

Mutation of *C. elegans* demethylase *spr-5* extends transgenerational longevity

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Complex organismal properties such as longevity can be transmitted across generations by non-genetic factors. Here we demonstrate that deletion of the *C. elegans* histone H3 lysine 4 dimethyl (H3K4me2) demethylase, *spr-5*, causes a trans-generational increase in lifespan. We identify a chromatin-modifying network, which regulates this lifespan extension. We further show that this trans-generational lifespan extension is dependent on a hormonal signaling pathway involving the steroid dafachronic acid, an activator of the nuclear receptor DAF-12. These findings suggest that loss of the demethylase SPR-5 causes H3K4me2 mis-regulation and activation of a known lifespan-regulating signaling pathway, leading to trans-generational lifespan extension.

Keywords: transgenerational inheritance; longevity; *C. elegans*; steroid signaling

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Introduction

An increasing number of studies report phenotypes regulated by inheritance of non-Mendelian information from ancestors. For the most part, the molecular mechanisms behind these transgenerational epigenetic effects are still unknown [1–3]. In worms, deletion of SPR-5, the ortholog of the human H3K4me1/2-specific demethylase LSD1 [4], initially do not exhibit phenotypes, however, successive generations lacking this demethylase display increasing infertility concomitant with a global accumulation of DNA methylation of adenines (6mA) and euchromatic histone H3 lysine 4 dimethyl (H3K4me2) and a global decline in heterochromatic H3K9me3 [4–7]. These progressive phenotypes can be reversed by the addition of a single copy of *spr-5* [4]. Typically, H3K4me2 is associated with gene activation while H3K9me3 is

associated with transcriptional repression [3]. We, and others, previously showed that H3K4me2, H3K9me, and 6mA regulatory proteins regulate the epigenetic memory induced by *spr-5* deletion [6–8]. Here we show that lack of SPR-5 not only causes progressive infertility, but also leads to a trans-generational increase in lifespan. The chromatin regulators that suppressed the progressive fertility defect and H3K4me2 and 6mA accumulation also suppressed the trans-generational lifespan extension. Interestingly, we found that the extended trans-generational longevity of *spr-5* mutant worms was not simply a byproduct of lowered reproduction but rather a regulated process, involving the known DAF-36/DAF-12 longevity signaling pathway. This DAF-36/DAF-12 signaling pathway did not regulate the inherited H3K4me2 accumulation or reduced egg-laying capacity of *spr-5* mutant worms. We further found that addition of dafachronic acid, the steroid which activates DAF-12, was sufficient to extend wild-type (WT) but not late generation *spr-5* mutant longevity. Our findings suggest that 6mA, H3K4me2 and H3K9me3 misregulation causes an activation of a known germline to soma signaling pathway which regulates trans-generational effects on longevity.

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Results

spr-5 mutant worms display a transgenerational extension of lifespan

Longevity is regulated by genetic and environmental factors [9] and has recently been shown, in *C. elegans*, to be regulated by transgenerational epigenetic inheritance [10, 11]. How epigenetic information is inherited in these instances and how it regulates longevity is still unknown. Because of the role SPR-5 has been shown to play in regulating fertility, the inverse relationship between reproduction and longevity [9] and the importance of histone methylation in regulating longevity [12], we hypothesized that SPR-5 may also play a role in lifespan regulation. Consistently, one of the top GO enrichment categories for genes regulated by SPR-5 in whole worm expression analyses across generations (generations 1, 13, and 26) [4] was ‘determination of adult lifespan’ (P

$= 3.4 \times 10^{-4}$). We found that for the first five generations, two independent *spr-5* mutant worm strains, *spr-5(by101)* and *spr-5(by134)* (generation 5, G5) had a normal lifespan (Figure 1A and Supplementary information, Table S1). However, after 10 and 20 generations bearing this mutation, *spr-5(by101)* and *spr-5(by134)* mutant worms (G10 and G20) displayed extended lifespan by 19%-44% (Figure 1B and 1C, Supplementary information, Tables S1-S5). Both *spr-5* alleles are predicted functional null alleles, the *by101* allele has a transposon insertion next to the catalytic residue and the *by134* allele has a nonsense mutation which eliminates the majority of the enzymatic domain [4, 5]. This trans-generational lifespan extension, similar to the progressive fertility defect and H3K4me2 accumulation, was reverted when worms were backcrossed to provide a single WT copy of *spr-5*. However, the increase in lifespan was not progressive, i.e., generation 10 *spr-5(by101)* mutant worms

