CRISPR/Cas paper model: snail shell coiling

CRISPR/Cas: a revolutionary genome editing tool

The CRISPR/Cas system involves two main components: a Cas nuclease and a guide RNA. Nucleases are enzymes that cut nucleic acids like molecular scissors. There are several Cas nucleases, but Cas9 is most commonly used in genome editing. Cas9 is a powerful tool because it can be programmed to cut nearly any DNA sequence.

Where Cas9 will cut is determined by a short RNA molecule called a guide RNA that binds to the Cas protein (Figure 1). After the guide RNA binds to Cas9, the complex scans the genome for a three base sequence called the PAM. The Cas9 PAM sequence is 5' NGG 3', where N can be any base. When Cas9 encounters a PAM sequence, it unzips the DNA, separating it into single strands. Cas9 then uses the guide RNA to establish whether or not to cut the DNA. At one end of the guide RNA are ~20 bases that determine which DNA sequence Cas9 will cut. If this ~20 base sequence in the guide RNA is complementary to the DNA, then Cas9 will cut both strands of the DNA. If the guide RNA does not match the DNA, then the complex will move on to the next PAM site, and the double helix will re-zip into double stranded form. The trick to using Cas9 as a gene editing tool is that scientists can customize this ~20 base sequence to target Cas9 to a specific region of DNA, basically allowing them to program where Cas9 will cut.

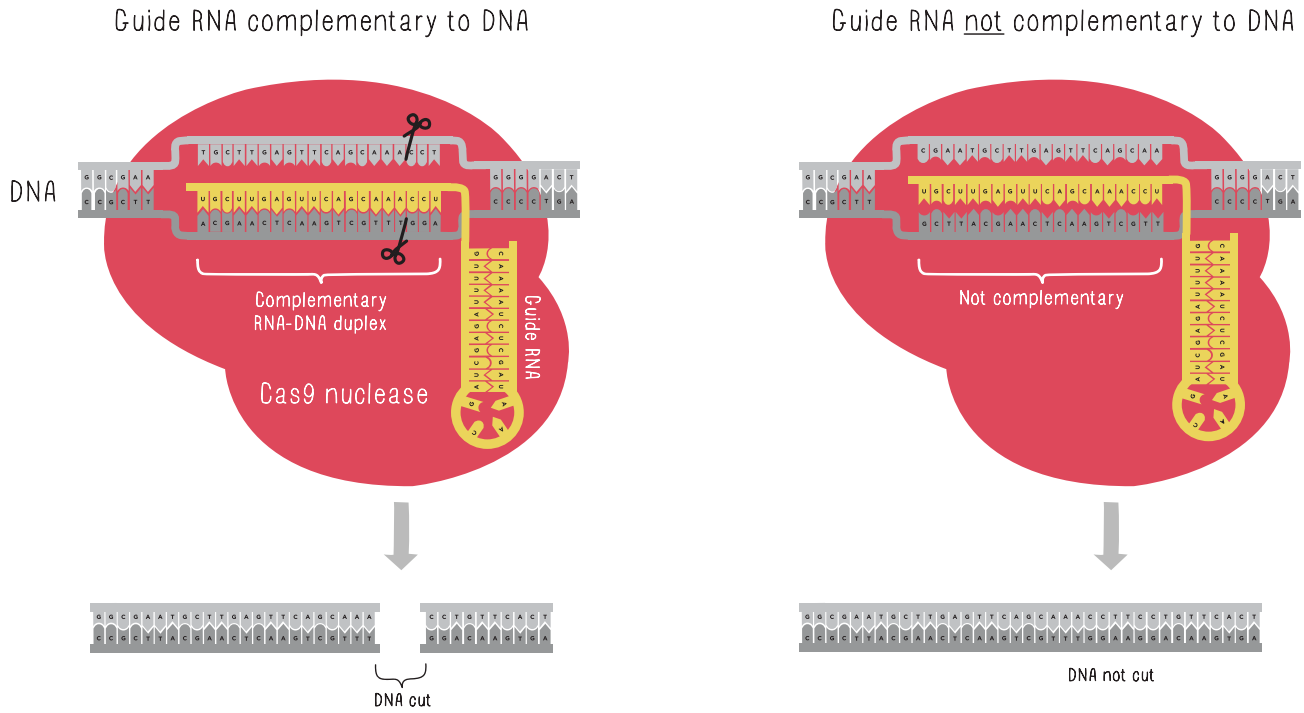

Figure 1. Cas9 cuts DNA as instructed by a guide RNA. When the guide RNA encounters a complementary DNA sequence, Cas9 will cut the DNA (left). When the guide RNA is not complementary to the DNA, Cas9 will not cut (right).

