A Forward Genetic Screen for Suppressors of Somatic P Granules in Caenorhabditis elegans

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ABSTRACT In Caenorhabditis elegans, germline expression programs are actively repressed in somatic tissue by components of the synMuv (synthetic multi-yulva) B chromatin remodeling complex, which include homologs of tumor suppressors Retinoblastoma (Rb/LIN-35) and Malignant Brain Tumor (MBT/LIN-61). However, the full scope of pathways that suppress germline expression in the soma is unknown. To address this, we performed a mutagenesis and screened for somatic expression of GFP-tagged PGL-1, a core P-granule nucleating protein. Eight alleles were isolated from 4000 haploid genomes. Five of these alleles exhibit a synMuv phenotype, whereas the remaining three were identified as hypomorphic alleles of known synMuv B genes, lin-13 and dpl-1. These findings suggest that most suppressors of germline programs in the soma of C. elegans are either required for viability or function through synMuv B chromatin regulation.

Cancer cells acquire a number of traits normally restricted to germline stem cells, including cellular immortality and the ability to self-renew. A subset of proteins exclusively found in the germ cells of the testis and/or ovary is overexpressed in most melanomas and is frequently found in breast, bladder, lung, and hepatocellular cancers (Sahin et al. 2005; Whitehurst 2013). Germline-enriched proteins can be highly antigenic when expressed outside of the germline and are often associated with malignancy and poor patient prognosis (Simpson et al. 2005; Blanchard et al. 2013).

Research in Caenorhabditis elegans is providing much needed insight into how germ line programs are repressed in the soma. One remarkable discovery is that components of the synMuv B chromatin remodeling complex, which include homologs of the tumor suppressors Retinoblastoma (Rb or LIN-35) and Malignant Brain Tumor (MBT or LIN-61), actively repress the somatic expression of germ-line-specific ribonucleoprotein aggregates called germ granules (Unhavaithaya et al. 2002; Wang et al. 2005; Cui et al. 2006; Petrella et al. 2011; Wu et al. 2012). Germ granules are found in the germ-cell cytoplasm of many species, where they are central to the pluripotent and immortal potential of the germ line (Strome and Updike 2015). In C. elegans, germ granules are called P granules, and when they are depleted both sperm-specific transcription and somatic differentiation are initiated in germ cells (Updike et al. 2014; Campbell and Updike 2015). Observations in various species suggest that the presence of germ granules outside of the germline could favor conditions that promote pluripotency and cell proliferation.

The somatic repression of germ-granule components by the synMuv B chromatin regulation complex is not exclusive to C. elegans (Georlette et al. 2007). In Drosophila, brain tumors in MBT mutants overexpress conserved germ-granule components like PIWI, VASA, and AUBERGINE, which are necessary for brain tumor formation in mbt mutant flies (Janic et al. 2010). Because of its promise in elucidating cancer signaling cofactors of Rb and MBT, the contribution of the synMuv B pathway to the repression of somatic P granules has been thoroughly investigated. However, it is still unclear how germ granules might promote conditions that favor oncogenesis, or whether other tumor-suppressor pathways, apart from synMuv B chromatin regulators, actively repress somatic germ-granule expression.

Through a genome-wide RNAi screen in C. elegans, we previously found several genes required to suppress somatic P-granule expression during embryogenesis and the first larval stage of development (Updike and Strome 2009). To determine if additional pathways in the soma suppress expression of germline programs, we took an unbiased approach using forward genetics to screen adult worms for ectopic P granules. Here we report that most, if not all, suppressors of germline programs in the soma are either required for viability or...
function through synMuv B chromatin regulation, and not some other pathway. We also describe new alleles of the synMuv B genes lin-13 and dpl-1 that express somatic P granules but do not readily exhibit a synMuv phenotype.

