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Two SET domain containing genes link epigenetic changes and aging in *Caenorhabditis elegans*

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Summary

Changes in epigenetic status and chromatin structure have been shown to associate with aging in many organisms. Here, we report an RNAi screen of putative histone methyltransferases and demethylases in wild-type *Caenorhabditis elegans* using reproduction inhibitor. We identified six genes that when inactivated by RNAi, consistently extend lifespan. Five of these genes do not require germline proliferation to affect lifespan. We further characterized two of these genes, the highly homologous SET domain containing genes, *set-9* and *set-26.* They share redundant functions in maintaining normal lifespan, while exhibiting differential tissue expression patterns. Furthermore, we found that *set-9* and *set-26* partially act through the Forkhead box O (FOXO) transcription factor, DAF-16, to modulate lifespan. Interestingly, inactivation of somatic SET-26 alone results in a robust lifespan extension and alters the levels of histone H3 protein and the repressive histone marks, H3K9me3 and H3K27me3, in an age-dependent manner. We hypothesize that inactivation of SET-26 triggers compensation mechanisms to restore repressive chromatin structure and hence affects chromatin stability to promote longevity.

Key words: aging; *Caenorhabditis elegans*; chromatin; epigenetics; histone modification; SET domain.

Introduction

Epigenetic controls include DNA methylation, post-translational modifications of histone peptides, and others. These modifications can have a direct and complex impact on the chromatin structure, gene expression, and other DNA biology (Bonasio *et al.*, 2010). However, there are a few widely accepted characteristics of some modifications (Ruthenburg *et al.*, 2007). For example, histone tail hyperacetylation is generally associated with chromatin decondensation and active transcription (Hebbes *et al.*, 1988). Tri-methylated histone H3 lysine-4 and lysine-36 (H3K4me3 and H3K36me3) are usually coupled with gene activation and elongation, while tri-methylated histone H3 lysine-9 and lysine-27 (H3K9me3 and H3K27me3) usually associate with gene silencing and highly compacted heterochromatin (Bonasio *et al.*, 2010).

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Many studies have pointed to the possibility that epigenetic changes have a direct and functional impact on aging (Calvanese *et al.*, 2009). Vertebrate studies showed that older age is often associated with global decrease in DNA methylation, which opens up the heterochromatin (Singhal *et al.*, 1987; Wilson *et al.*, 1987). In addition to global changes, many individual histone modification or DNA methylation sites on specific gene promoters or chromosomal regions are also linked to aging and aging-related diseases, like cancer (Calvanese *et al.*, 2009). Presumably, sophisticated changes of the balance among many modifiers are responsible for these age-associated chromatin structure alterations. Interestingly, unbiased screens have identified genes that are functionally implicated in chromatin modifications to affect aging in the roundworm *Caenorhabditis elegans* (Hamilton *et al.*, 2005).

To investigate whether and how chromatin status is associated with aging in *C. elegans* and whether there are other specific chromatin modifiers directly linking epigenetic changes and lifespan, we carried out a small-scale RNAi screen of the majority of the putative histone methyltransferases and demethylases in worms. From the screen, we identified six longevity genes that, when inactivated by RNAi, can extend the worm lifespan. Among the six genes, we further characterized two highly homologous genes, *set-9* and *set-26*, and found that they have differential expression patterns but overlapping functions in normal lifespan maintenance. We also observed that *set-9* and *set-26* influence longevity partially dependent on *daf-16*, a prominent longevity gene that encodes the worm ortholog of the highly conserved Forkhead transcription factor Forkhead box O (FOXO) (Kenyon *et al.*, 1993; Antebi, 2007). Furthermore, inactivation of somatic *set-26* alone is sufficient to extend worm lifespan and affects histone protein levels and two repressive histone modifications in an age-dependent manner.

Results

Six putative histone methyltransferases and demethylases identified as longevity determinants by an RNAi screen

Our previous genome-wide RNAi screen revealed several putative chromatin modifiers to be important for longevity determination in *C. elegans*. In particular, we identified proteins containing characteristic domains that are important for either histone methylation or methylated histone recognition (Hamilton *et al.*, 2005). As high-throughput RNAi screens are prone to false negatives (Ni & Lee, 2010), we carried out a small-scale RNAi screen targeting putative histone methyltransferases and demethylases to search for additional epigenetic regulators involved in aging modulation. For the histone methyltransferases, we focused on genes predicted to contain a SET domain, a signature motif of most histone methyltransferases (Dillon *et al.*, 2005). For the histone demethylases, we concentrated on both the LSD1/amine oxidase family and the jmjC-domain containing family (Klose *et al.*, 2006). Through bioinformatics and literature search, we identified 38 SET domain containing genes (Andersen & Horvitz, 2007), 6 LSD1/amine oxidase genes, and 14 jmjC-domain containing genes in *C. elegans* (Klose *et al.*, 2006). We were able to confirm 50 (out of 58) correct RNAi clones (Supporting Information).

To assay the lifespan phenotype associated with knockdown of each of the 50 genes, wild-type N2 worms were fed individual RNAi bacteria

starting at the L1 stage of development, and their adult lifespans were monitored in the presence of the mitotic inhibitor 5-fluoro-2′-deoxyuridine (FUDR), which inhibits progeny production (Mitchell *et al.*, 1979). Whereas the majority of the RNAi treatments resulted in shortening or no significant change of lifespan, we found that RNAi knockdown of six genes reproducibly extended lifespan of the animals (Table 1A and Table S1). Four of the six genes, *set-9*, *set-26*, *rbr-2*, and *utx-1,* have recently been identified as longevity-associated genes in worms through either high-throughput or targeted screening methods (Lee *et al.*, 2003; Hamilton *et al.*, 2005; Greer *et al.*, 2010; Jin *et al.*, 2011; Maures *et al.*, 2011), thus validating the efficacy of our screen. It is important to note that *set-9* and *set-26* genes are > 95% identical and their corresponding RNAi clones likely target both genes simultaneously (Fig. 1A). We also discovered two new worm longevity genes, *mes-2* and *jmjd-2*, that have not previously been implicated in worm aging. From the screen, we also found four RNAi clones that caused obvious developmental defects or slow growth. We retested these RNAi clones by exposing worms to RNAi treatment after they had reached adulthood but did not observe any lifespan increase (Table S1).

While this work was underway, Greer *et al.* (2010) published an RNAi screen of a similar set of putative histone methyltransferases with a major distinction being that no FUDR was used in their lifespan assays. As a result, they identified several genes that require germline proliferation and reproduction to affect lifespan, in addition to *set-9* and *set-26* that are the only common genes from the two screens. To test whether our positive candidates from the screen can affect lifespan independent of germline proliferation, we utilized *glp-1(e2141)* mutant worms, which lack a germline at nonpermissive temperatures (Austin & Kimble, 1987). We found that five of the six RNAi clones (except *jmjd-2*) continued to extend the lifespan of *glp-1(e2141)* worms to a similar extent as in wild-type worms (Table 1B), suggesting that germline proliferation is not required for these genes to modulate lifespan. Our data suggest that *jmjd-2* requires germline proliferation but not continuous progeny production as blocked by FUDR to affect lifespan (Table 1A and Table S1).

