Loss of CNDP causes a shorter lifespan and higher sensitivity to oxidative stress in Drosophila melanogaster

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ABSTRACT

Increasing oxidative stress seems to be the result of an imbalance between free radical production and antioxidant defenses. During the course of aging, oxidative stress causes tissue/cellular damage, which is implicated in numerous age-related diseases. Carnosinase (CN or CNDP) is dipeptidase, which is associated with carnosine and/or glutathione (GSH) metabolism, those are the most abundant naturally occurring endogenous dipeptide and tripeptides with antioxidant and free radical scavenger properties. In the present study, we generated Drosophila cndp (dCndp) mutant flies using the CRISPR/Cas9 system to study the roles of dCndp in vivo. We demonstrate that dCndp mutant flies exhibit shorter lifespan and increased sensitivity to paraquat or hydrogen peroxide (H₂O₂) induced oxidative stress. These results suggest that dCndp maintains homeostatic conditions, protecting cells and tissues against the harmful effects of oxidative stress in the course of aging.

INTRODUCTION

Aging is generally defined as the progressive decline in physiological integrity and function due to the accumulation of diverse deleterious changes in cells and tissues (Harman 2006; Tosato et al. 2007; Rando and Chang 2012). Consequently, aging is a major risk factor for a wide range of chronic, metabolic, and neurodegenerative diseases (Kennedy et al. 2014). Although the underlying mechanisms of aging have not been fully elucidated, oxidative stress has long been proposed as a significant driver of aging and age-related diseases (Harman 2006; Tosato et al. 2007).

In all aerobic organisms, normal physiological processes involving oxygen result in the production of reactive oxygen species (ROS). ROS play an important role in immune-mediated defenses against bacterial infection and the activation of cellular signaling to regulate various biological processes (Finkel and Holbrook 2000; Schieber and Chandel 2014). However, ROS also damage a wide variety of macromolecules, including proteins, lipids, and DNA (Finkel and Holbrook 2000; Tosato et al. 2007; Rando and Chang 2012). Therefore, aerobic organisms have a defense system that includes endogenous enzymatic and non-enzymatic antioxidant reactions to protect them against oxidative damage (Finkel and Holbrook 2000; Schieber and Chandel 2014).

Naturally occurring endogenous dipeptides containing histidine, such as carnosine, anserine, and homocarnosine, have antioxidant and free radical-scavenging properties (Bellia et al. 2014; Song et al. 2014). Carnosine, comprised of β-alanine and L-histidine, is the most abundant dipeptide. It is mainly present in neuronal tissue and skeletal muscle of...
vertebrates (Boldyrev et al. 2013). Carnosine has been shown to possess a number of critical properties including proton buffering capacity, antioxidant activity and chelating ability (Gaunitz and Hipkiss 2012; Boldyrev et al. 2013; Cararo et al. 2015; Hipkiss et al. 2016).

Carnosine is synthesized from β-alanine and histidine by carnosine synthase in many tissues and selectively degraded by carnosinase (CN), an intra- and extracellular dipeptidase (Boldyrev et al. 2013; Bellia et al. 2014). It has been reported that the dyshomeostasis of carnosine and CN causes several physiological dysfunctions and diseases, such as diabetes, ischemia, and neurological disease (Janssen et al. 2005; Bellia et al. 2011, 2014; Gaunitz and Hipkiss 2012; Boldyrev et al. 2013; Caruso et al. 2019). In humans, two types of CN have been identified, carnosine dipeptidase (CNDP) 1 and CNDP2 (also called cytosolic nonspecific dipeptidase) (Teufel et al. 2003; Bellia et al. 2014). CNDP1 protein is a secreted peptidase primarily found in the brain, liver, and plasma, and it has a substrate preference for carnosine and carnosine-like dipeptides (homocarnosine and anserine) (Bellia et al. 2014). In contrast, CNDP2 has a lesser activity toward carnosine and carnosine-like dipeptides compared with CNDP1. It exhibits a broad specificity toward various dipeptides, and is a ubiquitously expressed cytosolic enzyme (Teufel et al. 2003; Bellia et al. 2014).

Although the precise function of CNDP2 has not been defined, it may be a major Cys-Gly peptidase of the γ-glutamyl cycle and could play an essential role in glutathione (GSH) metabolism (Kaur et al. 2012; Bachhawat and Yadav 2018). GSH contributes to the scavenging of free radicals and peroxides, the chelation of heavy metals, and detoxification. It is present in all eukaryotic organisms, from yeasts to humans (Wu et al. 2004; Zhang et al. 2005).