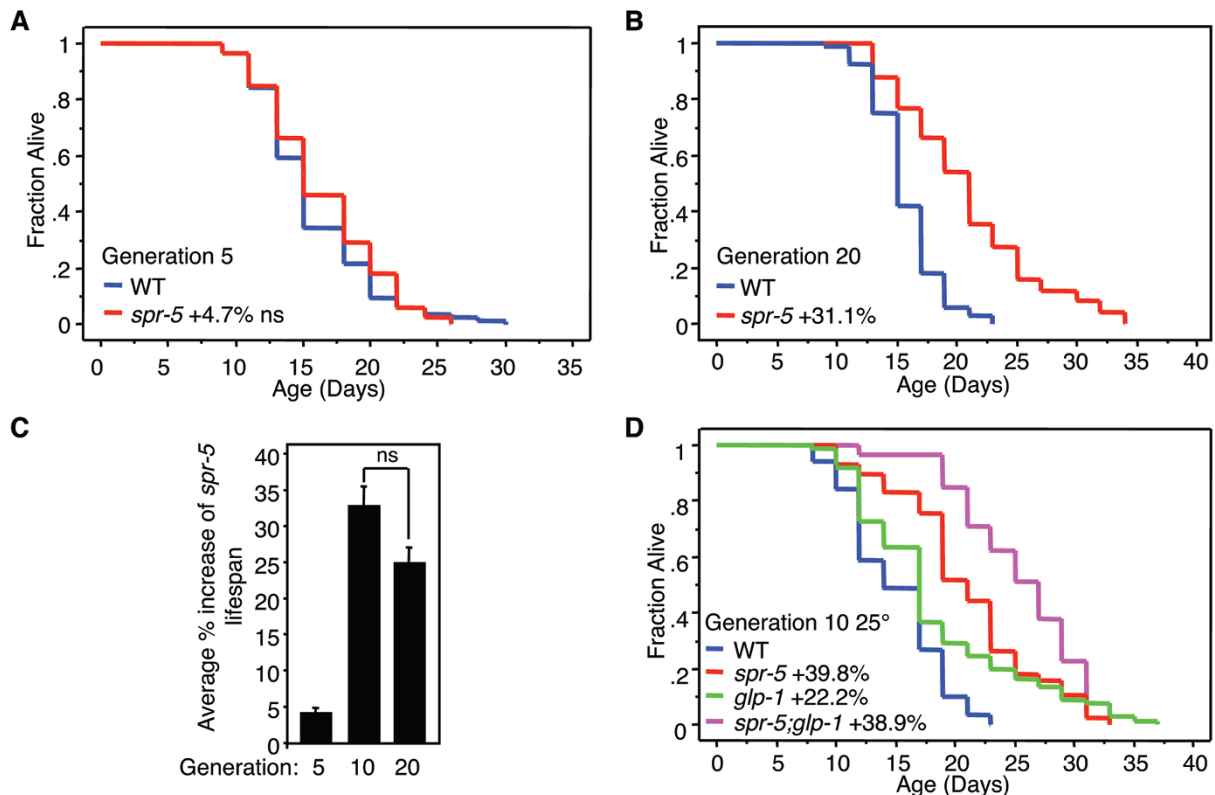


Figure 1 *spr-5* mutant worms have extended transgenerational longevity. **(A)** Early generation (G5) *spr-5(by101)* mutant worms live as long as wild-type worms ($P = 0.3737$). Statistics are presented in Supplementary information, Table S1. **(B)** Late generation (G20) *spr-5(by101)* mutant worms live longer than wild-type worms ($P < 0.0001$). Statistics are presented in Supplementary information, Table S1. **(C)** *spr-5(by101)* mutant worms do not display a progressive extension in lifespan (bars represent mean \pm SEM for two experiments for generation 5, four experiments for generation 10, and 11 experiments for generation 20). Statistics are presented in Supplementary information, Tables S1-S4. **(D)** *spr-5;glp-1* double mutant worms live significantly longer than *glp-1* mutant worms similarly to the extension of *spr-5* mutant worms lifespan compared with wild-type worms (two-way ANOVA $P = 0.3726$). *glp-1* lifespan extension is compared with WT worms and *spr-5;glp-1* lifespan extension is compared with *glp-1* mutant worms. Statistics are presented in Supplementary information, Table S2.

lived as long as generation 20 mutant worms (Figure 1C and Supplementary information, Tables S1 and S2). To determine whether there was a specific generation at which longevity was extended, we examined the lifespan of *spr-5(by134)* mutant worms at every generation between generation 5 and 10 (Supplementary information, Figure S1 and Table S1). The lifespan extension occurred at generation 7 or generation 8 but was always present by generation 10 (Supplementary information, Figure S1 and Tables S1-S5).

To determine whether this trans-generational lifespan extension was simply a byproduct of lowered reproduction or the consequence of alterations of a specific signaling pathway, we examined the lifespan of *spr-5(by101)* mutant worms whose reproduction was inhibited by either chemical or genetic means. We first treated worms with 5-fluorodeoxyuridine (FUdR), which inhibits proliferation of germline stem cells, the production of intact eggs in adults, and extends longevity [13, 14]. We found FUdR had similar effects on the lifespan of WT and early generation *spr-5(by101)* mutant worms (G5) (Supplementary information, Figure S2A). However, *spr-5(by101)* mutant worms after 10 and 20 generations displayed further lifespan extension compared with the WT worms when both were treated with FUdR (Supplementary information, Figure S2B and Table S2), suggesting that this extended longevity was not simply a byproduct of lowered reproduction. To test this hypothesis further, we crossed *spr-5(by101)* mutant worms with *glp-1(e2141ts)* mutant worms, which have a somatic germline but fail to develop a meiotic germline and are sterile when maintained at the restrictive temperature after the L1 stage [15]. *spr-5(by101)* mutation further extended the long lifespan of *glp-1(e2141ts)* mutant worms at the restrictive temperature when all strains were carried out to generation 10 (Figure 1D), once again suggesting that the extended trans-generational longevity is not a byproduct of lowered reproduction. Similar results were found when *spr-5(by134)* mutants were crossed with the temperature sensitive sterile *pgl-1(bn101)* mutant strain [16] (Supplementary information, Table S2). Together, these results suggest that a functional germline is necessary for the transmission of epigenetic information but becomes dispensable for the extended longevity.

Transgenerational longevity of spr-5(by101) mutant worms is dependent on chromatin regulators which control fertility and H3K4me2 and 6mA accumulation

We next investigated whether the extended trans-generational longevity was dependent on the same molecular components that regulate trans-generational fertility defects of *spr-5* mutant worms [6, 7]. RNAi inheritance has

been implicated in trans-generational epigenetic inheritance in several species [17]. In *C. elegans*, exogenous and endogenous RNAi pathways require the argonaute genes *rde-1* and *ergo-1*, respectively [18, 19], although additional argonautes do exist and could be required for specific RNA inheritance events [20]. We found that, similar to the progressive fertility defects [6], the trans-generationally extended longevity of *spr-5* mutant worms was independent of the main exogenous (Supplementary information, Figure S3A) and endogenous (Supplementary information, Figure S3B) RNAi pathways mediated by these argonautes. It remains to be determined whether any of the additional 25 argonautes [19], particularly those implicated in heritable RNA [21-23], could play a role in the trans-generational inheritance of longevity in *spr-5(by101)* mutant worms. Mutants of some of the untested argonautes display fertility defects and they regulate trans-generational inheritance [21] raising the possibility of coordinated regulation of heritable material.

