CAF-1 is essential for *Drosophila* development and involved in the maintenance of epigenetic memory

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Abstract

DNA synthesis during S-phase and upon DNA repair is accompanied by chromatin assembly. The chromatin assembly factor CAF-1 has been biochemically well-characterized to deposit histones onto newly synthesized DNA. To gain insights into the *in vivo* functions of CAF-1 in *Drosophila*, we generated null mutants of the largest subunit of dCAF-1, *dCAF-1-p180*. We show that, unlike *CAF-1* mutant yeast, *dCAF-1-p180* mutant flies are hemizygous lethal. Removal of maternal *dCAF-1-p180* activity by germline clones blocks oogenesis. Tissue-specific deletion of *dCAF-1-p180* in the eye primordia disrupts eye development. In addition, reduction of *dCAF-1-p180* activity suppresses gene silencing at heterochromatin and antagonizes *Polycomb*-mediated cell fate determination. Furthermore, heterozygous *dCAF-1-p180* mutant flies display an increased sensitivity to γ-irradiation and a reduced efficiency in recombinational double strand break (DSB) repair. Our experiments also show that human *hCAF-1-p150* can rescue the *dCAF-1-p180* mutant flies, demonstrating a functional conservation of eukaryotic CAF-1 activities *in vivo*. Together, our results establish that *dCAF-1-p180* is an essential gene for *Drosophila* development and further underscore the importance of dCAF-1 in regulating gene expression and DNA repair *in vivo*.
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Keywords: Chromatin assembly; dCAF-1; Germline clone; Gene silencing; PEV effect; Heterochromatin; Genome stability; DSBs

Introduction

Chromatin, a protein–DNA complex, is the packaged form of the eukaryotic genome found inside the nucleus. The majority of chromatin is assembled immediately following DNA synthesis, a process that is mediated in part by the histone chaperone chromatin assembly factor 1 (CAF-1) (Smith and Stillman, 1989; Shibahara and Stillman, 1999). CAF-1, a heterotrimeric protein conserved throughout eukaryotes (Kaufman et al., 1995; Tyler et al., 1996, 2001; Verreault et al., 1996; Kaufman et al., 1997), facilitates chromatin assembly by depositing histones H3 and H4 to newly synthesized DNA (Smith and Stillman, 1989; Shibahara and Stillman, 1999). Consistent with its critical role in chromatin functions, CAF-1 interacts with a variety of proteins including PCNA (Moggs et al., 2000), MBD1 (Reese et al., 2003; Sarraf and Stancheva, 2004), HP1 (Murzina et al., 1999; Quivy et al., 2004), ASF1 (Mello et al., 2002; Tamburini et al., 2006), BLM (Jiao et al., 2004), WRN (Jiao et al., 2007) and Cdc7 (Gerard et al., 2006). Reduction of CAF-1 activity in tissue culture cells leads to reduced and delayed packaging of the genome into chromatin, replication defects, S-phase arrest and check point activation defects (Krude, 1999; Hoek and Stillman, 2003; Ye et al., 2003; Nabatiyan and Krude, 2004; Takami et al., 2006). Several lines of evidence also suggest roles of CAF-1 in DNA repair

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pathways: (1) CAF-1 is capable of assembling chromatin coupled with NER (nucleotide excision repair) *in vitro* (Gaillard et al., 1996), (2) CAF-1 is localized to DNA templates undergoing NER and deposits histones H3 and H4 onto the repaired DNA (Martini et al., 1998; Moggs et al., 2000; Green and Almouzni, 2003; Polo et al., 2006) and (3) very recent reports indicate that CAF-1 is involved in the repair process of double-strand DNA breaks (Linger and Tyler, 2005; Nabatiyan et al., 2006).

Previous studies of the biological functions of CAF-1 have been primarily limited to yeast and plants. Yeast cells with mutations affecting CAF-1 are viable but exhibit several defects including increased UV sensitivity (Kaufman et al., 1997), impaired gene silencing at telomeres (Monson et al., 1997) and mating loci (Enomoto and Berman, 1998), gross chromosomal rearrangements (Myung et al., 2003) and global under-assembly of their genome into chromatin (Adkins et al., 2004). In *Arabidopsis*, genes encoding all three subunits of CAF-1 are non-essential, but mutant plants exhibit many developmental defects affecting shoot apical meristems, seedling architecture, leaf size and trichome differentiation (Exner et al., 2006; Kirik et al., 2006; Endo et al., 2006; Schonrock et al., 2006). The findings that *CAF-1* genes are not essential in yeast and plants appear to be at odds with the biochemically established role of CAF-1 in packing chromatin, an activity that might have been expected to be essential for all eukaryotic cells. Indeed, Houlard and colleagues recently reported that *CAF-1* mutant mice arrest developmentally at the 16-cell stage with severe alterations in the nuclear organization of constitutive heterochromatin (Houlard et al., 2006). However, it is not known whether the findings that *CAF-1* is essential in mice are applicable to other animals.

Drosophila CAF-1 (dCAF-1) was first biochemically identified as a chromatin assembly factor about a decade ago (Bulger et al., 1995; Kamakaka et al., 1996). There are two distinct CAF-1 complexes in *Drosophila* each with three subunits of p180, p105, p55 or p180, p75, p55. p75 is a C-terminally truncated form of p105 *in vivo* (Tyler et al., 2001). While the p105-containing complex has a well-established function in nucleosome assembly, the function of the p75-containing complex remains uncharacterized (Tyler et al., 2001). dCAF-1 interacts with dASF1 through its p105 subunit (Tyler et al., 2001). The smallest subunit of dCAF-1, p55, is also found in several different protein complexes, including the NURF chromatin-remodeling complex, PRC2 and dREAM (Tie et al., 2007; Beall et al., 2007). To analyze the *in vivo* functions of dCAF-1, we generated two null alleles of the gene encoding the largest subunit of dCAF-1, *dCAF-1-p180*. We show that the mutants are hemizygous lethal and dCAF-1 activity is essential to oogenesis. Our experiments of tissue-specific deletion of *dCAF-1-p180* reveal an essential role of CAF-1 in eye development. We also show that *dCAF-1-p180* mutations suppress gene silencing at heterochromatin and *Polycomb*-mediated cell fate determination. In addition, heterozygous *dCAF-1-p180* animals had an increased sensitivity to γ-irradiation and a reduced efficiency in the homologous recombination-mediated gap repair. Together, these results establish an essential role of dCAF-1 in *Drosophila* development and reveal biological processes that are affected by *dCAF-1-p180* mutations *in vivo*.

