The H3K27 demethylase UTX-1 regulates C. elegans lifespan in a germline-independent, insulin-dependent manner

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Summary

Aging is accompanied by alterations in epigenetic marks that control chromatin states, including histone acetylation and methylation. Enzymes that reversibly affect histone marks associated with active chromatin have recently been found to regulate aging in Caenorhabditis elegans. However, relatively little is known about the importance for aging of histone marks associated with repressed chromatin. Here, we use a targeted RNAi screen in C. elegans to identify four histone demethylases that significantly regulate worm lifespan, UTX-1, RBR-2, LSD-1, and T26A5.5. Interestingly, UTX-1 belongs to a conserved family of histone demethylases specific for lysine 27 of histone H3 (H3K27me3), a mark associated with repressed chromatin. Both utx-1 knockdown and heterozygous mutation of utx-1 extend lifespan and increase the global levels of the H3K27me3 mark in worms. The H3K27me3 mark significantly drops in somatic cells during the normal aging process. UTX-1 regulates lifespan independently of the presence of the germline, but in a manner that depends on the insulin-FoxO signaling pathway. These findings identify the H3K27me3 histone demethylase UTX-1 as a novel regulator of worm lifespan in somatic cells.

Key words: histone demethylase; aging; lifespan; UTX; H3K27me3; epigenetic; insulin pathway; FoxO transcription factor; germline; soma; Caenorhabditis elegans.

Introduction

Aging is accompanied by a dramatic loss of epigenetic control over repressed regions of chromatin in various species (Wareham et al., 1987; Gaubatz & Cutler, 1990; Kennedy et al., 1995; Smeal et al., 1996; Shen et al., 2008). Furthermore, cells isolated from old individuals (> 80 years) or from patients with Hutchinson-Gilford Progeria Syndrome, a premature aging disorder, exhibit a reduction in repressed chromatin marks (Scaffidi & Misteli, 2005, 2006). Age-dependent loss of chromatin repression is correlated with alterations in gene expression patterns, which have been documented in species ranging from Caenorhabditis elegans to humans (Lee et al., 2000; Lund et al., 2002; Bennett-Baker et al., 2003; Lu et al., 2004). While there is an age-dependent loss of chromatin repression in a range of organisms, whether and how maintenance of repressed chromatin affects longevity is only starting to be elucidated.

The repressed or active state of chromatin is governed by an array of epigenetic modifications, including modification of the core histones through phosphorylation, ubiquitination, acetylation, and methylation (Berger, 2007). Histone methylation at specific residues is particularly important for repressed chromatin (Strahl & Allis, 2000). For example, trimethylated lysine 27 on histone H3 (H3K27me3) is generally associated with facultative heterochromatin and transcriptionally silenced chromatin regions (Cao et al., 2002; Bernstein et al., 2006). On the other hand, trimethylated lysine 4 on histone H3 (H3K4me3) is associated with active chromatin and is present at transcriptional start sites (Bernstein et al., 2002; Santos-Rosa et al., 2002). Histone methylation is controlled by the counteracting action of two classes of enzymes: methyltransferases and demethylases (Shi & Whetstone, 2007). The reversibility of histone methylation raises the possibility that this epigenetic mark is an important control point for different processes, including aging.

Regulators of epigenetic marks associated with active chromatin have been implicated in lifespan regulation. For example, Sir2 was found to be important for yeast replicative lifespan by regulating acetylated H4K16, a mark associated with active chromatin (Dang et al., 2009). The H3K4 demethylase LSD-1 has also been identified as regulator of worm lifespan (McColl et al., 2008). Furthermore, a targeted RNAi screen for methyltransferases that regulate longevity identified members of the ASH-2 H3K4me3 regulating complex (Greer et al., 2010). Indeed, attenuation of members of the ASH-2 complex extends lifespan and decreases H3K4me3 levels in worms (Greer et al., 2010). Consistently, ectopic expression of RBR-2, an H3K4me3 demethylase that counteracts the effect of the ASH-2 methyltransferase complex, also extends worm lifespan (Greer et al., 2010). The ability of members of this H3K4me3 regulatory complex to regulate lifespan is dependent on the presence of an intact germline (Greer et al., 2010), but independent of the insulin-FoxO signaling pathway, a conserved pathway that has been well characterized in the regulation of aging (Johnson, 1990; Kenyon et al., 1993; Morris et al., 1996; Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997). Thus, marks associated with active chromatin are emerging as important regulators of the aging process. However, much less is known about epigenetic marks associated with repressive chromatin. While our previous targeted RNAi screen for longevity did not identify known methyltransferases of repressive marks (Greer et al., 2010), this screen was only focused on methyltransferases. Thus, the possibility remained that histone demethylases that reversibly erase repressive marks play an important role in lifespan.