In eukaryotic cells, once Cas9 cuts the target DNA, the cell will try to repair the break. A common fix is for the cell to reattach the broken strands of DNA through a process called non-homologous end joining (NHEJ). When the cell does this, it often ends up adding or removing a few DNA bases. These act much like typos in the DNA. While we often think of mutations like these as being harmful, they can sometimes be used as a tool by scientists. For example, mutations that disable a gene can help scientists understand that gene's function. For this reason, disabling, or 'knocking out,' a gene is a common use of the CRISPR/Cas system.

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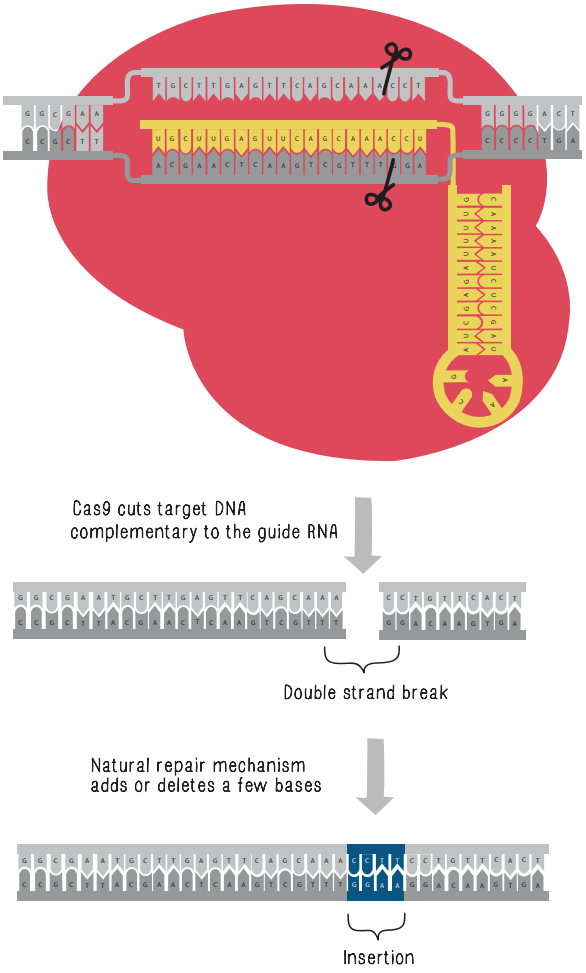


Figure 2. DNA breaks can lead to mutations. Breaks in the DNA can be repaired by two different mechanisms in eukaryotic cells. The more common repair mechanism, non-homologous end joining, reattaches the broken strands of DNA, but in the process often adds or removes a few random DNA bases. These insertions and deletions often disable the gene.

Using CRISPR/Cas to knock out genes in snails

Have you ever noticed that snail shells make a spiral shape? Have you ever wondered which direction those shells spiral and why? Probably not! But some scientists have, and they used CRISPR/Cas9 to answer the question.

Scientists had long observed that the shells of most snails spiral clockwise, but a few rare snails have shells that spiral counterclockwise (Figure 3). Because of snail anatomy, snails can usually only mate with other snails whose shells spiral in the same direction. Previous research suggested that a gene called *Lsdia1* might determine whether snail shells would spiral clockwise or counterclockwise. Unfortunately, without a way to efficiently alter the DNA in snails, this could not be directly tested and remained a hypothesis. CRISPR/Cas genome editing has finally allowed scientists to test if the *Lsdia1* gene controls which way snail shells coil (Abe and Kuroda, 2019).

Figure 3. Snail shells can spiral clockwise or counterclockwise. Most snails have shells that spiral clockwise (snail on the right). However, some snails have shells that spiral counterclockwise (snail on the left) Image reprinted with permission from Development (2019) 146.

Abe, M., and Kuroda, R. (2019). The development of CRISPR for a mollusc establishes the formin *Lsdia1* as the long-sought gene for snail dextral/sinistral coiling. Development 146.

Modeling CRISPR/Cas

Imagine you are a scientist testing the hypothesis that the *Lsdia1* gene controls which way snail shells coil. Your goal is to disrupt the *Lsdia1* gene using CRISPR/Cas9. You will use paper to model how CRISPR/Cas9 can be used to disrupt the *Lsdia1* gene!

