Cell. Mol. Life Sci. (2010) 67:1477–1490
DOI 10.1007/s00018-010-0260-7

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Role of *Drosophila* alkaline ceramidase (Dacer) in *Drosophila* development and longevity

Qiong Yang · Zhong-Jun Gong · Ying Zhou · Jing-Qun Yuan · Jiaan Cheng · Lin Tian · Sheng Li · Xin-Da Lin · Ruijuan Xu · Zeng-Rong Zhu · Cungui Mao

Received: 9 October 2009 / Revised: 31 December 2009 / Accepted: 6 January 2010 / Published online: 30 January 2010

Abstract Ceramidases catalyze the hydrolysis of ceramides to generate sphingosine (SPH) and fatty acids, and ceramide metabolism is implicated in various biological responses in *Drosophila melanogaster*. Here we report the cloning, biochemical characterization, and functional analysis of a *Drosophila* alkaline ceramidase (Dacer). Dacer, a membrane-bound protein of 284 amino acids, shares homology with yeast and mammalian alkaline ceramidases. Overexpression of Dacer in High Five insect cells increases ceramidase activity in the alkaline pH range, indicating that Dacer is a *bona fide* alkaline ceramidase. Dacer mRNA is highly expressed in the midgut and at the pupal stage. An inactivation of Dacer by insertional mutagenesis increases the levels of ceramides in both *Drosophila* pupae and adult flies. Dacer inactivation increases *Drosophila* pre-adult development time, lifespan, and anti-oxidative stress capacity. Collectively, these results suggest that Dacer plays an important role in the *Drosophila* development and longevity by controlling the metabolism of ceramides.

Keywords Alkaline ceramidase · *Drosophila* · Development · Lifespan

Introduction

Sphingolipids, important components of eukaryotic cell membranes, play an essential role in maintaining the

Q. Yang · Z.-J. Gong · Y. Zhou · J. Cheng · Z.-R. Zhu
State Key Laboratory of Rice Biology,
Key Laboratory of Molecular Biology of Crop Pathogens and Insects, the Ministry of Agriculture of China,
Hangzhou, Zhejiang 310029, China

Q. Yang · Z.-J. Gong · Y. Zhou · J. Cheng · Z.-R. Zhu (✉)
The Institute of Insect Sciences, Zhejiang University,
Hangzhou, Zhejiang 310029, China
e-mail: zrzhu@zju.edu.cn

J.-Q. Yuan
Center for Chemical Analysis and Detection,
Zhejiang University, Hangzhou, Zhejiang 310029, China

L. Tian · S. Li
Institute of Plant Physiology and Ecology,
Shanghai Institutes for Biological Sciences,
Chinese Academy of Sciences, Shanghai 200032, China

X.-D. Lin
College of Life Sciences, China Jiliang University,
Hangzhou, Zhejiang 310018, China

R. Xu · C. Mao (✉)
Department of Medicine and Molecular Biology,
Medical University of South Carolina,
Charleston, SC 29425, USA
e-mail: maoc@musc.edu

R. Xu · C. Mao
Department of Biochemistry and Molecular Biology,
Medical University of South Carolina,
Charleston, SC 29425, USA

Present Address:
Q. Yang
Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu 210014, China

structural and functional integrity of the biological membrane systems [1]. In addition, sphingolipids serve as a reservoir of many signaling molecules, such as ceramides, sphingosine (SPH), and sphingosine-1-phosphate (S1P), all of which have been implicated in a variety of cellular responses, such as cell proliferation, survival, differentiation, senescence, and apoptosis [2].

Ceramidases are a group of enzymes that catalyze the hydrolysis of ceramides to generate SPH, which is phosphorylated to form S1P. Ceramidases have been cloned from various organisms, including bacteria [3, 4], yeast [5, 6], *Drosophila melanogaster* [7], zebra fish [8], mice [9], rats [10], and humans [11]. Ceramidases have been shown to play an important role in regulating ceramides, sphingosine, and S1P, and thereby cellular responses mediated by these sphingolipid metabolites.

Five human ceramidases encoded by five distinct genes were cloned: the acid ceramidase (ASAH1), neutral ceramidase (ASAH2), alkaline ceramidase 1 (ACER1), alkaline ceramidase 2 (ACER2), and alkaline ceramidase 3 (ACER3). Each human ceramidase has a mouse counterpart. ACERs have similar protein sequences, but they have no homology to ASAH1 and ASAH2, which also have distinct protein sequences [2]. These human ceramidases have been shown to play an important role in the metabolism of sphingolipids and biological responses. Genetic deficiency in ASAH1 causes Farber disease, characterized by accumulation of ceramides and sphingolipids in lysosomes [12]. The human ASAH2 has been implicated in cell survival against TNF-α [13]. ACER1 is highly expressed in the epidermis and has a role in the differentiation of epidermal keratinocytes likely by regulating the metabolism of ceramides, SPH, and S1P [14]. In vitro studies suggest that ACER2 expression is important for cell proliferation and survival, likely by enhancing S1P signaling and/or reducing that of ceramides, but its overexpression may lead to cell growth arrest and/or cell death because of accumulation of cytotoxic levels of SPH in cells [11]. Our recent studies suggest that ACER3 may act as a house-keeping enzyme that maintains unsaturated long-chain ceramides, such as D-*erythro(e)*-$C_{18:1}$- and $C_{20:1}$-ceramides, at low levels in cells. Because of the existence of multiple isozymes in mammals, studies on the physiological and pathological roles of mammalian ceramidases are challenging.

The metabolic pathway of sphingolipids appears to be conserved between *Drosophila* and mammals. As in mammals, a tight regulation of the metabolism of sphingolipids in *Drosophila* is required for a variety of biological processes, such as reproduction and development [1, 15, 16]. However, the number of enzymes for each metabolic step is much smaller than that in mammals. Because of this simplicity, we define the physiological role of alkaline ceramidases using this model organism. By analyzing the sequence of the genome of *Drosophila melanogaster*, we found that only one putative protein encoded by the *Drosophila* brainwashing gene (*BWA*) shares similarity in protein sequence to known alkaline ceramidases. The *BWA* gene was identified as a gene whose mutation causes an abnormality in the structure of the corpora pedunculata (mushroom bodies) in the brain, although its biochemical properties remain unclear. In this study, we demonstrate that the protein product (Dacer) of the *BWA* gene has alkaline ceramidase activity and that Dacer plays an important role in the metabolism of ceramides in *Drosophila*. We further show that compared to the wild-type *Drosophila*, the *Drosophila* mutant deficient in Dacer had delayed pre-adult development, increased antioxidative stress capacity, and an extended lifespan, suggesting that Dacer plays an important role in various biological processes during the development and aging process of the fruit fly.

Materials and methods

Drosophila melanogaster stocks and maintenance

Wild-type w1118 and *bwa*^{e02081} (BL-18012) lines were obtained from the Bloomington *Drosophila melanogaster* Stock Center (Indiana University, Bloomington, IN). The annotated brainwashing gene (CG13969) localizes to the 38B2-3 region of the left arm of the second chromosome. A P-element insertion in this gene was identified (Fig. 1) and was subsequently mobilized to generate a mutant. Stocks were maintained at 25°C on a 12:12 h light:dark cycle at constant humidity by using standard cornmeal medium consisting of 8.6% sucrose, 1% yeast, 11.3% cornmeal, 1% agar, and 1% propionic acid.

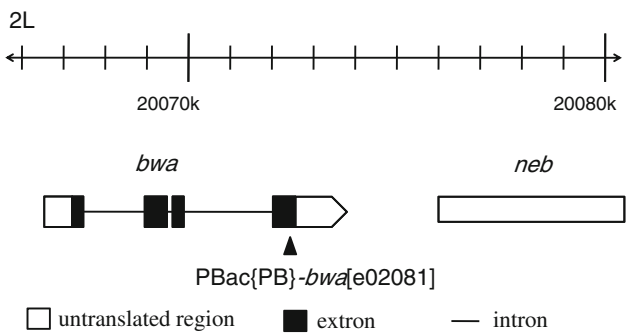

Fig. 1 Transposon location of the *dacer* mutant. The *Dacer/BWA* gene localizes to the 38B2-3 region of the left arm of the second chromosome. A P-element insertion (PBac(43)-*bwa*^{e02081}) was isolated and was subsequently mobilized to generate a *dacer/bwa* mutant that carries a transposon within the 4th extron of the Dacer/BWA gene

Lipid preparation

D-*e*-Sphingosine (C_{18}SPH) with 18 carbons and C_{17}SPH were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). C_{14}SPH, C_{16}SPH, and D-*e*-dihydrosphingosine with 14 (C_{14}DHS) or 16 (C_{16}DHS) carbons were from Matreya, LLC (Pleasant Gap, PA). All ceramides used in this study were synthesized as described [17] in the lipidomics core at the Medical University of South Carolina (MUSC), Charleston, SC.

cDNA cloning

A BLAST search of the *Drosophila melanogaster* genomic database using human alkaline ceramidases as queries revealed a putative protein encoded by the *BWA* gene (GenBank accession number: AF323976). To amplify the *BWA* coding sequence or open reading frame (ORF), total RNA was isolated from whole *Drosophila* adults using Trizol (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA using the SMART™ PCR cDNA synthesis kit (Clontech, TaKaRa). A small portion of transcribed cDNAs was subjected to PCR amplifications using the primer pair *BWA*F (5′-AGGATCCATGGGCGGGATGGGGGGCGGCGGGCT-3′) and *BWA*R (5′-AAAGCTTTTAGATGGTGTTG CGCA GAGCCTTAC-3′) under the condition of one cycle of 94°C for 60 s, 34 cycles of 94°C for 30 s, 63°C for 60 s, and 72°C for 60 s. The PCR products were cloned into the vector pGEM-T easy (Promega, USA). The resulting *BWA* ORF construct pGEMT-Dacer was sequenced to confirm the correctness of the *BWA* ORF.