In this study, we used a targeted RNAi screen focused on histone demethylases to identify four histone demethylases, UTX-1, RBR-2, LSD-1, and T26A5.5, that regulate C. elegans lifespan. We focused on UTX-1 because its mammalian counterpart, UTX, is known to function as a demethylase for the repressive mark H3K27me3 (Agger et al., 2007; Hong et al., 2007). We found that attenuation of utx-1 using RNAi or heterozygous mutants of utx-1 extends both mean and maximal worm lifespan and increases the global levels of H3K27me3. Interestingly, H3K27me3 levels strikingly decline during normal aging in somatic cells in worms. We also showed that the H3K27me3 demethylase UTX-1 controls lifespan independently of the germline, but genetically interacts with the insulin-FoxO pathway to regulate longevity. Our findings identify several histone demethylases as novel regulators of worm lifespan, including the
H3K27me3 demethylase UTX-1. Our results further indicate that loss of repressed chromatin is associated with aging in somatic cells.

**Results**

**A targeted RNAi screen identifies RBR-2, LSD-1, T26A5.5, and UTX-1 histone demethylases as regulators of lifespan in *C. elegans***

Histone methyltransferases have been implicated in the regulation of lifespan in both worms (Hamilton et al., 2005; Greer et al., 2010) and flies (Siebold et al., 2010). Because histone marks are dynamically controlled by both histone transferases and demethylases, there is the potential that both classes of enzymes affect the aging process. At present, however, only two histone demethylases, RBR-2 and LSD-1, which are known to demethylate the activating marks H3K4me3 and H3K4me2, have been implicated in the regulation of lifespan (McColl et al., 2008; Greer et al., 2010). To identify additional demethylases that regulate lifespan, and to potentially uncover regulators of repressive marks important for the aging process, we performed a directed RNAi screen against histone demethylases in fertile worms, starting RNAi upon hatching at the first larval L1 stage. The histone demethylases were selected on the basis that they contain an amine oxidase or a Jumonji C-terminal (JmjC) domain, which are hallmarks of the two classes of enzymes responsible for catalysis of histone demethylation (Shi et al., 2004; Tsukada et al., 2006). Knockdown of *rbr-2* significantly decreased lifespan, whereas knockdown of *lsd-1* extended lifespan, consistent with previous results (McColl et al., 2008; Greer et al., 2010). We also found that knockdown of two demethylases not previously known to regulate lifespan, *T26A5.5* and *utx-1*, significantly increased lifespan (17.07% ± 6.94 and 17.04% ± 3.09 increases in lifespan, respectively; Fig. 1A; Table S1). Thus, our targeted RNAi screen identified four histone demethylases that significantly altered lifespan of fertile worms, RBR-2, LSD-1, *T26A5.5*, and *UTX-1*, two of which (*T26A5.5*, and *UTX-1*) had not been previously identified to regulate the aging process.

*RBR-2* is orthologous to the human H3K4me3 demethylase RBP2 (JARID1A/KDM5A), and associates with the PR2 complex to repress gene transcription (Pasini et al., 2008; Fig. 1B). LSD-1 (T08D10.2) is orthologous to the lysine-specific demethylase 1 (LSD1) protein in mammals which demethylates H3K4me2/me1 as well as H3K9me2 and also functions as a repressor of gene transcription (Shi et al., 2004; Fig. 1B). *T26A5.5* encodes an orthologue of the JmjC domain-containing protein JHDM1B/KDM2B which specifically demethylates H3K36me3 (Tsukada et al., 2006; He et al., 2008), a mark associated with active gene transcription (Morrison et al., 2005; Rao et al., 2005; Fig. 1B). UTX-1 is orthologous to the members of a mammalian phylogenetic subgroup that contains the histone demethylases JMJD3, UTX and UTY, with closest homology to UTX. Interestingly, mammalian UTX specifically demethylates the repressive mark H3K27me3 and is required for the transcription of multiple target genes (Agger et al., 2007; Hong et al., 2007; Wang et al., 2010; Fig. 1B). The observation that several histone demethylases with different substrate specificities can control worm lifespan suggests interplay between epigenetic marks for the regulation of aging.

