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Supplemental Materials

for

A "Choose Your Own" Classroom-Based Activity That Promotes Scientific Inquiry about RNA Interference

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Appendix 1: Instructor guide

The activity is divided into two parts and is followed by an assessment. Here, I provide details for how to implement each part of the activity and the assessment and provide suggestions for instructors to modify this activity to fit a variety of different class sizes and levels.

Part I of activity

In the first part of the activity, students are presented with the puzzling observations from Napoli *et al.* 1990 [1] and are challenged to generate hypotheses about how an apparent overexpression of the *CHS* gene led to an opposite impact on expected phenotype. The activity is designed to build upon foundational content on chromatin remodeling and eukaryotic transcriptional control and before any formal introduction of RNA interference (RNAi), so it is likely that students will generate hypotheses relating to changes at those levels occurring prior to impacting mRNA stability. Student responses may be varied, and the instructor should emphasize that the hypotheses should be proposed *mechanistic* explanations that would explain the puzzling observation, i.e. what happened at the molecular level to lead to high transcription but a change in phenotype in the opposite direction. In addition, students will then be asked to think about possible experiments to test their hypotheses. This part of the activity presumes no prior knowledge of any experimental techniques, and instructors should again emphasize that students should focus on *what* they want their experiments to change and/or measure, and not worry about what molecular techniques they would use to do so. For instance, even if students have not learned about Western blots or similar techniques, students can still propose measuring the amount of protein in the control and experimental flowers. On the other hand, instructors who have already covered significant molecular techniques in class (or for instructors teaching upper-level classes where prerequisite classes have covered molecular techniques) may edit the activity to ask students to also propose specific molecular techniques in their experiment. Finally, students are asked to generate predictions of their expected results if their hypothesis is true and is not true.

Students should be working in small groups of 3-4 for this part of the activity, and the instructor (along with any teaching assistants, learning assistants, or supplemental instructors) should check in with groups to hear their ideas and provide feedback. At the end of this part of the activity, the instructor may wish to facilitate a class-wide discussion on the hypotheses and experiments, but should intentionally not provide any answers or additional insight into the mechanism to explain the observations, given that students will be exploring these themes in part II. This first part of the activity should take anywhere from 15-30 minutes, depending on the size of the class. For larger classes, instructors can print the discussion questions to facilitate groupwork.

Part II of activity

Students will continue to work in their groups in part II of the activity. Following the class-wide discussion on hypotheses from part I, students are then presented with a "choose-your-own-experiment" activity, where each group receives $20,000 of funding from the National Institutes of Health (NIH). To provide insight into the funding process, the activity states that the university's overhead costs for the grant will result in a 50% reduction of the funding available for lab use, leaving groups with $10,000 they are able to spend. Instructors may change the amount of funding available to groups (as well as the associated costs of each experiment) to

either speed up the activity or allow more discussion. A smaller amount of funding will result in groups being able to choose fewer experiments, thus shortening the amount of time this part of the activity takes, while increasing the funding will lengthen the discussion. With the current amount of funding, groups will on average be able to choose four different experiments over approximately 30 minutes.

The instructor should prepare and print the list of possible experiments and distribute to students to facilitate groupwork. The instructor will also need to decide on 1) how students keep track of their funding and 2) how to provide experimental results to the students. These decisions will be influenced by class size, classroom layout, and number of additional instructors (e.g. teaching assistants) present in the class. For small classes where students can easily access the front of the room, the instructor may wish to print "NIH fund" certificates in $1000 denominations as well as slips of paper that contain the results to each experiment. The instructor can then distribute the appropriate amount of funding to each group, and ask that each group send one person up to the front to convey which experiment they wish to run; the group will then exchange the appropriate amount of "NIH fund" certificates and receive the respective experimental result. For larger classes, the instructor can ask groups to keep track of their own funding on a separate page that lists what experiments they have chosen, how much those experiments cost, and the amount of remaining funds, so that there is no need to print out and distribute "NIH fund" certificates. Similarly, the instructor can have teaching assistants, if available, serve as runners and provide experimental results to the group to minimize the amount of student movement necessary in a large class. If this is not feasible, instructors can also either print a copy of experimental results for the students and ask them to only look at the results of the experiments they have chosen, or post them on a class webpage or learning management system and ask students to obtain their own experimental results online. It is recommended that instructors put a time restriction on when their NIH funding will "expire" to motivate groups to make decisions in a timely manner.