**MATERIALS AND METHODS**

**Strain maintenance**

*Caenorhabditis elegans* strains were maintained as per standard protocols (Brenner 1974). TH206 [pGL-1::EGFP + Cbr-unc-119(+)] I, MT10430 lin-35(n745)II, and the CB4856 Hawaiian isolate were obtained from the Caenorhabditis Genetics Center (CGC). DUP10 [PGL-1::GFP]; lin-13(sam4)III, DUP20 [PGL-1::GFP]; lin-13 (sam12)III, DUP21 [PGL-1::GFP]; dpl-1(sam13)III, DUP6 [PGL-1::GFP]; sam1, DUP14 [PGL-1::GFP]; sam8, DUP15 [PGL-1::GFP]; sam9, DUP16 [PGL-1::GFP]; sam10, DUP25 [PGL-1::GFP]; (sam17+), DUP52 samEx4(WRM0614dE05 + pCFJ104); [PGL-1::GFP]; dpl-1 (sam13)III, and DUP53 samEx5(WRM064aA06 + pCFJ104); [PGL-1::GFP]; lin-13(sam4)III were generated in this study.

**Fosmid rescue**

DUP10 was injected with the fosmid WRM064aA06 (20 ng/ul) to create DUP53, and DUP21 was injected with the fosmid WRM0614dE05 (20 ng/ul) to create DUP52. All injections used the myo-3::mCherry coinjection marker pCFJ104 (10 ng/ul) (Frokjaer-Jensen et al. 2008).

**Screen design**

EMS mutagenesis was performed on TH206 worms using the standard protocol (Kutscher and Shaham 2014). Two thousand F1 progeny were cloned to individual plates, and F2 grandchildren were examined for somatic PGL-1::GFP expression. This was in contrast to DUP10 and DUP20 backcrossed to TH206, where PGL-1::GFP was constrained to the germline in all cross-progeny.

**DNA sequencing**

CB4856 (Hawaiian) males were crossed into DUP10, DUP20, and DUP21 strains. F1 cross progeny were picked to new plates, and ap-rective PGL-1::GFP during the larval and adult stages. Fluorescence images were captured and tiled on a Leica DMI6000B inverted scope using a 40× air objective.

**Mapping**

EMDi (Hawaiian) males were crossed into DUP10, DUP20, and DUP21 strains. F1 cross progeny were picked to new plates, and approximately 50 F2s with the somatic P-granule phenotype were selected from each cross. The progeny of these F2 animals were pooled and then whole-genome—sequenced as previously described (Doitsidou et al. 2010). The three mutant strains (sam4, sam12, sam13) were multiplexed with nine additional mutants (unpublished), and all 12 samples were sequenced in a single lane on an Illumina HiSeq2500. The CloudMap pipeline was used to analyze mutant genome sequences, obtain map data, and find mutations as previously described (Minevich et al. 2012). The NCBI Sequence Read Archive is attached to BioProject #282736.

**Complementation**

DUP10 males were crossed into DUP20 hermaphrodites, and male cross progeny were examined for somatic PGL-1::GFP expression. This is in contrast to DUP10 and DUP20 backcrossed to TH206, where PGL-1::GFP was constrained to the germline in all cross-progeny.

**RNAi feeding**

RNAi feeding constructs were obtained from the Ahringer library (Kamath et al. 2003). The L4440 plasmid in HT115 bacteria was used as the RNAi control; RNAi experiments were performed at 20° unless otherwise stated. lin-15a RNAi for each strain was performed on L4 worms in three biological replicates and their progeny for Muv phenotypes were observed. To assay RNAi enhancement, three plates containing approximately 60 embryos each were placed on *his-44* RNAi feeding plates for each strain, and animals arrested during larval development were scored 2 d later. A t-test was used to calculate the significance of the enhancement compared to wild-type. To assay somatic P-granule suppression, L4s from DUP10, DUP20, DUP21, and TH206 were fed *mes-3*, *mes-4*, *mrg-1*, *lin-35*, *lin-61*, and control RNAi, and progeny were examined in the L3 and L4 larval stages. RNAi targets were blinded and three replicates (of 32 worms each) were quantified for somatic PGL-1::GFP expression in each strain. A t-test was used to calculate the significance of enhancement or suppression from control RNAi.

**RESULTS AND DISCUSSION**

Forward genetic screens provide an unbiased approach to identifying the most significant players in a given biological pathway. To further elucidate the pathways that repress germline programs in the soma, EMS mutagenesis was performed on a *Caenorhabditis elegans* strain expressing the constitutive P-granule component, PGL-1, tagged with GFP. The F2 generation was then screened for somatic expression of PGL-1::GFP granules (Figure 1), and eight independent alleles were isolated (Table 1). Three of these exhibited intestinal PGL-1 granule expression, whereas five expressed PGL-1 granules throughout the soma.