**T26A5.5 and UTX-1 histone demethylases regulate adult lifespan in *C. elegans***

The RNAi screen was performed by adding RNAi at the larval stage L1, which could affect developmental processes. We next asked whether knockdown of the identified histone demethylases regulates lifespan by affecting adult aging, or as a consequence of perturbing development. Depletion of *lsd-1* in adult worms increased lifespan (McColl et al., 2008), whereas depletion of *rbr-2* in adult worms decreased lifespan (Greer et al., 2010), indicating that both LSD-1 and RBR-2 regulate lifespan by...
affecting adult aging. To test whether T26A5.5 and utx-1 also extended lifespan by slowing adult aging, we treated the worms with the corresponding RNAi at the young adult stage upon reaching sexual maturity. Knockdown of T26A5.5 initiated at the L1 (Fig. 2A; Table S1) and young adult stage (Fig. 2B; Table S2) both increased lifespan by approximately 12% and 15% respectively compared to empty vector control. Knockdown of utx-1 initiated at the L1 stage extended worm lifespan by approximately 13% (Figs 1A and 2C; Tables S1 and S2). Interestingly, knockdown of utx-1 initiated at the young adult stage extended lifespan by approximately 29% (Fig. 2D; Table S2). These results indicate that knockdown of T26A5.5 and utx-1 regulate lifespan in adult worms. The fact that we observed a greater difference in the degree of lifespan extension when utx-1 knockdown was initiated at adulthood compared to the L1 stage suggests that utx-1 deficiency during development may partially impair the overall health of the worm.

**The H3K27me3 demethylase UTX-1 regulates lifespan in worms**

To further investigate whether T26A5.5 regulates aging, we assessed the lifespan of T26A5.5(ok2364) mutant worms. The longevity of T26A5.5(ok2364) mutant worms was not significantly increased compared with wild-type worms (Fig. 3A; Table S2). The fact that the RNAi-mediated knockdown of T26A5.5 extends lifespan but T26A5.5(ok2364) mutant worms are not long lived may be due to the allele of T26A5.5 used in this study, to the acute vs. chronic removal of T26A5.5, to an off-target effect of RNAi, or to the fact that T26A5.5(ok2364) may be a hypomorphic mutation that does not completely abrogate T26A5.5 activity.

To confirm that UTX-1 regulated longevity, we assessed the lifespan of utx-1 mutants. Of the available strains carrying utx-1 mutations, neither utx-1(tm3118) nor utx-1(tm3136) is viable when homozygous (Kemphues et al., 1988), precluding the examination of lifespan. Thus, we determined the lifespan of the viable utx-1(tm3118) heterozygous worms. To maintain the stability of the heterozygous utx-1 mutation across several generations, we crossed the utx-1(tm3118)/+ strain with a heterozygous balancer strain TU899 [stDp(2X;II)/+; uDf1 X] carrying a mutation proximate to the genomic position of utx-1. The lifespan of utx-1 heterozygous worms [stDp(2X;II)/+; utx-1(tm3118) X] was compared to that of the balancer strain. While both of these strains had a higher incidence of matricide (also called bagging) than wild-type (N2) worms, we observed that utx-1(tm3118) heterozygous worms have a significant increase in their mean lifespan compared with the control worms, ranging from 27 to 50% (Fig. 3B; Table S2). These results support our findings that utx-1 deficiency extends lifespan in worms. Thus, we focused on UTX-1 in the rest of the study.

**UTX-1 regulates H3K27me3 levels in the germline and the soma of C. elegans**

In mammals, UTX is known to specifically demethylate the H3K27me3 mark by acting antagonistically to the polycomb repressor complex 2

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**Fig. 2** Knockdown of two histone demethylases, T26A5.5. and UTX-1, extends lifespan when initiated either during development or in adulthood. (A–B) T26A5.5 RNAi extends lifespan at L1 (A) (12%, P < 0.0009), and young adults (B) (16.6%, P < 0.0005). (C) utx-1 knockdown initiated at the L1 stage extends worm lifespan by 12.6% (P < 0.0001) compared to control RNAi. (D) utx-1 knockdown initiated after worms have reached adulthood extends lifespan by 29.29% (P < 0.0001) compared to control RNAi. The efficacy of the RNAi-mediated knockdown of utx-1 was confirmed by quantitative RT–PCR (Fig. S1). Mean lifespan and statistics for independent experiments are presented in Tables S1 and S2 (Supporting information).
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Longevity induced by utx-1 knockdown does not require an intact germline

We have recently shown that the ASH-2 H3K4 trimethyltransferase complex regulates worm lifespan and does so in a manner that requires an intact germline (Greer et al., 2010). The H3K27me3 demethylase UTX was recently found to copurify with the ASH-2 methyltransferase complex in mammalian cells (Issavea et al., 2007), raising the possibility that the UTX-1 demethylase, like ASH-2, may regulate lifespan in a germline-dependent manner. To test whether the longevity induced by utx-1 knockdown also requires germline cells, we examined the effects of utx-1 knockdown in glp-1(e2141) mutant worms. When maintained at the restrictive temperature, the glp-1(e2141) worms fail to give rise to a functional germline (Pries et al., 1987) and exhibit an increased lifespan compared with wild-type worms (Arantes-Oliveira et al., 2002). Surprisingly, we found that utx-1 knockdown further extends the lifespan of both long-lived glp-1(e2141) worms (15.9%, P < 0.0006) and wild-type (N2) worms (23.6%, P < 0.0001; Fig. 5A). Analysis by two-way ANOVA confirmed that there was no significant interaction between the glp-1(e2141) genotype and utx-1 RNAi for lifespan extension (P = 0.574). Together, these results indicate that unlike the ASH-2 complex, UTX-1 regulates lifespan independently of the presence of an intact germline. These findings further imply that UTX-1 likely does not act together with the ASH-2 containing methyltransferase complex to regulate lifespan. These data also suggest that UTX-1 regulates lifespan by acting in somatic cells.

