



LET-418/Mi2 and SPR-5/LSD1 Cooperatively Prevent Somatic Reprogramming of *C. elegans* Germline Stem Cells

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SUMMARY

Throughout their journey to forming new individuals, germline stem cells must remain totipotent, particularly by maintaining a specific chromatin structure. However, the place epigenetic factors occupy in this process remains elusive. So far, “sensitization” of chromatin by modulation of histone arrangement and/or content was believed to facilitate transcription-factor-induced germ cell reprogramming. Here, we demonstrate that the combined reduction of two epigenetic factors suffices to reprogram *C. elegans* germ cells. The histone H3K4 demethylase SPR-5/LSD1 and the chromatin remodeler LET-418/Mi2 function together in an early process to maintain germ cell status and act as a barrier to block precocious differentiation. This epigenetic barrier is capable of limiting COMPASS-mediated H3K4 methylation, because elevated H3K4me3 levels correlate with germ cell reprogramming in *spr-5*; *let-418* mutants. Interestingly, germ cells deficient for *spr-5* and *let-418* mainly reprogram as neurons, suggesting that neuronal fate might be the first to be derepressed in early embryogenesis.

INTRODUCTION

To ensure that all lineages will develop after fertilization, germ cells must proceed through gametogenesis while maintaining totipotency and resisting somatic differentiation. After their induction, mammalian primordial germ cells (PGCs) express the transcription factors sufficient to not only maintain their pluripotency, such as *Oct4*, *Sox2*, or *Nanog*, but also activate the epigenetic changes essential to PGC specification, including chromosome X inactivation, histone H3K9 demethylation, and genome-wide erasure of methylated DNA (reviewed in Magnúsdóttir et al., 2012). The use of nonvertebrate systems such as *C. elegans* or *D. melanogaster* to study germ cell specification revealed that combinations of genetic and epigenetic events were the key to somatic fate repression. To maintain their unique status, *C. elegans* PGCs globally repress mRNA transcription and establish a specific chromatin structure and composition to tightly control gene expression (Wang and Seydoux, 2013). Recently, germline reprogramming was “artificially” obtained by the simultaneous ectopic expression of master somatic fate inducers (“terminal selector genes”) and the downregulation of chromatin repressors such as LIN-53/RbAP46-48 and the H3K27 methyl-transferase Polycomb (Patel et al., 2012; Tursun et al., 2011), implying that specific combinations of transcriptional and epigenetic factors were capable of controlling the germ cell program.

The ATP-dependent nucleosome remodeler Mi2 is the core component of the nucleosome remodeling and deacetylase complex (NuRD), a multisubunit transcriptional repressor complex known to play a major role in mamma-

lian cell fate determination and capable of different scopes of activities depending on its subunit content (reviewed in Bowen et al., 2004). Embryonic stem cells (ESCs) deficient for the NuRD subunit MBD3 are unable to undertake lineage commitment (Kaji et al., 2006). Conditional knockout mice models showed that Mi2/NuRD was essential in terminal differentiation programs, including T cell maturation (Williams et al., 2004) and Schwann cell-directed peripheral nerve myelination (Hung et al., 2012). In addition, recent findings propose that the NuRD repressive activity is required to limit pluripotency gene expression, thereby permitting ESC differentiation (Reynolds et al., 2012a).

Recently, histone H3 lysine 4 (H3K4) demethylase LSD1/KMD1A was identified as a de novo member of the NuRD complex in HeLa cell extracts (Wang et al., 2009) and in ESCs (Whyte et al., 2012), independently of the chromatin repressor complex CoREST, of which it is the core component (Lee et al., 2005). LSD1 carries differentiation-licensing functions in common with the NuRD complex. *Lsd1*^{-/-} ESCs fail to fully deactivate pluripotency gene enhancers to complete differentiation programs (Whyte et al., 2012). LSD1 silences the bivalent promoter of developmental genes, which combine activating H3K4me2/3 and repressing H3K27me3 histone marks, to maintain ESC pluripotency (Adamo et al., 2011). LSD1 is also involved in multiple developmental programs, including myoblast differentiation (Choi et al., 2010) and neuronal development (Ceballos-Chávez et al., 2012; Fuentes et al., 2012), and is a putative metastatic breast cancer suppressor (Wang et al., 2009). In *Drosophila*, loss of LSD1 leads to ovarian germline tumorigenesis, because somatic gonadal cells become unable to produce the lineage specification

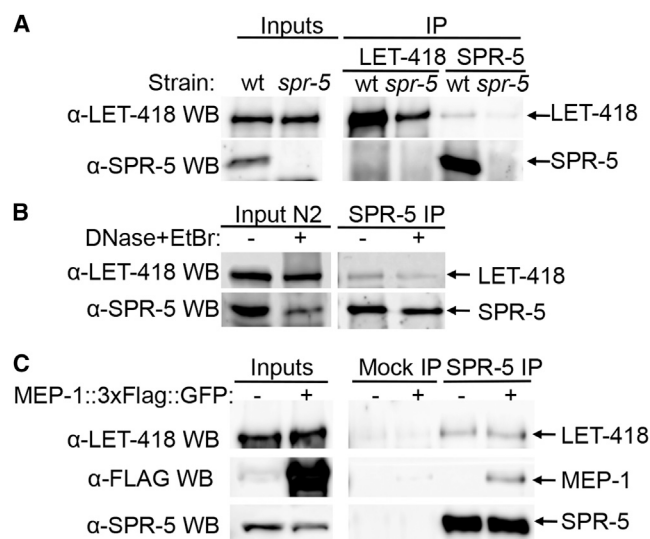


Figure 1. SPR-5 Interacts with LET-418 and MEP-1 In Vivo

(A) Coimmunoprecipitation of SPR-5 and LET-418 in embryonic extracts using anti-LET-418 or anti-SPR-5 antibodies. wt, wild-type; *spr-5*, *spr-5*(*by134*) null allele.

(B) Coimmunoprecipitation of LET-418 using anti-SPR-5 (SPR-5 IP) antibodies with (+) or without (–) DNaseI/ethidium bromide (DNase+EtBr) pretreatment.

(C) Coimmunoprecipitation of LET-418 and FLAG-tagged MEP-1, using anti-SPR-5 and anti-FLAG antibodies, in embryonic extracts of a control wild-type strain (–) or a strain stably expressing MEP-1::3xFLAG::GFP (+).

See also Figure S1.

signals required for germline stem cell differentiation (Eliazer et al., 2011).

In *C. elegans*, homologs for both the NuRD complex and LSD1 were identified. *C. elegans* LET-418/Mi2 is a subunit of a NuRD-like complex, together with Rb-binding protein LIN-53/RbAp48, histone deacetylase HDA-1/HDAC1, metastasis-associated protein homolog LIN-40/MTA1, and DCP-66/p66(α/β) (Passannante et al., 2010; Unhavaithaya et al., 2002; our unpublished data). The *C. elegans* NuRD-like complex was previously involved in controlling the vulval cell fate (von Zelewsky et al., 2000). In addition, similar to its *Drosophila* dMi2 homolog (Kunert et al., 2009), LET-418 interacts tightly with the zinc finger protein MEP-1 and HDA-1/HDAC1 in a distinct MEP-1-interacting complex (MEC) involved in repressing germline gene expression in somatic cells (Passannante et al., 2010; Unhavaithaya et al., 2002).