A Cys-Gly dipeptide is a catabolite resulting from the cleavage of extracellular GSH by γ-glutamyl transpeptidase, which contrary to GSH itself, is a redox-labile metabolite (Corti et al. 2005; Dominici et al. 2005). Auto-oxidation of Cys-Gly dipeptides leads to pro-oxidant species, such as thiol and oxygen radicals (Accaoui et al. 2000; Dominici et al. 2005). Therefore, the degradation of Cys-Gly dipeptides by CNDP2 may be important for protecting cells and tissues from oxidative stress. Additionally, the degradation of Cys-Gly dipeptides supplies extracellular cysteine for the intracellular synthesis of GSH (Zhang et al. 2005; Bachhawat and Yadav 2018). Therefore, it is possible that both CNDP1 and CNDP2 play essential roles in maintaining homeostatic conditions and protecting cells and tissues against the damaging effects of oxidative stress. Furthermore, in humans, the CNDP1 and CNDP2 genotypes have been reported as genetic risk factors for obesity, diabetic nephropathy, neurological disorders, and cancer (McDonough et al. 2009; Ahluwalia et al. 2011; Bellia et al. 2011, 2014; Kurashige et al. 2013; Yamakawa-Kobayashi et al. 2017; Zhang et al. 2019), although oxidative stress and subsequent inflammation increase the risk of developing such diseases (Savini et al. 2013; McMurray et al. 2016; Rani et al. 2016).

In the present study, we used Drosophila melanogaster to elucidate the role of CNDP in vivo. In the Drosophila genome, there is a single human CNDP ortholog-encoding gene, CG17337 (hereafter called dcndp). We created a dcndp deletion mutant using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, and obtained another mutant with the insertion of a transposon into dcndp to analyze its effect on the lifespan of Drosophila melanogaster and its sensitivity to oxidative stress.

**MATERIALS AND METHODS**

**Fly stocks.** Flies were cultured at 25°C under a 12-hour light/dark cycle with the standard Drosophila cornmeal/sucrose/yeast medium. w^1118^ (#5905 in Bloomington Drosophila Stock center) was used as the control strain in the experiment to examine lifespan and sensitivity to oxidative stress. Also w^1118^ was used for quantitative RT-PCR (qPCR) to examine dcndp expression in the adult tissues.

y w; dcndp^14,0706^, called as dcndp^p^ hereafter, in which PiggyBac transposon was inserted in dcndp gene region (see Fig. 2A), was obtained from Kyoto Drosophila Genomics and Genetic Resources (#142168). The dcndp^p^ mutant was outcrossed into the w^1118^ background through crossing with w^1118^; Sp/CyO. We also generated dcndp mutant line, dcndp^Δ35bp^, in which 35 bp-deletion was introduced within coding region of dcndp gene region (see below).

**Construction of the phylogenetic tree.** Amino acid sequences of CNDPs were obtained from NCBI databases, and alignment and construction of the phylogenetic tree of CNDPs were performed using Molecular Evolutionary Genetics Analysis Version 7.0 (MEGA7) software (Kumar et al. 2016). The unrooted phylogenetic tree was constructed by the neighbor-joining method with 1000 replications.
CNDPs used in our computational analysis are as follows: human (*Homo sapiens*) CNDP1 (NP_116038.4) and CNDP2-isoform2 (NP_060750.2); mouse (*Mus musculus*) Cndp1 (NP_100323.3) and Cndp2 (NP_075638.2); frog (*Xenopus tropicalis*) cndp1 (NP_001107394.1) and cndp2 (NP_0032518.1); zebrafish (*Danio rerio*) zgc:114181 (NP_001296962.1) and cndp2 (XP_005170061.1); fruit fly (*Drosophila melanogaster*) CG17337/dcndp-RA (NP_610181.2); worm (*Caenorhabditis elegans*) Y71H2A.11 (NP_497606.4) and pes-9 (NP_506610.1); Brewer’s yeast (*Saccharomyces cerevisiae*) DUG1 (NP_116702.1).

