

# Multiple Levels of Redundant Processes Inhibit *Caenorhabditis elegans* Vulval Cell Fates

Erik C. Andersen, Adam M. Saffer and H. Robert Horvitz<sup>1</sup>

Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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## ABSTRACT

Many mutations cause obvious abnormalities only when combined with other mutations. Such synthetic interactions can be the result of redundant gene functions. In *Caenorhabditis elegans*, the synthetic multivulva (synMuv) genes have been grouped into multiple classes that redundantly inhibit vulval cell fates. Animals with one or more mutations of the same class undergo wild-type vulval development, whereas animals with mutations of any two classes have a multivulva phenotype. By varying temperature and genetic background, we determined that mutations in most synMuv genes within a single synMuv class enhance each other. However, in a few cases no enhancement was observed. For example, mutations that affect an Mi2 homolog and a histone methyltransferase are of the same class and do not show enhancement. We suggest that such sets of genes function together *in vivo* and in at least some cases encode proteins that interact physically. The approach of genetic enhancement can be applied more broadly to identify potential protein complexes as well as redundant processes or pathways. Many synMuv genes are evolutionarily conserved, and the genetic relationships we have identified might define the functions not only of synMuv genes in *C. elegans* but also of their homologs in other organisms.

GLOBAL analyses of loss-of-function mutants have revealed that many null mutations do not cause obvious phenotypic abnormalities (PARK and HORVITZ 1986; WINZELER *et al.* 1999; FRASER *et al.* 2000; KAMATH *et al.* 2003). In some cases, the wild-type phenotypes of such mutants result from genetic redundancy, which has been suggested to confer phenotypic robustness to developmental and behavioral processes (HARRISON *et al.* 2007b; SIEBER *et al.* 2007). Genetic redundancy can be studied using synthetic interactions, in which an animal mutant in two genes displays a phenotype that is not seen in either single mutant. Genes that display such a synthetic interaction are typically thought to act in separate processes or pathways to mediate the same broad biological function. Numerous synthetic-lethal interactions have been identified in *Saccharomyces cerevisiae* (TONG *et al.* 2001, 2004; OOI *et al.* 2003). In the nematode *Caenorhabditis elegans*, mutations in the synthetic multivulva (synMuv) genes cause synthetic defects in the development of the hermaphrodite vulva (FERGUSON and HORVITZ 1989).

In *C. elegans*, three of six equipotent progenitor cells are specified to adopt vulval cell fates by three cell-signaling cascades involving receptor tyrosine kinase (RTK)/Ras, Notch, and Wnt pathways (SUNDARAM 2005; STERNBERG 2006). The three cells that adopt vulval fates

divide several times and give rise to the vulva. The remaining three cells divide once, and their progeny adopt nonvulval fates and fuse with the underlying hypodermis (SULSTON and HORVITZ 1977). The synMuv genes prevent the adoption of ectopic vulval cell fates (FERGUSON and HORVITZ 1989).

The synMuv genes originally were grouped into two classes, A and B; animals mutant for one or more genes within the same synMuv class do not have vulval abnormalities, but a multivulva (Muv) phenotype is observed in class A–B double mutants (FERGUSON and HORVITZ 1989). This synthetic interaction indicates that the class A and class B synMuv genes act in two redundant pathways or processes to inhibit the adoption of vulval cell fates. The class A synMuv genes encode nuclear proteins proposed to regulate transcription (CLARK *et al.* 1994; HUANG *et al.* 1994; DAVISON *et al.* 2005). Many class B synMuv genes encode homologs of regulators of transcription and chromatin, including a nucleosome remodeling and deacetylase (NuRD) complex (SOLARI and AHRINGER 2000; UNHAVAITHAYA *et al.* 2002), an Rb/E2F4/DP complex (LU and HORVITZ 1998; CEOL and HORVITZ 2001), two histone methyltransferases (ANDERSEN and HORVITZ 2007), heterochromatin protein 1 (HP1) (COUTEAU *et al.* 2002) and the DP/Rb/MuvB (DRM) complex (HARRISON *et al.* 2006). CEOL and HORVITZ (2004) proposed the existence of a third class of synMuv gene, called C. These genes encode proteins similar to members of the Tip60/NuA4 histone acetyltransferase complex. In this article,

<sup>1</sup>Corresponding author: Department of Biology, Room 68-425, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139. E-mail: horvitz@mit.edu

we show that these putative class C synMuv genes are not distinct from and should be considered class B synMuv genes. The gene *lin-3*, which encodes the EGF-like ligand of the RTK/Ras pathway required for the specification of vulval cell fates (HILL and STERNBERG 1992), is an apparent direct or indirect transcriptional target of at least some synMuv genes (CUI *et al.* 2006). Given this observation and the molecular nature of many synMuv proteins, the synMuv proteins likely act as transcriptional repressors to prevent the ectopic expression of the RTK/Ras pathway ligand LIN-3 and in this way prevent ectopic vulval induction.

To date, there are at least 4 class A synMuv genes and 25 class B synMuv genes (FERGUSON and HORVITZ 1989; LU and HORVITZ 1998; HSIEH *et al.* 1999; VON ZELEWSKY *et al.* 2000; COUTEAU *et al.* 2002; DUFOURCQ *et al.* 2002; UNHAVAITHAYA *et al.* 2002; THOMAS *et al.* 2003; POULIN *et al.* 2005; CEOL *et al.* 2006; ANDERSEN and HORVITZ 2007; TSENG *et al.* 2007). Two class B synMuv genes, *met-1* and *met-2*, act redundantly with each other to inhibit vulval cell fates (ANDERSEN and HORVITZ 2007), and the Muv phenotypes caused by mutations in the class B synMuv genes *let-418* and *hpl-2* are enhanced by mutations in other class B synMuv genes (VON ZELEWSKY *et al.* 2000; COUTEAU *et al.* 2002; ANDERSEN and HORVITZ 2007). These genetic interactions along with the molecular identities of the class B synMuv proteins led us to hypothesize that the class B genes do not act in a single pathway or process to inhibit vulval cell-fate specification.

By scoring the vulval phenotypes of strains sensitized using temperature and genetic background, we sought to determine whether genes within each of the two synMuv classes act together in the same pathway or process. In these assays, we compared the Muv phenotypes caused by single mutants with the Muv phenotypes caused by double mutants within the same synMuv class. We found that most but not all synMuv genes within a single class act in separate pathways or processes, as indicated by the more penetrant Muv phenotypes of double mutants within a class than those of the respective single mutants. The class C synMuv genes were originally postulated to define a separate class from the class B synMuv genes, because the putative class C mutations were weakly redundant with class B synMuv mutations. However, as our results show that nearly all class B synMuv genes are redundant with each other, the putative class C synMuv genes are not different from the class B synMuv genes. For the rest of this paper, we consider there to be only two synMuv classes, A and B.

In a few cases, we found that two genes within the same class did not show enhancement and hence might act in the same pathway or process. In several of these cases, the proteins encoded by these genes have been identified biochemically to be in a complex. We believe that such lack of genetic redundancy can identify sets of genes that mediate the same molecular function. Additionally, we showed that many synMuv proteins

repress *lin-3* transcription and that the redundancy observed in the vulval phenotype between class B synMuv genes likely results from an increase in *lin-3* expression.

## MATERIALS AND METHODS

**Strains and genetics:** *C. elegans* was cultured on the bacterial strain OP50 as described (BRENNER 1974). N2 was the wild-type strain. Mutant alleles used are listed below and in Table 1. Many alleles were described previously (RIDDLE *et al.* 1997) unless otherwise noted:

LG I: *lin-35*(n745) (LU and HORVITZ 1998), *lin-53*(n833) (LU and HORVITZ 1998), *lin-61*(n3447, n3809) (HARRISON *et al.* 2007a), and *met-1*(n4337) (ANDERSEN and HORVITZ 2007).  
 LG II: *lin-8*(n2731) (THOMAS *et al.* 2003), *dpl-1*(n3316) (CEOL and HORVITZ 2001), *trr-1*(n3712) (CEOL and HORVITZ 2004), *lin-56*(n2728) (THOMAS *et al.* 2003), and *lin-38*(n751).  
 LG III: *lin-37*(n4903) (ANDERSEN *et al.* 2006), *met-2*(n4256) (ANDERSEN and HORVITZ 2007), *lin-9*(n112) (BEITEL *et al.* 2000), *lin-52*(n771) (THOMAS *et al.* 2003), and *lin-36*(n766) (THOMAS and HORVITZ 1999).  
 LG IV: *ark-1*(sy247) (HOPPER *et al.* 2000).  
 LG V: *let-418*(n3536) (CEOL *et al.* 2006), *hda-1*(e1795) (DUFOURCQ *et al.* 2002), and *mys-1*(n3681) (CEOL and HORVITZ 2004).  
 LG X: *lin-15B*(n744), *lin-15A*(n433, n767), and *lin-15AB*(e1763) (FERGUSON and HORVITZ 1985).

The following balancer chromosomes were used: *mIn1* [*mIs14 dpy-10*(e128)] (EDGLEY and RIDDLE 2001), *hT2* [*qIs48*], *nT1* [*qIs51*] (MATHIES *et al.* 2003), and *qC1* [*nIs189*] (ANDERSEN *et al.* 2006).