We had previously found that trans-generational fertility defects and H3K4me2 accumulation of *spr-5* mutant worms were dependent not only on the euchromatin H3K4 methyltransferases SET-17 and SET-30 but also the H3K9me3/K36me3 demethylase JMJD-2 and the H3K9me binding protein EAP-1 [6]. We had also found that the trans-generational fertility defect of *spr-5* mutant worms was partially dependent on the potential 6mA methyltransferase DAMT-1 [7]. To determine whether these chromatin modifiers additionally controlled the trans-generational longevity extension that we observed in *spr-5* mutant worms, we crossed genetic null mutants of these various genes with *spr-5(by101)* or *spr-5(by134)* mutant worms and maintained homozygous double mutations with *set-17*, *set-30*, *jmjd-2*, *eap-1*, or *damt-1* for 20 generations. Mutation of *set-17*, *set-30*, *jmjd-2*, or *eap-1* was sufficient to completely suppress the extended longevity of *spr-5* mutant worms (Figure 2A-2D), as they had suppressed their progressive fertility defects and H3K4me2 accumulations [6]. Mutation of *damt-1* partially suppressed the extended longevity of *spr-5* mutant worms (Figure 2E), as it had partially suppressed their progressive fertility defects and H3K4me2 accumulations [7].

Transgenerational longevity but not fertility phenotypes of spr-5(by101) mutant worms is dependent on DAF-12 and DAF-36

Since the trans-generational longevity extension can be decoupled from the progressive fertility defect (Figure 1C and 1D, Supplementary information, Figure S2), we next wanted to determine whether specific signaling

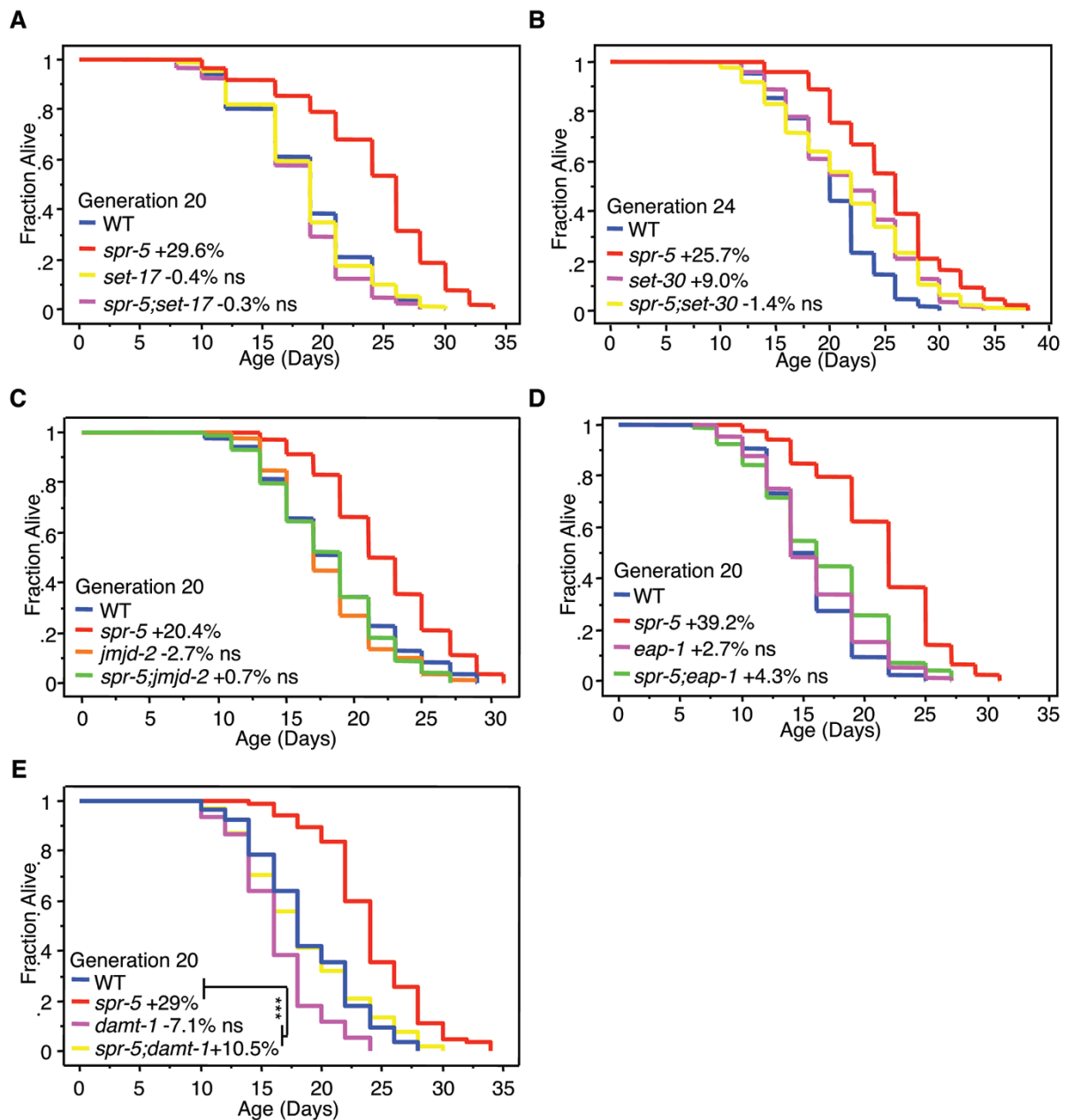


Figure 2 *spr-5* transgenerational longevity is dependent on chromatin regulators which control progressive fertility defect and H3K4me2 and 6mA levels. **(A)** *spr-5*(*by101*) mutant worms live longer than wild-type worms at generation 20 but *spr-5*;*set-17* double mutant worms do not live longer than wild-type worms (two-way ANOVA $P < 0.0001$). **(B)** *spr-5*(*by101*) mutation increases the lifespan of wild-type worms but does not increase the lifespan of *set-30*(*gk315*) mutant worms after 24 generations (2 way ANOVA, $P < 0.0001$). **(C)** *spr-5*(*by101*) mutant worms live longer than wild-type worms at generation 20 but *spr-5*;*jmd-2* double mutant worms do not live longer than *jmd-2*(*tm2966*) mutant worms (two-way ANOVA $P = 0.0002$). **(D)** *spr-5*(*by101*) mutant worms live longer than wild-type worms at generation 20 but *spr-5*;*eap-1* double mutant worms do not live longer than *eap-1*(*ok3432*) mutant worms (2 way ANOVA $P < 0.0001$). **(E)** *spr-5*(*by134*) mutant worms live longer than wild-type worms at generation 20 and this is partially dependent on *damt-1* (two-way ANOVA $P = 0.0004$). Statistics are presented in Supplementary information, Tables S3 and S4.

