Am J Cancer Res 2012;2(6):714-725
www.ajcr.us /ISSN:2156-6976/ajcr0000148

Original Article

DREF is involved in the steroidogenesis via regulation of *shadow* gene

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Received September 25, 2012; Accepted October 21, 2012; Epub November 20, 2012; Published November 30, 2012

Abstract: The *Drosophila* DNA replication-related element-binding factor (dDREF) has been identified as a master regulator of cell proliferation-related genes via its binding to the DRE sequence, 5′-TATCGATA. However, the biological roles of DREF are still to be clarified. Here, we show that DREF mutant females have steroid hormone ecdysone-deficient phenotypes, such as the loss of vitellogenic egg chambers. Furthermore, DREF knockdown in the prothoracic gland of larva prevented pupation and this was rescued via 20-hydroxyecdysone treatment. We found a DRE-like sequence (-625 to -632) in the 5′-flanking region of the *Drosophila shadow* gene, which catalyzes the conversion of 2-deoxyecdysone to ecdysone, and demonstrated that *shadow* is a novel target gene of dDREF using quantitative RT-PCR and Chip assays. In addition, we show that the level of dDREF protein correlated with age-related changes in the level of *shadow* mRNA in the ovaries of wild-type flies. Taken together, our data indicate that dDREF plays a key role in steroid synthesis via regulation of the *shadow* gene.

Keywords: *Drosophila*, DRE, DREF, transcriptional regulation, ecdysone, *shadow*, steroidogenesis

Introduction

Drosophila DNA replication-related element (DRE)-binding factor (dDREF) consist of an 80-kDa polypeptide homodimer that specifically binds to DRE sequences (5′-TATCGATA) [1]. The DRE is necessary for the promoter activity of genes such as PCNA, DNA polymerase α 180-kDa and 73-kDa subunits, raf, E2F, TBP, cyclin A, SkpA, dDREF itself, big brain, ketel, DmTTF, HP6, Mes4, p38b, p53 and warts [2-10]. The importance of dDREF in development has been reported in studies using transgenic flies [11-12]. A human homologue of DREF (hDREF) has been identified and shown to play a key role in the transcriptional regulation of human histone H1 and ribosomal protein (RP) genes via the human DRE (hDRE) sequence (5′-TGTCG(C/T)GA(C/T)A) [13-14]. Although many studies have demonstrated that the DRE/DREF system is a master regulatory mechanism for coordinated expression of many cell proliferation-related genes [2], the biological roles of DREF are still to be clarified.

Steroid hormones are known to control many aspects of development, reproduction, and homeostasis in higher organisms via regulation of proliferation and differentiation [15, 16]. It has been demonstrated that steroidogenesis in vertebrates and invertebrate have marked similarities in their catalysis process via cytochrome-p450s (CYPs), from dietary steroids to steroid hormones [17]. In *Drosophila*, steroidogenesis is activated by the neuropeptide prothoracicotropic hormone (PTTH), and 20-hydroxyecdysone (20E) is produced from sterol by the CYP family of enzymes [18]. The primary steroidogenic organs in *Drosophila* are the larval lateral ring gland, the prothoracic gland (PG), and the adult ovaries [19]. The halloween genes, which encode the CYP superfamily of enzymes including *CYP306A1* (*phantom*), *CYP302A1* (*disembodied*), and *CYP315A1*

Table 1. The number of vitellogenic egg chambers of the wild type and *Dref*^{KG09294}/+ females

	Wild-type (20day) n = 210 ovarioles	*Dref*^{KG09294} (20day) n = 197 ovarioles
Stage 7-9 (early vitellogenic)	210 (100%)	74 (37.6%)
Stage 10-13 (mid-late vitellogenic)	196 (93.3%)	31 (15.7%)
Stage 14 (mature)	67 (31.9%)	72 (36.5%)

All ovarioles contained several previtellogenic chambers.

(*shadow*), catalyze three sequential hydroxylations yielding ecdysone from dietary cholesterol [20]. Ecdysone is then converted into the principal molting hormone, 20-hydroxyecdysone (20E), by the P450 enzyme (CYP314A1; *shade*) in peripheral target tissues [21]. Steroid hormones such as 20E trigger the differentiation and morphogenesis of the imaginal discs, giveing rise to adult tissues, and the programmed cell death of larval cells [22]. Each hormone pulse leads to the induction of three early response genes, BR-C, E74, and E75, each of which encode multiple forms of related transcription factors [23-25]. Although steroid hormones play an essential role during an organism's lifespan, little is known about the regulatory mechanisms of steroidogenesis.

In this study, we show that adult females heterozygous for *Dref*^{KG09294} and larvae carrying DREF knockdown in the PG, induced hormone-deficient phenotypes and provided evidence that dDREF plays a role in the growth of the primary steroidogenic organs and in steroidogenesis via the regulation of a steroidogenesis-related gene *shadow*. Our results suggest that the conservation of DREF in human indicates a broad role for DREF proteins during steroidogenesis.

Results

DREF mutant has steroid hormone ecdysone-deficient phenotypes

Although individuals heterozygous for *Dref*^{KG09294} which having a P-element insertion in the 5′-flanking region of the dDREF gene have a normal phenotype during development [26], we observed that the ovaries of *Dref*^{KG09294}/+ females, more than 20 days old, lack vitellogenic (stage 7-13) egg chambers (**Figure 1A** and **Table 1**). We checked the fecundity of *Dref*^{KG09294}/+ females. The fecundity of *Dref*^{KG09294}/+ females decreased by 50% as compared with controls (**Figure 1B**).