### Generation of dcndp mutant line using CRISPR/Cas9 system

The CRISPR/Cas9 system was used to generate *dcndp* mutant line (Kondo and Ueda 2013; Bassett and Liu 2014). We designed the single-guide RNA (sgRNA) sequence using the Cas9 Target Finder (https://shigen.nig.ac.jp/fly/nigfly/cas9/). 5′-phosphorylated sense and antisense oligonucleotides corresponding to sgRNA target sequences were annealed and inserted into BbsI-digested pBFv-U6.2 vector. The vectors were injected into the embryos expressing Cas9 under the control of nanos promoter to induce germline mutations. Genetic crosses using *w1118*, Sp/CyO and PCR/sequencing screening were performed to isolate mutant lines with *w1118* background. We obtained *dcndp* mutant line in which the frame-shift 35 bp-deletion was introduced (see Fig. 2A and 2B). Oligonucleotides used in this experiment were as follows:

- **Sense**: 5′-ACCGTTGTGGGTATTCAGTCCG-3′
- **Antisense**: 5′-TGGATCCAGCACAGAACCAGGTC-3′

### qPCR

Total RNA was extracted from the adult whole body and seven tissues (brain, gut, malpighian tubule, ovary, testis, fat body, and muscle) using TRIzol (Thermo). Reverse-transcription was performed using SuperScript III (Invitrogen). cDNA was used as a template for qPCR using Quantifast SYBR Green PCR kit (QIAGEN) and Rotor-Gene Q (QIAGEN). The expression level of *dcndp* was normalized using an endogenous control, *ribosomal protein 49* (*rp49*), and the relative expression level was calculated (relative expression level = expression value of the gene of interest / expression value of *rp49*). The primer sets used for qPCR are as follows:

- **dcndp** *forward*, 5′-TTATCCTTGTGCCAGTGGGT-3′
- **dcndp** *reverse*, 5′-AGGCCGCAAGAAGTTTAGTG-3′
- **rp49** *forward*, 5′-CCACCAGTCGGATCGATA-3′
- **rp49** *reverse*, 5′-CACGTTGTGCACCAGGAAT-3′.

### Lifespan analysis

Newly eclosed male flies were collected within 24 h and divided into approximately 20 individuals per vial containing cornmeal/sucrose/yeast medium. Flies were transferred to new vials with cornmeal/sucrose/yeast medium every 4 days, and dead flies were counted every 2 days.

### Induction of the oxidative stress

Newly eclosed male flies were divided into approximately 20 individuals per vial, and maintained with cornmeal/sucrose/yeast medium for 5 days. Then, the flies were starved for 6 h on 1% agar, and transferred to vials containing 1.2% sucrose, 1% agarose, and 4 mM paraquat (1,1′-Dimethyl-4,4′-bipyridinium dichloride) (Sigma) or 0.1% hydrogen peroxide (H2O2). Paraquat and H2O2 induce oxidative stress by increasing the production of ROS in the body. Dead flies were counted every 12 h for the paraquat experiment or every 24 h for the H2O2 experiment.

### RESULTS

dcndp belongs to CNDP2 subgroup

First, we aligned the amino acid sequences of CNDPs in vertebrate, invertebrate, and yeast to construct the unrooted phylogenic tree. As shown in Fig. 1A, *Drosophila* CG17337/dcndp belongs to CNDP2 subgroup, suggesting the possibility that dcndp is an orthologue of vertebrate CNDP2. In addition, nematode and yeast CNDPs, Y71H2A.11 and pes-9 in *Caenorhabditis elegans* and DUG1 in *Saccharomyces cerevisiae*, were classified to CNDP2 subgroup. These results raise the possibility that invertebrate and unicellular organism possess only CNDP2.

In mammals, *CNDP1* is selectively expressed in the central nervous system and the liver, whereas CNDP2 displays ubiquitous expression (Teufel et al. 2003; Bellia et al. 2011, 2014; Oku et al. 2012). To examine the expression level of *dcndp* in the adult tissues, we performed qPCR using cDNA generated from RNA of adult tissues in *w1118* strain. As shown in Fig. 1B, *dcndp* expression was detected in all tissues tested (brain, gut, malpighian tubule, ovary, testis, fat body, and muscle), suggesting that *dcndp* is expressed ubiquitously in the adult stage. Together, these results suggest that *dcndp* is an orthologue of mammalian CNDP2.
To determine the role of \( dcndp \) in aging, we examined the lifespans of two \( dcndp \) mutant flies, along with \( w^{1118} \) as the control. We observed that both \( dcndp \) mutants had a short lifespan (Fig. 3). The average lifespan (± SD) of the control flies was 59.2 ± 16.0 days, while those of \( dcndp^{Δ35bp} \) and \( dcndp^P \) were 50.8 ± 12.7 and 40.3 ± 17.2 days, respectively (Fig. 3B). The differences in lifespans between the mutant and the control flies were statistically significant (\( P < 0.0001^{**} \), Log Lank test). This result suggests that aging in \( dcndp \) mutants may have accelerated.