Three *C. elegans* genes encode putative LSD1 homologs: Suppressor of Presenilin 5 (*spr-5*), T08D10.2/*lsd-1* and R13G10.2/*amx-1*. The SPR-5 protein displays a demonstrated biochemical H3K4 demethylase activity and functions in promoting fertility (Katz et al., 2009). SPR-5 is partially responsible for the specific erasure of H3K4me2

marks in the PGCs at their birth. *spr-5* mutants progressively accumulate H3K4me2 in PGCs throughout generations, correlating with the progressive “mortal germline” sterile phenotype peaking at 28–30 generations (Katz et al., 2009).

All these observations suggest that the functions of LSD1 and Mi2/NuRD in controlling cell lineage specification are ancient and well conserved across species. In order to decipher the molecular mechanisms by which LSD1 and NuRD determine cell fate in vivo, we set up to analyze their common functions in the developmental model organism *C. elegans*. Here, we describe an interaction between the *C. elegans* LET-418/Mi2-containing complexes and SPR-5/LSD1. In addition to the physical interaction between SPR-5, LET-418, and associated complexes, *spr-5* and *let-418* interact genetically to promote the normal development of germline stem cells. Concomitant loss of SPR-5 and LET-418 leads to immediate sterility, aberrant gonad development, and germline teratoma incidence. SPR-5 and LET-418 together maintain the germline stem cell status and form an epigenetic barrier to reprogramming. This infers the existence of a conserved link between LSD1 and Mi2-related complexes and shows that specific epigenetic regulators collaborate intricately to control cell fate during germ cell development.

RESULTS

LET-418 and SPR-5 Physically Interact In Vivo

To first test whether the *C. elegans* homologs of LSD1 and Mi2 interacted physically, coimmunoprecipitation (co-IP) assays of SPR-5 and LET-418 were performed in embryonic extracts of wild-type and *spr-5* null (*by134* allele) strains (Figure 1A). Although anti-LET-418 antibodies did not pull down visible amounts of SPR-5, anti-SPR-5 antibodies recovered detectable levels of the LET-418 protein in wild-type, but not *spr-5*-null-derived, samples (Figure 1A), demonstrating that SPR-5 and LET-418 interact in vivo.

To determine whether this interaction was bridged by DNA, the anti-SPR-5 immunoprecipitation was repeated using wild-type embryonic extract pretreated with DNaseI and ethidium bromide (EtBr), which separate all proteins from DNA (Figure 1B). LET-418 was still detectable in the DNase/EtBr-treated anti-SPR-5 eluate, ruling out DNA bridging as a cause for the interaction (Figure 1B).

An interaction was thereafter detected in embryonic extracts between 3×Flag-tagged MEP-1 and SPR-5 (Figure 1C) and between HDA-1 and SPR-5 (Figure S1A available online). Provided that HDA-1 is potentially a member of both the NuRD and MEC complexes, SPR-5 might be interacting preferentially with the MEC complex or with both. We therefore tested the interaction of SPR-5 with other NuRD complex members. A weak but reproducible

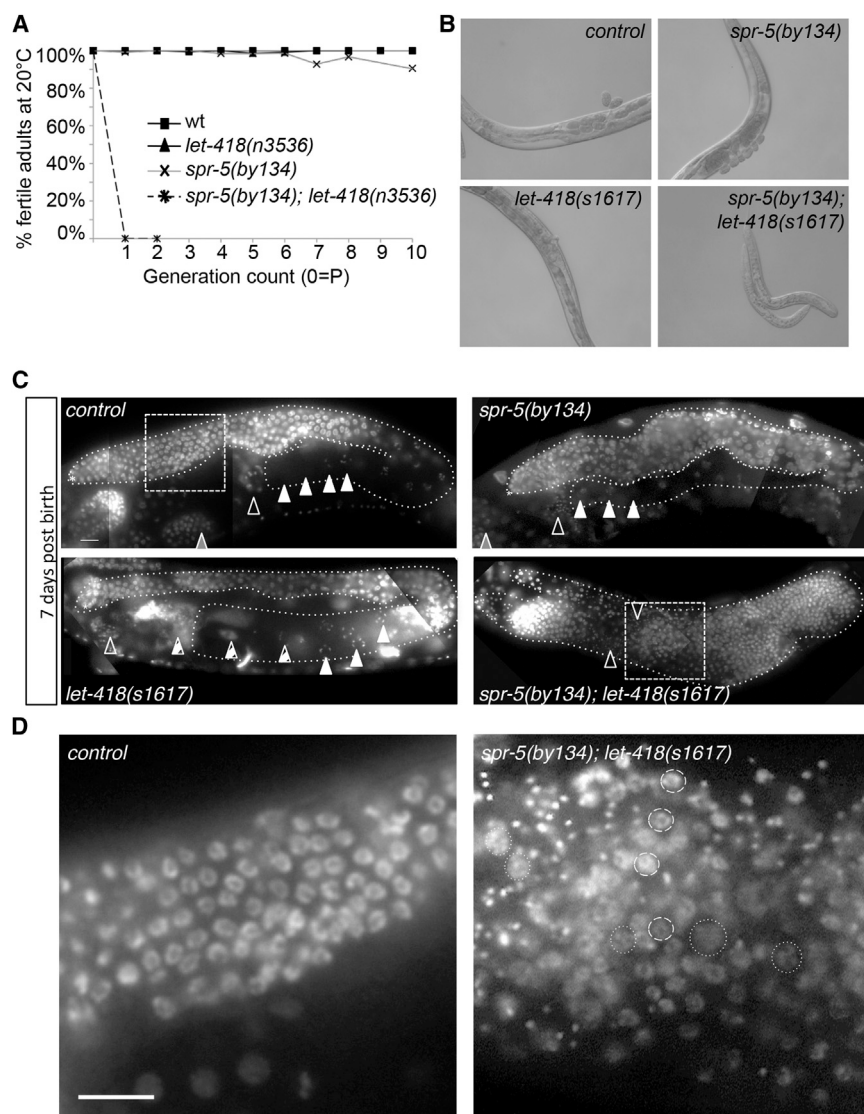


Figure 2. Simultaneous Downregulation of SPR-5 and LET-418 Causes Sterility and Germline Tumor Formation

(A) Double *spr-5(by134); let-418(n3536)* mutants are maternal-effect sterile. Percentage of fertile adults, at 20°C, over the indicated generations; ≥ 100 adults were counted per strain per generation.

(B) Nomarski pictures of the indicated genotypes 4 days postbirth.

(C) DAPI staining of the indicated mutant germlines 6 days postbirth. Double *spr-5; let-418* mutants develop a germline tumor. Plain arrows, oocytes; empty arrows, sperm; gray arrows, embryos; striped arrows, endomitotic oocytes. Scale bar = 20 μ m.

(D) Enlarged view of the squared zones from (C), showing DAPI-stained nuclei for the indicated genotypes. Fine dashed circles, larger nuclei with decondensed chromatin; long dashed circles, smaller nuclei different from wild-type germ cells. Scale bar = 20 μ m.

All strains in (B)–(D) displayed the *unc-46(e177)* allele in their background. See also Figure S2.

interaction was detected between SPR-5 and GFP-tagged LIN-53, a NuRD member homologous to mammalian RbAp48 (Harrison et al., 2006) (Figure S1B). Hence, SPR-5 interacts with both LET-418-containing NuRD and MEC complex subunits in vivo.