Most components of the synMuv B heterochromatin complex antagonize P-granule accumulation in somatic cells (Petrella et al. 2011). In *C. elegans*, components of this pathway are also known as synMuv B genes because they exhibit a synthetic (syn) multi-vulva (Muv) phenotype when combined with a mutation in a separate syn-Muv A class of genes. The class A synMuvS do not exhibit somatic P granules. Because screens for synMuv B mutants looking for the Muv phenotype have been completed to near saturation, we sought to distinguish mutations in the synMuv B pathway from those in a novel pathway. To do this, we used an RNAi feeding vector to knockdown expression of the synMuv A gene *lin-15a* in all eight of the new alleles. synMuv B mutants fed *lin-15a* RNAi exhibit a fully penetrant multi-vulva phenotype (Bosher et al. 1999), which can be observed in the *lin-35/Rb* mutant (positive synMuv B control) but not in wild-type worms (Figure 2A, arrowheads mark vulvae). We repeated *lin-15a* RNAi in triplicate and found that *sam1*, *sam8*, *sam9*, *sam10*, and *sam17* fall into the synMuv B class of mutants (3/3 replicates), validating the specificity of our screen (Table 1). However, *sam4*, *sam12,*
and sam13 alleles did not exhibit multiple vulvae after lin-15a RNAi (0/3 replicates), making them likely to contain mutations in genes that act in parallel or downstream of the synMuv B pathway.

Somatic PGL-1::GFP expression in sam4 and sam13 mutants is restricted to intestinal cells, whereas sam12 mutants express PGL-1 granules throughout the worm (Figure 2B). When these strains are backcrossed into the wild-type PGL-1::GFP parental strain, F1 progeny no longer express somatic PGL-1 granules. This suggests that each of these mutants is recessive for the somatic PGL-1::GFP-granule phenotype and are likely loss-of-function alleles.

To identify genetic lesions in the sam4, sam12, and sam13 alleles, Hawaiian Variant Mapping was used in combination with genome-wide sequencing, and mutations were identified using the CloudMap pipeline (Minevich et al. 2012). Linkage to somatic PGL-1::GFP was observed on chromosome II for sam13, and on chromosome III for sam4 and sam12 (Figure 3). We also observed some linkage to chromosome I at approximately 5 Mb for all three alleles, which most likely reflects the integration site of the PGL-1::GFP transgene that maps to chromosome I.

On chromosome III, we found four nonsynonymous mutations closely linked to sam4, one of which contained a G to A mutation that causes a G1583E substitution in LIN-13 (Figure 4A). RNAi depletion of the three other genes carrying nonsynonymous mutations did not cause somatic P-granule expression (data not shown). We generated a line with a fosmid containing lin-13 (marked with a myo-3::mCherry transgene) (Figure 4B) and observed rescue in 14 of 20 L4-staged worms carrying the transgene; 28/28 siblings without the transgene re-expressed intestinal PGL-1::GFP, suggesting that the amino acid substitution in LIN-13 is responsible for the phenotype. LIN-13 is a known lin-35/Rb pathway component that binds and recruits the heterochromatin protein HPL-2 to distinct nuclear foci (Meléndez and Greenwald 2000; Coustham et al. 2006) and also acts with LIN-35 and HPL-2 to dampen the ER stress response (Kozlowski et al. 2014). In addition to being a known synMuv B gene, lin-13 mutants were previously shown to exhibit somatic P-granule expression (Wang et al. 2005), suggesting that sam4 could be a hypomorphic allele of lin-13 that is not strong enough to cause a synMuv phenotype when combined with lin-15a RNAi. Despite sam4 and sam12 exhibiting different degrees of somatic P-granule expression, these two alleles failed to complement, suggesting that they contain mutations in the same gene (Table 1). sam4/sam12 cross progeny displayed an intermediate phenotype (100/100 cross progeny with somatic PGL-1::GFP expression).

