## DNA Methyltransferase Gene dDnmt2 and Longevity of Drosophila\*

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The DNA methylation program of the fruit fly *Drosophila melanogaster* is carried out by the single DNA methyltransferase gene *dDnmt2*, the function of which is unknown before. We present evidence that intactness of the gene is required for maintenance of the normal life span of the fruit flies. In contrast, overexpression of *dDnmt2* could extend *Drosophila* life span. The study links the *Drosophila* DNA methylation program with the small heatshock proteins and longevity/aging and has interesting implication on the eukaryotic DNA methylation programs in general.

DNA methylation at cytosines of the vertebrate genomes participates in the control of several interlinked biological processes, e.g. gene expression, cell growth, genomic imprinting, X chromosome inactivation, and embryogenesis, which in turn are mediated through chromatin remodeling (1, 2). The vertebrate DNA methyltransferases, including Dnmt1, Dnmt2, Dnmt3a, Dnmt3b, and Dnmt3L, are a family of proteins with highly conserved motifs in their carboxyl regions. Of these, Dnmt1 is the maintenance enzyme. Dnmt3a and Dnmt3b carry out the *de novo* methylation reaction. Unlike the above three enzymes, Dnmt2 lacks the N-terminal regulatory region (2). Although recent studies have demonstrated residual DNA methylation activities of the mammalian Dnmt2 proteins and the *Drosophila* ortholog dDnmt2 (3, 4), the latter of which is the single DNA methyltransferase responsible for genome methylation of the fruit flies (5-10), the functional role of the *Dnmt2* proteins has remained unclear. We present evidence below that *dDnmt2* is a regulator of the life span of Drosophila.

## EXPERIMENTAL PROCEDURES

Drosophila Strains and Culture Conditions— $dDnmt2^{GS12412}$  stock was a kind gift from Dr. T. Aigaki at Tokyo Metropolitan University. ywstrain, the recipient stock of Gene Search (GS) insertion, was used as an internal control in the longevity experiment. UAS-dDnmt2 transgene was inserted in pUAST vector as described (10). The reference background for transgenic lines was  $w^{1118}$ . UAS-dDnmt2 females were

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crossed with the *da-GAL4* males or  $w^{1118}$  males, and the life span of progeny was assessed. Flies were reared at 25 °C and maintained in vials containing standard cornneal agar medium. To monitor the life span, flies eclosed within 24 h were collected in single-sex groups of 20. They were then transferred to fresh medium and scored for survivorship once every 3 days until all of the flies died.

Stress Resistance—For thermal stress test, 4-day-old flies were transferred to new vials, maintained, and scored for survivorship at 36 °C. To examine the effect of starvation, flies were transferred to vials containing two 2.4-cm glass-fiber filter circles (Whatman) moisturized with 350  $\mu$ l of distilled water. The survivals were followed at 25 °C. Distilled water was added to keep the filters moist during the test. Paraquat resistance was performed as follows. Whatman glass-fiber filter circles soaked with 350  $\mu$ l of 20 mM paraquat (Sigma) in 5% sucrose solution were placed in clean empty vials. To minimize the variation generated by differential rates of paraquat intake, 3-day-old flies were first starved for 6 h and then transferred to vials containing the parauqat-wetted filters. The survival was scored at 25 °C in the dark.

 $RT^{I}$ -PCR Analysis—The expression levels of different genes were assayed by semiquantitative PCR. For this, total RNAs were isolated from adult flies of the four different genotypes UAS- $dDnmt2\langle I \rangle /+$ , UAS- $dDnmt2\langle I \rangle /+$ ;da-Ga14/+, yw, and  $dDnmt2^{GS12412}$ , respectively, and reverse transcribed for subscequent PCR reactions using DNA primers specific for different Drosophila genes. The PCR products were analyzed by agarose gel electrophoresis. Sequences of the primers are available upon request.

