SMG-ly Knocking Out Gene Expression in Specific Cells: An Educational Primer for Use with “A Novel Strategy for Cell-Autonomous Gene Knockdown in Caenorhabditis elegans Defines a Cell-Specific Function for the G-Protein Subunit GOA-1”

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SUMMARY A recent article by Maher et al. in GENETICS introduces an alternative approach to cell-type-specific gene knockdown in Caenorhabditis elegans, using nonsense-mediated decay. This strategy has the potential to be applicable to other organisms (this strategy requires that animals can survive without nonsense-mediated decay—not all can). This Primer article provides a guide and resource for educators and students by describing different gene knockdown methodologies, by assisting with the technically difficult portions of the Maher et al. article, and by providing conceptual questions relating to the article.

Related article in GENETICS: Maher, K. N., A. Swaminathan, P. Patel, and D. L. Chase, 2013 A novel strategy for cell-autonomous gene knockdown in Caenorhabditis elegans defines a cell-specific function for the G-protein subunit GOA-1. Genetics 194: 363–373.

Background

Most genes affect multiple cell types and they often function at different times during the development of an organism. As a consequence, most mutations are pleiotropic: that is, they cause a mix of phenotypes in different cells or tissues or at different times. While pleiotropy is useful for uncovering the relationships between phenotypes, investigators often want to be able to examine the effect of a gene in a single cell type or at a single time without the complicating effects caused by loss of gene function in other tissues or cells. To observe gene function in individual cell types, the gene itself has to be mutated or its expression has to be knocked down only in certain cells, while the normal activity of the gene remains uninterrupted in other cells.

Maher et al. (2013) have developed a novel method to study the mutant phenotype of a gene in a single cell type in the nematode Caenorhabditis elegans. Since the method relies on nonsense-mediated decay, a cellular process that affects gene expression in most or all eukaryotes, it has the potential for broader applicability to other organisms and other genes. In fact, Maher et al. also demonstrate that their method can be used for some genes and cell types that have proved to be refractory to other methods. To understand their method, we provide some background on other methods used to knock down gene function in specific cells and on the process of nonsense-mediated decay, which, despite its evolutionary conservation, may be unfamiliar to many students.

Mosaic analysis

Geneticists use a variety of methods to knock down or knock out the function of a gene in specific cells, each of which has its own strengths and weaknesses (Meneely 2009). Historically, the first method to be used was mosaic analysis, widely applied in Drosophila and C. elegans. In mosaic analysis, the wild-type allele of a gene is removed in a heterozygous individual only in particular tissues; upon subsequent mitosis, there are patches of cells that have only the mutant allele for the gene. That is, while most of the organism has the genotype...
a+/a, some patches of cells have the genotype a/a or a/-- (a single allele of the gene with no corresponding dominant wild-type allele on the other homologous chromosome). Two genetic methods have been used to create such mosaics. In Drosophila, mosaics are usually generated by mitotic recombination but this has not been found to work well in other organisms. Another method uses the loss of chromosomes or chromosome fragments, as summarized in Figure 1A. In worms, for example, small duplications (Dp) of parts of a chromosome are only somewhat stable during mitosis. Thus a worm whose overall genotype is Dp (a+)/a/a is wild type in most of its cells because of the dominant a+ allele, but might have patches of cells from which the duplication has been lost, resulting in a cell with the genotype a/a, which is mutant. Analogous methods with different chromosome rearrangements have been used in other organisms.

There is a rich history of scientists studying gene functions using mosaic analysis, particularly in Drosophila. These genetic methods have the great advantage that the investigator does not need a cloned version of the gene to perform the analysis; a mutant allele and a suitable set of genetic markers and chromosome rearrangements are sufficient to carry out the experiment. Mosaic analysis could not be used for every mutant or every cell type, however, and was not done in every model organism. The appropriate chromosome rearrangements were often hard to construct or work with, which limited its use. More significantly, the methods to generate the mosaics relied on random chromosome loss or somatic crossing over during mitosis and could not be targeted to specific cell types. That is, the investigator needed to examine many flies or worms with patches of mutant cells in different places to find the ones that had the mutant patches in the cells of interest. In addition, these methods worked best for cell types that could be easily observed, which limited their usefulness for internal structures in most organisms. (C. elegans, being transparent, was an exception.) More fundamentally, the ability to use only a mutant phenotype for the experiment was important in an age when obtaining a cloned version of the gene was difficult, but this advantage is no longer relevant in an era when genomes have been sequenced and all genes have been cloned.