Simultaneous Downregulation of SPR-5- and LET-418-Containing Complexes Leads to Synthetic Sterility

To understand the genetic relationship between *spr-5* and *let-418*, double mutants were generated. Similar to the *spr-5(101)* mutant allele phenotype reported previously (Katz et al., 2009), the *spr-5(by134)* null strain started to lose fertility after seven generations (Figure 2A). Conversely, *let-418(n3536)* temperature-sensitive (*let418ts*) hypomorphic worms maintain fertility over generations at 20°C, whereas they produce an L1-arrested progeny at

25°C (von Zelewsky et al., 2000; Figure 2A). Interestingly, combining *spr-5(by134)* with *let-418(n3536)* mutations at 20°C led to a maternal effect sterile phenotype (Figure 2A). The first generation of *spr-5(by134); let-418(n3536)* double homozygotes was fertile, whereas 97.8% (312/319) second-generation worms were sterile, with the remaining seven subfertile worms producing a total of 38 sterile third-generation progeny (Figure 2A). To determine whether this synergistic effect on fertility could also be obtained using RNAi-mediated gene targeting, *spr-5(by134)* L4 larvae were transferred onto *let-418(RNAi)* feeding plates at the semipermissive temperature of 20°C and their progeny observed. *spr-5(by134); let-418(RNAi)* mutants were synthetic sterile in the next generation, causing 100% sterility in *spr-5(by134); let-418(RNAi)* versus 27% in *let-418(RNAi)* animals at 20°C (Figure S2C). This effect was

**Table 1. Gonads of the *spr-5 let-418* Double Mutants Are Tumorous**

Strain	% Sperm	% Oocyte	% Embryos	% Endomitoses	% Tumors	n
<i>unc-46(e177)</i>	100	100	100	0	0	28
<i>spr-5(by134); unc-46(e177)</i>	97	92	92	0	0	39
<i>let-418(s1617) unc-46(e177)</i>	100	94	0	91	0	32
<i>spr-5(by134); let-418(s1617) unc-46(e177)</i>	17	0	0	0	100	36

Quantification of events occurring in the germlines of DAPI-stained 7-day-old adults of the indicated genotypes. % sperm, percentage of worms producing sperm; % oocyte, percentage of worms producing mature oocytes; % embryos, percentage of worms producing fertilized embryos; % endomitoses, percentage of worms containing endomitotic, nonfertilized oocytes; % tumors, percentage of worms presenting a tumorous germline; n, total number of worms counted.

also observed at 15°C, but to a lower extent (Figure S2B). Simultaneous loss of *spr-5* and *let-418* function therefore leads to synthetic sterility in the next generation.

This phenotype did not involve the other *C. elegans* LSD1 homologs, because *let-418ts* worms exposed to *lsd-1* and *amx-1(RNAi)* produced a 100% fertile progeny (Figure S2D).

Synthetic sterility was also observed in *spr-5(by134)* mutants grown on *mep-1(RNAi)* at 15°C and 20°C, on *hda-1(RNAi)* at 15°C, and on *dcp-66(RNAi)* at 15°C and 20°C (Figures S2B and S2C). No effect was observed on *lin-53(RNAi)* or on *hda-1(RNAi)* (at 20°C), due to embryonic lethality (Figures S2B and S2C; our unpublished data). These results imply that SPR-5 interacts with both the LET-418-containing NuRD and MEC complexes to promote fertility.

SPR-5 was first identified as a physical and functional member of the *C. elegans* CoREST complex (Eimer et al., 2002), a chromatin-remodeling complex with repressive transcriptional activities (Andrés et al., 1999; Lakowski et al., 2006). Three subunits were identified as putative CoREST complex members: *spr-1*, *spr-3*, and *spr-4* (Smialowska and Baumeister, 2006). *let-418ts* worms produced a 100% fertile progeny when fed on *spr-3* or *spr-4 (RNAi)* and 97.3% fertile progeny when fed on *spr-1(RNAi)* (Figure S2E), indicating that the CoREST complex was probably not involved in SPR-5/LET-418 function.

Overall, we found that *spr-5* interacts physically and genetically with NuRD and MEC complex members in a CoREST-independent manner to promote germline immortality over generations.

The Double *spr-5; let-418* Mutation Triggers Germline Tumor Formation

Morphologically, the sterile *spr-5(by134); let-418ts* sterile progeny was slow growing, shorter and thinner than wild-type, and displayed abnormally developed gonads (data not shown). DAPI (4',6-diamidino-2-phenylindole dihydrochloride) staining showed that the double-mutant gonads did not extend in the classical "U" shape but instead

formed a large oval shape invading the whole center of the worm, occasionally with a small gonadal protrusion on one side (Figure S2A). To confirm these observations, double-null *spr-5 let-418* mutants were generated from *spr-5(by134); let-418(s1617)/+* heterozygote parents. *let-418(s1617) M+Z-* single mutants are sterile, most likely due to precocious oocyte endomitosis (Figure 2C; Table 1). Double-null *spr-5(by134); let-418(s1617) M+Z-* mutants are also sterile, smaller than single mutants, and grow abnormally shaped oval gonads (Figures 2B and 2C). Similar to what we observed with the previous genotypes (Figure S2A), the sterility in double *spr-5(by134); let-418(s1617)* worm was due to abnormal germ cell progression and incomplete gametogenesis (Figure 2C). Their oval gonads retained normal germ cell nuclei at their extremities, whereas the central region contained both small condensed and large decondensed nuclei, which are normally not found in wild-type germlines (Figures 2C and 2D). Sperm was present in the central gonad, but oocytes were never observed, demonstrating a strong defect in completing gametogenesis (Figures 2C and 2D; Table 1). Seven days postbirth, 100% of the *spr-5(by134); let-418(s1617)* worms displayed a large disorganized germline (Figure 2C; Table 1). We are qualifying these abnormal *spr-5; let-418* germlines of "germline tumors," although they are different from the proximal or distal proliferative germline tumors previously observed in *C. elegans* (Francis et al., 1995; Subramaniam and Seydoux, 2003). Germline tumors were observed for all the combinations of *spr-5* and *let-418* alleles or RNAi tested; in addition, the progeny of *spr-5(by134); let-418(RNAi)* or *spr-5(by134); dcp-66(RNAi)* also developed abnormal germline tumors (Table S1), suggesting that the LET-418-containing NuRD and MEC complexes are involved in this abnormal germ cell progression.

spr-5 let-418 Germ Cells Lose Pluripotency while Maintaining Cell Division

To find out whether the tumorous germlines in *spr-5; let-418* worms were hyperproliferative, replication activity was

