

# Ceramide transfer protein function is essential for normal oxidative stress response and lifespan

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Communicated by Dan L. Lindsley, University of California at San Diego, La Jolla, CA, May 29, 2007 (received for review January 31, 2007)

**Ceramide transfer protein (CERT) transfers ceramide from the endoplasmic reticulum to the Golgi complex, a process critical in synthesis and maintenance of normal levels of sphingolipids in mammalian cells. However, how its function is integrated into development and physiology of the animal is less clear. Here, we report the *in vivo* consequences of loss of functional CERT protein. We generated *Drosophila melanogaster* mutant flies lacking a functional CERT (*Dcert*) protein using chemical mutagenesis and a Western blot-based genetic screen. The mutant flies die early between days 10 and 30, whereas controls lived between 75 and 90 days. They display >70% decrease in ceramide phosphoethanolamine (the sphingomyelin analog in *Drosophila*) and ceramide. These changes resulted in increased plasma membrane fluidity that renders them susceptible to reactive oxygen species and results in enhanced oxidative damage to cellular proteins. Consequently, the flies showed reduced thermal tolerance that was exacerbated with aging and metabolic compromise such as decreasing ATP and increasing glucose levels, reminiscent of premature aging. Our studies demonstrate that maintenance of physiological levels of ceramide phosphoethanolamine by CERT *in vivo* is required to prevent oxidative damages to cellular components that are critical for viability and normal lifespan of the animal.**

aging | *Drosophila* | membrane fluidity | sphingolipids | sphingomyelin

Sphingolipids are important constituents of the plasma membrane, providing structural framework for plasma membrane organization (1). As in mammals, *Drosophila* sphingolipids are critical for cellular homeostasis including membrane-associated events, in developmental processes such as embryogenesis and gametogenesis, in differentiation as in neurogenesis, and enzymes of sphingolipid biosynthesis have been associated with signal transduction cascades and phagocytosis (2, 3). However, there are some differences between *Drosophila* and higher mammals. The major sphingoid bases in *Drosophila* and other dipterans are tetradecasphingenine (C14) and hexadecasphingenine (C16) compared with octadecasphingenine (C18) in mammals (2, 3). Also the *N*-linked fatty acids are of shorter length in *Drosophila* sphingolipids, whereas they are generally longer in mammals. Likewise, phosphoglycerolipid chain lengths are also shorter. These characteristics predict that membranes would remain fluid even at lower temperature and correlates well with the requirement of lower ambient temperatures for *Drosophila* survival (between 18°C and 20°C). Also, in *Drosophila*, ceramide phosphoethanolamine (CPE), a structural analog of sphingomyelin, substitutes for sphingomyelin in the plasma membranes and is involved in segregation of lipids into liquid ordered structures that coordinate signaling across the plasma membrane by segregating proteins for mediating a variety of interactions between a cell and its environment (4–6). Ceramide constitutes the backbone of sphingomyelin and CPE.

Several lines of evidence indicate that ceramides are synthesized in the endoplasmic reticulum and are transported to the Golgi complex for biosynthesis of sphingomyelin (7–10). Sphin-

gomyelin is mostly synthesized in the luminal surface of the Golgi complex (7, 11, 12). The transport of ceramide from the endoplasmic reticulum (ER) to the site of sphingomyelin synthesis is thought to be mediated by both ATP-dependent and -independent pathways (13, 14). Efforts to generate cell lines with defects in sphingolipid metabolism led to the generation and characterization of a Chinese hamster ovary cell line, LY-A (15). When grown in sphingolipid-deficient media, these cells synthesize <40% sphingomyelin compared with the control cell line. They are defective in the ATP-dependent transport of ceramide from the ER to the Golgi complex. Efforts to rescue this defect by infecting these cells with a retroviral library containing a human cDNA library led to the identification of the ceramide transfer protein (CERT), responsible for the transport of ceramide from the ER to the Golgi complex (16). The transport of ceramide to the Golgi complex is essential for sphingomyelin biosynthesis and probably other sphingolipids (10, 13, 15, 17). CERT is a 600-aa protein that is conserved across many species, although a clear homolog cannot be identified in the yeast (16). A non-ATP-dependent minor pathway can transport ceramide from ER to the Golgi that will allow the synthesis of a small fraction of cellular sphingomyelin (14, 17). As mentioned above, several elegant studies have unraveled biochemical aspects of CERT function. However, its relevance in the context of development and physiological functions of an animal remains unknown (18).

We now report the generation and *in vivo* characterization of a *Drosophila* mutant lacking the functional CERT gene (*Dcert*). Although the mutant flies are viable and fertile, they have a dramatic decrease in lifespan. Lipidomic analyses demonstrate that the mutant flies have decreased CPE levels. Biophysical measurement and electron microscopic examinations reveal structural changes in the plasma membrane of the mutant animals. Biochemical experiments demonstrate increased susceptibility of the mutant membranes to oxidative damage and defective oxidative stress response that worsened with aging. These findings are associated with a progressive decline in motor activity of the mutant animals and metabolic imbalance including decreased ATP levels and increased glucose, resulting in premature aging and death.