**RNAi analyses:** RNAi of *efl-1* was performed by injection using clone yk617e4 to synthesize dsRNA, as previously described (ANDERSEN *et al.* 2006).

**Scoring the vulval cell fate:** Using a dissecting microscope, we scored the vulval phenotypes of each strain from at least three and in many cases nine independently grown cultures. At least 100 animals were scored for each genotype. Animals were scored as Muv if one or more ectopic ventral protrusions were observed. We scored vulval induction of a few strains using Nomarski optics and found the frequencies of ectopic inductions of vulval cell fates to be equivalent to the frequencies of the Muv phenotypes observed using a dissecting microscope. The penetrance of the Muv phenotype was calculated as the fraction of total animals that were Muv multiplied by 100. The average percentage of Muv and standard deviation of the replicates were calculated. A strain was scored as enhanced when the Muv phenotype penetrance of the double mutant was more than one standard deviation greater than the Muv phenotype penetrances of the respective single mutants. In some cases, enhancement was observed only when comparing the Muv phenotypes of double mutants to the respective single mutants at one of a number of temperatures tested.

**Quantitative PCR assay:** Synchronized wild-type and mutant animals were grown, and larvae were harvested at or near the L2-to-L3 larval transition, when vulval induction occurs. Total RNA was extracted using Trizol (Invitrogen). First-strand cDNA was prepared from 1 µg total RNA using the SuperScript III first-strand synthesis supermix for qRT-PCR (Invitrogen). Each real-time reverse transcriptase (RT) PCR mix contained 10 ng of RT products, SyBR Green PCR master mix (Applied Biosystems), and 0.4 µM of each primer. The real-time PCR was performed in triplicate on an Eppendorf Mastercycler ep realplex<sup>2</sup>. Two or three independent samples of each genotype were prepared, and levels of *lin-3* and *rpl-26* were quantified

**TABLE 1**  
synMuv alleles used in this study

	Allele	Mutation	Null?	Complex
Class A gene				
<i>lin-8</i>	<i>n2731</i>	Q113ochre	Yes	
<i>lin-15A</i>	<i>n767</i>	Deletion	Yes	
<i>lin-15A</i>	<i>n433</i>	A250V <sup>a</sup>	No	
<i>lin-38</i>	<i>n751</i>	R517C <sup>b</sup>	No <sup>c</sup>	
<i>lin-56</i>	<i>n2728</i>	Deletion	Yes	
Class B gene				
<i>ark-1</i>	<i>sy247</i>	Q528ochre	No	
<i>dpl-1</i>	<i>n3316</i>	Deletion	Yes <sup>d</sup>	DP/E2F/Rb, DRM
<i>efl-1</i>	RNAi <sup>e</sup>	NA	No	DP/E2F/Rb, DRM
<i>hda-1</i>	<i>e1795</i>	G182E	No <sup>c</sup>	NuRD
<i>let-418</i>	<i>n3536</i>	P675L	No <sup>c</sup>	NuRD
<i>lin-9</i>	<i>n112</i>	G341E	No <sup>c</sup>	DRM
<i>lin-15B</i>	<i>n744</i>	W485opal	Yes	
<i>lin-35</i>	<i>n745</i>	W151opal	Yes	DP/E2F/Rb, DRM
<i>lin-36</i>	<i>n766</i>	Y796ochre	No	
<i>lin-37</i>	<i>n4903</i>	Deletion	Yes	DRM
<i>lin-52</i>	<i>n771</i>	E36K	No <sup>c</sup>	DRM
<i>lin-53</i>	<i>n833</i>	L292F	No <sup>c</sup>	DRM, NuRD
<i>lin-61</i>	<i>n3809</i>	Q159ochre	Yes	
<i>lin-61</i>	<i>n3447</i>	S354N	No	
<i>met-1</i>	<i>n4337</i>	Deletion	Yes	
<i>met-2</i>	<i>n4256</i>	Deletion	Yes	
<i>mys-1</i>	<i>n3681</i>	G341R	No <sup>c</sup>	NuA4
<i>trr-1</i>	<i>n3712</i>	W2593amber	Yes <sup>d</sup>	NuA4

<sup>a</sup>E. DAVISON and H. R. HORVITZ, unpublished results.

<sup>b</sup>A. M. SAFFER, E. DAVISON and H. R. HORVITZ, unpublished results.

<sup>c</sup>This mutation is nonnull. Null alleles cause sterility or larval arrest.

<sup>d</sup>This mutation causes a recessive sterile phenotype. Homozygotes descended from heterozygous mothers could possess some maternally provided wild-type gene activity.

<sup>e</sup>For *efl-1*, we used RNAi to create a partial loss of gene function.

from each biological replicate. The  $\Delta C_T$  values for *lin-3* were determined using *rpl-26* as the internal reference, and the  $\Delta\Delta C_T$  values were calculated for each genotype by comparison with the wild type. All changes were normalized to the wild type. The error shown is the standard deviation of relative *lin-3/rpl-26* ratios for the biological replicates.

## RESULTS

To assess whether different synMuv genes function in the same process, we combined null or strong loss-of-function mutations (Table 1) and quantified the penetrance of the phenotype as compared to the phenotype of either single mutant. For simplicity below, we will use the word “process” instead of the phrase “pathway or process” to describe how groups of genes act together, but in each case such genes could act simultaneously (e.g., by encoding members of a protein complex) or sequentially (e.g., in a linear pathway). If a double synMuv mutant had a Muv phenotype equivalent in

penetrance to that of either of the single mutants, we concluded that the two genes could act in the same process. By contrast, if a double synMuv mutant had a Muv phenotype that was higher in penetrance than that of either single mutant, we concluded that the two genes function in separate processes. The only way to score an increase in penetrance was to score strains in which the penetrance of the Muv phenotype is incomplete. We used both temperature and the addition of partial loss-of-function alleles of synMuv genes to sensitize the vulval phenotype to incomplete penetrances, increasing our ability to detect enhancement.

**Temperature sensitizes the vulval phenotype:** As observed previously (FERGUSON and HORVITZ 1989; CLARK *et al.* 1994; LU and HORVITZ 1998; HSIEH *et al.* 1999; BEITEL *et al.* 2000; VON ZELEWSKY *et al.* 2000; CEOL and HORVITZ 2001; UNHAVAITHAYA *et al.* 2002; THOMAS *et al.* 2003; CEOL *et al.* 2006; ANDERSEN and HORVITZ 2007; HARRISON *et al.* 2007a), single null mutations of synMuv genes did not cause a Muv phenotype at 20° or 25°, but class A mutations combined with class B mutations caused Muv phenotypes. The Muv phenotype caused by synMuv mutations is temperature sensitive: at higher temperatures the penetrance of the Muv phenotype increases for all tested synMuv double mutants (FERGUSON and HORVITZ 1989) and see below. For example, the penetrance of the *lin-61(n3447)*; *lin-56(n2728)* Muv phenotype increased from 5% at 20° to 81% at 22.5°, and the penetrance of the *lin-35(n745)*; *lin-15A(n433)* Muv phenotype increased from 11% at 15° to 100% at 20°. Both *lin-56(n2728)* and *lin-35(n745)* are presumptive null alleles, and some synMuv class A–B null double mutants have been shown to have temperature-sensitive Muv phenotypes (CEOL and HORVITZ 2004; CEOL *et al.* 2006; ANDERSEN and HORVITZ 2007). Therefore, it is likely that it is the synMuv process and not the synMuv gene products that is temperature sensitive. Most synMuv double mutants have more penetrant Muv phenotypes at high temperatures and incompletely penetrant Muv phenotypes at low temperatures.

**Partial loss-of-function mutations in class A or class B synMuv genes can sensitize the vulval phenotype:** In addition to temperature, genetic background can sensitize the vulval phenotype of synMuv mutant strains. To seek redundancy between class A genes, we partially inhibited a class B synMuv gene using the missense mutation *lin-61(n3447)* (HARRISON *et al.* 2007a), which causes an incompletely penetrant synMuv defect in combination with strong or null class A mutations. This incompletely penetrant Muv phenotype might be enhanced by other null or strong class A mutations if the genes act in separate processes to inhibit vulval fates. To seek redundancy between class B genes, we partially inhibited a class A synMuv gene using the missense mutation *lin-15A(n433)*, which causes a weak class A synMuv defect (CLARK *et al.* 1994). The Muv phenotype

**TABLE 2**  
**Most class A synMuv double mutants have**  
**multivulva phenotypes**

Genotype <sup>a</sup>	% multivulva $\pm$ SD <sup>b</sup>	
	20°	25°
Wild type	0 $\pm$ 0	0 $\pm$ 0
<i>lin-8</i>	0 $\pm$ 0	0 $\pm$ 0
<i>lin-56</i>	0 $\pm$ 0	1 $\pm$ 0
<i>lin-38</i>	0 $\pm$ 0	1 $\pm$ 1
<i>lin-15A<sup>c</sup></i>	0 $\pm$ 0	0 $\pm$ 0
<i>lin-8 lin-56</i>	0 $\pm$ 0	0 $\pm$ 0
<i>lin-8 lin-38</i>	1 $\pm$ 1	7 $\pm$ 2
<i>lin-8; lin-15A<sup>c</sup></i>	0 $\pm$ 0	13 $\pm$ 6
<i>lin-56 lin-38</i>	0 $\pm$ 0	6 $\pm$ 4
<i>lin-56; lin-15A<sup>c</sup></i>	0 $\pm$ 0	1 $\pm$ 0
<i>lin-38; lin-15A<sup>c</sup></i>	0 $\pm$ 0	33 $\pm$ 10

<sup>a</sup>The alleles used are described in Table 1.

