A Nuclear RNA Degradation Pathway Helps Silence Polycomb/H3K27me3-Marked Loci in *Caenorhabditis elegans*

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In fission yeast and plants, RNA-processing pathways contribute to heterochromatin silencing, complementing well-characterized pathways of transcriptional repression. However, it was unclear whether this additional level of regulation occurs in metazoans. In a genetic screen, we uncovered a pathway of silencing in *Caenorhabditis elegans* somatic cells, whereby the highly conserved, RNA-binding complex LSM2-8 contributes to the repression of heterochromatic reporters and endogenous genes bearing the Polycomb mark H3K27me3. Importantly, the LSM2-8 complex works cooperatively with a 5′–3′ exoribonuclease, XRN-2, and disruption of the pathway leads to selective mRNA stabilization. LSM2-8 complex-mediated RNA degradation does not target nor depend on H3K9me2/me3, unlike previously described pathways of heterochromatic RNA degradation. Up-regulation of *lsm-8*-sensitive loci coincides with a localized drop in H3K27me3 levels in the *lsm-8* mutant. Put into the context of epigenetic control of gene expression, it appears that targeted RNA degradation helps repress a subset of H3K27me3-marked genes, revealing an unappreciated layer of regulation for facultative heterochromatin in animals.

Genomic DNA is organized into highly condensed, dark-staining heterochromatin, which correlates with reduced gene expression (Trojer and Reinberg 2007; Wenzel et al. 2011; Saksouk et al. 2015), and less condensed open chromatin, which is transcribed. Whereas the primary mechanism for both constitutive (enriched for H3K9me2/3) and facultative (enriched for H3K27me3) heterochromatic gene repression is thought to involve the inhibition of RNA polymerases, robust pathways that silence at the co- and posttranscriptional levels have also been documented in fission yeast and plants (Buhler 2009; Wang et al. 2016).

A genome-wide screen in our laboratory that monitored the derepression of a heterochromatic, multicopy reporter in *Caenorhabditis elegans* embryos identified 29 factors essential for silencing. Most of these were chromatin modifiers and transcription-related proteins (Towbin et al. 2012) (Figs. 1A and 2A), but we also recovered three subunits of the evolutionarily conserved like-SM protein (LSM) complex (Fig. 1A). A role for these proteins in the repression of heterochromatin was unexpected given the reported roles for the two LSM complexes, LSM1-7 and LSM2-8, in general RNA metabolism but not in transcriptional control per se (Kufel et al. 2004; Beggs 2005; Tharun 2009; Perea-Resa et al. 2012; Golisz et al. 2013; Cornes et al. 2015). Whereas pathways of RNA processing and degradation were reported to contribute to silencing in fission yeast and plants (Buhler et al. 2007; Vasiljeva et al. 2008; Keller et al. 2012; Zofall et al. 2012; Yamanaka et al. 2013; Egan et al. 2014; Chalamcharla et al. 2015; Tucker

et al. 2016), heterochromatic silencing through transcript degradation had not been documented in animals.

The RNA interference (RNAi) screen that identified *gut-2/lsm-2*, *lsm-5*, and *lsm-6* monitored the robust derepression or desilencing of a *C. elegans* heterochromatic reporter, which consisted of an integrated array of several hundred copies of a green fluorescent protein (GFP)-encoding reporter gene driven from a ubiquitously active promoter (Towbin et al. 2012). In a copy-number-dependent manner, these heterochromatic reporters carry the histone modifications H3K9me2/3 and H3K27me3, but lack H3K4 methylation, and are sequestered at the nuclear envelope, thereby mimicking many aspects of endogenous heterochromatin (Meister et al. 2010; Towbin et al. 2010). As expected, the loss of either of the two H3K9 methyltransferases (*met-2* and *set-25*) or the EZH2 homolog *mes-2*, which deposits H3K27me3, led to reporter derepression (Towbin et al. 2012). It was surprising, however, to detect three subunits of an RNA processing complex with the same phenotype. Therefore, we examined in more detail how LSM proteins regulate gene expression within heterochromatic domains in *C. elegans* (Mattout et al. 2020).

LSM PROTEINS REDUCE mRNA FROM HETEROCHROMATIC REPORTERS SELECTIVELY

The initial screen for reporter derepression monitored GFP expression in embryos (Fig. 1B). The elimination of LSM proteins by *lsm-2*, *lsm-5*, or *lsm-6* RNAi, on the other

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Figure 1. LSM proteins silence heterochromatic reporters integrated as high-copy-number gene arrays, but not euchromatic reporters. (A) The integrated, high-copy-number heterochromatic reporter *pkIs1582* used in the genome-wide screen (Towbin et al. 2012). RNA interference (RNAi)-based derepression monitored in progeny of all stages by increased green fluorescent protein (GFP) fluorescence. (B) An RNAi-based derepression screen was used to monitor derepression of *pkIs1582* (GFP) in embryos within young adult worms. The initial screen was reported in Towbin et al. (2012). (C) Fluorescence microscopy of *pkIs1582* (GFP) derepression in L4 larvae treated with the indicated RNAi clones, versus control (mock/L4440). Bar, 100 μ m. A + indicates relative intensity of GFP fluorescence. (D) Quantitation of GFP intensity of heterochromatic reporter *pkIs1582* in L1 larvae (GW306) following indicated RNAi by the worm sorter. L1 progeny were scored after RNAi of indicated genes, or control RNAi (mock: L4440). Shown are box plots of fluorescence intensity in arbitrary units (a.u.), with whiskers = 1st and 3rd quartiles; black lines, median; black circles, outliers. The notch around the median represents 95% confidence interval of the median. Quantification and statistical analysis were based on $n=375$ for each condition. (E) Quantitation of derepression of the heterochromatic (GW306) as well as the euchromatic (GW1108) reporters in L1 progeny under *lsm-6*, *lsm-7*, and control RNAi conditions, as in D. Numbers of L1 worms scored are $n=2000$ (GW306-mock), 1068 (GW306-*lsm6*), 613 (GW306-*lsm7*) and 875 (GW1108-mock), 1110 (GW1108-*lsm6*), 1026 (GW1108-*lsm7*), pooled from three independent experiments. Indicated P -values by two-tailed unpaired Student's t -test (n.s. = nonsignificant or $P>0.05$). Strains are described in Table 1. (Data in panels C,D are found in different formats in Mattout et al. 2020. Reproduced with permission.)

