. Asterisks indicate statistically significant differences between mutant and control (*P<0.05,**P<0.01). Error bars indicate standard deviations. Scale bar indicates 500 μm.

ing a vital role of dpolεp58. To gain more insight into its functional role during development we used the *dpolεp58* mutant flies, to investigate the effect of the loss of the protein on *Drosophila* development. Both homozygous *(yw; +; PBac{SAstopDsRed}LL07627*) and hemizygous (*yw; +; PBac{SAstopDsRed} LL07627/Df(3L)Exel6107*) *dpolεp58* mutant are pupal lethal, suggesting that dpolεp58 lead to defects in the normal development of *Drosophila* and is essential for viability in *Drosophila*. To further examine the phenotype of the dpolεp58 mutant, we investigated various tissue sizes in *dpolεp58* mutant flies. We compared the size of various imaginal discs, brain lobes and salivary glands in the wild type third instar larvae of the *dpolεp58* mutant (**Figure 3**). Most mutant tissues are significant-

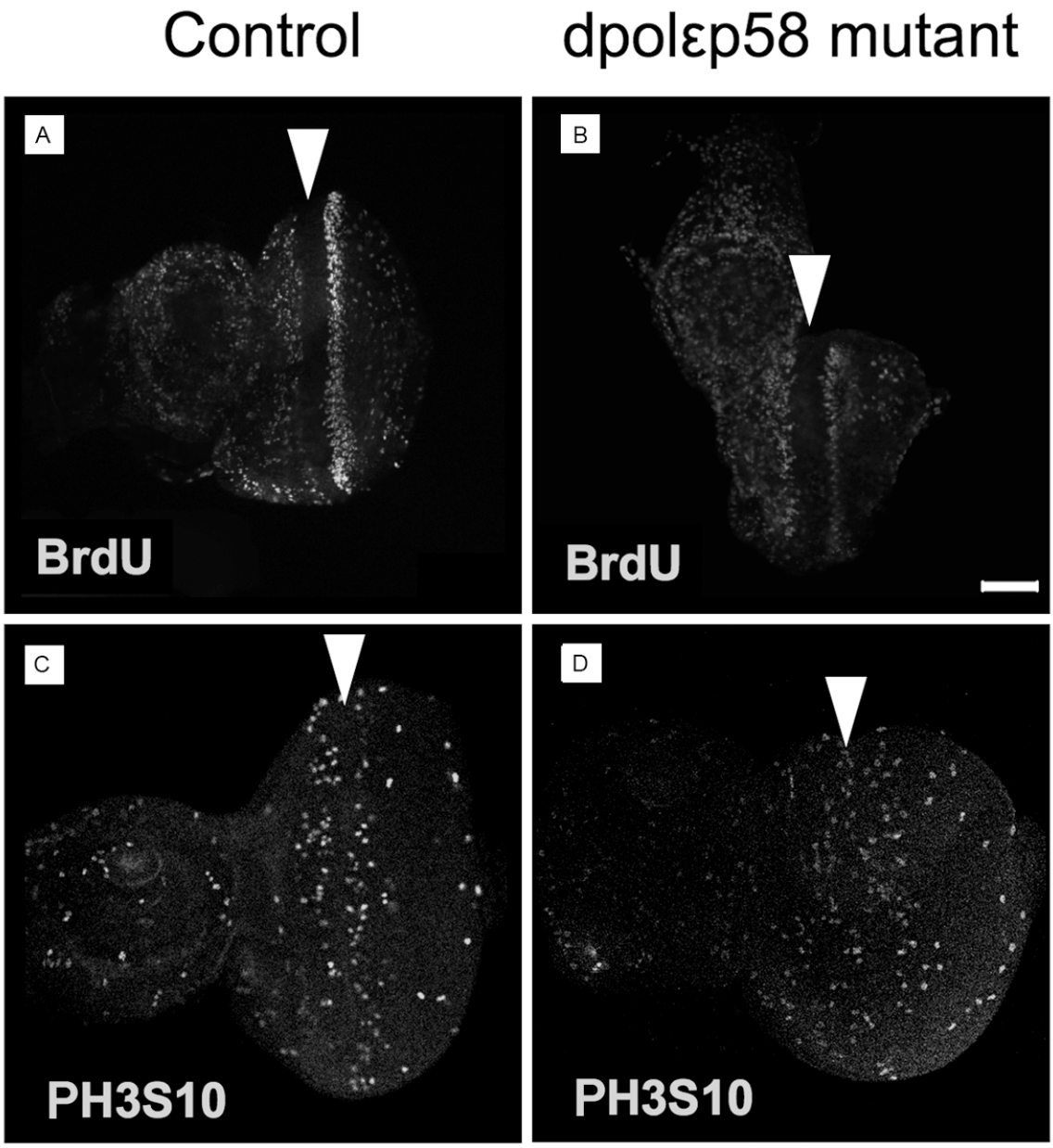

Figure 4. DNA polymerase ε p58 subunit plays an important role in progression of S phase during the G1-S-G2-M cell cycle in eye imaginal disc cells. A and C: Canton S. B and D: *yw; +; PBac{SAstopDsRed}/LL07627*. A and B: Patterns of BrdU incorporation in eye discs. C and D: Immunostaining of eye discs with anti-phosphorylated histone H3 Ser 10 (PH3S10) antibodies marking M phase cells. Arrowheads indicate the morphogenetic furrow. Scale bar indicates 100 μm.

ly smaller than those of wild type fly (**Figure 3A** and **3B**). The wing discs of dpolεp58 mutant are less than half the size of wild type discs (44.2% P>0.01). Similarly, the size of the eye discs of the dpolεp58 mutant are decreased to 78.3% (P>0.01) of those of wild type flies and size of salivary grand is also decreased (67.8%, P>0.01) (**Figure 3C**). These data indicate that dpolεp58 plays roles during development of larval tissue.

Roles of DNA polymerase ε p58 subunit in S phase progression

The observation that the *dpolεp58* mutation in flies reduced the size of various larval tissues (**Figure 3**), suggested a defect in cell cycle and proliferation. *Drosophila* provides a well-defined multi-cellular organism model with opportunities to study regulation of various types of cell cycle such as the mitotic G1-S-