Materials and methods

Fly stocks and genetics

Flies were cultured at 25 °C for all experiments unless noted otherwise. For generation of the *dCAF-1-p180* mutant alleles, we used the P-element insertion P{GT1}BG02681 obtained from the Bloomington stock center (BL-12864). Excision was done in both sexes using the Δ2–3 transposase. About 400 chromosomes with independent excision events were balanced with *Bin* flies (see below for more details). We identified two excisions with hemizygous lethality in males. These two alleles, designated *dCAF-1-p180[100]* and *dCAF-1-p180[270]*, were further characterized by DNA sequencing; the primers for PCR were 5′-GTGTACTTTCGCTTGCATGAGTAG-3′ and 5′-GGTGTCCGTA-TCCGCATCCATCT-3′, and the primer for DNA sequencing was 5′-GTGTAC-TTTCGCTTGCATGAGTAG-3′. Using *dCAF-1-p180* mutant stocks balanced with the *Kr-GFP* containing balancer chromosome (Casso et al., 2000), GFP-negative embryos and larvae were selected to determine the mortality at different stages of development. For generation of germline clones (GLCs), the *dCAF-1-p180* mutations were recombined with the X-chromosomal FRT insertion 9-2, balanced with *FM7c, Kr-GAL4, UAS-GFP* balancer and crossed with *ovo[D2], v[24], P{FRT(w[hs])}9-2, y[1], f[1]/Y; P{hsFLP}38* males. Offspring of this cross were given a heat shock (1 h 38 °C) at late third instar larval stage and virgin females with correct genotypes (Table 2) were crossed with wild type males.

Listed below are fly stocks used in this study and, where applicable, their sources:

1. *y w*;
2. *P{GT1}BG02681* (from Bloomington stock center);
3. *Bin* flies (*w, Bin/Y* for males and *w, Bin/w+, lethal; +/+* for females), *Bin* stands for *Binsinscy*;
4. *Df(1)JA27/FM7c, P{GAL4-Kr.C}DC1, P{UAS-GFP.S65T}DC5, sn[+]* (from Bloomington stock center);
5. *ovo[D2], v[24], P{FRT(w[hs])}9-2, y[1], f[1]/Y; P{hsFLP}38* (from Bloomington stock center);
6. *yw, f[1], Bar[36b], P{FRT(w[hs])}9-2* (from Bloomington stock center);
7. *w; Heidi/CyO* (kindly provided by François Karch);
8. *w; UAS-p35* (kindly provided by Markus Noll);
9. *S6, mus309[D2]/TM6B* (a kind gift from William Engels);
10. *w; UAS-asf1[102]* (kindly provided by François Karch);
11. *asf1[2]/TM6B* (kindly provided by François Karch);
12. *st[1], in[1], knt[ri-1], Scr[W], Pc[3]/TM3, sb* (from Bloomington stock center);
13. *w; UAS-hs-H_3-GFP* (kindly provided by Kami Ahamad);
14. *yw; UAS-dCAF-1-p180*;
15. *yw; UAS-DsRed-dCAF-1-p180*;
16. *yw; UAS-hCAF-1-p150*;
17. *yw; UAS-dBLM*;
18. *yw; pTARG-dCAF-1-p180[rescue]*;
19. *w; actin-Gal4*;
20. *w; tubulin-Gal4*;
21. *ey-Flp*;
22. *yw; ey-Gal4*;
23. *F-R5* flies (Takeuchi et al., 2007)
24. *314* (precise jump out line of P{GT1}BG02681)
25. *T(2;3)Sb[v]/In(2LR)SM1, Cy/In(3LR)TM6, Ubx* (kindly provided by Nicholas Dyson)
26. *35UZ-3* (kindly provided by Neel B. Brandsholt)

The efficiency of DSB repair was examined as described by Takeuchi et al. (2007). Females of *dCAF-1-p180[270]/FM7C; hs-I-Sce I* were crossed with *F-R5/Y*, and the offspring were given a heat shock at 38 °C for 1 h at second instar larval stage. Adult virgin females with mosaic y+ were selected and singly crossed to *yw* males. The number of crosses with y+ offspring was scored.

RT-PCR, plasmids and transgenic flies

The following primers were used in RT-PCR to quantitatively detect *dCAF-1-p180* mRNA in hemizygous and wild type embryos and larvae: forward 5′-ATGTTCAAGTGATAGATTAC-3′ and reverse 5′-TCGTAGTCGAAGAG-CACCTT-3′.

All constructs used in this study were generated according to standard procedures. To generate the *UAS-dCAF-1-p180* construct, *dCAF-1-p180* cDNA containing full-length coding sequence was cloned into *pUAST* vector at *Eco*RI/*Xho*I sites; *UAS-DsRed-dCAF-1-p180* fusion protein construct was generated as follows: *DsRed* PCR product was obtained using the primers 5′-ATAGAATTCCACCATGGCCTCCTCCGAGGACGT-3′ and 5′-ATA-GAATTCCAGGAACAGGTGGTGGCGG-3′ and cloned into *pUAST-dCAF-1-p180*. PCR product was sequenced to ensure that there is no mutation in the coding region; the *Eco*RI/*Sal*I fragment of *hCAF-1-p150* cDNA was cloned into *pUAST* to make *UAS-hCAF-1-p150* construct. *dBLM* cDNA was cloned at *Xho*I and *Xba*I sites of *pUAST* to get *UAS-dBLM*. To generate the *dCAF-1-p180* genomic rescue construct that is designated as *pTARG-dCAF-1-p180 [rescue]*, two PCR products were generated using the following primer pairs: forward 5′-AATTAGACGCGTTTCCGCGATCCAAC-3′, reverse 5′-TCCTG-CGGCCGCTGTTTTCCCCCAGCAAATC-3′ and forward 5′-ACAGCGGC-CGCAGGAAACAGGAAACAGG-3′, reverse 5′-CTCAACTAGTCTAACG-ACTGCCTTTTCTG-3′. P-element mediated germline transformation for all constructs was performed according to standard procedures.

Light and confocal microscopy

Images of larvae were captured using a Zeiss stereomicroscope stemi2000 equipped with an AxioCam MRc5 CCD camera. Images of fly eyes with different genotypes were taken under either the above Leica microscope or a Nikon stereomicroscope.

For visualization of cellular distribution of dCAF-1-p180 protein and its association with histone H3, salivary glands of the animals that carry corresponding transgene(s) were dissected, fixed and mounted in 80% glycerol and examined with an Olympus FV500 confocal microscope.

