The Synthetic Multivulval Genes of *C. elegans:* Functional Redundancy, Ras-Antagonism, and Cell Fate Determination

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Summary: Development of the *C. elegans* vulva requires coordination between a strikingly complex set of molecular regulators and pathways. In particular, the correct specification of vulval cell-fates requires both the activation of RTK/Ras/Map kinase members as well as negative regulation by a set of genes known as the SynMuvs. SynMuvs comprise two functionally redundant sets of genes that appear to antagonize Ras pathway signaling. In this way, SynMuv genes act to limit the number of cells adopting vulval fates. Recently, a number of SynMuv genes have been shown to encode worm homologs of the Rb transcriptional-regulatory complex. These and other results are discussed and we present several models for understanding the role of SynMuv genes in vulval development.   genesis 26:279–284, 2000.   © 2000 Wiley-Liss, Inc.

INTRODUCTION

Studies over the past few years have shown that knockout mutations in a significant percentage of *C. elegans* genes produce no obvious mutant phenotype. For some genes, their roles, which are not essential for viability or major aspects of development, may be revealed only under certain physiological conditions or by specific assays. For many other genes, their functions in developmental or cellular processes could be redundant and unmasked only when the activities of other genetic components are compromised.

Enhancer or suppressor screens, commonly used in genetic organisms such as *Drosophila* and *C. elegans*, are effective ways to reveal the functions of many of these genes. So-called "synthetic" mutant phenotypes have also been explored to study genes with redundant functions. When present as single mutations, synthetic mutants may appear completely wild type. However, when these same mutations are combined, the resultant double-mutant animals display an easily detectable phenotype. This redundancy itself may testify to the importance of such genes, since the organism may have evolved multiple strategies to ensure the proper functioning of an important biological process.

Perhaps the best example of this phenomenon in *C. elegans* is the Synthetic Multivulval genes, or SynMuvs. While wild-type *C. elegans* hermaphrodites contain a single vulva or "egg-laying" organ, SynMuv mutants may possess three or more vulval-like structures. SynMuv mutations fall into one of two classes, A or B. Neither A- nor B-class mutants generally display defects on their own or in combination with mutations of the same class (Ferguson and Horvitz, 1989). The SynMuv phenotype is revealed, however, when mutations are present in genes from both classes (Fig. 1) (Ferguson and Horvitz, 1989). Recently, several class B genes were identified as components of the Rb transcriptional regulatory-complex (Lu and Horvitz, 1998). This finding raises the possibility of cross talk between cell-cycle/transcriptional controllers and known regulators of vulval development including members of the RTK/Ras/Map kinase pathway. Below we will attempt to summarize recent findings as well as to suggest possible models for understanding the SynMuv phenotype.

VULVAL DEVELOPMENT

The wild-type *C. elegans* vulva is derived from three hypodermal precursor cells (P5.p, P6.p, and P7.p), which reside along the ventral midline in the region of the future vulva (Sulston and Horvitz, 1977). Three adjacent cells, two anterior (P3.p, P4.p) and one posterior (P8.p), also possess the ability to generate vulval tissue but normally acquire hypodermal fates. Collectively, P3.p–P8.p are referred to as vulval precursor cells (VPCs), reflecting their shared developmental potential.

The event that induces specific VPCs to adopt vulval fates is triggered by the EGF-like ligand, LIN-3, which

Genotype	Phenotype
Class A + Class B +	Wild type
Class A + Class B −	Wild type
Class A − Class B +	Wild type
Class A − Class B −	Muv
Ras(gf)	Muv
Ras(lf)	Vul
Class A − Class B − Ras(lf)	Vul

FIG. 1. Phenotypes of single and combination mutants. Muv, multivulval; Vul, vulvaless; gf, gain-of-function; lf, loss-of-function. Animals containing single mutations in either class A or class B SynMuv genes are wild type in appearance. Animals with mutations in both class A and B SynMuv genes are Muv, as are animals containing Ras(gf) mutations. In contrast, Ras(lf) alleles result in animals that are Vul. The Vul phenotype of Ras(lf) mutations is epistatic to the Muv phenotype of class A and B SynMuv double mutants, i.e., triple mutants are Vul.

