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LETTERS

Members of the H3K4 trimethylation complex regulate lifespan in a germline-dependent manner in *C. elegans*

Eric L. Greer[1,2], Travis J. Maures[1], Anna G. Hauswirth[1], Erin M. Green[4], Dena S. Leeman[1,2], Géraldine S. Maro[3], Shuo Han[1], Max R. Banko[1], Or Gozani[2,4] & Anne Brunet[1,2]

The plasticity of ageing suggests that longevity may be controlled epigenetically by specific alterations in chromatin state. The link between chromatin and ageing has mostly focused on histone deacetylation by the Sir2 family[1,2], but less is known about the role of other histone modifications in longevity. Histone methylation has a crucial role in development and in maintaining stem cell pluripotency in mammals[3]. Regulators of histone methylation have been associated with ageing in worms[4–7] and flies[8], but characterization of their role and mechanism of action has been limited. Here we identify the ASH-2 trithorax complex[9], which trimethylates histone H3 at lysine 4 (H3K4), as a regulator of lifespan in *Caenorhabditis elegans* in a directed RNA interference (RNAi) screen in fertile worms. Deficiencies in members of the ASH-2 complex—ASH-2 itself, WDR-5 and the H3K4 methyltransferase SET-2—extend worm lifespan. Conversely, the H3K4 demethylase RBR-2 is required for normal lifespan, consistent with the idea that an excess of H3K4 trimethylation—a mark associated with active chromatin—is detrimental for longevity. Lifespan extension induced by ASH-2 complex deficiency requires the presence of an intact adult germline and the continuous production of mature eggs. ASH-2 and RBR-2 act in the germline, at least in part, to regulate lifespan and to control a set of genes involved in lifespan determination. These results indicate that the longevity of the soma is regulated by an H3K4 methyltransferase/demethylase complex acting in the *C. elegans* germline.

Genome-wide RNAi screens for genes that regulate lifespan in *C. elegans* have been performed previously in worms in which progeny production was inhibited[4,10,11], which could mask the effect of some genes on lifespan. We performed a targeted RNAi screen in fertile worms by selecting genes that encode known worm methyltransferases, proteins containing the enzymatic domain of methyltransferases (SET domain), or orthologues of regulators of histone methylation. As reported previously[4], *set-9* and *set-15* knockdown extended worm lifespan (Fig. 1a). We also found that *ash-2*, *set-2* and *set-4* knockdown extended fertile worm lifespan, with *ash-2* knockdown having the most significant effect (23.1–30.9%, $P < 0.0001$) (Fig. 1a, Supplementary Fig. 1a). ASH-2 is a member of an H3K4 trimethylation (H3K4me3) complex in yeast, flies and mammals[9,12,13] and is important for the conversion of H3K4 dimethylation (H3K4me2) to H3K4me3 in mammals[14]. *ash-2* knockdown decreased global H3K4me3 levels at larval stage L3 (Fig. 1b), indicating that ASH-2 promotes histone H3 trimethylation at lysine 4 in *C. elegans*. WDR-5/TAG-125 is a WD40-repeat protein that interacts with ASH-2 in mammals, and is important for the mono-, di- and trimethylation of H3K4 in mammals and worms[15,16]. *wdr-5/tag-125* knockdown or *wdr-5/tag-125* deletion also decreased H3K4me3 levels (Fig. 1b) and significantly extended *C. elegans* lifespan (~30%, $P < 0.0001$ and 16–28%, $P < 0.0001$, respectively) (Fig. 1c, Supplementary Fig. 1b). Thus, ASH-2 and WDR-5 promote H3K4 trimethylation and normally limit lifespan in *C. elegans*.

In mammals, ASH-2 and WDR-5 form a complex with several H3K4me3 methyltransferases of the SET1/mixed lineage leukaemia (MLL) family[17]. There are four SET1/MLL orthologues in *C. elegans*: SET-1, SET-2, SET-12 and SET-16 (Supplementary Fig. 1c). Of these, only SET-2, which is similar to mammalian SET1A/SET1B, regulated worm lifespan (*set-2* knockdown: ~20%, $P < 0.0001$; *set-2* deletion *set-2(ok952)*: 17–26%, $P < 0.0001$) (Fig. 1a, d; Supplementary Fig. 1d, e). As reported previously[16], *set-2(ok952)* mutant worms or worms treated with *set-2* RNAi had reduced H3K4me3 levels (Fig. 1e). SET-2 was relatively specific in regulating lifespan and H3K4me3 in worms, as neither SET-9 nor SET-15 affected global H3K4me3 levels (Supplementary Fig. 1f), even though they both regulate lifespan[4]. The worm SET-2 methyltransferase domain expressed in bacteria directly methylated histone H3 at lysine 4 *in vitro* (Fig. 1f). SET2 generated H3K4me2 but not H3K4me3 *in vitro* (Supplementary Fig. 1g), consistent with the fact that ASH-2 is required for the conversion of H3K4me2 to H3K4me3 (ref. 18). To test if ASH-2, WDR-5 and SET-2 act together to regulate lifespan, we performed epistasis experiments. Lifespan extension by *ash-2* or *wdr-5/tag-125* knockdown was significantly less pronounced in *set-2(ok952)* mutants than in wild-type worms (Fig. 1g, combined two-way ANOVA $P < 0.0001$). Similarly, *ash-2* knockdown did not extend further the lifespan of *wdr-5/tag-125(ok1417)* mutant worms (Supplementary Fig. 1h, $P = 0.1534$). Thus, ASH-2, WDR-5 and SET-2 probably act in the same pathway, perhaps in a complex, to limit lifespan. As *ash-2* and *wdr-5/tag-125* knockdown slightly extended *set-2(ok952)* mutant worm lifespan (Fig. 1g, Supplementary Table 2), other methyltransferases may also complex with ASH-2 and WDR-5 to regulate lifespan.

