Suppressors, Screens, and Genes: An Educational Primer for Use with "A Network of Genes Antagonistic to the LIN-35 Retinoblastoma Protein of *Caenorhabditis elegans*"

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SUMMARY An article by Polley and Fay in this issue of *GENETICS* provides an excellent opportunity to introduce or reinforce concepts of reverse genetics and RNA interference, suppressor screens, synthetic phenotypes, and phenocopy. Necessary background, explanations of these concepts, and a sample approach to classroom use of the original article, including discussion questions, are provided.

Related article in *GENETICS*: Polley, S. R. G., and D. S. Fay, 2012 A network of genes antagonistic to the LIN-35 retinoblastoma protein of *Caenorhabditis elegans*. Genetics **191:** 1367–1380.

Background

Polley and Fay use the model genetic organism *Caenorhabditis elegans* to discover how the retinoblastoma (Rb) protein and other gene products can affect intestinal function. The authors employ an elegant and effective reverse genetic approach that identifies gene products that function in the same cellular pathways as the Rb protein. This article exemplifies ways in which a powerful genetic approach can be employed to discover new genetic and cellular interactions.

C. elegans as a model system

C. elegans is a small (~1 mm long) roundworm that lives in soil and compost, where it eats bacteria. It has been studied in the laboratory since the 1970s, where it also feeds on bacteria. The worm, as it is affectionately known, develops from a fertilized egg that hatches as a tiny worm at the first larval stage, called L1 larva. The L1 larva grows by cell division and then sheds its outer cuticle to become an L2 larva. This process repeats through L3 and L4 larval stages until the worm becomes an adult capable of reproduction.

The DNA sequence of the genome of *C. elegans* is known, and researchers can add extra genes to worms by microinjecting DNA into the ovary, where eggs are developing. Some of the progeny of the microinjected worm will incorporate this DNA into their nuclei in the form of an extrachromosomal array—many end-to-end copies of the added gene(s)—that is not in a chromosome. Perhaps because the array of genes does not have a centromere, as many as 30% of the offspring of a transgenic worm will not contain the added genes due to loss of the array during meiosis or in the early cell divisions of gametogenesis. Polley and Fay use gene array loss to their advantage in this experiment.

C. elegans is a simple animal, with an invariant number of cells that compose several types of tissues, including muscle, sensory and nonsensory neurons, intestine, gonads, and epithelial tissue. Because *C. elegans* is a self-fertile hermaphrodite (the same animal produces haploid sperm and haploid eggs) genetic experiments are easily designed and matings with (rare) males are not strictly required.

A word about nomenclature: gene names are written in lowercase italic letters (*lin-35*), and genes are often named on the basis of the phenotype seen when the gene is inactivated (*e.g.*, "Lin" stands for cell ***lin***eage defect). Protein names are given in uppercase non-italic letters, *e.g.*, LIN-35. Genes that have not been studied in detail are labeled with a clone number, *e.g.*, W07E6.2.

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Transcriptional control of gene expression and Rb function

To transcribe a gene in a multi-cellular organism, tissue-specific transcription factors bind to DNA sequences, such as enhancers, and recruit general transcription factors and RNA polymerase to the promoter of the gene. Tissue-specific transcription factors may also recruit proteins that alter chromatin structure, thereby making the promoter and enhancer more or less accessible to other transcription factors. Tissue-specific transcription factors usually have at least three important protein domains: a DNA-binding domain, an activation domain that recruits general transcription factors, and a modulator domain that can be bound by a regulator that can, as the name suggests, alter the activity of a transcription factor by various mechanisms.

Polley and Fay studied the role of a transcriptional regulator called Rb in mammals and LIN-35 in *C. elegans*. These two proteins have similar amino acid sequences and therefore likely have similar functions. Such similar proteins, and their genes found in different species, are called orthologs. The Rb protein binds to tissue-specific transcription factors (E2F proteins among them) to block the E2F activation domain and thereby inhibit transcription of genes that are activated by E2F. In mammals, this is one key function of Rb that leads to cell cycle arrest (and inhibition of carcinogenesis).

