DNA Methylation & Cadmium Exposure *in utero*

An Epigenetic Analysis Activity for Students

UNC-Chapel Hill's Superfund Research Program

Cadmium is one of the highest priority chemicals regulated under the EPA's Superfund program and can enter soil, water, and air from mining and industrial processes, burning coal and household wastes, where it can be taken up by fish, plants and animals. This activity features the research of a scientist who is funded by UNC-Chapel Hill's Superfund Research Program to investigate the link between exposure to cadmium and associated human health effects through the mechanism of epigenetic modification to DNA.

This activity is targeted to students in Advanced Placement (AP) Biology or college-level biology courses.

Curriculum Alignment

Advanced Placement (AP) Biology

Big Idea 2: Biological systems utilize free energy and molecular building blocks to grow, to reproduce, and to maintain dynamic homeostasis.

Enduring understanding 2.D: Growth and dynamic homeostasis of a biological system are influenced by changes in the system's environment.

Essential knowledge 2.D.1: All biological systems from cells to organisms to populations, communities and ecosystems are affected by complex biotic and abiotic interactions involving exchange of matter and free energy.

Essential knowledge 2.D.3: Biological systems are affected by disruptions to their dynamic homeostasis.

Enduring understanding 2.E: Many biological processes involved in growth, reproduction, and dynamic homeostasis include temporal regulation and coordination.

Essential knowledge 2.E.1: Timing and coordination of specific events are necessary for the normal development of an organism, and these events are regulated by a variety of mechanisms.

Essential knowledge 2.E.2: Timing and coordination of physiological events are regulated by multiple mechanisms.

Big Idea 3: Living systems store, retrieve, transmit, and respond to information essential to life processes.

Enduring understanding 3.A: Heritable information provides for continuity of life.

Essential knowledge 3.A.4: The inheritance pattern of many traits cannot be explained by Mendelian genetics.

Enduring understanding 3.B: Expression of genetic information involves cellular and molecular mechanisms.

Essential knowledge 3.B.1: Gene regulation results in differential gene expression, leading to cell specialization.

Big Idea 4: Biological systems interact, and these systems and their interactions possess complex properties.

Enduring understanding 4.C: Naturally occurring diversity among and between components within biological systems affects interactions with the environment.

Essential knowledge 4.C.2: Environmental factors influence the expression of the genotype in an organism.

Next Generation Science Standards (NGSS)

Disciplinary Core Ideas	**Science and Engineering Practices**	**Crosscutting Concepts**
From Molecules To Organisms: Structures And Processes (Ls1) Heredity: Inheritance and Variation of Traits (Ls3) Links among engineering, technology, science, and society (ETS2)	Analyzing and interpreting data Constructing explanations Developing and using models Obtaining, evaluating, and communicating information Using mathematics and computational thinking	Cause and effect Patterns Scale, proportion, and quantity Stability and change Structure and function Systems and system models
Performance Expectations Students who demonstrate understanding can: HS-LS3-1. Ask questions to clarify relationships about the role of DNA and chromosomes in coding the instructions for characteristic traits passed from parents to offspring.		

Activity Description

This activity focuses on the research of one scientist who is studying the link between disease and one well characterized epigenetic mechanism, DNA methylation.

UNC-Chapel Hill scientist Rebecca Fry, PhD, and her research team study children's health effects related to prenatal exposure to cadmium in North Carolina. Cadmium is a toxic metal that, like arsenic, poses a threat to children's health. Pregnant women can be exposed to cadmium through either smoking cigarettes or inhaling secondhand cigarette smoke, breathing contaminated air or by ingesting cadmium-contaminated drinking water or food (e.g., shellfish, kidney or liver meats). **"There is evidence that maternal exposure to cadmium may leave marks on the baby's DNA that could be passed on to future generations,"** Fry explains. "We looked at DNA collected from newborns' cord blood at birth to see if there is a relationship between prenatal exposure to cadmium and epigenetic changes to their DNA." In the *Resources* section, you will find the hyperlink to a 2 minute video titled *Elucidating mechanisms of cadmium-induced toxicity and disease* that describes Dr. Fry's research.

In this activity, students interpret scientific data from a recent peer-reviewed scientific publication to assess the relationship between prenatal exposure to cadmium and epigenetic changes to newborns' DNA in an effort to learn about the research taking place to understand the link between prenatal exposure to environmental contaminants and disease arising from altered DNA methylation patterns.

Background Information: Fry and her research team are investigating the role of DNA methylation in disease. DNA methylation occurs when an enzyme called DNA methyltransferase (DNMT) covalently attaches a methyl ($-CH_3$) group to a cytosine base that is typically adjacent to a guanine base (see Figure 1). Such sites where a cytosine is adjacent to guanine via a phosphodiester bond are called CpG sites. Scientists have observed that DNA methylation occurs predominately along places on the DNA strand that are rich in CpG sites. A region rich in CpG sites is referred to as a CpG island. CpG islands are associated with approximately 60-70% of mammalian genes, and most CpG islands are unmethylated in normal mammalian cells. Thus, changes in methylation patterns at CpG islands can interfere with normal gene expression by altering the transcriptional competency of a gene's promoter.