Table 1. Strains and reporters used in this study

Strain	Original name/OC	Genotype	Reference
GW1	N2 ^a	Wild-type, Bristol isolate	
GW76		<i>gwl54 [baf-1p::GFP-lacI::let-858 3'UTR; myo-3p::RFP] X</i>	Meister et al. 2010
GW306	NL2507	<i>pkIs1582[let-858p::GFP::let-858 3'UTR; rol-6(su1006)]V</i>	Towbin et al. 2012
GW566		<i>gwl539 [baf-1p::GFP-LacI::let-858 3'UTR; vit-5p::GFP] III; gwl54 [baf-1p::GFP-lacI::let-858 3'UTR; myo-3p::RFP] X</i>	Towbin et al. 2012
GW694		<i>gwl585[his-72p::mcherry-set-25::his-72 3'UTR; unc-119(+)] unc-119(ed3) ttTi5605 II</i>	Towbin et al. 2012
GW299		<i>gwl525 [tbb-1p::wmCherry-LacI::tbb-2 3'UTR unc-119(+)] unc-119(ed3)</i>	Mattout et al. 2020
GW637		<i>met-2(n4256) set-25(n5021) III; gwl54 [baf-1p::GFP-lacI::let-858 3'UTR; myo-3p::RFP] X</i>	Towbin et al. 2012
GW214		<i>hpl-2(tm1489) III; gwl54 [baf-1p::GFP-lacI::let-858 3'UTR; myo-3p::RFP] X</i>	Towbin et al. 2012
GW468		<i>mes-2(bn11) unc-4(e120)/mnC1 dpy-10(e128) unc-52(e444)II; gwl54[myo-3::RFP baf-1::GFP-lacI let-858] X.</i>	Towbin et al. 2012
GW1004		<i>lsm4::GFP-FLAGx3</i>	Mattout et al. 2020
GW1109	HW1390 oxc4	<i>lsm-8 (xe17 [myo2p::mcherry::unc54 3'UTR])IV</i>	Mattout et al. 2020
GW1125	ocx6	<i>lsm-8 (xe17 [myo2p::mcherry::unc54 3'UTR])IV/nT1[qIs51](IV;V)</i>	Mattout et al. 2020
GW1119	ocx6	<i>lsm-8 (xe17 [myo2p::mcherry::unc54 3'UTR])IV/nT1[qIs51](IV;V); pkIs1582 [let-858::GFP rol-6(su1006)]V</i>	Mattout et al. 2020
GW1120	ocx6	<i>lsm-8 (xe17 [myo2p::mcherry::unc54 3'UTR])IV; pkIs1582[let-858::GFP rol-6 (su1006)]V</i>	Mattout et al. 2020
GW1148	ocx6	<i>met-2(n4256) set-25(n5021) III; lsm-8 (xe17 [myo2p::mcherry::unc54 3'UTR]) IV/nT1[qIs51](IV;V)</i>	Mattout et al. 2020
GW1080	VC2785* oxc5	<i>lsm-4&ada-2(ok3151)/mln1[mls14 dpy-10(e128)]II</i>	Mattout et al. 2020

Strains with deletion alleles and reporters obtained from the *Caenorhabditis elegans* knockout consortium or made by the CRISPR–Cas9 system were outcrossed two to six times to the N2 (WT) strain. Worms were grown on OP50 and maintained at 22.5°C.

^a(CGC) *Caenorhabditis* Genetics Center, (OCx4) outcrossed four times.

hand, was detectable throughout worm development—that is, in larvae and in adult worms (L4 larvae shown in Fig. 1C; see also Mattout et al. 2020). This was also true for other RNAi hits from the initial screen—namely, *sams-3* RNAi and *mes-4* RNAi (Fig. 1C). Elevated GFP expression from the heterochromatic array after RNAi treatment was scored at all stages of worm development and in nearly every somatic cell type, except germline cells (see below). Given that Piwi-interacting RNAs (piRNAs) are known to contribute to silencing uniquely in germline cells, we hypothesize that the effect of *lsm* gene down-regulation may be masked by redundant silencing pathways, like piRNAs, that act specifically in the gonad. Alternatively, the LSM-mediated silencing pathway may not be functional in germ cells.

To obtain a quantitative value for reporter expression, we monitored the increase in GFP fluorescence intensity using the COPAS BioSort, which measures fluorescence on a worm-by-worm basis for hundreds to thousands of worms, using flow cytometry. Using the worm sorter, we compared the effects of *lsm-6*, *lsm-2/gut-2*, and *lsm-5* RNAi with the knockdown of further shared subunits of the two LSM complexes: LSM-3, LSM-4, and LSM-7 (Fig. 1D). The RNAi clone we used against *lsm-3* had several predicted targets; therefore, the results were inconclusive (data not shown). However, in case of both *lsm-4* and *lsm-7* RNAi, a significant ($P < 10^{-13}$) increase in average GFP fluorescence of twofold to fourfold was scored (see Mattout et al. 2020). The variable degrees of GFP derepression following RNAi against these LSM subunits likely stems from differences in RNAi efficiency.