Why is this gene called *lin-35* in worms? As mentioned above, the "*lin*" part of the name is short for cell ***lin***eage defect while "35" indicates that it was the 35th gene in worms named for this phenotype. In the case of *lin-35*, the associated lineage defect leads to an increase in the number of cells that are programmed to become part of the egg-laying structure, termed the vulva. *lin-35* is a bit unusual, however, in that inactivation of both chromosomal copies of *lin-35* does not lead to a vulval cell-lineage defect. Rather, one of several additional genes must be inactivated in conjunction with *lin-35* to produce excess vulval cells. Because the lineage defect is observed only when two homozygous mutations are combined, the phenotype is classified as "synthetic." Here the term "synthetic" means synergistic because the phenotype is the result of a synergistic genetic interaction between two independent mutations, neither of which cause the vulval phenotype alone. For this reason, *lin-35* is referred to as a synthetic multivulval mutant (synMuv) because it leads to a multivulval phenotype when combined with certain other mutations. Throughout this article, the mutant genotypes considered are homozygous, meaning that both alleles of the gene contain the same mutation. Thus, a double mutant refers to an animal containing homozygous mutant alleles of two different genes.

Background research

David Fay's laboratory has been addressing several questions. In what cellular or organismal process is *lin-35* involved? What other genes and proteins are directly or indirectly involved in processes with *lin-35*? Researchers had earlier exposed *lin-35* mutant worms to mutagens to discover additional *lin-35* synthetic phenotypes (Fay *et al.* 2002). This forward genetic screen revealed an early larval (L1) arrest phenotype that Fay *et al.* learned is due to deficient intestinal function leading to starvation. One such second mutant gene was identified as *slr-2*, encoding a protein containing a zinc-finger domain typically found in transcription factors. It is important to note that the alleles of *lin-35* and *slr-2* used by Polley and Fay are null alleles—versions of each gene that make no functional protein product: the *lin-35* allele is a G→A base substitution that produces an in-frame stop codon; the *slr-2* allele alters an mRNA splice site that produces aberrantly spliced transcripts with premature stop codons (Kirienko *et al.* 2008). Kirienko and co-workers demonstrated that LIN-35 and SLR-2 have redundant functions in the intestine because intestinal deficiency is seen only in double-mutant animals (another synthetic phenotype). Polley and Fay (2012) again harnessed the power of a genetic approach to identify other genes involved in intestinal function by isolating genes that, when downregulated, allow the survival of *lin-35*; *slr-2* double-mutant worms. These genes are thus termed "suppressors of the *lin-35*; *slr-2* synthetic lethal phenotype." Remember that suppression is seen only when the product of the suppressor gene is downregulated or missing.

Unpacking the Difficult Bits

The RNA interference screen

Polley and Fay use RNA interference (RNAi) to decrease the expression of other genes in the worm while looking for worms that grow and develop past the L1 stage, thus escaping (or suppressing) the *lin-35*; *slr-2*-induced L1 arrest. This approach is called a "suppressor screen."

What is RNAi? RNAi is a process by which RNA molecules that can base-pair with a particular mRNA are expressed in a cell, initially as double-stranded RNA. The double-stranded RNA is then processed by cellular machinery, and complementary base pairing between one strand of the double-stranded RNA and an mRNA leads to degradation of the mRNA and a drastic reduction in the amount of protein product made from that mRNA. The worm has a cellular pathway (using RISC and Dicer, etc.) to use double-stranded RNA to degrade other RNAs as an antiviral response (Wilkins *et al.* 2005). Andy Fire and co-workers found that *C. elegans* can be fed bacteria expressing double-stranded RNA molecules and that the RNA in the ingested bacteria is passed to cells of the worm, where it is processed and then downregulates mRNAs to which it can base-pair (Timmons *et al.* 2001). Julie Ahringer and co-workers designed an RNAi library—a set of bacterial strains each expressing a different double-stranded RNA molecule, one strand of which is complementary to mRNA encoded by a single *C. elegans* gene (Fraser *et al.* 2000). Polley and Fay fed each bacterial strain to *lin-35*; *slr-2* mutant worms and screened their