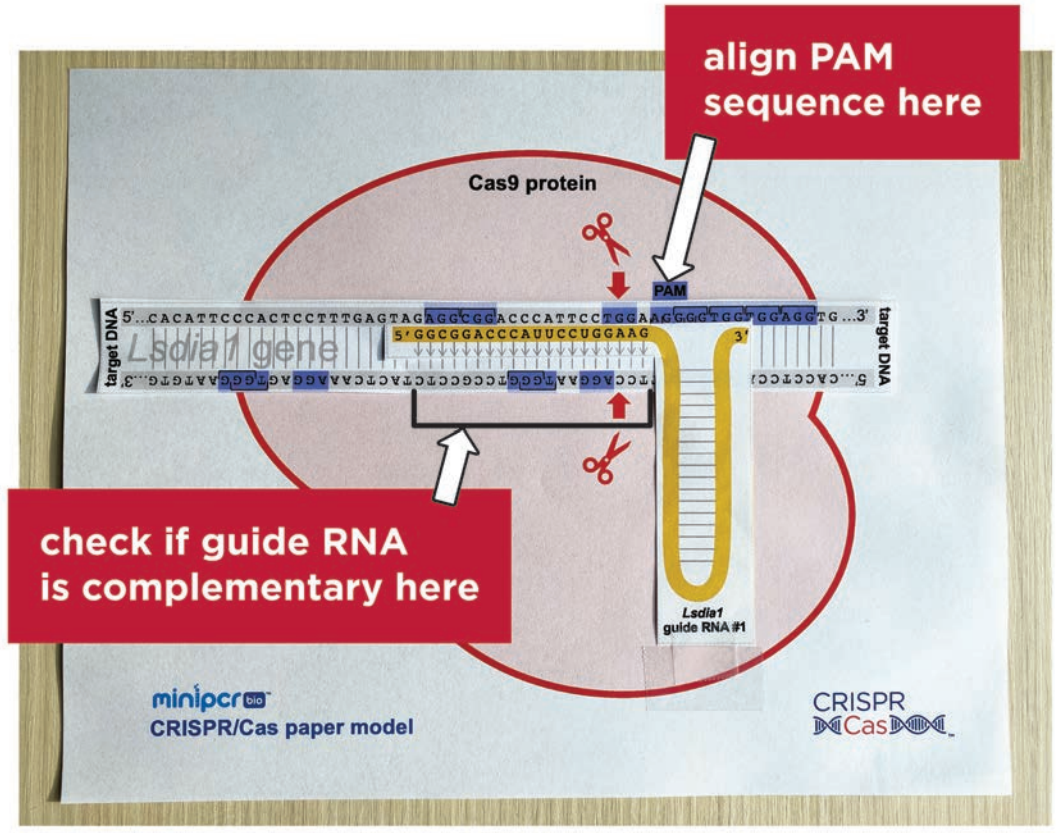
Supplies needed: tape, scissors, single-sided printout of CRISPR/Cas9 paper model (pages 11-12)

1. Cut out the two long strips of DNA labeled “target DNA” (page 12). This sequence includes a section of the snail *Lsdia1* gene.
2. Cut out Guide RNA #1 (page 12). This guide has been designed to target Cas9 to cut the *Lsdia1* gene.
3. Cut out Guide RNA #2 (page 12). This guide is blank. You will fill in a sequence that you design to mutate the *Lsdia1* gene.

You inject the *Lsdia1* guide RNA and Cas9 into early snail embryos whose parents had shells that spiral clockwise. Use the paper model to see what will happen in the cells of the snail embryos.

4. First, the guide RNA associates with Cas9 to form a complex.
    - Place the ***Lsdia1* guide RNA #1** onto the yellow zone in the Cas9 protein.
    - Be sure to line up the RNA carefully.
    - Place a piece of tape across the guide RNA where it says “guide RNA” to hold it in place.

5. Next, the guide RNA/Cas9 complex scans the genome for a PAM sequence.
    - Place one copy of the ***Lsdia1* DNA** on the white stripe on the Cas9 protein. The target DNA should be under the guide RNA so that you can still read the sequence on both strands of the DNA (see picture below).
    - It doesn’t matter which way you put the DNA in since Cas9 will check each strand of DNA for PAM sequences in turn.
    - Slide the target DNA until the purple PAM box on the Cas9 protein aligns with a purple PAM sequence on the target DNA.
    - Potential PAM sequences are highlighted in purple. When multiple PAM sequences are in a row or overlapping, they are also marked with brackets.