SET-9 and SET-26 have similar gene structure but differential tissue expression patterns

As *set-9* and *set-26* knockdown exhibited strong effects on longevity independent of the reproductive status of the worms (Table 1), we sought to further characterize their functions. In addition to sharing very high identity at the level of nucleotide (97%) and amino acid (96%) sequences in the coding regions, *set-9* and *set-26* also have the same SL1 trans-splicing sites at their 5′ ends (Fig. 1A and Supporting Information). Furthermore, they share high homology in the noncoding sequences flanking the coding regions (near 90% identity in the +/− 500 bp regions). Although both SET-9 and SET-26 proteins contain a SET domain, the signature motif of most histone methyltransferases (Dillon *et al.*, 2005), the position of the SET domain within the polypeptides, and the absence of several critical residues important for enzymatic activities suggest that SET-9/26 likely lack direct methyltransferase activities (Qian & Zhou, 2006).

We examined the expression patterns of SET-9 and SET-26 using an antibody raised against a polypeptide downstream of the plant homeodomain (PHD) and SET domains (Fig. 1A). Because the two proteins are 96% identical, we predicted that the antibody would recognize both proteins. Consistent with this, immunoblotting results indicated that a approximately 250 kD band (apparent MW of SET-9 and SET-26) showed diminished signal, but was not absent, in the *set-26(tm2467)* mutant that

Table 1 Lifespan effects by the six positive candidates from the RNAi screen. (A) Six positive RNAi clones from the lifespan screen extend wild-type worm lifespan (20 °C). Representative data from three or more independent experiments are shown. (B) Five of the six positive RNAi clones extend *glp-1(e2141)* mutant worm lifespan at the nonpermissive temperature (25 °C). Data are pooled from two independent experiments

(A) Positive candidates from RNAi lifespan screen on wild-type (20 °C)

RNAi	Mean LS ± SEM	*N*	*P*-value vs. EV	% of EV LS	Domain	Homolog (species)
EV (empty vector)	19.05 ± 0.27	88	–	100	–	–
set-9/26	21.40 ± 0.33	95	< 0.001	112	SET	MLL5 (hs)
mes-2	20.29 ± 0.29	49	< 0.002	106	SET	E(Z) (hs, dm)
utx-1	21.63 ± 0.37	104	< 0.001	113	JmjC	UTX (hs, dm)
jmjd-2	21.55 ± 0.29	94	< 0.001	113	JmjC	KDM4 (hs, dm)
rbr-2	26.12 ± 0.34	105	< 0.001	137	JmjC	JARID1 (hs) Lid (dm)

(B) RNAi lifespan on wild-type (N2) and *glp-1(e2141ts)* worms (25 °C)

Strain/RNAi	Mean LS ± SEM	*N*	*P*-value vs. N2/EV	% of N2/EV LS	*P*-value vs. *glp-1*/EV	% of *glp-1*/EV LS
N2/EV	16.16 ± 0.18	193	–	100		
N2/*set-9/26*	19.65 ± 0.25	176	< 0.001	122		
N2/*mes-2*	17.20 ± 0.20	179	< 0.001	106		
N2/*utx-1*	19.25 ± 0.25	175	< 0.001	119		
N2/*jmjd-2*	18.04 ± 0.19	176	< 0.001	112		
N2/*rbr-2*	17.98 ± 0.23	168	< 0.001	111		
glp-1/EV	19.24 ± 0.22	171	< 0.001	119	–	100
glp-1/set-9/26	22.57 ± 0.32	182			< 0.001	117
glp-1/mes-2	20.61 ± 0.30	129			< 0.001	107
glp-1/utx-1	22.60 ± 0.28	160			< 0.001	117
glp-1/jmjd-2	19.09 ± 0.18	160			0.201	99
glp-1/rbr-2	21.07 ± 0.41	108			< 0.001	110

All survival analyses were performed with SPSS software using Kaplan–Meier analysis and log-rank test to compute *P*-values. A *P*-value ≤ 0.05 is considered statistically significant.

