H3K4 demethylase activities repress proliferative and postmitotic aging

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Summary
Homeostasis of postmitotic and proliferating cells is maintained by pathways that repress stress. We found that the Caenorhabditis elegans histone 3 lysine 4 (H3K4) demethylases RBR-2 and SPR-5 promoted postmitotic longevity of stress-resistant daf-2 adults, altered pools of methylated H3K4, and promoted silencing of some daf-2 target genes. In addition, RBR-2 and SPR-5 were required for germ cell immortality at a high temperature. Transgenerational proliferative aging was enhanced for spr-5; rbr-2 double mutants, suggesting that these histone demethylases may function sequentially to promote germ cell immortality by targeting distinct H3K4 methyl marks. RBR-2 did not play a comparable role in the maintenance of quiescent germ cells in dauer larvae, implying that it represses stress that occurs as a consequence of germ cell proliferation, rather than stress that accumulates in nondividing cells. We propose that H3K4 demethylase activities promote the maintenance of chromatin states during stressful growth conditions, thereby repressing postmitotic aging of somatic cells as well as proliferative aging of germ cells.

Key words: Caenorhabditis elegans; cellular aging; chromatin; histone demethylase; life span.

Introduction
Cellular aging has been attributed to the dysfunction of multiple maintenance mechanisms. As humans age, global changes in epigenetic mechanisms occur, which alters the regulation of gene expression (Bocklandt et al., 2011). Further, in the accelerated aging disorder Hutchinson–Gilford Progeria syndrome, nuclear lamin A defects lead to dramatic changes in epigenetic modifications prior to alterations in nuclear morphology associated with disease progression (Shumaker et al., 2006). Because epigenetic modifications are reversible and could represent attractive therapeutic targets, it is important to understand how such modifications influence aging (Stillings & Fischer, 2011).

An epigenetic mark that is strongly correlated with transcriptional activation is the methylation state on histone 3 lysine 4 (H3K4), which can be mono (Me1)-, di (Me2)-, or tri (Me3)-methylated. The methylation status of any nucleosome is determined by a balance of the activities of specific histone lysine methyltransferases (KMTs) and histone lysine demethylases (KDMs) (summarized in Allis et al., 2007). Two classes of enzymes remove lysine-methyl marks: amino oxidases (KDM1) and Jumonji C (JmJC) domain-containing proteins. KDM1 proteins only remove Me1 and Me2 in a reaction that requires the cofactor flavin adenine dinucleotide (FAD) (Hou & Yu, 2010). Demethylases containing the JmJC domain can catalyze the removal of all methyl marks from specific lysine residues in vivo (Allis et al., 2007). The KDM5 class of JmJC proteins has specificity for H3K4. In humans, there are four KDM5 proteins, but many lower organisms possess only one member of this class (Allis et al., 2007).

A well-established system for studying the genetics and epigenetics of aging is the nematode Caenorhabditis elegans. Although H3K4 methylation can impact aging, KDM1 and KDM5 demethylases do so in opposing ways. The sole C. elegans KDM5 member, RBR-2, can remove both Me3 and Me2 marks from H3K4, but is more efficient at removing Me3 (Christensen et al., 2007). RBR-2 was originally identified as being transcriptionally down-regulated in long-lived daf-2 insulin/IGF-1 signaling mutants, suggesting that H3K4 demethylation may promote aging (Lee et al., 2003). However, overexpression of rbr-2 in the germline represses aging and extends lifespan, while the rbr-2(m1231) deletion mutant is short-lived (Greer et al., 2010). Furthermore, reduction in function of components of the ASH-2 trithorax complex, which results in reduced levels of H3K4 trimethylation, increases lifespan, and this effect is suppressed by deficiency for rbr-2 (Greer et al., 2010). Together, these results suggest that H3K4 methylation can be a pro-aging chromatin mark.

Opposing effects were observed when KDM1 demethylase activity was compromised. In C. elegans, there are three KDM1 genes. amx-1 encodes the sole KDM1B enzyme, while there are two paralogous KDM1A enzymes encoded by the spr-5 and lsd-1 genes (Eimer et al., 2002; Jarriault & Greenwald, 2002). Animals with a reduced function for lsd-1 have a longer life compared to wild-type strains (McColl et al., 2008; Maures et al., 2011). Thus, KDM1 and KDM5 enzymes may exert opposite effects on postmitotic aging by targeting enzyme class-specific substrates for H3K4 demethylation.