Given that the LSM complex has been implicated in RNA processing (Kufel et al. 2004; Beggs 2005; Tharun

2009; Perea-Rese et al. 2012; Golisz et al. 2013; Cornes et al. 2015), it was possible that somatic cell derepression was specific to the reporter construct used in the screen. We ruled this out by monitoring the effects of *lsm-2*, *lsm-5*, or *lsm-6* RNAi on the expression of four different heterochromatic reporters each integrated in the *C. elegans* genome as a large array (200–300 copies) at a single locus (Mattout et al. 2020). Each reporter carried a different promoter, gene, and 3' UTR, yet all showed reporter up-regulation following *lsm-2*, *lsm-5*, or *lsm-6* RNAi (Mattout et al. 2020). In contrast, two euchromatic reporters, which were single-copy transgenes integrated into a nonheterochromatic region of the genome, showed no change in expression under the same conditions (data shown for GW1108, *eft-3p::gfp cb-unc-119(+)* chr II in Fig. 1E). This was true for euchromatic reporters with both high and low levels of expression, indicating that LSM complex subunits are selectively involved in the repression of loci that have heterochromatic, but not euchromatic features. Derepression levels were comparable or greater than the loss of our positive control, MES-4, a histone H3K36 methyltransferase recovered in the original screen (Towbin et al. 2012), and persisted during somatic cell differentiation and in post-mitotic cells.

We confirmed that the up-regulation of GFP in these worms indeed reflects changes on the mRNA level, rather than GFP protein synthesis or turnover. This was achieved by quantifying the reporter mRNA by quantitative polymerase chain reaction using reverse transcription (RT-qPCR) following RNAi against various LSM subunits, notably *lsm-6* or *lsm-7* down-regulation (Mattout et al. 2020).

Figure 2. LSM-8 is required for heterochromatic reporter silencing, implicating the LSM2-8 complex. (A) The two main LSM complexes and functions (Beggs 2005; Tharun 2009). (B) Schematic view of the *lsm-8* deletion/gene replacement created by CRISPR-Cas9. (C) Differential interference contrast (DIC) images of L1/L2 larvae (upper panel) and young adults (lower panel) together with dual-channel fluorescence for *lsm-8*^{+/-} (yellow pharynx in merge) and *lsm-8*^{-/-} (red pharynx only) worms. Red and green channels are merged to distinguish heterozygous from homozygous worms. The inset in the upper panel shows the protruding vulva of an *lsm-8*^{-/-} adult (marked with white triangle) and the accumulating vesicles (black triangles). The normal vulva is boxed in the lower panel. Bars = 100 μm. (D) Red and green fluorescence channels are merged and coupled with DIC images of one *lsm-8*^{-/-} worm and one *lsm-8*^{+/-} heterozygote bearing the *pkIs1582* reporter (GW1120). Note that this strain has no nT1 balancer with the green fluorescent pharynx marker. GFP fluorescence in nuclei throughout the *lsm-8*^{-/-} worm is visible. Bar, 100 μm. (E) Merged DIC images with fluorescent images as in D. *lsm-8*^{-/-} mutant L4 larvae bearing *pkIs1582* reporter is shown with fully developed gonad (encircled in red). The *lsm-8*^{-/-} mutant is 100% sterile. Somatic nuclei and somatic cells of the gonad show GFP expression from the heterochromatic array. Bar, 50 μm. (This image also appeared in Mattout et al. 2020. Reproduced with permission.) (F) Quantification of the length, width, and gonad nuclei count from the DAPI staining of L3 WT and *lsm-8*^{-/-} larvae. *n* = 8.

LSM2-8 CONTRIBUTES TO HETEROCHROMATIC REPORTER SILENCING

Studies in yeast and plants have established that there are two LSM complexes (LSM1-7 and LSM2-8) (Fig. 2A) that share six subunits, whereas the seventh subunit in each complex, LSM-1 or LSM-8, participates only in one (Beggs 2005; Tharun 2009). The two complexes also in-

teract with distinct cofactors. RNAi against *lsm-1*, one of the nonshared subunits, did not derepress the heterochromatic reporter nor did the depletion of the 5' → 3' exoribonucleases, XRN-1, which is reported to act together with LSM1-7 in the cytoplasm to mediate cytoplasmic RNA decay (Figs. 1D and 2A). On the other hand, RNAi against *xrn-2*, a nuclear RNase that is thought to degrade RNA together with LSM2-8 in budding yeast (Tharun 2009),

did show significant heterochromatic reporter derepression (Fig. 1D). Null-allele worms lacking *lsm-1* or the decapping factor *dcap-2* also failed to show array derepression (Mattout et al. 2020). This argued against a role for LSM1-7 in heterochromatic reporter silencing and suggested instead that LSM2-8 and an evolutionarily conserved nuclear 5' → 3' exoribonuclease called XRN-2 (HsXRN2, SpDhp1, or ScRat1) are involved (Miki et al. 2016).

Because LSM-8, the only subunit unique for the nuclear LSM2-8 complex, was unresponsive to RNAi in our hands, it was essential to create a genomic deletion allele of the *lsm-8* gene. A full *lsm-8* deletion was generated by CRISPR–Cas9, and simultaneously we inserted a red fluorescent marker gene with pharynx-specific expression. We could track the null allele by fluorescence microscopy, but found that the homozygous *lsm-8*^{−/−} worms were 100% sterile. To propagate the *lsm-8* deletion, the mutation was balanced with a green fluorescence–marked balancer *nT1 [qIs51]*, which is also expressed in the pharynx. With dual fluorescence, we could distinguish offspring that are heterozygous for *lsm-8*^{+/−} (i.e., that have red and green fluorescence in the pharynx) from those that are homozygous (*lsm-8*^{−/−}; red pharynx only) (Fig. 2C). GFP fluorescence from the heterochromatic array was then monitored in red pharynx–marked homozygous, *lsm-8*^{−/−} animals with or without (Fig. 1D) the *nT1* balancer. Indeed, deficiency for LSM-8 strongly derepressed the heterochromatic array, yielding GFP fluorescences at levels similar to those observed after *lsm-2*, *lsm-5*, *lsm-6*, or *lsm-7* RNAi (Fig. 2D). Although mutant germline and gonad development up to L3 and L4 larval stages was similar to that in wild-type (N2) worms (Fig. 2E,F), there was no oocyte maturation in the *lsm-8*^{−/−} mutant. Nonetheless, we could monitor GFP derepression in all somatic tissues of larvae and adults, including the somatic cells of the gonad, but not in the germline itself (Fig. 2E).