offspring for growth beyond the L1 stage. If reduction or knockdown of expression of gene X allows the *lin-35*; *slr-2* double mutants to survive past the early larval stage, then gene X would be a suppressor of *lin-35*; *slr-2*-induced lethality. Loss of the gene X product reverses the mutant phenotype. Because all three genes affect the same phenotype, their gene products might function in the same cellular pathway, or they may be part of a multi-protein complex. This kind of suppressor screen is therefore a way to identify genes whose products function together in the same pathway or in compensatory pathways.

Alert readers will have realized that larval lethality is a pretty difficult phenotype with which to work. Because the *lin-35*; *slr-2* double-mutant worms die before they can mature and produce offspring, how does one collect double-mutant worms that have ingested the RNAi-inducing bacteria and that can produce offspring whose phenotype can be scored? Polley and Fay's solution was to add a wild-type copy of *lin-35* to the double-mutant worms on an extrachromosomal array. The array also carries another gene tagged with green fluorescent protein (GFP). Worms that contain the array can live past the larval stage due to the wild-type copy of *lin-35*, and some of their cells glow green due to GFP expression. Because the extrachromosomal array is not always stably inherited during meiosis, some offspring will lose it. About 30% of the offspring lose the array and are not green and die as young L1 larvae, *unless the lethality is suppressed by RNAi knockdown of another gene*.

Thus Polley and Fay placed several GFP-positive worms on 16,757 agar plates, each plate containing a bacterial strain expressing a different double-stranded RNA. They then looked for non-green offspring (that lost the gene array) that survived beyond the L1 larval stage. Because they knew which gene is targeted by the double-stranded RNA in each bacterial strain in the RNAi library, they automatically knew which gene is the (putative) suppressor of *lin-35*; *slr-2* larval arrest.

Suppressors

Although it is easy to understand the concept of suppression as a reversal of a mutant phenotype, understanding mechanisms of suppression is more difficult. The suppressors isolated by Polley and Fay are extragenic: they are mutations in genes other than *lin-35* and *slr-2*. One way to suppress null or loss-of-function alleles is with gain-of-function alleles in other genes. This is similar to having a "mutation" in a light switch that turns off all the lights in a room and then finding a dimmer switch (a "suppressor") at the other end of the room that can turn the lights up. Both the original light switch and the dimmer switch operate in the same circuit and therefore control the same outcome (phenotype). In this article, however, the suppressor phenotype is the result of knocking down gene expression with RNAi. In this case, imagine that there is a bracket holding a second light switch in our circuit in the "off" position. Removal of the bracket allows the switch to automatically pop into the "on" position. In this metaphor, the bracket could be a repressor of a gene or a negative regulator of a protein function. Removal of that repressor or regulator by RNAi would reverse the initial phenotype. Again, the suppressor screen identifies another component in the same circuit.

Similar circuits, such as signal transduction cascades, operate in cells, and the components of those cascades are often identified by suppressor and enhancer screens. The screen designed by Polley and Fay could identify suppressors of this type. Another type of suppressor was isolated by Polley and Fay. Returning to our metaphor of a darkened room as the "phenotype" to be suppressed, removing the cover from a lit lamp in the same room would do the trick. In this case, a parallel pathway of light generation is discovered by the suppressor screen. Polley and Fay identified genes that act as suppressors in pathways that are different from the *lin-35*; *slr-2* cellular circuit, as well as several that might be in the same functional circuit. An excellent on-line review by Hodgkin (2005) presents examples of other types of suppressors.