Although adult somatic tissue is postmitotic in C. elegans, aging of proliferating cells is relevant to organisms where somatic stem cell populations continue to divide throughout life. The germline is capable of maintaining itself in a nonaging pluripotent state over the generations, despite indefinite proliferation. Genes that promote proliferative immortality of germ cells have been identified in screens for C. elegans mortality germline (mrt) mutants that are initially fertile and become sterile after propagation for a number of generations, including several mutants that are defective for telomerase-mediated telomere replication (Ahmed & Hodgkin, 2000; Meier et al., 2006).
H3K4 methylation plays important roles in the germ cells of many organisms: H3K4 methylation is absent in the pole cells found in the Drosophila germline but accumulates with age, whereas removal of H3K4me2 occurs during the birth of germ cell precursors in *C. elegans* and remains absent until these cells begin to divide in the L1 larval stage (Schaner et al., 2003). KDM1 members play a role in this process, as mutations in *spr-5* could elicit fertility defects after many generations, and this was correlated with increasing retention of H3K4 methylation in germline precursor cells (Katz et al., 2009). *spr-5* elicited variable and progressive fertility defects, where high levels of sterility occurred after growth for 20 generations, an effect that was enhanced in *spr-5; amx-1* double mutants (Katz et al., 2009).

To characterize the role of H3K4 demethylases in the maintenance of somatic and germ cells, we examined the impact of *rbr-2* deficiency on somatic longevity in adults, as well as in germ cell maintenance as they proliferate over many generations, and in the maintenance of nondividing germ cells in long-lived dauer larvae. We also examined the function of *spr-5*. Both *rbr-2* and *spr-5* extended longevity in a manner that depended on reduced insulin/IGF-1 signaling, implying that demethylation of H3K4 by RBR-2 and SPR-5 contributes to the effect of insulin/IGF-1 signaling on longevity. Further, roles for the H3K4 demethylases *rbr-2* and *spr-5* were detected in germ cell maintenance over the generations under conditions of high temperature stress, which was exacerbated in strains where function of both genes was reduced. Thus, H3K4 demethylation may repress proliferative and postmitotic aging in response to stressful circumstances that include high environmental temperatures as well as genetic activation of a stress response pathway.

## Results

### Deficiency for *rbr-2* does not affect somatic morphology

In order to characterize *rbr-2*, we utilized two deletion alleles of *rbr-2*, *tm1231*, and *ok2544*. Both mutations cause in-frame deletions in exon 5 that remove the JmjC domain and therefore should lack demethylase activity (Fig. 1A). These deletions were verified by PCR genotyping and outcrossed four times in order to remove unlinked mutations that were generated during the creation of each deletion. Previous analysis of *rbr-2* (*tm1231*) revealed a high percentage of animals with vulval defects (Christensen et al., 2007). Moreover, RNAi knockdown of *rbr-2* in a background that displays a highly penetrant Multivulva phenotype at high temperature, *lin-15(n765ts)*, elicited Multivulva and Vulvalves phenotypes at low temperature, suggesting that the RBR-2 demethylase may interact with chromatin factors involved in the synthetic multivulva-B pathway (Ferguson & Horvitz, 1989; Lu & Horvitz, 1998; Solari & Ahringer, 2000; Christensen et al., 2007). In our hands, the initial lines of *rbr-2(tm1231)* exhibited weakly penetrant Proruding Vulva and Vulvalves phenotypes and continued to do so after being outcrossed. However, while initial *rbr-2(ok2544)* lines showed a weakly penetrant Vulvalves phenotype (observed in 29% of animals), outcrossed lines did not exhibit vulval defects. In addition, we observed that *tm1231* adults had a strong Small phenotype, whereas *ok2544* animals were normal in size (Table S1).

As the *tm1231* and *ok2544* deletions had different phenotypes, we examined whether the *rbr-2* deletion isolate *tm1231* had an unlinked mutation that elicits vulval or body size defects. We attempted to separate *rbr-2(tm1231)* from tightly linked mutations by singling Unc-non-Dpy recombinants from *+/rbr-2(tm1231) +/unc-24+ dpy-20* heterozygotes to create *unc-24 rbr-2 (tm1231)* double mutants. One

Fig. 1 H3K4 demethylases and somatic longevity. (A) Gene structure of *rbr-2* deletion alleles ok2544 and tm1231. The Jumonji C domain is shown in gray. Interaction of (B) *rbr-2(ok2544)* with *daf-16(mu16)* and (C) *daf-2(e1370)* with *rbr-2* or *spr-5* on lifespan. For (C), strains with solid lines were examined in the same experiment, while strains with a dotted line were examined together in a separate experiment. Mean lifespan and statistics are presented in Table S2. unc-24 *rbr-2(tm1231)* strain was generated and continued to exhibit Vulvalves worms in 17% of the animals examined. However, *unc-24 rbr-2(tm1231) +/+ rbr-2(ok2544) dpy-20* transheterozygotes failed to display vulval morphology defects or a Small phenotype in adults (Table S1), both of which are easily observed for outcrossed *tm1231* homozygotes using a dissecting microscope. Because both deletions remove the JmjC domain of RBR-2, with *ok2544* being the larger deletion (Fig. 1A), deficiency for *rbr-2* demethylase activity is unlikely to perturb vulval morphology or body size.