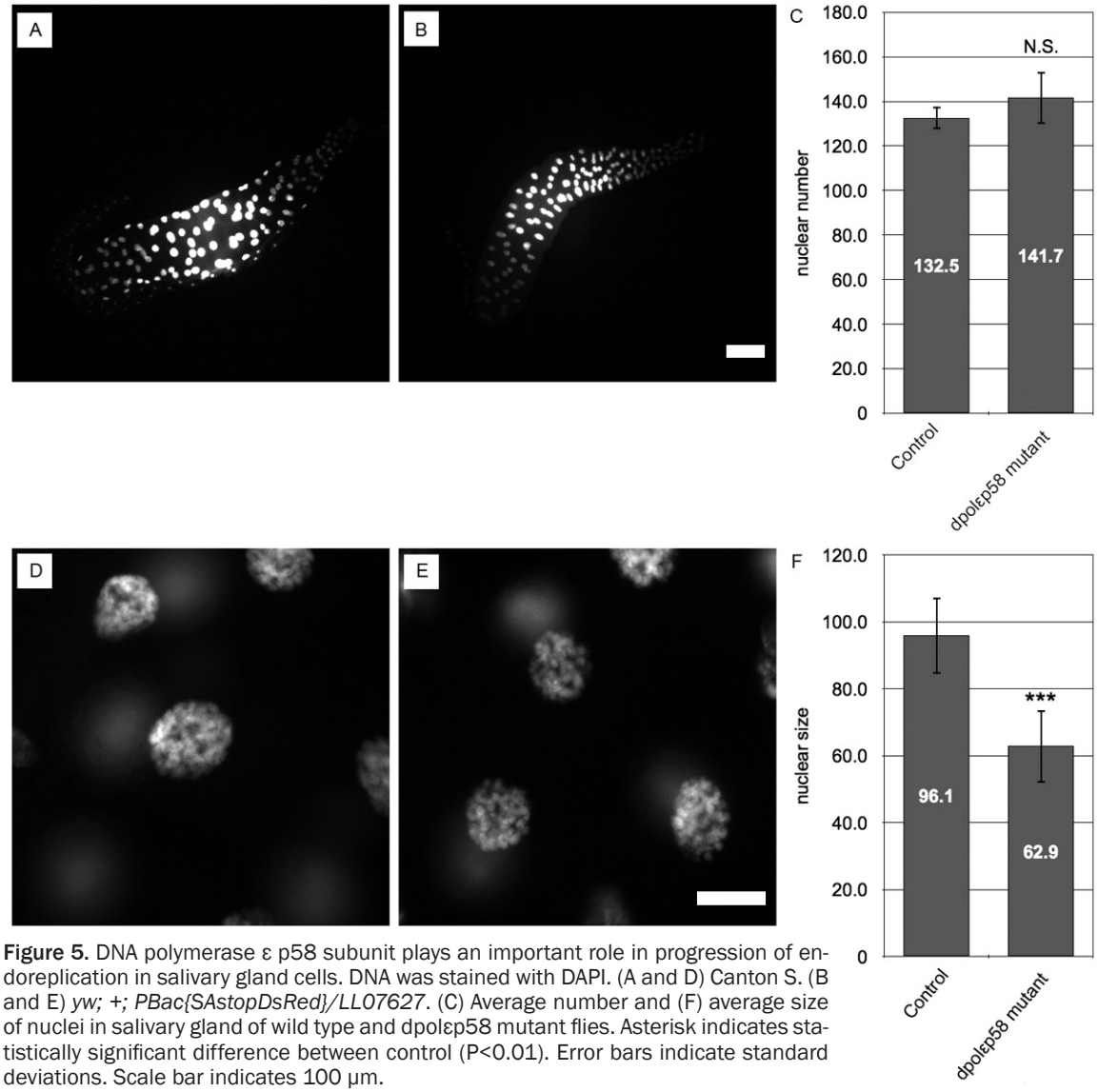

Figure 5. DNA polymerase ε p58 subunit plays an important role in progression of endoreplication in salivary gland cells. DNA was stained with DAPI. (A and D) Canton S. (B and E) *yw; +; PBac{SAstopDsRed}/LL07627*. (C) Average number and (F) average size of nuclei in salivary gland of wild type and dpolεp58 mutant flies. Asterisk indicates statistically significant difference between control (P<0.01). Error bars indicate standard deviations. Scale bar indicates 100 μm.

G2-M cell cycle in imaginal discs and the G-S endocycle in salivary gland cells. To investigate the underlying mechanism that directs the small tissue size phenotype of *dpolεp58* mutant flies, we first carried out the BrdU incorporation assays with eye imaginal discs (**Figure 4A** and **4B**). In wild type eye discs, cells divide asynchronously anterior to the morphogenetic furrow. As they enter the furrow, they are arrested in G1 phase and synchronously enter the last round of mitotic cell cycle [47]. Therefore, when eye discs of wild type flies are marked with BrdU, the cells entering S phase appear as a clear stripe posterior to furrow (**Figure 4A**). In contrast incorporation of BrdU in the S phase zone was dramatically reduced in eye imaginal discs of the *dpolεp58* mutant (**Figure 