RBR-2 is an H3K4me3 demethylase involved in vulva formation in worms[19] and is homologous to human RBP2 and PLU-1 (also known as KDM5A and KDM5B, respectively), two H3K4me3 demethylases of the JARID family[19]. Consistent with a published report[19], *rbr-2(tm1231)* mutant worms showed increased H3K4me3 levels (Fig. 2a). Both *rbr-2(tm1231)* mutation and *rbr-2* knockdown decreased lifespan significantly (15–25%, $P < 0.0001$ and 10–24.2%, $P < 0.0001$, respectively) (Fig. 2b, Supplementary Fig. 2a), indicating that RBR-2 is necessary for normal longevity. The decrease of H3K4me3 induced by *ash-2* knockdown was less pronounced in *rbr-2(tm1231)* mutant worms than in wild-type worms (Fig. 2c). In addition, *ash-2* knockdown no longer extended *rbr-2(tm1231)* mutant worm lifespan (Fig. 2d, $P = 0.4673$). Similarly, the lifespan of *wdr-5/tag-125(ok1415); rbr-2(tm1231)* and *set-2(ok952); rbr-2(tm1231)* double mutants was similar to that of

[1]Department of Genetics, Stanford University, 300 Pasteur Drive, Stanford, California 94305, USA. [2]Cancer Biology Graduate Program, Stanford University, Stanford, California 94305, USA. [3]Departments of Biology and Pathology, Stanford University, Stanford, California 94305, USA. [4]Department of Biology, Stanford University, Stanford, California 94305, USA.

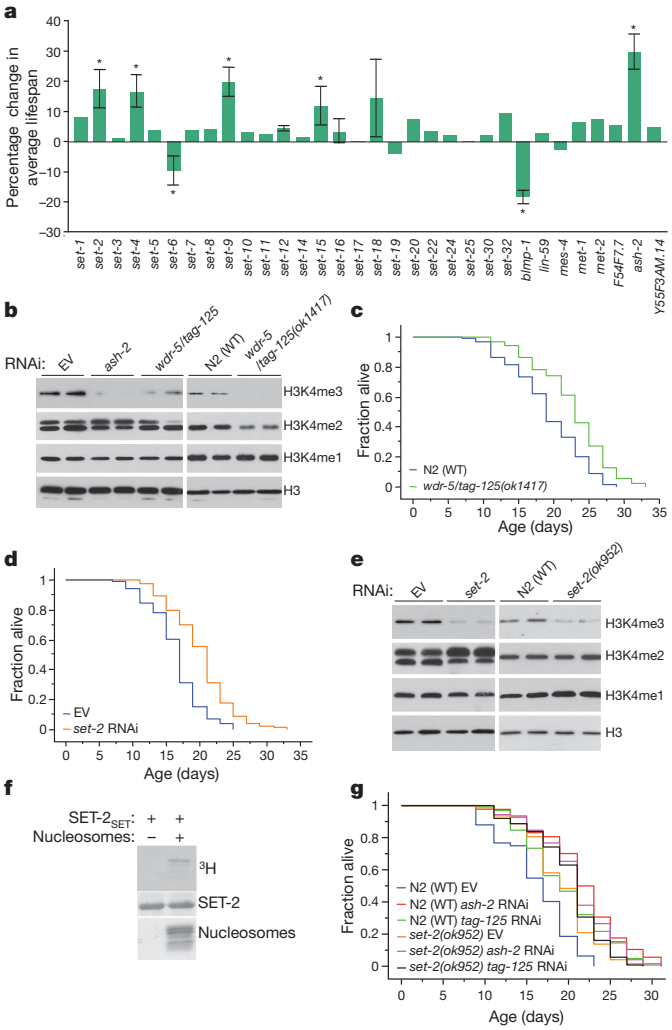

Figure 1 | ASH-2, WDR-5 and SET-2 function together to regulate H3K4me3 and lifespan in *C. elegans*. **a**, Percentage change in average lifespan in worms treated with RNAi to the indicated genes compared with empty vector. Mean ± s.d. of two or three independent experiments is shown. * $P < 0.05$. **b**, Western blots on L3 worm extracts (representative of four independent experiments). EV, empty vector; WT, wild type. **c**, *wdr-5/tag-125(ok1417)* mutant worms have an extended lifespan. **d**, *set-2* knockdown extends lifespan. **e**, Western blots of L3 worm extracts after *set-2* knockdown or deletion (representative of three independent experiments). **f**, The methyltransferase domain of worm SET-2 ($SET\text{-}2_{SET}$) methylates histone H3 *in vitro*. **g**, *ash-2* and *wdr-5/tag-125* knockdown slightly extend the lifespan of *set-2(ok952)* mutant worms, but significantly less than in the N2 (WT) background. *ash-2* mRNA was efficiently knocked-down by RNAi in *set-2(ok952)* mutant worms (Supplementary Fig. 3a). Statistics are presented in Supplementary Tables 1 and 2.