### H3K4 demethylation promotes longevity of *daf-2* adults

It was previously reported that RNAi knockdown of *rbr-2* in wild-type adults results in an increase in lifespan at 25 °C (Lee et al., 2003; Ni et al., 2012). However, the *rbr-2(tm1231)* strain has been reported to display reduced longevity (Greer et al., 2010), and overexpression of a GFP::rbr-2 fusion protein can extend lifespan of adult wild-type animals at 20 °C (Greer et al., 2010). At 20 °C, our outcrossed *rbr-2(ok2544)*
strain exhibited both a longer mean and maximum lifespan ($P < 0.05$) (Table 1; Fig. S1). At 25 °C, both mean and maximum lifespan were also extended for rbr-2(ok2544) adults ($P < 0.01$), as well as for unc-24 rbr-2 (tm1231) +/- rbr-2(ok2544) dpy-20 transheterozygotes ($P < 0.01$), in comparison with wild-type controls (Table 1; Fig. 1B). Log-rank analysis showed that these findings are significant, and two-way ANOVA showed no difference in the effect at two different temperatures ($P < 0.0178$). Finally, daf-16 single mutants were modestly short-lived in comparison with wild-type ($P < 0.001$), consistent with previous reports (Kenyon et al., 1993; Lakowski & Hekimi, 1996; Lin et al., 2001; Larsen et al., 2005), and rbr-2 deficiency did not modify lifespan in a daf-16 background ($P < 0.1$). Thus, the longevity of rbr-2 single mutants may be mediated via DAF-16 (Fig. 1B).

Although rbr-2 has not been identified in many studies examining downstream targets of DAF-2/DAF-16 signaling (Jones et al., 2001; McLewee et al., 2003; Murphy et al., 2003; Halaschek-Wiener et al., 2005). Lee et al. (2003) identified putative DAF-16 target sites genomewide, one of which corresponded to the rbr-2 locus, and then rigorously showed that rbr-2 was down-regulated in daf-2 adults. We therefore assessed the lifespans of daf-2(e1370); rbr-2(ok2544) and daf-2(e1370); rbr-2(tm1231) double mutants. Sharp reductions in mean lifespan were observed for daf-2(e1370); rbr-2 double mutant strains in comparison with wild-type ($P < 0.001$), consistent with previous reports (Kenyon et al., 1993; Lakowski & Hekimi, 1996; Lin et al., 2001; Larsen et al., 2005), and rbr-2 deficiency did not modify lifespan in a daf-16 background ($P < 0.1$). Thus, the longevity of rbr-2 single mutants may be mediated via DAF-16 (Fig. 1B).

To find targets of RBR-2 or SPR-5 in daf-2 mutants, we examined a total of 17 genes previously shown to be silenced when daf-2 is deficient, or de-silenced when rbr-2 or spr-5 is deficient (Murphy et al., 2003; Katz et al., 2009; Greer et al., 2010). RNA was harvested from wild-type, daf-2 single mutants, and spr-5; daf-2 or rbr-2; daf-2 double mutants, and cDNA was made and normalized by RT–PCR using act-1.

We found that six genes were repressed in daf-2, but not in wild-type RNA samples: cav-1A, T09B4.5, F08G5.6, dod-22, dod-24 and T24B8.5 ($n = 2$ RT–PCR experiments for two independently created cDNA aliquots per genotype) (Fig. 2B). cav-1A and T09B4.5 were consistently up-regulated in spr-5; daf-2 or rbr-2; daf-2 double mutants (Fig. 2B). Expression of the remaining four genes was restored for only a subset of spr-5; daf-2 or rbr-2; daf-2 genotypes. In addition, we analyzed the twelve sperm genes that were previously shown to be repressed by SPR-5 (Katz et al., 2009). We confirmed that nine of these genes were desilenced in daf-2; spr-5 mutants compared to daf-2 alone, but did not show changes in daf-2(e1370) versus wild-type as represented by C10G11.9 (Fig. 2B). Further, many were also consistently elevated in daf-2; rbr-2 (tm1231) double mutants, which was not observed for daf-2; rbr-2 (ok2544) (Fig. 2B). Only one of the 12 sperm genes, CO2FS.5.5, was repressed in daf-2 compared to wild-type. CO2FS.5.5 exhibited an expression profile similar to T24B8.5 (Fig. 2B). Together, our data imply that a subset of genes that are silenced when daf-2 is deficient may be targeted for silencing by RBR-2 and SPR-5 demethylases.