\( dcndp \) mutants were highly sensitive to oxidative stress

Subsequently, we investigated whether the \( dcndp \) mutant flies exhibited higher sensitivity to oxidative stress. Flies were cultured on the medium with 4 mM...
**dcndp mutant and oxidative stress**

Fig. 2 Mutant alleles of *dcndp* generated by CRISPR/Cas9-mediated genome editing and Piggybac insertion. (A) The schematics of *dcndp* gene region. Dark and light gray boxes indicate coding sequences (CDS) and untranslated region (UTR), respectively, and black line indicates intron. Piggybac insertion (LL07964) and CRISPR/Cas9-mediated deletion sites are indicated by triangle and gray box, respectively. Note that *dcndp* encodes two putative transcriptional variant, *dcndp*-RA and *dcndp*-RC. (B) Sequences of the target site in wild type (WT) and deleted region in obtained mutant line, *dcndp*Δ35bp. The target site and the neighboring protospacer adjacent motif (PAM) are indicated by arrow and underline, respectively. Deleted sequences are shown by hyphen. (C) The expression level of *dcndp* in the control (*w*1118) and *dcndp*Δ35bp, in which Piggybac transposon is inserted in the first intron of *dcndp*-RA (see Fig. 2A), measured using qPCR. The expression level of *dcndp* was normalized by *rp49* expression level. Average values of five (control) and three (*dcndp*Δ35bp) independent analysis are shown with standard errors. Asterisk indicates the statistically significant difference (*P* < 0.001, Welch’s *t*-test).

Fig. 3 Lifespan was shortened in *dcndp* mutants. (A) Survival curves of the *w*1118 (rhombus), *dcndp*Δ35bp (square), and *dcndp*Δ (triangle). Adult male flies for the control and two *dcndp* mutants were maintained in cornmeal/sucrose/yeast medium, and dead flies were counted every 2 days. Sample sizes: Control (*w*1118), *n* = 103; *dcndp*Δ35bp, *n* = 254; *dcndp*Δ, *n* = 199. (B) The mean lifespan (± SD) of *w*1118, *dcndp*Δ35bp, and *dcndp*Δ. The differences in lifespan between each *dcndp* mutant and the control (*w*1118) were statistically significant (***P* < 0.0001, Log-rank test).
under 0.1% H$_2$O$_2$ exposure, both dcndp$^{Δ35bp}$ and dcndp$^P$ had a shorter survival time compared to the controls (Fig. 5A). The average survival time (± SD) post exposure to H$_2$O$_2$ was 136.5 ± 24.6 h in controls, whereas those of dcndp$^{Δ35bp}$ and dcndp$^P$ mutants were 118.3 ± 14.4 and 115.2 ± 16.8 h, respectively (Fig. 5B). The differences in survival time during paraquat (4 mM) or H$_2$O$_2$ (0.1%) exposure between mutant (dcndp$^{Δ35bp}$ and dcndp$^P$) and the control flies were statistically significant (*P < 0.01, **P < 0.0001, Log-rank test).

**Fig. 4** dcndp mutants were highly sensitive to paraquat induced oxidative stress. (A) Survival curves of $W^{1118}$ (rhombus), dcndp$^{Δ35bp}$, (square), and dcndp$^P$ (triangle) under paraquat exposure. Adult male flies for the control and two dcndp mutants were maintained in the medium containing 4 mM paraquat, 1.2% sucrose, and 1% agarose, and dead flies were counted every 12 h. Sample sizes: Control ($W^{1118}$), n = 74; dcndp$^{Δ35bp}$, n = 90; dcndp$^P$, n = 118. (B) The mean survival time during paraquat exposure (± SD) of $W^{1118}$, dcndp$^{Δ35bp}$, and dcndp$^P$. The differences in survival time between each dcndp mutant and the control ($W^{1118}$) were statistically significant (*P < 0.01, **P < 0.0001, Log-rank test).