Although the *lsm-8*^{−/−} animals can develop to adulthood, the adults have protruding vulva (see inset, Fig. 2C, white arrowhead), show empty cavities or vacuoles in differentiated tissues (black triangle), and die prematurely (Mattout et al. 2020). Other *lsm* mutants, such as *lsm-2* and *lsm-5*, are phenotypically similar to *lsm-8* mutants, with clear protruding vulva, the presence of vacuoles, and 100% sterility. Adult populations of the *lsm-8* homozygous phenotype started dying after 6 days, whereas a similar loss of viability in wild-type worms would not occur before 10–12 days. Worms mutant for *lsm-1* did not present these phenotypes (Cortes et al. 2015), confirming the distinct roles played by the two LSM complexes.

LSM2-8 IS REQUIRED TO MAINTAIN SILENT ENDOGENOUS HETEROCHROMATIN

Although LSM2-8 was clearly implicated in heterochromatic array silencing, it was important to establish whether or not it also controls the expression of endogenous transcripts. The data sets for total RNA-seq of WT and homozygous *lsm-8*^{−/−} sorted L3 and L1 larvae are presented in Mattout et al. (2020) and data are deposited with the NCBI Gene Expression Omnibus (GSE92851).

The *lsm-8* data sets were compared with RNA-seq data from similarly sorted *met-2*^{−/−} *set-25*^{−/−} L3 larvae, which lack detectable H3K9 methylation (Towbin et al. 2012), and from L3 worms of the triple mutant, *met-2*^{−/−} *set-25*^{−/−} *lsm-8*^{−/−}, to determine if the LSM2-8 silencing pathways would be epistatic with the classic H3K9 methylation–mediated repression. In each case, the mutant and wild-type worms were sorted by fluorescence (nongreen pharynx indicating homozygosity) and by size, to generate a uniform population of L3 stage larvae. Mutant and wild-type L1 populations were also examined following sorting for L1 size larvae (Mattout et al. 2020). For the L3 populations, shifts in developmental timing arising during the sorting process were compensated by comparing matched replicates by making use of a characteristic, temporal fluctuation in gene expression characterized by the Großhans laboratory (Hendriks et al. 2014). With developmentally matched mutants and control larvae, we analyzed the RNA-seq data using edgeR, to determine the genes that changed significantly in each of three genetic backgrounds and WT strains (Mattout et al. 2020). Two or three analytical criteria were used, with the most stringent being the combined criteria of a false discovery rate (FDR) < 0.05 and a fold change (Fc) in average RNA level > 4 (Mattout et al. 2020). In Figure 3A, we highlight genes with average altered expression rates of Fc > 4 only.

We found that the deletion of *lsm-8* led to the strong and reproducible up-regulation of 122 genes (FDR < 0.05 and Fc > 4), with very few genes being down-regulated (Mattout et al. 2020). With less stringent cutoff values, even higher numbers of loci were selectively derepressed in *lsm-8*^{−/−} (Fig. 3A): Only FDR < 0.05 identified 147 genes up-regulated; only Fc > 4 identified 204 genes; and only Fc > 2 identified 1332 genes. Interestingly, a significant number of transcripts sensitive to *lsm-8*^{−/−} were involved in the innate immune response and the regulation of cell shape, but they did not reflect a single developmental stage. Genes derepressed in the mutant in L1 larvae overlapped only partially (5%–10% based on the thresholds used) with those derepressed in the L3 stage, and among those overlapping, many are involved in the innate immune response (Mattout et al. 2020). We confirmed the *lsm-8*^{−/−} larvae results by performing RNA-seq experiments on the *lsm-4* mutant (Fig. 4A). The patterns of genes derepressed were overlapping but not identical, in *lsm-4* and *lsm-8* mutants (Fig. 4B). This is to be expected, given that *lsm-4* also eliminates the activity of the LSM1-7 complex as well as the LSM2-8. Indeed, the distribution of LSM4-GFP is diffuse in both cytoplasm and nucleus, and in the nucleus it appears to be excluded from foci that bind the H3K9me3 histone methyltransferase (HMT), SET-25 (labeled with mCherry, Fig. 4C).

In yeast and plants, RNA-binding complexes feed back to establish H3K9 methylation on centromeric and other heterochromatin (Buhler et al. 2007; Vasiljeva et al. 2008; Keller et al. 2012; Zofall et al. 2012; Yamanaka et al. 2013; Egan et al. 2014; Chalamcharla et al. 2015; Tucker et al. 2016). It was therefore of interest to compare RNA-seq changes provoked by the loss of LSM2-8 with loss of canonical heterochromatin marks, H3K9me2 or me3. We