pathways, which function downstream of the chromatin-regulating enzymes, regulated the trans-generational longevity. To address this, we examined the *spr-5*

trans-generational longevity. Interestingly, the trans-generational longevity in *spr-5* mutants analyzed using whole worm extracts [4] display

significant overlap with *daf-12*- and *daf-16*-regulated genes [24] (Supplementary information, Figure S4A, hypergeometric probability $P = 3.37 \times 10^{-9}$). All of these *daf-12*- and *daf-16*-regulated genes increased expression between generation 13 and generation 1 of *spr-5* [4]. DAF-12 is a nuclear hormone receptor, which has been shown to be necessary for the longevity extension regulated by a germline to soma longevity signaling pathway [25]. DAF-12 is activated by the steroid dafachronic acid [26], which is synthesized from exogenous cholesterol by a complex biosynthesis process whose first committed step requires the Rieske oxygenase, DAF-36 [27-29]. A second major germline to soma signaling pathway that regulates longevity has been reported, which culminates with the transcription factor DAF-16/FOXO [9, 30].

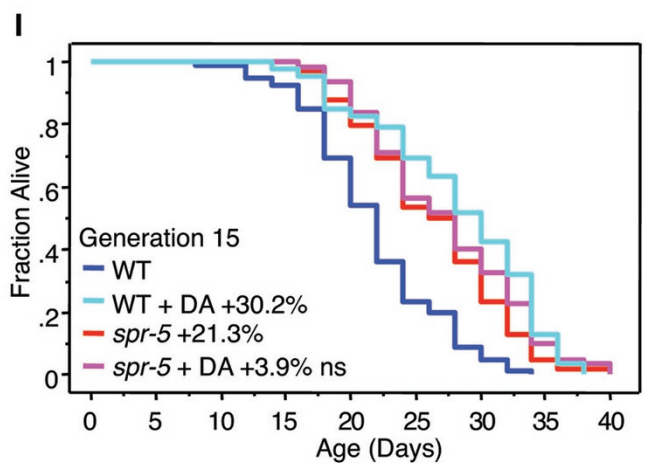
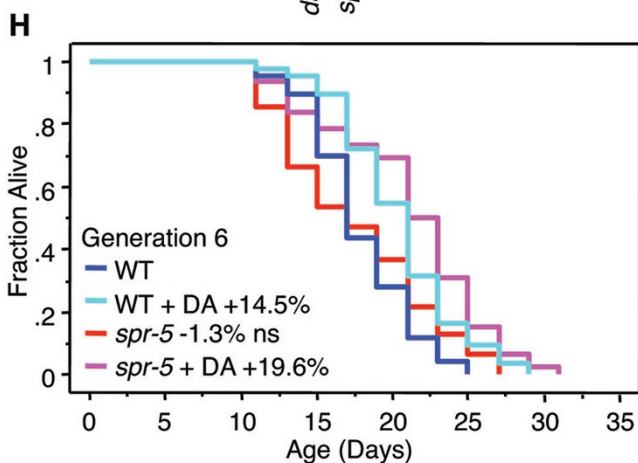
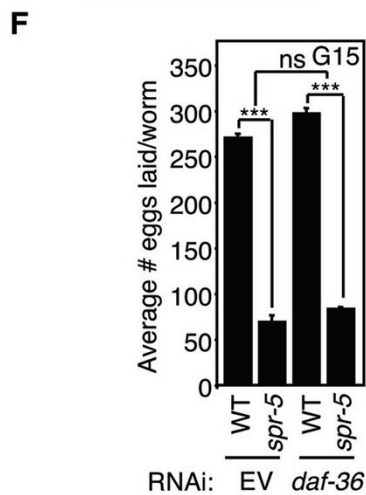
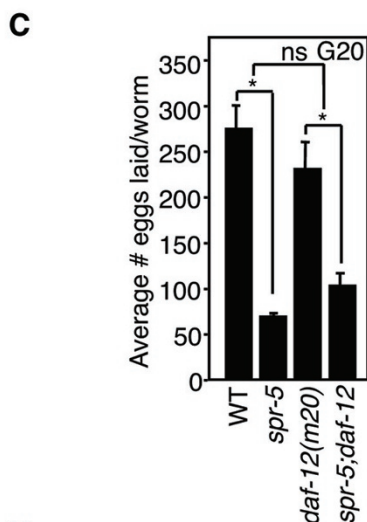
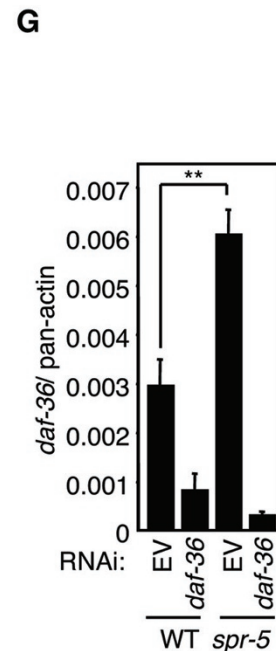
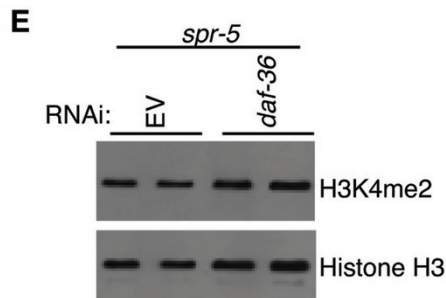
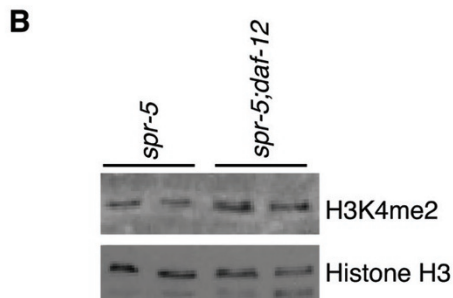
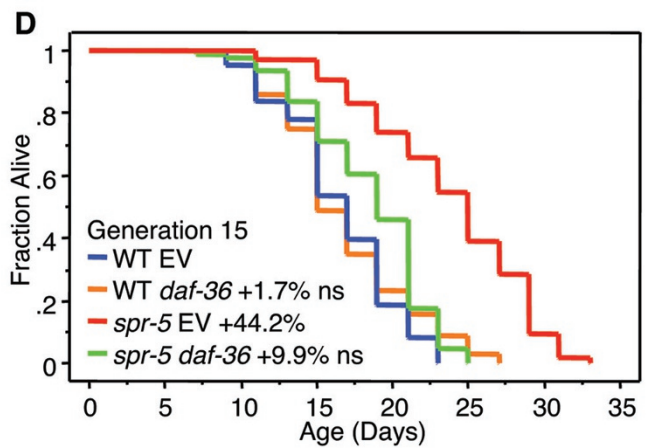
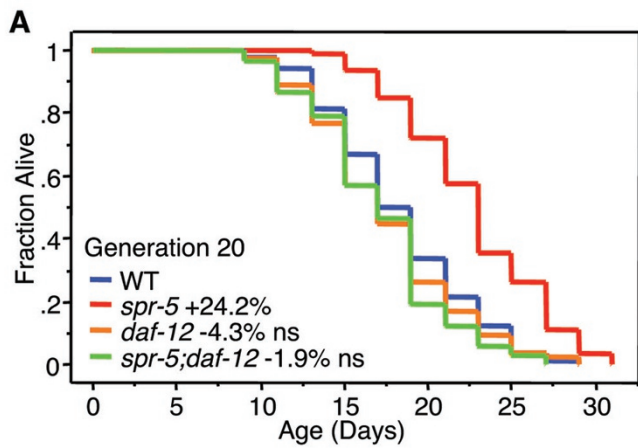
To address the involvement of these signaling pathways in the extended longevity of *spr-5(by101)* mutant worms, we first crossed *daf-12(m20)* with *spr-5(by101)*. We found that late generation *spr-5(by101)* mutants live longer than their WT counterparts (24.2% longer, $P < 0.0001$) but the *spr-5;daf-12* double mutants did not live significantly longer than *daf-12(m20)* mutant worms (Figure 3A). Importantly, although removing DAF-12 eliminated the trans-generational extension of lifespan, it had no effect on the global H3K4me2 accumulation (Figure 3B) or the progressive fertility defect (Figure 3C) of *spr-5* mutant worms. Taken together, these findings suggest that DAF-12 plays a role in the *spr-5*-induced lifespan extension but not other progressive phenotypes, suggesting that DAF-12 functions downstream of the chromatin reorganization. Because all of the *daf-12*-regulated genes increased expression between generation 13 and generation 1 of *spr-5* [4], we would hypothesize that prolonged *spr-5* loss activates DAF-12.