Plasmid construction

pGEMT-Dacer was digested with the restriction enzymes *BamHI* and *HindIII* to release the Dacer ORF, which was subcloned in-frame with the 6 × histidine (HIS) tag into the baculovirus transfer vector pFastBacHT. DH10 *Escherichia coli* Bac cells (Gibco-BRL) were then transformed with the plasmid pFastBacHT-Dacer. Site-specific transposition of the expression cassette from the pFastBacHT-Dacer plasmid to the bacmid DNA of DH10 Bac cells disrupts the lacZ gene, and the recombinant clones are $lacZ^{-}$ and gentamycin resistant. The positive colonies (white) containing the recombinant bacmid (Bac-Dacer) and wild-type (WT) bacmid (Bac-WT) were cultured to isolate the bacmid DNA.

Dacer overexpression in High Five cells

High Five cells (Tn5B-1-4) were cultured in TNM-FH insect medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum (Gibco, BRL). The bacmid DNA of Bac-Dacer and Bac-WT were transfected by Lipofectamine 2000 (Invitrogen) into High Five cells to yield the A*utographa californica* nuclear polyhedrosis virus (AcNPV) carrying the Dacer ORF and control bacmid. A baculoviral stock with a suitable titer was generated and used to infect High Five cells. Forty-eight hours after infection, the cells were harvested. After being washed three times with PBS, the cells were resuspended in buffer A (20 mM Tris–HCl, pH 7.4), containing 0.25 M sucrose and 20 g/ml protease inhibitor mixture (CLAP; Roche Applied Science), and then were sonicated for 5 s at a power level of 35% on a microtip-equipped Sonic Dismembrator. The total cell lysates were centrifuged at 1,000 $\times g$ for 10 min. The post-nuclear supernatants were centrifuged for 10 min at 10,000$\times g$ to obtain the Mr = 10,000 membrane fraction. The resulting supernatant was centrifuged for 1 h at 100,000 $\times g$ to obtain microsomes (the *M*r = 100,000 membrane fraction), which were washed three times with buffer A. Microsomes (40 μg protein per lane) prepared from the High Five cells containing the bacmid of Bac-Dacer as well as Bac-WT were resolved by SDS-PAGE and then transferred to a nitrocellulose membrane. The HIS-tagged Dacer was detected by an anti-HIS antibody using an ECL Plus detection kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Protein concentration determination

Protein concentrations were determined with BSA as a standard using a bicinchoninic acid (BCA) protein determination kit (Beyotime) according to the manufacturer's instructions.

Ceramidase activity assay

Ceramidase activity was determined by the release of sphingosine from ceramides according to Xu et al. [11]. Briefly, microsomes (20 μg protein) were incubated with ceramide substrate (75 μM) in 40 μl assay buffer (25 mM Tris-HCl, pH 6–9, containing 5 mM $CaCl_2$, 0.15% Triton X-100) at 37°C for 30 min. The reactions were stopped by extraction with chloroform and methanol. Sphingoid bases in the lipid extracts were determined by HPLC with C_{17}SPH as an internal standard as described [17].

ESI/MS/MS lipid analysis

Electrospray ionization (ESI)/MS/MS analysis of sphingolipids was performed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer, operating in a multiple reaction monitoring (MRM) positive ionization mode, according to published methods [18]. Each of the samples of 48-h-old *Drosophila* pupae and 3-day-old adult flies was suspended in 4 ml of the ethyl acetate/isopropanol/water

(60/30/10%; v/v) solvent system in a 15-ml tube, to which was added 50 µl of a mixture (1 µM) of internal sphingolipid standards (ISs) including C_{17}SPH, D-*e*-C_{16}-ceramide (d17:1/16:0), and D-*e*-C_{18}-ceramide (d17:1/18:0). *Drosophila* pupae or adults were then homogenized for 5 min using a homogenizer set at the maximal power mode, followed by 1 h sonication in a water bath sonicator. After centrifugation, 1 ml of the lipid extract was used for phospholipid (Pi) determination as described [19], and the remaining was used for ESI/MS/MS. The lipid extracts were dried under a stream of nitrogen gas. For ESI/MS/MS, dried lipid extracts for each sample were dissolved in 100 µl of acidified (0.2% formic acid) methanol, and were injected on the HP1100/TSQ 7000 LC/MS system and gradient-eluted from the BDS Hypersil C8, 150 × 3.2 mm, 3-µm particle size column, with a 1.0 mM methanolic ammonium formate/2 mM aqueous ammonium formate mobile phase system. Peaks corresponding to the target analytes and IS were collected and processed using Xcalibur software. Quantitative analyses of endogenous sphingolipids (SPLs) were based on calibration curves generated by spiking an artificial matrix with known amounts of the target analyte synthetic standards and an equal amount of the IS. The target analyte/IS peak area ratios were compared to the calibration curves using a linear regression model. Levels of particular SPLs were normalized to Pi and expressed as SPLs/Pi (pmol/µmol).

Developmental time analysis

Fly eggs were laid on 90-mm petri dishes containing 20 ml of standard cornmeal medium. The dishes were monitored at 12-h intervals, and the numbers of embryos, larvae, and pupae were recorded; emerging adults were collected, sexed, and counted. The data recoding continued until no flies emerged for 3 consecutive days. The developmental time of egg, larval, and pupal stages of immature flies were calculated.

Juvenile hormone acid methyltransferase (JHAMT) activity assay

Juvenile hormone III (JH III) was purchased from Sigma, and [^{3}H]-SAM was from PerkinElmer. JH III acid (JHA) was produced from their corresponding methylesters by saponification [20]. Purified JHA and [^{3}H]-SAM were dissolved in sodium phosphate solution (0.1 M Na_2HPO_4-NaH_2PO_4, pH 7.2). Corpus allatums (CA) were isolated from ten wandering larvae, which were homogenized in the sodium phosphate buffer. After centrifugation, the supernatant (50 µl) was transferred to an equal volume of reaction buffer containing JHA and [^{3}H]-SAM. For negative controls, only the phosphate solution buffer was added to the same reaction mixtures. After incubation at 28°C for 90 min, the enzymatic reaction was stopped by the addition of an equal volume of methanol. The radiolabeled product was extracted with 1 ml of hexane and counted with a liquid scintillation counter [21].

Lifespan analysis

Eclosing (insects emerging from pupal case) adults (0–12 h) were collected. Approximately 200 male and female flies were maintained separately in individual vials (with 20 flies per vial) and transferred to fresh vials every 3 days, and survivors were counted every 24 h. For statistical analysis, the mean and maximum lifespan of each strain was calculated from the time (in days) at which survival reached 50 and 10% of the starting population.

Oxidative stress assay

Three-day-old flies (100 male and 100 female of each strain) were transferred to vials containing standard cornmeal medium supplemented with 20 mM paraquat (with 20 flies per vial) [22]. To measure the lifespan on oxidative stress, the numbers of dead flies were counted every 6 h.

Mitochondrial hydrogen peroxide measurement

Hydrogen peroxide production was measured in isolated fly mitochondria under normal respiration conditions as described [23]. Adult flies at different ages living on normal food or paraquat-containing food were collected on ice before mitochondria were isolated using Tissue Mitochondria Isolation Kit (Beyotime) according to the manufacturer's instructions. Protein concentration was determined using a BCA Protein Determination Kit (Beyotime) with bovine serum albumin as standard. Mitochondria (about 0.15 µg protein/µl) were incubated at 25°C in 50 µl assay medium containing 20 mM sn-glycerol 3-phosphate (Sigma) as substrate. Mitochondrial hydrogen peroxide production was determined using the Hydrogen Peroxide Assay Kit (Beyotime) according to the manufacturer's instructions. The absorbance at 560 nm was recorded on a μQuantMicroplate spectrophotometer (Biotek Instruments). All tests were done in triplicate, and more than 100 flies were used in each assay.