Author contributions: R.P.R. and J.K.A. designed research; R.P.R., C.Y., J.C.A., S.S.R., M.B.E., X.W., U.A., and J.K.A. performed research; J.K.A. contributed new reagents/analytic tools; R.P.R., A.H.M., U.A., and J.K.A. analyzed data; and R.P.R. and J.K.A. wrote the paper.

The authors declare no conflict of interest.

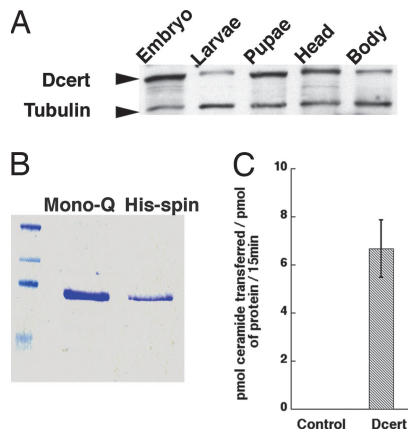
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Abbreviations: CERT, ceramide transfer protein; CPE, ceramide phosphoethanolamine; HNE, 4-hydroxy-2-nonenal.

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This article contains supporting information online at [www.pnas.org/cgi/content/full/0705049104/DC1](http://www.pnas.org/cgi/content/full/0705049104/DC1).

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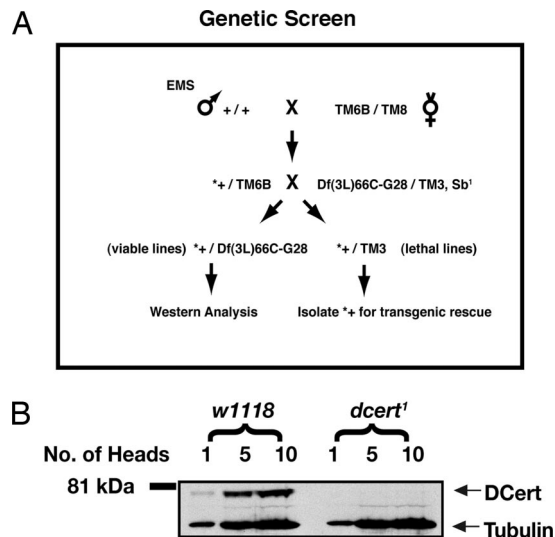
**Fig. 1.** *Drosophila melanogaster* encodes an authentic CERT (Dcert). (A) Dcert is ubiquitously expressed. Extracts were prepared from *Drosophila* during various stages of development as indicated, loaded on gel, and probed for Dcert protein. Blots were probed for tubulin as loading controls. (B) Dcert expression in S2 cells. The *Dcert* cDNA was cloned into pRMHAC vector, expressed, and purified by using ion-exchange Mono Q and nickel column (44). (C) Dcert has ceramide transfer activity *in vitro*. The assay was performed essentially as described (16). Dcert showed robust transfer of ceramide between liposomes ( $n = 3$ ).

## Results and Discussion

**Identification and Characterization of *Drosophila* CERT (Dcert).** Blast search of the *Drosophila melanogaster* genome at the Fly Base identified CG7207 as the *Drosophila* homolog of the CERT gene and was named *Dcert*. *Dcert* localized to 66C3–4 on the left arm of the third chromosome. The *Dcert* is predicted to encode a 604-aa protein that is 43% identical and 60% similar to the mammalian proteins. Like the mammalian proteins, Dcert contains an N-terminal pleckstrin homology (PH) domain (amino acids 40–134), a C-terminal START (amino acids 375–596) domain and a middle coiled-coil-rich domain with an FFAT region (16) [see supporting information (SI) Fig. 7]. The PH domain in mammalian protein binds phosphatidylinositol 4-phosphate. The START domain, a member of the lipid-binding STAR domain family, binds to ceramide. The FFAT region can bind to the VAP protein implicated in trafficking from the ER (8). We generated polyclonal antibodies against the Dcert protein both in rabbit and chicken and also obtained monoclonal antibodies. Western blot analysis of the wild-type *Drosophila* demonstrated that the protein was expressed ubiquitously (Fig. 1A) and throughout development. Immunofluorescence localization studies showed that the Dcert protein localized to endoplasmic reticulum and the Golgi complex (SI Fig. 8A–C). The expression profile of the protein is consistent with the proposed role in general sphingolipid biosynthesis and transport of ceramide from the ER to the Golgi complex.

**Biochemical Function of Dcert.** To evaluate whether the *Drosophila* protein was an authentic CERT, we expressed a C-terminally FLAG-tagged Dcert in S2 cells. The protein was purified by using a two-step ion exchange and affinity chromatography purification protocol (Fig. 1B). The freshly purified protein demonstrated ceramide transfer activity comparable with that reported for the human protein (16) (Fig. 1C). The Dcert protein identified in this study is therefore an authentic ceramide transfer protein.