stimulates a conserved RTK/Ras/Map kinase pathway in the targeted cells (reviewed by Greenwald, 1997). VPC fates are determined in part by their proximity to the source of the signal, the anchor cell, which is positioned directly over P6.p. This results in P6.p receiving the greatest LIN-3 signal, while P5.p and P7.p receive lower but sufficient doses to induce vulval fates. In contrast, P3.p, P4.p, and P8.p presumably receive subthreshold doses of LIN-3 and consequently fail to acquire vulval fates (Sternberg and Horvitz, 1986; Hill and Sternberg, 1992) (Fig. 2A). Removal of the LIN-3 signal by laser ablation of the anchor cell (Kimble, 1981) or by loss-of-function mutations in the *lin-3* gene (Horvitz and Sulston, 1980) results in all VPCs adopting epidermal fates (Fig. 2B).

In SynMuv double mutants, all six VPCs adopt vulval fates, leading to the Multivulval (Muv) phenotype (Fig. 2c) (Ferguson and Horvitz, 1989). This finding has led researchers to postulate that SynMuv genes comprise two redundant pathways that function to inhibit the expression of vulval cell fates. In the case of P5.p–P7.p, these inhibitory effects are overcome by LIN-3 activation. Muv phenotypes are also observed in animals that contain gain-of-function (gf) mutations in downstream effectors of the LIN-3 signal such as *let-60/*Ras (reviewed by Sternberg and Han, 1998).

The initial identification of SynMuv mutants by Horvitz and colleagues is a testimony to both serendipity and the power of a well-executed genetic analysis. In one screen, a Muv animal was identified whose phenotype was shown to depend on two unlinked (and otherwise silent) mutations (Horvitz and Sulston, 1980). This *lin-8;*

lin-9 double-mutant strain demonstrated that a Muv phenotype could result from synthetic interactions. Ferguson and Horvitz (1989) and others have subsequently screened systematically for additional SynMuv genes, leading to the identification of the majority of SynMuv mutations known today (Fig. 3).

An additional side note of interest was the discovery of *lin-15a* and *lin-15b.* These two genes are positioned tandemly in an operon on the X chromosome and encode distinct proteins of class A and B types, respectively. Several of the early *lin-15* alleles (Ferguson and Horvitz, 1985) turned out to be deletions that abolished or reduced the activities of both genes, leading to a Muv phenotype (Clark *et al.,* 1994; Huang *et al.,* 1994). Given the unusually tight linkage displayed by *lin-15a/b,* it was not until single mutations had been isolated in each gene that the dual nature of the original mutations, and their classification as SynMuv genes, became apparent (Ferguson and Horvitz, 1989).

REQUIREMENT FOR RAS PATHWAY FUNCTION

Epistasis analyses indicate that an intact RTK/Ras/Map kinase pathway is essential for expression of the SynMuv phenotype (Lu and Horvitz, 1998). For example, in triple mutants where class A and B SynMuv mutations are combined with a Ras loss-of-function mutation (lf), animals are vulvaless (Vul) (Fig. 1). In fact, many mutations in the RTK/Ras/Map kinase pathway were isolated as suppressors of the *lin-15a/b* Muv phenotype (reviewed by Sternberg and Han, 1998). Curiously, inhibition of LIN-3 signaling, either through lf mutations (Ferguson *et al.,* 1987; P. Sternberg, personal communication) or by removal of its source (Sternberg and Horvitz, 1989), does not block expression of the SynMuv phenotype (Fig. 2D). This has been interpreted to mean that in the absence of SynMuv gene function, basal (non-ligand-induced) levels of Ras signaling are sufficient to induce vulval fates (Thomas and Horvitz, 1999). The function of SynMuv genes is therefore to oppose basal levels of Ras pathway signaling in VPCs, thereby necessitating the induction event by LIN-3.