ATP measurement

ATP levels in the flies were quantified by a luciferin-luciferase system using the ATP Assay Kit (Beyotime) according to the manufacturer's instructions. Newly eclosed flies were sexed and reared at a density of 20 flies per vial. The flies were aged to different days at 25°C on the normal diet. The flies were washed thoroughly in PBS and homogenized in the same buffer. The levels of ATP were determined by luminometry using the SpectraMax M5 luminometer.

Statistic analyses

Statistical significance of the data from the qRT-PCR were analyzed using Tukey's student range test. Differences in development time, JHAMT activity, and ATP levels between w1118 and *dacer* mutant flies were tested using Student's *t* tests. Data were handled using SAS, and the significance level was $P < 0.05$ in all tests. Asterisk (*) indicates the significant difference between two means.

Results

BWA has alkaline ceramidase activity

A BLAST search of the *Drosophila melanogaster* genomic database using human alkaline ceramidases as queries revealed a putative protein encoded by the *BWA* gene (GenBank accession number: AF323976). This putative protein exhibited a 35, 46, and 26% identity in protein sequence to the human alkaline ceramidases ACER1, ACER2, and ACER3, respectively. The *BWA* gene product was also found to contain multiple domains conserved among these alkaline ceramidases (Fig. 2a). This putative ORF was amplified by RT-PCR from adult *Drosophila* RNA as described in "Materials and methods." DNA sequencing indicated that the cloned ORF has the predicted sequence. The pSORTII program predicted that the *BWA* gene consists of five putative transmembrane domains (TM1-5) (Fig. 2b). According to its homology to the human alkaline ceramidases, we postulated that the *BWA* gene product may be a *Drosophila* alkaline ceramidase (Dacer).

To determine whether Dacer indeed encodes alkaline ceramidase activity, the Dacer coding sequence was cloned

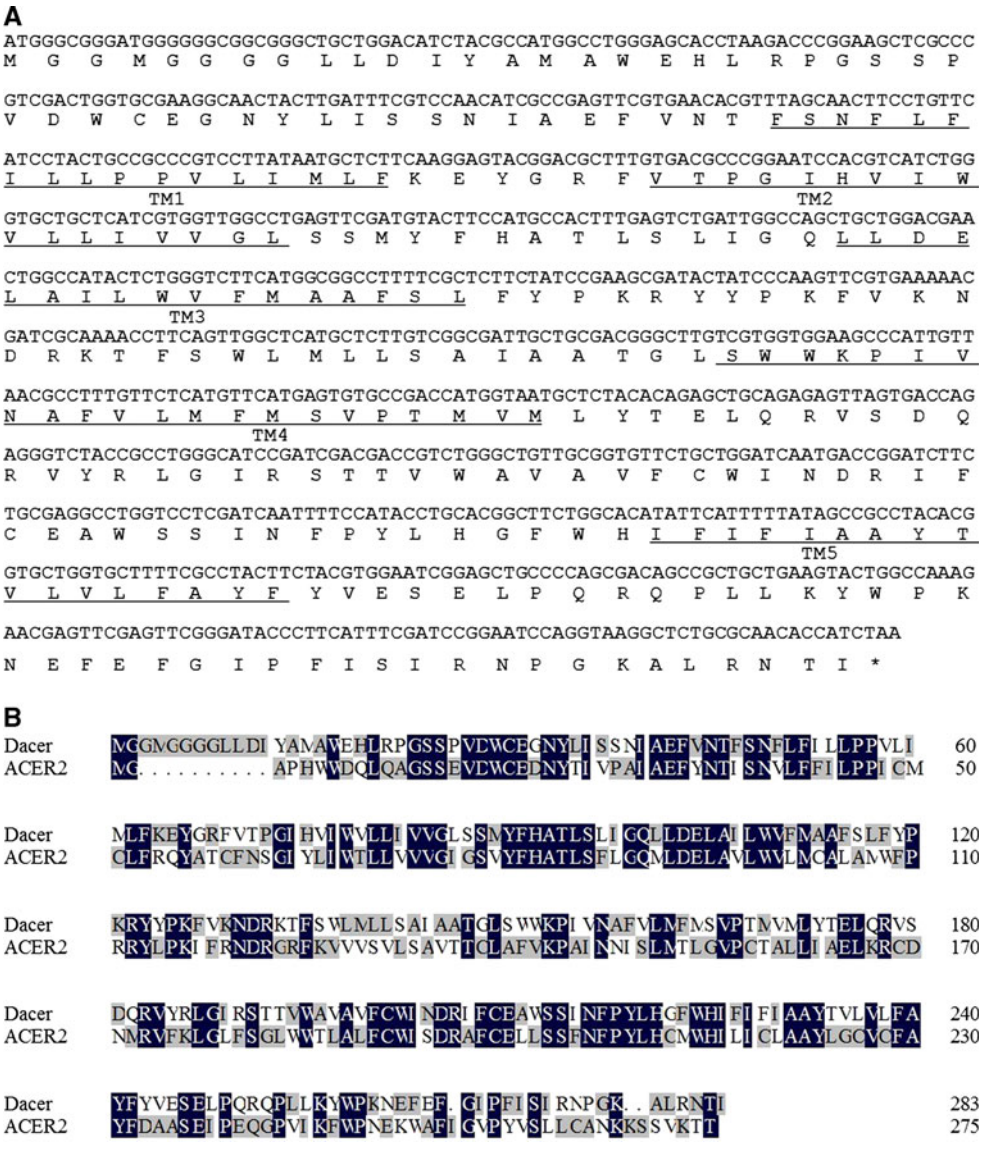

Fig. 2 Coding and protein sequences of Dacer. **a** The Dacer coding sequence (GenBank accession number: AF323976) was cloned from adult *Drosophila* flies. The amino acid sequence was derived from the coding sequence using the DNAman software. Putative transmembrane domains (TMs1–5) of Dacer were predicted by the pSORT II program. **b** Protein sequence alignment of Dacer and ACER2 was performed using the DNAman software. Identical amino acid residues among the aligned proteins are shaded in *dark grey* and similar amino acid residues *light grey*

into a Baculoviral vector (pFastBacHT B), and the recombinant bacmid DNA (Bac-Dacer) or the WT bacmid DNA (Bac-WT) was transfected into High Five cells. The microsomes were isolated from High Five cells transfected with Bac-Dacer or Bac-WT. Western blot analysis with the anti-6xHIS tag antibody detected a protein band (apparent molecular mass ~34 kDa) in the microsomes isolated from the cells transfected with Bac-Dacer but not in the microsomes isolated from cells transfected with Bac-WT. In vitro activity assays demonstrated that, compared to the Bac-WT microsomes, the Bac-Dacer microsomes had a ~twofold increase in ceramidase activity on D-*e*-C_6, D-*e*-C_{12}, D-*e*-C_{16}, D-*e*-C_{18}, and D-*e*-$C_{24:1}$-ceramide at pH 9.0 (Fig. 3b), suggesting that Dacer indeed possesses ceramidase activity.

To determine whether, similar to its mammalian homologues, Dacer has an alkaline pH optimum for its in vitro activity, we measured ceramidase activity of the recombinant Dacer at different pH values. The activity of the recombinant Dacer was determined by subtracting endogenous ceramidase activity of High Five cells in Bac-WT microsomes from ceramidase activity in Bac-Dacer microsomes. We found that recombinant Dacer has the highest activity at pH 8.0 (Fig. 3c), suggesting that Dacer has an alkaline pH optimum for its in vitro activity.

Dacer/BWA is highly expressed in the midgut and at the pupal stage of the life cycle

To better understand the physiological roles of Dacer, qRT-PCR was performed to quantify the temporal and spatial expression of Dacer based on the normalized relative quantification $2^{-\Delta\Delta Ct}$ method using *Drosophila* tubulin cDNA as a control. Dacer mRNA levels were much higher in the midgut than in other organs of *Drosophila* (Fig. 4a). We also found that Dacer mRNA levels are upregulated at the pupal stage compared to the larval or adult stage of the life cycle. These results suggest that Dacer mRNA is expressed highly in the midgut, moderately in the brain, and only slightly in other organs, and that Dacer expression is upregulated at the pupal stage during the development of *Drosophila* (Fig. 4b).