There are currently 13 possible experiments or collaborations listed; instructors should feel free to customize this list of experiments to tailor this activity to their own class. Experiments can be easily removed or added; new experiments require a brief description of what the experiment will be, a short summary of the results, and an amount of funding needed to run the experiment. Funding amounts for the 13 experiments do not reflect actual costs of these experiments (nor are they proportional to each other) but were instead chosen so that there were a mix of experiments costing different amounts and so that the more seemingly informative experiments cost more money.

A brief summary of the experiments and results are provided here; instructors should review these experiments and results ahead of time.

1. In experiment 1, students irradiate flowers of both control groups to introduce mutations. However, all the flowers die as a result of the irradiation, providing no additional insight into distinguishing between proposed hypotheses. This experiment is designed to show students that hastily thrown together experiments are unlikely to succeed; students may have chosen this experiment thinking that they could knockout certain genes, but any resulting mutations from this experiment are random and not specific to any gene.
2. In experiment 2, students inject more of the CHS pigment directly into flowers. The flowers turn more purple, indicating that there is nothing wrong with the CHS pigment itself.

3. Experiment 3 presents a simplified version of an experiment from Weintraub and Groudine in 1976 [2], which confirmed that condensed chromatin leads to no expression and that decondensed chromatin allows for gene expression, as applied to the *CHS* case study. In this simplified experiment, students add DNase to the isolated chromatin for the *CHS* gene and run out the results on a gel. The inclusion of this experiment is dependent on the instructor having covered this experiment in past lectures, and otherwise more context may be needed for this experiment. DNase is more likely to degrade DNA if the chromatin is decondensed, thus allowing for the testing of chromatin condensation and decondensation. The results show a smear of bands, indicating that DNase has cut the chromatin and that the chromatin for *CHS* is likely decondensed.
4. Option 4 presents a collaboration rather than a true experiment and is an homage to the fact that the RNAi mechanism was first elucidated in *C. elegans* [3]. The results show that a similar result of overexpression leading to decreased mRNA levels is seen in *C. elegans*, providing additional context for students.
5. Experiment 5 allows students to measure the protein levels of *CHS*. The results are consistent with what should students should expect: the experimental group with very low levels of mRNA has very low amounts of the corresponding protein.
6. In experiment 6, students can sequence the *CHS* gene and compare the sequences between the experimental and control groups. The results indicate no sequence differences. While this is again a simplified result, it indicates that the differences in mRNA levels between the groups is not due to a sequence difference in the *CHS* gene itself.
7. Experiment 7 presents a commonly used bioengineering technique of placing the *GFP* gene next to a target gene in order to measure expression of the target gene. The simplified results - which presume that a *CHS-GFP* mRNA fusion would be entirely degraded in RNAi – reflect that if the mRNA of a gene is degraded, then the protein cannot be made either, i.e. if the *GFP* mRNA is degraded then no GFP protein will be made.
8. In experiment 8, students can experimentally manipulate the level of acetylation for the corresponding histones for *CHS*. This experiment presumes that the instructor has covered the basic mechanisms of epigenetics. Increasing acetylation of histones can increase expression of the corresponding genes, but since RNAi would degrade any *CHS* mRNA in the experimental group, there would be no functional change in amount of pigments produced.
9. Experiment 9 is an entirely fictitious experiment and result that involves crossing flowers of different colors. This experiment may be an attractive option for students who are wondering about how color may be inherited and who wish to explore if color is inherited in a simple Mendelian fashion, but the results are not meaningful given that the color of the flower is being changed through RNAi, rather than in a classical Mendelian fashion.
10. In experiment 10, students are provided with the (rather difficult in practice) option to insert more *CHS* mRNA directly into the control flowers. The results show that the control flower now changes color to white, indicating a decrease in amount of CHS pigment produced. These results should serve as a major hint for students that the overexpression of a gene (in this case, an overabundance of mRNA for a gene) can trigger some mechanism to lower its expression.

11. Experiment 11 is perhaps the most critical one if students are to confirm the presence of a mechanism that degrades mRNA. This experiment provides one of the main findings from the Napoli *et al.* paper [1], that of a 50-fold reduction of CHS mRNA in the experimental group. This thus presents a clear contrast where increased transcription of the gene has led to a decreased amount of mRNA, leading to the conclusion of mRNA degradation.
12. Experiment 12 is another fictitious, simulated experiment where students can inject transcription factors to increase the level of transcription for CHS. The results indicate no change in the experimental group since RNAi would degrade any additional CHS mRNA produced, while in the control there is a slight change in phenotype since RNAi may not be triggered yet by the increase in *CHS* transcription.
13. The final experiment, experiment 13, provides students with the option to use CRISPR-Cas9 to randomly edit base pairs in the *CHS* gene, knocking out the gene. A non-functional CHS pigment means that the resulting flowers are all white, providing students with validation of the connection between this gene and its impact on phenotype.