CONNECTION TO TRANSCRIPTION

Recent work by Lu and Horvitz (1998) has implicated a *C. elegans* Rb homolog as well as several associated factors in the SynMuv pathway. *lin-35,* a class B SynMuv gene, encodes a predicted protein with 15% identity to Rb and about 20% identity to Rb-associated proteins p107 and p130. Another class B SynMuv gene, *lin-53,* encodes a protein closely related to the Rb binding proteins RbAp46 and RbAp48 (70% and 72% identity, respectively). In mammalian systems, Rb forms a protein complex that inhibits the activity of E2F transcription factors, which are required for the expression of DNA synthesis genes and entry into S-phase (reviewed by Kaelin, 1999). Lu and Horvitz (1998) further showed that inactivation of an additional component of the Rb com-
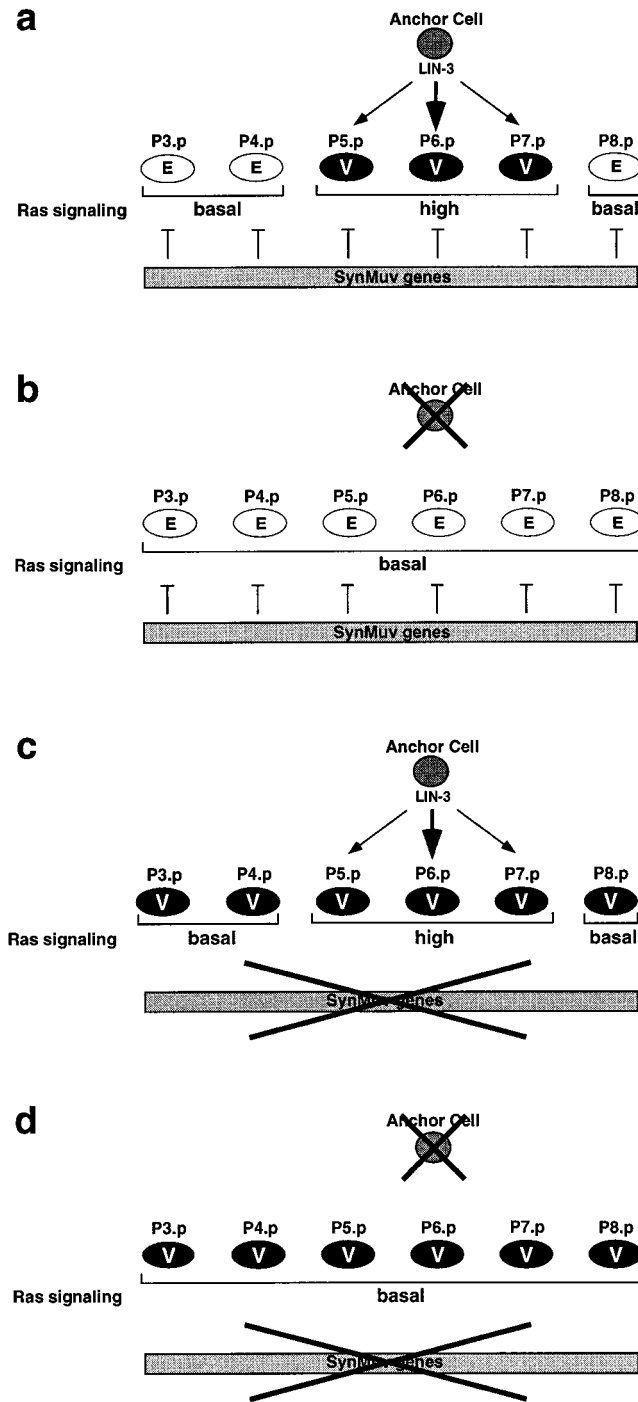
FIG. 2. VPC specification in wild-type and mutant backgrounds. **(a)** During wild-type vulval development, LIN-3 signaling stimulates Ras/Map kinase pathway activity in P5.p–P7.p, leading to the adoption of vulval cell fates (V). Basal (noninduced) levels of Ras/Map kinase activity in P3.p, P4.p, and P8.p are not sufficient to overcome the inhibitory effects of SynMuv genes, leading to adoption of epidermal fates (E). **(b)** Removal of the anchor cell abolishes LIN-3 signaling, resulting in basal levels of Ras/Map kinase activity in P3.p–P8.p and the uniform adoption of epidermal fates. **(c)** In the absence of SynMuv class A and B functions, basal levels of Ras/Map kinase activity in P3.p, P4.p, and P8.p are sufficient to induce vulval cell fates. **(d)** In the absence of both the LIN-3 signal and SynMuv function, basal levels of Ras/Map kinase activity are sufficient to induce vulval fates in P3.p–P8.p.

plex, histone deacetylase (*hda-1*), also caused a SynMuv phenotype when coupled to a class A mutation. Taken together, these results suggest that class B SynMuv genes act through transcriptional inhibition.