Suppressor characterization

WormBase (http://www.wormbase.org), a carefully maintained database of published knowledge about *C. elegans* genes, including what is known about orthologs of those genes in other organisms, was used to sort the suppressor genes into three functional classes: (1) genes involved in ribosome production; (2) genes already known to suppress a synMuv phenotype; and (3) genes encoding prohibitins, proteins that have multiple functions including cell cycle regulation and mitochondrial function. In addition, Polley and Fay found suppressors that did not fit into any of these categories.

Studying the genetic interactions of suppressors can provide information about the pathways in which they act. By undertaking RNAi of a suppressor gene in worm strains that contain the *lin-35*; *slr-2* mutations plus a null allele of another known suppressor, Polley and Fay determined whether reductions of suppressor proteins have additive effects. That is, they determined whether suppression is seen in a larger number of offspring when the products of two suppressor genes are reduced by mutation or RNAi compared to when only one mutant suppressor allele is present. Suppressors with additive effects likely act in slightly different pathways; suppressors with non-additive effects likely have the same mechanism of suppression. Using the light circuit metaphor again, non-additive suppressors each might remove the same light switch bracket while additive suppressors might remove brackets controlling different lights. To determine whether suppression is specific for a particular cellular process or is due to a fairly direct interaction with *lin-35* target genes, Polley and Fay measured the degree of suppression of six other *lin-35* synthetic phenotypes, for example, defects in gonad or germline development or defects in pharynx development. A suppressor gene with a direct interaction with *lin-35* target genes might be expected to suppress all or most *lin-35* synthetic phenotypes.

Connections to Genetics Concepts

The concept of suppression is often a difficult one for students, but it is worth teaching in some depth. Suppressor (and enhancer) screens contribute greatly to our understanding of biological processes by identifying genes with functions that we might otherwise never suspect. The suppressor screen is an excellent example of the power of the genetic approach. Polley and Fay mention a forward genetic screen (from phenotype > gene) undertaken in previous work and describe a reverse genetic screen (from gene > phenotype) using RNAi. The reverse genetic (RNAi) screen is the centerpiece technique of the article, followed by the initial characterization of suppressors identified by RNAi.

Polley and Fay use a synthetic phenotype, an unusual and sometimes difficult concept. The article provides an opportunity to discuss the utility of transgenic organisms and extrachromosomal arrays (used in worms) as opposed to artificial chromosomes (used in yeast) or plasmids (more familiar to students). Instructors can also use the article to reinforce the need for controls in all experiments (*e.g.*, the RNAi vector used the in the RNAi screen; figure 1A in the accompanying article) and the use of a statistical test and *P*-values. Finally, instructors can use the Polley and Fay article to illustrate the utility of model organisms, the ease with which they can be genetically modified, the ease of constructing strains with multiple mutant alleles, and the ability to knock-down gene expression experimentally.

Approach to Classroom Use

Genetics instructors are encouraged to provide this primer article to students concurrently with Polley and Fay (2012). Students would be expected to read carefully the *Introduction* and *Results* sections of Polley and Fay as well as the first paragraph of the *Discussion*. The article can certainly be used as a teaching aid in isolation, and instructors may want to apply the C.R.E.A.T.E. approach (*c*onsider, *r*ead, *e*lucidate hypotheses, *a*nalyze and interpret the data, and *t*hink of the next *e*xperiment) of Hoskins *et al.* (2007) with this article alone, or instructors may use the discussion questions supplied below. If an instructor wants to apply the method of Hoskins *et al.* (2007) in which students sequentially read several articles from one lab, a useful sequence would be Fay *et al.* (2002) for the genetic screen for synthetic phenotypes, Kirienko *et al.* (2008) that describes the forward genetic screen that identified additional *lin-35* synthetic phenotypes and the genes that confer those phenotypes, followed by Polley and Fay (2012). To provide a contrasting approach to discerning the role of *lin-35*, instructors could add an article by Kirienko and Fay (2007) in which the transcriptomes of *lin-35* mutant and wild-type worms are compared. A review by Fay and Yochem (2007) of SynMuv genes and an article describing synMuv suppression (Cui *et al*. 2006) may also be helpful.