If time permits, instructors may wish to allow groups to collaborate at the end of this activity, where groups can share which experiments they chose and their results with other groups. Following this, instructors should then spend about 15-20 minutes recapping the activity and asking groups to share their results. Many groups will likely have ruled out their initial hypotheses based on the experimental results, while other groups may not be able to make as many inferences, depending on their hypotheses and choice of experiments. Instructors should use this discussion to highlight how – based on measuring mRNA levels (experiment 11) – there is most likely some mechanism that is degrading mRNA of a target gene. Instructors can then introduce RNAi and the mechanisms and relevant proteins involved in this process.

Abbreviated version of activity

If short on time, instructors may choose to implement a shortened version of this activity, which should take between 10-15 minutes. In this abbreviated version, parts I and II are condensed into a guided think/pair/share. Instructors first present the same puzzling observation as used in part I of the activity; instructors can use the same text as in the introduction of part I on their Powerpoint slides. Instructors should discuss why this is puzzling and then, in the next slide, present the main findings from Napoli *et al.* [1] of a *decrease* in CHS mRNA levels despite an attempt to overexpress this gene. Instructors should then conduct a think/pair/share, where students are asked to think individually first about what could explain these apparent paradoxical results, then pair with a partner to discuss, and share with the class their ideas. Depending on the size of the class, instructors can facilitate a class-wide discussion where each group shares their idea or ask several groups to do so. Instructors should then introduce RNAi and the mechanisms and relevant proteins involved in this process.

Assessment

Following coverage of the mechanisms of RNAi, instructors can give the included activity as a means of formative assessment, which should take approximately 20 minutes. Depending on time, instructors can ask students to work individually or to do so in groups, and instructors should facilitate discussion of the answers after the assessment. The assessment is designed to explicitly counter the misconception that RNAi directly impacts the rate of transcription. Instead, degradation of mRNA has no direct impact on transcription levels of that gene. The assessment

thus asks students to critically think and predict transcription, translation, and mRNA levels for three genes. No inferences can be made about two of the three genes since no additional information is provided to indicate if these genes are even expressed or not in the given cell type. However, for the third gene, endogenous RNAi has been triggered to target this gene, meaning that the transcription levels should putatively be high and thus the amounts of mRNA and translation should be low or zero. There are also questions that ask students to connect these observations to make a prediction about the level of methylation in the DNA, which requires that the instructor have previously covered epigenetics. The final question requires knowledge of the RNAi mechanism and how microRNA is complementary to a portion of the target mRNA; as such, students should be able to make an inference that the complementary regions of mRNA (and the corresponding region of DNA) must be exonic given that no introns are found in mature mRNA.

References for instructor guide

1. Napoli C, Lemieux C, Jorgensen R. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. The Plant Cell 2: 279-289.
2. Weintraub H, Groudine M. 1976. Chromosomal subunits in active genes have an altered conformation. Science 193: 848-856.
3. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391: 806-811.

Appendix 2: Student handout for activity

You decide to bioengineer petunias by using *Agrobacterium* to insert extra copies of *chalcone synthase* (*CHS*) gene, which you know produces a pigment that makes petunias purple. This method of gene insertion has worked previously for other genes in this plant, leading to greater expression of those genes. You thus expect that by inserting this extra copy of the gene that your experimental petunia will become even more purple, as compared to a control petunia where you do not do anything. To your surprise, you find that your resulting experimental flowers are either all white or have patterns of white and purple. None of them are more purple than before or more purple than the control petunias. **DO NOT TURN TO THE NEXT PAGE UNTIL INSTRUCTED TO DO SO** – it has some spoilers!

1. Working with your group, develop a hypothesis for this unexpected result. Remember that a good hypothesis will be a complete, thorough explanation for the puzzling observation.

2. Once you have a good hypothesis, come up with one or two other alternate hypotheses.

3. Once you have come up with good hypotheses, design an experiment to test your hypothesis. You do not need to know exact protocols or techniques (or need to know what's feasible), but instead focus more on what you would want to do or measure.

4. Describe your predicted results if your hypothesis were correct, as well as your predicted results if your hypothesis were incorrect. What about for your alternate hypotheses?

5. Once you are done coming up with your experiment and predicting your results, now turn to the next page.

Part II – DO NOT READ THIS PART UNTIL YOU ARE DONE WITH PART I. Now read through the following lab options that describe feasible lab techniques and answer the following questions. Do any of these options align with the experiment you proposed?