Cre-lox

Once cloned genes were widely available, investigators could use the tools of molecular genetics to delete a gene or knock down its expression in particular tissues. In mice, the most common method uses the site-specific recombination enzyme Cre, which produces targeted crossovers at sequences known as loxP sites (Capecchi 2005). Cre and lox are found in the bacteriophage P1 and serve to integrate the phage into the bacterial genome. The highly efficient site-specific interaction is key for their use in making targeted mutations. Thus, if a gene or a region of a gene is flanked by loxP sites—“floxed” in the jargon of mouse genetics—a Cre-mediated recombination between the lox sites results in deletion of the gene or that portion of the gene. Cre-lox-mediated targeted insertions, deletions, and rearrangements have been widely used to construct transgenic mice for many genes (summarized in Figure 1B).

The specificity of the Cre-lox interaction also provides the basis for making a gene deletion in only certain cells. Constructs in which parts of the gene are flanked by loxP sites can be made in vitro, introduced into embryonic stem (ES) cells, and used to produce mice in which the gene has been floxed. In a different strain of mice, the Cre recombinase has been inserted under the control of a regulatory region that confers cell- or tissue-specific expression. When the two strains are bred, the offspring will have the floxed gene deleted only in the cells that express Cre—for example, only in the pancreas (and not the liver) if Cre has been placed under the control of a pancreas-specific regulatory region. Many mouse strains now exist with Cre expression in only certain specific cell types or at certain times, which allows the effects of a particular floxed gene to be studied in each cell type separately. Despite its utility in mice, the Cre-lox system has not been widely used in other model organisms, however.

RNA interference

Other than working with mutant alleles, the most widely used method to knock down gene activity is RNA interference (RNAi). To carry out RNAi, the investigator introduces double-stranded RNA (dsRNA) corresponding to part of the transcript of the gene of interest. The dsRNA is processed to a 22-nucleotide small interfering RNA (siRNA), which forms a double-stranded hybrid with the target transcript. This double-stranded RNA between one strand of the siRNA and the messenger RNA (mRNA) targets the mRNA for destruction by cellular machinery and/or blocks the translation of the mRNA into protein (Boutros and Ahiringer 2008).

RNAi takes advantage of the same cellular pathway as used by microRNAs for the regulation of gene expression. This is a significant advantage. Because it uses the normal machinery of a cell rather than introducing sequences from another source, RNAi is extremely versatile and has been used to knock down the expression of many thousands of genes in dozens of plants and animals. It allows genetic analysis of mutant phenotypes in organisms for which traditional genetic approaches have not been used or are not possible, such as regeneration in Planaria (Alvarado 2003).

With some small changes, RNAi can also be used to knock down gene expression in specific cells (Figure 1C). The key molecule for RNAi is the double-stranded RNA that produces the siRNA. One method to produce this dsRNA is to introduce into cells a DNA sequence that, when transcribed, forms a hairpin that is primarily double stranded. Cell specificity is provided by the identity of the promoter that regulates transcription only in certain cells. When the sequence is transcribed, the hairpin has the siRNA as its stem so processing of this sequence by normal cellular machinery results in RNA interference. If the vector has a regulatory region that allows transcription only in certain cells, only those cells are expected to express the siRNA and show the effects of RNAi.
As powerful as RNAi is, some limitations have been encountered. First, some genes and tissues are refractory to RNAi. In worms genes expressed in the nervous system or the germline are relatively insensitive to RNAi (although see Calixto et al. 2010). Second, RNAi knocks down gene expression but may not be knocking it out completely and we do not have controls to demonstrate how well RNAi is working for a particular gene in most cases. Third, and potentially...
Thus, the knockdown might not be confined to the cell type expressing the RNAi sequence. For these reasons, Maher et al. (2013) turned to a different approach to knock down transcription of a gene in specific cells. They used the cellular machinery of nonsense-mediated decay (NMD) and demonstrated its effectiveness with genes expressed in the nervous system in worms. Because NMD is a normal cellular process in eukaryotes, the approach should be generally applicable, as with RNAi. The approach requires an understanding of the process of nonsense-mediated decay, which itself requires a few comments about stop codons.