Once students demonstrate that they understand the RNAi screen by providing a clear explanation of the approach and rationale, analysis of the data contained in the figures in the accompanying article should be the main focus of discussion. Many core genetics concepts are reinforced well with data in figures 1–4 in the accompanying article by Polley and Fay. The figures are quite accessible to undergraduate students; it is the connection to molecular and cellular mechanisms of suppression that will require repetitive explanation. The data from figure 5 in the accompanying article can be omitted for simplicity. To thoroughly engage an entire class with the article, small groups of students can be assigned to present portions of the article to the class as a whole or can be assigned to answer the particular discussion questions below. One reasonable division of material for discussion is the following:

Group 1: Diagram the reverse genetic screen and provide an answer to question 1 below.

Group 2: Describe the data shown in figure 1A in the accompanying article. Define and describe the three classes of suppressor genes indicated in the figure.

Group 3: For what purpose did Polley and Fay produce the double- and triple-mutant animals indicated in figure 1, B–D, in the accompanying article? How do you read these graphs? What do these data suggest?

Group 4: What research question is addressed by the data in figure 2 in the accompanying article? How does the effect of G418 compare to genetic suppression shown in figure 1A in the accompanying article? How might you interpret these data? How do the authors interpret these data?

Group 5: How did Polley and Fay assess the mechanism by which the prohibitins suppress L1 lethality?

Group 6: Explain the data shown in figure 4 in the accompanying article, including the reason the authors collected these data. Pick out several interesting points that you can interpret for the class.

Groups 7 and 8: Address question 8 below, choosing different genes.

Questions for Further Exploration

1. Can you organize the data collection for the RNAi suppressor screen? For example, if you were to create a spreadsheet onto which you placed the data, how would you label the columns and rows? What would you measure to produce quantitative data?
2. How did Polley and Fay confirm that their results were both significant and then biologically meaningful?
3. How might you interpret the fact that *dpl-1;slr-2* mutant worms also have an L1 arrest phenotype?
4. What does it mean that the penetrance of the *dpl-1;slr-2* phenotype differs from that of *lin-35*; *slr-2* double mutants? How does penetrance differ from expressivity?
5. What is an informational suppressor? See Hodgkin (2005) for more details about suppressors. Why do

the authors conclude that they did not isolate informational suppressors?

6. What conclusions can you draw about the minimal number of mechanisms of suppression by comparing the data shown in figure 1, B–D, to figure 1A in Polley and Fay (2012)?
7. How might RNAi knockdown of genes involved in ribosome biogenesis lead to suppression of L1 lethality? Be sure to include in your answer the cellular role of *lin-35* and the cellular effect of a *lin-35* null allele. Do the data shown in figure 2 of Polley and Fay (2012) support or refute your model? Could you say that G148 treatment phenocopies the suppressor phenotype?
8. Describe a possible mechanism of suppression of just one or two *lin-35* synthetic phenotypes (from figure 4 in the accompanying article). Be sure to include in your answer the cellular role of *lin-35* and the cellular effect of a *lin-35* null allele. Why do some suppressors alleviate only the *lin-35*; *slr-2* phenotype but not other *lin-35* synthetic phenotypes?
9. If you were a graduate student charged with choosing to study further a single suppressor gene identified in this screen, which gene would you choose? Be prepared to defend your answer using the data provided.
10. What further experiment might you undertake to test the hypothesis that differences in tissue specificity of RNAi might account for the patterns of suppression seen in figure 4 in the accompanying article?
11. Prepare a paragraph in which you summarize the work of Polley and Fay so that a beginning biology student could understand the significance of the work and the findings.

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