**Effects of rbr-2 on fertility**

Deficiency for spr-5 has been previously reported to result in high levels of sterility after growth for many generations at 20 °C (Katz et al., 2009). rbr-2(ok2544) and rbr-2(tm1231) strains that had been outcrossed a single time had lower brood sizes in comparison with

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**Table 1** An rbr-2(ok2544) deficiency results in increased lifespan at 20 and 25 °C

| Strains                        | Temperature (°C) | Mean lifespan (days) | Maximum lifespan (days) | No. death/censored (no. trial) |
|-------------------------------|------------------|----------------------|-------------------------|--------------------------------|
| Wild-type                     | 20               | 19.52 ± 1.03         | 33                      | 332 (1)                        |
| rbr-2(ok2544)                 | 20               | 22.25 ± 0.97*        | 37                      | 64/11 (1)                      |
| Wild-type                     | 25               | 12.94 ± 0.33         | 25                      | 305/87 (3)                     |
| rbr-2(ok2544)                 | 25               | 14.91 ± 0.73**       | 40                      | 74/26 (1)                      |
| rbr-2(tm1231); rbr-2(ok2544)  | 25               | 14.22 ± 0.61**       | 36                      | 105/45 (2)                     |
| daf-16(mu86)                  | 25               | 11.29 ± 0.21†        | 21                      | 334/101 (3)                    |
| daf-16(mu86); rbr-2(ok2544)   | 25               | 10.88 ± 0.41†        | 19                      | 66/34 (2)                      |

*Days ± SEM.

* $P < 0.050$ compared to the wild-type control using Mantel–Cox log-rank test.

** $P < 0.010$ compared to the wild-type control using Mantel–Cox log-rank test.

$P < 0.001$ compared to the wild-type control using Mantel–Cox log-rank test.
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wild-type controls (Fig. 3A), whereas four outcrosses led to wild-type levels of fertility for ok2544, but not for tm1231 (Fig. 3A). Thus, the effects of tm1231 on brood size are independent of the histone demethylase activity of rbr-2.

Transgenerational effects of rbr-2 deficiency on fertility were studied by propagating strains for many generations at 20 °C, or at the higher stressful temperature of 25 °C, using the mortal germline assay, in which small population bottlenecks of 6 L1 larvae are transferred every two generations (Smeklic & Ahmed, 2005). In early generations, as well as after many generations of growth at 20 °C, outcrossed rbr-2 strains displayed very few or no sterile animals when assessed either by singling cohorts of 40 L4 larvae or by scanning large populations of adults for sterile animals with a germline proliferation (Glp) defect (sterile adult animals displaying an empty uterus that is clearly visible under a dissecting microscope). However, growth at 25 °C resulted in immediate drops in fertility for both ok2544 and tm1231. Subsequent propagation at 25 °C resulted in large numbers of Glp animals for both alleles, ultimately leading to complete sterility for all rbr-2 tm1231 lines by generation 16 (n = 17 lines). In contrast, only 90% of rbr-2(ok2544) lines became sterile during 30 generations of growth at 25 °C (n = 20 lines) (Fig. 3B). Thus, deficiency for rbr-2 causes a Mortal Germline (Mrt) phenotype that is incompletely penetrant after 30 generations of growth (Ahmed & Hodgkin, 2000).

The morphology of sterile adult hermaphrodite germlines of tm1231 and ok2544 homozygotes as well as unc-24 rbr-2(tm1231) +/+ rbr-2 (ok2544) dpy-20 transheterozygotes was visualized by fluorescence microscopy using the DNA-intercalating dye 4′,6-diamidino-2-phenylin- dole (DAPI). Fertile rbr-2 transheterozygotes in early generations at 25 °C had wild-type-sized germlines (Fig. 4A), whereas late-generation sterile adults often exhibited small or empty germline arms (Fig. 4B,C). Consis- tently, sterile late-generation rbr-2(tm1231) adults exhibited empty or small germlines (42%, n = 128), but also displayed a germline overpro- liferation phenotype not seen in the transheterozygotes in late generations (Table 2). High-resolution analysis of the number of DAPI spots in oocytes arrested in diakinesis revealed 5.69 ± 0.1 for wild-type controls, 5.74 ± 0.1 for sterile rbr-2(tm1231) adults, and 5.89 ± 0.1 for sterile transheterozygotes (Table 2), indicating that sterility is not caused by chromosome fusions, as occurs in C. elegans telomerase mutants (Meier et al., 2006).