Figure 3. LSM2-8 silences hundreds of genes and 95% of these carry the Polycomb mark H3K27me3. (A) Deletion of *lsm-8* (*lsm-8^{-/-}*) derepresses significantly more than 200 genes ($F_c > 4$). Relative gene expression profiles are shown as scatter plots, with fold change ($F_c > 4$) for two RNA-seq replicates of L3 sorted worms of the indicated genotype versus WT. Each dot corresponds to a gene. The original RNA-seq data are available at NCBI Gene Expression Omnibus number GSE92851. The same data set was analyzed in Mattout et al. (2020), but with ($FDR < 0.05$ and $F_c > 4$). (B) Scatter plot comparing the relative gene expression between the *lsm-8* (x-axis) and the *met-2 set-25* double mutant (y-axis). Common up-regulated genes are shaded yellow; 36% of genes up-regulated in the *lsm-8* mutant ($FDR < 0.05$ and $F_c > 4$) are also up-regulated ($FDR < 0.05$ and $F_c > 4$) in the *met-2 set-25* mutant. *lsm-8^{-/-}*-specific up-regulated genes are shaded in pink; *met-2^{-/-} set-25^{-/-}*-specific are in blue. (This panel is reproduced from Figure 3C in Mattout et al. 2020 with permission.) (C) LSM2-8-mediated silencing of the heterochromatic reporter is independent of H3K9 methylation. Quantitation of derepression of GFP expressed from the *gwIs4* heterochromatic reporter in L1 progeny in WT and *met-2 set-25* mutant genotypes, after control or *lsm-7* RNAi. Quantitation of GFP derepression expressed from the *gwIs4* heterochromatic reporter in L1 progeny in WT and *met-2 set-25* mutant genotypes, respectively, from strains GW76 and GW637, after control or *lsm-7* RNAi. P -values indicated; two-tailed unpaired t -test. Quantitation and statistical analyses were based on $n = 1460, 2399, 1593$, and 1189 worms for conditions indicated from left to right, pooled from three independent experiments. $N = 339, 1004, 426, 673$ same after *lsm-7* RNAi in the *hpl-2^{-/-}* mutant background. (D,E) Scatter plots compare the average gene expression changes in *lsm-8^{-/-}* worms (x-axis in $\log_2$, RNA-seq L3 stage) versus enrichment for the indicated euchromatic or heterochromatic histone modification (y-axis in $\log_2$, ModEncode data of WT L3 stage). Up-regulated genes ($FDR > 0.05$ and $F_c > 4$) in the *lsm-8^{-/-}* mutant are in red to the right of the black line, and genes enriched for the histone mark are above the red line (enriched over input). % indicates genes in upper right zone. (The panels in E are reproduced from Fig. 4B of Mattout et al. 2020, with permission.) (F) Scatter plot of absolute gene expression ($\log_2$) of *lsm-8^{-/-}* versus WT. Red dots as in E and each dot corresponds to a gene. Values < 6 ($\log_2$) are considered as very low expression: 6 ($\log_2$) corresponds to about 60 RNA-seq reads per gene, whereas 10 ($\log_2$) corresponds to about 1000 reads per gene. Most *lsm-8^{-/-}* derepressed genes have low steady-state level expression in WT worms but moderate expression levels in the mutant (pink shading). (This panel is reproduced from Fig. 4C of Mattout et al. 2020, with permission.)

Figure 4. *lsm8* and *lsm4* deletions derepress overlapping gene sets in L1 and L3 larvae. (A) Deletion of *lsm4* (*lsm4*^{-/-}) leads to L1 larval arrest and derepresses more than 100 genes significantly. Relative gene expression profiles are shown as scatter plots of fold change (Fc) in log₂ of two RNA-seq replicates of L1 stage worms, *lsm4*^{-/-} versus WT. Each dot corresponds to a gene. The class of genes that were most significantly affected control body morphogenesis ($P=0.0072$). (B) Scatter plot comparing the relative gene expression between the *lsm8* (x-axis) and the *lsm4* mutant (y-axis). Common up-regulated genes are shaded yellow; 53% of genes up-regulated in *lsm8* (FDR < 0.05 and Fc > 4) are also up-regulated to some extent (50% increase) in *lsm4*. Genes up-regulated in *lsm8*^{-/-} only are shaded pink, and those in *lsm4*^{-/-} only in blue. (C) Z-projection of live confocal images of embryonic progeny issued from the cross between the strain (GW1004) expressing LSM-4-Flag-GFP (GFP, green channel) and the strain (GW694) SET-25, the H3K9me3 HMT, fused to mCherry. The merged image shows no significant overlap on the brightest foci of SET-25. The boxed area is enlarged to show nuclear foci in bottom panels. Bars, 10 μ m.

found that 36% of the genes derepressed because of the loss of H3K9 methylation (*met-2*^{-/-} *set-25*^{-/-}) overlapped with those depressed in the *lsm8*^{-/-} mutant at the L3 stage (Fig. 3B; Mattout et al. 2020). To test the additivity of the LSM2-8 and H3K9me-mediated repression pathways, we compared the RNA-seq data of the single mutants with the triple mutant (*lsm8*^{-/-}; *met-2*^{-/-} *set-25*^{-/-}) (Fig. 3A) and found that there were clear subsets of up-regulated genes that were unique to either the *lsm8* or the *met-2 set-25* mutant, and that genes showing mild up-regulation upon loss of either *lsm8* or *met-2 set-25* (Fc ~ 2) were more strongly expressed when combined (FDR < 0.05 and Fc > 4) (Mattout et al. 2020). Thus,

the LSM2-8 pathway of silencing for endogenous loci is distinct from that mediated by H3K9me2/3, even though some genes are targeted by both pathways. Consistently using the heterochromatic reporter pkIS1582, we could show that *lsm7* RNAi derepresses in an additive fashion with the loss of either H3K9me3 (*met-2 set-25*) or the loss of the H3K9me3 reader, HPL-2 (Fig. 3C).

LSM2-8 TARGETS POLYCOMB-MARKED GENES

To ask what the *lsm8*-sensitive genes have in common, we compared our RNA-seq data to the normalized ChIP-

seq data of common histone modifications generated by ModEncode (Fig. 3D–E). In worms, as in most organisms, H3K4me2, H3K4me3, and H3K27ac are associated with active genes (Liu et al. 2011; Wenzel et al. 2011; Ho et al. 2014), whereas H3K9me2/3 and H3K27me3 are repressive histone marks that colocalize with heterochromatin (Wenzel et al. 2011). The genes that were up-regulated in the *lsm-8*^{-/-} mutant were depleted for active marks (H3K4me3) (Fig. 4E) in WT L3 larvae, and showed no particular enrichment for H3K9me2 nor for H3K9me3 (Mattout et al. 2020). Strikingly, however, 95% of genes that were derepressed by loss of LSM-8 were enriched for the repressive Polycomb mark, H3K27me3 (Fig. 3E). This was true not only for strongly up-regulated genes, but also for genes that were mildly up-regulated upon loss of LSM-8 (less than fourfold) and for windows of 500 bp across the genome. Thus, the overriding feature of genes or domains silenced by LSM2-8 was their enrichment for H3K27me3.