Longevity induced by utx-1 knockdown does not entirely require the DR pathway induced by eat-2

Environmental interventions such as dietary restriction (DR) extend the mean and maximal lifespan in worms, flies, rodents [for review, (Mair & Dillin, 2008)], and even primates (Colman et al., 2009). There are several ways to elicit DR in worms (Klass, 1977; Hosono et al., 1989; Houthoofd et al., 2002; Kaeberlein et al., 2006; Lee et al., 2006; Bishop & Guarente, 2007; Greer et al., 2007; Honjo et al., 2009). eat-2(ad1116) mutant worms provide a genetic way to mimic DR as they exhibit a significantly reduced rate of pharyngeal pumping and have an extended lifespan (Avery, 1993; Lakowski & Hekimi, 1996). We tested whether utx-1 functions in the same genetic pathway as eat-2 mutants to regulate lifespan. As shown in Fig. 5B, utx-1 knockdown further extended the lifespan of both long-lived eat-2(ad1116) mutant worms (16.7% (P < 0.0001) and wild-type (N2) worms (23.2%, P < 0.0001), suggesting that utx-1 and eat-2 do not function in the same genetic pathway to regulate lifespan. However, analysis by two-way ANOVA revealed a significant interaction between the eat-2(ad1116) genotype and utx-1 RNAi (P < 0.0001). Although interpretation of these results is limited by the absence of independent replication and by variation in longevity in strains with different genetic background (Gems & Riddle, 2000), these results suggest that utx-1 regulates lifespan in a manner that does not require the DR pathway induced by the eat-2 mutation, but that utx-1 may partially overlap with this DR pathway.

Longevity induced by utx-1 deficiency requires an intact insulin-FoxO signaling pathway

Mutations that decrease the activity of the insulin receptor DAF-2 more than double the lifespan of the worm (Kenyon et al., 1993; Kimura et al., 1997). The long lifespan of eat-2 is further extended by deficiencies in the germline pathway (Hsin & Kenyon, 1999), similar to that of utx-1.
deficient worms. Thus, we asked whether longevity induced by utx-1 knockdown is dependent on the insulin/IGF-1 signaling pathway. utx-1 knockdown extends the lifespan of wild-type (N2) worms (14%, \( P < 0.0001 \)), but does not further extend the long lifespan of daf-2(e1370) mutant worms (−1.4%, \( P = 0.5367 \); Fig. 6A). Analysis by two-way ANOVA revealed a statistically significant interaction between the daf-2(e1370) genotype and utx-1 RNAi (\( P = 0.039 \)). Although epistasis experiments for longevity can be difficult to interpret when the mutant allele, such as daf-2(e1370), is not null (Gems & Partridge, 2008), these results suggest that the longevity induced by utx-1 knockdown requires intact insulin/IGF-1 signaling.

We further tested the requirement of the insulin pathway in longevity regulation by UTX-1. Extended longevity of the daf-2 mutants is in large part mediated by DAF-16, the worm FoxO transcription factor (Lin et al., 1997; Ogg et al., 1997). We next examined the effect of utx-1 knockdown in daf-16(mu86) mutant worms, which carry a null mutation for the FoxO gene. While utx-1 knockdown significantly extended the lifespan of wild-type (N2) worms (28.2%, \( P < 0.0001 \); utx-1 knockdown no longer extended the lifespan of the daf-16(mu86) mutant worms (2.4%, \( P = 0.6034 \)). Analysis by two-way ANOVA revealed a significant interaction between the daf-16(mu86) genotype and utx-1 RNAi (\( P < 0.0001 \); Fig. 6B). These results indicate that the longevity induced by utx-1 RNAi requires the pro-longevity transcription factor FoxO/DAF-16.