Results

The Drosophila CAF-1 is essential for development

To investigate the *in vivo* functions of dCAF-1, we generated *dCAF-1-p180 Drosophila* mutants by imprecise excision of a P-element insertion upstream of the *dCAF-1-p180* locus. Two alleles, *dCAF-1-p180²⁷⁰* and *dCAF-1-p180¹⁰⁰*, were recovered, which had a deletion of 576 bp and 1803 bp of DNA, respectively (Figs. 1A and B). Both alleles deleted the promoter and the N-terminal portions of the *dCAF-1-p180* coding region including the putative PCNA interaction domain (Moggs et al., 2000). While the mutator P-element stock was homozygous viable, neither of the *dCAF-1-p180* alleles could be maintained in a hemizygous state in males, indicating that *dCAF-1-p180* is an essential gene.

To further characterize the phenotypes of the mutant animals, we separated hemizygous from heterozygous *dCAF-1-p180* animals at the early embryonic stages using a GFP-
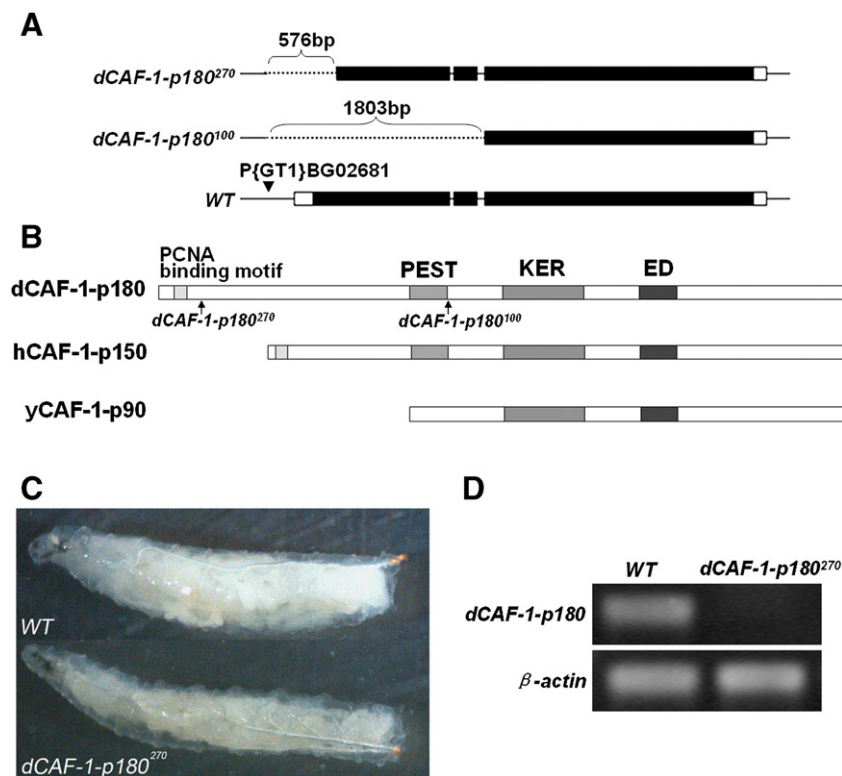

Fig. 1. *dCAF-1-p180* is essential for *Drosophila* development. (A) Schematic presentation of two *dCAF-1-p180* alleles, *dCAF-1-p180¹⁰⁰* and *dCAF-1-p180²⁷⁰*. Dotted lines represent the deletions. Filled bars indicate the coding regions of *dCAF-1-p180* gene, while open bars indicate the 5′ and 3′ UTRs. The P-element insertion site BG02681 is marked with the arrowhead. (B) Comparison of orthologs of the largest subunit of CAF-1 from humans, flies and yeast: hCAF-1-p150, dCAF-1-p180 and yCAF-1-p90, respectively. Conserved domains of PCNA binding motif, PEST domain, KER domain and ED domain are indicated above. 3′ break points of the deletions of *dCAF-1-p180²⁷⁰* and *dCAF-1-p180¹⁰⁰* that delete parts of the coding region are marked by arrows. (C) Third instar larvae of wild type and *dCAF-1-p180²⁷⁰* mutant. (D) RT-PCR to detect *dCAF-1-p180* mRNA in total RNA preparations from wild type and *dCAF-1-p180¹⁰⁰* mutant embryos. *β-actin* is loading control.

Table 1
Statistics of survival ratio

Developmental stages	Survival rates	
	dCAF-1-p180^{270}/FM7C and FM7C	dCAF-1-p180^{270}/Y
Embryonic stage	464 (75%)	157 (25%)
1st instar stage	419 (78%)	116 (22%)
2nd instar stage	396 (83%)	80 (17%)
3rd instar stage	377 (97%)	11 (3%)
Adult stage	301 (100%)	0 (0%)

dCAF-1-p180^{270}/FM7C, Kr-GFP virgins were crossed to FM7C, Kr-GFP/Y males. GFP positive and negative embryos were separated at the embryonic stage for scoring and further development. The number of animals scored at the indicated stages is given. The percentage in the parenthesis represents the percentage of all animals at the indicated stage.

marked balancer chromosome. Death of dCAF-1-p180 hemizygous males occurred progressively over the three larval instars, but predominantly during the second instar (Table 1). Generally, dCAF-1-p180 mutant larvae were developmentally arrested 1–2 days prior to their death. Rarely, a few escapers could develop into third instar larvae where they persisted up to the 18th day of life. Such arrested larvae appeared normal but remained smaller than the wild type and displayed a smaller fat body (Fig. 1C).

Two sets of experiments further indicate that the observed defects were caused by mutations in dCAF-1-p180. First, all defects can be completely rescued by either a genomic fragment of dCAF-1-p180 or by a GAL4-UAS-mediated dCAF-1-p180 expression (see below for further details). Second, Fig. 1D shows that in dCAF-1-p180 mutant embryos the dCAF-1-p180 mRNA is undetectable by RT-PCR, indicating that the mutations, which delete the gene promoter and portions of its coding sequence, abolish dCAF-1-p180 transcription.