To examine this signaling pathway more thoroughly, we knocked down the Rieske oxygenase *daf-36*, in *spr-5(by101)* mutant worms. DAF-36 is required for the production of the DAF-12 activating steroid dafachronic acid [27, 28]. Consistently, we found that late generation *spr-5(by101)* mutant worms treated with *daf-36* RNAi eliminated the trans-generational lifespan extension (Figure 3D, two-way ANOVA $P < 0.0001$). Similar to knockout of *daf-12*, knockdown of *daf-36* also had no effect on the increased H3K4me2 level (Figure 3E) or the progressive fertility decline (Figure 3F). We also found that later generation *spr-5* mutant worms displayed elevated mRNA levels of *daf-36* (Figure 3G), suggesting that SPR-5 may function to repress *daf-36* gene expression. Similar to lifespan extension, elevated *daf-36* expression varied from replicate to replicate but was evident by generation 9 (Supplementary information, Figure S4F). Together, these results suggest that the trans-generational

longevity extension is separable from the decreased reproduction and requires the DAF-36/DAF-12 signaling pathway.

Since this DAF-36/DAF-12 signaling pathway was required for the extended trans-generational longevity, we next investigated whether all germline to soma longevity signaling pathways were necessary for the extended trans-generational longevity of *spr-5* mutant worms. In contrast to DAF-36/DAF-12, our results suggest that DAF-16 was not involved in the extended trans-generational longevity of *spr-5* mutant worms. We found that knockdown of *daf-16* decreased the longevity of both WT and *spr-5(by101)* mutant worms by a similar percentage (-13.5% and 16.3%, respectively, two-way ANOVA $P = 0.3361$) (Supplementary information, Figure S4B). *daf-16* knockdown also had no effect on *spr-5(by101)* egg laying (Supplementary information, Figure S4C). Because results obtained with RNAi are not as conclusive as null alleles [31] this experiment leaves open the possibility that the DAF-16 signaling pathway may be involved. To examine this signaling pathway more thoroughly, we also knocked down the intestinal ankyrin-repeat protein *kri-1*, which is required for DAF-16 nuclear localization and longevity in germline-deficient animals [32], in *spr-5(by101)* mutant worms. We similarly found that late generation *spr-5(by101)* mutant worms treated with *kri-1* RNAi were still long lived compared with WT worms treated with *kri-1* RNAi (Supplementary information, Figure S4D, two-way ANOVA $P = 0.1831$), suggesting that KRI-1 is not involved in *spr-5*-induced lifespan extension. *kri-1* knockdown, like *daf-16* knockdown, had no effect on *spr-5(by101)* egg laying (Supplementary information, Figure S4E). Together, these results suggest that the extended trans-generational longevity, but not other trans-generational phenotypes of *spr-5* mutant worms, is dependent on the DAF-36/DAF-12 and not the KRI-1/DAF-16 signaling pathway.