**Nonsense-mediated decay**

The last exon of a eukaryotic gene includes one of the stop codons, UAG, UAA, or UGA. No transfer RNA (tRNA) has an anticodon complementary to these codons; thus, when one of these codons is encountered by the ribosome, the release factor eRF-1 recognizes the unpaired stop codon, the ribosome is released from the mRNA, and translation terminates. An mRNA typically has a single stop codon (or several clustered together near the 3’ end of an mRNA).

However, mutations from a sense codon to a stop codon occur regularly; mutations that generate a stop codon are known as nonsense mutations. Nonsense mutations have been recognized and used in prokaryotic genetics for decades. For many years, it was assumed that a transcript with a nonsense mutation (as well as its normal stop codon) was translated until the first stop codon is encountered, at which point translation terminated. Such a system would produce short peptides consisting of the sequence from the start codon to the nonsense mutation.

About 20 years ago, it was found that this is not what happens in eukaryotes. Interestingly, eukaryotes have a surveillance system known as NMD to identify and degrade mRNA molecules with more than one stop codon (Chang et al. 2007). Because the mRNA with multiple stop codons is degraded before translation begins, short peptides are not produced from messages with nonsense mutations. The exact mechanism by which NMD recognizes that one of the stop codons is a premature termination codon (PTC) is unknown, although it seems to involve a persistent association between the pre-mRNA and some ribonucleoproteins from the splicing complex. By whatever mechanism it works, NMD is quite efficient so that genes with a nonsense mutation produce little or no mature mRNA.

NMD is a highly conserved pathway among eukaryotes and has been well studied in *C. elegans*. For historical reasons, genes encoding the NMD machinery in *C. elegans* are known as *smg* genes, encoding seven SMG proteins, all with mammalian orthologs (Figure 2). One highly conserved protein is UPF1 (SMG-2 in worms), a helicase that is recruited to the mRNA when a stop codon is recognized. When UPF1/SMG-2 interacts with two other proteins (SMG-3 and SMG-4), the mRNA is rapidly degraded. As with many other biological processes, UPF1/SMG-2 is itself regulated by a cycle of phosphorylation and dephosphorylation. Four conserved proteins, SMG-1, SMG-5, SMG-6, and SMG-7, are responsible for phosphorylation and dephosphorylation, and all of them are required for NMD. A mutation that eliminates the activity of any of these genes in worms results in the loss of NMD and the persistence of transcripts with premature stop codons; similar results have been observed with the knockdown or inhibition of the mammalian orthologs of these genes.

**Maher et al.’s Results**

**Organism-wide gene knockdown using nonsense-mediated decay**

To test whether the NMD machinery is an effective mechanism by which to knock down the expression of a gene, Maher et al. (2013) designed a tripartite mCherry transgene in which the neuronal *rab-3* promoter was positioned upstream of the mCherry coding region with its own stop codon. Immediately downstream of the mCherry reporter, a 4.1-kb region of the *let-858* gene, with several exons and introns from *let-858* and including its own stop codon, was inserted. NMD has been shown to work well with a distance of at least 500 bp between the stop codons, and this region of *let-858* had previously been shown to confer NMD when inserted downstream of other stop codons. Since the first stop codon in the transcript, the stop codon from the mCherry gene in this case, is detected as a premature termination signal, the SMG genes activate NMD and the entire transcript is degraded.

To determine whether their transgene targeted a NMD response, Maher et al. (2013) compared the expression of their transgene in wild-type and *smg-5* (NMD-defective) mutant animals by injecting equal concentrations of their transgene and an untagged *rab-3p::GFP* reporter gene into both animals types. GFP was expressed in both wild-type and *smg-5* animals, indicating the protein expression from the injected transgenes was effective, but the mCherry protein was expressed only in *smg-5* mutants. These findings suggested to the researchers that the mRNA from their *rab-3p::mCherry:*let-858 transgene was persisting in the NMD-defective animals and being degraded in animals with functional NMD machinery.