In comparison to the mouse, where developmental arrest occurs at a very early embryonic stage in mCAF-1-p150 mutants (Houlard et al., 2006), the completion of embryonic development and considerable larval development in mutant Drosophila stands as a contrast. Drosophila embryos are known to contain highly active chromatin assembly factors that are deposited during oogenesis into the egg (Varga-Weisz and Becker, 1998). To study the role of maternal dCAF-1 activity in development, we eliminated maternal contribution by generating dCAF-1-p180 germline clones (GLCs) in females. Such females, when crossed to wild type males,

Table 2
Statistics of germline clone analysis

Maternal genotypes	Number of mothers tested	Number of mothers with eggs
dCAF-1-p180^{270}, P{FRT(w[hs])}9-2/ovo^{D2}, P{FRT(w[hs])}9-2	48	0
FM7C/ovo^{D2}, P{FRT(w[hs])}9-2	47	0
P{FRT(w[hs])}9-2/ovo^{D2}, P{FRT(w[hs])}9-2	50	32

yielded no eggs (Table 2), suggesting that dCAF-1 is essential for oogenesis. Analysis of the ovaries of females with GLCs revealed that oocytes lacking maternal dCAF-1-p180 contribution failed to develop beyond stage 10 of oogenesis (Supplementary Fig. 1). Furthermore, the observation that the dCAF-1-p180^{270}, P{FRT(w[hs])}9-2 flies rescued by a genomic copy of dCAF-1-p180 are fertile indicates that the failure of normal oogenesis for dCAF-1-p180 GLCs is caused by the dCAF-1-p180 mutation.

Tissue-specific deletion of dCAF-1-p180 disrupts eye development

To further study the role of dCAF-1 during development, we established a tissue-specific knockout analysis. We generated a transgenic line, pTARG-dCAF-1-p180, that harbors a dCAF-1-p180 genomic segment (containing 5 kb of upstream regulatory elements and the entire coding region of dCAF-1-p180) flanked by two FRTs (see Materials and methods for details). This transgene can completely rescue the mutant animals to adulthood without any visible defects (Fig. 2B). Taking advantage of such rescued flies, we used ey-FLP to specifically delete the rescuing genomic segment at the onset of eye–antenna primordial development. Figs. 2C and D show that tissue-specific knockout of dCAF-1-p180 causes severe developmental defects in the eye without affecting other aspects of development. While some flies lacked eyes completely, others exhibited extremely small eyes with misorganized ommatidia (Figs. 2C and D). These results, together with the demonstrated role of dCAF-1 in oogenesis, further show that dCAF-1-p180 is an essential gene for development in Drosophila.
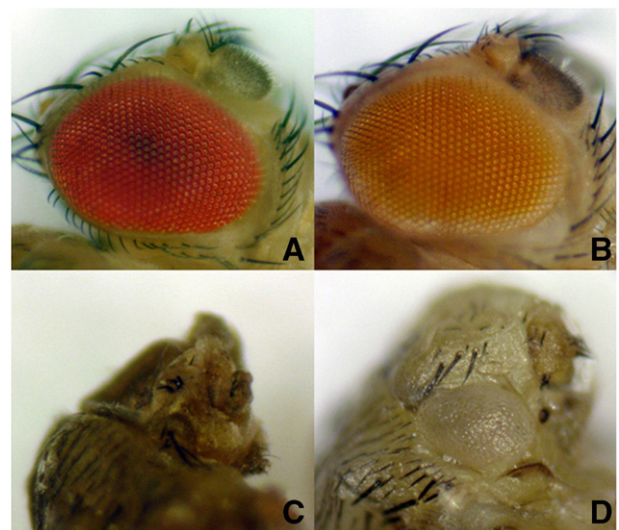

Fig. 2. dCAF-1-p180 is essential for the eye development. The dCAF-1-p180 mutant flies that were rescued by the pTARG-dCAF-1-p180[rescue] transgene were crossed to ey-FLP to specifically delete dCAF-1-p180 in the eye (see text for further details). Such tissue-specific deletion of dCAF-1-p180 caused eyeless or small-eye flies (C and D, miniwhite gene flipped out). (A) Wild type eye, endogenous w$^+$. (B) Rescued fly eye, miniwhite gene. Anterior up and dorsal left.

dCAF-1 is involved in heterochromatin-induced gene silencing and affects Pc-mediated cell fate determination

In yeast, it has been shown that *yCAF-1* (*Cac1, Cac2, Cac3*) mutants exhibit loss of gene silencing at the mating foci and telomeric regions (Zhang et al., 2000). In humans CAF-1 is associated with heterochromatin organizer HP1 (Murzina et al., 1999). Furthermore, another histone chaperone in *Drosophila*, dASF1, regulates position effect variegation (PEV) (Moshkin et al., 2002). These findings led us to hypothesize that *dCAF-1-p180* mutation may also affect gene expression in *Drosophila*. To test this hypothesis, we crossed *dCAF-1-p180* mutations into one of the well established PEV models, the *Heidi* flies, which harbor a chromosomal rearrangement that juxtaposes the *miniwhite* reporter gene next to the centromeric heterochromatin on the second chromosome (Seum et al., 2000). Therefore, heterozygous *Heidi* flies show random inactivation of the *miniwhite* reporter gene, exhibiting mosaic eyes (Fig. 3A,a). When one copy of