Even if they don't, it's your lucky day! You receive a small grant from the National Institutes of Health for $20,000 of research funding. You can only spend $10,000 of this funding, however – your university's overhead rate (for facilities and administrative costs) is over 50%! (This is very normal for most universities.) Assume that you know each of the lab techniques below is feasible and will work. The cost of each experiment is indicated in brackets; choose carefully, since you have limited funding! Once your group has decided on an option, come up to the instructor with your research money and in exchange you'll receive the results of that experiment! After you are done spending your research money, work together to discuss the results – what, if anything, can you determine from these data? Do the data support or reject any of your hypotheses?

Your lab options:

14. You can irradiate both your control and experimental petunias in the hopes of introducing mutations to this gene and observe what happens. [$2000]
15. You can inject the CHS protein (the pigment) directly into the plant and see what happens. [$2000]
16. You can isolate the chromatin for the CHS gene, add DNase, and run the results out on a gel. [$1000]
17. You can fly to a conference and consult with some collaborators, who you know have been working on a similar project of inserting in DNA and recording the phenotype. You know your collaborators work in *C. elegans*, a worm commonly used in lab studies. [$1000]
18. You can insert an antibody that will bind to the CHS protein, thus allowing you to quantify the amount of CHS protein in both the control and experimental groups. [$3000]
19. You can sequence the original CHS gene of the petunia as well as the CHS gene you are bioengineering to insert inside the petunia to compare the DNA sequences for this gene. [$3000]
20. You can bioengineer the gene GFP (green fluorescence protein) to be under the control of the same promoter as CHS in both your control and experimental petunias. If the GFP gene is expressed (and the protein is made), the cell will glow a distinctive green. You can tell whether or not a given cell (and thus the flower) is glowing green or not. [$4000]
21. You can insert histone acetyltransferases that you know will acetylate the corresponding histones for the CHS gene. [$2000]
22. You can cross the resulting white flowers with each other and check the color of their offspring, and also cross the resulting white flower with a control, purple flower. [$1000]
23. You can choose to inject mRNA of the CHS gene directly into control flowers and observe the resulting phenotype. [$2000]
24. You can isolate the mRNA from the total RNA of both the control and experimental petunias, and measure the amount of mRNA. [$2000]
25. You can artificially inject more of the activators that you know control CHS transcription [$2000].
26. You can use a DNA editing technique (called CRISPR-Cas9) to randomly edit base pairs in the CHS gene in your control petunia and observe what happens. [$3000]

Appendix 3: Experimental results

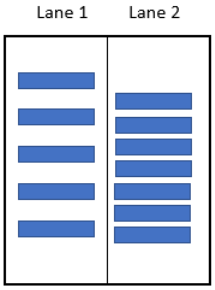
1. You can irradiate both your control and experimental petunias in the hopes of introducing mutations to this gene and observe what happens. [$2000]

   Results: your flowers all die after radiation.

2. You can inject the CHS protein (the pigment) directly into the plant and see what happens. [$2000]

   Results: your flowers turn more purple.

3. You can isolate the chromatin for the CHS gene, add DNase, and run the results out on a gel. [$1000]



   Results: you obtain the following results, where the first lane is the ladder and the second lane is your experimental sample.

4. You can fly to a conference and consult with some collaborators, who you know have been working on a similar project of inserting in DNA and recording the phenotype. You know your collaborators work in *C. elegans*, a worm commonly used in lab studies. [$1000]

   Results: your colleagues tell you that when they insert *unc-22*, a gene for a myofilament, they get fewer myofilaments then before.

5. You can insert an antibody that will bind to the CHS protein, thus allowing you to quantify the amount of CHS protein in both the control and experimental groups. [$3000]

   Results: you find that your control petunia has substantial amounts of the protein, while your experimental petunias do not have any of this protein.

6. You can sequence the original CHS gene of the petunia as well as the CHS gene you are bioengineering to insert inside the petunia to compare the DNA sequences for this gene. [$3000]

   Results: you find no differences in the CHS gene in the petunia as well as the CHS gene you inserted.

7. You can bioengineer the gene GFP (green fluorescence protein) to be under the control of the same promoter as CHS in both your control and experimental petunias. If the GFP gene is expressed (and the protein is made), the cell will glow a distinctive green. You can tell whether or not a given cell (and thus the flower) is glowing green or not. [$4000]

   Results: you find that your control flowers glow, but your experimental flowers do not.

8. You can insert histone acetyltransferases that you know will acetylate the corresponding histones for the CHS gene. [$2000]

   Results: you find that your control petunias are still purple, and that your experimental petunias are still white.