In many studies, ecdysone has been known to regulate vitellogenesis and egg chamber maturation during mid-oogenesis [17, 27]. dDREF is strongly expressed in the nucleus of follicle cells [1, 28], which produce ecdysone hormone. Therefore, to determine whether the phenotypes of *Dref*^{KG09294}/+ females are associated with ecdysone hormone signaling, we checked the level of the ecdysone target genes, E74A and E74B, in *Dref*^{KG09294}/+ females. The mRNA levels of both genes in 20-day-old *Dref*^{KG09294}/+ females decreased as compared with those of 20-day-old wild-type females (**Figure 1C**). These results indicate that dDREF may be involved in an ecdysteroid hormone signaling pathway in adults.

DREF knockdown in the larval prothoracic gland affect the pupariation and the expression of ecdysone target gene

To assess whether dDREF is involved with the ecdysone signaling pathway, we first investigated the expression of dDREF in the ecdysone producing prothoracic gland (PG) of larvae using an anti-DREF antibody (mAb4). dDREF was detected in the nucleus of larval PG (**Figure 2A**). Next, to investigate the function of dDREF in the PG, we manipulated the levels of dDREF in the PG by crossing *P0206-Gal4*, a PG and corpora allata (CA) specific driver of Gal4 expression [29-30], with flies carrying UAS constructs that allowed expression of double stranded DREF RNA (*dref-IR*). The timing of embryonic and larval development in these animals was compared with those of controls (**Figure 2B**). Interestingly, animals carrying one copy of *UAS-dref-IR* and *P0206-Gal4* failed to undergo pupation (**Figure 2C**). The larvae were also as much as 210% the size of wild type at AEL 3 weeks (**Figure 2C**). At AEL 6 weeks, the larval volume decreased and melanotic tumors were detected in the gut, lymph gland, and epidermis (data not shown). These results indicate that DREF knockdown in the PG affects pupariation.

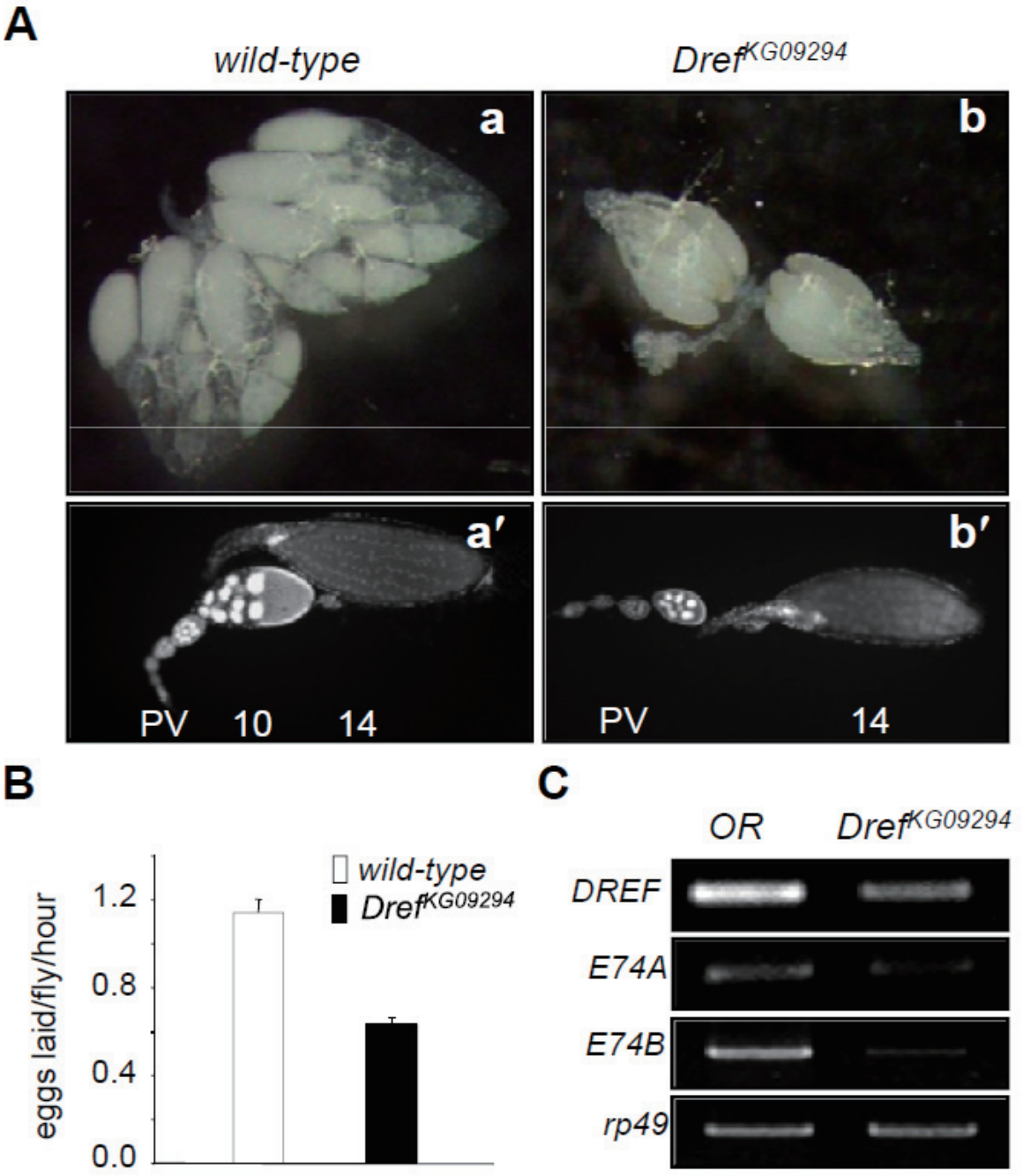

Figure 1. Ecdysone-deficient phenotypes of *Dref*^{KG09294}/+ females. A. *Dref*^{KG09294}/+ females lack vitellogenic egg chambers. Ovaries of wild-type (a, a′) or *Dref*^{KG09294}/+ (b, b′) 20-day-old females are shown. PV indicates earlier previtellogenic stage. B. Fecundity defects of *Dref*^{KG09294}/+ heterozygote females. Fecundity was expressed as the number of eggs laid/fly/hour. Twenty females of each genotype were mated to 20 wild-type (*Oregon-R*) males at 25 °C for 2 days and then egg were collected for 6 h and counted. C. Decreased expression of steroid hormone target genes in the ovaries of *Dref*^{KG09294}/+ females. Total RNA was prepared from the whole body of wild-type and *Dref*^{KG09294}/+ females. RT-PCR was performed to determine E74A, E74B, and *rp49* mRNA levels.