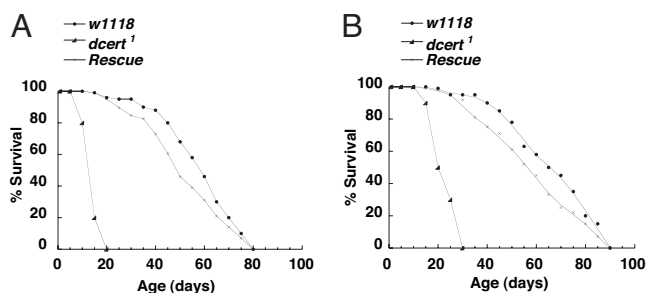
To evaluate the *in vivo* significance and relevance of the transport function of Dcert, we generated *Drosophila* flies lacking a functional Dcert protein and undertook a systematic phenotypic analysis of the mutant flies.



**Fig. 2.** Generation and isolation of *dcert<sup>1</sup>* mutant. (A) The genetic scheme to isolate *dcert<sup>1</sup>* mutant is outlined. In short, individual mutagenized viable lines were established over a deficiency uncovering the *Dcert* region and a representative fly from each was screened by Western blot analysis to isolate the mutant. (B) Western blot analysis of the *dcert<sup>1</sup>* mutant is shown. One, 5, and 10 head extracts were loaded on the gel and probed with the chicken anti-Dcert polyclonal antibody. The blot was probed for *Drosophila* tubulin as loading control. EMS, ethylmethane sulfonate.

**Generation and Isolation of a Functional Null for Dcert.** We performed a Western blot-based genetic screen and obtained functional null mutants, (Fig. 2A). It is an unbiased screen, does not rely on any phenotype, and is designed to isolate mutations in genes that result in either viable or lethal phenotypes (19, 20). Three thousand fifty-nine viable lines were established, and a single head from each of these lines was analyzed for lack of protein by Western blots. One of the lines lacked a detectable Dcert protein of expected size (Fig. 2B). Sequence analysis of this mutant showed a stop codon at amino acid position 376, and we predicted it to code for a truncated protein. The truncated protein lacks the ceramide binding START domain. Only 2–5% of comparable signal from the truncated protein was seen in long exposures of the blots (SI Fig. 9A). RT-PCR of *Dcert* from the mutant indicated that the mRNA was unstable, probably because of nonsense-mediated RNA degradation (21) (SI Fig. 9B). The observed decrease in mutant protein levels could thus be due to instability of both RNA and protein. We expressed and purified the truncated protein in S2 cells and found it had no demonstrable ceramide transfer activity (SI Fig. 9C and D). We called this mutant *dcert<sup>1</sup>*. We detected no difference in morbidity or viability between mutants homozygous for *dcert<sup>1</sup>* and *dcert<sup>1</sup>* transheterozygous flies over the deficiency [Df (3L) 66C-G28], indicating that *dcert<sup>1</sup>* was a functional null. Also, introduction of the *Dcert* gene into the mutant flies by transgenic rescue reversed all of the phenotypes described below, demonstrating that the residual protein had no dominant-negative effect or any other effect that could be detected in our study.

**dcert<sup>1</sup> Mutants Are Viable and Fertile but Have a Very Short Lifespan.** The mutant flies were viable and fertile and gave rise to apparently healthy progeny. However, we found that *dcert<sup>1</sup>* mutant animals had a greatly shortened lifespan compared with controls. The mutant flies began to die by  $\approx 10$  days of age, and all of the male flies were dead between 10 and 20 days of life (Fig. 3A). Although some of the females lived up to 30 days of age, they too underwent premature death (Fig. 3B). The decreased lifespan was completely rescued in *dcert<sup>1</sup>* flies carrying the