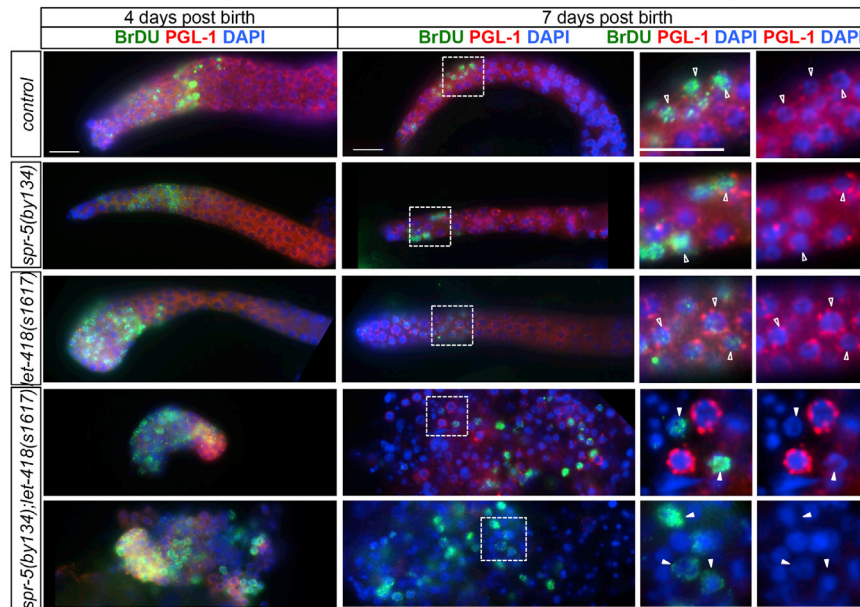


Figure 3. *spr-5 let-418* Germline Tumors Replicate Rapidly while Losing Pluripotency

BrdU incorporation assay in single or double *spr-5(by134) let-418(s1617)* mutants 4 or 7 days postbirth at 20°C. Gonad immunostaining against BrdU (green) and the P granule component PGL-1 (red) plus DAPI staining of the DNA content (blue). Empty arrows, PGL-1-positive, BrdU-positive cells; plain arrows, PGL-1-negative, BrdU-positive cells; right panel squares, enlargements of the boxed areas on their left. All these strains contained the *unc-46(e177)* mutation in their genetic background. Scale bar = 20 μm. See also Figure S3 and Table S2.

monitored using a BrdU incorporation assay (Figure 3; Table S2). In parallel, the germ cell status was checked by immunostaining PGL-1, a component of the cytosolic P granules (Kawasaki et al., 1998) cosegregating specifically with germ cells (Hird et al., 1996). Wild-type and *spr-5(by134)* or *let-418(s1617)* single-null mutants displayed comparable amounts of BrdU-positive cells in the distal mitotic region of the gonads at 4 and 7 days after birth (Figure 3). All their germ cells were P granule positive and progressed normally toward gametogenesis (Figure 3; Table S2). On the contrary, double *spr-5(by134); let-418(s1617)* mutants held disorganized, BrdU-positive cells in every part of their abnormally oval gonad (Figure 3). Strikingly, 78% of the BrdU-positive cells were P granule negative (Figure 3; Table S2), indicating that the double-mutant germ cells lost pluripotency while maintaining an active replication.

The mitotic potential of gonadal cells was also assessed by immunostaining phosphorylated histone H3 serine 10 (PH3) (Hendzel et al., 1997) in 7-day-old worms (Figure S3; Table S3). Wild-type and single *spr-5* or *let-418* mutant gonads were entirely P granule positive, with a few PH3-positive cells, reflecting the low mitotic rate at this age (Figure S3; Table S3). Strangely, germlines of *spr-5(by134); let-418(s1617)* double mutants contained two distinct populations of cells, separable by the intensity of their PH3 staining. The first population of “high-PH3-signal” cells was defined as cells displaying PH3 levels comparable to the control and single mutants; these cells were all devoid of P granules (Figure S3; Table S3). On the other hand, a second population of “low-PH3-signal” cells, never observed in the control strains, was composed essentially of P-granule-positive cells and might indicate the presence

of a slowly dividing population (Figure S3; Table S3). Altogether, our data suggest that *spr-5; let-418* germlines are tumoral, because replication and mitosis are maintained in the whole gonad and are not restricted to a mitotic zone. In addition, cells in the central tumors keep dividing but fail to maintain their germ cell status, which is evocative of cells undertaking a somatic fate.

spr-5 let-418 Germ Cells Reprogram into Neurons

To detect the likely activation of somatic differentiation pathways, reporter constructs expressing GFP or red fluorescent protein under the control of tissue-specific promoters were introduced in the *spr-5(by134)* background and ectopic expression was examined in *let-418(RNAi)*-treated worms. Strikingly, strong ectopic expression of pan-neuronal reporter *unc-119p::gfp* was detected specifically within the *spr-5(by134); let-418(RNAi)* worm gonads starting at the young-adult stage (Figure 4A). This ectopic expression increased progressively between 4 and 7 days after birth and was detected in a large majority of worms (73%–75% at day 7; Figure 4A). Moreover, the pan-cellular expression of the transgene allowed us to observe that the GFP-positive cells adopted a neuron-like morphology and developed cellular projections similar to neuronal axons (Figure 4A). Nomarski pictures confirmed that the *unc-119p::GFP*-positive cells had lost the “pan-fried egg” shape of normal germ cells, became flatter, and projected axon-like structures (Figure 4B). Neuronal transformation was further confirmed by testing the expression of two other pan-neuronal transgenes, *rab-3p::nls::rfp* and *unc-33p::gfp*, in the *spr-5(by134); let-418(RNAi)* mutant background. In both cases, these transgenes were found ectopically expressed in the mutant

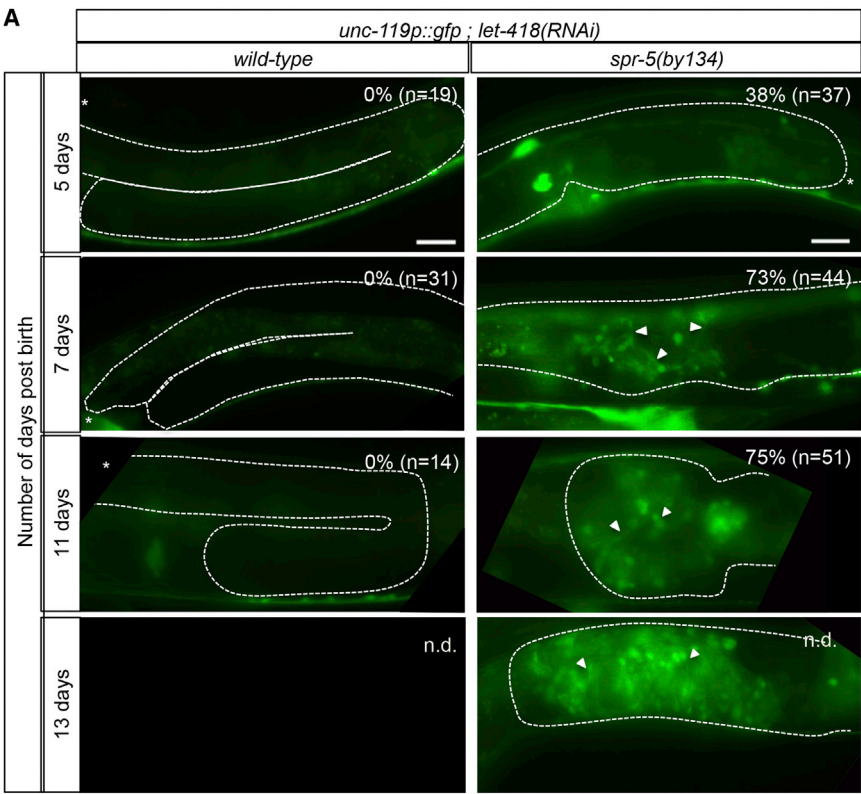
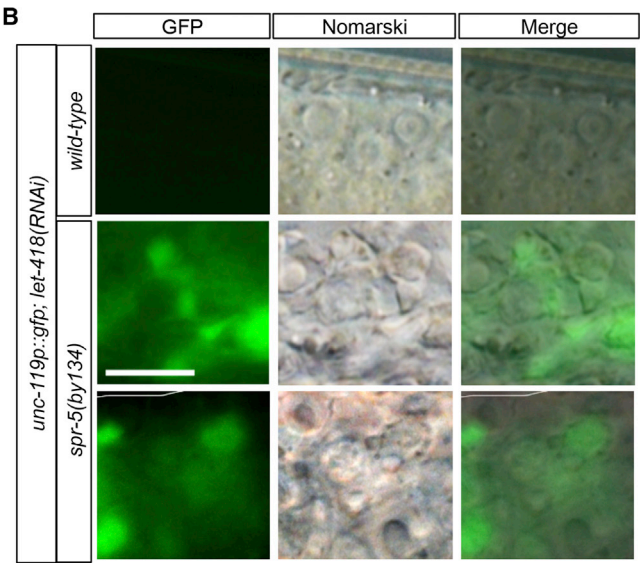