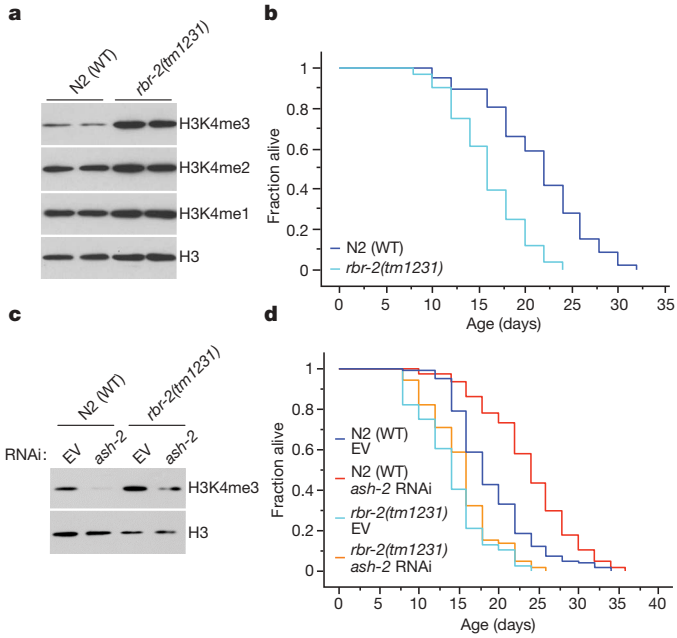

Figure 2 | RBR-2 is an H3K4me3 demethylase that counteracts the effect of the ASH-2 methyltransferase complex. **a**, Western blots of *rbr-2(tm1231)* mutant worms (representative of three independent experiments). **b**, *rbr-2(tm1231)* mutant worms have a decreased lifespan. **c**, Western blots of *rbr-2(tm1231)* mutant worms treated with *ash-2* RNAi (representative of two independent experiments). **d**, *ash-2* knockdown does not extend the lifespan of *rbr-2(tm1231)* mutant worms. *ash-2* mRNA was efficiently knocked down by RNAi in *rbr-2(tm1231)* mutant worms (Supplementary Fig. 3a). Statistics are presented in Supplementary Table 3.

rbr-2(tm1231) single mutants (Supplementary Fig. 2b, c, $P = 0.2121$ and $P = 0.6943$, respectively). Together, these results indicate that RBR-2 counteracts the effects of ASH-2/WDR-5/SET-2 on H3K4me3 and lifespan.

Whole-mount immunocytochemistry with a monoclonal ASH-2 antibody (Supplementary Fig. 4a) showed that ASH-2 is expressed in the nuclei of cells from many tissues, and is highly expressed in the germline (Fig. 3a). Visualization of an ASH-2–GFP (green fluorescent protein) fusion driven by the endogenous *ash-2* promoter in low-copy or high-copy transgenic lines confirmed that ASH-2 is highly expressed in the germline and in newly formed eggs (Supplementary Fig. 4b, c). Visualization of an RBR-2–GFP fusion driven by the endogenous *rbr-2* promoter in low-copy or high-copy transgenic lines showed expression of RBR-2 in the nuclei of cells from many tissues, although RBR-2 was not particularly enriched in the germline or eggs (Supplementary Fig. 4d, data not shown). The H3K4me3 mark was present in the nuclei of cells from all tissues, with high abundance in the germline in L3 and young adult worms (Fig. 3b, Supplementary Fig. 4e), consistent with the high levels of ASH-2 and SET-2 (ref. 20) in the germline. As expected, *wdr-5/tag-125(ok1417)* and *set-2(ok952)* mutant worms had markedly reduced H3K4me3 levels (Fig. 3b, Supplementary Fig. 4e). Conversely, *rbr-2(tm1231)* mutant worms showed increased H3K4me3 levels, particularly in the germline (Fig. 3b, Supplementary Fig. 4e). These results confirm that members of the ASH-2 complex and RBR-2 regulate H3K4me3, and that this mark is abundant in the germline.

To determine if the presence of an intact germline is necessary for lifespan regulation by ASH-2, we examined the effects of *ash-2* knockdown in *glp-1(e2141ts)* mutant worms, which develop only 5–15 meiotic germ cells instead of ~1,500 when shifted to the restrictive temperature at the L1 stage[21,22]. *ash-2* knockdown did not extend further the long lifespan of *glp-1(e2141ts)* mutant worms (Fig. 3c). We verified that at the permissive temperature, *ash-2* knockdown extended *glp-1(e2141ts)* mutant worm lifespan (Supplementary Fig. 5a, b). Consistent with these observations, *ash-2* knockdown did not extend the lifespan of sterile *glp-4(bn2ts)* or *pgl-1(bn101ts)* mutant worms (Supplementary Fig. 5c, d). The presence of an intact germline was also necessary for lifespan regulation by WDR-5, SET-2 and RBR-2 (Fig. 3d, Supplementary Fig. 5e–g). In contrast, lifespan regulation by SET-9 and SET-15 did not depend on an intact germline (Supplementary Fig. 6a, b), indicating that not all methyltransferases require the germline to control lifespan.