Ecdysone hormone leads to the direct induction of early response gene such as E74, E75, and BR-C, in non-steroidogenic tissues [23]. Next, we investigated the expression levels of early ecdysone response genes in the target tissues of larvae carrying one copy each of *UAS-dref-IR* and *P0206-Gal4*. In the larval fat body, an ecdysone target tissue, the expression

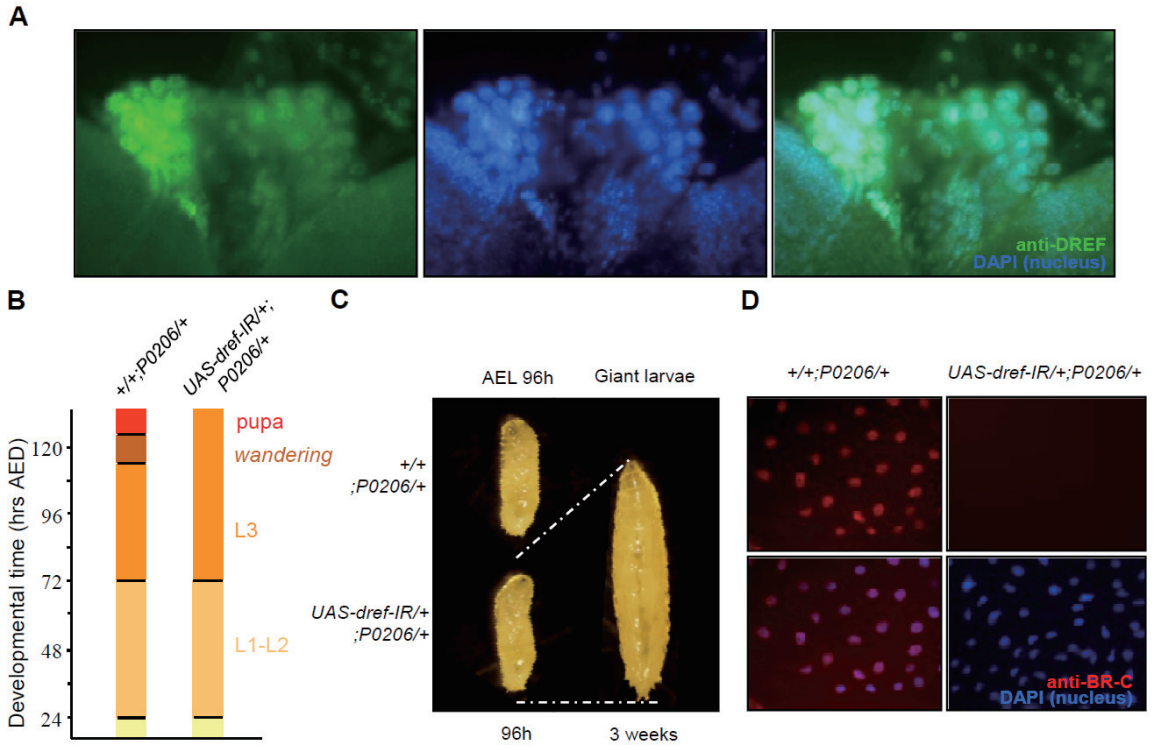

Figure 2. The phenotypes of larvae having dDREF knockdown in the prothoracic glands. A. Expression of dDREF in the PG. The dDREF expression in the PG was detected using an anti-DREF antibody (mAb4). PG of 3rd instar larvae were stained with anti-DREF antibody (green) and DAPI (blue). Original magnification is ×400. B. Effect of dDREF knockdown in the PG on timing of larval development. Transition timing (horizontal bars) was determined when 50% of the animals had passed a given transition (n>100). Reduced dDREF under *P0206-Gal4* affected pupation. C. Reduction of dDREF in the PG caused a giant larvae phenotype. The larvae carrying one copy of both *UAS-dref-IR* and *P0206-Gal4* grew continuously for 3 weeks. D. The BR-C level in the fat bodies determined by anti-BR-C in larvae carrying one copy of *P0206-Gal4* with or without *UAS-dref-IR*. Reduction of dDREF level in the PG reduced the ecdysone target gene BR-C levels in nonsteroidogenic tissues. Original magnification of the fat body is ×400. red, anti-BR-C; blue, DAPI.

of BR-C significantly decreased compared to that of larvae carrying only *P0206-Gal4* (**Figure 2D**). This result indicates that DREF levels in the PG can modulate expression of ecdysone target genes in non-steroidogenic tissues.

The phenotypes of larvae having DREF knockdown PG were rescued via 20E treatment

It was reported that the non-pupariating phenotype of *ecd*^{1} (*ecdysoneless* mutant) larvae was rescued by feeding with 20-hydroecdysone (20E) [31]. We tested whether the phenotype of larvae having DREF knockdown in the PG could also be rescued by feeding 20E at AEL 72 h. Interestingly, the pupation defects of giant larvae that were fed 20E at 250 μg/*ml* and 500 μg/*ml* was rescued up to 72% and 98%, respectively (**Figure 3A**).