6. Once Cas9 encounters a PAM sequence, the guide RNA will have a chance to pair with the DNA sequence (see picture on previous page, U pairs with A and C pairs with G).
    - If the guide RNA and the target DNA are not complementary, slide the target DNA to the next PAM site and repeat this step.
    - Remember: The PAM sequence is three bases long. Sometimes, however, there can be multiple PAM sequences in a row. These are indicated on the DNA with brackets.
    - If you find no matches on one strand of DNA, take the DNA out and turn it around to try the other strand.
    - When you find a complementary sequence, circle the PAM site on the DNA that is aligned with the PAM box on Cas9, and then advance to step 7.

7. If the guide RNA and the target DNA are complementary, Cas9 will cut the DNA
    - Mark both strands of the target DNA where the pink arrows point.
    - Take the DNA out and use scissors to cut both strands where you marked the DNA.

Questions

A. What is the Cas9 PAM sequence?
5’ __ __ __ 3’

B. How does the requirement that Cas9 bind to a PAM sequence affect the ability of scientists to target Cas9 to the exact DNA sequence they are interested in?

You have successfully cut the *Lsdia1* DNA in the snail embryo, but it isn’t mutated yet…

8. When the cell’s DNA repair machinery detects that the DNA has been cut, it will move in to repair the break. In eukaryotes, the most common repair mechanism (NHEJ) adds or removes some bases from the cut ends of the DNA, then re-attaches the two ends.
    - Cut off between 0-5 bases from each end of the DNA at the cut site. You get to pick how much DNA to cut off—there is no right or wrong answer here.
    - Tape the two ends of the DNA back together where you cut them.

Questions

Once the snails have matured, you sequence their DNA and find that several of the snails carry mutations in the *Lsdia1* gene.

Below is a section of the original (wild-type) *Lsdia1* sequence and four different mutant sequences that scientists actually generated in snails when they used this guide RNA (Abe and Kuroda, 2019). The PAM is marked in purple. The mutant sequences represent four successful CRISPR/Cas DNA modifications. If there is a "-" it means those bases were removed from the sequence, much like you did with scissors. If a letter is written in red, it means there was an additional base added to the sequence.

Wild-type *Lsdia1*	5' GGCCACATTCCCACTCCTTTGAGTAGAGGCGGACCCATTCCTGGAAGGGGTGGTGGAGGTG 3'
Mutant 1	5' GGCCACATTCCCACTCCTTTGAGTAGAGGCGGA----------GGGGTGGTGGAGGTG 3'
Mutant 2	5' GGCCACATTCCCACTCCTTTGAGTAGAGGCGGACCCATTCCT----GGGGTGGTGGAGGTG 3'
Mutant 3	5' GGCCACATTCCCACTCCTTTGAGTAG----------GGGTGGTGGAGGTG 3'
Mutant 4	5' GGCCACATTCCCACTCCTTTGAGTAGAGGCGGACCCATTCCTGGTAAGGGGTGGTGGAGGTG 3'

C. Compare the repaired DNA sequence from your paper model to wild-type sequence. On the "your result" sequence below (indicated by the arrow), cross out the DNA bases that were deleted when you repaired the DNA.

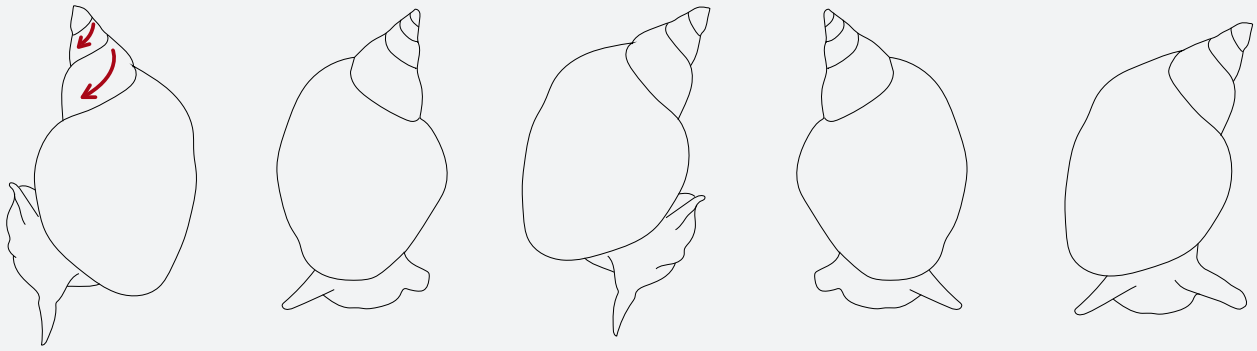
Wild-type *Lsdia1*	5' GGCCACATTCCCACTCCTTTGAGTAGAGGCGGACCCATTCCTGGAAGGGGTGGTGGAGGTG 3'
➡ Your result	5' GGCCACATTCCCACTCCTTTGAGTAGAGGCGGACCCATTCCTGGAAGGGGTGGTGGAGGTG 3'