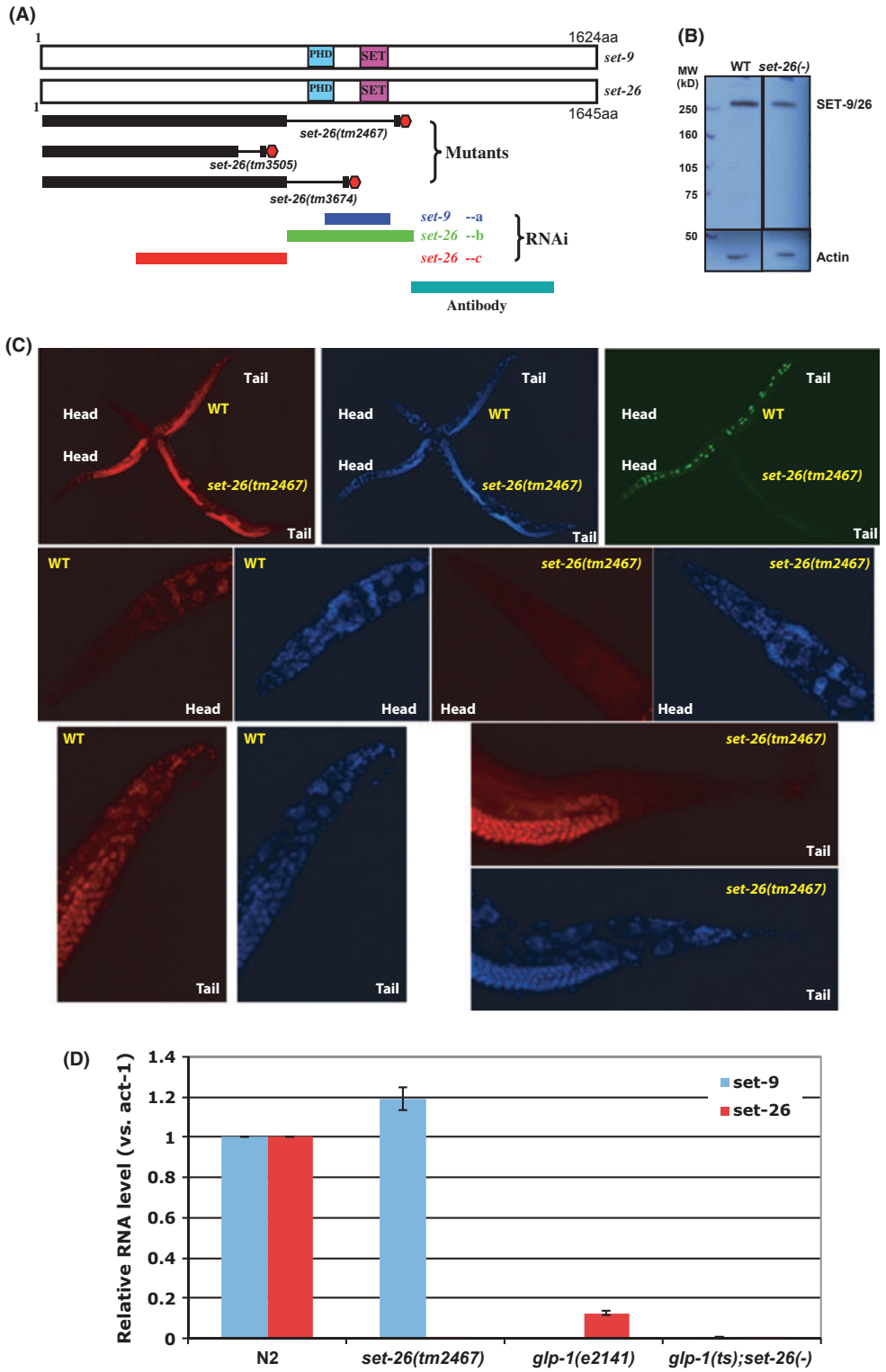

Fig. 1 *set-9* and *set-26* genes share high homology but have differential expression patterns. (A) Schematic linear protein structures of SET-9 and SET-26. Deleted regions of the three *set-26* mutant alleles were depicted as thin lines within thick black lines that indicate remaining coding regions. Premature stop codons caused by the deletions were depicted as '⬣'. Three RNAi constructs (a, b, and c) are shown aligned to their targeted sequences. The polypeptide used for antibody production is also shown. (B) Western Blot of equal amount of whole worm lysate from wild-type and the *set-26(tm2467)* mutant worms using SET-9/26 antibody. Actin is used as a loading control. (C) SET-9 and SET-26 are nuclear proteins. The wild-type worms (labeled with an irrelevant GFP transgene *sur-5::gfp*, Green) and the *set-26(tm2467)* mutant worms (no GFP) were co-stained with a purified SET-26 antibody (Red) and Hoechst (Blue, DNA). Zoom-in frames of the heads and tails were shown at the bottom. (D) Total RNA of young adult worms raised at 25 °C were subject to reverse transcription and qPCR using primer sets and thermocycles that can distinguish *set-9* and *set-26* transcripts. The RNA levels of *act-1* gene were used for normalization. Error bars show the difference between two independent experiments, and the pairwise two-tailed *t*-test was used to calculate the *P*-values.

was expected to have no expression of the antigenic region (Fig. 1B). The remaining signal likely represented SET-9 protein. Immunostaining in wild-type worms using the same antibody showed nuclear signal in most tissues, except spermatids (Fig. 1C and Fig. S1A, and data not shown). In contrast, signal was only found in the germline in the *set-26(tm2467)* mutant worms (Fig. 1C). The same pattern was observed in all four larval stages (data not shown). These suggest that SET-9 protein is predominantly expressed in the germline with very little to no expression in the somatic tissues. However, we could not directly test this because no true *set-9* loss-of-function mutant exists, as the only *set-9* mutant available contains a tandem deletion/duplication (Wormbase). Similar as *set-26(tm2467)*, two other *set-26* mutants that have deletions of the PHD and SET domains and premature stop codons (Fig. 1A and Supporting Information) exhibit the same expression pattern (data not shown).

To further distinguish the differential expression patterns between *set-9* and *set-26* genes, we designed primers and strategies to specifically amplify either *set-9* or *set-26* transcripts (Fig. 1D). In the *set-26(tm2467)* mutant, *set-26* transcript was undetectable, and the level of *set-9* RNA remained largely the same as in wild-type worms (*P*-value 0.184). In the germlineless *glp-1(e2141)* worms raised at a nonpermissive temperature, consistent with the notion that SET-9 is likely to be a germline protein (Fig. 1C), we detected very little *set-9* RNA expression (Fig. 1D). Interestingly, we also detected a dramatic decrease of the *set-26* transcript level (down to approximately 12% of WT, *P*-value 0.01), suggesting that the majority of *set-26* is expressed in the germline with a small but significant amount expressed in the somatic tissues, as was observed by immunostaining (Fig. 1C). As a further corroboration, we observed that an RFP-tagged *set-26* transgene was expressed in both the germline and somatic tissues (Fig. S1B).

set-9 and *set-26* genes have redundant functions in maintaining normal lifespan

To rule out possible off-target effects associated with the *set-9/26* RNAi clones from the screen, we designed an additional RNAi construct (c) targeting a region of *set-9/26* that is not overlapping with the two existing RNAi constructs (a and b) from the screen (Fig. 1A). We found that all three constructs resulted in similar life extension effect in wild-type worms (> 20% extension, Fig. 2A and Table S2). The RNAi-treated worms showed no obvious growth or developmental phenotypes (Fig. S2E), suggesting that the longevity effect of *set-9/26* is not secondary to other developmental impairments.

We next examined whether the *set-26* mutation has a lifespan extension phenotype similar to what was seen with the *set-9/26* RNAi treatment. We found that the three *set-26(-)* single mutant worms all live moderately longer than wild-type worms (Fig. 2B and Fig. S2A,B). For the remainder of this study, we will focus on the *set-26(tm2467)* mutant. We note that the *set-26(tm2467)* single mutants did not live as long as wild-type worms that were fed *set-9/26* RNAi bacteria (21% vs. 34% in Fig. 2B, 7% vs. 22% in Fig. S2B), suggesting that *set-9* likely has a redundant role in lifespan determination. Consistent with this prediction, we found that *set-9/26* RNAi can further extend the lifespan of the *set-26(tm2467)* mutant, to a degree similar to that of wild-type worms treated with *set-9/26* RNAi (Fig. 2B and Fig. S2B). Taken together, our results suggest that *set-9* functions redundantly with *set-26* in lifespan maintenance.

Considering both the expression and lifespan data discussed thus far, we conclude that deletion of *set-26* alone in both the germline and soma has a small but significant effect on lifespan (Fig. 2B and Fig. S2A,B) and that the germline-expressed *set-9* also contributes to normal lifespan maintenance (Fig. 2B). Because neither *set-9* nor *set-26* were detected in *glp-1(e2141);set-26(tm2467)* double mutants cultured at the nonpermissive temperature (Fig. 1D and Fig. S1D), we reasoned that this double mutant strain can serve as a mimic of double deletion of both *set-9* and *set-26*, and that by comparing *glp-1(e2141)* vs. *glp-1(e2141);set-26(tm2467)*, we could deduce the functions of somatic SET-26. We found that deletion of *set-26* in the *glp-1(e2141)* background substantially extended the lifespan of *glp-1(e2141)* worms (19%, Fig. 2C). This finding is consistent with our earlier observation that *set-9/26* RNAi could prolong the lifespan of germlineless mutants (Table 1B, Fig. 2C) and suggests that somatic *set-26* alone plays a significant role in modulating longevity. As a further validation of this notion that somatic *set-26* has a direct role in longevity modulation, we found that somatic over-expression of a *set-26* genomic fragment in the *glp-1(e2141);set-26(tm2467)* double mutant was able to reverse the prolonged lifespan of the double mutant as compared to the *glp-1(e2141)* single mutant (Fig. 2D). Further experiments confirmed that this rescue of the lifespan phenotype is because of over-expression of wild-type SET-26 because RNAi knockdown of *set-26* expression can abolish the rescue effects by the transgene (Fig. S2C).