Next, we checked expression of the hormone early response gene BR-C in these larvae. The expression of BR-C in the fat bodies of larvae carrying one copy of *UAS-dref-IR* and *P0206-Gal4* increased after 20E feeding (**Figure 3B**). The BR-C expression detected at 6 h after 20E feeding increased with time, reaching a maximum level at 24 h. These results indicate that the phenotypes of larvae having DREF knockdown in the PG are associated with the levels of ecdysone.

DREF can regulate the growth of the PG and the transcription of the drosophila shadow gene

To investigate the effect of dDREF knockdown on the level of ecdysone, we hypothesized that dDREF could be involved in two ways: a dDREF-

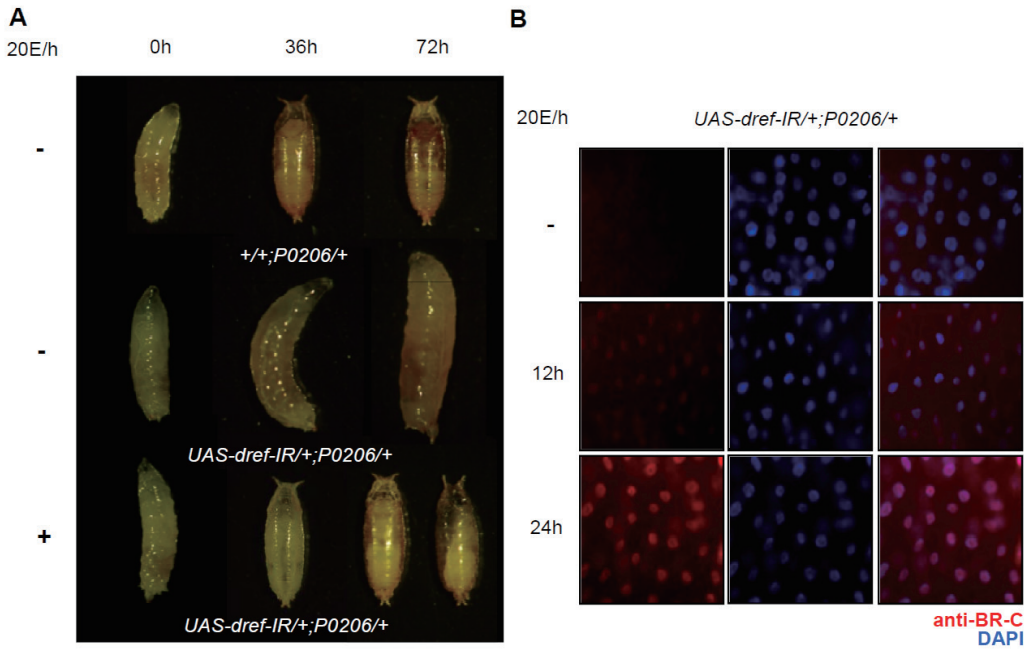

Figure 3. Effect of 20E treatment on the phenotypes of larvae having dDREF knockdown in the PG. A. The failed pupation of 3rd instar larvae carrying one copy of *UAS-dref-IR* and *P0206-Gal4* was rescued by 20E treatment. The larvae carrying one copy of *UAS-dref-IR* and *P0206-Gal4* at AEL 72±2 h were incubated for 2 days in the media with or without 500 μg/ml 20E. The larvae carrying one copy of *P0206-Gal4* is a positive control. B. Increased expression of the ecdysone early response gene BR-C by 20E treatment in the fat bodies of larvae carrying one copy of *UAS-dref-IR* and *P0206-Gal4*. The larval fat bodies were dissected on the ice and stained with anti-BR-C antibody. Original magnification is ×400. red, anti-BR-C; blue, DAPI.

dependent effect promoting the growth of PG cells and/or a dDREF-dependent effect on the transcriptional regulation of ecdysone biosynthetic genes. To assess the first possibility, we examined the size of the PG in larvae harboring *P0206-Gal4* with or without *UAS-dref-IR*. The dDREF knockdown under *P0206-Gal4* control affected the growth of the ring gland, particularly that of the PG (**Figure 4A**).

For the second possibility, we performed a search of dDREF-binding sites (5′-TATCGATA) within the promoter regions of *Drosophila* P450 genes. Interestingly, we found DRE-like sequences (5′-TATCGTTA) in the 5′-flanking region (-625 to -632 with respect to the transcription initiation site) of the *Drosophila shadow* gene (**Figure 4B**). It was reported that the expression of *shadow* is strong in the ecdysone producing tissues, the larval PG and adult ovaries [21]. To investigate whether dDREF binds to the DRE located in the 5′-flanking region of the *shadow* gene, we performed chromatin immunoprecipitation assays. Primers for amplifying the region containing the DRE were used for chromatin immunoprecipitation in conjunction with an anti-DREF polyclonal antibody. Based on the threshold cycle (C_T) values for each real-time PCR reaction, the relative proportion of immunoprecipitated DNA fragments was determined (**Figure 4C**). It was reported that the *elf4a* promoter contains a DRE site and that dDREF specifically binds to it [32]. Thus, an *elf4a* primer pair was used in this experiment as a positive control and the *Act 5C* gene, which has no DRE sequence, was used as a negative control. As is the case with the *elf4a* positive control (7.97-fold higher), the amplification of the DNA fragment containing DRE region of the *shadow* promoter from the anti-DREF immunoprecipitates was 5.33-fold higher than that from the immunoprecipitates using the control IgG. In contrast, the amplification of the *Act 5C* negative control was only 0.3-fold higher. These results indicate that dDREF binds to the genomic region containing the DRE of the *shadow* gene *in vivo*.