D. Is your resulting sequence the same as any of the mutant sequences that scientists found after using this guide RNA?

E. Compare your mutation to those made by other students. Did any groups create the same mutants? Using just this one guide RNA, how many different mutations were created?

F. NHEJ introduces random mutations. Considering this, why do you think it is important for scientists to sequence the cell's DNA after CRISPR/Cas genome editing?

G. You also breed these genetically modified snails to generate offspring that are homozygous for each mutation. Below are sketches of these homozygous mutants (Abe and Kuroda, 2019).



Wild-type *Lsdia1*
Shell coils clockwise

Mutant 1

Mutant 2

Mutant 3

Mutant 4

Use the DNA sequences and the images of the homozygous snails to characterize each mutation in the table below

Mutant	Mutation type (insertion or deletion)	# of bases changed	Shell coils clockwise or counterclockwise?
1			
2			
3			
4			

H. In the snails above, the DNA that was altered will still be transcribed into mRNA, and that mRNA will still be translated by the ribosome. The sequence observed in Mutant 3 is the most severe mutation in terms of the total number of bases changed compared to the wild-type *Lsdia1* sequence. However, this mutation does not affect the direction in which the snail's shell spirals. Can you think of a reason why this might be the case? Hint: think about the number of bases deleted and how the instructions for making protein are encoded in DNA.

I. Remember that the reason scientists wanted to knock out the *Lsdia1* gene in snails was to test their hypothesis that the *Lsdia1* gene determined the direction in which a snails shell spiraled. Does the experimental evidence support this hypothesis? Explain your reasoning.

J. DNA is said to have a universal genetic code. Cas9 is a bacterial protein. Explain why a universal genetic code allows us to use any organism, including snails, to express the *cas9* gene as protein.

Now that you've got some experience with the CRISPR/Cas system, try to design your own guide RNA to mutate the *Lsdia1* DNA! When scientists want to use CRISPR/Cas to disrupt a target DNA sequence, they typically test multiple guide RNAs.

9. Design another guide RNA that could be used to disrupt this region of the *Lsdia1* gene. There is more than one acceptable answer to this question.
    - Remove guide RNA #1 and place the blank ***Lsdia1* guide RNA #2** onto the Cas9 protein. Be sure to line up the RNA carefully. Place a piece of tape across the guide RNA where it says "guide RNA" to hold it in place.
    - Place the uncut ***Lsdia1* DNA** strip on the white stripe on the Cas9 protein. The target DNA should be under the guide RNA but placed so that you can still read the sequence on both strands of the DNA.
    - Slide the target DNA so that a purple PAM sequence aligns with the purple PAM box on the Cas9 protein.
    - Where the pink arrows align on the DNA is where Cas9 will cut if this PAM is used.
    - Once you have chosen a PAM sequence, use the *Lsdia1* sequence to fill in the appropriate guide RNA sequence in the empty boxes. The guide RNA should be complementary to the bottom strand of DNA in this model. Remember that RNA uses U instead of T.
    - Also, record your guide sequence here:
      5' __ 3'