As discussed, the production of dafachronic acid, which is essential for the activation of steroid hormone receptor DAF-12, is dependent on DAF-36. Given that DAF-36 inhibition suppresses the trans-generational longevity phenotype associated with *spr-5(by101)*, we speculate that SPR-5 might regulate lifespan through dafachronic acid via controlling the expression of DAF-36. Consistent with this speculation, the late generation *spr-5(by101)* worms have elevated levels of *daf-36* mRNA (Figure 3G and Supplementary information, Figure S4F). To address this further, we fed WT and *spr-5* mutant worms dafachronic acid. We found that dafachronic acid extended the lifespan of WT worms (14.5%, $P < 0.0001$) and early generation *spr-5* mutant worms (19.6%, $P = 0.0005$) to a similar extent (two-way ANOVA $P = 0.4326$)



(Figure 3H). We found that dafachronic acid extended the lifespan of WT worms (30.2%, $P < 0.0001$) but not that of generation 15 *spr-5(by101)* or *spr-5(by134)* mutant worms (3.9%, $P = 0.2372$ or 1.8%, $P = 0.5661$) (Figure 3I). Collectively, these results suggest that increasing dafachronic acid levels extend the lifespan of WT but not late generation *spr-5* mutant worms.

Discussion

This study identified a role of SPR-5 in the regulation of trans-generational lifespan. The extended trans-generational longevity is dependent on the same set of chromatin methylation regulators, which control the progressive fertility defect, but is not a byproduct of the reduced fertility. Rather, SPR-5 impacts lifespan by controlling the DAF-12 signaling pathway. Our findings thus lay the framework for a molecular model where the interplay between H3K4 versus H3K9 methylation impacts trans-generational epigenetic inheritance upstream of a specific longevity pathway in *C. elegans*.

In *C. elegans*, a reduction in germline stem cell number leads to increased lifespan [9], therefore at first glance, it is perhaps not surprising that *spr-5(by101)*, which lay fewer eggs in later generations also live longer. However, egg-laying is not always linked to lifespan [14, 15]. Indeed, we found that *spr-5(by101)* mutant worms carrying this mutation for 10 generations already reached maximal lifespan extension (Figure 1C). This is different from the trans-generational fertility defect phenotype, which is progressive, i.e., the phenotype continues to worsen until generation 20. Our finding that

inhibition of reproduction, either chemically or through genetic removal of the meiotic germline, had no effect on the trans-generational extension of longevity further supports the separation of the fertility and longevity phenotypes. The meiotic germline is required to pass epigenetic information to descendants but after this epigenetic memory has been transmitted it appears dispensable for the extended trans-generational longevity. These experiments do not rule out the possibility that the longevity signal could originate from the somatic gonad which is unaffected by FUdR treatment or the *glp-1* or *pgl-1* mutations. Since DAF-12 has been shown to be necessary for a germline to soma longevity signaling pathway [9, 25, 30] it could be playing a similar role here. However, this is not necessarily the case since DAF-12 is expressed ubiquitously and the dafachronic synthesis components appear to be expressed throughout the worm in different tissues [25]. Therefore, the longevity signal could originate from the somatic gonad, from inherited hormones, or from a somatic mis-regulation of chromatin inherited from the parental generation. It will be interesting, in future studies, to determine where this altered longevity cue originates from.

Given that the longevity extension of *spr-5* mutants was dependent on a known germline to soma signaling pathway involving DAF-36 and DAF-12 (Figure 3), we speculate that SPR-5, in conjunction with the aforementioned histone methylation regulators, plays a role in the regulation of the DAF-12 target genes. The fact that DAF-36 and DAF-12 manipulation have no effect on reducing global H3K4me2 levels or fertility in *spr-5* mutant worms suggests that the chromatin regulation by

Figure 3 *spr-5(by101)* transgenerational longevity is dependent on *daf-12* and *daf-36*. **(A)** *spr-5(by101)* mutant worms live longer than wild-type worms at generation 20 but *spr-5;daf-12* double mutant worms do not live longer than *daf-12(m20)* mutant worms (two-way ANOVA $P < 0.0001$). Statistics are presented in Supplementary information, Table S4. **(B)** *spr-5;daf-12* double mutant worms show similar levels of H3K4me2 as *spr-5(by101)* mutant worms at generation 20 as assessed by western blots of whole worm lysates of L3 worms. **(C)** *spr-5;daf-12* double mutant worms lay similar numbers of eggs as *spr-5(by101)* mutant worms at generation 20 (graph is the mean \pm SEM of three independent experiments: each experiment consists of average eggs laid for 10 worms of each genotype performed in triplicate). * $P < 0.05$ by paired *t*-test; ns, not significant by two-way ANOVA. **(D)** *daf-36* knock down eliminates the *spr-5(by101)* lifespan extension at generation 15 (2 way ANOVA $P < 0.0001$). *spr-5* EV lifespan extension is compared with WT EV and *spr-5 daf-36* lifespan extension is compared with WT *daf-36*. Statistics are presented in Supplementary information, Table S5. **(E)** *daf-36* knock down does not affect the increased H3K4me2 of *spr-5(by101)* mutant worms at generation 15 as assessed by western blots of whole worm lysates of L3 worms. **(F)** *daf-36* knock down does not significantly alter the reduced egg laying capacity of generation 15 *spr-5(by101)* mutant worms. Graph represents the mean \pm SEM of 2 independent experiments: each experiment consists of 3 replicates of 10 worms each. *** $P < 0.001$ by *t*-test; ns, not significant by two-way ANOVA. **(G)** Generation 15 *spr-5(by101)* mutant worms display higher levels of *daf-36* mRNA compared with wild-type worms. Graph represents the mean \pm SEM of two independent experiments: each experiment consists of 2 biological replicates of 100 worms each. ** $P < 0.01$ by *t*-test. **(H)** Dafachronic acid addition extends wild-type worm lifespan (14.5% $P < 0.0001$) and generation 6 *spr-5(by101)* mutant worm lifespan (19.6% $P = 0.0005$). Statistics are presented in Supplementary information, Table S5. **(I)** Dafachronic acid addition extends wild-type worm lifespan (30.2% $P < 0.0001$) but does not extend generation 15 *spr-5(by101)* mutant worm lifespan (3.9% $P = 0.2372$). Statistics are presented in Supplementary information, Table S5.