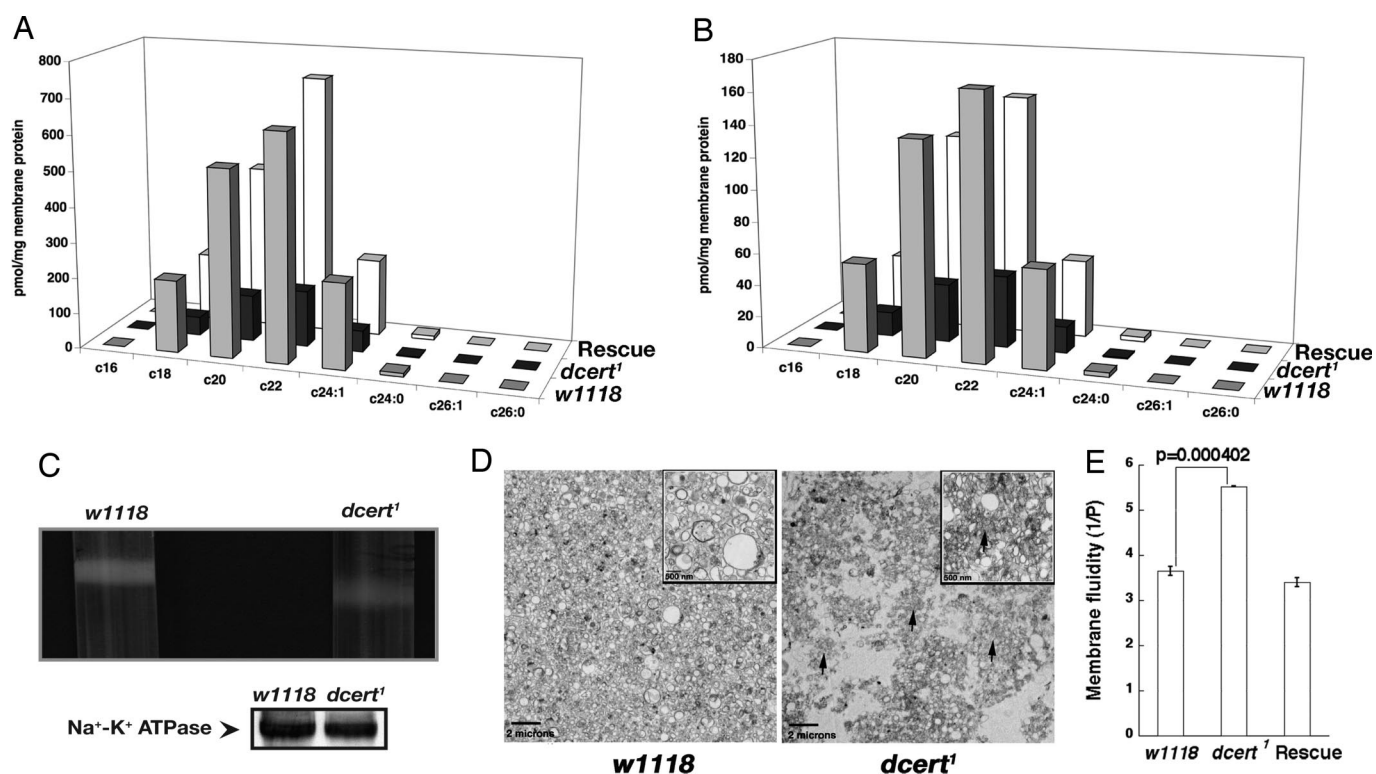
**Fig. 3.** *dcert1* have reduced lifespan. (A) *dcert1* mutant male flies have reduced lifespan, with most flies dead by day 20, whereas the controls live up to 80 days at 25°C. (B) Reduced viability of the *dcert1* female flies compared with *w1118* controls. The control female flies live up to 90 days of age, whereas *dcert1* were dead by 30 days of age. Introduction of the transgene rescued the phenotype in both instances (200 flies each time,  $n = 3$ ).

transgene. Thus, the loss of Dcert, a protein involved in sphingolipid metabolism, affected the lifespan of the flies. How does the loss of Dcert function that can potentially affect transfer of ceramide lead to a phenomenon of premature aging and death? The following paragraphs delineate our efforts linking the biochemical defect to the observed phenotype.

***dcert1* Mutants Have Defective Sphingolipid Metabolism.** The transfer of ceramide mediated by CERT is not a catalytic event, and the

transport is in molar equivalents (with respect to Dcert and ceramide, Fig. 1C), and therefore it is impractical to measure and compare ceramide transfer rates by using crude extracts from the control *w1118* and *dcert1* mutants. Instead, we measured the metabolite that is predicted to depend on the transport, namely CPE (the sphingomyelin equivalent in *Drosophila*) as a measure of CERT function. Additionally, we examined ceramide levels from control and mutant animals to evaluate whether defective transfer resulted in altered ceramide levels. We used liquid chromatography electrospray ionization tandem mass spectrometry for estimating CPE. As seen in Fig. 4A, the total CPE levels were decreased by >70% in the mutant animals. The ceramide levels were also decreased by >70% (Fig. 4B). The decrease in total ceramide suggests that transfer of ceramide from the ER to the Golgi complex by CERT tightly regulates ceramide levels *in vivo*.