| Mutant Allele | Somatic P Granules   | synMuv with lin-15a RNAi | Complementation |
|---------------|----------------------|--------------------------|----------------|
| sam1          | Whole worm           | Yes                      |                |
| sam4          | Intestinal           | No                       | sam12          |
| sam8          | Whole worm           | Yes                      |                |
| sam9          | Intestinal           | Yes                      |                |
| sam10         | Whole worm           | Yes                      |                |
| sam12         | Whole worm           | No                       | sam4           |
| sam13         | Intestinal           | No                       |                |
| sam17         | Whole worm           | Yes                      |                |

Table 1 Somatic PGL-1::GFP alleles from the mutagenesis

Figure 2 Characterization of mutant alleles. (A) Images of wild-type and lin-35 mutant worms fed lin-15a RNAi. Arrowheads point to vulvae. (B) Fluorescent images of PGL-1::GFP expressed in the germ-line (wild-type), intestine (sam4 and sam13), and throughout the soma (sam12).
expression), suggesting that sam12 represents a stronger loss-of-function when compared to sam4 in an allelic series. On chromosome III, whole genome sequencing found four nonsynonymous mutations and one stop-gained mutation closely linked to sam12; however, RNAi depletion of these genes did not cause somatic P-granule expression (not shown). Sequence coverage was low and incomplete across lin-13 in sam12 worms, so we sequenced lin-13 and found a C to T mutation that introduced a stop codon (Q1585*). (Figure 4A). Low broods

**Figure 3** Hawaiian Variant Mapping and CloudMap analysis. Ratios of Hawaiian to N2 SNPs show linkage in the center of chromosome III for sam4 and sam12, and linkage on chromosome II for sam13. Green vertical bars indicate the position of lin-13 on III and of dpl-1 on II. Even after SNP normalization, some degree of linkage was observed at 5 Mb on chromosome I for all three mutants. Chromosome IV, which is unlinked, is shown for comparison.
(~10/worm) in lin-13(sam12) mutants prevented us from obtaining transgenic lines to test rescue. The predicted lin-13(n387) null allele is homozygous sterile in the absence of maternal LIN-13 (Meléndez and Greenwald 2000). Although lin-13(sam12) worms exhibit very slow growth and low broods, sam12 is an unlikely null because it is possible to maintain homozygous mutants.

On chromosome II, we found one splice site donor, three frameshift, and three nonsynonymous mutations closely linked to sam13. The splice-donor mutation is a G to A base pair substitution in the first nucleotide of intron 2 in dpl-1 (Figure 4A). Of the seven genes mutated in sam13, only dpl-1 RNAi causes somatic P-granule expression. This result was anticipated as DPL-1, also a synMuv B protein in the LIN-35/Rb complex, was previously reported to repress somatic P-granule expression (Ceol and Horvitz 2001; Wang et al. 2005). We generated a line with a fosmid containing dpl-1 (Figure 4B); 16/16 progeny with the transgene rescued, whereas 32/32 siblings without the transgene reexpressed intestinal PGL-1::GFP, suggesting the splice-donor mutation in dpl-1 is responsible for the phenotype. DPL-1 encodes a homolog of human DP, the heterodimerization partner of the E2F transcription factor (Ceol and Horvitz 2001). In C. elegans, DPL-1 initiates spermatheca dilation to promote ovulation and fertilization, and strong loss-of-function mutations inhibit ovulation and oocytes undergo endomitosis (Chi and Reinke 2006, 2009). dpl-1(sam13) animals do not appear to have defects in ovulation, suggesting that this allele causes a splicing defect that only weakly compromises DPL-1 function.