SPR-5 occurs upstream of the DAF-36/DAF-12 signaling pathway. Consistent with this model, mis-regulated genes in *spr-5* mutants display significant overlap with *daf-12*-regulated genes (Supplementary information, Figure S4A) and *spr-5* mutant worms show elevated levels of *daf-36* mRNA (Figure 3G). However, it remains to be seen whether SPR-5 directly regulates histone methylation at *daf-12*-regulated genes. In the future, it will be important to determine if SPR-5 is recruited to these target genes, and if and how SPR-5 deletion modulates the H3K4 methylation regulatory network to promote DAF-12 gene transcription. Finally, does SPR-5 directly inhibit *daf-36* gene expression or does it regulate this DAF-36/DAF-12 signaling pathway indirectly through the control of alternative *daf-36* regulators? Collectively, our findings suggest that the maintenance of an appropriate chromatin balance is important not only for the generation in which it occurs but also for the longevity of descendants. Our findings provide some of the first mechanistic insight into how epigenetic information from ancestors can influence the longevity of descendants.

It is interesting to note that the previous example of trans-generational epigenetic inheritance of longevity that we identified [10] was caused by deletion of an H3K4 trimethylase complex while the example presented here was caused by deletion of an H3K4 mono/dimethyl demethylase. These two examples are very different in that in the first case longevity extension occurs immediately upon deletion of the H3K4 trimethylase complex [14] and persists even when WT copies of the H3K4 trimethylase complex are returned and then reverts only after three WT generations to WT longevity [10]. However, this study finds that initial mutants live as long as WT worms but a longevity extension appears after 7 or 8 generations without the H3K4me2 demethylase. The pathways mediating these two trans-generational longevity phenotypes are distinct: the lifespan extension caused by deletion of the H3K4 trimethylase complex depends on the presence of a functional germline and is independent of *daf-12* [10, 14] while the *spr-5* deletion induced trans-generational lifespan extension does not require a functional germline (Figure 1D) and is dependent on *daf-12* (Figure 3A). In addition, the genes, which are trans-generationally mis-regulated in these two paradigms, do not overlap (data not shown). At the global level, deletion of the H3K4 trimethylase does not affect H3K4me1/me2 levels [14] and while H3K4me3 levels are elevated in *spr-5* mutant worms they do not become mis-regulated in a trans-generational manner [6]. H3K4me3 is associated primarily with gene promoters of actively transcribed genes or with genes poised for transcription while H3K4me2 is associated with both

promoters and enhancers [33]. However, both enzymes regulate the methylation of lysine 4 on histone H3. It remains to be determined whether this residue is particularly susceptible to transmitting heritable material or for regulating longevity signaling pathways.

While this provides another example where genetic mis-regulation of a chromatin regulator causes trans-generational lifespan extension, it remains to be seen whether environmental manipulations, which naturally regulate LSD1, can affect trans-generational phenotypes including lifespan. There are increasing examples of how environmental manipulations can have multi- and trans-generational effects on a wide variety of phenotypes [1-3]. There are also correlative reports that environmental alterations can regulate human longevity trans-generationally [34]. It will be exciting to determine whether these altered trans-generational lifespan phenotypes in people are due to the mis-regulation of H3K4 regulators that we have identified in *C. elegans*.

Materials and Methods

Lifespan assays

Worm lifespan assays were performed at 20 °C, without 5-fluoro-2'-deoxyuridine (FUdR), as described previously [35] unless noted otherwise. When FUdR was used worms were placed on FUdR containing plates at the L4 stage. For each lifespan assay, ~90 worms per condition were used in three plates to begin the experiment (30 worms per plate). Worms that underwent matricide, exhibited a ruptured vulva, or crawled off the plates, were censored. Statistical analyses of lifespan were performed on Kaplan-Meier survival curves in StatView 5.0.01 by log rank (Mantel-Cox) tests. The values from the Kaplan-Meier curves are included in the Supplementary Tables. For dafachronic acid experiments, (25S)- Δ^7 -dafachronic acid (Cayman Chemical: 100 μ l of 1 mM in EtOH added to 1 liter of media) was added to plate media directly before pouring and worms were kept on dafachronic acid from birth.

Worm strains

The N2 Bristol strain was used as the WT background. The following mutations were used in this study: LGI: *spr-5(by101)*, *spr-5(by134)*; LGII: *jmjd-2(tm2966)*, *eap-1(ok3432)*, *set-17(n5017)*; *damt-1(gk961032)*; LGV: *rde-1(ne219)*, *ergo-1(tm1860)*; and LGX: *set-30(gk315)*, *daf-12(m20)*. Some of these strains have been previously used in [4, 6, 18, 19, 36]. In this paper mutant worms were backcrossed: *jmjd-2(tm2966)*: 2 times, *daf-12(m20)*: 3 times, *rde-1(ne219)*: 3 times, *set-17(n5017)*: 2-7 times, *set-30(gk315)*: 2-8 times, *damt-1(gk961032)*: 5 times, *ergo-1(tm1860)*: 7 times, and *eap-1(ok3432)*: 2-13 times. Generation 1 *spr-5* mutant worms were obtained by crossing later generation (generation 10-20) homozygous mutant worms with WT males to obtain P0 heterozygous mutants. Individual P0 heterozygous mutants were picked to plates and allowed to lay generation 1 progeny after which the P0 heterozygous mutant genotype was confirmed by single worm PCR genotyping. Generation 1 progeny were picked to individ-