Because the lifespan extension effect by *set-9/26* RNAi is not germline dependent, we wondered whether initiating such RNAi treatment after development might still exert a lifespan extension phenotype. Interestingly, worms fed any of the three RNAi bacteria showed very little to no lifespan extension when RNAi treatment was started at the young adult stage (Fig. S2D). This suggests that the functions of *set-9/26* during development are important for normal adult lifespan maintenance.

SET-9 and SET-26 regulate lifespan in a partially *daf-16*-dependent manner

The FOXO family of transcription factors are highly conserved key converging points of several longevity pathways (Antebi, 2007). *Caenorhabditis elegans* has a single FOXO ortholog, *daf-16*, and mutants with *daf-16* deficiency exhibit shortened lifespan compared with wild-type animals (Kenyon *et al.*, 1993; Antebi, 2007). Very recent studies showed that *daf-16* functions downstream of a histone modifier to influence lifespan (Jin *et al.*, 2011; Maures *et al.*, 2011). Thus, we examined whether *set-9* and *set-26* interact with *daf-16* to modulate lifespan.

We found that *daf-16(mgDf47)* mutation could partially but not completely suppress the lifespan extension phenotype induced by *set-9/26* RNAi [19% extension in WT vs. 10% in *daf-16(mgDf47)*, *P*-value < 0.001, Fig. 3A], suggesting that SET-9/26 maintain normal lifespan partly by repressing DAF-16 activity. We note that our previous studies showed that *set-9/26* RNAi cannot significantly extend the lifespan of *daf-16(mgDf47)* mutant worms in the RNAi enhancing mutant *rrf-3(pk1426)* background (Hamilton *et al.*, 2005). Strain differences as well as the more frequent scoring and the increased statistical power gained by pooling multiple experiments in the current study likely explain the slight discrepancy. In addition, we observed that the *set-26(tm2467)* mutation also extended lifespan in a partially *daf-16*-dependent manner [19% extension in WT compared to 10% in *daf-16(mgDf47)* background, *P*-value < 0.001, Fig. 3B]. Similarly, we found that somatic *set-26* partially depends on *daf-16* to affect lifespan as well [18% vs. 6% extension by *set-26(tm2467)* in *glp-1(e2141)* vs. *glp-1(e2141);daf-16(mgDf47)*, *P*-value 0.001, Fig. 3C; 24% vs. 20% extension by *set-26(tm2467)* in *glp-1(e2141)* treated with control RNAi compared with *daf-16* RNAi, *P*-value < 0.001, Fig. S3A]. Overall, our data support the model that *set-9* and *set-26* act through *daf-16* to achieve part of its function in longevity modulation.