We attempted to determine whether *shadow* expression is regulated by dDREF in living flies

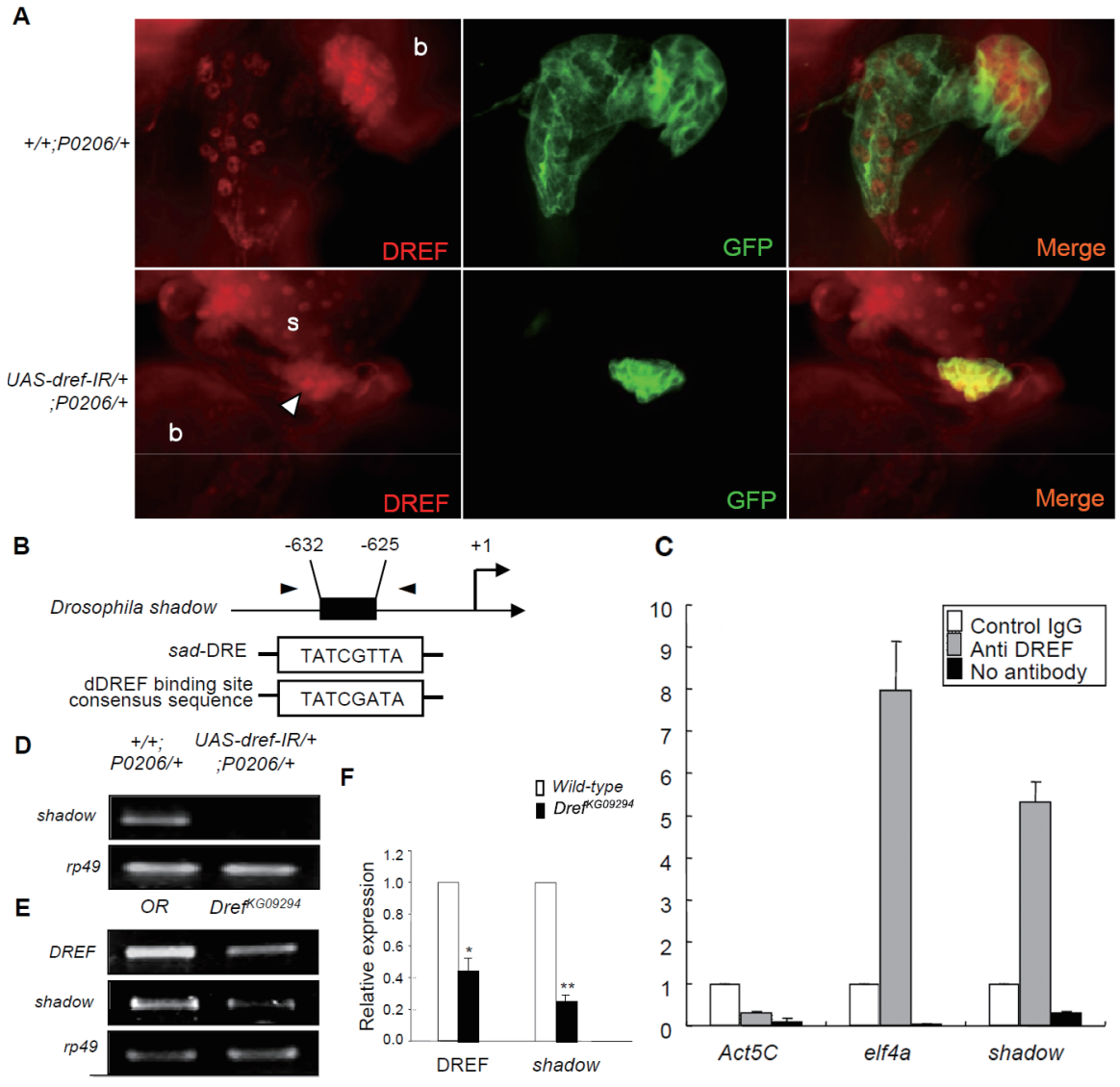

Figure 4. Transcriptional regulation of the *Drosophila shadow* gene by dDREF. A. Modulation of dDREF levels in the PG produces autonomous growth effects (green, GFP; red, DREF). Original magnification is ×200. b, brain; s, salivary gland; arrowhead, PG. B. Structure of the 5′-upstream region of the *shadow* gene. The transcription initiation site is indicated by the arrowhead and numbered as +1. The closed boxes indicate the dDREF binding site within the *shadow* gene. The nucleotide sequences of the *sad*-DRE and its consensus binding sites are shown in boxes below. Arrowheads indicate positions of the primers used for chromatin immunoprecipitation assays. C. Examination of dDREF-binding in the 5′-flanking region of the *shadow* gene by chromatin immunoprecipitation. The data shown are derived from quantitative real-time PCR analysis of the *shadow* gene promoter, the *elf4a* gene promoter, or the *Act 5C* gene, as shown at the bottom. Chromatin from the Kc cells was immunoprecipitated with either anti-DREF IgG or control rabbit IgG. The fold differences in values are from comparison of the anti-DREF immunoprecipitated sample (shown as anti-DREF IgG column) with the corresponding control IgG immunoprecipitated sample (control IgG column). A sample without antibody addition was also included as a negative control (no antibody column). D. Decreased expression of *shadow* by expression of dDREF double stranded RNA under *P0206-Gal4*. Total RNA was prepared from 3rd instar larvae carrying one copy of both *P0206-Gal4* and *UAS-dref-IR*. RT-PCR was performed to determine dDREF, *shadow,* and *rp49* mRNA levels. E. Decreased expression of *shadow* in the *Dref*^{KG09294}/+ larvae. Total RNA was prepared from the whole bodies of wild-type and *Dref*^{KG09294} mutant 3rd instar larvae. RT-PCR was performed to determine dDREF, *shadow,* and *rp49* mRNA levels. F. Decreased expression of *shadow* in the ovaries of *Dref*^{KG09294}/+ females. Total RNA was prepared from ovaries of wild-type and *Dref*^{KG09294}/+ 20-day-old females. Real-time RT-PCR was performed to determine dDREF, *shadow,* and *rp49* mRNA levels. A single asterisk represents P<0.01 and double asterisk represents P<0.001 when compared to the control.