We performed a genome-wide analysis to identify ASH-2-regulated genes that are dependent on the presence of an intact germline. *ash-2* knockdown led to changes in the expression of 220 genes at the L3 stage and 847 genes at day 8 of life (day 5 of adulthood) in wild-type worms (Fig. 3e, Supplementary Tables 6–9). The majority of ASH-2-controlled genes were regulated in a germline-dependent manner, as their expression was affected in wild-type worms, but not in *glp-1(e2141ts)* mutant worms (93.6% at L3 and 69.8% at day 8 of life)

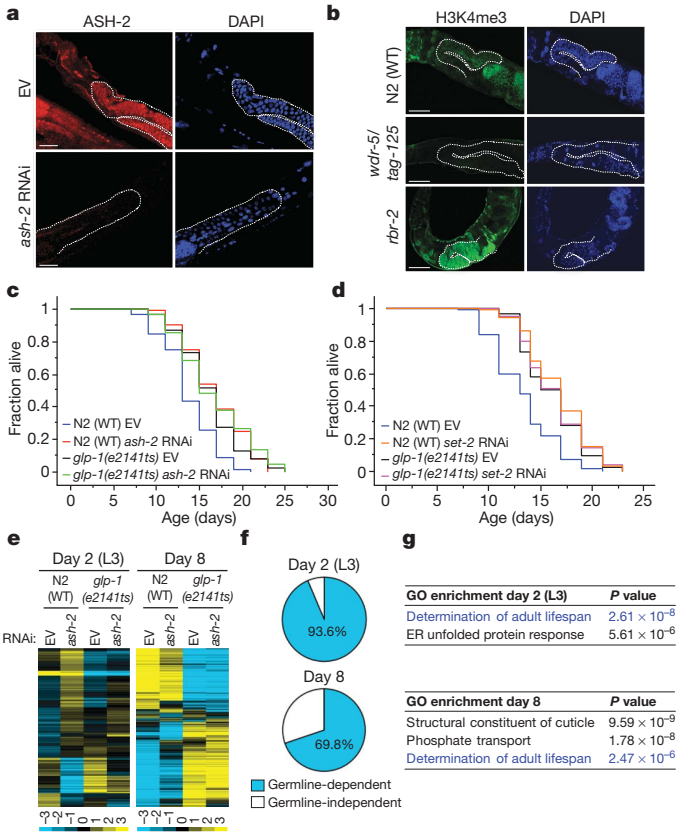

Figure 3 | An intact germline is necessary for longevity and gene expression control by the H3K4me3 regulatory complex. **a**, **b**, Whole-mount immunofluorescence of young adult worms stained with an ASH-2 antibody (**a**) or an H3K4me3 antibody (**b**). Dashed lines mark the germline. Scale bars, 50 μm. **c**, *ash-2* knockdown does not extend further the long lifespan of *glp-1(e2141ts)* mutant worms that were shifted to the restrictive temperature at the L1 stage. **d**, *set-2* knockdown does not extend further the long lifespan of germline-deficient *glp-1(e2141ts)* mutant worms that were shifted to the restrictive temperature at the L1 stage. Statistics are presented in Supplementary Table 4. **e**, Microarray clusters of genes that change significantly on *ash-2* knockdown in wild-type worms, but not in *glp-1(e2141ts)* mutants. Experimental values are presented in Supplementary Tables 8 and 9. **f**, Percentage of genes regulated by ASH-2 only in wild-type worms, but not in *glp-1(e2141ts)* mutants. **g**, Top gene ontology (GO) terms for genes regulated by ASH-2 in wild-type worms, but not in *glp-1(e2141ts)* mutants.

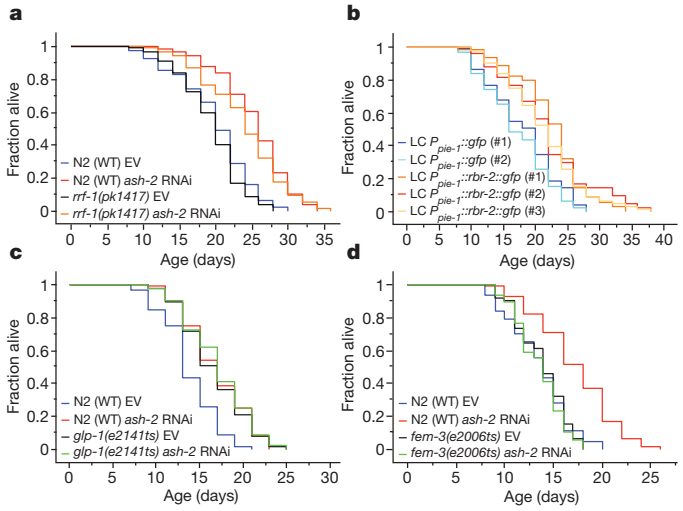

Figure 4 | ASH-2/RBR-2 function primarily in the germline to regulate lifespan and require the continuous production of mature eggs for lifespan extension. **a**, *ash-2* RNAi extends the lifespan of *rrf-1(pk1417)* mutant worms, which are deficient for RNAi in the soma, to a similar extent as in N2 (WT) worms. **b**, Three independent lines of low-copy (LC) integrant $P_{pie\text{-}1}$*::rbr-2::gfp* transgenic worms have increased lifespan compared to two independent lines of $P_{pie\text{-}1}$*::gfp* transgenic worms. **c**, *ash-2* knockdown does not extend further the long lifespan of *glp-1(e2141ts)* mutant worms that were shifted to the restrictive temperature at the young adult stage. **d**, *ash-2* knockdown does not extend the lifespan of *fem-3(e2006ts)* mutant worms, which do not produce mature eggs. *ash-2* mRNA was efficiently knocked down by RNAi in *fem-3(e2006ts)* mutants (Supplementary Fig. 3b). Statistics are presented in Supplementary Tables 4 and 5.