**Fig. 5** dcndp mutants were highly sensitive to H$_2$O$_2$ induced oxidative stress. (A) Survival curves of $W^{1118}$ (rhombus), dcndp$^{Δ35bp}$, (square), and dcndp$^P$ (triangle) under H$_2$O$_2$ exposure. Adult male flies for the control and two dcndp mutants were maintained in the medium containing 0.1% H$_2$O$_2$, 1.2% sucrose, and 1% agarose, and dead flies were counted every 24 hours. Sample sizes: Control ($W^{1118}$), n = 255; dcndp$^{Δ35bp}$, n = 203; dcndp$^P$, n = 196. (B) The mean survival time during paraquat exposure (± SD) of $W^{1118}$, dcndp$^{Δ35bp}$, and dcndp$^P$. The differences in survival time between each dcndp mutant and the control ($W^{1118}$) were statistically significant (**P < 0.0001, Log-rank test).
were statistically significant (\(P < 0.01*\) or \(P < 0.0001**\), Log Lank test). Mutant flies were more sensitive to paraquat or \(\text{H}_2\text{O}_2\) exposure. These data suggest that \(dcndp\) is required for proper antioxidative responses.

**DISCUSSION**

Here, we demonstrated that two \(dcndp\) mutants (\(dcndp^{35bp}\) and \(dcndp^8\)) exhibited shorter lifespans, and increased sensitivity to paraquat- or \(\text{H}_2\text{O}_2\)-induced oxidative stress. Genetic mutations and environmental interventions that shorten the lifespan of organisms are often associated with a decreased resistance to oxidative stress in that organism (Tosato et al. 2007; Schieber and Chandel 2014). Therefore, our results suggest the possibility that a defect of \(dcndp\) can modify antioxidant status and influence the maintenance of homeostatic conditions in flies.

\(CNDP\) belongs to the M20 family of metallopeptidases, which are highly conserved between species, from bacteria to mammals (Teufel et al. 2003; Bellia et al. 2014). In humans, there are two types of structurally related \(CNDP\)s (\(CNDP1\) and \(CNDP2\)) that possess different characteristics (Teufel et al. 2003; Bellia et al. 2014). \(CNDP1\) is expressed in the brain and liver and has a narrow substrate spectrum. On the other hand, \(CNDP2\) is expressed in many tissues and has broad substrate specificity, including Cys-Gly dipeptide (Teufel et al. 2003; Kaur et al. 2012; Bellia et al. 2014; Bachhawat and Yadav 2018).

In the \(Drosophila\) genome, only one human \(CNDP\) ortholog-encoding gene, \(CG17337\), has been detected. Additionally, \(CNDP1\) enzyme activity has never been detected in invertebrate species (Oku et al. 2011) and the \(CNDP2\) sequence was generated. Although it was viable and developed normally, it showed metabolic alterations in its amino acid profile and increased intracellular carnosine levels (Schmohl et al. 2019). However, there is no report on knockout model animals for \(CNDP2\).

In the present study, we created a \(dcndp\) defective mutant using CRISPR/Cas9 and obtained another mutant with the insertion of a transposon into \(dcndp\) to investigate the roles of \(dcndp\) in vivo. \(Drosophila\) is an excellent model organism because most of its disease-causing genes and fundamental physiological processes are conserved in humans (O’Kane 2003; Smith et al. 2014; Wangler et al. 2015). Additionally, \(Drosophila\) has a very short lifecycle compared with other animals such as mice and Zebrafish. Therefore, \(Drosophila\) is an invaluable model organism for studying the complexity of the aging process (He and Jasper 2014). We expect that endogenous ROS levels increase in \(dcndp\) mutant flies. However, at present, we have no direct evidence that the loss of \(dcndp\) alters the endogenous carnosine and GSH levels, and effects on oxidative stress status. Further studies are required to assess the functional consequences of \(dcndp\) mutations in \(Drosophila\), as well as its role in antioxidative responses and the aging process. Additionally, investigating whether \(dcndp\) overexpression in \(Drosophila\) induces lifespan extension or resistance to oxidative stress would be insightful to understand the role of \(dcndp\) in the maintenance of homeostasis.

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**CONFLICT OF INTERESTS**

The authors declare that they have no conflict interests.

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