<sup>b</sup>Percentage of multivulva was determined as described in MATERIALS AND METHODS. SD, standard deviation.

<sup>c</sup>The null allele *lin-15A(n767)* was used for these analyses.

caused by a partial loss of *lin-15A* function in combination with a null class B synMuv mutation might be enhanced by other null or strong class B mutations if these two class B synMuv genes act in separate processes to inhibit vulval fates.

**Most class A–A double mutants have Muv phenotypes:** To look for enhancement, we scored strains at temperatures in which the Muv phenotypes were <100% penetrant and phenotypic enhancement could be quantified easily. We observed the vulval phenotypes of animals with null mutations in *lin-8*, *lin-15A*, and *lin-56* (Table 1). We used a partial loss-of-function allele of *lin-38*, because a null mutation caused larval lethality, which precluded scoring of the vulval phenotype (A. M. SAFFER and H. R. HORVITZ, unpublished results). Whereas class A single mutants do not have Muv phenotypes at 20° (FERGUSON and HORVITZ 1989; THOMAS *et al.* 2003; DAVISON *et al.* 2005), some class A synMuv mutations caused very weak Muv phenotypes at 25°, with <1% penetrance (Table 2). We also observed the vulval phenotypes of class A–A double mutants (Table 2). Most strains were not Muv at 20°, but many had Muv phenotypes at 25°, ranging from 1 to 33% penetrance. Only the class A–A double mutants *lin-8 lin-56* and *lin-56; lin-15A* did not have a Muv phenotype greater than that of either single mutant at 25°.

Therefore, not only do the class A and B synMuv genes act in separate processes, but, given these results, most class A synMuv genes can act in processes separate from those of other class A synMuv genes. Given that *lin-8 lin-56* and *lin-56; lin-15A* double mutants were not enhanced when compared to their respective single mutants, the two genes in each of these gene pairs might act in the same process. Alternatively, they might act in different processes, with the decrease in synMuv gene

function in these experiments being insufficient to reach the threshold necessary to cause a Muv phenotype, just as certain synMuv gene double mutants were Muv only if strong loss-of-function alleles were used, *e.g.*, *lin-8(n2741); lin-15B(n2245)* was 1% Muv at 15°, while *lin-8(n2376); lin-15B(n2245)* was 98% Muv at 15° (DAVISON *et al.* 2005). Because the *lin-8; lin-15A* double mutant had an enhanced vulval phenotype, indicating that *lin-8* and *lin-15A* act in separate processes, one or both of the class A–A synMuv gene pairs *lin-8 lin-56* and *lin-56; lin-15A* must act in parallel.

***lin-15A* and *lin-56* act in the same process to inhibit the specification of vulval cell fates:** For class A–A double mutants that contained at least one null mutation and for which no enhancement was observed, either the two synMuv genes act in the same process or they act in different processes and loss of function of both genes did not cause sufficient loss of inhibition of vulval cell fates to result in a Muv phenotype. Addition of a partial loss-of-function mutation in a synMuv gene from a different synMuv class could allow the detection of subtle redundancy by increasing the loss of inhibition of vulval fates to result in a Muv phenotype. We combined class A single or class A–A double mutations with the class B synMuv mutation *lin-61(n3447)*.

We found that single class A synMuv mutations in combination with *lin-61(n3447)* caused Muv phenotypes at 20° and 22.5° (Table 3). In a *lin-61(n3447)* background, the Muv phenotypes caused by *lin-15A* or *lin-38* mutations were more penetrant than those caused by *lin-8* or *lin-56*, indicating that *lin-15A* and *lin-38* cause a stronger inhibition of vulval cell fates. Because *lin-8* and *lin-56* mutations each cause a relatively weak inhibition of vulval cell fates, the lack of enhancement observed in *lin-8 lin-56* and *lin-56; lin-15A* double mutants at 25° could be because neither class A–A double mutant had sufficient loss of vulval cell-fate inhibition to cause a Muv phenotype.

To address possible redundancy between the class A synMuv genes more sensitively, we constructed triple mutants with two class A synMuv genes and the class B synMuv gene *lin-61* (Table 3). The group of class A–A mutants that with an added *lin-61* mutation had Muv phenotypes greater than that of each respective class A; *lin-61* double mutant included the group of class A–A double mutants that had Muv phenotypes at 25°; thus, the two class A synMuv genes in each of these double mutant combinations were shown to act in separate processes by two different observations. The Muv phenotype of the *lin-61; lin-8 lin-56* triple mutant was stronger than the Muv phenotypes of either *lin-61; lin-8* or *lin-61; lin-56* animals at 20° and 22.5°. Therefore, *lin-8* and *lin-56* act in different processes. By contrast, the Muv phenotype of the *lin-61; lin-56; lin-15A* triple mutant was not stronger than *lin-61; lin-56* or *lin-61; lin-15A*. Because all other class A; *lin-15A; lin-61* and class A; *lin-56; lin-61* triple mutants had Muv phenotypes

TABLE 3

**Mutations in most class A synMuv genes enhance the Muv phenotypes caused by mutations in other class A synMuv genes in a class B synMuv mutant background**

	<i>lin-8</i>	<i>lin-15A</i>	<i>lin-38</i>	<i>lin-56</i>
20°				
<i>lin-8</i>	8 ± 6	60 ± 7	69 ± 8	21 ± 11
<i>lin-15A</i>		21 ± 8	78 ± 10	<b>14 ± 7</b>
<i>lin-38</i>			32 ± 18	49 ± 13
<i>lin-56</i>				5 ± 1
22.5°				
<i>lin-8</i>	69 ± 3	100 ± 1	100 ± 0	95 ± 2
<i>lin-15A</i>		97 ± 2	100 ± 0	<b>97 ± 2</b>
<i>lin-38</i>			99 ± 1	100 ± 0
<i>lin-56</i>				81 ± 8

Each strain was grown at the temperature shown. All strains were homozygous for the class B mutation *lin-61(n3447)*. The alleles used are described in Table 1. *lin-15A(n767)* was used in these analyses. Class A–A–B triple mutants with Muv phenotypes not enhanced when compared to the respective class A–B double mutants are in boldface type. The percentage of Muv was determined as described in MATERIALS AND METHODS, and the average percentage of Muv and standard deviation are shown.

enhanced as compared to the respective class A; *lin-61* double mutants, the lack of enhancement observed in the *lin-61; lin-56; lin-15A* triple mutant suggests that *lin-56* and *lin-15A* act in the same process.

**Most class B–B double mutants do not have Muv phenotypes:** We also assayed redundancy among the class B synMuv genes using temperature to sensitize the vulval cell-fate decision. We used null mutations of the class B synMuv genes *dpl-1*, *lin-35*, *lin-61*, *lin-37*, *met-1*, *met-2*, and *trr-1* (Table 1). We used partial loss-of-function alleles of *let-418*, *lin-52*, and *mys-1*, because null alleles cause larval lethality or sterility. We studied the pairwise interactions among this set of 10 class B synMuv genes comprehensively. We did not test all 378 pairwise interactions involving all 28 class B genes. Unlike the class A genes, most class B single and class B–B double synMuv mutants did not have Muv phenotypes at 20° or 25° (Table 4). The exceptions were *let-418(n3536)* and *trr-1(n3712)*, each of which caused very low penetrance Muv defects as single mutants. Many class B–B double mutants had larval-lethal phenotypes at 25°, precluding scoring vulval phenotypes at high temperature. These larval-lethal phenotypes indicate that in the control of viability the class B synMuv genes act redundantly, but this redundancy might not be indicative of functions in vulval development. Also, the class B–B double null mutants *lin-61(n3809) lin-35(n745)* and *lin-61(n3809); lin-37(n4903)* had very low penetrance Muv phenotypes. Because most class B–B double mutants were not appreciably Muv at high temperature, the class B synMuv genes might all act in the same process. Given that there are a number of examples of redundancy