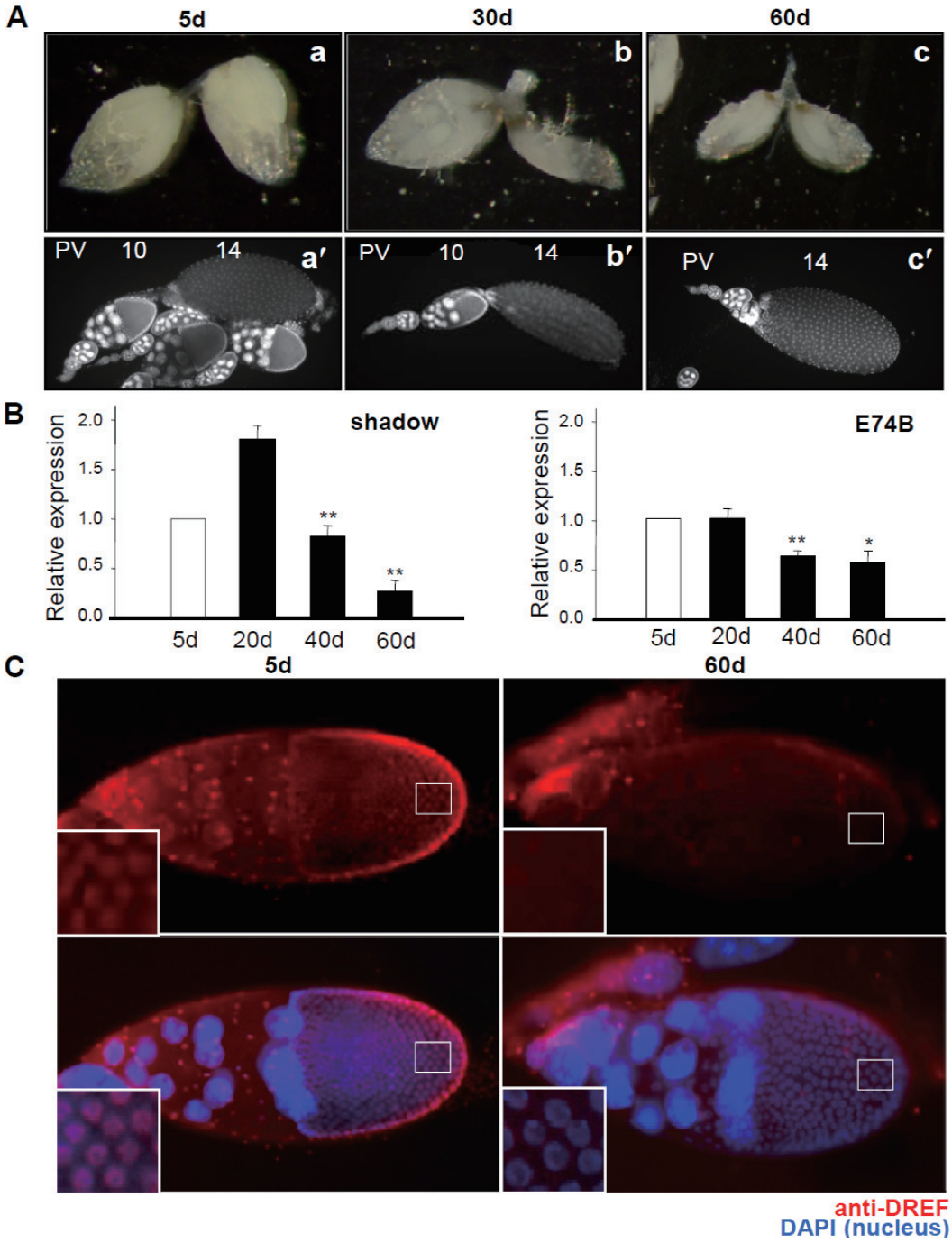

Figure 5. Age-related changes in dDREF and *shadow* expression in the ovaries of wild-type females. A. The ovaries of wild-type females shrank with age. Five- (a, a′), 30- (b, b′), and 60-day-old (c, c′) ovaries (n= 32, 29, or 27, respectively). B. Age-related changes in *shadow* and E74B mRNA levels within the ovaries of wild-type females. Total RNA was prepared from 5-, 20-, 40-, and 60-day-old ovaries. Real-time RT-PCR was performed to determine *shadow*, E74B, and *rp49* mRNA levels. A single asterisk represents $P<0.01$ and double asterisk represents $P<0.001$ when compared to the control. C. DREF dramatically decreased with age in the nucleus of follicle cells. Ovaries of both young (5-day-old) and old (60-day-old) wild-type females were stained with anti-DREF antibody (red) and DAPI (blue). Original magnification is ×200.

Table 2. Age-related change in the number of vitellogenic egg chambers of wild type females

	Wild-type (5day) n = 207 ovarioles	Wild-type (30day) n = 200 ovarioles	Wild-type (60day) n = 184 ovarioles
Stage 7-9 (early vitellogenic)	207 (100%)	200 (100%)	79 (42.9%)
Stage 10-13 (mid-late vitellogenic)	199 (96.1%)	157 (78.5%)	28 (15.2%)
Stage 14 (mature)	81 (38.6%)	83 (41.5%)	45 (24.5%)

All ovarioles contained several previtellogenic chambers.