either *dCAF-1-p180* mutation was crossed into these flies, the *miniwhite* reporter gene was expressed in all cells producing almost homogeneously red-eyed flies (Figs. 3A,d and f). In control experiments of crossing *Heidi* flies to either *yw* flies or a precise jump outline (314) from our jump out experiment in which *dCAF-1-p180^{270}* and *dCAF-1-p180^{100}* were recovered, we did not detect any suppression of the PEV effect (Figs. 3A,b and Supplementary Fig. 2B). In addition to *Heidi*, we also tested the effect of *dCAF-1-p180* mutation on another PEV model. *T(2;3)Sb^v* (hereafter referred to *Sb^v*) translocation juxtaposes the dominant *Sb* mutation (causing stubble bristles) and the centric heterochromatin of the second chromosome, resulting in mosaic flies with stubble and normal bristles (Di Stefano et al., 2007; Supplementary Fig. 3B). When *Sb^v* was crossed to *dCAF-1-p180^{270}*, we observed a significant decrease in the frequency of normal bristles (Supplementary Table 1). This decrease was rescued partially by introducing one copy of the *pTARG-dCAF-1-p180[rescue]* transgene (Supplementary Table 1). Together, these results
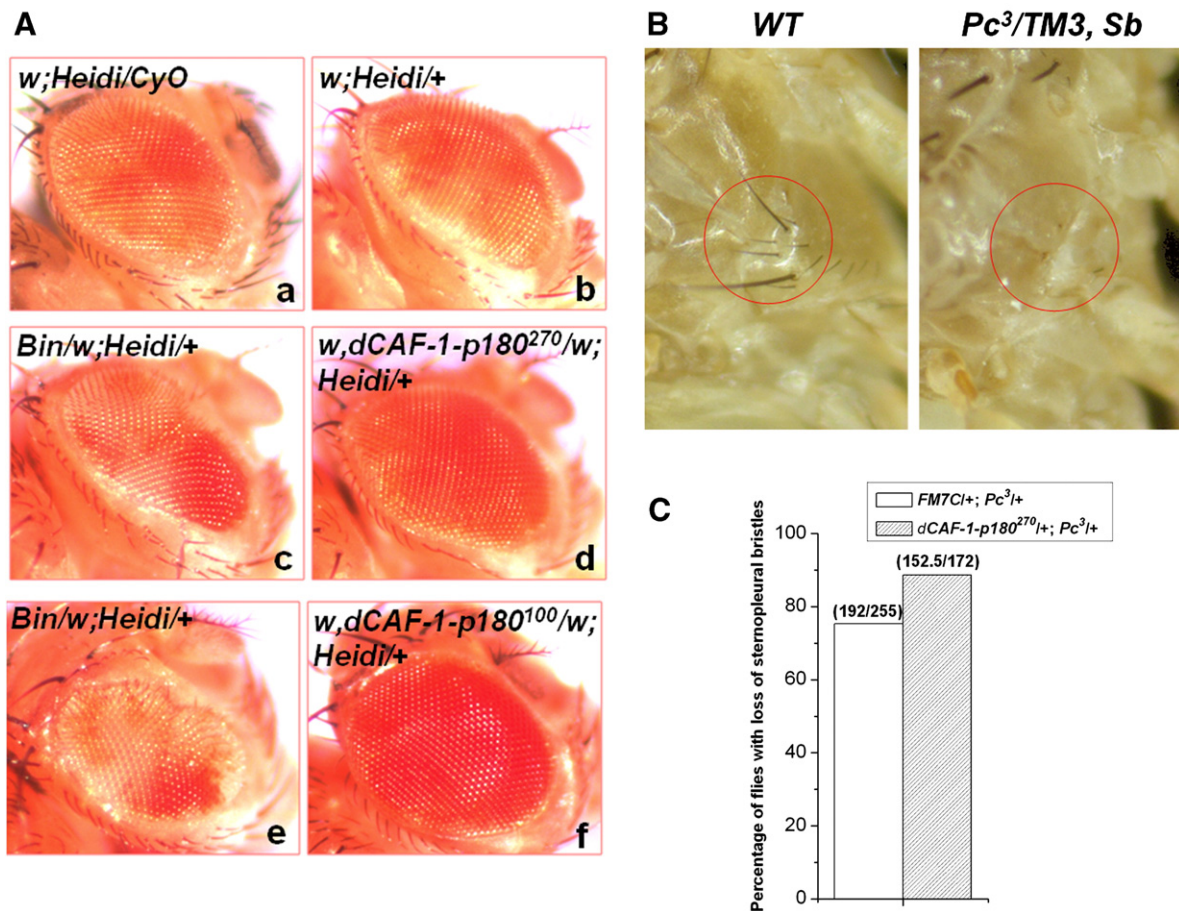

Fig. 3. *dCAF-1-p180* is involved in heterochromatin-mediated gene silencing. (A) *dCAF-1-p180* mutations as a suppressor of position effect variegation. Heterozygous mutation of *dCAF-1-p180* suppresses the *Heidi* mosaic eye phenotype caused by position effect variegation (see text for more details). All eyes are from females. (a). *w; Heidi/CyO* stock, (b) offspring of the cross: *y, w × w; Heidi/CyO*. (c and d) Offspring from the cross: *w, dCAF-1-p180^{270}/FM7C × w; Heidi/CyO*. Panel c is endogenous control for panel d. (e and f) Offspring from the cross: *w, dCAF-1-p180^{100}/FM7C × w; Heidi/CyO*. Panel e is endogenous control for panel f. (B and C) *dCAF-1-p180* mutation modulates the loss-of-sternopleural-bristle phenotype of heterozygous *Pc* mutant. Loss of sternopleural bristles in *Pc* mutants represents a transformation of thoracic segment T2 to T1 (B). Heterozygous *dCAF-1-p180* mutation enhances the loss-of-sternopleural-bristle phenotype (C). Loss-of-sternopleural-bristle phenotype on one side or both sides of a fly was scored as 0.5 and 1, respectively. The numbers in the parenthesis on top of each column are flies with loss-of-sternopleural-bristle/total number of flies counted.

show that *dCAF-1-p180* mutations can affect PEV presumably by reorganizing the heterochromatin–euchromatin boundary.

It is known that *Polycomb* group genes repress gene expression to maintain cell identity via sustaining particular chromatin states (Sparmann and van Lohuizen, 2006; Schwartz and Pirrotta, 2007). We conducted experiments to determine whether *dCAF-1-p180* mutations could influence *Pc*-mediated cell identity determination. The development of sternopleural bristles on ventral T2 segment is dependent on proper *Pc* function. One of the characteristics of *Pc* heterozygous mutants is the loss of sternopleural bristles due to the transformation of T2 segment to T1 segment in the absence of proper *Pc* function (Furuyama et al., 2004; Fig. 3B, right panel). In such flies, when one copy of the $dCAF\text{-}1\text{-}p180^{270}$ mutation was introduced, the loss-of-sternopleural-bristle phenotype was enhanced (Fig. 3C). Similarly, another phenotype of *Pc* mutation, namely the wing-to-haltere partial transformation due to *Ubx* de-repression (Smolik-Utlaut, 1990), was also enhanced by one copy of *dCAF-1-p180* mutation (Supplementary Table 2 and Supplementary Fig. 4). These data suggest that *dCAF-1-p180* can synergistically interact with *Pc* in maintaining special chromatin states and epigenetic memory to control specific cell fate determination.