We next asked whether knockdown of utx-1 affects the nuclear translocation of FoxO/DAF-16, using a strain of worms carrying an integrated DAF-16::GFP transgene (Henderson & Johnson, 2001). In standard culture conditions, DAF-16::GFP was predominately cytoplasmic (Fig. 6C). Interestingly, utx-1 knockdown increased DAF-16::GFP nuclear localization (Fig. 6D), similar to the knockdown of age-1, an insulin signaling pathway effector (phosphatidylinositol-3 kinase; Rahman et al., 2010) (Fig. 6E), or to 1h heat shock at 35 °C (Henderson & Johnson, 2001; Fig. 6F). The effect of utx-1 knockdown on FoxO/DAF-16 nuclear localization was relatively specific, since knockdown of another gene involved in longevity, clk-1, did not result in increased nuclear localization of DAF-16::GFP, as shown previously (Henderson & Johnson, 2001; Fig. 6F). Together with our genetic interaction results, these findings suggest that UTX-1 regulates lifespan at least in part by modulating FoxO/DAF-16 nuclear localization. UTX-1 may normally prevent the FoxO/DAF-16 transcription factor from accessing its target genes.

Global somatic H3K27me3 levels decrease with age in germline-deficient worms

Because deficiency in the H3K27me3 demethylase UTX-1 leads to both an extended lifespan and an increase in global H3K27me3 levels, we asked whether the levels of H3K27me3 changed during the normal aging

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**Fig. 4** The demethylase UTX-1 regulates H3K27me3 levels in the germline and soma of worms. (A) Western blot on whole-worm extracts of wild-type (N2) worms at the L3 stage treated with empty vector control RNAi or utx-1 RNAi using an H3K27me3 antibody. An antibody to total histone H3 (H3) was used to control for equal loading. Each lane represents an independent cohort of approximately 200 worms. The blots presented are representative of two independent experiments. (B) Quantification of relative H3K27me3 levels compared to H3. Mean ± SD of two independent experiments. *\( P < 0.05 \) by paired t-tests. (C) Whole-worm immunofluorescence of worms treated with empty vector control RNAi or utx-1 RNAi and costained with an H3K27me3 antibody (green) and an actin antibody (red) as a control for antibody accessibility. DAPI staining (blue) was used to visualize nuclei and is shown in the merged image from all three channels (bottom panel). The worms are oriented anterior (left) to posterior (right). Yellow arrow: intestinal cell; orange arrow: germline cell. These results are representative of four independent experiments. The dashed line indicates the germline. Scale bar, 25 \( \mu m \).

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process in the worm. We initially assessed the H3K27me3 mark in wild-type worms maintained on solid media. We observed a slight decrease in the H3K27me3 mark after day 12, which corresponds to middle age in these worms (Fig. S3A,B). However, the interpretation of these experiments was limited by the fact that growth on solid media made it difficult to obtain enough worms at later stages of life (e.g. day 20, when more than 80% of worms are already dead), thereby resulting in variability in H3K27me3 levels. To obtain sufficient amounts of synchronized worms, especially for later ages, we grew worms in liquid cultures. Given that UTX regulates lifespan in a germline-independent manner, we sought to monitor H3K27me3 levels in somatic tissues by assessing this mark in germline-deficient worms (glp-1(e2141) mutant worms at the restrictive temperature; Fig. 7). Western blot experiments revealed that H3K27me3 levels were not significantly affected between youth and middle age in these worms (11 days in liquid culture; Fig. 7A,B). Interestingly, H3K27me3 levels drastically dropped to almost undetectable levels in glp-1(e2141) worms older than day 11 (day 14, 17 and 20, Fig. 7A,B). The late-stage loss of the H3K27me3 mark in glp-1(e2141) worms is consistent with our observation that increased H3K27me3 is associated with longevity. These results also suggest that high somatic levels of H3K27me3 are a biomarker of youthfulness. Collectively, these data support the possibility that utx-1 deficiency extends lifespan by maintaining high levels of H3K27me3, perhaps allowing a better control of chromatin repression.

**Discussion**

Our targeted screen for histone demethylases regulating lifespan in *C. elegans* confirms a role for the histone demethylases RBR-2 and LSD-1 in the control of longevity and identifies potential novel regulators of lifespan (T26A5.5 and UTX-1). In particular, our study reveals that the H3K27me3 demethylase, UTX-1, regulates lifespan in an insulin pathway-dependent manner. Because histone demethylases and their functions are highly conserved in more complex animals, including mammals, their effect on lifespan in *C. elegans* may likely be extended to other species.

The reason UTX-1 was not identified earlier in previous large-scale RNAi screens (Lee et al., 2003; Hansen et al., 2005; Chen et al., 2007; Curran & Ruvkun, 2007) is unclear. It is possible that the increase in lifespan upon utx-1 knockdown was not large enough to be reproducibly detected in a large screen. One clear difference between this screen and its predecessors is that our screen was performed using fertile worms in the absence of the DNA synthesis inhibitor, 5-fluorodeoxyuridine (FUdR). While longevity induced by utx-1 knockdown is independent of the worm’s fertility and should not be affected by FUdR, this drug can increase the lifespan of wild-type worms (Aithladh & Sturzenbaum, 2010). Thus, FUdR treatment may have masked the effects on lifespan of utx-1 RNAi in previous screens.