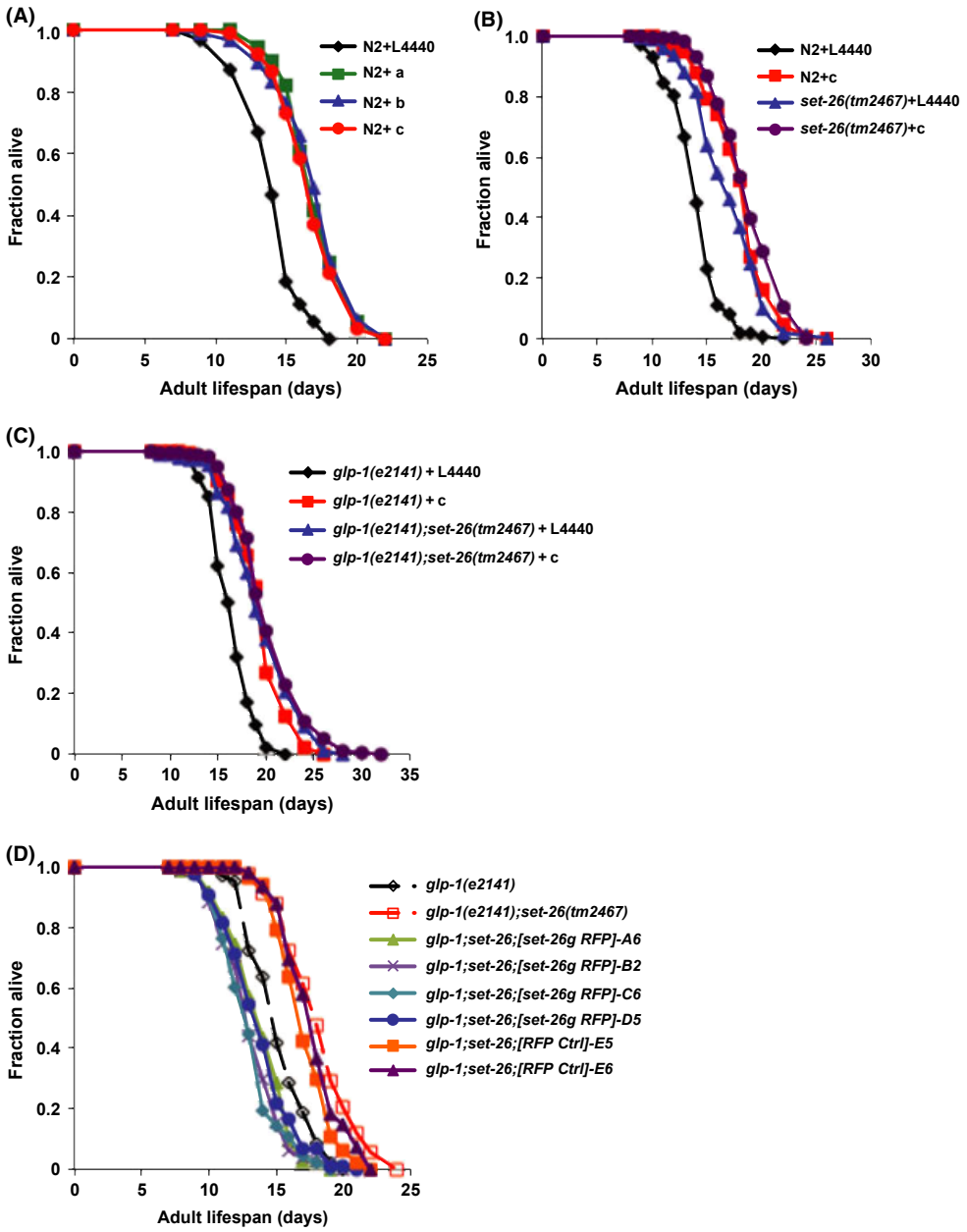

Fig. 2 *set-9* and *set-26* genes have redundant functions in maintaining normal lifespan. (A) Knockdown of *set-9* and *set-26* by three RNAi constructs (as shown in Fig. 1A) prolongs the lifespan of wild-type worms to a similar extent at 25 °C. (B) *set-26(tm2467)* mutant lives moderately longer than wild-type N2 worms when fed control RNAi bacteria (L4440), but has a similar lifespan as wild-type worms when fed *set-9/26* RNAi bacteria. (C) *set-26(tm2467)* mutation and *set-26* RNAi similarly extend the lifespan of the germlineless *glp-1(e2141)* worms at the nonpermissive temperature 25 °C. (D) Transgenic worms over-expressing the genomic *set-26* gene in *glp-1(e2141);set-26(tm2467)* background live shorter than their corresponding transgenic controls. Multiple lines of transgenic worms are used. The *set-26* over-expression lines are *glp-1(e2141);set-26(tm2467);rwEx21[pJKL702-unc-119(mini)-set-26g mec-7::RFP pBSK]* (lines A6, B2, C6, and D5) and the transgenic control lines are *glp-1(e2141);set-26(tm2467);rwEx22[pJKL702-unc-119(mini) mec-7::RFP pBSK]* (lines E5 and E6).

Somatic SET-26 affects global levels of histone expression and repressive histone modifications in an age-dependent manner

To directly examine whether chromatin structure is affected when *set-9/26* is inactivated, we monitored the global levels of several histone modifications that are well-characterized markers of chromatin structure. To avoid the complications by having hundreds of germ cells that are going through dramatic chromatin changes in wild-type worms (Kimble & Crittenden, 2005; Schaner & Kelly, 2006), we used *glp-1(e2141)* mutants at a nonpermissive temperature harboring either the wild-type or *tm2467* allele of *set-26* gene to investigate the effect on histone modifications caused by loss of somatic SET-26. Extracts from synchronized worms at Day 1 (young adult) and Day 12 (old adult) were subjected to immunoblotting to compare the levels of SET-26, histone H3, and several specific modifications of histone H3 in both genetic backgrounds and age groups (Fig. 4). We found that actin protein levels do not significantly change with age or with the *set-26* mutation and serves as an appropriate

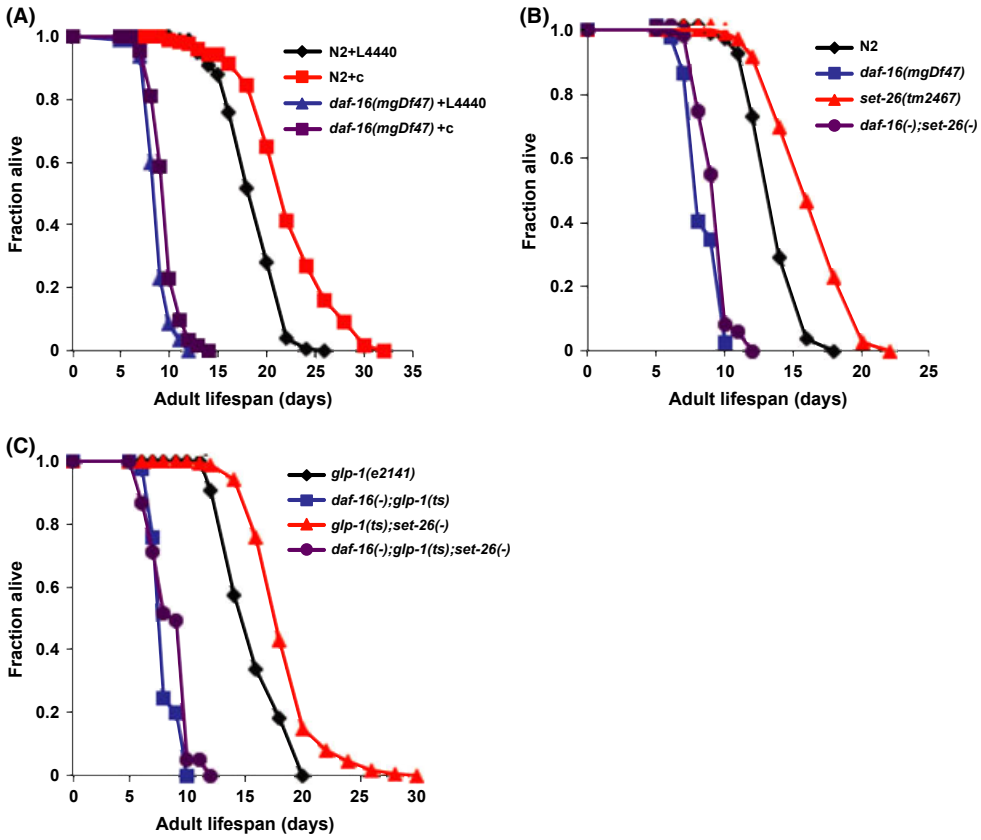

Fig. 3 *set-9* and *set-26* partially function through *daf-16* to influence lifespan. (A) *daf-16(mgDf47)* mutation partially suppresses the lifespan extension by *set-9/26* RNAi in wild-type N2 worms (20 °C). (B,C) *daf-16(mgDf47)* mutation partially suppresses the lifespan extension conferred by *set-26(tm2467)* single mutation in wild-type animals (B) and in the germlineless *glp-1(e2141)* mutant worms (C) at 25 °C.

loading control. As shown in Fig. S1 (Supporting information), SET-26 protein level is undetectable in *glp-1(e2141);set-26(tm2467)* double mutant. Interestingly, we detected a large reduction in SET-26 protein level with age in *glp-1(e2141)* single mutant worms. Given that deletion of *set-26* results in a robust lifespan extension in the *glp-1* mutant background (Fig. 2), this finding was somewhat unexpected. Further work is required to determine why SET-26 levels decrease with normal aging.

We found that total histone H3 protein level in somatic tissues decreased dramatically with age irrespective of the *set-26(tm2467)* mutation [2.63- and 3.33-fold in *glp-1(e2141)* and *glp-1(e2141);set-26(tm2467)* worms, respectively, Fig. 4]. Curiously, we found that deletion of *set-26* moderately increased the histone H3 protein levels (1.83-fold for D1 adults), indicating that *set-26(-)* deletion causes accumulation of core histones.

Then we measured the abundance of several well-studied histone modifications (using total histone H3 level for normalization), including the open chromatin markers AcH4 (pan-acetylated histone H4), H3K4me3, and H3K36me3, as well as the repressive chromatin markers H3K9me3 and H3K27me3 (Bonasio *et al.*, 2010). Statistical analysis showed that neither aging nor the *set-26(tm2467)* mutation had a significant effect on the global levels of the two active gene expression markers H3K4me3 and H3K36me3. We did observe a dramatic increase in the AcH4 levels with age, indicating an increase in open chromatin formation at older age. However, we did not detect any significant effect on AcH4 by the *set-26(tm2467)* mutation.