Dacer inactivation increases *Drosophila* ceramide levels

Dacer/*bwa* mutant flies have been reported to have a fusion of the beta lobes in the central brain [24]. The mutant flies from the Bloomington *Drosophila* Stock Center were identified to harbor a transposon within the 4th extron of

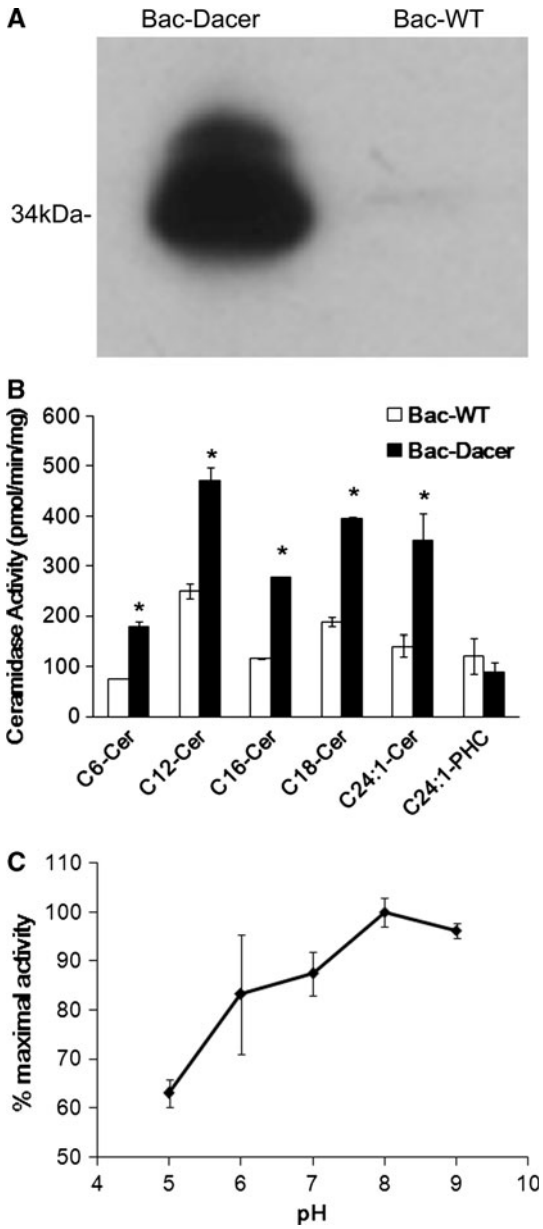

Fig. 3 Dacer encodes alkaline ceramidase activity. **a** Microsomes were prepared from Bac-Dacer and Bac-WT cells. A portion of the microsomes was subjected to Western blot analysis with the anti-HIS antibody (1:1,000). The relative molecular mass (Mr) (~34 kDa) was estimated according to standard proteins. **b** Another portion of the above microsomes was assayed for ceramidase activity at pH 9.0 using indicated ceramides as substrates. **c** Microsomal ceramidase activity was determined using D-*e*-C_{16}-ceramide as substrate at different pH values. Ceramidase activity of the recombinant Dacer at each pH was computed by subtracting ceramidase activity in Bac-WT microsomes from that in Bac-Dacer microsomes. The Dacer ceramidase activity at pH 8 is highest and set as 100%, and ceramidase activity at other pH values is expressed as % of the maximal activity. All data represent the mean value of three independent experiments performed in duplicate

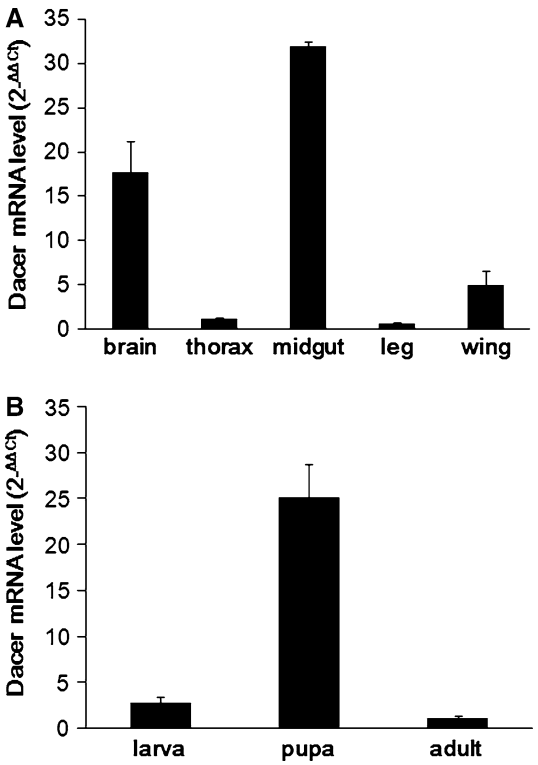

Fig. 4 Dacer is highly expressed in the ovary and at the pupal stage. qRT-PCR analysis was performed with cDNAs reversely transcribed from RNA isolated from different *Drosophila* organs (**a**) or from flies at different developmental stages (**b**). Data represent the mean value ± SE of three independent experiments performed in duplicate

the *Dacer/BWA* gene (designated bwa^{e02081}) (Fig. 5a). Because Dacer/BWA is a *Drosophila* alkaline ceramidase that catalyzes the hydrolysis of ceramides, we examined ceramide levels in 48-h-old pupae and 3-day-old adults of wild-type w1118 flies and *dacer* mutant flies. ESI/MS/MS demonstrated that compared to w1118 controls, both *dacer* mutant pupae (Fig. 5b, c) and adults (Fig. 5d, e) had much higher levels of most ceramide species containing either C_{14}SPH or C_{16}SPH, suggesting that Dacer plays a role in controlling the levels of ceramides in *Drosophila* at both pupal and adult stages.

Dacer inactivation delays *Drosophila* pre-adult development

We showed that Dacer mRNA levels are markedly increased at the pupal stage, indicating that Dacer may have a role in the pre-adult development of the *Drosophila*. To investigate this possibility, the pre-adult development time of w1118 or *dacer* mutant flies was determined. We found that like w1118 control embryos, the embryos of the mutants developed normally and eventually emerged as adults without obvious abnormalities. However, the development time from egg to adult eclosion increased significantly ($P < 0.05$) in *dacer* mutants (10.49 ± 0.06 days; $n = 138$), compared to w1118 flies (8.85 ± 0.06 days; $n = 136$) (Table 1). Data analysis further showed that the larval development time was 6.92 ± 0.08 ($n = 242$) days for *dacer* mutants and 4.69 ± 0.04 ($n = 179$) days for w1118 controls, suggesting that differences in the total immature development rate result in the longer larval development time of *dacer* mutants compared to w1118 controls. In addition, the immature development time was 8.85 ± 0.08 days for both w1118 male ($n = 90$) and female flies ($n = 48$), but small differences were observed between immature mutant male (10.69 ± 0.08 days; $n = 68$) and female flies (10.29 ± 0.09 days; $n = 68$). These results demonstrate that the Dacer inactivation significantly delays the pre-adult development, especially the larval development in *Drosophila*.

Dacer inactivation increases JHAMT activity

Juvenile hormones (JHs) are a family of sesquiterpenoid hormones that play a central role in the control of insect's development and growth [25]. Juvenile hormone acid methyltransferase (JHAMT) of the silkworm *Bombyx mori* methylates the carboxyl group of JH I, II, and III acids (JHAs) to generate hormonally active JHs, and it has been shown to be critical in JH biosynthesis [20, 26]. JHAMT activity positively correlates with levels of JHs in insects at the larval stage [27]. Based on these observations, we determined whether Dacer inactivation affected JHAMT activity in *Drosophila* larvae. As shown in Fig. 6, JHAMT activity was significantly increased in *dacer* mutant flies (0.66 ± 0.04 fmol/CA/h) compared with w1118 control flies (0.49 ± 0.05 fmol/CA/h), suggesting that Dacer inactivation may increase the levels of JHs in the *Drosophila* at the larval stage.

Dacer inactivation increases *Drosophila* lifespan

To get more insight in the physiological role of Dacer in mature flies, we analyzed the effect of Dacer inactivation on the lifespan in *Drosophila*. The rationale for this study is that ceramides have been shown to be important for *Drosophila* longevity [28]. We found that the average lifespan (50% survival) and maximum lifespan (10% survival) were 38.07 ± 0.85 ($n = 213$) and 53.19 ± 0.27 ($n = 21$) days, respectively, for the *dacer* mutant female flies, and 24.65 ± 0.73 ($n = 232$) and 44.00 ± 0.58 ($n = 23$) days, respectively, for the w1118 female flies (Fig. 7a). The average lifespan and maximum lifespan were 41.27 ± 1.04 ($n = 182$) and 55.44 ± 0.23 ($n = 18$) days, respectively, for the *dacer* mutant male flies, and 27.82 ± 0.73 ($n = 173$) and 41.82 ± 0.85 ($n = 17$) days, respectively, for the