Four additional class B genes and one class A gene have been identified. Two of the class B genes (*tam-1* and *lin-13*) appear to encode transcriptional regulators (Hsieh *et al.,* 1999; Melendez and Greenwald, 2000), while two others (*lin-15b* and *lin-36*) encode novel proteins (Huang *et al.,* 1994; Clark *et al.,* 1994; Thomas and Horvitz, 1999). Analysis of *tam-1* mutations suggests that in certain contexts *tam-1* may function as a positive regulator of transcription. Specifically, expression levels of transgenes contained within repetitive extrachromosomal arrays were dramatically reduced in lf *tam-1* mutants (Hsieh *et al.,* 1999). In contrast, the expression of genes within nonrepetitive arrays, as well as several endogenous genes tested, showed little or no change. This finding led the authors to speculate that *tam-1* may normally promote the transcription of genes that reside in chromosomal regions containing repetitive elements. Interestingly, several other class B mutants, including *lin-35,* displayed a similar defect (Hsieh *et al.,* 1999). It is unclear how class B genes might act positively in the case of transgene expression and negatively in the case of genes required for vulval specification. Therefore, the role of class B genes in this process is at present unresolved.

Another putative transcriptional regulator, *lin-13,* encodes a predicted protein with 14 zinc-fingers as well as a putative Rb binding domain (Melendez and Greenwald, 2000). Whether *lin-13* acts as a positive or negative regulator of transcription is currently unknown. Unlike other class B genes, which do not display a Muv phenotype on their own, *lin-13* single mutations confer a temperature-sensitive Muv phenotype (Ferguson *et al.,* 1985; Ferguson and Horvitz, 1987; Melendez and Greenwald, 2000). The function of *lin-13* as a class B SynMuv gene is revealed when the Muv phenotype is dramatically enhanced at the permissive temperature (15°C) by the presence of a class A mutation. It is thus possible that *lin-13* possesses both class A and B functions, and if that is the case, class A gene functions may also relate to transcriptional control. At present, only a single class A gene has been reported to be cloned; *lin-15a* encodes a predicted novel protein and thus the biochemical function of this class of proteins remains unknown (Huang *et al.,* 1994; Clark *et al.,* 1994).

SITES OF ACTION

Mosaic analysis using a deletion mutation that abolishes both *lin-15a* and *lin-15b* activities had previously implicated the hypodermis as the site of SynMuv gene function (Herman and Hedgecock, 1990). Namely, *lin-15a/b* function was not required autonomously in VPCs for repression of vulval fates, but rather appeared to be required in the surrounding hypodermal cells. Similar results were also obtained in a mosaic analysis of the class B SynMuv gene *lin-37* (Hedgecock and Herman,

Gene	Molecular Identity
Class A	
lin-8	Unknown
lin-15A	Novel (Clark et al., 1994; Huang et al., 1994)
lin-38	Unknown
lin-56	Unknown
Class B	
lin-9	Unknown
lin-13	Zinc-finger protein w/Rb binding motif (Melendez and Greenwald, 2000)
lin-15B	Novel
lin-35	Similar to Rb, p107, p130 (Lu and Horvitz, 1998)
lin-36	Novel (Thomas and Horvitz, 1999)
lin-37	Unknown
lin-51	Unknown
lin-52	Unknown
lin-53	Similar to Rb-binding proteins RbAp46 and RbAp48 (Lu and Horvitz, 1998)
lin-54	Unknown
lin-55	Unknown
hda-1	Histone deacetylase (Lu and Horvitz, 1998)
tam-1	Ring Finger/B-box factor (Hsieh et al., 1999)

FIG. 3. Gene names and molecular identities of class A and B SynMuv mutants. A lf mutation in any class A gene combined with a lf mutation in any class B gene results in animals that display a Muv phenotype. For further details, see text.