TABLE 4

**Class B synMuv single and class B–B double mutants do not have appreciable Muv phenotypes**

Genotype <sup>a</sup>	% multivulva ± SD <sup>b</sup>	
	20°	25°
Wild type	0 ± 0	0 ± 0
<i>lin-35</i>	0 ± 0	0 ± 0
<i>lin-61<sup>c</sup></i>	0 ± 0	0 ± 0
<i>dpl-1<sup>d</sup></i>	0 ± 0	0 ± 0
<i>trr-1<sup>d</sup></i>	2 ± 1	3 ± 2
<i>lin-37</i>	0 ± 0	0 ± 0
<i>lin-52</i>	0 ± 0	0 ± 0
<i>let-418</i>	0 ± 0	1 ± 0
<i>mys-1</i>	0 ± 0	0 ± 0
<i>efl-1</i>	0 ± 0	0 ± 0
<i>lin-35 lin-61<sup>c</sup></i>	0 ± 0	1 ± 0
<i>lin-35; lin-37</i>	0 ± 0	0 ± 0
<i>lin-35; lin-52</i>	0 ± 0	0 ± 1
<i>lin-35; let-418</i>	0 ± 0	Lvl
<i>lin-35; mys-1</i>	0 ± 0	Lvl
<i>lin-35; efl-1</i>	0 ± 0	0 ± 0
<i>lin-61<sup>c</sup>; lin-37</i>	0 ± 0	1 ± 1
<i>lin-61<sup>c</sup>; lin-52</i>	0 ± 0	0 ± 0
<i>lin-61<sup>c</sup>; let-418</i>	2 ± 2	Lvl
<i>lin-61<sup>c</sup>; mys-1</i>	0 ± 0	Lvl
<i>dpl-1; efl-1<sup>d</sup></i>	0 ± 0	0 ± 0
<i>trr-1; mys-1<sup>d</sup></i>	4 ± 3	Lvl
<i>lin-37; let-418</i>	0 ± 0	0 ± 0
<i>lin-37; mys-1</i>	0 ± 0	Lvl
<i>lin-37; efl-1</i>	0 ± 0	0 ± 0
<i>lin-52; let-418</i>	0 ± 0	0 ± 0
<i>lin-52; mys-1</i>	0 ± 0	Lvl
<i>let-418 mys-1</i>	0 ± 0	Lvl

Lvl, larval lethality.

<sup>a</sup> The alleles used are described in Table 1.

<sup>b</sup> Percentage of multivulva was determined as described in MATERIALS AND METHODS. SD, standard deviation.

<sup>c</sup> The null allele *lin-61(n3809)* was used for these analyses.

<sup>d</sup> These animals were descended from synMuv mutant heterozygotes, because the mutations cause recessive sterility, as described in Table 1.

within the class B synMuv genes (VON ZELEWSKY *et al.* 2000; COUTEAU *et al.* 2002; ANDERSEN and HORVITZ 2007), it is more likely that many of these genes act in separate processes, and the loss of inhibition of vulval cell fates caused by the class B synMuv mutations was insufficient to reach the threshold necessary to cause a Muv phenotype.

**Most class B synMuv genes act redundantly with genes of the same class to inhibit vulval cell fates:** To observe subtle redundancies among the class B synMuv genes and to assess the relative input of each of the class B genes into vulval cell-fate inhibition, we combined class B single and class B–B double mutants with the weak class A mutation *lin-15A(n433)* (Tables 5 and 6). We tested many class B genes with viable null phenotypes. *dpl-1*, *lin-35*, *lin-37*, and *met-2* caused the strongest

TABLE 5

Mutations of most class B and C genes enhance the Muv phenotypes caused by mutations in other class B genes in a class A synMuv mutant background

	<i>lin-35</i>	<i>met-1</i>	<i>dpl-1</i> <sup>a</sup>	<i>trr-1</i> <sup>a</sup>	<i>lin-37</i>	<i>met-2</i>	<i>lin-52</i>	<i>lin-61</i>	<i>let-418</i>	<i>mys-1</i>
15°										
<i>lin-35</i>	11 ± 3	93 ± 5	<b>13 ± 3</b>	Lvl	72 ± 6	98 ± 3	23 ± 3	97 ± 3	50 ± 5	67 ± 9
<i>met-1</i>		0 ± 0	33 ± 15	65 ± 6	100 ± 0	62 ± 10	<b>2 ± 1</b>	4 ± 3	<b>1 ± 1</b>	<b>1 ± 1</b>
<i>dpl-1</i>			0 ± 0	55 ± 13	69 ± 5	90 ± 11	<b>0 ± 0</b>	46 ± 21	<b>0 ± 0</b>	<b>0 ± 0</b>
<i>trr-1</i>				1 ± 1	Lvl	61 ± 6	<b>11 ± 12</b>	18 ± 10	<b>6 ± 9</b>	<b>1 ± 2</b>
<i>lin-37</i>					4 ± 3	100 ± 0	22 ± 16 <sup>b</sup>	95 ± 3	33 ± 5	29 ± 5
<i>met-2</i>						7 ± 7	79 ± 7	67 ± 20	39 ± 4	<b>20 ± 10</b>
<i>lin-52</i>							0 ± 1	17 ± 7	<b>0 ± 0</b>	<b>1 ± 1</b>
<i>lin-61</i>								0 ± 0	<b>1 ± 1</b>	<b>1 ± 1</b>
<i>let-418</i>									0 ± 0	<b>1 ± 1</b>
<i>mys-1</i>										0 ± 0
17.5°										
<i>lin-35</i>	65 ± 10	96 ± 4	<b>60 ± 3</b>	Lvl	100 ± 0	100 ± 0	93 ± 10	100 ± 1	100 ± 0	99 ± 1
<i>met-1</i>		0 ± 0	81 ± 5	90 ± 8	100 ± 0	95 ± 3	26 ± 18	16 ± 3	<b>2 ± 2</b>	15 ± 16
<i>dpl-1</i>			2 ± 2	90 ± 2	100 ± 0	99 ± 2	13 ± 3	91 ± 2	<b>3 ± 1</b>	<b>0 ± 1</b>
<i>trr-1</i>				4 ± 5	Lvl	79 ± 4	64 ± 2	88 ± 3	<b>3 ± 4</b>	<b>5 ± 7</b>
<i>lin-37</i>					59 ± 11	100 ± 1	92 ± 5 <sup>b</sup>	100 ± 0	96 ± 6	92 ± 7
<i>met-2</i>						59 ± 19	86 ± 14	98 ± 1	89 ± 7	95 ± 6
<i>lin-52</i>							0 ± 0	58 ± 18	<b>1 ± 2</b>	<b>6 ± 6</b>
<i>lin-61</i>								1 ± 1	9 ± 2	9 ± 6
<i>let-418</i>									0 ± 0	9 ± 2
<i>mys-1</i>										0 ± 0
20°										
<i>lin-35</i>	100 ± 0	100 ± 0	100 ± 0	Lvl	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
<i>met-1</i>		8 ± 4	99 ± 1	100 ± 1	100 ± 0	100 ± 1	86 ± 10	96 ± 6	<b>6 ± 1</b>	88 ± 6
<i>dpl-1</i>			70 ± 7	100 ± 0	100 ± 0	100 ± 0	87 ± 2	99 ± 1	97 ± 1	84 ± 5
<i>trr-1</i>				12 ± 5	Lvl	100 ± 0	98 ± 2	100 ± 0	53 ± 11	<b>6 ± 6</b>
<i>lin-37</i>					100 ± 0	100 ± 0	100 ± 0 <sup>b</sup>	100 ± 0	100 ± 0	100 ± 0
<i>met-2</i>						99 ± 1	100 ± 0	100 ± 0	100 ± 0	100 ± 0
<i>lin-52</i>							15 ± 6	100 ± 0	89 ± 10	97 ± 1
<i>lin-61</i>								19 ± 17	94 ± 5	93 ± 6
<i>let-418</i>									0 ± 0	31 ± 7
<i>mys-1</i>										2 ± 1

All strains were homozygous for the class A mutation *lin-15A(n433)*. Each strain was grown at the temperature shown. The alleles used are described in Table 1. *lin-61(n3809)* was used in these analyses. Class A–B–B triple mutants with Muv phenotypes not enhanced when compared to the respective class A–B double mutants are in boldface type. The percentage of Muv was determined as described in MATERIALS AND METHODS, and the average percentage of Muv and standard deviation are shown.

<sup>a</sup>These animals were descended from synMuv mutant heterozygotes, because the mutations cause recessive sterility, as described in Table 1.

<sup>b</sup>RNAi of *lin-52* was used in the *lin-37(n4903)*; *lin-15A(n433)* strain for this experiment.

inhibition of vulval cell fates, as mutants in each of these genes had highly penetrant Muv phenotypes at 17.5° and 20° in combination with *lin-15A(n433)*. Mutants in *lin-61*, *lin-52*, *let-418*, *met-1*, *mys-1*, and *trr-1* did not have appreciably Muv phenotypes at 17.5° or 20° in combination with *lin-15A(n433)*, suggesting that these genes caused a weaker inhibition of vulval cell fates.

We found using a sensitized genetic background that most class B genes act in separate processes to inhibit vulval cell fates (Tables 5 and 6). In combination with the weak class A mutation *lin-15A(n433)*, most combinations of class B–B double mutants had more highly penetrant Muv phenotypes than those found in the respective single mutants. This difference was observed

readily in strong double mutant combinations at 15°, intermediate combinations at 17.5°, and weak combinations at 20°. For example three weak synMuv genes, *let-418*, *lin-52*, and *met-1*, show insignificant enhancement at 17.5°, but at 20° enhancement is observed between *let-418* and *lin-52* but not between *let-418* and *met-1*.