## RESULTS AND DISCUSSION

Ubiquitous Expression of UAS-dDnmt2 transgene driven by daughterless(da)-GAL4 resulted in 2–4-fold higher amounts of dDnmt2 in three independent UAS-dDnmt2/+;da-GAL4/+ lines. RT-PCR data of one of the lines, UAS-dDnmt2 $\langle 1 \rangle$ /+;da-GAL4/+, is shown in lanes 2 and 4 of Fig. 1A in comparison with the controls (lanes 1 and 3, Fig. 1A). The UAS-dDnmt2 $\langle 1 \rangle$ /+;da-GAL4/+ flies had a great increase of their mean life span (58%) in comparison with the da-GAL4/+ flies (Fig. 1A). Their mean life span was also significantly extended, by 16%, when compared with the UAS-dDnmt2 $\langle 1 \rangle$ /+ flies (Fig. 1A). The other two dDnmt2-overexpressing lines had a longer life span as well (data not shown).

In contrast to the above is a P element line  $dDnmt2^{GS12412}$ , in which a Gene Search vector (11) was inserted in exon 1 of the dDnmt2 gene.  $dDnmt2^{GS12412}$  is homozygous viable and the level of its dDnmt2 RNA is at least 50% lower than the control yw line, which is the recipient stock of the GS insertion (compare lanes 1 and 2, Fig. 1B; see also Fig. 2A). While no obvious phenotypes could be seen during development, homozygous  $dDnmt2^{GS12412}$  flies died more quickly than the heterozygotes with balancer. We followed up on this observation by examining the possible relationship between dDnmt2 gene expression and fly aging. As exemplified in Fig. 1B, life span measurements in comparison with yw showed that homozygous  $dDnmt2^{GS12412}$  line lived, on the average, 27% shorter than ywflies, with the average life spans being 54 days and 75 days, respectively.

Longevity genes in the *Drosopila* also mediate increased resistance to various stresses (12). We have first carried out the thermotolerance measurement but found no difference of the average survival at 36 °C, either between yw and homozygous  $dDnmt2^{GS12412}$  or between UAS-dDnmt2/+ and UAS-dDnmt2/+;da-GAL4/+. We then compared the different dDnmt2 lines

<sup>1</sup> The abbreviations used are: RT, reverse transcription; GS, Gene

Search; SOD, the superoxide dismutase gene.

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FIG. 1. A, life span extension by overexpression of dDnmt2. Median life spans and sample sizes (n) are as follows: UAS- $dDnmt2\langle 1 \rangle / +; da$ -GAL4 / +, 87 days and n = 300; UAS- $dDnmt2\langle 1 \rangle / +$ , 76 days and n = 205; da-GAL4 / +, 55 days and n = 200. B, the survival curves of homozygous  $dDnmt2^{GS12412}$  and control yw flies. The average life spans of homozygous  $dDnmt2^{GS12412}$  and yw are 57 days (n = 117) and 78 days (n = 134), respectively. C, flies were exposed to paraquat for examination of the sensitivity to oxidative stress. The survival percentages of different lines at 24 and 36 h of dieting are expressed by the bar histograph.



FIG. 2. RT-PCR anylysis. The data from RT-PCR analysis of six Drosophila genes, dDnmt2 (A), tubulin (B), Spatzle (C), Hsp22 (D), Hsp23 (E), and Hsp26 (F), are shown here. The PCR products loaded in different lanes were derived from total RNAs isolated from flies of the genotypes  $UAS-dDnmt2\langle 1\rangle + (lanes 1), UAS-dDnmt2\langle 1\rangle + ; da-Gal4/ + (lanes 2), yw (lanes 3), and dDnmt2^{GS12412} (lanes 4), respectively.$ 

described above with their respective controls for the resistance to starvation. In *Caenorhabditis elegans*, the mutant daf-2 has extensive fat accumulation and exhibits marked increase in longevity, suggesting the link between metabolism and longevity (13). In Drosophila, some long life flies like the *mth* and *chico* mutants also showed higher resistance to starvation (12, 14). A 20% decrease of survival of the homozygous  $dDnmt2^{GS12412}$  females relative to the vw females was found. Also, the *dDnmt2*-overexpressing transgenic flies, when compared with the UAS control lines, showed an increase of 20% in the females and 15% in the males (data not shown).