4B**). This resulted in the size of eye imaginal disc being reduced in mutant flies (**Figure 3C**). These data indicate that dpolεp58 is important for entry and/or progression though S phase during the G1-S-G2-M cell cycle in eye imaginal disc cells.

Next, we monitored Ser 10 phosphorylated histone H3 (PH3) in eye imaginal discs to detect the M phase which follows the S phase (**Figure 4C** and **4D**). PH3 staining representing the synchronized M phase zone in the wild type eye disc was also reduced in the mutant disc, but somewhat increased in the posterior region (**Figure 4C** and **4D**). These results suggest that delayed S phase induced delayed entry into M

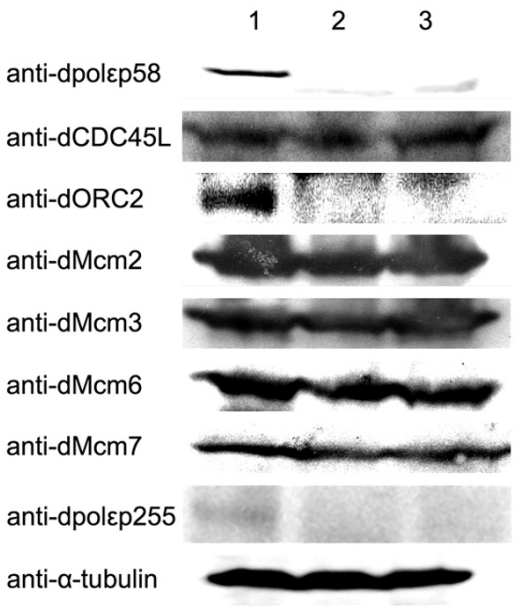

Figure 6. Level of dORC2 protein was specifically decreased in dpolεp58 mutant. Protein extracts were prepared from third instar larvae with the following flies: Canton S (lane 1), *yw; +; PBac{SAstopDSRed}LL07627/PBac{SAstopDSRed}LL07627* (lane 2), and *yw; +; PBac{SAstopDSRed}LL07627/Df(3L)Exel6107* (lane 3). They were probed with the indicated antibodies. α-tubulin is a loading control.

phase in cells in the posterior region and indicate that delayed cell proliferation causes the defect in eye imaginal disc development to induce the small eye disc phenotype in *dpolεp58* mutant flies.