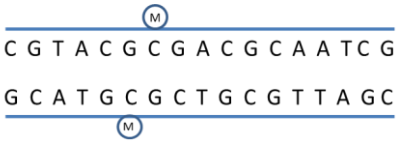

Figure 1: This DNA schematic depicts two methyl groups (M) covalently attached to cytosine (C) bases on complementary DNA strands. DNA methylation patterns can be preserved during DNA replication.

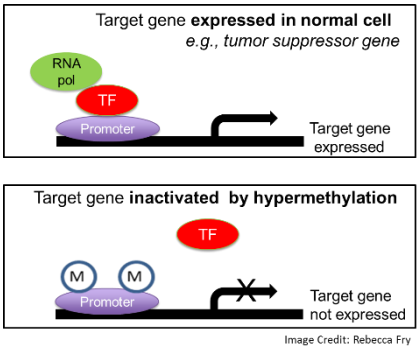

Figure 2: The addition of methyl groups to CpG islands common to promoters (shown in purple) is <u>one</u> mechanism for silencing gene transcription by preventing access to one or more transcription factors (TF, shown in red).

While DNA methylation is involved in normal control of gene expression, changes in the extent of DNA methylation can contribute to cancer or disease by silencing genes that that should otherwise be active (or expressed) or by causing expression of genes that are usually inactive. DNA methylation is one mechanism for suppressing (or silencing) gene transcription by preventing one or more transcription factors (TF) and thus RNA polymerase (RNA pol) from accessing a gene's promoter, which is required for transcription (see Figure 2). These DNA methylation changes do not involve changes in DNA sequence and are therefore described as 'epigenetic' changes, meaning 'above the genome', the literal translation of 'epigenome.' Epigenetic changes can be maintained and inherited by daughter cells during mitosis and meiosis. Therefore, epigenetic modifications that occur *in utero* can be passed on to subsequent generations.

It is worth noting that an epigenetic modification--in this case DNA methylation--will not necessarily result in a *functional change* in gene expression. In fact, it is possible that only a very small fraction of differentially methylated genes will exhibit a functional change in gene expression. There is still much to be learned about epigenetics. Dr. Fry's research is taking her into previously unchartered territory thanks to advances in knowledge and technology.

Teacher Preparation

- Students should have a basic understanding of DNA structure and function prior to introducing the concept of epigenetics. **Students should already be familiar with the following terminology:**
    - Deoxyribonucleic acid (DNA)
    - Gene
    - Gene expression/regulation
    - Nitrogenous base
    - Phosphodiester bond
    - Promoter
    - Ribonucleic acid (RNA)
    - Transcription
    - Transcription factors (TF)
    - Translation
- Prior to this activity, conduct the lesson *DNA Wrap: Packaging Matters (*See *Resources* section) or at a minimum, introduce your students to the concept of ***epigenetics - the notion that phenotypes can be inherited without alterations to the nucleotide sequence of DNA.*** The *Background Information* presented here and on page 1 of the *DNA Wrap: Packaging Matters* lesson will be useful.

Materials

- Copies of student worksheet, one per student
- Color copies of heat map, one per student or student pair
- Red and blue colored pencils or pens, one set per student or student pair
- Computer with MS Office Software (including PowerPoint)
- PowerPoint slide set that accompanies this lesson
- Projector

Key Terms

- **Students will be introduced to the following terminology by conducting this activity:**
    - Cadmium
    - CpG sites/CpG islands
    - DNA methylation
    - DNA methyltransferase (DNMT)
    - Differential methylation
    - Epigenetics
    - Heat map
    - Transcription factor occupancy theory

Activity Procedure

1. If you have not done so already, introduce your students to the concept of epigenetics. You may find it useful to describe one or more examples (see *Resources*) that have been well studied by scientists:

   Dutch Hunger Winter
   - Famine (starvation) induced epigenetic modifications in individuals exposed *in utero;* those individuals surviving into adulthood exhibited higher rates of obesity and cardiovascular disease.

   Diethylstilbestrol (DES) Exposure
   - Exposure to a synthetic form of estrogen, diethylstilbestrol (DES), induced epigenetic modifications that resulted in increased fertility problems and cancer risks in women exposed to this chemical *in utero*.