using the GAL4-UAS system. In RT-PCR experiments, the level of *shadow* mRNA in the whole larvae having *UAS-dref-IR* and *P0206-Gal4* was lower than those observed in the controls (**Figure 4D**). We also assessed the level of *shadow* mRNA in *Dref*^{KG09294}/+ larvae. The level of *shadow* mRNA in the 3rd instar larvae of the *Dref*^{KG09294}/+ heterozygous was reduced in comparison with that of wild type (**Figure 4E**). To confirm the RT-PCR data, *shadow* mRNA levels in the ovaries of the *Dref*^{KG09294}/+ females were examined by real-time RT-PCR. The expression level was significantly reduced in comparison with that of wild type (**Figure 4F**). These results indicate that dDREF positively regulates the expression of *shadow*. Taken together, we conclude that dDREF can regulate ecdysteroidogenesis via regulation of PG growth and transcriptional regulation of the *Drosophila shadow* gene.

dDREF is involved in age-related decrease in steroidogenesis

It was reported that dDREF was expressed in the adult ovary [28]. First, we observed the ovary phenotype of 5-, 30-, and 60-day-old wild-type females. The size of ovary shrank with age (**Figure 5A**). The number of wild-type ovariole having vitellogenic (stage 8-13) egg chambers (**Table 2**) also decreased with age. To know whether these phenotypes were associated with age-related changes in steroidogenesis, we analyzed whether the expression of *shadow* is also modulated with age. The level of E74B mRNA was also examined. The level of *shadow* and E74B mRNA in wild-type ovaries significantly decreased with age (**Figure 5B**). Next, to investigate the relationship between the DRE/DREF system and age-related changes in steroidogenesis, we checked the level of dDREF in adult ovaries. Interestingly, dDREF expression in the nucleus of follicle and nurse cells of older females, as measured using anti-DREF antibodies, was decreased when compared to that of young females (**Figure 5C**). These results indicate that age-related decreases in steroidogenesis may be associated with dDREF function.

Discussion

In *Drosophila*, it has been demonstrated that ectopic expression of dDREF in the imaginal discs can induce abnormal DNA synthesis, apoptosis, and failures in differentiation [11]. In contrast, the expression of the dDREF dominant negative N-terminal region in the larval salivary gland reduced the occurrence of endoreplication [33]. Furthermore, RNA interference-mediated knockdown of dDREF *in vivo* demonstrated that dDREF is required for the normal progression through the cell cycle and consequently for growth of the imaginal discs and endoreplicating organs [12]. Recently, it was reported that DREF is required for cell and organismal growth in *Drosophila* and functions downstream of the nutrition/TOR pathway [34]. Here, we showed that dDREF is expressed in the larval prothoracic gland (PG), a primary steroidogenic organ (**Figure 2**), and that dDREF knockdown in the PG decreased its size significantly (**Figure 3A**). Our data suggest that dDREF is required for the growth of the steroidogenic organ, PG.

Many studies have reported that the dDREF homo-dimer binds specifically to the DRE sequence (5′-TATCGATA) within the promoters of many DNA replication and cell proliferation-related genes that require the DNA polymerase α subunits, CycA, Draf, PCNA, E2F and p38b MAPK to activate their transcription [2, 8]. Here, we also show that DREF is involved in steroidogenesis via the transcriptional regulation of the *Drosophila shadow* gene. We identified

the DRE-like sequence (5′-TATCGTTA) in the region between -625 and -632 with respect to the transcription initiation site of the *shadow* gene. Using a chip assay and quantitative RT-PCR, we demonstrated that dDREF binds to the genomic region containing DRE in the *shadow* gene promoter and regulate the levels of *shadow* mRNA. Therefore, our data indicate that *shadow* is a novel target of dDREF. It was demonstrated that hDREF is an important transcription factor that plays a role in cell cycle-dependent regulation of RP genes and histone H1 [13, 35]. Interestingly, we found an 8 out of 10 nucleotides match within the hDRE-like sequence (5′-TGTCGCCCA) in the region between -524 and -533 with respect to the transcription initiation site of the human CYP19A1 gene. This raises the question of whether the human CYP19 gene is also regulated by hDREF. Whether hDREF is involved in the human steroidogenesis would be an interesting avenue of research.

In humans, many reports have shown that abnormal levels of steroid hormones cause diverse diseases including hormone-related cancer, osteoporosis, and neurodegenerative diseases [36-38]. It was reported that an estrogen deficient state is characterized by accelerated aging [39]. Here, we demonstrated that the number of ovariole having vitellogenic egg chambers and the expression of *shadow* and E74B mRNAs at the adult stage both decreased with age. These data indicate an age-related decrease of steroidogenesis in *Drosophila*. We found that the ovaries of 20-day-old *Dref*^{KG09294}/+ females demonstrated phenotypes similar to those of 60-day-old wild-type females. Furthermore, we found that the dDREF levels within the follicle cell nucleus of 60-day-old flies were markedly lower than that of 5-day-old flies. Our data suggest that dDREF may also be involved an age-related decrease in steroidogenesis and that *Dref*^{KG09294} would be an excellent model for studying steroid hormone function in adults. Recently, it was reported that hDREF is associated with importin β1 during nuclear transport via the hATC domain [35]. It was also reported that the protein levels of nucleocytoplasmic transport factors (NCT) containing importin β1 were reduced with age [40]. Interestingly, we found that *Drosophila* DREF has an ATC sequence on its 3′-carboxyl-terminal region (data not shown). Further studies investigating the molecular mechanisms by which dDREF levels in the nucleus of follicle cells are modulated with age are necessary.

Experimental procedures

Fly stocks

Fly stocks were cultured at 25°C on standard media. The *Oregon-R* flies were used as the wild-type strain. The *P0206-Gal4* fly stock was kindly provided by Drs W. Janning and L. Pierre. *Oregon-R* and *Dref*^{KG09294} stocks used in this study were obtained from the Bloomington *Drosophila* stock center.