(Fig. 3f). Interestingly, one of the top gene ontology (GO) enrichment categories for genes regulated by ASH-2 at L3 and day 8 was 'determination of adult lifespan' (Fig. 3g, $P = 2.61 \times 10^{-8}$ and $P = 2.47 \times 10^{-6}$, respectively). Furthermore, at both L3 and day 8 of life, ASH-2-regulated genes were also significantly enriched (twofold, $P = 5 \times 10^{-6}$ and 1.5-fold, $P = 6 \times 10^{-6}$, respectively) for genes whose expression changed during ageing[23].

To determine if members of the ASH-2 complex function in the germline itself to regulate lifespan, we used *rrf-1(pk1417)* mutant worms in which RNAi is efficient in the germline, but not in somatic cells[24]. *ash-2* or *set-2* knockdown still extended lifespan in *rrf-1(pk1417)* mutants (23.6%, $P < 0.0001$ and 22.5%, $P < 0.0001$, respectively) (Fig. 4a, Supplementary Fig. 7a), indicating that ASH-2 and SET-2 function in the germline to regulate lifespan. Conversely, to test if RBR-2 expression in the germline was sufficient to extend lifespan, we generated low-copy integrants of RBR-2–GFP driven by the germline-specific *pie-1* promoter ($P_{pie\text{-}1}$*::rbr-2::gfp*). Three independent $P_{pie\text{-}1}$*::rbr-2::gfp* lines had an extended lifespan compared to two independent control $P_{pie\text{-}1}$*::gfp* lines (17.9–30.7%, $P < 0.005$) (Fig. 4b), indicating that expression of RBR-2 in the germline is sufficient to prolong worm lifespan. High-copy $P_{rbr\text{-}2}$*::rbr-2::gfp* transgenic worms also had a modest increase in lifespan (10–14%, $P < 0.005$) (Supplementary Fig. 7b). Because high-copy transgenic worms tend to have suppression of transgene expression in the germline[25], these results indicate that expression of RBR-2 in somatic cells may also contribute to lifespan extension, or that very low amounts of RBR-2 expressed in the germline in these high-copy transgenics may be sufficient to extend lifespan. Thus, the ASH-2 methyltransferase complex and the RBR-2 demethylase act in the germline, at least in part, to regulate lifespan.

The ASH-2 complex and RBR-2 do not seem to regulate lifespan by simply affecting progeny production. *ash-2* knockdown worms were fertile and did not have significant differences in number of eggs laid (Supplementary Fig. 8a), number of live progeny (Supplementary Fig. 8b), germline cell number (Supplementary Fig. 8c) and germline morphology (data not shown). Similarly, *set-2(ok952)*, *wdr-5/tag-125(ok1417)* and *rbr-2(tm1231)* mutant worms did not have significant differences in egg number and were mostly fertile (Supplementary Fig. 8d).

We next investigated whether ASH-2 and RBR-2 require an intact adult or developing germline to regulate lifespan. Knocking-down *ash-2* and *rbr-2* in young adults, after the germline has developed, still affected lifespan (*ash-2*: +26%, $P < 0.0001$; *rbr-2*: −13.5%, $P < 0.0001$) (Supplementary Fig. 9a, b). Furthermore, *ash-2* knockdown no longer extended lifespan in *glp-1(e2141ts)* mutant worms switched to the restrictive temperature at the young adult stage (Fig. 4c), whereas *ash-2* knockdown extended lifespan in *glp-1(e2141ts)* mutant worms that were switched to the restrictive temperature after the egg laying period (Supplementary Fig. 9c). Similar observations were made with *glp-4(bn2ts)* mutant worms (Supplementary Table 4). Consistently, *ash-2* knockdown no longer extended lifespan in another *glp-1* mutant, *glp-1(e2142ts)*, which has a fully developed germline, but no adult germline stem cells or eggs (Supplementary Fig. 9d). Finally, *ash-2* knockdown did not extend further the long lifespan of worms treated with 5-fluorodeoxyuridine (FUdR), a drug that inhibits the proliferation of germline stem cells and the production of intact eggs in adults[26] (Supplementary Fig. 9e). Similarly, *rbr-2* knockdown did not decrease—and

even slightly extended—worm lifespan in the presence of FUdR (Supplementary Fig. 9f), consistent with a previous study[27]. Thus, ASH-2 regulates lifespan in adult worms by a mechanism that depends on the presence of adult germline stem cells and/or the continuous production of eggs.

To distinguish if the germline stem cells or the continuous production of eggs is required for the regulation of lifespan by ASH-2, we used *fem-3(e2006ts)* mutant worms, which have functional germline stem cells, but do not produce fertilized eggs[28]. *ash-2* knockdown no longer extended the lifespan of *fem-3(e2006ts)* mutant worms (Fig. 4d), indicating that the production of mature eggs, rather than germline stem cells, is necessary for *ash-2* knockdown to extend lifespan. Furthermore, *ash-2* knockdown still significantly extended the lifespan of *daf-16(mu86)* and *daf-12(m20)* mutant worms, two genes involved in signalling pathways that are critical for germline stem cells to regulate lifespan[21,22,29] (Supplementary Fig. 10a, b). Consistently, *set-2(ok952)* mutant worms displayed lifespan extension even in the presence of *daf-16* RNAi (Supplementary Table 2). These results indicate that the ability of members of the ASH-2 complex to regulate lifespan depends on the continuous production of mature eggs, but not on the germline stem cell signalling pathway.