The H3K27me3 mark is associated with regions of facultative heterochromatin. By demethylating the H3K27me3 mark, UTX may relieve chromatin repression (Agger et al., 2007). As shown in this study, knockdown of utx-1 resulted in a corresponding increase in H3K27me3 levels. Coupled with our observation that utx-1 knockdown extends lifespan, these results suggest that the rate of aging may be subject to the regulation of the H3K27me3 mark. Indeed, a loss of epigenetic control over transcriptional silencing has been observed during aging (Wareham et al., 1987; Gaubatz & Cutler, 1990; Kennedy et al., 1995; Smeal et al., 1996; Shen et al., 2008) and may be explained, at least in part, by the drop in H3K27me3 levels we observed in the late stages of the worm’s life. Thus, the RNAi-mediated reduction of UTX-1 may promote longevity through the continued maintenance of the repressive H3K27me3 mark, preventing spurious and/or detrimental gene transcription late in life. Our data do not exclude the possibility that utx-1 deficiency at the beginning of adult life sets a different level of H3K27me3, which may have consequences on longevity later in life. Changes in H3K27 methylation status may also be an indirect consequence of UTX-1 depletion in worms. For example, sir-2.1 depletion has been found to indirectly increase H3K27 methylation (Wirth et al., 2009). Our results do not rule out the possibility that UTX-1 also has non-histone targets.

The specific genes that may be derepressed by loss of H3K27me3 during aging are not known yet. UTX is thought to control the expression of HOX genes in mammalian cells (Agger et al., 2007; Lan et al., 2007) and a recent genome-wide study identified 2000 genes that are occupied by UTX in mammalian cells, including the retinoblastoma (Rb) gene (Wang et al., 2010). In fact, it is likely that histone demethylases, such as UTX-1, regulate the expression of many genes, making it difficult to identify precisely which ones are important for longevity. In worms, the requirement of the insulin-FoxO pathway for longevity induced by UTX-1 deficiency raises the intriguing possibility that UTX-1 directly influences the expression of regulators of the insulin-FoxO pathway. This is consistent with the
observation that utx-1 knockdown triggers FoxO nuclear translocation. Collectively, our results suggest that utx-1 is genetically upstream of FoxO/daf-16, perhaps directly regulating genes that affect the activity of the insulin signaling pathway. However, our study does not exclude the possibility that UTX-1 regulation and H3K27me3 levels are also affected by insulin-FoxO signaling.

In *Drosophila*, the heterozygous mutation of E(Z), a member of the PRC2 H3K27 trimethyltransferase complex, has been recently found to extend longevity (Siebold et al., 2010). One explanation for the fact that attenuation of an H3K27me3 methyltransferase [E(Z)] in flies or of an H3K27me3 demethylase (UTX-1) in worms both extend lifespan is that UTX-1 and E(Z) may not function in the same tissue or cell in the organism to regulate lifespan. Moreover, UTX-1 may not work in opposition of every single E(Z) target gene. It is also possible that optimal levels of H3K27me3 are required for proper lifespan extension and that either excess or dearth of H3K27me3 is detrimental for optimal fitness and lifespan. While there is a striking degree of conservation in the histone methylation pathway across species, it is also possible that there exist species-specific differences in how epigenetic marks regulate lifespan.

H3K27 demethylation was recently found to be accompanied with H3K4 trimethylation in mammalian cells (Issaeva et al., 2007) and in *C. elegans* (Fisher et al., 2010). The H3K4me3 methyltransferase complex, that contains the subunits ASH2L, WDR5, and the H3K4me3 methyltransferase MLL2 copurifies with UTX in mammalian cells (Issaeva et al., 2007). In *C. elegans*, homologous proteins responsible for H3K4 trimethylation, ASH-2, WDR5, and the methyltransferase SET-2 were recently found to regulate lifespan in a germline-dependent manner (Greer et al., 2010). As we have shown in this study, however, knockdown of utx-1 did not require the presence of the germline to extend lifespan. Furthermore, utx-1 knockdown extends lifespan in a manner that depends on the insulin-FoxO pathway, while the longevity induced by set-2 knockdown was only partially dependent upon FoxO/daf-16 to regulate lifespan (Greer et al., 2010). Collectively, our results suggest that the UTX-1 demethylase and the SET-2 trimethyltransferase complex impact lifespan by acting in distinct tissues, somatic vs. germline, respectively. It is possible, however, that there is coordinated regulation of H3K4 trimethylation and H3K27 demethylation at genes that regulate lifespan. Indeed, evidence from a recent study in *C. elegans* suggests that UTX-1 and the SET-16 methyltransferase function together in an MLL-like complex (Fisher et al., 2010). Although SET-16 does not appear to regulate lifespan under the conditions tested (Greer et al., 2010), UTX-1 and SET-16 may still cooperate to regulate lifespan under specific circumstances. UTX-1 could also associate with other methyltransferase com-