The two lin-13 alleles and the splice site donor mutation in dpl-1 are not synMuv with lin-15a RNAi at 20°, so we asked if these alleles are RNAi defective or whether they demonstrate enhanced RNAi sensitivity associated with known lin-13, dpl-1, and other synMuv B mutants. The presence of P granules makes the C. elegans germline exceptionally sensitive to RNAi, and germline RNAi is defective when P-granule assembly is compromised in the absence of PGL-1 (Robert et al. 2005). Somatic P granules in synMuv B mutants cause enhanced RNAi sensitivity throughout the body of the worm (Wang et al. 2005). To determine if sam4, sam12, and sam13 exhibit enhanced or defective RNAi sensitivity, we performed feeding RNAi on his-44. his-44 RNAi feeding has been shown to cause only 12% early larval arrest in wild-type worms, but it causes 86% arrest in the rrf-3(pk1426) RNAi sensitive strain (Wang and Ruvkun 2004). Like other synMuv B mutants, all three alleles showed enhanced larval arrest on his-44 RNAi (Figure 5A), suggesting that the absence of a synMuv phenotype with lin-15a RNAi cannot be attributed to defective RNAi. Both Rb/lin-35 and MBT/lin-61 RNAi further enhanced somatic P-granule expression in all three alleles, suggesting that they are hypomorphic, and not amorphic, alleles (Figure 5B). Somatic P-granule expression is suppressed in known synMuv B mutants by RNAi depletion of the chromatin regulators mes-3, mes-4, and mrg-1 (Unhavaithaya et al. 2002; Wang et al. 2005; Cui et al. 2006; Andersen et al. 2006; Takasaki et al. 2007; Rechtsteiner et al. 2010). We also observed mild suppression when these chromatin regulators were depleted in sam4, sam12,
and sam13 mutants (Figure 5B). Some alleles of lin-13 have been shown to exhibit a Muv phenotype independent of synMuv A at elevated temperatures (Ferguson and Horvitz 1985), so lin-15a RNAi was performed on lin-13(sam4), lin-13(sam12), and dpl-1(sam13) mutants at 25°C. At this temperature, Muv animals were still not observed with control or lin-15a RNAi on lin-13(sam4) and lin-13(sam12) worms (0/3 replicates). However, at 25°C Muv worms were observed on 3/3 replicates of dpl-1(sam13) worms fed lin-15a RNAi (average 39.9% Muv; SD = 4.5%), but not control RNAi (0/3 replicates). Collectively, these results suggest that sam4, sam12, and sam13 share features with previously described loss-of-function lin-13 and dpl-1 alleles, with the exception that they do not readily exhibit synMuv phenotypes.

By screening 4000 haploid genomes, we expected to get one to two loss-of-function mutations of each gene required to suppress the somatic expression of P granules (Jorgensen and Mango 2002). Of the eight mutations we obtained from the screen, only synMuv B genes were isolated. Because this screen was not performed to saturation, it does not exclude the possibility that non-synMuv B components repress the somatic expression of germ line programs, but it does suggest that if they exist, then they are either rare or essential for early development. Notably, we did not recover alleles of daf-2, a gene whose depletion was previously shown to cause ectopic expression of GFP::PGL-1 driven behind a pie-1 promoter (Curran et al. 2009). This may be due to the limited number of haploid genomes screened, or because the PGL-1::GFP transgene used in our screen is not driven by pie-1 but rather by the pgl-1 promoter. Due to the limited scope of this screen, little can be inferred concerning the significance of isolating one dpl-1 and two lin-13 alleles instead of other synMuv B genes; however, isolating these alleles may implicate DPL-1 and LIN-13 in a more central role because they do not have to be completely inhibited to cause somatic P-granule suppression.

Although it is assumed that germ line components expressed in the soma induce germ cell characteristics that could lead to oncogenesis (i.e., pluripotency and the ability to self-renew), it is unclear how germ granules themselves may contribute to this process. Recently, it was discovered that stress granules, another ribonucleoprotein aggregate that shares some components with germ granules, are important for tumor progression; knockdown of the stress granule nucleator G3BP1 reduces local invasive capacity in tumor xenografts (Somasekharan et al. 2015). Interestingly, knockdown of the C. elegans homolog, g3bp-1/k08f4.2, suppresses synMuv A/B phenotypes (Cui et al. 2006). Whether stress granules exert protective effects to cancer cells during chemotherapy and radiation or whether stress granules sequester mRNAs encoding factors that inhibit oncogenesis is unknown. However, these findings highlight the importance of translational regulation in cancer and may help to explain how the presence of germ-line-enriched proteins in tumors can be associated with malignancy and poor patient prognosis.

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Figure 5 RNAi enhancement and PGL-1::GFP suppression. (A) sam4, sam12, and sam13 mutants exhibit increased larval arrest on his-44 RNAi. (B) Somatic PGL-1::GFP intensity is enhanced with lin-35 and lin-61 RNAi, but partially suppressed with mes-3, mes-4, and mrq-1 RNAi. Error bars = SD. p values: ***<0.0005, **<0.005, and *<0.05.
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