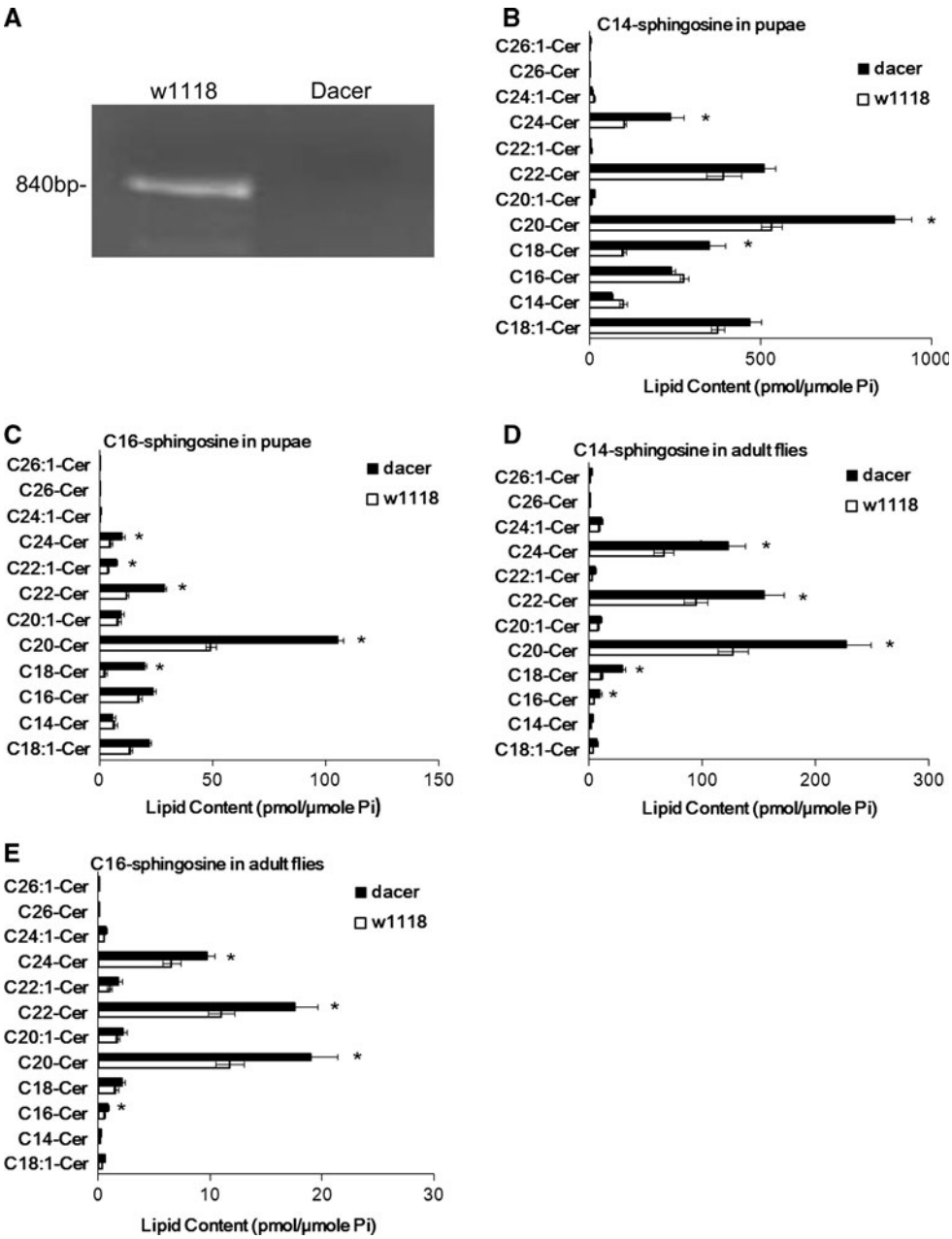

Fig. 5 Dacer inactivation increases *Drosophila* ceramides. **a** Total RNA isolated from *dacer* mutant and control flies was reversely transcribed into cDNAs. The cDNAs were subjected to RT-PCR analysis for Dacer mRNA using the primer pair BWA-5′ and BWA-3′ corresponding to the 5′ and 3′ ends of the coding sequence, respectively. Note that the full-length coding sequence (840 bp) was amplified from controls' RNA, but not from mutants' RNA. **b**, **c.** Pupae of mutant and control flies were harvested at 48 h and subjected to ESI/MS/MS analysis for C_{14}SPH- and C_{16}SPH-ceramides. **d, e.** Adults of mutant and control flies were harvested at day 3 and subjected to ESI/MS/MS analysis for C_{14}SPH- and C_{16}SPH-ceramides. The contents of sphingolipids were normalized to total phospholipids (Pi) in flies. Data represent mean value ± SE of three independent experiments performed in duplicate

w1118 male flies (Fig. 7b). These results clearly indicate that Dacer inactivation significantly increases the *Drosophila* lifespan (Student's t test, $P < 0.0001$ for all comparisons).

The *dacer* mutant flies are resistant to oxidative stress damage

Enhanced resistance to various stresses is often associated with interventions that extend lifespan. We then examined the ability of w1118 and mutant flies to resist oxidative stress. The rationale behind this study is that ceramides have been shown to be important in anti-oxidative stress in *Drosophila* [28]. Because paraquat, upon uptake by *Drosophila*, was shown to kill insects by generating reactive oxygen species (ROS) [29], the period that an insect survives paraquat should correlate to the insect's capacity for anti-oxidative stress. Based on this assumption, we determined whether there is a difference in the survival time between w1118 and *dacer* mutants. We demonstrated that in the presence of 20 mM paraquat in the food, the average lifespan and maximum lifespan were 154.4 ± 6.7 ($n = 100$) and 267.0 ± 7.8 ($n = 10$) h, respectively, for the mutant female flies, 78.2 ± 3.9 ($n = 100$) and 156.0 ± 11.0 ($n = 10$) h, respectively, for the w1118 female flies (Fig. 8a). The average and maximum lifespan

Table 1 Dacer inactivation delays pre-adult development in *Drosophila*

	w1118		*dacer* mutant		
	Mean ± SE	*n*	Mean ± SE	*n*	*P*-value
Total	8.85 ± 0.06	138	10.49 ± 0.06	136	0.01
Female	8.85 ± 0.06	48	10.29 ± 0.09*	68	0.01
Male	8.85 ± 0.06	90	10.69 ± 0.08*	68	0.01

Pre-adult development time of the *dacer* mutant and w1118 flies was assayed. The development time of a fly in days was calculated as the time interval between the midpoint of the egg collection period and the midpoint of the 12-h period during which the fly emerged as an adult

P-value, the probability of significant difference in the developmental durations between control and mutant *Drosophila* flies (*t* test)

n number of individuals observed

* Significant difference between developmental duration of females and males of the *dacer* mutant (*t* test, $P < 0.05$)

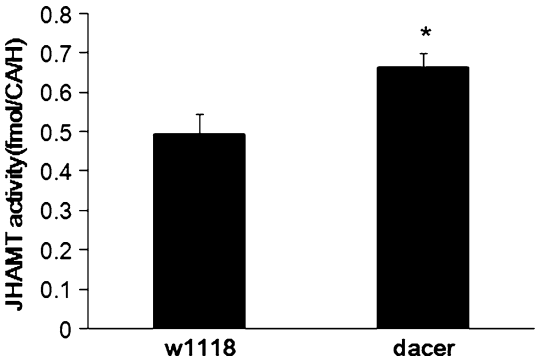

Fig. 6 The *dacer* mutant flies have enhanced JHAMT activity. Purified JH III acid and [^{3}H]-SAM were dissolved in sodium phosphate solution. CAs were isolated from wandering larvae and homogenized in the sodium phosphate buffer. After centrifugation, the supernatant was transferred to an equal volume of reaction buffer containing JHA and [^{3}H]-SAM. The enzymatic reaction was incubated at 28°C for 90 min and then was stopped by the addition of an equal volume of methanol. The radiolabeled product was extracted with 1 ml of hexane and counted with a liquid scintillation counter. Data represent the mean value ± SE of JHAMT activity (fmol/CA/h). Three independent experiments were performed in duplicate

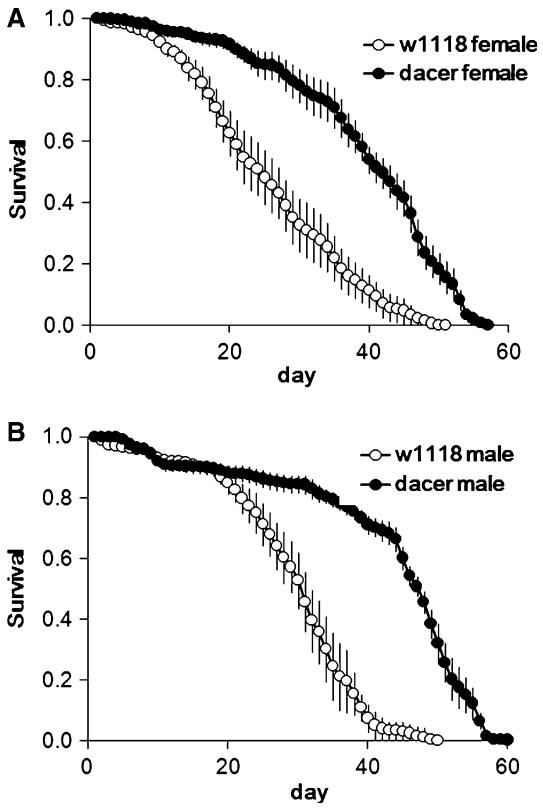

Fig. 7 The *dacer* mutant flies have extended lifespans; 213 mutant and 232 control female as well as 182 mutant and 173 control male eclosion flies were collected. Male and female flies were maintained separately in individual vials on normal food and transferred to fresh vials every 3 days. Survivors were scored every 24 h. Longevity curves of w1118 control flies (*open circles*) and of *dacer* mutant flies (*filled circles*) are shown. For statistical analysis, the mean and maximum lifespan of each strain was calculated from the time (in days) at which survival reached 50 and 10% of the starting population

were 128.0 ± 6.0 ($n = 100$) and 235.8 ± 4.7 ($n = 10$) h, respectively, for the mutant male flies and 60.5 ± 2.1 ($n = 10$) and 97.2 ± 1.2 ($n = 10$) h, respectively, for w1118 male flies (Fig. 8b). These results indicated that Dacer inactivation imparts resistance to oxidative stress for both male and female flies.