ual plates and allowed to lay progeny. Generation 1 worms were subsequently genotyped by single worm PCR and homozygous mutant worms were perpetuated for subsequent generations. For double mutant crosses WT males were crossed with either *spr-5* or second mutant homozygous mutant hermaphrodites. Male progeny from these crosses were then crossed with homozygous mutant or *spr-5* hermaphrodites, respectively. Hermaphrodite progeny from these crosses were picked to individual plates and allowed to lay progeny. The parental generation was then genotyped by single worm PCR at the mutant loci used in the male strain. Progeny of heterozygous parental generation worms were picked to individual plates and after laying progeny were genotyped at both mutant loci. Subsequent single or double homozygous mutant progeny were maintained until the appropriate generation and longevity or egg laying assays were performed.

RNA interference

E. coli HT115 (DE3) bacteria containing the vectors of interest were grown at 37 °C and seeded on standard nematode growth medium plates containing ampicillin (100 mg/ml) and isopropylthiogalactoside (IPTG; 0.4 mM). Worms were maintained on dsRNA expressing bacteria starting at generation 2.

Single worm genotyping

Single worms were placed in 5 µl of worm lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl₂, 0.45% NP40, 0.45% Tween-20, 0.01% gelatin (w/v) and 60 mg/ml proteinase K), and incubated at -80 °C for 1 h, 60 °C for 1 h, and then 95 °C for 15 min. PCR reactions were performed using the following primers: *rde-1* F: 5'-TTCATTGAGTTTCCCCACCTACC-3'; *rde-1* R: 5'-CTCCTCTGTTTTTCATTGGCACC-3'; *ergo-1* F: 5'-GCAG-GCTTTTAGCGATTTCAGAC-3'; *ergo-1* R: 5'-CGACGGT-CAACTCAACTCCATC-3'; *set-30* F: 5'-CTCCGTTAGAAGT-GGTAGGGGTG-3'; *set-30* R: 5'-GAAGTTGCCTCCAAATG-CCG-3'; *eap-1* F: 5'-TCCATTCAAGTTCGCAATCC-3'; *eap-1* R: 5'-CTCTCCATTAGCATCATTCCCG-3'; *spr-5* (by101) F: 5'-AACACGTGCCTCCATGAATATCT-3'; *spr-5* (by101) R: 5'-GAACACGTGTGTTCTCCAGCAA-3'; *spr-5* (by101) I: 5'-CCTATAGAAGTTTCCCACAGTG-3'; *spr-5* (by134) F: 5'-CCAATTGTGCTCCAACC-3'; *spr-5* (by134) R: 5'-AACTTC-GAAGAGCACGGA-3' (PCR reactions for *spr-5*(by134) were cut with *PsiI* to distinguish WT from mutant genotype), *set-17* F: 5'-ACCATCTTGCTGTGAAACGAGG-3'; *set-17* R: 5'-TGAACGGATTCTGGCTGGC-3'; *jmjd-2* F: 5'-TTTACGC-CGCAAAAAGTGC-3'; *jmjd-2* R: 5'-TCTACGATGCTCAAGT-GGAAGAGTG-3'; *daf-12* F: 5'-GAAAGTTCTGGTGCTTGT-GGCTC-3'; *daf-12* R: 5'-TGTGGTACTGCTGATTCCCTG-3'; *damt-1* F: 5'-CGGTTATGGAGGAAAGAAGGG-3'; *damt-1* R: 5'-TTTTATGGGTATCGGGAACGG-3'; *damt-1* I 5'-GCA-CATCCCAGGAATGAGATTG-3'. PCR reactions were performed according to the manufacturer's protocol (Invitrogen: Platinum PCR supermix) and PCR reactions were resolved on agarose gels.

Antibodies

The H3K4me2 (07-030) was obtained from Millipore. The Histone H3 (ab1791) antibody was obtained from Abcam.

Protein analysis by western blot

Worms were grown synchronously to appropriate stages and

washed off plates with M9 buffer. Worms were washed several times in M9 buffer and snap frozen in liquid N₂. 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 N₂. Worm extracts were sonicated three times for 30 s at 15 W (VirSonic 600) and boiled for 2 minutes before being resolved on SDS-PAGE (15%) and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies (H3K4me2, 1:2 000; 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).

Worm RNA extraction and reverse transcription followed by Quantitative PCR

RNA was extracted by addition of 1 ml of Trizol (Invitrogen) for 100 µl worm pellets of young adult worms. Six freeze thaw cycles were performed in liquid nitrogen. The RNA extraction was performed according to the Trizol protocol. The expression of target genes was determined by reverse transcription of 1 µg total RNA with the Superscript III kit (Invitrogen) followed by quantitative PCR analysis on a Roche Lightcycler 480 II with SYBR Green I Master (Roche) with the following primers: *pan-actin* F: 5'-TCGGTATGGGACAGAAGGAC-3'; *pan-actin* R: 5'-CATCCCAGTTGGTGACGATA-3'; *daf-36* F: 5'-GGAGGATAGACATTGTGGAGTTATGC-3'; *daf-36* R: 5'-CGGGAGTTACTGTTTGAATAACGAC-3'. The results were expressed as 2^{-(gene of interest number of cycles - actin number of cycles)}. Control PCR reactions were also performed on total RNA that had not been reverse-transcribed to test for the presence of genomic DNA in the RNA preparation.

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Author Contributions

ELG and YS conceived and planned the study and wrote the paper. ELG performed the experiments. BB, CL, and AA analyzed steroid concentrations.

Competing Financial Interests

YS is a co-founder of Constellation Pharmaceuticals, Inc and a member of its scientific advisory board. The remaining authors declare no conflict of interest.

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