**Cell-type-specific gene knockdown using nonsense-mediated decay**

To test whether they could induce a cell-specific knockdown of the transgene using NMD activity, the researchers injected *smg-5* and *smg-5*; *unc-17p::SMG-5* mutant animals with their mCherry transgene and an *unc-17p::GFP* reporter gene. (*unc-17p* is used as a promoter to express GFP in all cholinergic neurons.) When the expression of mCherry was analyzed, the researchers found that the *smg-5* mutants without the *unc-17p::SMG-5* NMD-rescuing transgene expressed mCherry in all types of neurons, while mutants containing the *unc-17p::SMG-5* rescuing construct contained detectable levels of mCherry only in...
(noncholinergic neurons) GABAergic neurons (Maher et al. Figure 1, B and C) (The authors note that all noncholinergic neurons expressed mCherry in this experiment, but only the GABA neurons are visible in the figure that is focused on the ventral cord). This indicates that the NMD response was induced specifically in cholinergic-type neurons, but not in other neurons—including GABAergic-type neurons. This experiment indicates that the NMD mechanism can be successfully employed to knock down genes in specific cell types.

**Testing the efficacy of nonsense-mediated decay, using goa-1 phenotypic indicators**

Having used the mCherry and GFP reporter genes to show that NMD works very well to knock out gene expression, Maher et al. (2013) then investigated the role of the G-protein subunit GOA-1. GOA-1 is expressed widely in the nervous system of worms, and goa-1 mutants exhibit a range of neurological and behavioral defects. The investigators designed a construct that included the goa-1 gene upstream of the let-858 NMD degradation signal. This gene construct was then stably integrated into the genome of goa-1 mutants under the control of the goa-1 regulatory region, and rescue of Goa-1 mutant phenotypes was compared in wild-type (smg+ and smg−, not really wild type as goa-1 is gone) and smg-5 mutants. In goa-1; smg-5 double-mutant animals with the goa-1 transgene, the transgene rescues the goa-1 mutant phenotype and the worm behaves like wild type; this occurs because NMD is defective in smg-5 mutant worms so the rescuing transgene is expressed. This shows that the transgene appears to confer goa-1 activity throughout the worm. On the other hand, in smg-5+ worms in which NMD occurs, the transgene cannot rescue the goa-1 mutant. This result suggests that regulating the expression of smg-5+ can be used to make cell-specific knockouts.

**Conclusion**

Their strategy definitely worked. Maher et al. (2013) began their experiments by creating a transgene they hoped would elicit an NMD response in smg-5+ worms and become degraded. Using mCherry protein to show expression of the transgene, Maher et al. did, in fact, observe a lack of mCherry expression in smg-5+ worms compared to smg-5 mutant animals. The researchers were also able to show that in NMD-defective animals, NMD could be rescued in specific cell types to elicit a targeted cell-type-specific knockdown of a single gene; in particular, this was highlighted using goa-1 mutant animals and a goa-1 rescue transgene. By placing smg-5+ under the control of a regulatory region that directs transcription in four specific neurons, two neurons in the pharynx and two neurons involved in egg-laying hermaphrodite specifics (HSNs), the researchers tested the ability of the goa-1::let-858 transgene to rescue different phenotypes of the goa-1 mutant (Figure 3). Remember that rescue of the mutant phenotype occurs only when NMD is inactive. Phenotypes such as rate of movement and the ability to back up, which are affected in goa-1 mutants, are rescued in this strain, showing that smg-5+ is not being transcribed in the neurons regulating these traits. On the other hand, defective egg laying is not rescued when smg-5+ is expressed in the HSNs, again indicating that when NMD is active, the transcript from the complementary transgene is degraded and no rescuing GOA-1 function occurs.