For the two repressive chromatin markers, H3K9me3 and H3K27me3, we detected strong age-dependent effects overall. In *glp-1(e2141)* worms, we observed a strong age-dependent decrease in both modifications (4.17- and 3-fold for H3K9me3 and H3K27me3, respectively), which suggests a loss of chromatin compaction during aging. In contrast, when *set-26* is deleted, no significant age-dependent loss of either repressive histone mark was detected. This demonstrated a significant age-dependent *set-26(tm2467)* effect on the levels of H3K9me3 and H3K27me3 (two-way ANOVA *P*-values are 0.012 and 0.036, respectively). It is of note that the H3K9me3 level was somewhat lower in young *glp-1(e2141);set-26(tm2467)* double mutant compared to young *glp-1(e2141)* single mutant, but the level of this histone mark did not significantly decrease with increasing age in the double mutants. Remarkably, the levels of H3K27me3 in young and old *glp-1(e2141);set-26(tm2467)* double mutants remained at a high level similar to young *glp-1(e2141)* single mutant animals. These data suggest that inactivation of somatic *set-26* prevents the age-dependent loss of histone modifications that are normally associated with highly compacted heterochromatin.

Discussion

In our attempt to discover new histone modifiers that modulate longevity, we identified six putative histone methyltransferases and demethylases that, when inactivated by RNAi, extend wild-type worm lifespan. The chromatin players we identified, with the exception of *jmjd-2*, do not require germline proliferation to affect normal lifespan. These results indicate that our screening method that uses FUDR creates a bias toward identification of germline-independent longevity genes, distinct from a

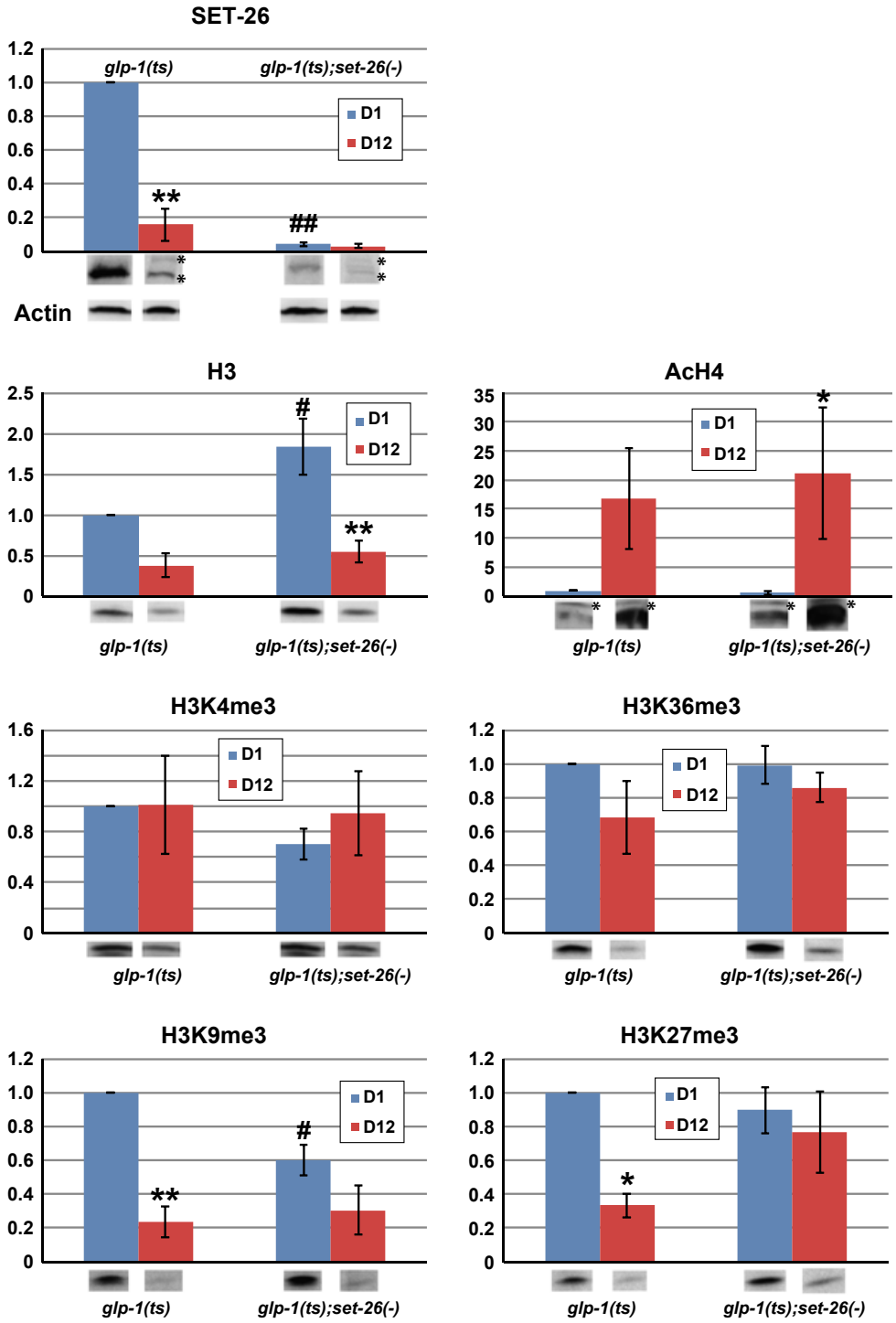

Fig. 4 Deletion of somatic *set-26* affects the levels of histone H3 protein and histone modifications in an age-dependent manner. Synchronized *glp-1(e2141)* and *glp-1(e2141ts);set-26(tm2467)* worms were harvested at Day 1 (D1) and Day 12 (D12) adulthood at 25 °C. Equal amount of worm total lysates were subjected to electrophoresis and Western blot. A representative Western blot is shown under each quantification chart, except that ACTIN blot is shown separately. Nonspecific bands were labeled with '*'. SET-26 and histone H3 protein levels were normalized to actin levels. AcH4, H3K4me3, H3K36me3, H3K9me3, and H3K27me3 were normalized to H3 levels (Supporting information). The amount of each epitope from the D1-*glp-1(e2141)* sample was set to 1. Error bars show standard error of mean (SEM) from 3 to 5 independent experiments. Two-way ANOVA was used to analyze the significance of the effect by genetic background (Mut), age (Age), and the interaction between Mutation and Age (Mut*Age). The set of *P*-values of (Mut, Age, Mut*Age) for each epitope is as following: SET-26 (< 0.001, < 0.001, < 0.001), H3 (0.024, < 0.001, 0.107), AcH4 (0.640, 0.001, 0.578), H3K4me3 (0.302, 0.462, 0.502), H3K36me3 (0.401, 0.042, 0.358), H3K9me3 (0.061, < 0.001, 0.012), H3K27me3 (0.168, 0.005, 0.036). A *P*-value ≤ 0.05 is considered statistically significant. Tukey (HSD) pairwise comparison was also used. When comparing D1 and D12 samples of the same genetic background, we use '*' and '**' to denote $P < 0.05$ and $P < 0.005$, respectively. When comparing the two genetic mutants of the same age group, we use '#' and '##' to denote $P < 0.05$ and $P < 0.005$, respectively.