**The Plasma Membrane in *dcert1* Mutants Have Altered Physical Properties.** Like sphingomyelin in mammalian cells, *Drosophila* CPE also localizes mostly to the plasma membrane (6, 22). Like sphingomyelin, it is a structural component of the plasma membrane. Decreased CPE levels in the mutants can therefore affect the integrity of plasma membrane. Hence, we examined plasma membranes isolated from the control and mutant flies. Plasma membranes were prepared from the control *w1118* and *dcert1* mutant flies by using density gradient centrifugation as described in *Methods*. Plasma membrane preparations from mutants were less dense (Fig. 4C) and less stable, readily diffusing into the surrounding Opti-prep medium. Transmission



**Fig. 4.** Plasma membrane preparations from *dcert1* demonstrate physical and biochemical alterations. (A and B) *dcert1* have reduced CPE levels (d14:1, CPE) (A) and reduced ceramide levels (d14:1) (B). The total CPE and ceramide levels were measured as described in *Methods*. These levels are restored in the mutants carrying the transgenic construct ( $n = 4$ ). (C) Density gradient centrifugation of the plasma membrane preparations of *dcert1* appears less compact and less dense when compared with those from *w1118* controls. The lower image shows Western blot analysis of the aliquots for plasma membrane marker, Na<sup>+</sup>-K<sup>+</sup> ATPase. In both instances, the two preparations contained equal amounts of total protein. (D) Transmission electron microscopic examination of plasma membrane vesicles from *w1118* controls show uniformly sized relatively homogenous population of vesicles in contrast to the less dense and relatively nonhomogenous population in the *dcert1* mutants. In several areas of the section, the plasma membrane vesicles from the *dcert1* mutant are collapsed (arrows). (Insets) The vesicles at higher magnification. (E) *dcert1* plasma membrane shows increased membrane fluidity as evidenced by decreased polarization of TMA-DPH ( $n = 7$ ). The increased fluidity is corrected by the introduction of a copy of the *Dcert* gene.

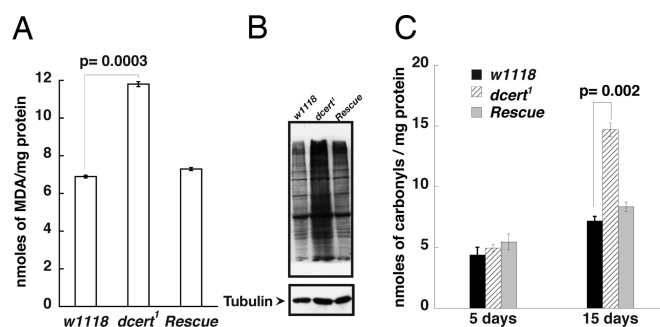


electron microscopic examination revealed fairly homogenous and well defined vesicles in the control *w1118* plasma membrane preparations (Fig. 4D). In contrast, the mutant plasma membrane vesicle preparations were loosely packed, less homogeneous, and nondiscrete, and a significant fraction of the vesicles appeared collapsed (Fig. 4D and *Inset*).

The less dense plasma membrane suggested a decreased viscosity of these membranes. Membrane viscosity is inversely proportional to its fluidity. Membrane fluidity is proportional to the rotational and lateral diffusion rates of membrane components and can be measured by using fluorescent dyes and fluorescence anisotropic/polarization measurements (23). TMA-DPH [1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate] is an excellent fluorescent polarization probe, and the depolarization of its fluorescence can be easily monitored. It is routinely used for plasma membrane fluidity determinations (23, 24). Hence, we performed membrane polarization studies using TMA-DPH in plasma membrane preparations from *dcert<sup>1</sup>* and control flies. Membrane polarization measurements indicated an increase in fluidity of the plasma membrane from *dcert<sup>1</sup>* flies as seen in Fig. 4E. This was restored to normal levels in the rescued flies, confirming that loss of CERT function with the decreased CPE levels was responsible for increased membrane fluidity. These experiments suggest that lack of CPE in *dcert<sup>1</sup>* mutant plasma membranes alters the composition and renders them more fluid than controls.

***dcert<sup>1</sup>* Mutants Are Oxidatively Stressed.** Many of the properties of the plasma membrane can be attributed to its dynamic, fluid lipid bilayer structure. A recent study indicated that increased fluidity of the plasma membrane renders them susceptible to free radicals and oxidative damage (25). Because the *dcert<sup>1</sup>* mutants had increased membrane fluidity, we asked whether they were more susceptible to free radicals (reactive oxygen species). Isolated plasma membranes can be subjected to attack by radicals *in vitro*, and subsequent lipid peroxidation can be monitored by color reaction. The amount of lipid peroxide formed will be a function of susceptibility of the membranes to radical attack. Accordingly, we exposed isolated plasma membranes from the control *w1118* and the *dcert<sup>1</sup>* to iron/ascorbate-induced lipid peroxidation, and the lipid peroxidation products were estimated as thiobarbituric-reactive substances (TBARS) (26, 27). As seen in Fig. 5A, the plasma membrane preparations of *dcert<sup>1</sup>* were more susceptible to reactive oxygen radicals and generated more lipid peroxide products compared with the *w1118* controls. In contrast, the *dcert<sup>1</sup>* mutants carrying the transgene were comparable to the wild-type controls. Thus, our data supports the notion that decreased CPE in *dcert<sup>1</sup>* mutant flies correlates with increased membrane fluidity and renders them susceptible to actions of free radicals and reactive oxygen species.