**Connections to Genetics Concepts**

While some of the molecular constructs that are used for these experiments require a bit of patience to understand, Maher et al. (2013) highlights many important concepts that are worthwhile for introductory genetics students. For example, the ability to perform cell-specific knockdown of a particular gene of interest in model organisms (specifically *C. elegans* in Maher et al. 2013) allows geneticists to study the role of genes in ways that traditional mutant analysis does not allow. This provides an opportunity to discuss the origins and importance of pleiotropy. The ability to examine the function of genes in a cell-specific manner has afforded researchers the opportunity to observe that genes expressed in individual cell types can result in different phenotypes compared to organism-wide expression of a gene. It also allowed researchers to observe the effects of mutations that are lethal when expressed in the organism as a whole. This could work well to introduce strategies other than the much more widely used Cre-lox and RNAi approaches.

Maher et al. (2013) is an excellent resource to demonstrate how researchers are able to use particular features of the model organism to their advantage. Specifically, Maher et al. take advantage of the naturally occurring NMD machinery present in wild-type *C. elegans* and lacking in smg-5-defective animals to knock down genes in a cell-autonomous manner. It also introduces the concept of NMD itself, which reinforces how stop codons work and how nonsense mutations arise. Another feature of Maher et al. (2013) that should be highlighted for genetics students is the importance of choosing relevant and meaningful controls for experiments. Maher...
et al. (2013) contains a number of such relevant controls corresponding to the specific type of experiment the researchers conducted.

Questions for Classroom Use

These questions are designed to reinforce important concepts and technical and methodological areas as well as to foster additional thoughts regarding the broader goals and implications of this kind of research. We recommend that students be given all of these questions in advance and given time to prepare answers. When they arrive in class, each student can be assigned a question and students can be divided into small groups based on their assignment. Groups can then be given time to organize and compare their answers. Each group then presents its ideas to the class, after which the question can be opened up to the entire class to discuss since every student has prepared an answer to every question.

1. What does “cell autonomously” mean? What might be examples of genes that do not act cell autonomously?
2. When you consider the regulatory steps in gene expression and gene activity, what are some other strategies that could be used to knock out or knock down gene expression in specific cells or tissues for genes that work autonomously?
3. What function does NMD machinery have in wild-type organisms and why is this function thought to be important?
4. Nonsense-mediated decay occurs in most, if not all, eukaryotes, but not in bacteria. Based on your knowledge of the biological differences in gene expression between bacteria and eukaryotes, why is it not surprising that NMD does not occur in bacteria?
5. What are some of the advantages of using an evolutionary conserved process (such as NMD) as the basis for cell-specific gene knockouts? What might be some of the disadvantages?
6. How did the authors utilize NMD for the experiments described in the article? Diagram the gene constructs with appropriate labels for different experiments.

Figure 3 The use of NMD for cell-specific gene knockouts. The smg-5+ gene, which is needed for NMD, is integrated under the control of the tph-1 regulatory region, which results in transcription in only four neurons, including the HSNs. The goa-1+ gene with the termination tag from let-858 is integrated in the genome as well. In the HSNs, smg-5 is transcribed, so NMD occurs, and the goa-1 transcript with the termination tag is degraded. This results in the failure to rescue the goa-1 mutant phenotype in the HSNs. In other neurons, smg-5 is not transcribed, so there is no NMD, and the goa-1 transcript persists. In these cells, the goa-1 mutant phenotype is rescued.

7. The authors chose to use goa-1 to demonstrate how their NMD construct worked. What features of goa-1 made it a good gene to select to test the efficacy of their strategy?
8. Why was it important that researchers used a null mutation in goa-1? Could they have used other types of mutations? Why or why not?
9. What are some examples of controls used in the authors’ experiments? What do the experimental controls “control” for? Be specific.
10. What did the quantitative RT-PCR comparing goa-1 wild-type mRNA expression to goa-1 null mutant expression demonstrate that the authors’ previous experiments had not?
11. What do the authors contend are the benefits of using their NMD-dependent method?
12. Do you foresee any drawbacks or complications to using the NMD machinery to knock down gene expression in C. elegans?
13. The authors used NMD to investigate the function of goa-1 in different cell types. How would you modify the strategy to investigate the activity of a gene that works at different times in development? Consider all of the component parts of their strategy using goa-1 and smg-5 and try to think specifically about which of these components would need to be replaced and which would still be used. For a component that needs to be replaced, think about what it would be replaced with.
14. What would be necessary to use this approach in model organisms other than C. elegans?

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