1995). These findings suggested that the hypodermis may normally prevent VPCs from acquiring vulval cell fates, possibly via a cell signaling pathway. Consistent with this, *lin-13* is expressed strongly in the hypodermis and only weakly in the vulval precursor cells (Melendez and Greenwald, 2000), although mosaic analysis will be needed to clearly determine the site of *lin-13* function. However, the finding that other class B genes (*lin-35, lin-36,* and *lin-53*) are expressed strongly in VPCs is more consistent with an autonomous function (Lu and Horvitz, 1998; Thomas and Horvitz, 1999). Moreover, mosaic analysis of *lin-36* indicates that its activity is required within VPCs (Thomas and Horvitz, 1999). One possible resolution is that such genes may act downstream of the hypodermal signal, which requires *lin-37* and *lin-15a/b* function. However, no SynMuv genes have yet been identified that would be predicted to function in a cell signaling pathway. Because expression of class B genes has been observed in a large number of cell types during development, it is possible that class A genes may confer specificity to VPCs in particular. However, the expression pattern of class A genes has not yet been reported.

ADDITIONAL ASPECTS

Two other properties of SynMuv genes are worth noting. SynMuv mutants often display a more pene-

trant phenotype at higher temperatures (Ferguson and Horvitz, 1989). This effect appears to be dependent on the specific combination of class A and B genes assayed; some double mutants are nearly wild type at 15°C and 100% Muv at 20°C, while other combinations are 100% Muv at both temperatures. In the case of *lin-13,* the temperature-sensitive nature of the Muv phenotype is associated with null mutants, indicating that the process in which *lin-13* functions is itself temperature sensitive (Melendez and Greenwald, 2000). In addition, many SynMuv double mutants display lethality or sterility when grown at 25°C, suggesting that these genes also function in other developmental processes (Ferguson and Horvitz, 1989).

An additional feature of SynMuv mutants is that they generally show pronounced maternal effects (Ferguson and Horvitz, 1989). Specifically, the double-mutant progeny of heterozygous parents usually display a less severe phenotype than double-mutant animals derived from homozygous mutant parents. That maternal products should survive into late larval stages to affect developmental events has been observed for a number of genes in *C. elegans,* including cell-cycle regulators and genes involved in DNA synthesis and mitosis (Albertson *et al.,* 1978; Sulston and Horvitz, 1981; D.F., J. Yoder., W. Zhang, and M.H., unpublished observations).

MODELS AND CONCLUSIONS

Given the established role of several class B genes in transcriptional repression, Horvitz and colleagues have proposed that SynMuv genes act by directly inhibiting the expression of genes required for the generation of vulval cells (Lu and Horvitz, 1998) (Fig. 4A). In this model, SynMuv genes would oppose RTK/Ras/Map kinase pathway signaling at a common target. However, the effect of SynMuv genes on transcription would most likely be global and not limited to a single class of genes such as those involved in vulval specification. Therefore, a reasonable extension of this model is that a nonspecific reduction in gene expression is sufficient (or at least necessary) to prevent vulval cell differentiation.

It is also possible that SynMuv genes, particularly class B genes, act on vulval development through an indirect mechanism. For example, Rb and its associated proteins have well-documented roles in the regulation of the G1-S-phase transition (reviewed by Kaelin, 1999). Therefore, mutations in class B genes could perturb certain aspects of the cell cycle or cell growth, which in turn may predispose VPCs to adopt vulval fates (Fig. 4B). In fact, mutations in cyclin E lead to an increase in the adoption of vulval cell fates and enhance the Muv phenotype of a *lin-15a/b* temperature-sensitive allele (Fay and Han, unpublished observations). Mutations in such genes could in theory act by extending the length of the cell-cycle window during which VPCs are susceptible to receiving signals or adopting vulval fates.

Another possibility is that SynMuv genes may function to ensure the fusion of noninduced VPCs with the hypodermal syncitium (Fig. 4C) (Herman and Hedgecock, 1990). In wild-type animals, the daughters of noninduced VPCs (P3.p, P4.p, and P8.p) normally fuse to the hyp-7 syncitium, while the daughters of induced VPCs (P5.p, P6.p, and P7.p) do not undergo fusion and continue dividing (reviewed by Greenwald, 1997). Thus stated, LIN-3 induction may prevent P5.p–P7.p from fusing with the surrounding syncitium. Nonfused VPCs would then execute a "default" differentiation pathway, namely vulval development. If SynMuv mutants fail to execute the fusion step, then all VPCs would adopt vulval fates. This hypothesis is appealing in that the hyp-7 syncitium has been implicated as one of the sites for SynMuv gene function.