**Some synMuv genes within the same class function nonredundantly to inhibit vulval cell fates:** Several class B–B double mutants had Muv phenotypes that were not enhanced as compared to the single class B mutants in a *lin-15A(n433)* background, indicating that these gene pairs likely act in the same process to inhibit vulval cell fates (Tables 5 and 6). Specifically, three pairs of

TABLE 6

Mutations in most class B synMuv genes enhance the Muv phenotypes caused by mutations of other class B synMuv genes in a class A synMuv mutant background

Genotype <sup>a</sup>	% multivulva $\pm$ SD <sup>b</sup>			
	15°	17.5°	18.5°	20°
Wild type	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
<i>ark-1</i>	0 $\pm$ 0	0 $\pm$ 1		4 $\pm$ 2
<i>dpl-1<sup>c</sup></i>	0 $\pm$ 0	2 $\pm$ 2		70 $\pm$ 7
<i>efl-1</i>	0 $\pm$ 0	8 $\pm$ 12		92 $\pm$ 7
<i>hda-1/+</i>	0 $\pm$ 0	0 $\pm$ 0		0 $\pm$ 0
<i>let-418</i>	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
<i>lin-9</i>	4 $\pm$ 3	63 $\pm$ 11	81 $\pm$ 6	100 $\pm$ 0
<i>lin-35</i>	10 $\pm$ 3	65 $\pm$ 10	86 $\pm$ 5	100 $\pm$ 0
<i>lin-36</i>	0 $\pm$ 0	0 $\pm$ 0		5 $\pm$ 2
<i>lin-37</i>	4 $\pm$ 3	59 $\pm$ 11	32 $\pm$ 9	100 $\pm$ 0
<i>lin-52</i>	0 $\pm$ 1	0 $\pm$ 0	0 $\pm$ 0	15 $\pm$ 6
<i>lin-53</i>	0 $\pm$ 0	1 $\pm$ 1	7 $\pm$ 3	100 $\pm$ 1
<i>lin-61<sup>d</sup></i>	0 $\pm$ 0	1 $\pm$ 1		19 $\pm$ 17
<i>mys-1</i>	0 $\pm$ 0	0 $\pm$ 0		2 $\pm$ 1
<i>trr-1<sup>c</sup></i>	1 $\pm$ 1	4 $\pm$ 5		12 $\pm$ 5
<i>lin-35; ark-1</i>	37 $\pm$ 23	92 $\pm$ 3		100 $\pm$ 0
<i>lin-35; hda-1/+</i>	36 $\pm$ 2	97 $\pm$ 1		100 $\pm$ 0
<i>lin-36; let-418</i>	1 $\pm$ 2	1 $\pm$ 0		99 $\pm$ 1
<i>lin-36; mys-1</i>	1 $\pm$ 1	15 $\pm$ 15		99 $\pm$ 1
<i>lin-53; let-418</i>	0 $\pm$ 1	4 $\pm$ 2	46 $\pm$ 13	99 $\pm$ 1
<i>lin-53; lin-9</i>	13 $\pm$ 4	67 $\pm$ 9	93 $\pm$ 5	100 $\pm$ 0
<i>lin-53; lin-37</i>	13 $\pm$ 1	42 $\pm$ 14	100 $\pm$ 0	100 $\pm$ 0
<i>lin-53; lin-52</i>	0 $\pm$ 0	3 $\pm$ 2	16 $\pm$ 5	99 $\pm$ 1
<i>lin-53; mys-1</i>	8 $\pm$ 13	47 $\pm$ 11		100 $\pm$ 1
<i>lin-61<sup>d</sup>; ark-1</i>	7 $\pm$ 8	34 $\pm$ 16		84 $\pm$ 26
<i>lin-61<sup>d</sup>; hda-1/+</i>	1 $\pm$ 1	7 $\pm$ 5		100 $\pm$ 0
<i>lin-61<sup>d</sup>; lin-9</i>	86 $\pm$ 12	100 $\pm$ 0		100 $\pm$ 0
<i>lin-61<sup>d</sup>; lin-36</i>	1 $\pm$ 1	21 $\pm$ 16		99 $\pm$ 1

<sup>a</sup> These strains were homozygous for the class A synMuv mutation *lin-15A(n433)*. The alleles used are described in Table 1.

<sup>b</sup> Percentage of multivulva was determined as described in MATERIALS AND METHODS. SD, standard deviation.

<sup>c</sup> These animals were descended from synMuv mutant heterozygotes, because the mutations cause recessive sterility, as described in Table 1.

<sup>d</sup> *lin-61(n3809)* was used in these analyses.

genes—*lin-35* and *dpl-1*; *let-418* and *met-1*; and *mys-1* and *trr-1*—did not show synthetic enhancement in a *lin-15A(n433)* background. This lack of genetic enhancement was detected even though these combinations involve alleles that in other combinations are sufficiently strong to cause Muv phenotypes. We suggest that our findings identify sets of genes that act nonredundantly, *i.e.*, in the same processes.

*lin-35* and *dpl-1* encode the *C. elegans* homologs of human Rb and DP, respectively. Rb mediates transcriptional repression of the genes bound by the heterodimeric transcription factor E2F, which is composed of the proteins DP and E2F (KREK *et al.* 1993). In *C. elegans*, DPL-1 DP and EFL-1 E2F interact *in vitro* (CEOL and HORVITZ 2001), and their homologs interact both

*in vitro* and *in vivo* in other organisms (BANDARA *et al.* 1993; HELIN *et al.* 1993; KREK *et al.* 1993). We tested whether RNAi of the *C. elegans* homolog of E2F, *efl-1* (CEOL and HORVITZ 2001), would enhance the Muv phenotypes of *lin-35*; *lin-15A* and *dpl-1*; *lin-15A* mutants. RNAi of *efl-1* in a *lin-15A(n433)* background caused a Muv phenotype that was  $8 \pm 12\%$  penetrant at 17.5° (average  $\pm$  standard deviation, see MATERIALS AND METHODS). At 17.5°, *dpl-1(n3316)*; *lin-15A(n433)* and *lin-35(n745)*; *lin-15A(n433)* animals had  $2 \pm 2\%$  and  $65 \pm 10\%$  penetrant Muv phenotypes, respectively. RNAi of *efl-1* in *dpl-1(n3316)*; *lin-15A(n433)* or *lin-35(n745)*; *lin-15A(n433)* mutants did not enhance the Muv phenotypes at 17.5° ( $7 \pm 10\%$  Muv and  $72 \pm 11\%$  Muv, respectively). These data indicate that *efl-1* E2F and *dpl-1* DP, which together encode a heterodimeric transcription factor, act to inhibit vulval fates in the same molecular process both with each other and with *lin-35* Rb.

We note that because null mutations of *dpl-1* and *trr-1* cause sterility, the *dpl-1* and *trr-1* homozygous animals we scored were descended from heterozygous mothers. Therefore, these animals could have maternal rescue of *dpl-1* or *trr-1*. Additionally, RNAi of *efl-1* and *mys-1(n3681)* likely caused partial loss of gene function, as null alleles of each gene cause sterility. We believe that the loss of gene function of each of these four genes was sufficient to allow us to detect enhancement of the Muv phenotype by our assays, because each allele or RNAi enhanced the Muv phenotypes of other synMuv combinations. For example, RNAi of *efl-1* enhanced the Muv phenotype of *lin-37(n4903)*; *lin-15A(n433)* mutants from 59 to 100% at 17.5°, and *mys-1(n3681)* enhanced other Muv phenotypes (Tables 5 and 6).

We chose several other class B synMuv genes to test in a limited number of class B–B double mutant combinations in a *lin-15A(n433)* background. Null or strong mutations in *ark-1*, *lin-9*, or *lin-36* enhanced the Muv phenotypes caused by other class B genes (Table 6). The mutation *hda-1(e1795)* causes a recessive Muv phenotype (DUFOURCQ *et al.* 2002), and the Muv phenotype is enhanced by both class A and class B synMuv mutations (E. C. ANDERSEN and H. R. HORVITZ, unpublished results). Heterozygous *hda-1(e1795)* in three different mutant class B synMuv backgrounds caused enhancement of those Muv phenotypes, indicating that *lin-35*, *lin-37*, and *lin-61* function in separate processes from *hda-1*. Using a strong partial loss-of-function allele of the class B synMuv gene *lin-53* RbAp48 (LU and HORVITZ 1998), we found that in a *lin-15A(n433)* background *lin-53(n833)* failed to enhance the Muv phenotypes caused by *lin-9(n112)*, *lin-37(n4903)*, *lin-52(n771)*, and *let-418(n3536)* at 17.5° or 20° but did enhance the Muv phenotype caused by *mys-1(n3681)*. Because four class B; *lin-53*; *lin-15A* triple mutants were weakly Muv at 17.5° and completely Muv at 20°, we scored each of these strains at the intermediate temperature 18.5°. At this

temperature, weak to moderate enhancement of the Muv phenotype was observed (Table 6), indicating that these genes act in separate processes.