similar chromatin gene screen previously done without FUDR (Greer *et al.*, 2010). We further characterized the two highly homologous genes *set-9* and *set-26* that strongly extend the worm lifespan in both wild-type and the germlineless *glp-1(e2141)* background. We found that *set-9* and *set-26* share redundant functions in maintaining normal lifespan despite their differential tissue expression patterns, and they function partially through the well-known longevity determinant *daf-16*. We found that inactivation of somatic *set-26* alone is sufficient to extend lifespan, as well as to abrogate the age-dependent changes in the abundance of H3K9me3 and H3K27me3, two H3 modifications widely associated with compact chromatin.

Identification of histone modifiers that affect lifespan in a germline-independent pathway

Among the six longevity genes, we found that *rbr-2, utx-1*, and *jmjd-2* encode proteins with the JmjC-domain, a functional motif representative of a large class of histone demethylases (Klose *et al.*, 2006). Intriguingly, previous reports showed that inactivation of *rbr-2* results in a moderate lifespan extension when reproduction is inhibited by FUDR or *glp-1* mutation but shortens lifespan in wild-type reproductive worms (Table 1) (Lee *et al.*, 2003; Greer *et al.*, 2010). Previous studies suggest that *C. elegans* RBR-2 (an H3K4me3 demethylase) antagonizes the activity of the methyltransferase SET-2/ASH-2 COMPASS complex in reproductive worms (Greer *et al.*, 2010). Inactivation of *rbr-2* is believed to deregulate H3K4me2/3 maintenance and impair germline stem cell proliferation (Greer *et al.*, 2010; Li & Kelly, 2011), thus likely contributing to the longevity phenotype associated with the *rbr-2* inactivation. How RBR-2 inactivation in nonreproductive worms increases lifespan is unknown. We speculate that RBR-2 may antagonize other H3K4 methyltransferases and/or affect a different set of downstream target genes in the somatic tissues to restrict normal lifespan. The second jmjC gene we identified is *utx-1*, a member of the UTX/UTY family that demethylates H3K27me3 and H3K27me2 to H3K27me (Agger *et al.*, 2007; Hong *et al.*, 2007). We found that *utx-1* RNAi substantially increases the lifespan of both wild-type and germlineless worms in a *daf-16*-dependent manner (Table 1 and data not shown). This is consistent with two recent studies reporting that inactivation of *utx-1* increases worm lifespan regardless of germline proliferation and likely acts through repression of the components of Insulin/Insulin-like growth factor signaling pathway by increasing H3K27me3 levels on those genes and possibly other loci (Jin *et al.*, 2011; Maures *et al.*, 2011). The third gene we identified encodes JMJD-2 protein, a histone demethylase that affects global H3K9me3 levels and localized H3K36me3 levels in *C. elegans* (Whetstine *et al.*, 2006). Nevertheless, the physiological role of JMJD-2 is poorly understood other than that it was found to regulate cell cycle progression by changing chromatin accessibility (Black *et al.*, 2010). It is interesting to note that *jmjd-2* is the only gene identified in this screen that requires germline proliferation to affect longevity but is not affected by FUDR, which inhibits mitosis therefore gradually inhibiting progeny production. This observation and some previous studies point to multilayer signaling from the reproduction system to influence worm lifespan (Arantes-Oliveira *et al.* 2002, Greer *et al*., 2010).

We also identified three SET domain containing longevity genes, including *set-9*, *set-26*, and *mes-2*. MES-2 is a well-known Polycomb group protein that is essential for germline development and patterning. It functions in a repressive complex to maintain X-chromosome inactivation and somatic homeotic gene repression (Strome, 2005; Schaner & Kelly, 2006). Our *mes-2* RNAi treatment did not yield apparent developmental defects but revealed a new function of this gene in maintaining normal worm lifespan in a germline-independent manner. Interestingly, heterozygous mutants of the *E(z)* gene (*Drosophila* homolog of *mes-2*) live longer than control flies and show derepression of some Polycomb target genes (Siebold *et al.*, 2010). Our results suggest an intriguing possibility that MES-2 has conserved functions in longevity in diverse species.

In addition to the RNAi treatments that extended lifespan, our screen identified 36 RNAi (out of the 50) that resulted in significantly shortened lifespan (Table S1). Interestingly, among these 36 genes, 23 of them are known to have germline-enriched expression (Kim *et al.*, 2001) and/or functions in normal development (Wormbase). Considering the known importance of these genes in development, it is not surprising that their RNAi knockdown leads to shortened lifespan.

SET-9 and SET-26 have differential tissue expression patterns but share redundant functions in lifespan modulation

Our lifespan assays suggest that germline-expressed SET-9 shares redundant functions with germline- and soma-expressed SET-26 to determine a normal lifespan, and the small fraction of SET-26 in the somatic tissues also plays a significant role in lifespan determination (Fig. 2). Because the *set-9* and *set-26* genes are so similar in sequences and there is only one homologue gene in the close relative *C. briggsae*, it is likely that *set-9* and *set-26* are derived from a recent gene duplication event (Woollard, 2005). It will be interesting to tease out whether the different expression patterns of *set-9* and *set-26* contribute to some specialized biological functions, how their functions in germline and soma affect aging differently, and how they function in comparison with their homologous genes in other species during evolution.

SET-9 and SET-26 are largely but not completely dependent on *daf-16* to modulate lifespan

Our *daf-16* lifespan epistasis analyses suggest that SET-9 and SET-26 work through DAF-16 to modulate lifespan (Fig. 3). As *daf-16* transcript levels are not affected by *set-26* status in *glp-1(e2141)* worms (Fig. S3B) and that SET-26 regulates global histone modifications (Fig. 4), an interesting possibility is that SET-26 acts as a cofactor of DAF-16 in target gene regulation via changes in local chromatin accessibility. To test this, we selected twelve known DAF-16 regulated genes (McElwee *et al.*, 2003; Murphy *et al.*, 2003) to test their transcript levels in response to *set-26(tm2467)* and *daf-16(mgDf47)* mutations in either wild-type or *glp-1(e2141)* background (Fig. S4). The results showed that five of the genes we tested showed substantial expression changes in *set-26(tm2467)* mutants compared to wild-type worms. However, their expression changes appeared to be independent of *daf-16* status, as they showed similar down-regulation in both *set-26(tm2467)* and *daf-16(mgDf47);set-26(tm2467)* mutants. It is important to note that SET-26 may impact only a subset of DAF-16 targets and we may not have examined the appropriate DAF-16 target genes. Future genome-wide expression profiling will provide a clearer picture of whether and how SET-26 regulates DAF-16-mediated gene transcription.