Consistent with the fact that H3K27me3 is the hallmark of Polycomb-repressed genes (Liu et al. 2011; Margueron and Reinberg 2011; Grossniklaus and Paro 2014; Conway et al. 2015), the absolute steady state level of the *lsm-8*-sensitive mRNAs was very low or almost undetectable in WT larvae (Fig. 3F). Although H3K27me3 is deposited in *C. elegans* by a PRC2-like complex comprising MES-2/E(z)/Ezh2, MES-3, and MES-6/Esc (Ketel et al. 2005; Yuzyuk et al. 2009; Gaydos et al. 2014) and mediates cell type-specific repression of developmentally regulated genes, in worms H3K27me3 and H3K9me3 ChIP-seq signals colocalize at many sites across the *C. elegans* genome (Ho et al. 2014). Interestingly, based on the ModEncode data sets from WT L3 larvae, 83% of the genes that are reproducibly enriched for H3K9me3 are also enriched for H3K27me3, and 41% of the genes enriched for H3K27me3 also carry H3K9me3. Among the genes up-regulated by *lsm-8* mutation, we find that 40%–43% also carry H3K9me3 (Mattout et al. 2020). Because this rate is the same as the genome-wide coincidence of paired K9/K27 methylation, it appears that nucleosomes or domains bearing both repressive marks are not significantly enriched among *lsm-8* targets. Most likely, H3K9me2 or me3 marks are irrelevant, for LSM2-8 recruitment or silencing.

LSM2-8 SILENCES THROUGH RNA DEGRADATION

Given that the LSM2-8 complex is known to stabilize and bind U6 snRNA in yeast and plants (Beggs 2005; Perea-Resea et al. 2012; Zhou et al. 2014), and that the LSM2-8 complex coprecipitates both with a factor involved in U6 snRNA stability (Ruegger et al. 2015) and with U6 snRNA itself in *C. elegans* (Mattout et al. 2020), we analyzed the RNA-seq data for splicing defects. We compared the exon–exon junction reads in the *lsm-8* RNA-seq data with those from WT worms, to see if LSM2-8-mediated silencing coincides with defects in splicing, but we found irregular splicing events at only 18 exon–exon junctions out of 134,836 splice junctions

(<0.02%) (Mattout et al. 2020). Thus, impaired splicing is most likely not the cause of *lsm-8*^{-/-}-triggered derepression.

If *lsm-8*^{-/-} worms reduced the expression of PRC2 or PRC1 homologs, then it would follow that histone H3K27me3 levels would be reduced, and Polycomb-target genes would be derepressed. By checking the relevant mRNA levels, we could rule this out as a mechanism (Mattout et al. 2020). Nonetheless, to explore the relationship of LSM2-8-mediated silencing to H3K27me3, we treated the heterochromatic reporter-bearing *lsm-8*^{-/-} worms with RNAi against either *mes-2* (EZH2) or *xrn-2*. As controls, we combined the *lsm-8* mutant with RNAi against *lsm-7* for epistasis and with *mes-4* (H3K36me HMT) or *set-25* (H3K9me HMT), which both show additivity with *lsm-8* (Fig. 5A). Although *xrn-2* RNAi is epistatic with the *lsm-8* background, the depletion of *mes-2* increased reporter expression, albeit less than that observed for *set-25* or *mes-4* RNAi (Fig. 5A). This argues that Polycomb-mediated silencing is not exclusively dependent on LSM-8. Again, this can be explained by the fact that H3K27me3 is known to repress on the level of transcription. The strong epistasis observed with XRN-2, on the other hand, argued that LSM2-8 may silence through RNA turnover or degradation (Fig. 5A). Comparison of RNA-seq data from worms treated with *xrn-2* RNAi at L4 stage (Miki et al. 2016) and *lsm-8* mutant worms at L3 confirms significant overlap of up-regulated endogenous genes (Fig. 5B). Indeed, 71% of the genes up-regulated by *lsm-8*^{-/-} were also up-regulated by *xrn-2* RNAi (Fig. 5B, yellow) and 95% of those genes are enriched for H3K27me3. Again, this argues that LSM-8 and XRN-2 function on the same pathway. Nonetheless, a subset (<1/3) of LSM2-8 target genes (pink) were not affected by the down-regulation of XRN-2. It is unclear if those reflect experimental differences or a subset of genes silenced by LSM2-8 through another mechanism.

LSM2-8 ACTS TOGETHER WITH RNA DEGRADATION ENZYMES

To find other genes implicated in this potential nuclear RNA decay function, we tested candidate cofactors for heterochromatic reporter derepression and examined their genetic relationship with *lsm-8*^{-/-}. Heterochromatic array derepression at the L1 stage was monitored by GFP expression, following a targeted RNAi screen against genes that are co-regulated with LSM2-8 subunits and/or functionally implicated in RNA processing—namely, RNA Pol II subunits (*rpb-12*, *rpb-7*) and the type II poly(A) binding protein (*pabp-2*). Like *xrn-2* RNAi, RNAi against *rpb-12*, *rpb-7*, or *pabp-2* on their own derepress the heterochromatic reporter (Fig. 5A), whereas the depletion of six additional genes that are implicated in aspects of RNA metabolism does not (*F49C12.11*, *T13F2.2*, *cgh-1*—the human ortholog of DDX6, *B0495.8*, *C50D2.8*, and *pab-2*—the human ortholog of PABPC1) (Mattout et al. 2020). Important, nonetheless, is the epistasis between derepression by *xrn-2* RNAi, *rpb-12*, *rpb-7*, and *pabp-2*