In short, we identified four sets of synMuv genes—*lin-15A* and *lin-56*; *lin-35*, *dpl-1* and *efl-1*; *let-418* and *met-1*; and *mys-1* and *trr-1*—for which the genes within each set likely act together to inhibit vulval fates.

**The penetrances of synMuv vulval phenotypes correlate with the level of *lin-3* mRNA expression:** CUI *et al.* (2006) reported that the *lin-3* gene, which encodes the EGF ligand of the Ras pathway is a transcriptional target of some synMuv proteins. Three double mutants carrying a class A and a class B synMuv mutation had increased *lin-3* EGF mRNA levels. We determined whether each of the class A genes and the class B genes we studied repress *lin-3* transcription and whether the redundancy we found to control the vulval phenotype also controls *lin-3* transcription.

We found that all class A–B double mutant strains between mutations in each of the four class A genes and *lin-15B*(n744) had increased *lin-3* mRNA levels as compared to the wild type (Figure 1A). These results suggest that all four class A synMuv proteins repress the transcription of *lin-3* EGF. All class A–B double mutant strains between mutations in each of the 13 class B genes used in this study and *lin-15A*(n767) also had increased *lin-3* mRNA levels as compared to the wild type (Figure 1B). This analysis greatly expands the number of genes implicated in the transcriptional repression of *lin-3*.

The level of increased *lin-3* transcription in the class A–B synMuv double mutants roughly correlated with the phenotypic strength of the respective Muv phenotypes (supplemental Figure S1). For example, double mutants between *lin-9* or *lin-35* and a class A gene had very strong Muv phenotypes and high levels of *lin-3* transcription. By contrast, *mys-1*; *lin-15A* animals had a weak Muv phenotype and a lower increase in *lin-3* transcription.

Notably, *ark-1*(sy247); *lin-15A*(n767) had an increased level of *lin-3* transcription, even though *ark-1* encodes a protein proposed to directly downregulate Ras pathway activity through SEM-5 (HOPPER *et al.* 2000). One possibility is that there is positive feedback through the Ras pathway during vulval induction, so that Ras pathway activity elevates levels of *lin-3* mRNA. We measured the level of *lin-3* transcription in mutants carrying the gain-of-function *let-60* Ras allele *n1046* and found *lin-3* transcription was slightly increased (2.8-fold  $\pm$  0.9-fold) as compared to the wild type. Therefore, it is possible that at least some of the increase in *lin-3* mRNA levels in the *ark-1*(sy247); *lin-15A*(n767) mutant is caused by positive feedback.

We found that the Muv phenotypes of many class A–B–B synMuv triple mutants were enhanced as compared to the respective class A–B double mutants. We wondered if this redundancy extends to the control of *lin-3* transcriptional repression. We compared the *lin-3* transcription levels of *lin-61*(n3809); *lin-15A*(n433), *lin-37*(n4903); *lin-*

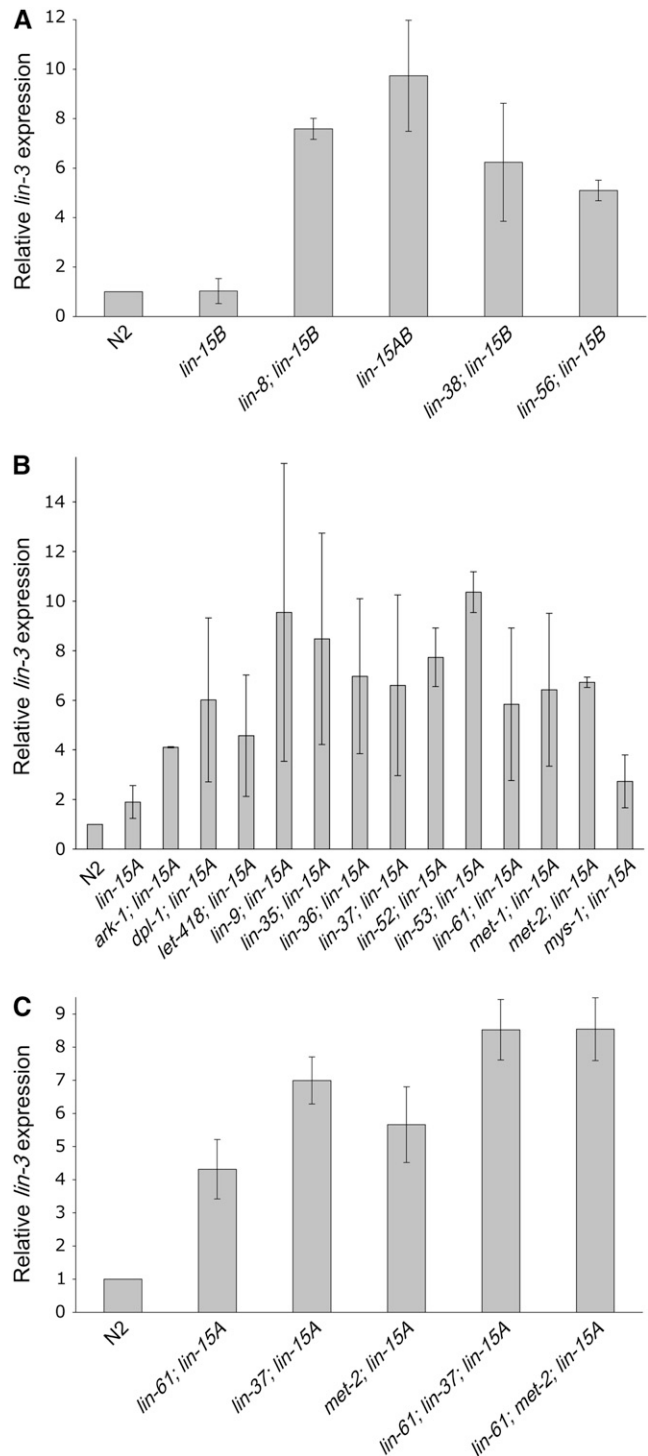


FIGURE 1.—Regulation of *lin-3* transcription by the synMuv genes. Real-time RT–PCR experiments were performed on the strains shown. Mean  $\Delta\Delta C_T$  values were used to calculate relative changes in *lin-3* expression normalized to levels of *rpl-26*. Mean values and standard deviations of relative *lin-3*/*rpl-26* ratios for the trials are shown. (A) Mutation of each class A synMuv gene in a class B synMuv mutant background has increased *lin-3* transcription. (B) Mutation of each class B synMuv gene in a class A synMuv mutant background has increased *lin-3* transcription. *lin-15A*(n767) and *lin-61*(n3809) were used for this experiment. (C) Two class B–B–A synMuv triple mutants have increased levels of *lin-3* transcription compared to their respective class B–A double mutants. *lin-15A*(n433) and *lin-61*(n3809) were used for this experiment.



*lin-15A(n433)*, and *met-2(n4256); lin-15A(n433)* double mutants to the *lin-3* expression of *lin-61(n3809); lin-37(n4903); lin-15A(n433)* and *lin-61(n3809); met-2(n4256); lin-15A(n433)* triple mutants. We found that in each case there was an increase in *lin-3* transcription in the triple mutants as compared to the respective double mutants (Figure 1C), indicating that for these synMuv genes there is redundant control of *lin-3* transcriptional repression.

## DISCUSSION

**Genetic enhancement tests can distinguish processes that act in series or in parallel:** Genetic enhancement experiments are most simply interpreted when one or both of the mutations used in the study are null. If a null mutation is combined with another mutation, the phenotype can be more severe than the phenotypes of the respective single mutants if the genes act in parallel (redundant) processes but cannot be more severe if the genes act in the same process. At least one of the two mutations must be null for such a test, because enhancement of the mutant phenotype of a double mutant between two partial loss-of-function mutations could result even if the genes act in the same process. Additionally, the penetrance of the mutant phenotype must be incomplete, so that differences can be observed.

This type of genetic pathway analysis of biological systems defines logical relationships equivalent to “and” *vs.* “or” statements in mathematics and to “series” *vs.* “parallel” circuits in electrical engineering. If a double mutant has a phenotype more severe than that of either single mutant, the two genes must provide separate functions, *e.g.*, act in parallel as a logical “or”; if either gene is active, some function is provided. By contrast, if a double mutant has a phenotype equivalent to that observed in either single mutant, the two genes might provide the same function, *e.g.*, act together in series as a logical “and”; if either gene is inactive, no function is provided.

By these criteria, the class A and B synMuv genes act in parallel processes, as class A–B double mutants have almost completely penetrant Muv phenotypes at 20° (FERGUSON and HORVITZ 1989). It has been previously thought that within a synMuv gene class there is no enhancement in double mutants, in which case all of the synMuv genes within each synMuv class could act in a single process. However, we found that there are multiple levels of redundant processes involving the functions of both the class A and class B synMuv gene classes in inhibiting vulval cell fates (Figure 2). Most class A–A or class B–B double mutants either had Muv phenotypes or showed enhancement in a sensitized genetic background or at higher temperatures. By contrast, a few sets of genes within a single synMuv class failed to show enhancement and hence might act within the same process.