Oxidative damage to DNA and proteins has also been implicated in a variety of degenerative diseases. Accumulations of oxidative damages are associated with aging too. To test the resistance to oxidative stress, we fed flies with the free radical generator, paraquat. At 36 h after being fed with diet containing the paraquat, the *dDnmt2*-overexpressing flies exhibited higher resistance to oxidative stress than the controls (Fig. 1C), but no obvious difference was observed between  $dDnmt2^{GS12412}$ and yw. Transgenic flies carrying extra copies of SOD and catalase genes, which function to eliminate reactive oxygen species, had an increased life span (see Ref. 15 and references therein). A null mutation in chico was homozygous viable, and the flies exhibited an increased life span as well as a higher level of the superoxide dismutase activity (14). Although our dDnmt2 overexpression flies showed more resistance to oxidative stress, the level of SOD gene expression in these flies was not altered (data not shown).

*dDnmt2* could regulate the *Drosophila* life span by modulating the expression of a set of genes, which may or may not consist of genes previously demonstrated to affect the life spans of yeast, C. elegans, mice, and Drosophila (15-19). We have tested by RT-PCR the levels of expression of several of these "longevity" genes including InR, an insulin-like receptor; chico, an insulin-like receptor substrate protein; mth, a G-proteincoupled receptor; and SOD, the CuZn superoxide dismutase, as mentioned above. No significant differences of expression of these genes were found among the Drosophila lines analyzed in Fig. 1 (data not shown). Interestingly, however, we found that the expression levels of several small heat shock proteins (sHsp)-encoding genes (Hsp22, Hsp23, and Hsp26) were coordinately changed in flies with altered amounts *dDnmt2* (Fig. 2). The levels were up-regulated, all by  $\sim$ 3-fold, in flies overexpressing *dDnmt2* (compare *lanes 2* with *lanes 1* in *D*–*F* of Fig. 2), and they were down-regulated, by 3-, 2-, and 3-fold, respectively, in flies with decreased amount of dDnmt2 (compare lanes 4 with lanes 3 in D-F of Fig. 2). It should be noted here that life span analysis of a precise excision line of  $dDnmt2^{GS12412}$  would conclusively demonstrate that the shortened life span of  $dDnmt2^{GS12412}$  is not due to sporadic mutation(s) generated during construction of the original P element library. However, the parallel effects on the Drosophila by dDnmt2 (this study) and by Hsp22 (22, 23) and our finding that the *Hsp22* gene is regulated by *dDnmt2* (Fig. 2) strongly suggest that the lengthening-shortening effects on the fly life span observed by us more likely resulted from the up- and-down-regulation of the dDnmt2 gene instead of sporadic mutation(s).

Small Hsps, like other Hsps, are molecular chaperones that are coordinately regulated by thermal stress and, more relevantly, they are involved in the aging processes (reviewed in Ref. 18). The levels of Hsp22, which is a mitochondrial protein, and the cytosolic Hsp26 are both higher in aged flies (20, 21). More importantly, overexpression of Hsp22 and the two cytosolic small Hsps, Hsp23 and Hsp26, all increased the life span of Drosophila (11, 18, 22). Decrease of Hsp22 expression due to P element insertion led to shortened Drosophila life span (23). Also, overexpression of Hsp22 and Hsp26 increase the resistance to oxidative and heat stress (22, 24). These studies all point to the beneficial effects of the small Hsps on longevity, presumably by preventing the aggregation of damaged and/or oxidized proteins (25). Our RT-PCR data in Fig. 2 suggest that one of the mechanisms for *dDnmt2* to regulate the *Drosophila* life span is by acting upstream of the expression of the small Hsp genes, at least those encoding Hsp22, Hsp23, and Hsp26. Thus, the dDnmt2 gene has emerged as a newly discovered player in the regulation of *Drosophila* life span.

The regulation of the animal life span is modulated by multiple, and likely interconnected, cellular signaling pathways (see Refs. 12-18 and 23 and references therein). While the details of the molecular and cellular basis of *dDnmt2* being a longevity gene awaits further investigation, our finding has uncovered a new function of the eukaryotic DNA methyltransferase gene family. Namely, a threshold level of expression of dDnmt2, a gene conserved among the flies, mammals, and yeast Schizosaccharomyces pombe (2, 5, 6), appears to be essential for the maintenance of a normal life span of Drosophila. While overexpression of exogenous mammalian DNA methyltransferases Dnmt1 and Dnmt3a in Drosophila caused fly lethality (26), elevation of the endogenous *dDnmt2* level actually increased the life span of the flies, as shown in this study. Whether the same function is carried out by the mammalian *Dnmt2* genes remains an intriguing question.

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