Figure 4. *spr-5 let-418* Germ Cells Reprogram toward a Neuronal Fate

(A) Ectopic expression of the pan-neuronal reporter *unc-119p::gfp* within the *spr-5(by134) let-418(RNAi)* germinal gonad. *spr-5(by134)* or control worms (wild-type) expressing the *unc-119p::gfp* reporter were grown on *let-418(RNAi)* at 20°C and monitored for ectopic GFP expression in their gonads at 5, 7, 11 and 13 days postbirth. The percentage of worms ectopically expressing *unc-119p::GFP* in their gonads is indicated within each representative picture. Dashed lines, gonad limits; n.d., not determined; *, distal tip (when observable). Scale bar = 20 μm.

(B) Nomarski pictures of *unc-119p*-driven GFP-positive cells in the *spr-5(by134) let-418(RNAi)* germlines 7 days postbirth. Scale bar = 10 μm.

See also [Figure S4](#).



tumorous germlines ([Figure S4A](#)). Strikingly, this phenomenon was also observed when the *unc-119p::gfp* transgene was expressed into *spr-5(by134)* worms exposed to *dcp-66(RNAi)* or *mep-1(RNAi)* worms ([Figure S4B](#); data not shown). Thus, downregulating one member of a LET-418-containing complex is therefore sufficient to produce synthetic cell-fate catastrophes in *spr-5* worm germlines.

SPR-5 and LET-418 Protect the Germline against Multiple Somatic Fates

To determine whether other cell fates were induced in the *spr-5; let-418* mutants, muscle- and intestinal-specific transgenic reporters were tested in our system. Among them, only the muscular *unc-97::gfp* reporter was found to be ectopically expressed in a subset of *spr-5(by134)*;



let-418(RNAi) germlines (54%; Figure S4A; data not shown). To confirm this, ectopic expression of the MYO-3 muscular marker expression (Miller et al., 1983) was followed in early and late adult tumors (Figure S5A). Cytoplasmic expression of MYO-3, which is distinct from the signal generally observed in sheath cells around wild-type gonads, was detected in PGL-1-negative cells in a small amount of old-adult tumorous germlines (Figure S5A). These cells formed distinct “clonal” groups (Figure S5A), substantiating our previous finding that cells kept dividing after losing P granules (Figure 3). Because of its late occurrence, this germ-to-muscle reprogramming might be general of germline tumors in aging worms, although this phenomenon was not documented in the literature. To rule out this possibility, worms with mutations causing proliferative tumors (*mog-6(q465)*; *gld-3(q730)* (Belfiore et al., 2004) and *glp-1(ar202)* gain of function (Pepper et al., 2003) mutations, respectively) were grown to old age and ectopic expression of MYO-3 was assessed by immunostaining (Figure S5B). Although the tumorous germlines of both mutants were filled with proliferative germ cells, there was no P granule loss or ectopic MYO-3 expression, confirming that muscular differentiation was not simply an age-related process but was specific to the *spr-5*; *let-418* tumorous germlines (Figures S5A and S5B).

Altogether, our data infer that the *spr-5*; *let-418* germline forms a type of teratoma, in which germ cells keep dividing but fail to complete meiosis, lose their pluripotent status, and reprogram into neurons, muscles, and possibly other undetermined cell types.

Germline Tumor Formation in *spr-5 let-418* Mutants Is Linked to a COMPASS-Complex-Dependent Increase in H3K4 Methylation Levels in Chromatin

In *spr-5* mutants, the loss of H3K4 demethylase activity is accompanied by an increase in H3K4me2/3 levels in the PGCs (Katz et al., 2009). To determine whether germline defects in the double *spr-5*; *let-418* mutants were caused by ectopic accumulation of methylated H3K4 on chromatin, we first undertook a genetic approach that consisted of decreasing H3K4 methyltransferase activity in those mutants. In *C. elegans*, most of the H3K4 methylation is ensured by the SET1 homolog SET-2 (Greer et al., 2010; Xiao et al., 2011; Xu and Strome, 2001), in association with homologs of the yeast SET1/COMPASS complex components such as WDR-5.1 (Li and Kelly, 2011), DPY-30 (Pferdehirt et al., 2011), ASH-2 (Greer et al., 2010), and RbBP5 (Li and Kelly, 2011). Endogenous levels of SET-2 or WDR-5.1 were therefore downregulated by RNAi in the double-null mutants *spr-5(by134)*; *let-418(s1617)*, and germline tumor formation was monitored (Figure 5A). *control(RNAi)*-treated, *spr-5(by134)*; *let-418(s1617)* worms developed germline tumors in a majority (91%) of individ-

uals, whereas *set-2(RNAi)*-treated worms were partially rescued, with 36% worms harboring two normally shaped gonads, and 64% developed only one unilateral tumor (Figure 5A; Table S4). *wdr-5.1(RNAi)*-treated worms were also partially rescued, but to a lesser extent (Figure 5A; Table S4). The rescued gonads, although smaller, presented a normal mitosis-through-meiosis progression, up to spermatogenesis; however, there was no oogenesis (Figure 5A), indicating that gametogenesis was only partially rescued.

To confirm these results, we generated a double *spr-5(by134)*; *set-2(ok952)* mutant in which the SET-2 enzymatic activity is mildly compromised (Simonet et al., 2007; Xiao et al., 2011). Strikingly, 96% of the *spr-5(by134)*; *set-2(ok952)*; *let-418(RNAi)* progeny showed no germline tumor and contained two normally shaped gonads in which spermatogenesis took place; among these, 9% produced fertilized embryos (Figure S6, two first rows; Table S5).

In summary, reduction of the COMPASS H3K4 methyltransferase activity partially rescues the germline program in *spr-5*; *let-418* animals, allowing for the maintenance of germ cell status, progression through meiosis, and suppression of somatic differentiation.