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Figure 5. XRN-2 and other RNA factors show epistasis with *lsm-8* deficiency for heterochromatic array derepression. (A) RNAi experiments were performed in the *lsm-8^{-/-}* (GW1119) strain. The GFP derepression of the heterochromatic reporter *pkl51582* was quantified by semiautomated fluorescence microscopy on homozygous *lsm-8^{-/-}* progeny under indicated RNAi conditions. *P*-values (Students *t*-test) are indicated: (*) *P* < 0.005. This indicates additivity between the *lsm-8* derepression and derepression by the RNAi used. Bars = S.E.M., *N* = 2, *n* = 55, 45, 22, 11, 10, 23, 25, 25, 85. (These data are reproduced from Fig. 6F of Mattout et al. 2020, with permission.) (B) Scatter plot comparing relative gene expression changes of *lsm-8^{-/-}* L3 larvae and *xrn-2* RNAi treated L4 (Miki et al. 2016). Common up-regulated genes are shaded yellow; 71% of genes up-regulated in the *lsm-8* mutant ($F_c > 4$) are also up-regulated to some extent (50% increase) in *xrn-2* depleted worms. *lsm-8^{-/-}*-specific up-regulated genes are shaded pink. (This panel is reproduced from Fig. 6A of Mattout et al. 2020, with permission.) (C) WT and *lsm-8^{-/-}* worms were sorted, re-fed with OP50 in liquid culture for 1 h at room temperature, and treated with 50 μ g/mL final concentration of α -amanitin, which inhibits Pol II and Pol III transcription. RNA was isolated at time 0, 4.5, and 6 h, as indicated for each independent experiment. RNA levels of three transcripts affected by LSM-8 and two control transcripts (expression not affected by LSM-8) were determined by RT-qPCR and normalized to 18S rRNA levels, which are insensitive to α -amanitin. The value at 0 h is defined as 100%. Error bars represent standard deviation of the mean from at least three independent experiments. (Data are reproduced from Extended Data Fig. 8A in Mattout et al. 2020, with permission.)

and the *lsm-8* mutant. In conclusion, PABP-2, RPB-12, and RPB-7 may act with XRN-2 on the LSM2-8-mediated silencing pathway.

LSM2-8 CONTRIBUTES TO THE DEGRADATION OF mRNAs FROM POLYCOMB-MARKED GENES

The cooperation between the RNA-binding LSM2-8 complex and XRN-2 suggests that LSM2-8 may silence genes by triggering mRNA degradation. It would follow that *lsm-8* deletion might increase the RNA stability of specific LSM2-8-sensitive transcripts in vivo because of a failure to degrade these RNAs by XRN-2. This was confirmed for the mRNAs of three endogenous *lsm-8*-sensitive genes—*far-3*, *grl-23*, and *ZK970.2*—by monitoring mRNA stability after the addition of α -amanitin, an inhibitor of RNA Pol II and Pol III elongation. The rate of mRNA decay was monitored at the L3 stage of sorted WT and *lsm-8*^{-/-} worms over 6 h using RT-qPCR. RNA levels were normalized to that of the 18S rRNA. Deletion of *lsm-8* delayed the turnover of *far-3*, *grl-23*, and *ZK970.2* mRNAs, whereas the mRNA and pre-mRNA of two control genes (*eft-3* and *F08G2.8*) showed no change in turnover rates (Fig. 5C; Mattout et al. 2020). Consistently, transcript levels from these *lsm-8*-sensitive genes, *far-3* and *ZK970.2*, were also strongly up-regulated in *xrn-2* RNAi treated worms as well, with a fold change in log₂ of 4.9 and 4.0, respectively (Mattout et al. 2020). The decay of the three *lsm-8*-sensitive mRNAs was also attenuated in the Polycomb-deficient strain (*mes-2*) (Mattout et al. 2020). Thus, our data suggest that MES-2-mediated repression of LSM-8 regulated genes acts at least in part through mRNA degradation (Mattout et al. 2020).

LSM2-8 ACTION FEEDS BACK TO MAINTAIN H3K27me3 LEVELS

Although *mes-2* and *lsm-8* depletion appeared to be additive with respect to derepression, one cannot conclude that the LSM2-8 complex acts independently of MES-2 and H3K27me3, especially considering the striking correlation of LSM-8-sensitivity with H3K27me3 (Fig. 3E). We initially tested for global changes in repressive epigenetic marks, H3K9me3 and H3K27me3, by immunostaining of embryos following *lsm-7* RNAi. However, we found no global drop in staining (Fig. 6A). Nonetheless, when we performed quantitative chromatin immunoprecipitation (ChIP) for H3K27me3 on genes that are sensitive to *lsm-8* ablation (i.e., genes silenced by the LSM2-8 complex in WT worms), we observed a significant decrease (>50%) in H3K27me3 levels (Fig. 7B). This was not detected for *lsm-8*-insensitive H3K27me3-marked genes. This suggests that the LSM2-8 complex selectively feeds back to maintain H3K27me3 levels, either directly or indirectly, at a subset of H3K27me3-silenced loci. The fact that it is not detectable by immunostaining argues that H3K27me3 lev-

Figure 6. LSM2-8 helps maintain full H3K27me3 levels on silenced loci. (A) Z-projection of confocal images of wild-type (GW566) embryos treated with control or *lsm-7* RNAi (GFP, live imaging) or stained with antibodies against H3K9me3 and H3K27me3, as indicated. Nuclei are enlarged in the insets. Bar, 5 μ m. (B) H3K27me3 ChIP-qPCR on target genes in WT and *lsm-8*^{-/-} worms. Three categories of genes were assessed. Five genes were monitored that are up-regulated in *lsm-8*^{-/-} worms and enriched for H3K27me3 mark in WT (*lsm-8* target genes), three genes were monitored that are enriched for the H3K27me3 mark in WT but do not change in expression in the *lsm-8* mutant (nontarget genes), and the control category is represented by *ska-1*, a gene not enriched for H3K27me3 in WT and with no expression change (control). $N = 3$, $n = 3$, bars (mean $\pm$ S.E.M.). (Data appear in Fig. 7A of Mattout et al. 2020 and are reproduced with permission.)

els may not be affected globally, although many Polycomb targets do show significant derepression.