DNA polymerase ε p58 subunit is essential for endoreplication in salivary glands

Salivary glands of the *dpolεp58* mutant are significantly smaller than those of wild type (**Figure 3C**). Since it is known that cells in the salivary gland proliferate by endoreplication consisting of a G-S cell cycle [48], to gain further insight into the role of *dpolεp58* during the endoreplication cycle, we stained salivary gland nuclei of wild type and dpolεp58 mutant flies with DAPI to examine their number and size (**Figure 5**). There was no significant difference in the number of nuclei between wild type and dpolεp58 mutant (**Figure 5A-C**). However, the size of nuclei was reduced by 34.5% ($P<0.01$) (**Figure 5D-F**). These data suggest that dpolεp58 plays a role in progression of endoreplication.

Similar phenotypes have been observed in knockdown flies of *dpolεp255* by the *eyeless-GAL4* driver or *Act25-GAL4* driver [37] and *dpolεp255* mutant flies [38]. Therefore our observations further confirm an important role of dpolε in both S phase entry and/or progression during mitotic cell cycle and progression of endoreplication, and suggest that this role likely requires at least two of the four polymerase epsilon subunits.

The dpolεp58 protein may interact with the dOrc2 protein

In yeast depletion of one replication protein can efficiently decrease the level of other replication proteins with which it interacts [6]. In addition it is suggested that the yeast polε plays a role not only in elongation but also in initiation of DNA replication [29, 32]. We therefore performed the Western blot analysis with third instar larval extracts from Canton S, the homozygous *dpolεp58* mutant, and the hemizygote with the deficiency chromosome which lacks a genomic region including the entire *dpolεp58* locus, to examine level of various replication factors (**Figure 6**). Levels of cdc45, MCM2, MCM3, MCM6 and MCM7 are not changed in extracts from the *dpolεp58* mutants. However, level of the band representing dORC2 was extensively decreased in the *dpolεp58* mutants. The level of dpolεp255 was decreased in the *dpolεp58* mutants as expected (**Figure 6**, lanes 2 and 3) [6, 36]. This suggested an interaction between the two proteins. To confirm this we crossed flies expressing dsRNA against *dOrc2* and *dpolε* with flies expressing the *MS1096-GAL4* driver, which drives GAL4 expression in the wing. Reduction of DNA polymerase ε p58 (*MS1096/w; +; +/UAS-dpolεp58-IR*) or Orc2 (*MS1096/w; +; +/UAS-dOrc2-IR*) alone in the wing disc induced an atrophied wing phenotype (data not shown). Double knockdown of polεp58 and dOrc2 (*MS1096/w; +; UAS-dpolεp58-IR/UAS-dOrc2-IR*) in wing disc strongly enhanced the atrophied wing phenotype (data not shown). These data indicate that *dpolεp58* genetically interacts with *dOrc2*.

The origin recognition complex (ORC), a heteromeric six-subunit protein (ORC1-ORC6), is a central component for DNA replication and highly conserved from yeast to human [49-51]. ORC binds to DNA at replication origins and serves as a scaffold to assemble other key initiation factors such as Cdc6, Cdt1, MCM complex and Cdc45 [52]. The complex, including

ORCs, regulates the initiation of DNA replication. The *Drosophila k43* gene, which encodes the ORC2 subunit, has been identified in two independent screens: a late-larval lethal mutation with small or missing imaginal discs in a lethality screen and a female-sterile allele with thin and fragile egg shells in a female sterility screen [53, 54]. It is reported that in budding yeast, interaction of *dpb2* subunit of polε with Psf1 subunit of GINS essential for assembly of the CMG helicase during the initiation of chromosome replication and also serves subsequently to integrate polε into the replisome at DNA replication forks [32]. Based on these reports and the experimental results described here, we therefore suggest that polε is involved in regulation of replication initiation by interaction with ORC complex or replisome complex in *Drosophila*.

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