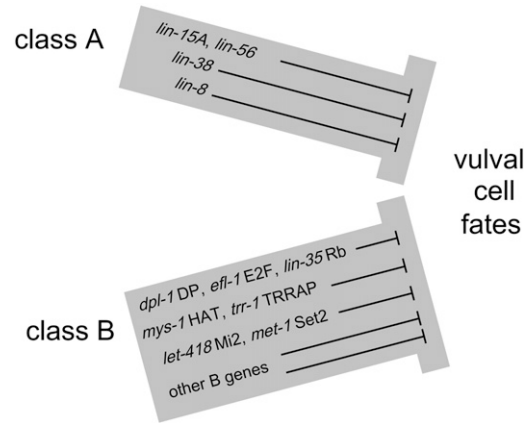


FIGURE 2.—Model: The synMuv classes function in two redundant pathways or processes, each composed of separate molecular pathways or processes, to mediate the inhibition of vulval cell fates. The class A and B synMuv genes act in two distinct pathways to inhibit the expression of vulval cell fates. Additionally, each class is composed of many separate pathways as defined by genetic redundancy tests. A few synMuv genes act nonredundantly with each other: *lin-15A* and *lin-56* act together within the class A genes, while within the class B genes *dpl-1 DP*, *efl-1 E2F*, and *lin-35 Rb*; *mys-1 HAT* and *trr-1 TRRAP*; and *let-418 Mi2* and *met-1 Set2* act together to inhibit vulval fates.

**The synMuv genes define two distinct classes:** The class B synMuv genes *met-1*, *met-2*, *hda-1*, *hpl-2*, and *let-418* cause a Muv phenotype in combination with either class A or class B synMuv mutations (VON ZELEWSKY *et al.* 2000; COUTEAU *et al.* 2002; DUFOURCQ *et al.* 2002; CEOL and HORVITZ 2004; ANDERSEN and HORVITZ 2007). Mutations in each of these genes cause stronger Muv phenotypes when combined with class A synMuv mutations than with class B synMuv mutations. The class C genes were considered a separate class because of their redundancy with both class A and class B genes. However, our findings indicate that the class C synMuv genes are not distinctive in this respect, as most class B synMuv genes are similarly weakly redundant with each other. Furthermore, many class A–A double mutants had weak Muv defects comparable in strength to or stronger than those of class B–C double mutants.

We propose that the synMuv genes should be classified on the basis of the strength of the Muv phenotype observed in a double mutant with a class A synMuv mutation as compared to a double mutant with a class B synMuv mutation. The double mutant combination with the more penetrant Muv phenotype distinguishes the class. For example, if a synMuv mutation when combined with a class A synMuv mutation caused an 80% penetrant Muv phenotype and when combined with a class B synMuv mutations caused a 15% penetrant Muv phenotype, this synMuv gene would be assigned to class B, because it is more Muv in combination with class A mutations than with class B mutations. Because mutations in *met-1*, *met-2*, *hda-1*, *hpl-2*, *lin-13*, *let-418* or

any of the class C genes all exhibit strong Muv phenotypes when combined with mutations in class A genes and weak Muv phenotypes when combined with mutations in class B genes, we suggest that each of these genes should be assigned as class B synMuv genes.

**Lack of genetic enhancement can identify proteins that function in a complex or a process:** A few sets of synMuv genes in the same class did not display synthetic interactions. While all synMuv genes act redundantly with some other synMuv genes to inhibit vulval cell fates, a set of synMuv genes that acts nonredundantly might act together in a more specific molecular process, as discussed below.

Specifically, some sets of synMuv genes that did not show synthetic enhancement encode proteins expected or known to function together *in vivo*. For example, we found that *dpl-1* DP, *eft-1* E2F, and *lin-35* Rb define a single process in the inhibition of vulval cell fates. The corresponding *C. elegans* proteins likely form a complex (CEOL and HORVITZ 2001), and their homologs function together in a complex (BANDARA *et al.* 1993; HELIN *et al.* 1993; KREK *et al.* 1993). Similarly as shown previously (CEOL and HORVITZ 2004), *mys-1* HAT and *trr-1* TRRAP define a single process, and their homologs have been identified together in a histone acetyltransferase complex (IKURA *et al.* 2000). Our data also indicate that the class A genes *lin-15A* and *lin-56* act together in the same process. This observation is consistent with recent molecular data that indicate LIN-56 and LIN-15A are each required for the stability of the other *in vivo* and bind each other *in vitro*, supporting the hypothesis that LIN-15A and LIN-56 function in a protein complex (E. M. DAVISON and H. R. HORVITZ, unpublished results). We suggest that our genetic enhancement tests have identified groups of genes that function together in protein complexes or that act sequentially in a pathway.

From our genetic enhancement tests of class B synMuv genes, we identified one pair of genes that had not been found previously to act together, *met-1* and *let-418*. The two histone methyltransferases *met-1* and *met-2* act redundantly to control vulval cell-fate inhibition; *met-1* is similar to the yeast histone H3 lysine 36 methyltransferase Set2p (ANDERSEN and HORVITZ 2007). *let-418* encodes the *C. elegans* homolog of human Mi2, an ATP-dependent chromatin-remodeling enzyme found in the NuRD complex (VON ZELEWSKY *et al.* 2000). To date, neither MET-1 nor its homologs have been found in NuRD-like complexes. Perhaps the NuRD-like complex requires MET-1 to function in the inhibition of vulval fates. For example, MET-1 might methylate histone H3 lysine 36 at genes that normally promote vulval cell fates, leading to recruitment of the NuRD-like complex and transcriptional repression.

It is possible that members of a protein complex could display synthetic enhancement. However, genes that display synthetic enhancement cannot encode

proteins that work as essential components of the same complex (*i.e.*, if either component is absent, the complex has no function) or as members of a linear pathway in which each step requires the prior step for activity (*i.e.*, if any component is absent, the pathway has no output). Many class B synMuv proteins, including LIN-9, LIN-35, LIN-37, LIN-52, LIN-53, LIN-54, DPL-1, and EFL-1, are components of the *C. elegans* DRM complex (HARRISON *et al.* 2006). Nonetheless, most double mutants among *lin-9*, *lin-35*, *lin-37*, *lin-52*, and *lin-53* showed weak redundancy, suggesting that most proteins in the DRM complex have redundant functions during vulval development. These results suggest that either the DRM complex is composed of members that can functionally substitute for each other or the complex (which was isolated from embryos) does not function in the inhibition of vulval cell fates. The synthetic interactions between many DRM complex members were much weaker than interactions between non-DRM complex members, suggesting that complex components might have subtle redundant functions.

**Genetic enhancement tests can identify functionally related groups of evolutionarily conserved but uncharacterized genes:** The gene *lin-3*, which encodes the EGF ligand for the receptor tyrosine kinase RTK/Ras signal transduction cascade that drives vulval development, is a transcriptional target of some synMuv proteins (CUI *et al.* 2006). We found that all of the synMuv genes we examined repress *lin-3* transcription. Each set of synMuv genes we hypothesize to act together in a single process to inhibit vulval cell fates might mediate a specific mechanism of *lin-3* transcriptional repression. For example, two sets of class B synMuv genes, *dpl-1*, *eft-1*, and *lin-35*; and *mys-1* and *trr-1*, probably act redundantly to control two different processes of transcriptional repression, namely the DNA binding of a transcriptional repressor and histone acetylation, respectively. We suggest that a third set of class B synMuv genes, *let-418* and *met-1*, mediates a movement and methylation of histones important for transcriptional repression in a process distinct from those of the previous two examples.

An understanding of the distinct mechanisms used by different sets of synMuv genes in *lin-3* repression could define how the homologs of these genes control repression of target genes in other organisms. We suggest that genetic enhancement studies of additional synMuv gene interactions might implicate the homologs of conserved but uncharacterized synMuv genes in well-characterized processes and in this way facilitate the understanding of how these genes act and of how they interact with other gene processes in both development and disease.