9. You can cross the resulting white flowers with each other and check the color of their offspring, and also cross the resulting white flower with a control, purple flower. [$1000]

   Results: the offspring of both the white x white cross and the white x purple cross are both white.

10. You can choose to inject mRNA of the CHS gene directly into control flowers and observe the resulting phenotype. [$2000]

    Results: the control flower now becomes white.

11. You can isolate the mRNA from the total RNA of both the control and experimental petunias and measure the amount of mRNA. [$2000]

    Results: the control petunias have about 50-fold more mRNA for CHS than the experimental petunias.

12. You can artificially inject more of the activators that you know controls CHS transcription [$2000].

    Results: your control petunias become a little bit more purple, while your experimental petunias remain white.

13. You can use a DNA editing technique (called CRISPR-Cas9) to randomly edit base pairs in the CHS gene in your control petunia and observe what happens. [$3000]

    Results: your flowers all are white.

Appendix 4: Assessment for activity

You are conducting some experiments in lab and notice that a certain microRNA gene (mmu-miR-712) is being transcribed at high levels in your study organism. You happen to have sequenced a portion of three other genes and find that gene 3 has 22 bases that happen to be complementary to the microRNA gene, but that neither gene 1 nor gene 2 have any bases complementary to the microRNA gene.

1. What type of cell are you likely working with here – *E. coli* or a mouse cell? Explain.

2. What information, if any, can you predict about the level of methylation of the promoters of genes 1, 2, and 3?

3. What information, if any, can you predict about the level of transcription for genes 1, 2, and 3?

4. What information, if any, can you predict about the amount of mRNA for genes 1, 2, and 3?

5. What information, if any, can you predict about the level of translation for genes 1, 2, and 3?

6. What information, if any, can you provide about the portion of sequences you looked at for genes 1, 2, and 3? I.e. can you tell if they are intronic or exonic sequences?

Appendix 5: Assessment key

1. What type of cell are you likely working with here – *E. coli* or a mouse cell? Explain.

The cell is likely from a mouse, given that RNA interference is found predominantly in eukaryotic organisms.

2. What information, if any, can you predict about the level of methylation of the promoters of genes 1, 2, and 3?

There is no information provided about genes 1 and 2, so thus we cannot determine if the genes are expressed or not. As such, we cannot make predictions about the level of DNA methylation in these genes. However, for gene 3 it is likely that RNAi has been triggered, meaning that there are putatively high levels of mRNA that is leading to RNAi. With high levels of transcription, it is likely that there is no (or low levels of) DNA methylation given that chromatin must be decondensed for transcription to take place.

3. What information, if any, can you predict about the level of transcription for genes 1, 2, and 3?

There is no information provided about genes 1 and 2, so thus we cannot determine if the genes are expressed or not. As such, we cannot make predictions about the level of transcription for these genes. However, for gene 3 it is likely that RNAi has been triggered, meaning that there are putatively high levels of mRNA that is leading to RNAi. This means that there are putatively high levels of transcription for this gene.

4. What information, if any, can you predict about the amount of mRNA for genes 1, 2, and 3?

There is no information provided about genes 1 and 2, so thus we cannot determine if the genes are expressed or not. As such, we cannot make predictions about the amount of mRNA for these genes. However, for gene 3 it is likely that RNAi has been triggered, meaning that there are putatively high levels of mRNA that is leading to RNAi. However, RNAi will lead to degradation of gene 3's mRNA, causing gene silencing. It is thus likely that there is no (or low levels of) gene 3 mRNA following RNAi.

5. What information, if any, can you predict about the level of translation for genes 1, 2, and 3?

There is no information provided about genes 1 and 2, so thus we cannot determine if the genes are expressed or not. As such, we cannot make predictions about the level of translation for these genes. However, for gene 3 it is likely that RNAi has been triggered, meaning that there are putatively high levels of mRNA that is leading to RNAi. RNAi is thus likely degrading gene 3's mRNA, and with no (or low amounts of) mRNA, it is likely that there is no (or low) translation of gene 3's mRNA.

6. What information, if any, can you provide about the given sequences for genes 1, 2, and 3? I.e. can you tell if they are intronic or exonic sequences?

There is no information provided about genes 1 and 2, so thus we cannot determine if the sequences we are examining are intronic or exonic. However, for gene 3 it is likely that RNAi has been triggered. We know that the microRNA is complementary with a portion of our sequence of gene 3; since RNAi involves the binding of mature mRNA, the complementary portion must be exonic since mature mRNA does not include introns.