Fig. 6 Longevity induced by utx-1 deficiency requires the insulin-FoxO pathway. (A) utx-1 knockdown did not further extend the lifespan of the long-lived daf-2(e1370) mutant worms (−1.42%, *P* = 0.5267) (*P* = 0.039 by two-way ANOVA) to test for an interaction between the daf-2(e1370) genotype and utx-1 RNAi). (B) utx-1 knockdown did not significantly increase the lifespan of the short-lived daf-16(mu86) mutant worms (2.4%, *P* = 0.6034; *P* < 0.0001 by two-way ANOVA) to test for a statistical interaction between the daf-16(mu86) genotype and utx-1 RNAi). Mean lifespan and statistics for independent experiments are presented in Table S2 (Supporting information). (C–F) Representative pictures of FoxO/DAF-16 localization in daf-16::GFP transgenic worms. The percentage of worms with nuclear FoxO expression was determined by counting more than 40 worms per genotype and condition. (A) control RNAi (20%, *n* = 20); (B) utx-1 RNAi (80%, *n* = 20) (E) age-1 RNAi (100%, *n* = 20); (F) clk-1 RNAi (20%, *n* = 20); and (G) control RNAi, 35°C heat shock for 1 h (100%, *n* = 50). Scale bar, 100 μm.
plexes to regulate aging within the soma. In fact, two other methyltransferases (SET-9 and SET-15) regulate lifespan in a germline-independent manner (Greer et al., 2010), raising the possibility that UTX-1 regulates lifespan together with one or both of these methyltransferases. Our results suggest that different chromatin-modifying complexes, involving both methyltransferases and demethylases, regulate lifespan in the germline and in the soma. Understanding the interplay between these reversible epigenetic modifications in different tissues will give insights into mechanisms that slow – or possibly reverse – the aging process in multicellular organisms.

Experimental procedures

Worm strains and RNA interference

Wild-type (N2) and daf-16(mu86) strains were provided by Dr Man-Wah Tan. The daf-2(e1370) and eat-2(ad1116) were provided by Dr Cynthia Kenyon. The utx-1(tm3718) strain was provided by Dr Shohei Mitani. TU899 stDp2II(X;II)+ II; uDf1 X was backcrossed three times to our lab’s N2 strain, except that was not backcrossed. The strain was provided by Dr Shohei Mitani. IsDAF-16::GFP (TJ356) was provided by Dr Stuart Kim. The balancer strain, IsDAF-16::GFP (TJ356) was provided by Dr Stuart Kim. The T356 strain was provided by Dr Shohei Mitani.

Western blot analysis

Worms were synchronously grown to appropriate stages and washed off of plates with M9 buffer (KH2PO4, 22 mM; K2HPO4, 34 mM; NaCl, 86 mM; MgSO4, 1 mM). Worm pellets were resuspended in Trizol (Invitrogen, Carlsbad, CA, USA), followed by six freeze–thaw cycles in liquid nitrogen. One μg of total RNA was reverse transcribed with oligo dT primers using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer’s protocol. Real-time PCR was performed on a Bio-rad iCycler using iQ SYBR green (Bio-rad, Hercules, California, USA) with the following primers: pan-actin F: TCGGTATGGGACAGAAGGAC, pan-actin R: CATCCCAATTTGGTGACGATA, utx-1 F: TCTGAGTAGCTT CGGTTAGG, utx-1 R: TCTGAGATGTCGTTAGG. The experiments were conducted in duplicate, and the results were expressed as 2(-ΔΔCt).
antibodies to H3K27me3 (Upstate 07449, 1:2500), or histone H3 (1:1000; Abcam ab1791, Cambridge, Massachusetts, USA). The primary antibodies were visualized using horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:7500; Calbiochem, San Diego, California, USA) and ECL Plus (Amersham Biosciences, Piscataway, New Jersey, USA).