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## LITERATURE CITED

- ANDERSEN, E. C., and H. R. HORVITZ, 2007 Two *C. elegans* histone methyltransferases repress *lin-3* EGF transcription to inhibit vulval development. *Development* **134**: 2991–2999.
- ANDERSEN, E. C., X. LU and H. R. HORVITZ, 2006 *C. elegans* ISWI and NURF301 antagonize an Rb-like pathway in the determination of multiple cell fates. *Development* **133**: 2695–2704.
- BANDARA, L. R., V. M. BUCK, M. ZAMANIAN, L. H. JOHNSTON and N. B. LA THANGUE, 1993 Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. *EMBO J.* **12**: 4317–4324.
- BEITEL, G. J., E. J. LAMBIE and H. R. HORVITZ, 2000 The *C. elegans* gene *lin-9*, which acts in an Rb-related pathway, is required for gonadal sheath cell development and encodes a novel protein. *Gene* **254**: 253–263.
- BRENNER, S., 1974 The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71–94.
- CEOL, C. J., and H. R. HORVITZ, 2001 *dpl-1* DP and *egl-1* E2F act with *lin-35* Rb to antagonize Ras signaling in *C. elegans* vulval development. *Mol. Cell* **7**: 461–473.
- CEOL, C. J., and H. R. HORVITZ, 2004 A new class of *C. elegans* synMuv genes implicates a Tip60/NuA4-like HAT complex as a negative regulator of Ras signaling. *Dev. Cell* **6**: 563–576.
- CEOL, C. J., F. STEGMEIER, M. M. HARRISON and H. R. HORVITZ, 2006 Identification and classification of genes that act antagonistically to *let-60* Ras signaling in *Caenorhabditis elegans* vulval development. *Genetics* **173**: 709–726.
- CLARK, S. G., X. LU and H. R. HORVITZ, 1994 The *Caenorhabditis elegans* locus *lin-15*, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. *Genetics* **137**: 987–997.
- COUTEAU, F., F. GUERRY, F. MULLER and F. PALLADINO, 2002 A heterochromatin protein 1 homologue in *Caenorhabditis elegans* acts in germline and vulval development. *EMBO Rep.* **3**: 235–241.
- CUI, M., J. CHEN, T. R. MYERS, B. J. HWANG, P. W. STERNBERG *et al.*, 2006 SynMuv genes redundantly inhibit *lin-3*/EGF expression to prevent inappropriate vulval induction in *C. elegans*. *Dev. Cell* **10**: 667–672.
- DAVISON, E. M., M. M. HARRISON, A. J. WALHOUT, M. VIDAL and H. R. HORVITZ, 2005 *lin-8*, which antagonizes *Caenorhabditis elegans* Ras-mediated vulval induction, encodes a novel nuclear protein that interacts with the LIN-35 Rb protein. *Genetics* **171**: 1017–1031.
- DUFOURCQ, P., M. VICTOR, F. GAY, D. CALVO, J. HODGKIN *et al.*, 2002 Functional requirement for histone deacetylase 1 in *Caenorhabditis elegans* gonadogenesis. *Mol. Cell. Biol.* **22**: 3024–3034.
- EDGLEY, M. L., and D. L. RIDDLE, 2001 LG II balancer chromosomes in *Caenorhabditis elegans*: *mT1(II/III)* and the *mIn1* set of dominantly and recessively marked inversions. *Mol. Genet. Genomics* **266**: 385–395.
- FERGUSON, E. L., and H. R. HORVITZ, 1985 Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode *Caenorhabditis elegans*. *Genetics* **110**: 17–72.
- FERGUSON, E. L., and H. R. HORVITZ, 1989 The multivulva phenotype of certain *Caenorhabditis elegans* mutants results from defects in two functionally redundant pathways. *Genetics* **123**: 109–121.
- FRASER, A. G., R. S. KAMATH, P. ZIPPERLEN, M. MARTINEZ-CAMPOS, M. SOHRMANN *et al.*, 2000 Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408**: 325–330.
- HARRISON, M. M., C. J. CEOL, X. LU and H. R. HORVITZ, 2006 Some *C. elegans* class B synthetic multivulva proteins encode a conserved LIN-35 Rb-containing complex distinct from a NuRD-like complex. *Proc. Natl. Acad. Sci. USA* **103**: 16782–16787.
- HARRISON, M. M., X. LU and H. R. HORVITZ, 2007a LIN-61, one of two *Caenorhabditis elegans* malignant-brain-tumor-repeat-containing proteins, acts with the DRM and NuRD-like protein complexes in vulval development but not in certain other biological processes. *Genetics* **176**: 255–271.
- HARRISON, R., B. PAPP, C. PAL, S. G. OLIVER and D. DELNERI, 2007b Plasticity of genetic interactions in metabolic networks of yeast. *Proc. Natl. Acad. Sci. USA* **104**: 2307–2312.
- HELIN, K., C. L. WU, A. R. FATTAEY, J. A. LEES, B. D. DYNLACHT *et al.*, 1993 Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. *Genes Dev.* **7**: 1850–1861.
- HILL, R. J., and P. W. STERNBERG, 1992 The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans*. *Nature* **358**: 470–476.
- HOPPER, N. A., J. LEE and P. W. STERNBERG, 2000 ARK-1 inhibits EGFR signaling in *C. elegans*. *Mol. Cell* **6**: 65–75.
- HSIEH, J., J. LIU, S. A. KOSTAS, C. CHANG, P. W. STERNBERG *et al.*, 1999 The RING finger/B-box factor TAM-1 and a retinoblastoma-like protein LIN-35 modulate context-dependent gene silencing in *Caenorhabditis elegans*. *Genes Dev.* **13**: 2958–2970.
- HUANG, L. S., P. TZOU and P. W. STERNBERG, 1994 The *lin-15* locus encodes two negative regulators of *Caenorhabditis elegans* vulval development. *Mol. Biol. Cell* **5**: 395–411.
- IKURA, T., V. V. OGRYZKO, M. GRIGORIEV, R. GROISMAN, J. WANG *et al.*, 2000 Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. *Cell* **102**: 463–473.
- KAMATH, R. S., A. G. FRASER, Y. DONG, G. POULIN, R. DURBIN *et al.*, 2003 Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421**: 231–237.
- KREK, W., D. M. LIVINGSTON and S. SHIRODKAR, 1993 Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members. *Science* **262**: 1557–1560.
- LU, X., and H. R. HORVITZ, 1998 *lin-35* and *lin-53*, two genes that antagonize a *C. elegans* Ras pathway, encode proteins similar to Rb and its binding protein RbAp48. *Cell* **95**: 981–991.
- MATHIES, L. D., S. T. HENDERSON and J. KIMBLE, 2003 The *C. elegans* Hand gene controls embryogenesis and early gonadogenesis. *Development* **130**: 2881–2892.
- OOI, S. L., D. D. SHOEMAKER and J. D. BOEKE, 2003 DNA helicase gene interaction network defined using synthetic lethality analyzed by microarray. *Nat. Genet.* **35**: 277–286.
- PARK, E. C., and H. R. HORVITZ, 1986 Mutations with dominant effects on the behavior and morphology of the nematode *Caenorhabditis elegans*. *Genetics* **113**: 821–852.
- POULIN, G., Y. DONG, A. G. FRASER, N. A. HOPPER and J. AHRINGER, 2005 Chromatin regulation and sumoylation in the inhibition of Ras-induced vulval development in *Caenorhabditis elegans*. *EMBO J.* **24**: 2613–2623.
- RIDDLE, D. L., T. BLUMENTHAL, B. J. MEYER and J. R. PRIESS, 1997 *C. elegans II*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- SIEBER, P., F. WELLMER, J. GHEYSSELINCK, J. L. RIECHMANN and E. M. MEYEROWITZ, 2007 Redundancy and specialization among plant microRNAs: role of the MIR164 family in developmental robustness. *Development* **134**: 1051–1060.
- SOLARI, F., and J. AHRINGER, 2000 NURD-complex genes antagonise Ras-induced vulval development in *Caenorhabditis elegans*. *Curr. Biol.* **10**: 223–226.
- STERNBERG, P. W., 2006 Pathway to RAS. *Genetics* **172**: 727–731.
- SULSTON, J. E., and H. R. HORVITZ, 1977 Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev. Biol.* **56**: 110–156.
- SUNDARAM, M. V., 2005 The love-hate relationship between Ras and Notch. *Genes Dev.* **19**: 1825–1839.
- THOMAS, J. H., C. J. CEOL, H. T. SCHWARTZ and H. R. HORVITZ, 2003 New genes that interact with *lin-35* Rb to negatively regulate the *let-60* ras pathway in *Caenorhabditis elegans*. *Genetics* **164**: 135–151.
- THOMAS, J. H., and H. R. HORVITZ, 1999 The *C. elegans* gene *lin-36* acts cell autonomously in the *lin-35* Rb pathway. *Development* **126**: 3449–3459.
- TONG, A. H., M. EVANGELISTA, A. B. PARSONS, H. XU, G. D. BADER *et al.*, 2001 Systematic genetic analysis with ordered arrays of yeast deletion mutants. *Science* **294**: 2364–2368.

- TONG, A. H., G. LESAGE, G. D. BADER, H. DING, H. XU *et al.*, 2004 Global mapping of the yeast genetic interaction network. *Science* **303**: 808–813.
- TSENG, R. J., K. R. ARMSTRONG, X. WANG and H. M. CHAMBERLIN, 2007 The bromodomain protein LEX-1 acts with TAM-1 to modulate gene expression in *C. elegans*. *Mol. Genet. Genomics* **278**: 507–518.
- UNHAVAITHAYA, Y., T. H. SHIN, N. MILIARAS, J. LEE, T. OYAMA *et al.*, 2002 MEP-1 and a homolog of the NURD complex component Mi-2 act together to maintain germline-soma distinctions in *C. elegans*. *Cell* **111**: 991–1002.
- VON ZELEWSKY, T., F. PALLADINO, K. BRUNDSCHWIG, H. TOBLER, A. HAJNAL *et al.*, 2000 The *C. elegans* Mi-2 chromatin-remodelling proteins function in vulval cell fate determination. *Development* **127**: 5277–5284.
- WINZELER, E. A., D. D. SHOEMAKER, A. ASTROMOFF, H. LIANG, K. ANDERSON *et al.*, 1999 Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* **285**: 901–906.

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