To verify whether the observed tumoral phenotypes correlated with a COMPASS-dependent increase in H3K4 methylation levels, germlines of *spr-5(by134)*; *let-418(s1617)* double-null mutants treated with *set-2* or *wdr-5.1(RNAi)* were immunostained for trimethylated H3K4 (H3K4me3) (Figure 5B). Strikingly, elevated levels of H3K4me3 were specifically observed in the P-granule-negative cells of *spr-5(by134)*; *let-418(s1617)* mutants (Figures 5B and 5C). Coherent with the previous observations that downregulation of COMPASS activity rescued the *spr-5(by134)*; *let-418(s1617)* mutant phenotypes, *set-2* and *wdr-5.1(RNAi)* treatment resulted in a reduction not only of the number of P-granule-negative cells but also of the global H3K4me3 levels in those cells (Figures 5B and 5C; data not shown). Hence, simultaneous targeting of SPR-5 and LET-418 functions leads to an abnormal, COMPASS-dependent, increase in H3K4me3 levels in germ cells, which tightly correlates with the loss of germ cell status.

Germ Cell Pluripotency Is Maintained via H3K4 Methylation Control

Our results strongly support the hypothesis that a strong increase in H3K4 methylation can lead to germ cell reprogramming. Sterile *spr-5(by134)* late-generation worms, which display a high H3K4 methylation level in their germ cell chromatin, should therefore show neuronal differentiation of their germline (Katz et al., 2009). To test this hypothesis, sterile *spr-5(by134)* worms, which stochastically appear at each generation, were analyzed for ectopic expression of the pan-neuronal *unc-119p::gfp* reporter and

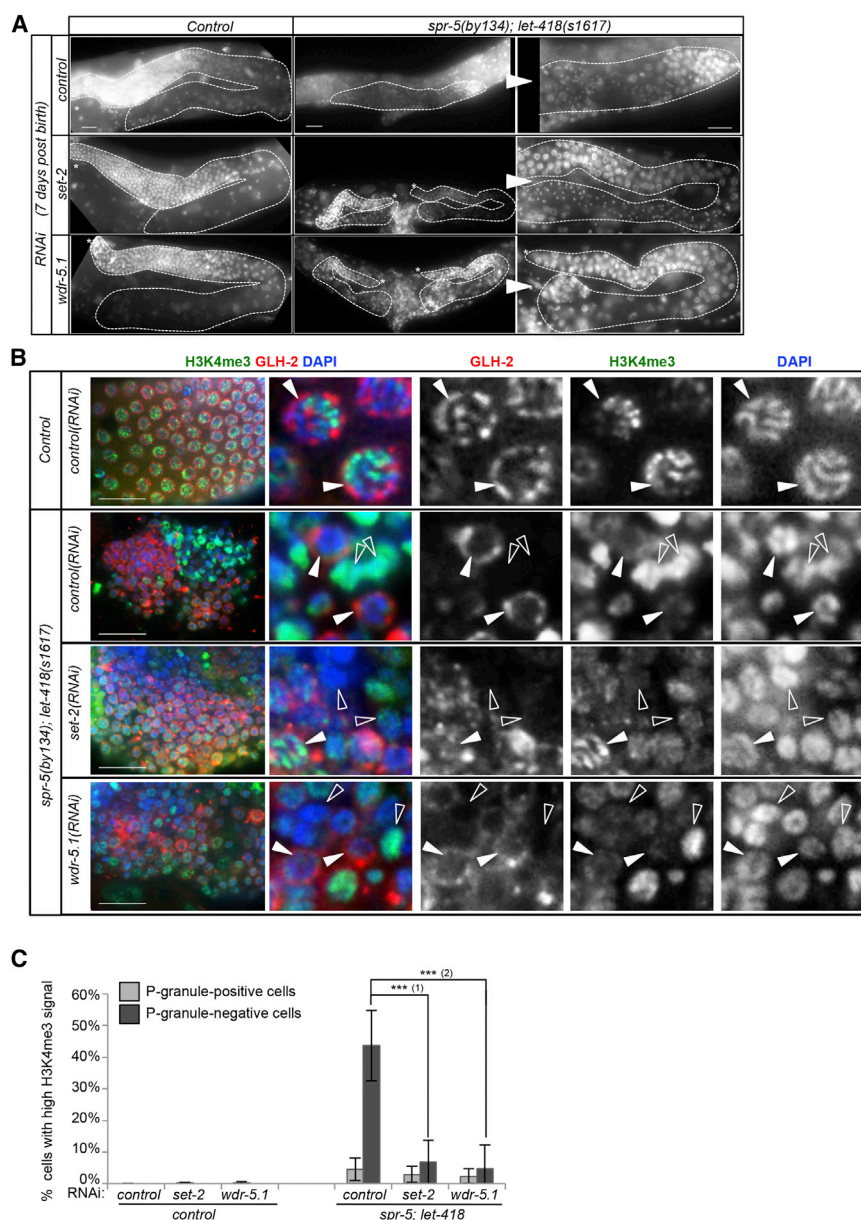


Figure 5. The Cooperative Control of COMPASS-Dependent H3K4 Methylation Levels by SPR-5 and LET-418 Is Linked to Germ Cell Status

(A) Germline tumorigenesis of *spr-5 let-418* mutants is partially rescued by *set-2* and *wdr-5.1(RNAi)*. DAPI staining of wild-type and double *spr-5(by134) let-418(s1617)* null mutants grown on control, *set-2*, or *wdr-5.1(RNAi)* 7 days postbirth at 20°C. Right panels show an enlarged view of the right arm of *spr-5; let-418* mutant gonads. Dashed lines show the position of the somatic gonad. *, distal tip (when visible). Scale bar = 20 μm.

(B) P-granule-negative cells of the *spr-5(by134); let-418(s1617)* germlines show elevated H3K4me3 levels, which are partially rescued by *set-2(RNAi)* and *wdr-5.1(RNAi)*. Immunostaining of control or *spr-5(by134); let-418(s1617)* germlines after exposure to control, *set-2*, or *wdr-5.1(RNAi)* 7 days postbirth at 20°C. Full arrows show P-granule (GLH-2)-positive germ cells, whereas empty arrows point to P-granule-negative cells. Scale bar = 20 μm.

(C) Quantification of (A), showing the percentage of cells with elevated H3K4me3 signal in P-granule-positive (light gray bars) or P-granule-negative (dark gray bars) cells. Results are represented as the mean percentage of cells with high H3K4me3 signal, counted manually using the ImageJ cell counting tool on immunostaining pictures, between two biological replicates. Error bars = SD; p values: t test (1), p = 0.00025; t test (2), p = 0.00012. All the strains present in this figure contained the *unc-46(e177)* mutation in their genetic background.

compared to their fertile counterparts (Figure 6A). Strikingly, ectopic *unc-119p::gfp* expression was detected in the germline of more than three-quarters of the sterile *spr-5(by134)* population, although it was never observed in fertile worms (Figure 6). This ectopic expression was accompanied by loss of germ cell shape toward a neuron-like morphology, including axonal extensions (Figure 6A; data not shown). Side-by-side immunostaining of fertile and sterile *spr-5(by134); unc-119p::gfp* dissected gonads confirmed that the H3K4me3 level in sterile, P-granule-negative *spr-5(by134)* germlines (Figure 6B, right gonad) is visibly higher than in the P-granule-positive, fertile *spr-5(by134)* germline (Figure 6B, left gonad). Hence, the pro-

gressive accumulation of H3K4 methyl marks on germ cell chromatin over generations directly leads to loss of pluripotency, germ cell reprogramming, and sterility in *spr-5(by134)* null worms.