Here we show that members of an H3K4me3 methyltransferase complex have a pivotal role in the regulation of longevity in *C. elegans*. Our results indicate that the ASH-2 complex and RBR-2 regulate ageing at least in part by controlling trimethylation of H3K4, although they might also act by controlling the methylation of non-histone proteins. ASH-2 regulates the expression of a specific subset of genes enriched for lifespan determination genes, but more global changes in chromatin state and gene expression—and therefore quantity of protein produced—may also be responsible for the modulation of lifespan by ASH-2. The observation that the continuous production of mature eggs throughout adulthood is necessary for ASH-2 to regulate lifespan in *C. elegans* may explain why the ASH-2 trimethylase complex was not identified in earlier screens for ageing genes in *C. elegans*, in which the production of mature eggs was inhibited[4,10,11]. ASH-2/RBR-2 may function in the germline—perhaps in the maturing eggs—to control endocrine hormones that would, in turn, regulate longevity of the soma. Alternatively, ASH-2/RBR-2 may control the expression of genes involved in lifespan determination, and additional signals from the germline/mature eggs may be required to allow lifespan extension. The regulation of H3K4me3 by specific methyltransferase complexes may regulate ageing in a germline-dependent manner in other species. Male flies heterozygous for the H3K4me3 methyltransferase *trx* had a normal lifespan[8], but *trx* may not be the specific H3K4me3 methyltransferase that regulates lifespan, or the H3K4me3 regulatory complex may be more important in females/hermaphrodites than in males for controlling longevity. The finding that ageing can be regulated epigenetically raises the intriguing possibility that aspects of the ageing process could be reversed.

METHODS SUMMARY

Lifespan assays. Worm lifespan assays were performed at 20 °C, without FUdR, as described previously[30] unless noted otherwise. For each lifespan assay, 90 worms per condition were used in three plates (30 worms per plate), except for the initial RNAi screen, which was performed with 30–60 worms per condition.

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, Fisher's combined probability tests were performed. To compare the interaction between genotype and RNAi, two-way ANOVA tests were performed.

Microarray analysis. N2 (wild type) and *glp-1(e2141ts)* mutant worms were hatched at 16 °C and switched to bacteria with control empty vector or *ash-2* RNAi and moved to the restrictive temperature of 25.5 °C at the L1 stage. RNA was isolated at larval stage L3 or day 8 of life. Each condition was done in independent triplicate with ~1,000 worms per triplicate for L3 worms, and ~200 worms picked every day after adulthood to fresh plates per triplicate for day 8 worms. Total RNA was isolated using an RNAqueous kit (Ambion). Microarray hybridization was performed at the Stanford Protein and Nucleic Acid facility with oligonucleotide arrays (Affymetrix, GeneChip *C. elegans* Genome Arrays). Background adjustment and normalization was performed with RMA (Robust Multiarray Analysis).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions E.L.G. and A.B. conceived and planned the study. E.L.G. performed the experiments and wrote the paper with the help of A.B.; T.J.M. completed Fig. 3a, b, Supplementary Fig. 4c, e and Supplementary Fig. 8c, and generated all low-copy integrant transgenic worm lines. A.G.H. helped with Fig. 1b, e and Supplementary Fig. 1f. E.M.G. was advised by O.G. and completed Fig. 1f and Supplementary Fig. 1g. D.S.L. helped with Fig. 2a. G.S.M. generated all high-copy transgenic worm lines. S.H. helped with Supplementary Fig. 8c. M.R.B. generated the $P_{rbr\text{-}2}$*::rbr-2::gfp* construct. All authors discussed the results and commented on the manuscript.

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METHODS

***C. elegans* strains.** N2 and *daf-16(mu86)* strains were provided by M. W. Tan. *glp-1(e2142ts)* and *rrf-1(pk1417)* strains were provided by A. Fire. *unc-119(ed3)* strain was provided by S. Kim. *wdr-5/tag-125(ok1417)*, *set-2(ok952)*, *rbr-2(tm1231)*, *fem-3(e2006ts)*, *pgl-1(bn101ts)*, *glp4(bn2ts)*, *glp-1(e2141ts)* and *daf-12(m20)* strains were provided by T. Stiernagle and the *Caenorhabditis* Genetics Center. Double mutants were constructed by crossing *rbr-2(tm1231)* males with *glp-1(e2141ts)* hermaphrodites, *set-2(ok952)* males with *rbr-2(tm1231)* hermaphrodites, *wdr-5/tag-125(ok1417)* males with *rbr-2(tm1231)* or *pgl-1(bn101ts)* hermaphrodites. P_{rbr-2}*::rbr-2::gfp* high-copy or low-copy integrant transgenic lines contain 2.3 kilobases of the *rbr-2* promoter driving the full-length *rbr-2* cDNA fused to GFP at the carboxy-terminus. P_{ash-2}*::ash-2::gfp* high-copy or low-copy integrant transgenic lines contain 1.6 kb of the *ash-2* promoter driving the full-length *ash-2* cDNA fused to GFP at the C terminus. P_{pie-1}*::rbr-2::gfp* low-copy integrant transgenic line contains 2.4 kb of the germline-specific *pie-1* promoter[31] driving the full-length *rbr-2* cDNA fused to GFP at the C terminus. P_{pie-1}*::gfp* low-copy integrant transgenic line contains 2.4 kb of the germline-specific *pie-1* promoter driving GFP.