CONCLUSION

We have shown that in nematodes a conserved nuclear RNA-binding complex, LSM2-8, contributes to full repression of heterochromatic reporters and genomic regions that carry the H3K27me3 epigenetic mark. A related, cytoplasmic complex, LSM1-7, appears to have no specific role in heterochromatic silencing. Unlike heterochromatin-linked RNA degradation pathways in plants and fission yeast, LSM-2-8-mediated repression is inde-

Figure 7. Model for the LSM2-8 pathway of silencing. We showed that the LSM2-8-mediated silencing pathway makes use of XRN-2 ribonuclease and involves other factors, such as PABP-2 (*H.s.*: PABPN1). We hypothesize that RNA arising from H3K27me3 genomic regions that are controlled by the LSM2-8 complex acquire a specific feature during transcription (e.g., a specific structure, RNA modification, 3' UTR, poly(A/U) tail, or specific RNA-binding protein(s)), which allows recognition and processing by LSM2-8. LSM2-8-mediated silencing also feeds back to regulate H3K27me3 levels on LSM2-8-regulated genes, although it is unclear if the interaction with PRC2 complex is direct (dotted arrow). LSM2-8-mediated silencing of H3K27me3-bound regions defines a new mechanism for selective post-/cotranscriptional silencing for facultative heterochromatin through RNA decay.

pendent of and additive with H3K9me3 and H3K9me HMTs. Our data suggest that the LSM2-8 complex acts primarily on RNA stability, selectively mediating the decay of transcripts from H3K27me3-tagged genes through the 5'–3' exonuclease XRN-2. The pathway does not, however, involve XRN-1 nor the decapping enzyme DCAP-2. The LSM2-8-mediated silencing occurs at Polycomb H3K27me3-marked genes, with only ~40% of the LSM2-8 targets also carrying H3K9me3. This represents the stochastic frequency of H3K27me3 and H3K9me3 coincidence on genes in L3 stage larvae, suggesting that H3K27me3 and not H3K9me3 is the recognition signal for mRNA degradation. As sketched in our model in Figure 7, we propose that the LSM2-8 complex mediates gene silencing primarily through the exonuclease XRN-2, which would degrade the transcripts from a subset of H3K27me3-marked genes either co- or posttranscriptionally. This represents a novel pathway for Polycomb-mediated gene silencing in animals, which appears to act in addition to transcriptional repression.

The fact that only a subset of transcripts arising from H3K27me3-marked genes is found to be significantly derepressed in *lsm-8* mutant worms suggests that there may be additional criteria that provide specificity for LSM2-8 control. Using a less stringent cutoff for derepression in the *lsm-8* RNA-seq data set (Mattout et al. 2020), we find many more genes up-regulated, and >93% still bear H3K27me3. It is important to note that the derepression of tissue-specific transcripts (i.e., those that are expressed in only a few cells of larvae or adult worms) will not be detectable by our RNA-seq methods that monitor RNA in whole worm extracts. This was confirmed to be the case for one HOX gene called *egl-5*, which is known to be

regulated by MES-2 (EZH2) (Mattout et al. 2020). By analyzing the cell type-specific derepression of *egl-5* using fluorescence microscopy, we were able to show similar levels of derepression of this gene in male tail cells, in both *lsm-8* and *mes-2* mutants (Mattout et al. 2020), although its derepression level by RNA-seq was only ~1.5-fold.

The molecular basis of LSM2-8 specificity for H3K27me3 provides a framework for future studies exploring how the epigenetic information can be transferred from the chromatin to an RNA-binding complex. We propose that the RNAs arising from H3K27me3-marked genomic regions are recognized and bound selectively by the LSM2-8 complex, and that this likely requires a feature that is acquired during transcription from that locus—for example, a specific structure, RNA modification, cap, a poly-A/U tail, or specific RNA-binding protein(s) recognized by LSM2-8. It is important to note that we scored a drop in H3K27me3 specifically on LSM2-8-sensitive genes in *lsm-8*^{−/−} animals, even though the overall staining level of H3K27me3 appeared unchanged (Fig. 6). This suggests that RNA degradation helps maintain H3K27me3 levels. In other words, transcriptional repression by this epigenetic mark may be transferred from the RNA-binding complex LSM2-8 to chromatin.

An RNA decay-mediated pathway involving the cofactors PABP-2, RPB-12, and RBP-7 appears to function independently of DACP-2 and LSM-1 and additively with HPL-2 and the H3K9me3 HMT, SET-25. The epistasis with type II poly(A) binding protein (PABP-2) (Hirschler et al. 2011) (*H.s.*: PABPN1 and *Sp*Pab2) is particularly interesting, because it appears to regulate 3' UTR and poly(A) tail length in the nucleus (Kuhn et al. 2009). Moreover, it binds nascent RNAs early during the elongation step

(Lemieux and Bachand 2009; Beaulieu et al. 2012). Given that the LSM2-8 complex is known to bind to the 3' oligo(U) tail of the U6 snRNA (Zhou et al. 2014), as well as the 3' poly(A⁺) tail of nuclear RNAs (Kufel et al. 2004), PABP-2 may regulate its specificity by modulating the 3' end of mRNAs at H3K27me₃-marked domains. The participation of RNA Pol II subunits RPB-7 and -12 in LSM2-8-mediated gene silencing is also suggestive of an involvement of RNA Pol II in silencing, just as the *Schizosaccharomyces pombe* RBP-7 homolog has been implicated in centromeric repeat RNAi-directed silencing (Djupedal et al. 2005). Interestingly, fission yeast lacks H3K27me₃; thus, the link to chromatin status may have evolved at a later stage. In *S. cerevisiae*, the same subunit plays a different, but direct role in Pat1/Lsm1-7-mediated mRNA decay in the cytoplasm (Lotan et al. 2007; Haimovich et al. 2013). One speculative scenario, based on functions described in other organisms, is that *rpb-7* and *rpb-12* RNA Pol II subunits might be loaded on LSM2-8-regulated transcripts to signal to that these should be degraded by XRN-2. An unknown decapping enzyme(s) may also be involved, which would allow RNA degradation by XRN-2 in the nucleus.

We conclude that facultative heterochromatin in metazoans can be silenced through a mechanism of selective transcript degradation and not only by transcriptional repression. LSM2-8 may mediate gene silencing by linking a specific epigenetic state to transcript degradation, adding an additional layer of control over differentiation and development.

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