To confirm these results, we studied the impact of depleting other H3K4 demethylases on germ cell development in the absence of LET-418. H3K4 histone demethylases (KDM) can be organized in two groups relative to their functional domain (reviewed in Rotili and Mai, 2011). In *C. elegans*, only four H3K4 KDMs were identified. The amine-oxidase family includes the three LSD1 homologs *spr-5*, *lsd-1*, and *amx-1* (Katz et al., 2009; Maures et al., 2011), whereas the JumonjiC (JmJC)/JARID family is only

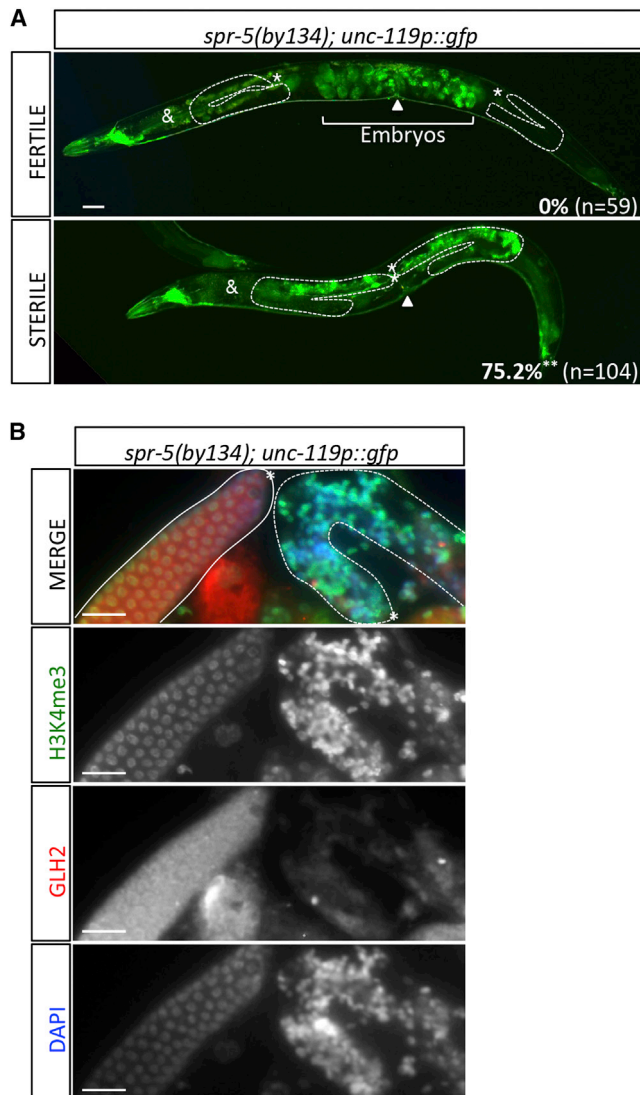


Figure 6. Loss of Fertility in *spr-5(by134)* Single Mutants Is Also Associated with Germ Cell Reprogramming and Elevated H3K4me3 Levels

(A) Percentage of worms ectopically expressing the pan-neuronal reporter *unc-119p::gfp* in the germline of *spr-5(by134)* fertile and sterile individuals of a same generation 7 days postbirth at 20°C. Two representative pictures are presented. n, total number counted. t test p value = 0.0025 (**). White dashed lines show gonad borders. *, distal tip; &, background fluorescence of the intestine; white arrow, position of the vulva. Scale bar = 20 μ m.

(B) Side-by-side immunostaining showing H3K4me3 (green) and GLH2 (red) levels in dissected gonads from fertile and sterile *spr-5(by134)* individuals from (A). Left gonad (white full line), fertile worm; right gonad (white dashed line), sterile worm. Scale bar = 20 μ m.

See also Figure S6 and Table S5.

represented by the RBP2 homolog *rbr-2* (Christensen et al., 2007). As mentioned above, depletion of *lsd-1* or *amx-1* via RNAi did not reduce the fertility of *let-418ts* worms at semi-

permissive temperature (Figure S2D), implying that none of these LSD1 homologs were involved in the SPR-5/LET-418-dependent mechanisms of germ cell protection. Similarly, the *rbr-2(tm1231)* mutation did not lead to increased sterility when combined to *let-418(RNAi)* (Figure S6). However, double *spr-5(by134); rbr-2(tm1231)* mutants were synthetic lethal when exposed to *let-418(RNAi)* (Figure S6; Table S5), suggesting that *spr-5* and *rbr-2* might function principally in separate and complementary pathways. This synthetic lethality was largely rescued when downregulating SET-2 activity, to an higher extent with respect to fertility than in *spr-5(by134); let-418(RNAi)* worms (Figure S6; Table S5). Thus, the combined loss of two H3K4 KDMs, SPR-5 and RBR-2, in combination with LET-418 might generate a global, COMPASS-mediated increase in H3K4 methylation levels incompatible with embryonic development.

Altogether, we believe that the LET-418-containing NuRD and MEC complexes specifically interact with the H3K4 demethylase SPR-5, but not with RBR-2, AMX-1, or LSD-1, to form an epigenetic barrier to germ cell reprogramming. A deficiency in SPR-5 activity, accompanied by loss of NuRD or MEC function, leads to inappropriate levels of H3K4 methylation on germ cell chromatin due to uncontrolled COMPASS activity and triggers loss of germ cell status and somatic differentiation.

DISCUSSION

Here, we describe the identification of an epigenetic mechanism necessary and sufficient to maintain pluripotency and/or avoid precocious differentiation of the germ cell lineage in *C. elegans*. Histone H3K4 demethylase SPR-5/LSD1 physically interacts with the LET-418-containing chromatin-remodeling NuRD and MEC complexes. This is accompanied by a genetic interaction, leading to a synthetic sterile phenotype in double mutants and indicating that SPR-5 and LET-418 have a collaborative role in promoting fertility. We then discovered that *spr-5; let-418* germ cells progressed anarchically, eventually lost their pluripotent status, and undertook somatic differentiation, leading to teratoma formation. Most of the reprogramming germlines contained a large number of neuron-shaped cells, suggesting that the neuronal fate is a major target for combined SPR-5 and LET-418-complex regulation.

The LET-418/SPR-5 Physical Interaction Might Potentiate Their Biochemical Function

Our finding that NuRD and SPR-5 work together to control germ cell fate suggests that the state of the chromatin linked to germ cell differentiation relies principally on the three biochemical activities of these complexes,



namely histone deacetylation, histone demethylation, and nucleosome remodeling. Identifying a physical LET-418/SPR-5 interaction was surprising considering the synthetic phenotypes of the double *spr-5; let-418* mutants, which would not be expected if those factors worked together in a single complex. A first, simple interpretation is that the SPR-5 and LET-418 complexes interact in embryonic and adult somatic cells, but not in the germline. Arguing against this idea, immunostaining experiments show that both proteins colocalize around the adult germ cell chromatin (data not shown). More excitingly, we postulate that the LET-418/SPR-5 interaction has a synergistic effect on their biochemical activities. As an illustration, Reynolds et al. recently demonstrated that NuRD-mediated deacetylation of histone H3K27 was necessary for Polycomb/PRC2 access and subsequent methylation of this residue to regulate the expression of poised developmental genes in ESCs (Reynolds et al., 2012b). Furthermore, the activity of human LSD1 depends on the histone code surrounding the H3K4 residue and is inhibited by H3K9 acetylation (Forneris et al., 2005). The NuRD complex's histone deacetylase activity could be necessary to deacetylate H3K9 and promote LSD1-dependent H3K4 demethylation. In our hands, concomitant loss of LET-418 and SPR-5/LSD1 increased the amount of methylated H3K4 on chromatin in cells that had lost their germline status. Downregulating members of the COMPASS H3K4 methyltransferase complex partially rescued this H3K4me3 increase, comforting the hypothesis that SPR-5 and LET-418 are synergistically limiting the COMPASS-dependent H3K4 methylation in germ cells.