RNA interference. *Escherichia coli* HT115 (DE3) transformed with vectors expressing dsRNA of the genes of interest were all obtained from the Ahringer library (a gift from M. W. Tan), except RNAi to *rbr-2*, *blmp-1*, *set-4* and *set-18* that were from the Open Biosystems library (a gift from K. Shen) and were grown at 37 °C and seeded on standard nematode growth medium (NGM) plates containing ampicillin (100 mg ml^{-1}) and isopropylthiogalactoside (IPTG; 0.4 mM). Adult worms were placed on standard NGM plates and removed after 4–6 h to obtain synchronized populations of worms. L1 worms obtained from these synchronized populations were placed on NGM plates containing ampicillin (100 mg ml^{-1}) and IPTG (0.4 mM) seeded with the respective bacteria. Worms placed on RNAi at different time points were placed on empty vector control RNAi at the L1 stage and shifted to the respective bacteria at the appropriate time.

Constructs. pSM, a derivative of the pPD49.26 plasmid[32] with additional cloning sites was used to generate transgenic constructs. P_{rbr-2}*::rbr-2::gfp* transgenic plasmid included 2.3 kb of *rbr-2* promoter driving the full-length *rbr-2* cDNA fused to GFP at the C terminus. The *rbr-2* promoter was amplified by PCR from wild-type (N2) genomic DNA using the following primers, F: 5′-ATAGCGGCCGCC CAACATTCTCTGGTGTGTATG-3′ and R: 5′-TATGGATCCTTTTCAGTTTG GCTTATAGCTTCATGAG-3′ and subcloned into pSM between NotI and BamHI. The *rbr-2* cDNA was amplified by PCR from *rbr-2* cDNA[19] with the following primers F: 5′-ATAGCTAGCATGCGTGCACGTCGTCAAGAGAAC AG-3′ and R: 5′-TATACCGGTAAATCGGTGGAAACACTCGAAGTTGGAGA GTCC-3′ and subcloned into pSM containing the *rbr-2* promoter, between NheI and AgeI.

P_{ash-2}*::ash-2::gfp* transgenic plasmid included 1.6 kb of *ash-2* promoter driving the full-length *ash-2* cDNA fused to GFP at the C terminus. The *ash-2* promoter was amplified by PCR from wild-type (N2) genomic DNA using the following primers, F: 5′-ATAGCTAGCATCTCGTTCTTCTTCTGCTCACGG-3′ and R: 5′-TATGTCGACAGTGCTGAAAATCTAGTTTTTTAG-3′ and subcloned into pSM between NheI and AccI. The *ash-2* cDNA isoform A (*C. elegans* ORFeome library, Open Biosystems) was amplified by PCR with the following primers, F: 5′-ATAGTCGACATGAGAAGCTCAAAAGGAGGTCGGGG-3′ and R: 5′-TA TGGTACCATAATTTCTTGTTTAATTTCTTTC-3′ and cloned into pSM containing the *ash-2* promoter, between AccI and KpnI. To generate the P_{pie-1}*::rbr-2::gfp* biolistic bombardment plasmid, *rbr-2::gfp* was subcloned between SpeI and XmaI into the pIC26 plasmid[33] containing 2.4 kb of the *pie-1* promoter[31]. To generate the P_{ash-2}*::ash-2::gfp* and P_{rbr-2}*::rbr-2::gfp* biolistic bombardment plasmids, the *unc-119* rescue fragment and a PacI linker: 5′-GGGGTTAA TTAACCCCGC-3′ were subcloned between SpeI and PacI in pSM P_{ash-2}*::ash-2::gfp* and pSM P_{rbr-2}*::rbr-2::gfp*. To generate a bacterially expressed form of SET-2, the SET domain of the *set-2* cDNA isoform B (*C. elegans* ORFeome library, Open Biosystems) was amplified by PCR with the following primers, F: 5′-ATAGGATCCCAACGTCGATTGCTTACGTCACTTGGTG-3′ and R: 5′-TATCTCGAGTCAATTAAGATATCCACGACACGTCTTCGC-3′ and cloned into pGEX 4T1 (Promega) between BamHI and XhoI. To generate a bacterially-expressed fusion protein between maltose-binding protein (MBP) and ASH-2, full-length *ash-2* cDNA was amplified by PCR with the following primers, F: 5′-ATAGAATTCATGAGAAGCTCAAAAGGAGGTCGGGGACG-3′ and R: 5′-TATTCTAGATTAAATTTCTTGTTTAATTTCTTTCTTG-3′ and then subcloned between EcoRI and XbaI into the pMCH vector[34]. All the fragments generated by PCR were entirely sequenced to verify that there were no mutations introduced by the PCR amplification steps.

High-copy transgenic worm generation. Extrachromosomal-array-carrying transgenic strains were generated using a microinjector, injecting 10 ng μl^{-1} of the transgene plasmid and 60 ng μl^{-1} of the co-injection plasmid P_{odr-1}*::dsred* (ref. 35). Two or three independent transgenic lines for each transgene were examined for fluorescence expression and lifespan assays. Fluorescence images were obtained using a confocal microscope Zeiss LSM 510 META.

Low-copy integrant transgenic worm generation by biolistic transformation. Transgenic worm strains were generated using standard biolistic transformation[36] by bombarding *unc-119(ed3)* worms with the constructs containing the indicated cDNA fused to GFP driven by either endogenous promoters or by the *pie-1* germline specific promoter, as well as the *unc-119* rescue gene. Approximately 2 weeks after bombardment, worms in which the uncoordinated phenotype was rescued were picked to individual NGM plates. Progeny were kept at 25 °C for 3–4 days and were examined for GFP genomic integration by PCR. GFP-positive worms were selected and expanded[37]. Two to three independent lines were generated per construct.