Age-matched cohorts of N2 worms were grown on NGM plates and transferred to new plates accordingly to exclude contaminating progeny. Living worms from age-matched cohorts were washed in M9 buffer and snap frozen in liquid nitrogen. Whole lysates were then extracted from worms at each stage (4, 6, 8, 10, 12, 14, and 16 days old), as described earlier. Cohorts of glp-1(e2141) mutant worms were grown in liquid culture at the restrictive temperature (25 °C) to generate large quantities of worms and to eliminate confounding methylation signals which may arise from the germline or progeny. Worms were grown in S Medium supplemented with Escherichia coli OP50 as a food source and shaken vigorously to ensure oxygenation (Lewis & Fleming, 1995). Age-matched cohorts were harvested from liquid culture, centrifuged in 60% sucrose density (OD) values for total histone H3 protein. Subtracted OD values for H3K27me3 were normalized to the background-emission. DAPI and Alexa Fluor 488 signals could be compared. DAPI and Alexa Fluor 488 antibody (A21042, 1:100; Invitrogen), and with the antibody (Chemicon MAB1501R, 1:100), followed by goat anti-mouse antibodies to H3K27me3 (Upstate 07449, 1:2500), or histone H3 (1:1000; Abcam ab1791, Cambridge, Massachusetts, USA). The primary antibodies were visualized using horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:7500; Calbiochem, San Diego, California, USA) and ECL Plus (Amersham Biosciences, Piscataway, New Jersey, USA).

Whole-worm immunofluorescence

Worms were washed several times to remove bacteria and resuspended in fixing solution (160 mM KCl, 100 mM Tris HCl pH 7.4, 40 mM NaCl, 20 mM Na2EGTA, 1 mM EDTA, 10 mM spermidine HCl, 30 mM Pipes pH 7.4, 1% Triton X-100, 50% methanol, 2% formaldehyde) and subjected to two rounds of snap freezing in liquid N2. The worms were fixed at 4 °C for 30 min and washed briefly in T buffer (100 mM Tris HCl pH 7.4, 1 mM EDTA, 1% Triton X-100) before a 1-h incubation in T buffer supplemented with 1% β-mercaptoethanol at 37 °C. The worms were washed with borate buffer (25 mM H3BO3, 12.5 mM NaOH, pH 9.5) and then incubated in borate buffer containing 10 mM DTT for 15 min. Worms were blocked in PBST (PBS, pH 7.4, 0.5% Triton X-100, 1 mM EDTA) containing 1% BSA for 30 min and incubated overnight first with the actin antibody (Chemicon MAB1501R, 1:100), followed by goat anti-mouse Alexa Fluor 488 antibody (A21042, 1:100; Invitrogen), and with the H3K27me3 antibody (Upstate 07449, 1:100) followed with goat anti-rabbit Alexa Fluor 594 antibody (A11012, 1:100; Invitrogen). DAPI (2 μg mL⁻¹) was added to visualize nuclei. For histone H3 staining, worms were incubated with the histone H3 antibody (Abcam ab1791, 1:1000) followed with goat anti-rabbit Alexa Fluor 594 antibody as described earlier. The worms were mounted on a microscope slide, and individual optical planes were visualized using a Leica SP2 confocal system. Image acquisition parameters were identical across conditions, so that the fluorescence signal could be compared. DAPI and Alexa Fluor 488 signals were sequentially imaged to eliminate the signal from overlapping emission.

Visualization of DAF-16 localization

The DAF-16::GFP transgenic worms were birthed on plates seeded with RNAi bacteria and grown to adulthood. Worms were then transferred to 1 mL of fixing solution and incubated for 5 min. Fixed worms were then washed with M9 buffer, mounted on a microscope slide, and imaged using a Zeiss Axiostop 2 plus fluorescence microscope with a 20x objective. Twenty to fifty animals per condition were scored as having predominantly nuclear vs. cytoplasmic DAF-16::GFP.

Statistical analysis

Statistical analyses of lifespan were performed on Kaplan–Meier survival curves in StatView 5.0.01 by Logrank (Mantel-Cox) tests. For statistical comparison of independent replicates, the Fisher’s combined probability test was performed. To compare the interaction between genotype and RNAi treatment, two-way ANOVA tests were performed in Prism 5 using the mean and standard error values obtained from the Kaplan–Meier survival curves. The values from the Kaplan–Meier curves, Fisher’s combined probability tests, and two-way ANOVA tests are included in the Tables S1 and S2 (Supporting information).

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Supporting information

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

Table S1 A targeted RNAi screen in fertile worms identifies histone demethylases that significantly affect worm lifespan.

Table S2 Attenuation of utx-1 extends lifespan in adult worms in a germ-line-independent, insulin-dependent manner.

Fig. S1 RT-qPCR analysis of utx-1 mRNA levels relative to pan-actin mRNA levels in wild-type (N2) worms treated in the absence (control) or presence of utx-1 RNAi.

Fig. S2 utx-1 RNAi does not alter the levels of total H3 in wild-type worms.

Fig. S3 H3K27me3 levels from whole worm lysates decrease after day 12 in wild-type worms.

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