Surprisingly, we did not observe any genetic interaction impairing germline development between *let-418* and the three other *C. elegans* H3K4 demethylases *rbr-2*, *lsd-1*, and *amx-1*, re-enforcing the idea that the *let-418/spr-5* interaction is specific and involved in promoting proper germ cell progression toward gametogenesis. However, *spr-5; rbr-2* double mutants, which grow normally and are fertile at low generation count, were synthetic lethal when exposed to *let-418(RNAi)*. This implies that in the absence of *spr-5* and *let-418* functions, *rbr-2* is essential to the worms' development and that SPR-5 and RBR-2 function primarily on different targets and possibly in different tissues. The fact that COMPASS downregulation rescues *spr-5; rbr-2* mutants on *let-418(RNAi)* confirms that all these phenotypes were generated by a deregulation of H3K4 methylation on chromatin, but the functions and tissue specificity harbored by *rbr-2* during development remain elusive. *rbr-2* was proposed to act mainly in somatic cells during early embryogenesis, *spr-5* being then considered as the main germline-specific H3K4 KDM (Wang et al., 2011). *rbr-2* also counteracts the effects of *ash-2* in the germline of aging worms (Greer et al., 2010). Finally, exten-

sive studies on two *rbr-2 null* alleles demonstrated that RBR-2 and SPR-5 are required to maintain germline immortality at high temperatures (Alvares et al., 2014). In our hands, the germline of *rbr-2(tm1231)* mutants did not display higher-than-normal H3K4me3 levels (data not shown). However, more extensive studies investigating the intricate relationship among SPR-5, RBR-2, and SET-2 will be necessary to understand their unique function in controlling germ cell status.

LET-418 and SPR-5: An Epigenetic Barrier to Somatic Programs in Germ Cells

In our model, SPR-5- and LET-418-containing complexes are the prime inhibitors of COMPASS-mediated H3K4 methylation, forming a strong epigenetic barrier against germ cell differentiation, for which the mechanistic of action start to become unraveled. The observation that late-generation *spr-5* sterile mutants also exhibit reprogrammed germlines directly links H3K4 methylation to somatic fate. In wild-type animals, LET-418 complexes might target SPR-5 to chromatin and potentiate SPR-5 activity by deacetylating histones but also maintain a certain level of competition with COMPASS for specific sites on chromatin. In the absence of SPR-5, this steric competition is at least partially acting to protect chromatin, explaining why only a small subpopulation of *spr-5* mutant worms, versus all the *spr-5; let-418* mutants, undergo germline reprogramming. An overdose of H3K4me2/3 marks on germ cell chromatin, above a defined threshold, would therefore represent an irreversible "somatic signal," condemning the germline to mortality.

Noticeably, among every single *let-418* mutant allele already studied in our laboratory, germ cell reprogramming was never obtained (our unpublished data). This might indicate that SPR-5 might be coupled to additional chromatin factors to protect germ cell chromatin against COMPASS activity.

Neuronal Differentiation: The "Default" Program?

A majority of sterile *spr-5* and *spr-5; let-418* worms developed neurons in their reprogrammed germline. Interestingly, the first asymmetric divisions of the *C. elegans* embryo produces one germ cell progenitor (P) and one somatic cell progenitor (AB), from which most (254/259) neurons originate (Sulston et al., 1983). SPR-5- and LET-418-containing complexes might constitute important guardians of the neuronal differentiation program within P-granule-positive germ cell progenitors. It would be of upmost interest to determine whether the LET-418/SPR-5 interaction is specific to germ cells and/or occurs specifically at the promoter of differentiation genes and gets disrupted once cells exit the "P" lineage, lose pluripotency, and enter somatic differentiation.



EXPERIMENTAL PROCEDURES

C. elegans strains, expression vectors, and antibodies used in these experiments are described in detail in the [Supplemental Information](#).

C. elegans Strains and Cultures

The Bristol N2 strain was used as the wild-type strain of reference in all experiments, which were performed at 20°C under standard worm culture conditions (Brenner, 1974), unless otherwise stated.

RNAi

RNAi constructs were all amplified from the Ahringer RNAi library (Kamath and Ahringer, 2003), except for the control RNAi control (pPD129.36, Fire lab L4440), LET-418, and MEP-1 RNAi vectors (pFG98 and pMP167 RNAi vectors; information available upon request). RNAi experiments were performed by feeding as described in Kamath et al. (2001). Briefly, L4 mothers of the indicated genotype were fed on RNAi plates at 20°C (unless otherwise stated) and allowed to lay fertilized embryos for 24–36 hr. Their F1 progeny were then analyzed at the indicated time of growth post-birth on the RNAi plates at 20°C (where “birth” = egg laying time). F1 worms were transferred to fresh plates every 2–3 days when necessary.

Coimmunoprecipitations

Co-IP assays were performed following standard protocols described in detail in the [Supplemental Information](#).

Transgenerational Fertility Assay

To start the transgenerational fertility assays at generation count zero, the *spr-5(bv134)* BR3417 strain was outcrossed with N2 and homozygote *spr-5(bv134)* mutants were selected by PCR. At each generation, six nonstarved, fertile adults were transferred to a fresh plate at 20°C, allowed to lay eggs for 24 hr, and eliminated. At least 100 worms of the next generation were scored per strain per generation. “Fertile” worms contained visible embryos in their uterus 4 days postbirth at 20°C.

DAPI Staining

Rapid DAPI staining protocol was applied as follows. Briefly, worms were harvested and washed in M9, fixed for 10 minutes in methanol at –20°C, washed in M9, stained with 2 µg/ml DAPI (Sigma-Aldrich), washed extensively in M9, and mounted in Vectashield mounting medium (Vector Laboratories) before being examined under a UV-light microscope (Zeiss Axioplan 2 microscope, Zeiss AxioCam Color camera, AxioVision 4.6 software).

BrdU Assays

BrdU assays were performed as described in Biedermann et al. (2009) with modifications, as described in the [Supplemental Information](#).

Cell Counting in Germline Tumors

Cells positive for PGL-1, GLH-2, BrdU, PH3, or H3K4me3 signal were counted manually using the Cell Counter application of

the ImageJ software (National Institutes of Health). In the BrdU and PH3 experiments, the amounts of counted cells are indicated in the corresponding tables. For the H3K4me3 experiment (Figure 6), a minimum of 500 DAPI-positive nuclei were counted in each category, totaled from at least five different pictures for each of the indicated strains.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2014.02.007>.

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