It is also possible that like UTX-1, SET-9 and SET-26 modulate lifespan by regulating expression of components of *daf-16*-dependent longevity pathways (Jin *et al.*, 2011). Several well-known longevity pathways impinge on *daf-16*, including the *daf-2*/insulin pathway and the germline pathway (Kenyon *et al.*, 1993; Hsin & Kenyon, 1999). From our current study, the germline pathway is not likely to be involved as both *set-9/26* RNAi and *set-26* mutation are able to significantly extend worm lifespan in the germlineless mutant worms (Fig. 2). It will be interesting to test

whether *set-9/26* genetically interacts with other known *daf-16*-dependent longevity pathways in the future.

Age-dependent decline of somatic SET-26

Even though SET-9 and SET-26 proteins may not have direct methyltransferase activity (Qian & Zhou, 2006), their nuclear localization and effects on histone modifications suggest that they do have critical roles in affecting chromatin status. Indeed, they may act similarly to MLL5, the closest homolog of SET-9/26 in mammals, to affect chromatin structure through interactions with other chromatin modifiers (Sebastian *et al.*, 2009). Interestingly, both worm SET-9/26 and mammalian MLL5 also contain a protein–protein interaction domain, PHD. Emerging studies have shown that PHD domains in some disease-linked chromatin remodeling factors can distinguish and bind certain forms of methylated histones to affect gene expression (Baker *et al.*, 2008). It would be interesting to find out whether SET-9/26 and MLL5 can interpret some methylated histone status and associate with other regulators accordingly to affect local chromatin structure.

In our study, the levels of SET-26 protein in the soma decrease dramatically as worms age, reminiscent of the significant decline in abundance of some chromatin regulators (Pegoraro *et al.*, 2009). We speculate that a dramatic age-dependent diminution in the levels of key chromatin modifiers leads to a more relaxed chromatin regulatory environment, which likely results in an increase in aging-associated genome instability. This is consistent with a previous study reporting that RNAi knockdown of *set-9* and *set-26* increased the rate of reporter gene frameshift (Pothof *et al.*, 2003). We hypothesize that the genotoxic stress resulted by *set-9/26* inactivation can trigger compensatory mechanisms that help promote chromatin health and therefore increase lifespan. This is similar to the hormesis responses from mild heat and oxidative stresses that can protect against later severe stresses to prolong worm lifespan (Lithgow *et al.*, 1995; Cypser & Johnson, 2002).

Inactivation of somatic SET-26 increases the histone H3 protein level

Similar to previous reports, we observed a clear age-dependent reduction in the levels of histone H3 protein (Maures *et al.*, 2011). This is reminiscent of a recent report that histone protein levels decline during yeast replicative aging and that over-expression of some of the core histones increases the yeast replicative lifespan (Feser *et al.*, 2010). Interestingly, we repeatedly observed a moderate increase in histone H3 protein level in the young *glp-1(e2141);set-26(tm2467)* vs. *glp-1(e2141)* mutant worms. This suggests that loss of somatic SET-26 in nondividing tissues directly or indirectly facilitates the accumulation of steady state levels of histone proteins, which might help promote chromatin maintenance and hence longevity. It will be informative to test whether over-expression of histone proteins in the adult worm soma can promote longer lifespan.

Somatic SET-26 affects the levels of repressive histone modifications in an age-dependent manner

We detected a strong increase in the levels of the open chromatin marker acetylated histone H4 in the aging adult worm soma. This finding suggests age-dependent opening of chromatin in worms, which might be related to the observation in vertebrates where DNA methylation, a repressive marker, decreases globally with age (Singhal *et al.*, 1987; Wilson *et al.*, 1987). On the same note, we also found clear decrease in the levels of two repressive histone marks, H3K9me3 and H3K27me3, during adult worm aging. Similar age-dependent trend of the two marks have been observed previously in worms and mammalian tissues (Sarg *et al.*, 2002; Maures *et al.*, 2011). These observations clearly indicate a global decrease in heterochromatin formation and gene repression with age, hence overall genome instability.

Interestingly, *set-26(tm2467)* alone also leads to a decrease in the levels of the repressive H3K9me3 mark in young worms, suggesting a decrease in heterochromatin formation (Fig. 4). This observation might be related to earlier findings that inactivation of *set-9/26* leads to increased genome instability (Pothof *et al.*, 2003). However, in the *glp-1(e2141);set-26(tm2467)* double mutant, we observed no further age-dependent decrease in the levels of the two repressive gene marks, H3K9me3 and H3K27me3, when compared to *glp-1(e2141)* single mutant worms. Taking altogether, we hypothesize that *set-26(-)* mutation induces mild DNA damage stress early on to activate a hormesis mechanism that elicits beneficial responses, including elevation of histone H3 protein level and prevention/minimization of the deleterious loss of chromatin compaction through histone modifications, to restore genome stability and the youthfulness of the worms. It will be enlightening to find out what players are downstream of *set-26(-)* to slow down the aging process. In addition, we only assayed for global changes in histone H3 protein level and histone modifications in this study. In the future, it will be important and informative to perform genome-wide studies to map the localization of histone occupancy and redistribution of different histone modifications, so as to reveal locus-specific changes with age.

In summary, our studies revealed several new longevity genes that link various histone modifications to lifespan modulation. Additional characterization of two SET domain containing genes, *set-9* and *set-26*, further confirms the notion that global epigenetic status and genome stability affect organismal aging. These results have important implications for future development of interventions that can alter global chromatin structure to promote healthy aging.

Experimental procedure

Lifespan assays

Lifespan assays were performed similarly as described (Rizki *et al.*, 2011). Details can be found in Supporting Information.

Immunostaining

Worm fixation and immunostaining were performed as previously described (Li *et al.*, 2008) and see details in Supporting Information.

Immunoblotting and quantification

Worm sample preparation was described in Supporting Information. The enhanced chemiluminescence was captured using ChemiDoc XRST (Bio-Rad, Hercules, CA, USA) and quantified using Image Lab Software.

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Author contributions

ZN and SSL conceived this project. ZN performed most of the experiments and data analyses. AE helped generate some of the transgenic worms and performed some lifespan experiments. EA helped with some strain crosses and performed some lifespan experiments. ZN and SSL wrote the paper.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 SET-26 is expressed in both germline and somatic tissues.

Fig. S2 *set-9* and *set-26* have redundant functions in affecting worm lifespan.

Fig. S3 *set-26(tm2467)* mutation partially depends on *daf-16* to extend *glp-1(e2141)* worm lifespan, but does not affect *daf-16* RNA level.

Fig. S4 *set-26(tm2467)* mutation affects the expression of some of the known DAF-16 target genes.

Table S1 Lifespan screen of 50 RNAi clones in wild type worms targeting putative histone methyltransferase and demethylase genes.

Table S2 Lifespan data for the figures and supplementary figures.

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