Immunohistochemistry

Immunohistology was performed as described previously [41]. We used the following primary antibodies: mouse anti-DREF (mAb4) 1:200 and mouse anti-BR-C 1:100 [Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA]. We used the following secondary antibodies: mouse-Cy3 (Jackson ImmunoResearch, West Grove, PA, USA) 1:400. DAPI (Molecular Probes, Eugene, OR, USA) was used at 1 μg*/ml*. We used Vectashield Mounting Medium (Vector Labs, Burlingame, CA, USA) for microscopic observation. Fluorescent samples were examined on a Karl Zeiss AxioPlan2 fluorescent microscope (Carl Zeiss Inc., Jena, Germany). Images were processed and assembled in Photoshop CS.

Fecundity analysis

Egg laying rate analysis was performed as described [42]. Fecundity was expressed as the number of eggs laid/fly/hour. Twenty 5-day-old wild-type or mutant virgin females were crossed to 20 wild-type males for 2 days and their eggs collected on a molasses agar plate and counted at 24 h intervals. This experiments were conducted at 25°C.

Quantification of vitellogenic egg chambers

Wild-type and heterozygous *Dref*^{KG09294} females were mated within 1 day of eclosion to Oregon-R males. Number of vitellogenic egg chambers was counted as described previously [43]. Ovaries were dissected, fixed in 4% paraformaldehyde for 30 min, stained in 1 μg*/ml* DAPI (Molecular Probes) for 20 min, washed three times with PBS, and mounted in Vectashield

Mounting Medium (Vector Labs). The number of ovarioles containing early vitellogenic (stage 7-9), mid-late vitellogenic (stage 10-13), or mature (stage 14) egg chambers were counted.

20E feeding

Hormone feeding experiment was performed as described previously [31]. Two hundred early third instar larvae (AEL 72±2 h) of each genotype were placed in vials with a sucrose-yeast medium containing 20-hydroxyecdysone (20E) (Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 250 or 500 μg/*ml*. This experiment was conducted at 25°C on dark conditions.

Quantitative RT-PCR

Real-time RT-PCR and RT-PCR were performed as described previously [32]. Total RNA from larvae was isolated with Trizol Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's protocol and cDNAs were synthesized with M-MLV-RT (reverse transcriptase) (Promega, Madison, WI, USA).

Oligonucleotide primers for real-time RT-PCR: dDREFsense:5´-ACACCTATCTGCGAGAGAAAAATATAC; dDREF antisense: 5´-CAAAGAACTTCAATGTTTCGTACAG; *shadow* Up2: 5´-CGGAGAGTGGTGAAATACGA; *shadow* Do1: 5´-TACGCTGTCAACGGGCATCT; Oligonucleotide primers for RT-PCR: E74A sense: 5´-TCCGAGAGCAACTTCGAGAT; E74A antisense: 5´-TTGATCAAATCGCCACAGAG; E74B sense: 5´-ATGTGTCCAGCTCCAGCTCT; E74B antisense: 5´-TAGTGACTCGGGGACTTTGG.

Oligonucleotide primers of ribosomal protein 49 (*rp49*) were described previously [44]. The real-time RT-PCR products were analyzed using OpticMonitor3.

Chip assay

Chromatin immunoprecipitation was performed using a Chip Assay kit as recommended by the manufacturer (Upstate Biotechnology Inc., Lake Placid, NY, USA) . Approximately 2 x 10^7 Kc cells were fixed in 1% formaldehyde at 37°C for 10 min and then quenched in 125 mM glycine for 5 min at 25°C. Cells were collected, washed twice in PBS containing protease inhibitors (1 mM PMSF, 1 μg/*ml* aprotinin and 1 μg/*ml* pepstatin A), and lysed in 2 ml of SDS lysis buffer (Upstate). Lysates were sonicated to break the DNA into fragments of less than 1 kb and centrifuged at 15,300 x g for 10 min at 4˚C. The sonicated cell supernatants were diluted 10 fold in chip dilution buffer (Upstate Biotechnology Inc., Lake Placid, NY, USA) and pre-cleared with 80 *μl* of salmon sperm DNA/Protein G agarose-50% slurry for 30 min at 4°C. After a brief centrifugation, supernatants were incubated with 1 μg of the normal rabbit-IgG antibody (Sigma) or 1 μg of anti-DREF polyclonal antibody for 16 h at 4°C. Salmon sperm DNA/Protein G agarose-50% slurry was added and incubated for 1 h at 4°C. After washing, immunoprecipitated DNA was eluted with buffer containing 1% SDS and 0.1 M $NaHCO_3$. The protein-DNA crosslinks were then reversed by heating at 65°C for 4 h. After deproteinization with Proteinase K, DNA was recovered by phenol-chloroform extraction and ethanol precipitation. The immunoprecipitated DNA fragments were then detected by quantitative real-time PCR using SYBR Green I (TaKaRa Dalian Biotechnology Co., Ltd. Dalian, China) and the Applied Biosystems 7500 Real Time PCR system. The ΔC_T value of each sample was calculated by subtracting the C_T value of the input sample from the C_T value obtained of the immunoprecipitated samples. Fold differences of each sample, relative to the control using non-immune IgG, were then calculated by raising 2 to the $\Delta\Delta C_T$ power. The $\Delta\Delta C_T$ was calculated by subtracting the ΔC_T value of the sample immunoprecipitated with control IgG.

Oligonucleotide primers for Chip assay: eif4adre1p: 5′-CTTTACCATACACACTGCGAAG; eif4aantidre1p: 5′-CAAAAGAGGCTCCATCTTGCAAAAG; actin5CP: 5′-CTCCATCATGAAGTGTGATGTG; actin5CantiP: 5′-CGTACTCCTGCTTGGACGTC; shadowFn:5′-GCGATTGCATGGGCTTAAAATCAGAGTCGAAAAAGG; shadow Rn: 5′-CTGTAAATAATGTTATCTATTGAATGCGTTTTTGG.

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