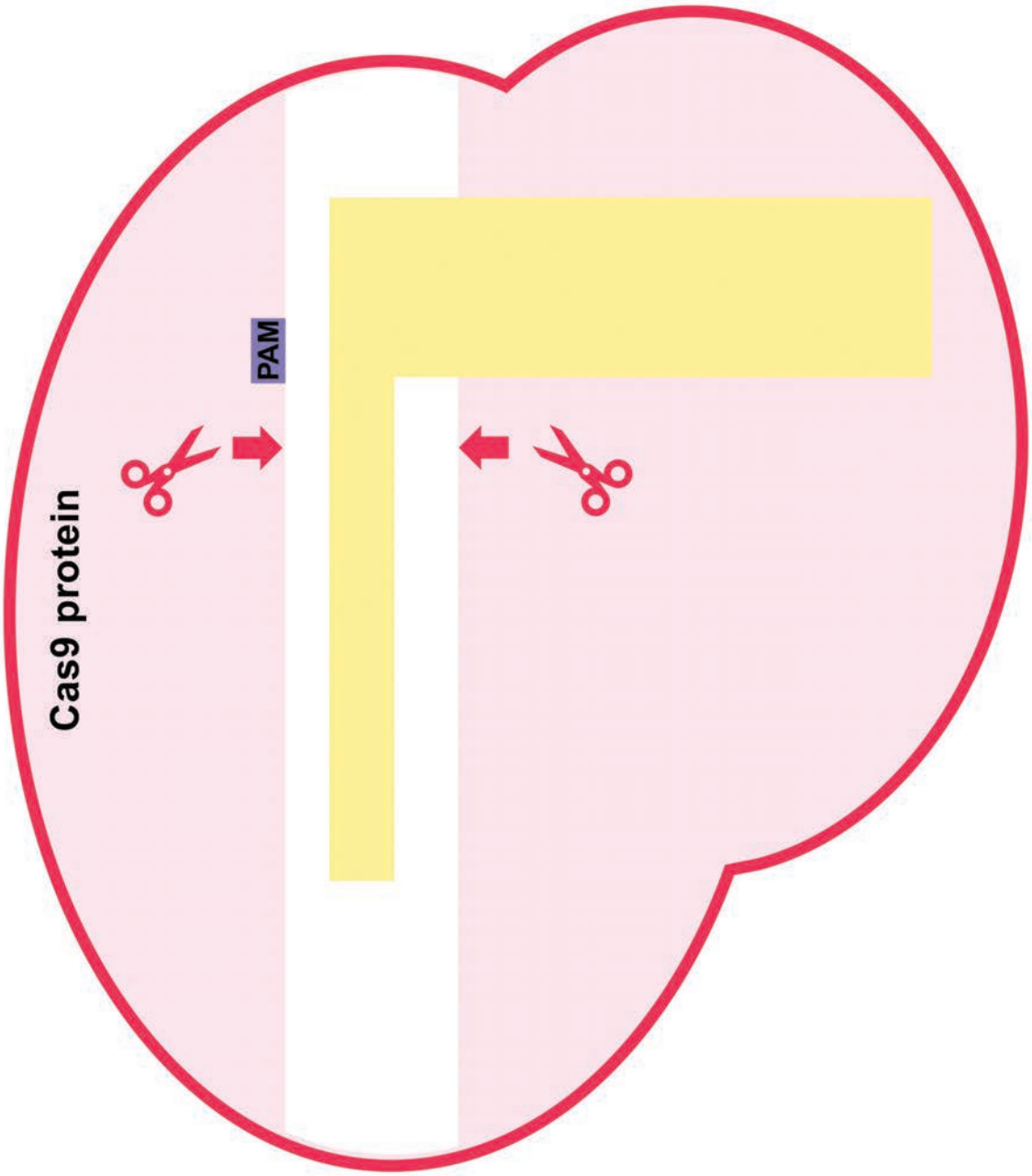
10. Remove the DNA and give your completed ***Lsdia1* guide RNA #2** to another group and have them test it to see if it will lead to Cas9 cutting the *Lsdia1* gene.

Just like that, you've designed a guide RNA that could be used to knock out a specific gene in snails! This exercise shows one of the main advantages of CRISPR/Cas for genome editing: that knowledge of the simple base-pairing rules is pretty much all you need to target a specific DNA sequence for modification.

Questions

K. Compare your guide RNA sequence with your classmates'. How many different guide RNAs did your class design to disrupt just this short region of the *Lsdia1* gene?

L. Why is it helpful for scientists that the Cas9 PAM sequence is relatively common in the genome?


PAM
Cas9 protein


target DNA
5'...CACATTCCCACTCCTTTGAGTAGAGGCGGACCCATTCCTGGAAGGGGTGGTGGAGGTG...3'
3'...GTGTAAGGGTGAGGAAACTCATCTCCGCCTGGGTAAGGACCTTCCACCACCCCTCCAC...5'
Lsdia1 gene
target DNA
target DNA
5'...CACATTCCCACTCCTTTGAGTAGAGGCGGACCCATTCCTGGAAGGGGTGGTGGAGGTG...3'
3'...GTGTAAGGGTGAGGAAACTCATCTCCGCCTGGGTAAGGACCTTCCACCACCCCTCCAC...5'
Lsdia1 gene
target DNA
5'GGCGGACCCAUUCCUGGAAG
3'
Lsdia1 guide RNA #1
3'
5'
Lsdia1 guide RNA #2