To visualize the cellular localization of dCAF-1-p180, we used a DsRed tag to make a fusion protein that was sufficient to rescue the mutant phenotypes. With this fluorescent protein, we could observe the *in vivo* distribution of dCAF-1-p180. Consistent with its chromatin-related functions, dCAF-1-p180 is localized to the nucleus of *Drosophila* cells (Fig. 4, upper panels), a finding similar to what has been reported in human cells (Krude, 1995). Our results also showed that dCAF-1-p180 is not evenly distributed in the nucleus but, rather, compart-

Table 3
Statistics of γ-irradiation sensitivity

Dose (Gy)	Survival ratio of offspring with different genotypes $dCAF\text{-}1\text{-}p180^{270}/+:+/+$
0	1.01 (579/572)
20	0.87 (398/458)
30	0.76 (278/366)
40	0.70 (191/273)

Females of $dCAF\text{-}1\text{-}p180^{270}/FM7C$, *Kr-GFP* were crossed to *Y/+* males. $dCAF\text{-}1\text{-}p180^{270}/+$ offspring could be readily identified at late third instar larval stage according to the GFP marker ($dCAF\text{-}1\text{-}p180^{270}/Y$ animals would not survive to this stage). Six hundred larvae with the genotype of $dCAF\text{-}1\text{-}p180^{270}/+$ or $+/+$ (wild type for *dCAF-1* regardless of sex) were collected and irradiated with the indicated dose. Surviving adults (numbers given in parenthesis) were counted 2 days after eclosion and survival ratio calculated.

mentalized forming punctuates. More interestingly, its localization overlaps, at least partially, with histone H3 that is tagged with GFP (Fig. 4, lower panels).

dCAF-1 is important for maintaining genome integrity

Previous work in mammalian cells and yeast from us and other laboratories has suggested that CAF-1 may be involved in DNA repair (Green and Almouzni, 2003; Jiao et al., 2004, 2007; Linger and Tyler, 2005). Here, we performed experiments to directly determine whether *dCAF-1-p180* mutants exhibit DNA repair defects. First, our experiments showed that heterozygous *dCAF-1-p180* mutants are more sensitive to γ-irradiation that is known to lead to double strand DNA breaks (DSBs) (Table 3). Second, we analyzed the role of *dCAF-1-p180* in the homologous recombinational gap repair. We introduced DSBs in a P-element that carries an incomplete *yellow* gene in a wild type or heterozygous *dCAF-1-p180* mutant background. Upon homologous recombination with the endogenous *yellow* locus, a complete and functional *yellow* gene is frequently restored in the transgene in the wild type background (Fig. 5A; Takeuchi et al., 2007). However, in the presence of one copy of a *dCAF-1-p180* mutation, the reconstitution of the *yellow* gene occurred at a significantly lower rate, indicating that the efficiency of homologous recombinational gap repair is reduced, though not completely abrogated (Fig. 5B). Together, these results suggest that dCAF-1 is involved in the process of DSB repair in *Drosophila*.

Ectopic expression of dCAF-1 causes developmental defects

Our results described thus far indicate that reduction of *dCAF-1-p180* activity can affect its normal functions *in vivo*. Our experiments reported in this section further revealed that ectopic expression of dCAF-1-p180 could also cause deleterious effects during development. In particular, when *ey-GAL4* was used to drive eye-specific expression of the *UAS-dCAF-1-p180* transgene, a small-eye phenotype was obtained (Fig. 6A). One additional copy of either *ey-GAL4* or *UAS-dCAF-1-p180* resulted in even smaller eyes, indicating that this phenotype is dependent on *dCAF-1-p180* dose. To determine whether the
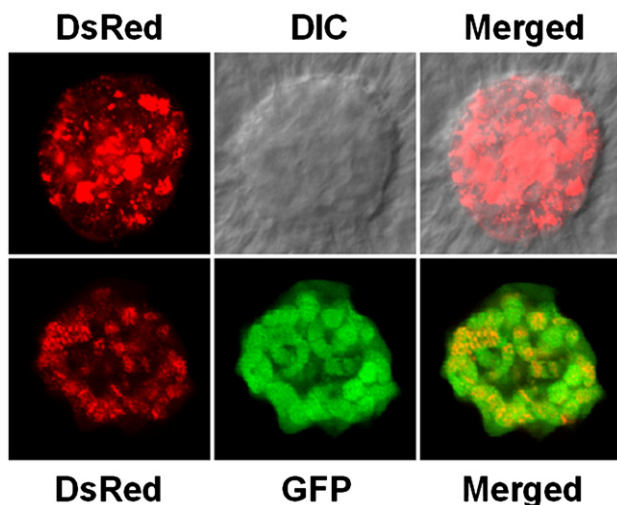

Fig. 4. Cellular distribution of dCAF-1-p180 protein and its co-localization with histone H3. The upper panels show the localization of dCAF-1-p180 protein, which is fused with DsRed tag, in the nucleus of a salivary gland cell. The lower panels show the co-localization of dCAF-1-p180 protein (red) and histone H3 (green) that is tagged by GFP. DIC, differential interference contrast.
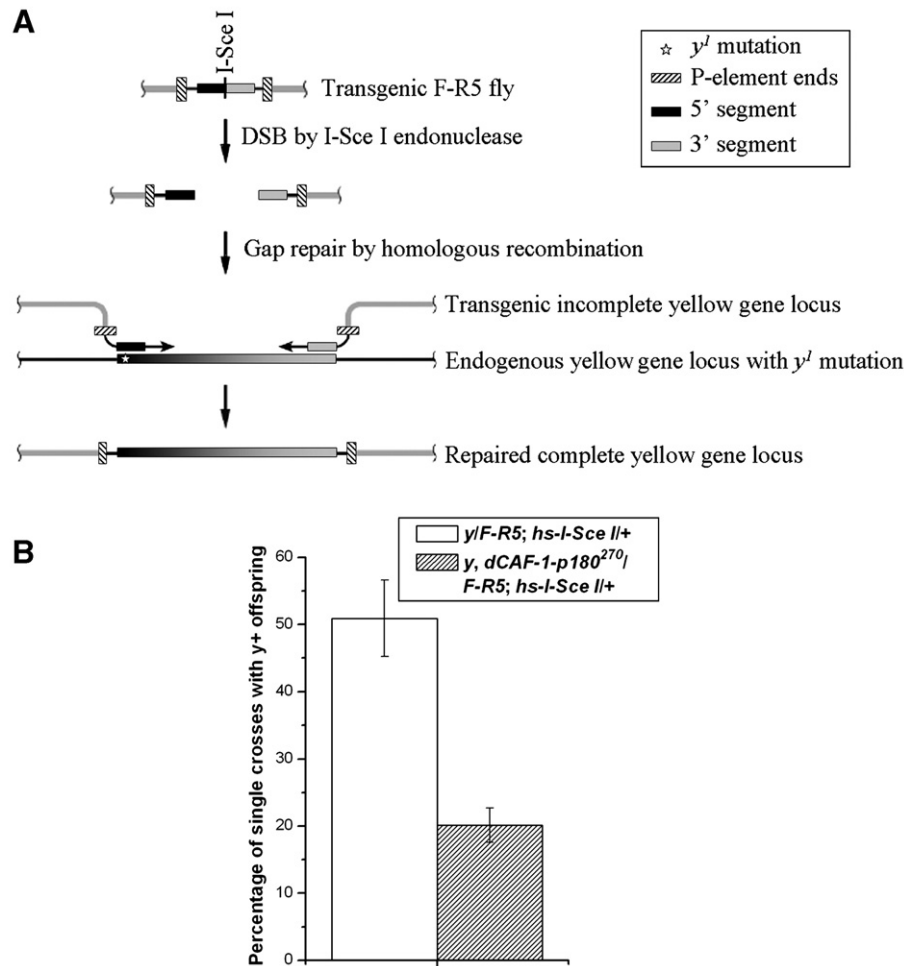
Fig. 5. *dCAF-1-p180* is involved in the homologous recombination-mediated gap repair. (A) Schematic illustration of the *in vivo* gap repair system. F-R5 incomplete *yellow* transgene is cut by the I-Sce I endonuclease to generate double strand breaks and repaired via homologous recombination with the endogenous *yellow* locus. (B) Repairing events that occurred in the germ cells were scored. White bar, wild type background. Shaded bar, *dCAF-1-p180* heterozygous background. Each experiment was conducted in triplet groups, with each group containing 70 or more single crosses between virgin females carrying mosaic somatic repair y^+ clones and *yw* males. In wild type background, the repair efficiency was 31/70, 41/72 and 37/72 for three independent groups, and in *dCAF-1-p180* heterozygous background, the efficiency was decreased to 12/72, 17/76 and 15/70. The numbers represent tubes with y^+ offspring/total tubes of single crosses.