A second H3K4 demethylase represses proliferative aging of germ cells at high temperature

The spr-5 (KDM1A) H3K4 demethylase allele by101 has been shown to cause high levels of sterility (~90%) when propagated at 20 °C for many generations (Katz et al., 2009), which was enhanced by the loss of amx-1 (KDM1B). However, loss of function of all C. elegans KDM1 genes did not prevent H3K4 demethylation in the germline precursor cells Z2 and Z3 in early generations. We therefore hypothesized that KDM1 fertility defects of spr-5 mutants might be exacerbated by deficiency for the KDM5 demethylase rbr-2. spr-5 alleles were isolated based on their ability to suppress the egg-laying defects seen in sel-12 presenilin mutants (Eimer et al., 2002; Jarriault & Greenwald, 2002). We did not use the spr-5 allele by101 because it was the first spr-5 allele isolated and was the longest cultured of the spr-5 alleles and because spr-5 strains have recently been shown to be deficient for meiotic double-strand break repair (Nottke et al., 2011). Thus, by101 may have a significant load of spontaneous background mutations due to extended culturing (Eimer et al., 2002; Jarriault & Greenwald, 2002). Furthermore, by101 is caused by a Tc3 transposon insertion and exhibits two spr-5 transcripts: one that is longer than the wild-type, presumably containing the Tc3 insertion, and a second transcript of approximately wild-type length that likely results from transposon excision (Eimer et al., 2002). Instability of the by101 Tc3 transposon insertion, possibly due to alterations in epigenetic marks or to novel mutations in the by101 strain, could have contributed to the fertility phenotypes previously reported for this allele (Katz et al., 2009). Because all spr-5 alleles have a very similar strong suppressor of sel-12 phenotype, we chose to examine the by134 allele, which has an early stop codon that severely reduces transcript levels and truncates the SPR-5 protein (Eimer et al., 2002). Thus, by134 is likely to be a very strong loss of function, or null, allele of spr-5 and should have no enzymatic activity (Eimer et al., 2002).

We observed a small number of Glp animals in outcrossed spr-5 (by134) strains that had been grown for over 60 generations at 20 °C, with 1 of 49 singled L4s exhibiting no progeny. Brood size counts of non- Glp animals revealed lower than wild-type brood sizes of 79.3 ± 8.22 progeny per worm (n = 27). However, passing 6 L1s at 20 °C every
Deficiencies in H3K4 demethylation promote increased fertility defects at 25 °C. (A) Brood size at 20 °C for N2 wild-type, rbr-2(tm1231) and rbr-2(ok2544) after one (1X) or four (4X) outcrosses. (B) The effect of rbr-2 and spr-5 mutations on fertility until the F30 generation when propagated at 25 °C.

Fig. 3 Late-generation rbr-2 transheterozygotes display atrophied germlines. (A) Wild-type germline of unc-24 rbr-2(1231)+/+ rbr-2(ok2544) dpy-24 F3 generation grown at 25 °C. (B) Atrophied germlines found in sterile late-generation unc-24 rbr-2(1231)+/+ rbr-2(ok2544) dpy-24 adults from independently propagated lines. (C) Magnified image of germline arm in (B). Dotted lines outline the germline. Bar represents 100 μm.

Deficiency for rbr-2 at high temperature elicited a late-onset Mrt phenotype (sterility after growth for many generations). spr-5 strains all became sterile by generation 14 at 25 °C (n = 17/17 lines tested) (Fig. 3B). This Mrt phenotype was more rapid and penetrant than for rbr-2 at 25 °C or for spr-5 mutations at 20 °C (Katz et al., 2009). Moreover, four independently derived spr-5(by134); rbr-2(ok2544) double-mutant strains became sterile by generation F8, indicating that deficiency for rbr-2 enhances the Mrt phenotype of spr-5 at 25 °C (P < 0.05; n = 19 lines in total, at least 2 lines tested per initial strain in addition to ten outcrossed lines) (Fig. 3B).

Analysis of sterile spr-5 or spr-5; rbr-2 adults by microscopy revealed phenotypes similar to those of sterile rbr-2 mutants. For example, 5.76 ± 0.2 bivalents were observed for spr-5; rbr-2 germlines that did contain oocytes (Table 2). Additional phenotypes were occasionally observed for spr-5(by134), including disorganized DAPI-stained chromosomes in oocytes at the germline bend (Fig. S4A), endomitotic oocytes (Iwasaki et al., 1996), as well as a modest reduction in the number of bivalents per oocyte (5.4 ± 0.1; P < 0.05) (Fig. S4B–C).
RBR-2 does not promote longevity of quiescent germ cells