Free radicals, including reactive oxygen species, are generated during metabolic reactions involving enzymes, autooxidation, heme proteins and mitochondrial electron transport (28). They are also generated by the interaction of the environment with the cell such as radiation, etc. (28). These reactive species will act on membrane lipids and generate lipid peroxidation products. The lipid peroxides can further aggravate oxidative stress by further oxidizing membrane lipids and other cellular constituents, including proteins. However, under physiological conditions, they are kept in check by antioxidant mechanisms and resistance of normal membranes to their action. If, indeed, the *dcert<sup>1</sup>* mutant plasma membranes were susceptible to attack by free radicals and reactive oxygen species as we have demonstrated *in vitro*, we should be able to demonstrate the products of increased lipid peroxidation and their effects on cellular components *in vivo*. A well characterized and extensively studied cytotoxic product of lipid peroxidation is 4-hydroxy-2-nonenal (HNE), which is thought to be responsible for many of the cytopathological

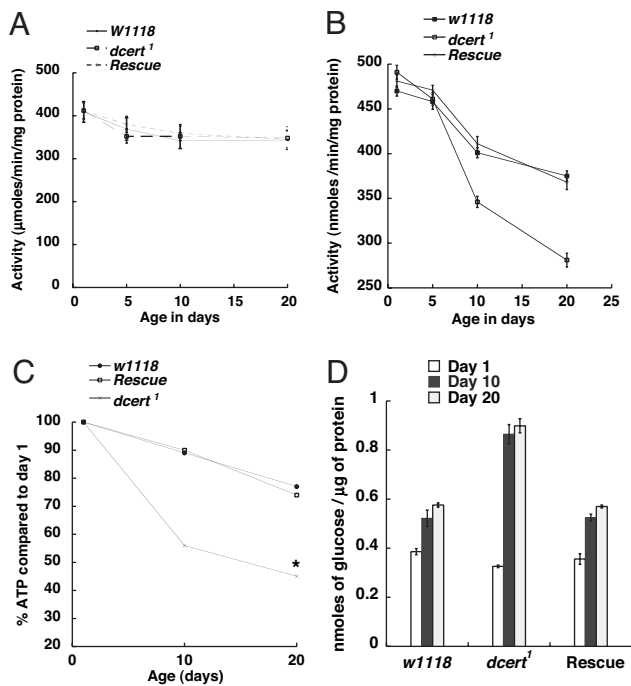


**Fig. 5.** Increased oxidative stress damage in *dcert<sup>1</sup>* cell extracts. (A) Plasma membranes were isolated by density gradient centrifugation as described in *Methods*, and the membrane fluidity was measured as the inverse function of fluorescence polarization. Each of these membrane preparations were subjected to lipid peroxidation by FeSO<sub>4</sub>/ascorbic acid, and the extent of lipid peroxidation was expressed as MDA equivalents per mg of membrane protein. (B) Western blot analysis of 15-day-old wild-type and *dcert<sup>1</sup>* flies with affinity purified antibody raised against 4-hydroxy-2-nonenal shows increased modification of cellular proteins in the *dcert<sup>1</sup>* mutants (seen as increased reactivity of all proteins in the extract to HNE antibody) compared with age-matched controls. The extracts were probed for tubulin as loading controls. (C) Although newly eclosed flies did not show significant difference in carbonylation of proteins, elevated levels were seen in 15-day-old *dcert<sup>1</sup>* extracts ( $n = 3$ ). In both experiments, the modification was corrected by the introduction of a copy of the wild-type gene.

effects observed during oxidative stress (29, 30). It acts in various ways, including hydroxynonenylation of proteins, a modification that can affect their physiological functions (31). Similarly, protein carbonylation is also an adverse consequence of oxidative stress, and its generation is aggravated by increased lipid peroxidation (32–34). We therefore examined the degree of hydroxynonenylation and protein carbonylation in *dcert<sup>1</sup>* mutants and control flies. There was a significant increase in hydroxynonenylation of mutant fly proteins compared with controls as seen in Western blots (Fig. 5B). Similarly, there was increased protein carbonylation in older mutant flies (Fig. 5C). These were corrected by the introduction of a copy of the wild-type gene (Fig. 5B and C). Thus lack of Dcert function leads to increased fluidity of the plasma membrane, as seen in the *dcert<sup>1</sup>* mutant, increased susceptibility of such membranes to reactive oxygen species, generation of lipid peroxidation products, reactive aldehydes that aggravate cellular stress by oxidizing cell membranes and proteins.

**Metabolic Dysfunction in *dcert<sup>1</sup>* Mutants.** Oxidative stress and modification of proteins by hydroxynonenal and protein carbonylation affect metabolic functions, and the details of the mechanisms involved are being extensively studied (35, 36). The *dcert<sup>1</sup>* mutant flies not only died early but also showed decreased mobility with increasing age. Older flies had a tendency to stay at the bottom of the vial and remain immobile for long periods of time. They also demonstrated decreased efficiency in a performance-evaluation test termed the negative geotaxis experiment (37, 38). *Drosophila* flies have an innate ability to climb up the walls of the vial when it is banged against a hard surface. This ability to climb up against the gravitational pull decreases with increasing age. We found that the *dcert<sup>1</sup>* mutant flies performed very poorly in this test, even in younger flies (SI Fig. 10).