small-eye phenotype might be caused by cell death through apoptosis, we introduced into such flies a *UAS-p35* transgene that expresses the anti-apoptotic protein p35. Our results revealed a suppression of the small-eye phenotype by p35 in these flies (Fig. 6B), suggesting that ectopic expression of dCAF-1-p180 in the eye may lead to, at least in part, apoptosis during development.

Previous studies have shown that CAF-1 can interact with BLM and ASF1 in humans. To determine whether and how the genes encoding the *Drosophila* counterparts interact genetically with *dCAF-1-p180*, we expressed them in the eye under the UAS control with the *ey-GAL4* driver along side with *UAS-dCAF-1-p180*. Simultaneous overexpression of either dBLM or dASF1 suppressed the small-eye phenotype caused by ectopic expression of *dCAF-1-p180* (Figs. 6C and D). In addition, the *dCAF-1-p180*-induced small-eye phenotype was further enhanced by a copy of mutation in either *dBLM* or *dASF1* (Figs. 6C and D). These results suggest that normal eye development requires a proper

concentration balance between dCAF-1 and its interacting proteins.

dCAF-1-p180 defects can be rescued by its human counterpart

The chromatin assembly activity of CAF-1 is highly conserved from yeast to humans, and the proteins of hCAF-1-p150, dCAF-1-p180 and yCAF-1-p90 share high degrees of homology at the amino acid level (Fig. 1B). To determine whether the human ortholog of *dCAF-1-p180* could rescue the *dCAF-1-p180* mutant defects, we generated transgenic flies of *UAS-hCAF-1-p150*. Our results showed that the transgenes *UAS-hCAF-1-p150* could rescue *dCAF-1-p180* mutant in combination with ubiquitously expressed GAL4 drivers. However, the rescue efficiency of *UAS-hCAF-1-p150* was about 40% of that of *UAS-dCAF-1-p180*. In addition, *UAS-hCAF-1-p150* transgene alone (all five lines tested) without any GAL4 drivers exhibited no rescuing capacity while two of the eight *UAS-dCAF-1-p180* transgenic lines tested were able to
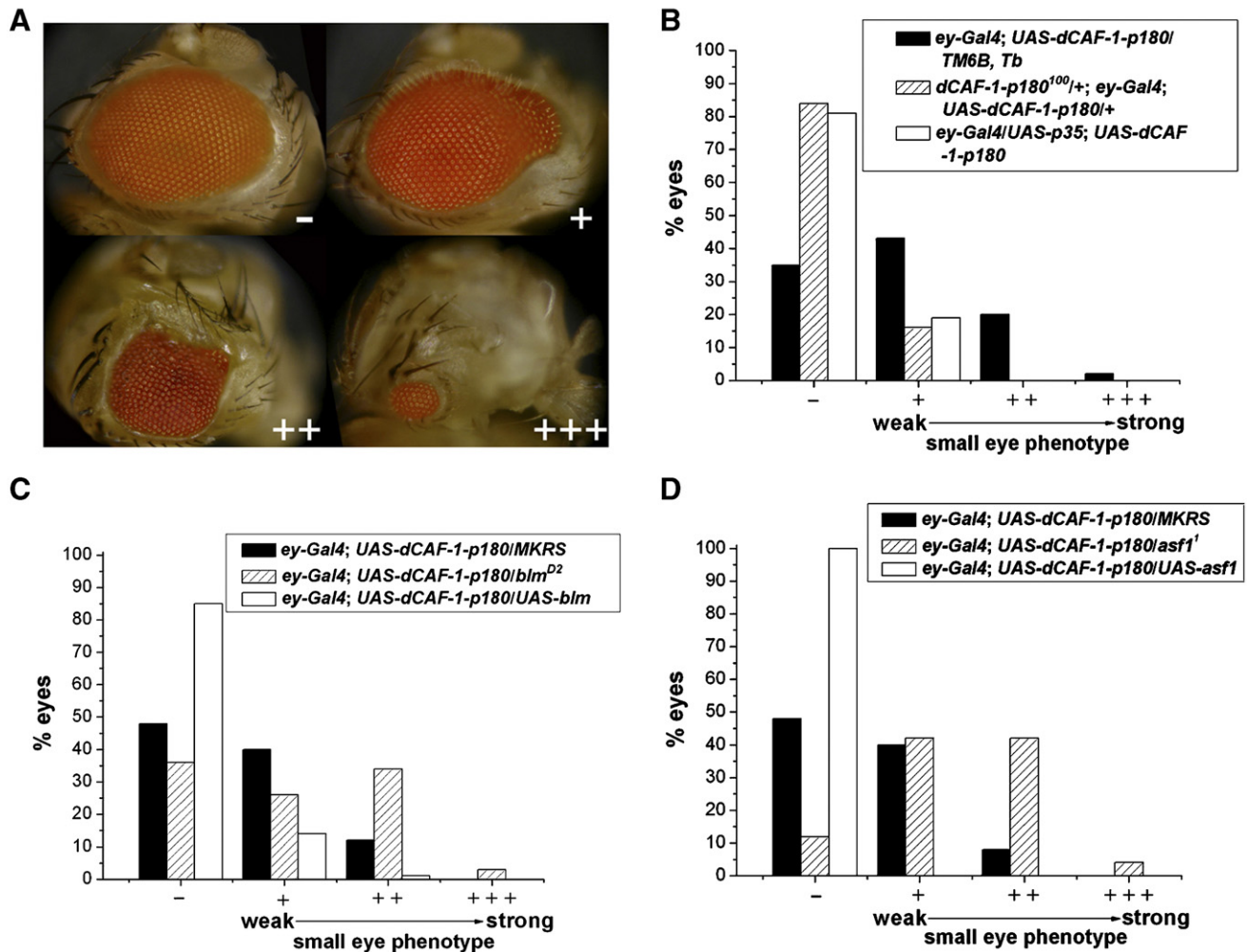
Fig. 6. Ectopic expression of *dCAF-1-p180* in the *Drosophila* eyes causes developmental defects. (A) Representatives of different categories of the small-eye phenotype ranging from no defect (−) to increasing severities of small eyes (indicated by increasing number of +'s). (B) Ectopic expression of *dCAF-1-p180*-induced small-eye phenotype, which can be partially suppressed by mutating an endogenous copy of *dCAF-1-p180* or by simultaneously expressing the anti-apoptotic protein p35. (C) Modulations of the small-eye phenotype by either simultaneous overexpression of *dBLM* or a mutation of the endogenous *dBLM* gene. (D) Modulations of the small-eye phenotype by either simultaneous overexpression of *dASF1* or a mutation of the endogenous *dASF1* gene.