rbr-2 deficiency results in a partially penetrant Mortal Germline phenotype at 25 °C, which suggests transmission and accumulation of a heritable form of damage, or ‘proliferative ageing’, of germ cells over multiple generations of growth. We tested for an analogous role of rbr-2 in the maintenance of quiescent germ cells over long periods of time using dauer larvae, a stress-induced larval stage that is long-lived. daf-2 mutants arrest development as dauer larvae at 25 °C but can resume development and produce progeny when transferred to 15 °C. Cohorts of dauer larvae for daf-2(e1370); rbr-2(ok2544) double mutants as well as daf-2(e1370) single-mutant controls were maintained at 25 °C for several months. Every 10–15 days, pools of dauers were single and transferred to 15 °C for recovery. Both strains showed comparable levels of high viability, development to adulthood, and fertility for 60 days (Fig. 5). At 75 days, although daf-2(e1370) dauer larvae appeared paralyzed, they moved slightly when single and showed movement on the recovery plate. However, they failed to recover at 15 °C. Unexpectedly, a high percentage of daf-2(e1370); rbr-2(ok2544) double-mutant larvae recovered and continued to display high levels of fertility when transferred to 15 °C until day 98, at which time the experiment was terminated. In contrast, most rbr-2(ok2544) strains that had been propagated continuously at 25 °C for 98 days (28 generations) became sterile (Fig. 3B). Thus, deficiency for rbr-2 failed to compromise the maintenance of quiescent germ cells in dauer larvae. The prospect that rbr-2 function could be detrimental to longevity of daf-2-mutant dauer larvae deserves further investigation, as most studies of the effects of daf-2 on longevity concern adults. We speculate that regulation of gene expression by RBR-2 during dauer entry or dauer diapause could promote an optimal life history strategy that results in a trade-off in dauer longevity.

To further investigate the interaction of H3K4 demethylases with reduced insulin/IGF-1 signaling, we examined a role for rbr-2, as well as spr-5, in constitutive dauer formation of daf-2(e1370) mutants at 25 °C. At 25 °C, a strong dauer formation phenotype was observed for daf-2(e1370); rbr-2 double mutants (Table S5). Thus, the defect in lifespan is not accompanied by a defect in dauer formation per se. At 20 °C, only a proportion of daf-2(e1370) larvae arrest as dauers, and no consistent effect was observed for both rbr-2 alleles (plenty of dauers occurred in both cases) (Table S5). However, spr-5(bly134); daf-2 double mutants exhibited few dauers at 20 °C (Tables S5 and S6), suggesting that spr-5 promotes dauer formation in conditions where dauer formation is weakly induced. Thus, while both rbr-2 and spr-5 promote longevity in daf-2 mutant adults, only spr-5 promotes dauer formation in daf-2 mutants, and rbr-2 promotes dauer aging. This reveals selective roles for rbr-2 and spr-5 on different aspects of diapause and longevity in IIS mutants.

### Discussion

Longevity assays in model invertebrates typically focus on adult somatic cells that are quiescent (C. elegans) or that only possess small populations of stem cells (Drosophila). Studies of proliferative aging in C. elegans have defined a number of mutants that are deficient for telomerase-mediated telomere replication. However, neither telomere length nor telomerase affects postmitotic aging of C. elegans adults (Meier et al., 2006). In this study, we define two proteins that can repress aging in both postmitotic somatic cells and proliferating germ
cells, RBR-2 and SPR-5. Therefore, these two forms of aging may be related, and histone demethylase activities could be broadly relevant to the regulation of longevity in diverse organisms (Misteli, 2010).

It has been suggested that RBR-2 has anti-aging functions, as rbr-2 (tm1231) adults were short-lived (Greer et al., 2010). However, it was also reported that RNAi knockdown of rbr-2 causes increased longevity of the enhanced RNAi strain rrf-3 (Lee et al., 2003) and of the N2 wild-type strain at both 20 and 25 °C (Ni et al., 2012). Consistently, our analysis of rbr-2(ok2544) revealed a significant, if modest, increase in mean lifespan. rbr-2(ok2544) failed to complement rbr-2(tm1231) for this increased longevity phenotype. Thus, the tm1231 allele is not neomorphic but has unusual phenotypes that could be due to additional closely linked mutations in this genetic background; such issues of genetic background have previously confounded studies of the biology of aging in Drosophila and C. elegans (Burnett et al., 2011; Viswanathan & Guarente, 2011). The differences between the tm1231 and ok2544 alleles are unlikely due to residual demethylase activity as both alleles delete the conserved ImjC domain (Fig. 1A). However, we cannot rule out the possibility that the tm1231 allele could have unique and possibly interesting properties that are not due to linked mutations, such as a direct effect on lifespan that suppresses the anti-aging effects caused by ash-2 RNAi (Greer et al., 2010). The tm1231 deletion is predicted to result in a protein product that retains some RBR-2 sequences, including a C3HC2 zinc finger domain, which are absent from the predicted protein product of ok2544. We conclude that rbr-2(ok2544) may be a better mutation for studying null RBR-2 function than rbr-2(tm1231).