Quantitative RT–PCR. Two hundred worms were picked to NGM plates with *E. coli* OP50 overnight two days in a row. Worms were then picked to NGM plates without bacteria and washed three times with M9 buffer (22 mM KH_2PO_4, 34 mM K_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$). Worm pellets were resuspended in TRIzol (Invitrogen), followed by six freeze-thaw cycles in liquid N_2. One microgram 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) with the following primers: pan-actin F: 5′-TCGGTATGG GACAGAAGGAC-3′, pan-actin R: 5′-CATCCCAGTTGGTGACGATA-3′, *ash-2* F: 5′-CGATCGAAACACGGAACGA-3′, *ash-2* R: 5′-TGCCGGAATCTGC AGTTTTT-3′. The experiments were conducted in duplicate and the results were expressed as $2^{(-(\text{target gene number of cycles}-\text{pan-actin number of cycles}))}$.

Microarray analysis. One-class analysis in significance analysis of microarrays (SAM)[38] was performed comparing empty vector to *ash-2* RNAi using a 5% false discovery rate (FDR). Resulting SAM lists of genes regulated by ASH-2 were compared for genes that were specifically changed in N2, but not in *glp-1(e2141ts)* mutant worms. A complete linkage hierarchical clustering was performed using Gene Cluster 3.0 (ref. 39). Clustering results were analysed further with Java Treeview[40]. GOstat[41] was used to identify overrepresented gene ontology categories in the list of ASH-2 regulated genes that were dependent on the presence of the germline.

Antibodies. The monoclonal ASH-2 antibody was generated by injection of a fusion protein between GST and amino acids 285–572 of *C. elegans* ASH-2 and was affinity-purified by Abmart (Shanghai, China). The H3K4me3 antibody (8580), the H3K4me1 antibody (8895), the H3K9me3 antibody (8898) and the H3 antibody (1791) were obtained from Abcam. The H3K4me2 antibody (07-030) was obtained from Upstate Biotechnology. The affinity-purified monoclonal GFP antibody was a gift from A. Villeneuve.

Whole-mount 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 Na_2EGTA, 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 N_2. 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 H_3BO_3, 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 with H3K4me3 antibody (1:100), the monoclonal ASH-2 antibody (1:100), or with an affinity-purified GFP antibody (1:1,000) and with Alexa Fluor 594 secondary antibody (1:25–1:100). 4′,6-diamidino-2-phenylindole (DAPI; 2 mg ml^{-1}) was added to visualize nuclei. The worms were mounted on a microscope slide and visualized using a Leica SP2 confocal system or a Zeiss Axioskop2 plus fluorescence microscope.

Protein analysis by western blot. Worms were grown synchronously to appropriate stages and washed off plates with M9 buffer (22 mM KH_2PO_4, 34 mM K_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$). Worms were washed several times in M9 buffer and snap frozen in liquid N_2. Sample buffer (2.36% SDS, 9.43% glycerol, 5% β-mercaptoethanol, 0.0945 M Tris-HCl pH 6.8, 0.001% bromophenol blue) was added to worm pellets and they were repeatedly snap frozen in liquid N_2. Worm extracts were sonicated three times for 30 s at ~15 W (VirSonic 600) and boiled for 2 min before being resolved on SDS–PAGE (14%) and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies (H3K4me3, 1:1,000; H3K4me2, 1:2,000; H3K4me1, 1:500; H3K9me3, 1:1,000; H3, 1:2,000) and the primary antibodies were visualized using horseradish peroxidase-conjugated anti-rabbit secondary antibody (Calbiochem 401393) and ECL Plus (Amersham Biosciences).

***In vitro* methylation assays.** Methyltransferase assays were performed as described previously[42]. Briefly, 10 μg of glutathione *S*-transferase (GST)-purified SET-2_{SET} was incubated with nucleosomes purified from HeLa cells in the presence of either 0.1 mM *S*-adenosyl-methionine (SAM) or 2 μCi [^{3}H]SAM at 20 °C overnight in a

methyltransferase reaction buffer (50 mM Tris-HCl pH 8.0, 10% glycerol, 20 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride (PMSF)). Reactions were subjected to SDS–PAGE and either autoradiography or western blot as described above.

Fertility assays. Wild-type (N2) worms treated with empty vector or *ash-2* RNAi bacteria or *set-2(ok952)*, *wdr-5/tag-125(ok1417)*, or *rbr-2(tm1231)* mutant worms were synchronized. From day 3 to day 8 of life, 10 worms were placed on NGM plates with OP50-1 in triplicate (30 worms total per condition). After 24 h, the adult worms were removed from each plate and the number of eggs was counted. For *ash-2* RNAi, the number of live progeny was also counted after 48 h. Statistical analyses of fertility were performed using two-way ANOVA tests with Bonferroni post-tests, or *t*-tests using the mean and standard error values.

Germline cell morphology. N2 worms were grown until either day 4 or day 8 on plates with bacteria expressing empty vector or *ash-2* RNAi. Worms were then fixed, permeabilized and DAPI-stained as described above. Worms were imaged on a Leica SP2 confocal microscope. *Z*-sections of germ cell nuclei were imaged, compiled to stacks and manually counted in a blinded manner using MetaView imaging software. For each condition, distal gonad germ cell numbers from three worms were counted and the results of two independent experiments were analysed using a two-tailed Student' *t*-test.

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