We also examined whether accumulating oxidative stress insults in the mutant flies leads to compromised metabolic functions that could potentially explain the progressive decline in motor activity and early death. The mitochondrion is the powerhouse of the cell and generates most of the ATP required for metabolic functions. It is one of the primary targets of oxidative stress, and its function has been shown to decline with



**Fig. 6.** Metabolic dysfunction in *dcert<sup>1</sup>* mutant flies. (A) Citrate synthase activity is comparable between *dcert<sup>1</sup>* mutants and *w1118* controls at all three time periods tested ( $n = 4$ ). (B) *dcert<sup>1</sup>* mutant flies show 40% reduction in cytochrome C oxidase activity in 20-day-old flies, whereas controls and rescued flies have >80% of activity compared with newly eclosed flies ( $n = 4$ ,  $P < 0.0001$  between control and mutant on day 10 and 0.0002 on day 20). (C) *dcert<sup>1</sup>* mutant flies show a significant decrease in ATP levels compared with *w1118* controls at day 20 ( $n = 4$ ). \*,  $P = 0.0002$  compared with the control. (D) *dcert<sup>1</sup>* mutant flies show accumulation of glucose by day 10 and remain high at all ages tested ( $n = 4$ ,  $P = 0.0012$  on day 10 and 0.0002 on day 20 between control and the mutant).

aging (28). We therefore examined activities of two representative enzymes of mitochondria, namely citrate synthase and cytochrome C oxidase, involved in the tricarboxylic acid cycle and the electron transport chain, respectively. Although activities of citrate synthase showed no difference between the control *w1118* and the *dcert<sup>1</sup>* mutant flies (Fig. 6A), cytochrome C oxidase showed significantly lower levels of activity in 10-day and older flies (Fig. 6B). We believe our investigations indicate that there is a chronic and gradual loss of mitochondrial function in *dcert<sup>1</sup>* mutants. Although some of the enzymes such as cytochrome C oxidase are more susceptible to these perturbations, others, such as citrate synthase, are not affected.

ATP measurements have been routinely used as an indicator of metabolic function in *Drosophila* (39, 40). There was significant decrease in ATP levels in the mutant animals compared with the controls with aging, and these defects were corrected with the introduction of the *Dcert* gene (Fig. 6C). This result correlates with the decreased activity of cytochrome C oxidase that catalyzes the transfer of electrons to oxygen to generate water while coupling it to ATP generation. Thus, cumulative damage from products of oxidative stress ultimately results in compromised ATP generation in *dcert<sup>1</sup>* mutant flies.

We also compared the levels of triglyceride and glucose in the control and mutant animals to evaluate the generality of metabolic imbalance in *dcert<sup>1</sup>* flies. There was no significant difference in the triglyceride levels between the control *w1118* and the mutant *dcert<sup>1</sup>* flies at all ages tested (data not shown). However, total glucose content was increased in older *dcert<sup>1</sup>* flies compared with age-matched controls (Fig. 6D). The increased

glucose levels peaked at day 10 and remained high thereafter. The altered glucose homeostasis is reminiscent of metabolic disorders such as hyperglycemia and diabetes that have been associated with chronic oxidative stress and aging.

Our findings are consistent with a progressive decline in metabolic function and their regulation. We believe accumulating oxidative stress and associated alterations in protein function in aging *dcert<sup>1</sup>* flies leads to metabolic dysfunction, including decreased ATP generation and glucose utilization, compromised motor function, and premature death.

***dcert<sup>1</sup>* Mutants Do Not Undergo Neuronal Degeneration.** Several *Drosophila* mutations with defects that eventually lead to neuronal degeneration also result in decreased mobility of the flies that worsens with increasing neurodegeneration (41, 42). Flies undergoing neuronal degeneration exhibit vacuolation in the central nervous system, accumulation of lipid bodies, and disruption of the anatomy of the brain. We assessed whether oxidative stress was affecting the nervous system selectively as opposed to a general metabolic dysfunction and also to evaluate whether such a mechanism was responsible for sluggish mobility of aging *dcert<sup>1</sup>* flies. We compared sections of heads from 10-day-old control and mutant flies by transmission electron microscopy. We saw no visible changes of degeneration in the brain of *dcert<sup>1</sup>* mutant flies (SI Fig. 11). We conclude that sluggish movements of aging flies in *dcert<sup>1</sup>* is not due to specific neuronal degeneration that could have developed from oxidative stress.