An important point is that multiple mechanisms could be operating in the generation of the SynMuv phenotype. This possibility seems even more likely given that two distinct classes of mutants are required for the expression of the phenotype. The basic observation that class A and B genes are genetically "redundant" does not mean that both classes carry out identical biological functions. Malfunctions in two distinct pathways could converge to produce the observed defect. For example, the combination of cell-cycle and transcriptional defects could interfere with cell fusion, if both timing and gene expression are critical to this process. Clearly more work
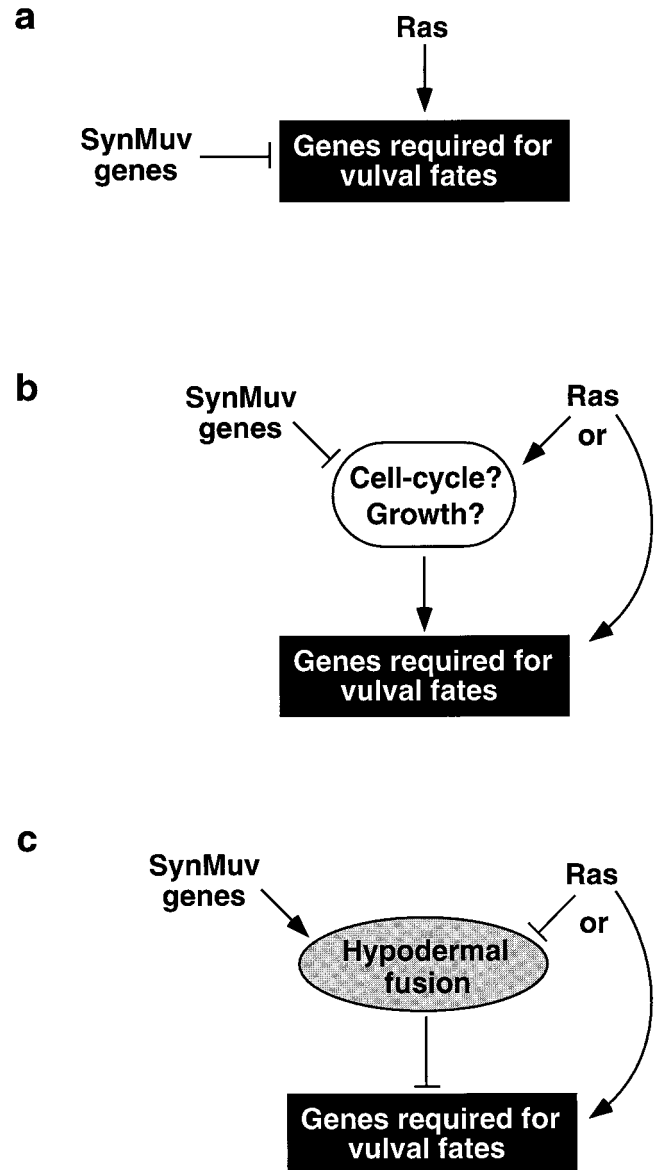

FIG. 4. Speculative models to explain SynMuv gene function. **(a)** SynMuv genes may prevent VPCs from adopting vulval fates by directly inhibiting the transcription of genes required for vulval cell-fate specification. **(b)** SynMuv genes may repress vulval fates through an indirect means such as cell-cycle regulation. **(c)** SynMuv genes may act by promoting fusion of VPCs to the hypodermis, thereby preventing VPCs from adopting vulval cell fates. In this model, SynMuv gene function may be required in hypodermal cells, VPCs, or both. For further details, see text.

will be necessary to sort out these possibilities and provide a more precise picture of how these processes relate to one another.

The era of facile genetic screens in *C. elegans* has largely been played out. Future progress will increasingly rely on the ability of researchers to exploit more complex and sophisticated phenotypes such as those revealed by silent or synthetic mutants. While this com-

plexity adds significantly to the difficulty of the analysis, such studies should lead to more detailed, albeit complex, descriptions of many important biological processes.

ACKNOWLEDGMENTS

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