Dacer inactivation reduces stress-induced ROS production in the mitochondria

Increasing studies demonstrated that ROS production is one of the key determinants of *Drosophila*'s lifespan [30]. Therefore, we measured the rate of production of hydrogen peroxide (H_2O_2) in live mitochondria from *dacer* and control adult flies living on normal diets at different ages. As shown in Fig. 9a, at day 1, the amount of H_2O_2 produced by *dacer* and control mitochondria was similar. However, by day 5, the amount of H_2O_2 was slightly lower in the *dacer* mitochondria as compared with control mitochondria. This trend continued until at least day 20. This suggests that during the aging process, the ROS production is slightly reduced in long-lived *dacer* mutant flies as compared to that in w1118 controls.

We showed that *dacer* mutants survived paraquat better than wild-type flies. To determine whether *dacer* mutants produce less ROS in response to paraquat than wild-type controls do, we compared the rates of H_2O_2 production in live mitochondria from *dacer* and control flies at 36 h after paraquat treatment. As shown in Fig. 9b, the production of mitochondrial H_2O_2 was significantly reduced in *dacer* mutants compared to w1118 controls, suggesting that

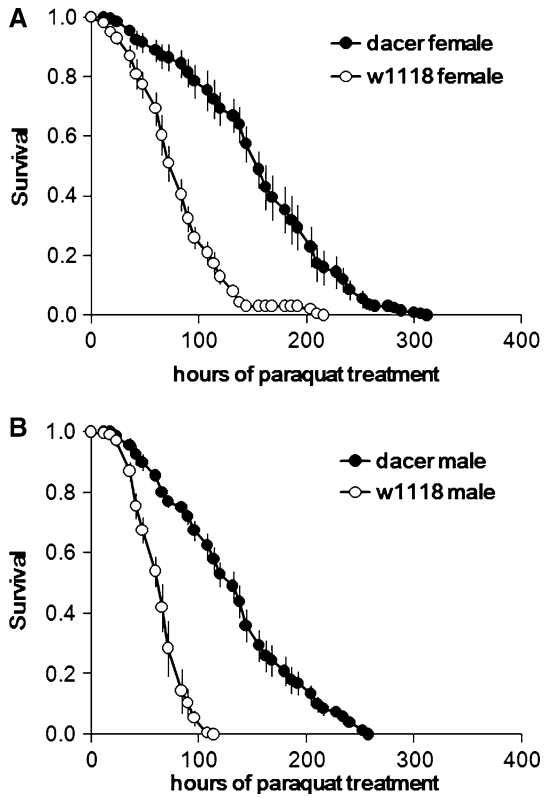

Fig. 8 The *dacer* mutant flies are resistant to oxidative stress. Three-day-old flies were transferred to vials containing standard cornmeal medium added with 20 mM paraquat (20 flies per vial). To measure the survival rate under oxidative stress, the lifespan of control and mutant flies on a paraquat diet was analyzed. Longevity curves of control w1118 flies (open circles) and of *dacer* mutant flies (*filled circles*) are shown. For statistical analysis, the mean and maximum lifespan of each strain was calculated from the time (in hours) at which survival reached 50 and 10% of the starting population

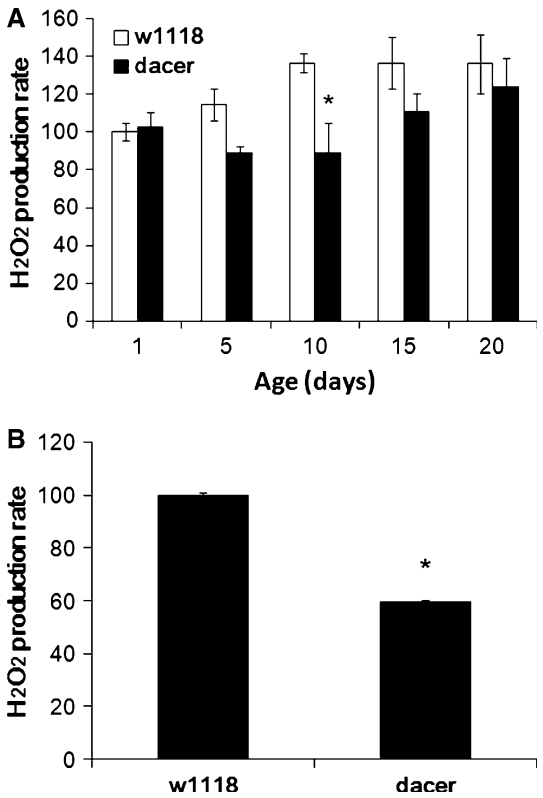

Fig. 9 The *dacer* mutant flies have decreased ROS production in the mitochondria. **a** Mutant and control eclosion flies were collected and maintained on normal food. Flies at different ages (1-, 5-, 10-, 15-, and 20-day-old) were kept on ice before isolation of mitochondria. Mitochondria isolated by using Tissue Mitochondria Isolation Kit were incubated at 25°C with 20 mM sn-glycerol 3-phosphate as substrate. Mitochondrial hydrogen peroxide production was measured at 560 nm using the Hydrogen Peroxide Assay Kit. The 100 value refers to control H_2O_2 production of mitochondria that was obtained from 1-day-old w1118 flies, and other values represent % of the control H_2O_2 production. **b** Three-day-old flies were collected and maintained on 20 mM paraquat-containing food. Mitochondria were isolated from *dacer* and control flies at 36 h after paraquat treatment. Mitochondrial hydrogen peroxide production was measured at 560 nm. The 100 value refers to control H_2O_2 production of mitochondria that was obtained from w1118 flies, and other values represent % of the control H_2O_2 production. All data represent the mean value of three independent experiments performed in duplicate

Dacer inactivation reduces the stress-induced production of ROS in mitochondria.

Dacer inactivation alters mitochondria metabolic function involved in motor activity

The mutant had greater vial wall-climbing ability than the wild type (data not shown). A recent study indicated that accumulating oxidative stress leads to compromised metabolic functions that induce the progressive decline on motor activity and early death [28]. Because *dacer* mutant flies have lower ROS production and an increased resistance to oxidative stress, we predicted that Dacer inactivation may alter mitochondrial metabolic functions that are involved in motor activity. Mitochondria are important organelles for cellular respiration and energy supply through ATP generation [31]. ATP levels have been routinely used as an indicator of metabolic function in *Drosophila* [32, 33]. Thus, ATP levels were measured in w1118 or *dacer* mutant adult flies. As shown in Fig 10, ATP levels were higher in both young and old *dacer* mutant flies compared to age-matched w1118 flies, suggesting that Dacer inactivation enhances *Drosophila* metabolic function.

Discussion

The *BWA* gene was identified as a gene whose mutations cause an abnormality in the structure of the mushroom body in *Drosophila*. However, the biochemical properties and physiological function of the product of the *BWA* gene

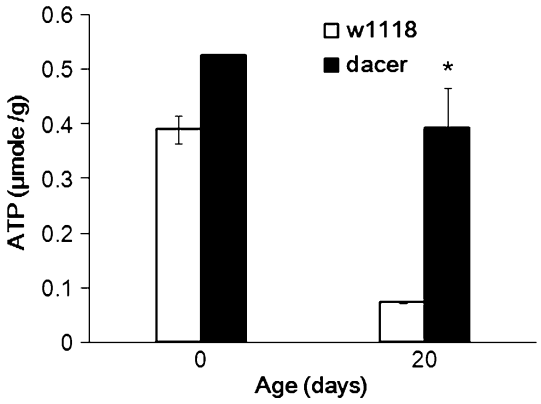

Fig. 10 The *dacer* mutant flies have increased ATP levels. ATP levels in the flies were determined by luciferin-luciferase system. Young (day 1) and old flies (day 20) on the normal diet were washed thoroughly in PBS and homogenized. The levels of ATP in homogenates were estimated by luminometer. The ATP levels in control (*open bars*) and mutant flies (*filled bars*) are shown. The error bars of the ATP levels in young *dacer* mutant and old w1118 flies are too small to see

have been unclear. Protein sequence alignment revealed that the protein product of the *BWA* gene shares sequence similarity with the alkaline ceramidases that we previously cloned from yeast (YPC1p and YDC1p), mice (Acer1, Acer2, and Acer3), and humans (ACER1, ACER2, and ACER3). BWA is closest in protein sequence to ACER2 and Acer2. Similar to ACER2, we demonstrated that BWA has the highest in vitro ceramidase activity at pH values around 8. Like ACER2 and other alkaline ceramidases, BWA has multiple putative transmembrane domains, suggesting that it is a membrane-bound protein. Based on its similarity in protein sequence and enzymatic activity to known alkaline ceramidases, we concluded that BWA is a *Drosophila* alkaline ceramidase, which we renamed Dacer. Unlike mammals that express three alkaline ceramidase genes, *Drosophila melanogaster* has no other homologues of Dacer. This simplicity makes *Drosophila* an ideal model for studying the physiological and pathological roles of alkaline ceramidases.