rescue the mutants at a low efficiency of about 2–3% without a GAL4 driver.

Discussion

Despite extensive previous studies of the histone chaperone CAF-1 *in vitro*, its biological functions during animal development have been poorly documented. In this report, we generated and characterized null mutants of the largest subunit of *Drosophila* CAF-1, *dCAF-1-p180*. Our results show that *dCAF-1-p180* is an essential gene: (1) mutant flies are hemizygous lethal, (2) removal of maternal *dCAF-1* activity blocks oogenesis and (3) tissue-specific deletion of *dCAF-1-p180* in the eye primordia disrupts eye development. It is generally thought that CAF-1 might be crucial for DNA replication because *in vitro* studies demonstrate that CAF-1 couples DNA synthesis and chromatin assembly. Indeed, although both yeast and plant mutants for CAF-1 are viable (Kaufman et al., 1997; Exner et al., 2006), cell proliferation defects have been reported (Hoek and Stillman,

2003). In mouse ES cells, *mCAF-1-p150* knock-down has been shown recently to lead to massive heterochromatin reorganization and eventual cell arrest and death and cell division could proceed only two rounds after maternal mCAF-1-p150 becomes undetectable (Houlard et al., 2006). Mouse *mCAF-1-p150* mutant embryos die at 16-cell stage (Houlard et al., 2006). In addition to cell proliferation, CAF-1 has also been shown to play a critical role in differentiation during plant development (Exner et al., 2006). Our finding that *dCAF-1-p180* is an essential gene in *Drosophila* further underscores the importance of CAF-1 in animal development. We propose that dCAF-1 controls development through its activities in both promoting cell proliferation and regulating proper expression of genes that control differentiation.

In *Drosophila*, several groups of genes have been shown to play a role in position effect variegation (PEV), including PCNA, a DNA replication clamp involved in DNA metabolism; Su(var)3−9, a histone methyl transferase important for heterochromatin induced silencing; and dASF1, a histone chaperone

playing a role in chromatin assembly and transcription. In this report, we provide evidence suggesting that *dCAF-1-p180* also plays an important role in chromatin-mediated regulation of gene expression. First, *dCAF-1-p180* heterozygous mutation suppresses the *Heidi* PEV phenotype. In addition, *dCAF-1-p180* interacts synergistically with *Polycomb* to specify sternopleural bristle development. These results suggest that a reduction of dCAF-1 activity can affect gene expression by shifting the balance of chromatin states in favor of a less repressive one.

Reports from yeast and human cells have suggested that CAF-1 is involved in NER and DSB DNA repair via non-homologous end joining (Kaufman et al., 1997; Green and Almouzni, 2003; Linger and Tyler, 2005; Polo and Almouzni, 2006; Jiao et al., 2007). Very recent work in plants (Kirik et al., 2006; Endo et al., 2006) also indicates that CAF-1 is important for maintaining genome stability by suppressing illegitimate homologous recombination. We show here that dCAF-1-p180 dose alteration affects directly the efficiency of homologous recombinational gap repair *in vivo*. However, the precise mechanism through which dCAF-1 participates in this DNA repair to maintain genome integrity remains to be elucidated. It is possible that CAF-1 activity may play a role in DNA synthesis during gap repair and/or homology search that enables pairing of the two alleles located at different chromosomal sites. It is also possible that CAF-1 may play a role in restoring the repaired DNA to its original chromatin state. Considering our finding that dCAF-1 affects both PEV and DSB repair, we propose that dCAF-1 is involved in establishing/maintaining epigenetic memory that is important for both gene expression and DNA repair.

One of the important findings in this report is that the dosage of dCAF-1-p180 is critical for dCAF-1 functions. Our results show that (1) null mutants are hemizygous lethal, (2) heterozygosity of *dCAF-1-p180* shows defects in DSB repair and repression of gene silencing and (3) overexpression of dCAF-1-p180 in the eye leads to inappropriate eye development. Our genetic interaction experiments based on the small-eye phenotype caused by *dCAF-1-p180* overexpression also suggest an importance of a concentration balance between dCAF-1-p180 and its interacting proteins. Finally, our preliminary experiments show that a reduction of *dCAF-1-p180* dose leads to male sterility preferentially. As mentioned in the Results section, the *UAS-dCAF-1-p180* transgene alone can rescue the *dCAF-1-p180* mutants to adulthood at a low efficiency. This is most likely due to a low-level leaky expression of *dCAF-1-p180* from the heat shock core promoter in the UAS vector, thus mimicking a hypomorphic allele of *dCAF-1-p180*. While such rescued flies did not exhibit any visible defects morphologically, all males were sterile. The underlying mechanisms of *dCAF-1-p180* in controlling the development of the male reproductive system remain to be elucidated.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.08.039.

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