**Reduced Thermal Tolerance in *dcert<sup>1</sup>* Flies.** The physical nature of the membrane–lipid matrix is thought to facilitate the perception of temperature, and plasma membranes are one of the primary biological sensors of temperature in an organism. Also, exposure to heat is known to increase oxidative stress (43). Because *dcert<sup>1</sup>* mutant flies have altered membrane fluidity and increased oxidative stress, we predicted they would be sensitive to effects of increasing environmental temperature. To test this, we exposed the control and mutant flies to 34°C and assessed their viability (flies grow optimally between 18°C and 25°C). Many 5-day-old control flies survived for up to 50 h at this temperature (SI Fig. 12A), whereas 25-day-old flies survived up to 25 h. However, 5-day-old *dcert<sup>1</sup>* flies survived to only ≈35 h and 15-day-old mutant flies were all dead by 15 h of exposure (SI Fig. 12B). Thus, the mutant flies showed increased susceptibility to thermal stress that worsened with aging. *dcert<sup>1</sup>* flies carrying the transgene were comparable with wild-type controls (SI Fig. 12C).

Although specific components of stress response signaling and antioxidant machinery are very important in controlling lifespan of higher organisms, our study highlights the significance of maintenance of the structural milieu of the cell. It demonstrates that perturbations in plasma membrane composition will have chronic and long-lasting effects on the viability of the organisms. These experiments demonstrate that decreased CPE levels in *dcert<sup>1</sup>* mutants resulted in increased membrane fluidity, and the altered physical nature of the membrane rendered them susceptible to environmental oxidative stress conditions.

A model depicting the etiopathogenesis for accelerated aging-like phenomenon and premature death of *dcert<sup>1</sup>* flies is illustrated in SI Fig. 13. Our study demonstrates that CERT-mediated transport of ceramide is critical for maintenance of normal CPE levels at the plasma membrane. This, in turn, ultimately has an impact on the quality and duration of lifespan of the animal. The changes in membrane properties, increased oxidative stress, and resulting decrease in physiological functions of mutant flies, including decreased locomotor activity, decreasing ATP levels, and increased glucose levels, are some of the commonly described features of aging.

## Methods

**Cloning, Expression, and Purification of Dcert in S2 Cells, Estimation of CPE and Ceramides.** *Dcert* gene was cloned in to pRMHA-C in-frame with the C-terminal FLAG tag and expressed in S2 cells by using standard techniques (44). CPE and ceramides were estimated from isolated membranes from *dcert*<sup>1</sup> and *w1118* flies by using a combination of HPLC and mass spectrometry. For details of the procedure see *SI Materials and Methods*.

**Assay for Ceramide-Transfer Activity.** Ceramide-transfer activity of Dcert was measured as described with the exception noted below (9). The reaction mixture containing the acceptor and donor vesicles was incubated with Dcert protein at 25°C instead of 37°C, because *Drosophila* thrive between 18°C and 25°C.

**Antibodies, Genetic Screen for Isolation of Dcert Mutants, and Negative Geotaxis Experiments.** The Western blot-based genetic screen and negative geotaxis assays were essentially done as described (19, 20, 45). For details see *SI Materials and Methods*.

**Isolation of Plasma Membrane, Fluorescence Polarization Measurements, Measurement of Plasma Membrane Peroxidation *in Vitro*, and Estimation of Carbonyl Content.** Plasma membrane was isolated by density gradient centrifugation (5). Fluorescence intensities in both parallel and perpendicular directions to the incident light were measured, and fluorescence polarization was calculated

(46). The susceptibility of plasma membranes from the wild-type control and mutant to lipid peroxidation was performed as described (26, 27). The protein carbonyl content of the flies was estimated as described (47, 48). For details see *SI Materials and Methods*.

**Preparation of Mitochondrial Fraction and Biochemical Measurements.** Flies were anesthetized, washed thoroughly in PBS, and homogenized in the homogenization buffer [20 mM Tris (pH 7.0) with 0.2 M sucrose]. The cell debris and nuclear fractions were removed by centrifuging at 1,500 × g for 10 min. The postnuclear supernatant was centrifuged at 13,000 × g for 15 min, and the mitochondrial pellet obtained was washed in the required assay buffer for the enzyme assays and lysed in the same buffer by freeze–thawing.

Commercially available kits were used in these measurements and performed according to the manufacturer's instructions. For details see *SI Materials and Methods*.

We thank Drs. Andrew Byrd and Sergey Tarasov for help with fluorescent polarization experiments; Kunio Nagashima and Jason De La Cruz for technical assistance; and Drs. Mark Fortini, Shyam Sharan, and Ira Daar for critical comments on the manuscript. This study was supported by the Intramural Research Program of the National Cancer Institute, National Institutes of Health (NIH), U.S. Department of Health and Human Services (R.P.R., C.Y., M.B.E., X.W., and J.K.A.), NIH Grant R01EY16469 (to S.S.R. and U.A.), and LIPID MAPS Consortium Grant GM69338 (to J.S.A. and A.H.M.).

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