Our previous studies demonstrated that mammalian alkaline ceramidases play an important role in regulating the metabolism of sphingolipids [11]. In this study, we showed that Dacer inactivation leads to an increase in ceramides in both pupae and adult flies, suggesting that, similar to mammalian alkaline ceramidase, the *Drosophila* alkaline ceramidase also plays an important role in controlling the metabolism of *Drosophila* ceramides. Because ceramides can be converted to glycosphingolipids, ceramide-phosphoethanolamines (sphingomyelin analogues), or ceramide-1-phosphate, Dacer inactivation may also result in increases in the levels of these ceramide-containing sphingolipids. This possibility is currently under investigation. Interestingly, Dacer inactivation did not cause a decrease in the levels of its products, free sphingoid bases, in either pupae or adult flies (data not shown). This indicates that Dacer inactivation may increase the generation of free sphingoid bases by a different ceramidase, such as the neutral ceramidase, or decreases the conversion of free sphingoid bases to their phosphates.

By qPCR analysis, we demonstrated that Dacer mRNA is highly expressed at much higher levels in pupae than in adult flies. One would expect that the levels of ceramides should be lower in pupae than in adult flies, but the opposite is true. This suggests that pupae may have a much higher capacity in the de novo synthesis of ceramides than adult flies do. The high expression of Dacer may prevent ceramides from being accumulated in the cellular compartment(s) where Dacer resides. This role of Dacer may be physiologically relevant because too much ceramide may cause an organelle stress.

It was shown that the structure of the ellipsoid body of *Drosophila melanogaster* was altered in *dacer* mutants [24]. Our preliminary studies with electron microscopy did not show obvious abnormalities in the structure of the brain in the *dacer* mutant (data not shown), although with qPCR we demonstrated that Dacer mRNA is expressed in the *Drosophila* brain. The effect of the Dacer mutation on the ellipsoid body and other functional zones warrants more thorough investigation.

In this study, we showed that the development time of the immature stages from egg to adult eclosion increased significantly in *dacer* mutants compared to w1118 controls. In *Drosophila*, the timing of adult emergence depends upon ecological factors, including temperature, photoperiod, humidity, food, intrinsic factors, and hormones [34]. JHs are synthesized de novo in specialized endocrine glands, corpus allatums (CA). A strict regulation of the titer of JHs throughout the insect's life is critical to its successful development. Previous studies showed that the titer of ecdysone and JHs in the developing insect control its developmental stage. At the development stage, as long as there are enough JHs, the ecdysone promotes larva-to-larva molts [35]. With lower amounts of JHs, the ecdysone promotes pupation, and complete absence of JH results in the formation of the adult [36]. JHAMT activity has been shown to positively correlate with levels of JHs in larval insects [27]. We found that JHAMT activity was significantly increased in *dacer* mutant larvae compared to the wild-type larvae, suggesting that Dacer inactivation may significantly increase JHs in larvae. These observations suggest that Dacer inactivation delays pre-adult development probably because of a delay in lowering JHs in developing insects. How Dacer inactivation increases JHAMT activity remains unknown.

In this study, we found that the lifespan of the *dacer* mutant flies was greatly extended compared to wild-type

flies. Interestingly, the yeast mutants deficient in alkaline ceramidase YDC1, the yeast homologue of Dacer, also have an increased chronological lifespan compared to wild-type yeast strains [37], whereas YDC1 overexpression leads to reduced chronological lifespan [38]. These results suggest that the role of alkaline ceramidase in controlling lifespan is conserved from low eukaryotes to high eukaryotes. We demonstrated that the inactivation of Dacer caused a significant increase in the levels of ceramides. Because the inactivation of Dacer leads to increased ceramides in *Drosophila* without affecting the levels of free sphingoid bases, the increased lifespan of the *dacer* mutant may be associated with altered levels of ceramides and/or ceramide-containing sphingolipids (gluosylceramides, ceramide-phosphoethanolamines, and ceramide-1-phosphate). In line with this concept, Rao et al. [28] reported that an inactivation of the ceramide transfer protein gene (*Dcert*) reduces levels of ceramides and ceramide-phosphoethanolamines and significantly decreases the *Drosophila* lifespan.

The mechanism by which ceramides and/or ceramide-containing sphingolipids affect *Drosophila* lifespan is complex. Our data suggest that increasing the levels of ceramides and/or ceramide-containing sphingolipids, via inactivation of the alkaline ceramidase, extends *Drosophila*'s lifespan, probably via reducing the oxidative damage to mitochondria. We found that, during the natural aging process, the mitochondrial ROS production was lower in long-lived *dacer* mutant flies than in age-matched control flies. The difference in ROS production was more significant when the flies were challenged with paraquat-induced oxidative stress. These results suggest that an increase of ceramides and ceramide-containing sphingolipids reduces the production of mitochondrial ROS, especially in response to stress. Mitochondria have been suggested to be the main targets of ROS-induced oxidative damage [31]; thus, there is an inverse relationship between the production of mitochondrial ROS and mitochondrial function [39]. In line with this notion, we demonstrated that the ATP levels decline much more slowly during the aging process in *dacer* mutant flies than in wild-type flies. This suggests that increasing ceramides and ceramide-related sphingolipids attenuates the age-related decline in mitochondrial function by reducing ROS production. The production of ATP in mitochondria declines with age during the human aging process [40]. This also holds true for *Drosophila* [41]. Therefore, a decline in mitochondrial function due to an increase in ROS production may limit an organism's lifespan.

In addition to a reduction in mitochondrial damage by ROS, Rao et al. [28] showed that reduced ceramides in *dcert1* mutants increase plasma membrane fluidity, resulting in enhanced oxidative damage to cellular proteins. Because Dacer inactivation may increase the content of ceramides and ceramide-containing sphingolipids in the plasma membrane, it is plausible that Dacer inactivation increases the stiffness of the plasma membrane, thus resulting in increased resistance to ROS. Therefore, increasing the levels of ceramide and ceramide-related sphingolipids due to Dacer inactivation increases *Drosophila*'s lifespan also by reducing oxidative damage to other organelles.

It is generally believed that ceramides are stressful molecules that induce cell apoptosis or cell death. However, our data support the notion that ceramides, especially those targeted to degradation by Dacer, have a protective role in stress response in *Drosophila*. Indeed, emerging studies suggest that ceramides could be important anti-stress molecules, especially in invertebrates. Menuz et al. [42] demonstrated that loss of one of the ceramide synthase genes, *hyl2*, makes *Caenorhabditis elegans* more susceptible to anoxia and heat shock, suggesting that ceramide species synthesized by hyl2 have a protective role for animals under the stresses of oxygen deprivation and heat. Even in mammals, some ceramide species may have an anti-stress role. A very recent study by Senkal et al. [43] showed that tumor cells with decreased levels of ceramide carrying 16-carbon acyl chain (D-*erythro*-C_{16}-ceramide) die because of ER stress, suggesting that this ceramide species has a protective role in ER stress.

In conclusion, we demonstrate that the product of the BWA is a *Drosophila* alkaline ceramidase that plays an important role in the metabolism of ceramide, *Drosophila* pre-adult development, the aging process, and oxidative stress. This study may suggest new directions for experiments designed to unravel the physiological role of mammalian alkaline ceramidases.

Acknowledgments This work was supported by China's National Basic Research 973 Program 2009CB119203 (ZZ), NSFC grants 30528024 (CM), and MOE project 111 (CM), and the United States National Institutes of Health grant R01CA104834 (CM). We thank Qiang Li, Dongdong Niu, Weihua Wang, and Wenjuan Jiao for their technical assistance in fly colony maintenance. We also thank Dr. Jennifer Schnellmann for English proof reading and editing of the manuscript.