We found that members of two classes of H3K4 demethylase are required for extended longevity in daf-2(e1370) adults. This suggests an anti-aging function for RBR-2, consistent with the observation that overexpression of rbr-2 in germ cells extends lifespan of otherwise wild-type adults (Greer et al., 2010). The interaction between rbr-2 and daf-2 in C. elegans may be conserved in mammals, as IGFI receptor and RBP-2 demethylase activities were shown to mediate the emergence and maintenance of a ‘drug-tolerant’ state of cancer cell lines (Sharma et al., 2010). A recent report showed that the UTX-1 H3K27 demethylase and the T26AS.5 H3K36 demethylase are pro-aging and that deficiency for utx-1 extends lifespan in a manner that mimics reduced levels of daf-2 signaling (Maures et al., 2011), which may be due to the regulation of daf-2 gene expression (Jin et al., 2011). We speculate that removal of H3K27 silencing marks by UTX-1 could promote aging in a manner that resembles the anti-aging effects of RBR-2 and SPR-5, whose H3K4 demethylase activities may promote silencing of pro-aging genomic loci, such as cav-1A and T09B4.5, in the context of daf-2 deficiency (Figs 1C and 2). daf-2-dependent epigenetic modifications could be relevant to the activity of the DAF-16/Foxo transcription factor, or some of its targets.

Although both rbr-2 deletions elicit a Mortal Germline phenotype at a higher temperature, the partially penetrant Mortal Germline phenotype of ok2544 is likely to reflect the true role of RBR-2 in repression of transgenerational aging in the germline. In addition, defects in either rbr-2 or spr-5 only affected germ cells that were continuously cycling at higher temperatures, as quiescent germ cells from dauer larvae did not exhibit strong fertility defects during an analogous length of time (Fig. 2). Thus, cell proliferation is likely required for the accumulation of transgenerational stress in rbr-2 and spr-5 mutants. An alternative explanation is that pathways that establish quiescence and longevity in dauer larvae (Narbonne & Roy, 2006) could repress the stress that is caused by deficiency for rbr-2 or spr-5 in germ cells.

A previous study showed that KDM1 enzymes, especially SPR-5, affect germline maintenance over many generations at 20 °C (Katz et al., 2009). However, we found that the severe spr-5 mutation by134 resulted in modest effects on fertility at 20 °C and that spr-5 mutants can be maintained for many generations, consistent with a recent report from the laboratory of M. Colaiacco (Nottke et al., 2011). We suggest that the variable effects of spr-5 on germline maintenance may be allele specific, but could also be explained by factors that affect the epigenetic landscape of spr-5 strains, such as laboratory growth conditions or genetic backgrounds that were employed to perform crosses. One clear difference in the way we conducted experiments from those reported by Katz et al. (2009) is that we maintained strains in a wild-type (N2) background as much as possible. Double-mutant strains were constructed by directly crossing single-mutant strains or by using a limited number of genetic markers. Katz et al. maintained spr-5(by101) strains in a heterozygous state, balanced over hT2, a marked reciprocal translocation, prior to starting experiments. The effects of using the hT2 balancer, which has been subjected to several rounds of mutagenesis, on quantitative aspects of aging and fertility over many generations are unknown.

In our hands, much stronger effects on fertility were observed for rbr-2 and spr-5 mutants at 25 °C. Mutations in these genes are not known to be temperature sensitive, suggesting that they uncover a temperature-sensitive requirement for H3K4 demethylase activity. Due to increased molecular motion at higher temperatures, chromatin may become more open to transcription, leading to a greater requirement for proteins that facilitate transcriptional repression such as H3K4 demethylases. Alternatively, high temperature might induce the expression of stress-related genes, such as heat-shock proteins, and expression of this stress program might be tempered by the action of genes that repress transcription. A recent study has shown that the bn129 allele of the H3K4 histone methyltransferase set-2 (KMT2) has a temperature-sensitive Mrt phenotype (Xiao et al., 2011). In addition, deficiencies in synMuvB proteins elicit a larval arrest phenotype caused by failure to remodel the germline chromatin program at higher temperature (Petrella et al., 2011). Thus, high temperature may be a sensitized condition capable of detecting biological functions of chromatin-modifying proteins, such as facilitating the dynamics and/or balance of H3K4 methylation in the maintenance of fertility.