References

1. Acharya U, Acharya JK (2005) Enzymes of sphingolipid metabolism in *Drosophila melanogaster*. Cell Mol Life Sci 62:128–142
2. Mao CG, Obeid LM (2008) Ceramidases: regulators of cellular responses mediated by ceramide, sphingosine, and sphingosine-1-phosphate. Biochimica et Biophysica Acta (BBA). Mol Cell Biol Lipids 1781:424–434
3. Okino N, Ichinose S, Omori A, Imayama S, Nakamura T, Ito M (1999) Molecular cloning, sequencing, and expression of the gene

encoding alkaline ceramidase from Pseudomonas aeruginosa. Cloning of a ceramidase homologue from Mycobacterium tuberculosis. J Biol Chem 274:36616–36622
4. Okino N, Tani M, Imayama S, Ito M (1998) Purification and characterization of a novel ceramidase from *Pseudomonas aeruginosa*. J Biol Chem 273:14368–14373
5. Mao CG, Xu RJ, Bielawska A, Obeid LM (2000) Cloning of an alkaline ceramidase from Saccharomyces cerevisiae: an enzyme with reverse (CoA-independent) ceramide synthase activity. J Biol Chem 275:6876–6884
6. Mao CG, Xu RJ, Bielawska A, Szulc ZM, Obeid LM (2000) Cloning and characterization of a *Saccharomyces cerevisiae* alkaline ceramidase with specificity for dihydroceramide. J Biol Chem 275:31369–31378
7. Yoshimura Y, Okino N, Tani M, Ito M (2002) Molecular cloning and characterization of a secretory neutral ceramidase of *Drosophila melanogaster*. J Biochem (Tokyo) 132:229–236
8. Yoshimura Y, Tani M, Okino N, Iida H, Ito M (2004) Molecular cloning and functional analysis of zebrafish neutral ceramidase. J Biol Chem 279:44012–44022
9. Mao CG, Xu RJ, Szulc ZM, Bielawski J, Becker KP, Bielawska A, Galadari SH, Hu W, Obeid LM (2003) Cloning and characterization of a mouse endoplasmic reticulum alkaline ceramidase: an enzyme that preferentially regulates metabolism of very long chain ceramides. J Biol Chem 278:31184–31191
10. Olsson M, Duan RD, Ohlsson L, Nilsson A (2004) Rat intestinal ceramidase: purification, properties, and physiological relevance. Am J Physiol Gastrointest Liver Physiol 287:G929–G937
11. Xu RJ, Jin JF, Hu W, Sun W, Bielawski J, Szulc Z, Taha T, Obeid LM, Mao CG (2006) Golgi alkaline ceramidase regulates cell proliferation and survival by controlling levels of sphingosine and S1P. FASEB J 20:1813–1825
12. Segui B, Bezombes C, Uro-Coste E, Medin JA, Andrieu-Abadie N, Auge N, Brouchet A, Laurent G, Salvayre R, Jaffrezou JP, Levade T (2000) Stress-induced apoptosis is not mediated by endolysosomal ceramide. FASEB J 14:36–47
13. Osawa Y, Uchinami H, Bielawski J, Schwabe RF, Hannun YA, Brenner DA (2005) Roles for C-16-ceramide and sphingosine 1-phosphate in regulating hepatocyte apoptosis in response to tumor necrosis factor-alpha. J Biol Chem 280: 27879–27887
14. Sun W, Xu RJ, Hu W, Jin JF, Crellin HA, Bielawski J, Szulc ZM, Thiers BH, Obeid LM, Mao CG (2008) Upregulation of the human alkaline ceramidase 1 and acid ceramidase mediates calcium-induced differentiation of epidermal keratinocytes. J Invest Dermatol 128:389–397
15. Kango-Singh M, Halder G (2004) *Drosophila* as an emerging model to study metastasis. Genome biology 5
16. Fyrst H, Zhang XY, Herr DR, Byun HS, Bitttnan R, Phan VH, Harris GL, Saba JD (2008) Identification and characterization by electrospray mass spectrometry of endogenous *Drosophila* sphingadienes. J Lipid Res 49:597–606
17. Usta J, El Bawab S, Roddy P, Szulc ZM, Hannun YA, Bielawska A (2001) Structural requirements of ceramide and sphingosine based inhibitors of mitochondrial ceramidase. Biochemistry 40:9657–9668
18. Bielawski J, Szulc ZM, Hannun YA, Bielawska A (2006) Simultaneous quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. Methods 39:82–91
19. Van Veldhoven PP, Bell RM (1988) Effect of harvesting methods, growth conditions and growth phase on diacylglycerol levels in cultured human adherent cells. Biochimica et Biophysica Acta (BBA). Lipids Lipid Metabol 959:185–196
20. Sheng ZT, Ma L, Cao MX, Jiang RJ, Li S (2008) Juvenile hormone acid methyl transferase is a key regulatory enzyme for juvenile hormone synthesis in the Eri silkworm, Samia cynthica ricini. Arch Insect Biochem Physiol 69:143–154
21. Liu Y, Sheng ZT, Liu HH, Wen D, He QY, Wang S, Shao W, Jiang RJ, An SH, Sun YN, Bendena WG, Wang J, Gilbert LI, Wilson TG, Song QS, Li S (2009) Juvenile hormone counteracts the bHLH-PAS transcription factors MET and GCE to prevent caspase-dependent programmed cell death in *Drosophila*. Development 136:2015–2025
22. Lee HS, Lee KS, Yu K, Hong SY (2008) Expression of genes related to Parkinson's disease after paraquat treatment in *Drosophila melanogaster*. Pestic Biochem Physiol 92:19–23
23. Neretti N, Wang PY, Brodsky AS, Nyguyen HH, White KP, Rogina B, Helfand SL (2009) Long-lived Indy induces reduced mitochondrial reactive oxygen species production and oxidative damage. Proc Natl Acad Sci USA 106:2277–2282
24. Boquet I, Hitier R, Dumas M, Chaminade M, Preat T (2000) Central brain postembryonic development in *Drosophila*: Implication of genes expressed at the interhemispheric junction. J Neurobiol 42:33–48
25. Gilbert LI, Granger NA, Roe RM (2000) The juvenile hormones: historical facts and speculations on future research directions. Insect Biochem Mol Biol 30:617–644
26. Shinoda T, Itoyama K (2003) Juvenile hormone acid methyltransferase: A key regulatory enzyme for insect metamorphosis. Proc Natl Acad Sci USA 100:11986–11991
27. Kinjoh T, Kaneko Y, Itoyama K, Mita K, Hiruma K, Shinoda T (2007) Control of juvenile hormone biosynthesis in Bombyx mori: cloning of the enzymes in the mevalonate pathway and assessment of their developmental expression in the corpora allata. Insect Biochem Mol Biol 37:808–818
28. Rao RP, Yuan C, Allegood JC, Rawat SS, Edwards MB, Wang X, Merrill AH, Acharya JK (2007) Ceramide transfer protein function is essential for normal oxidative stress response and lifespan. Proc Natl Acad Sci USA 104:11364–11369
29. Lin YJ, Seroude L, Benzer S (1998) Extended life-span and stress resistance in the *Drosophila* mutant methuselah. Science 282: 943–946
30. Sedensky MM, Morgan PG (2006) Mitochondrial respiration and reactive oxygen species in *C. elegans*. Exp Gerontol 41:957–967
31. Saretzki G (2009) Telomerase, mitochondria and oxidative stress. Exp Gerontol 44:485–492
32. Chang KT, Min KT (2005) *Drosophila melanogaster* homolog of Down syndrome critical region 1 is critical for mitochondrial function. Nat Neurosci 8:1577–1585
33. Mandal S, Guptan P, Owusu-Ansah E, Banerjee U (2005) Mitochondrial regulation of cell cycle progression during development as revealed by the tenured mutation in *Drosophila*. Dev Cell 9:843–854
34. Qiu J, Hardin PE (1996) Developmental state and the circadian clock interact to influence the timing of eclosion in *Drosophila melanogaster*. J Biol Rhythms 11:75–86
35. Nijhout HF (1998) Insect hormones. Princeton University Press, Princeton
36. Klowden MJ (2007) Physiological systems in insects, 2nd edn. Academic Press, London
37. Powers RW, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S (2006) Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes Dev 20:174–184
38. Aerts AM, Zabrocki P, Francois IEJA, Carmona-Gutierrez D, Govaerta G, Mao C, Smets B, Madeo F, Winderickx J, Cammue BPA, Thevissen K (2008) Ydc1p ceramidase triggers organelle fragmentation, apoptosis and accelerated ageing in yeast. Cell Mol Life Sci 65:1933–1942

39. Van Remmen H, Richardson A (2001) Oxidative damage to mitochondria and aging. Exp Gerontol 36:957–968
40. Krishnan KJ, Greaves LC, Reeve AK, Turnbull DM (2007) Mitochondrial DNA mutations and aging. N Y Acad Sci 1100:227–240
41. Dubessay P, Garreau-Balandier I, Jarrousse AS, Fleuriet A, Sion B, Debise R, Alziari S (2007) Aging impact on biochemical activities and gene expression of *Drosophila melanogaster* mitochondria. Biochimie 89:988–1001
42. Menuz V, Howell KS, Gentina S, Epstein S, Riezman I, Fornallaz-Mulhauser M, Hengartner MO, Gomez M, Riezman H, Martinou JC (2009) Protection of *C. elegans* from anoxia by HYL-2 ceramide synthase. Science 324:381–384
43. Senkal CE, Ponnusamy S, Bielawski J, Hannun YA and Ogretmen B (2010) Antiapoptotic roles of ceramide-synthase-6-generated C16-ceramide via selective regulation of the ATF6/CHOP arm of ER-stress-response pathways. FASEB J 24