Although strains deficient for both demethylases did not exhibit strong fertility defects at 20 °C, the Mrt phenotype of spr-5; rbr-2 double mutants at 25 °C was significantly faster than that of either single mutant. It is possible that these demethylases interact with common as well as distinct genomic loci to repress transgenerational aging. Despite being able to demethylate both Me3 and Me2 from H3K4, RBR-2 plays a less important role in repressing transgenerational stress at 25 °C than SPR-5, which demethylates Me2 and Me1 (Christensen et al., 2007; Katz et al., 2009). SPR-5 may be more important than RBR-2 for promoting germ cell immortality because RBR-2 has only weak activity in removing Me2 and is more efficient at removing Me3 (Fig. 2A) (Christensen et al., 2007). Alternatively, SPR-5 may have a stronger phenotype because KDM1A enzymes can also demethylate H3K9 (Metzger et al., 2005) and other nonhistone proteins (Nicholson & Chen, 2009). Given their overlapping mutant phenotypes and the reduced levels of H3K4me2 in daf-2; rbr-2 mutants (Fig. 2), we propose that RBR-2 and SPR-5 may function sequentially on distinct H3K4 methyl marks of common histone targets to repress proliferative or postmitotic aging.

Our findings that RBR-2 and SPR-5 impact cell proliferation and aging in C. elegans could be relevant to human homologs of these proteins, which have been implicated in the regulation of stem cell fate and in cancer biology. For example, in embryonic stem cells, LSD1 (the human homolog of SPR-5) helps to maintain the balance between differentiation and self-renewal (Adamo et al., 2011). LSD1 has also been implicated in many cancers, and selective inhibitors of LSD1 are being investigated as possible
anticancer agents (Wang et al., 2011). RBP-2, the human homolog of RBR-2, is released from promoters of genes that are expressed during cellular differentiation (Christensen et al., 2007), consistent with a role for heterochromatin in maintaining a pluripotent state. In addition, RBP-2 promotes cancer cell survival and proliferation (Roessch et al., 2010; Zeng et al., 2010; Blair et al., 2011). Given that the anti-aging roles of RBR-2 and SPR-5 in both proliferative and postmitotic aging occur under conditions of temperature stress or a daf-2 genetic background that may mimic stressful conditions, we speculate that a conserved function of H3K4 demethylases may be to repress cellular aging by modifying chromatin in response to physiological stress.

Experimental procedures

Strains

Unless noted otherwise, all strains were cultured at 20°C on nematode growth medium (NGM) plates seeded with Escherichia coli OP50. Mutations used include dpy-16(mg50) I, daf-16(mu86) I, spr-5(by134) I, glp-4(bn2) II, daf-2(e1368) III, daf-2(e1370) III, unc-24(e120) IV, rbr-2(tm1231) IV, rbr-2(ok2544) IV, dpy-20(e1282) IV. rbr-2 mutations were outcrossed vs. an unc-24 dpy-20 marker strain that itself had been outcrossed three times vs. N2 wild-type. Freshly time after outcrossing were used.

Generating the double-mutant strain is found in supplemental methods. Experimental procedures

Western blot

Worm extracts were made from the indicated daf-2 and wild-type strains by shifting 200 L4s from 15 to 25°C and allowing them to grow for 2 days. Worms were then collected and resuspended in 2× Laemmli buffer containing 5% beta-mercaptoethanol. Samples were boiled for 10 min, and proteins were separated in a 15% SDS-PAGE gel. The gel was transferred to nitrocellulose and blotted for H3 (abcam #ab1791) to make sure samples were normalized. The blot was then stripped and was transferred to nitrocellulose and blotted for H3 (abcam #ab1791) to

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Author contributions

SMcA and SA designed research; SMA, GAM, and EYJ performed research; BL communicated unpublished results and provided tools; SMA, BL, and SA wrote the paper; all authors analyzed the data.

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Conflict of interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Fig. S1 rbr-2(ok2544) affects longevity at higher temperatures.

Fig. S2 The effects of rbr-2 on daf-2(e1368)-mediated longevity.

Fig. S3 Mutations in H3K4 demethylases do not affect fertility for strains propagated at 20 °C.

Fig. S4 DAPI-stained oocytes with chromosome defects.

Data S1 Methods.

Table S1 Smaller body size and vulval defects in rbr-2(tm1231) but not rbr-2 (ok2544).

Table S2 Deficiencies for H3K4 demethylase activity decreases longevity in a daf-2(e1370) background.

Table S3 Dauer formation and L1 or L2 larval arrests of progeny from daf-2(e1370) and daf-2(e1370) H3K4 demethylase double mutants that were shifted from 15 °C to either 20 or 25 °C as L4 larvae.

Table S5 Quantification of H3K4me2 compared to total H3K4 levels.

Table S6 Quantification of H3K4me3 compared to total H3K4 levels.