   Agouti Mice
   - Mice with an unmethylated Agouti gene, which is constantly being expressed, exhibit yellow fur and obesity while mice with a methylated Agouti gene have brown fur and are not obese. Thus DNA methylation silences the Agouti gene. **This example clearly demonstrates that DNA methylation does not always lead to negative consequences for the individual.**

2. Introduce your students to the epigenetic research taking place in Dr. Rebecca Fry's lab. Conclude by asking your students to identify the central question guiding Dr. Fry's research: *Does maternal cadmium exposure alter DNA methylation patterns in newborns?*

3. Next, describe the experimental design that Fry and her team used to test the hypothesis that cadmium exposure results in alterations of DNA methylation patterns in infants exposed to cadmium *in utero*. Alternatively, you may wish to task students with coming up with an experimental design to test the hypothesis that maternal cadmium exposure alters DNA methylation patterns in newborns.

   Experimental Design: Fry and her team analyzed DNA from umbilical cord blood collected at birth from 17 infants born in Durham County, NC. These infants had mothers with documented exposure to cadmium, as indicated by levels in maternal blood. DNA, specifically, methylated CpG islands, from newborn cord blood leukocytes was isolated then amplified using a technique called *methylated CpG island recovery assay* (MIRA) (see *Resources*). Next, *microarray analysis* (see *Resources*) was used to assess over 16,000 CpG islands to determine which islands or promoter regions exhibited differential methylation in response to cadmium exposure (each promoter corresponds to a specific gene). A *visual representation* of the resulting microarray data, referred to as a heat map, allows for comparison of methylation patterns between individuals. *Note: the heat map does not show fluorescence but rather uses shades of red or blue to depict the relative abundance of methylated sites for the genes that exhibited differential methylation patterns in association with cadmium exposure in utero.*

   [Optional] **You may wish to review the following standard lab techniques/procedures that were utilized in this research:**

   DNA fragmentation (sonication)
   DNA isolation (column chromatography to isolate DNA of interest)
   DNA amplification (polymerase chain reaction (PCR))
   Fluorescent Labeling DNA of Interest
   Hybridization of DNA to Microarray (Gene Chip)

4. Next, direct your students' attention to their worksheet, which asks them to interpret a *visual representation* of the experimental data, referred to as a **heat map** (Figure 3). Fry and her team identified 61 specific genes that exhibited **differential methylation patterns** in association with cadmium exposure *in utero;* these genes exhibited a change in methylation in response to cadmium exposure and this response varied across individuals.

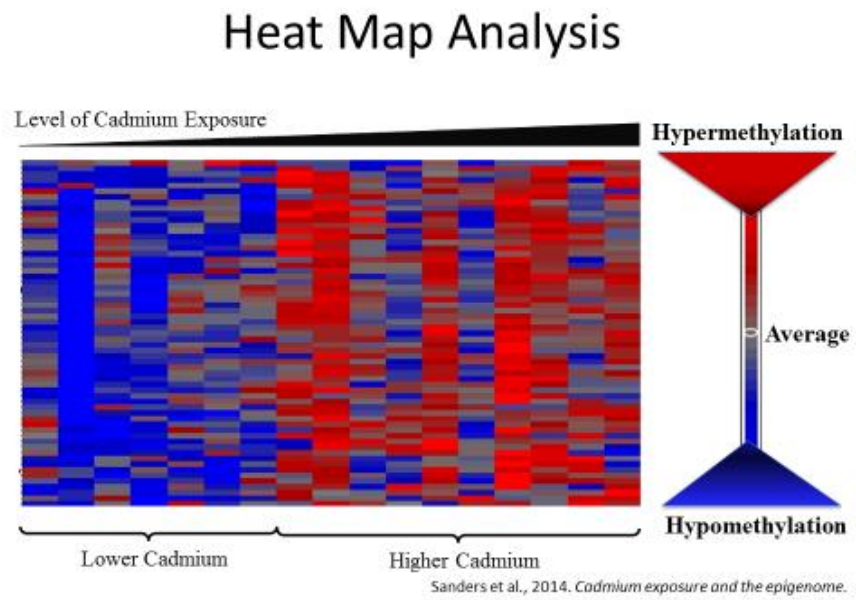

Figure 3. Changes in DNA methylation patterns associated with cadmium exposure *in utero*. The heat map is a color (qualitative) representation of quantitative methylation abundance data and illustrates the average DNA methylation levels in 61 genes (y-axis); each column of the x-axis corresponds to one infant. Red represents a relative increase in CpG island methylation level, and blue represents a relative decrease in methylation level. Maternal blood cadmium levels ranged from below detection to 1.05 µg/L with an average concentration of 0.44 µg/L. *See Resources section for research citation.*

5. Either project the image of the heat map at the front of the room and/or provide color copies of the heat map to student pairs. Orient the students to the heat map by informing them that each rectangle in this heat map corresponds to one gene in an individual; the vertical column (y axis) of rectangles represents 61 different genes within the same individual. The number of columns (x-axis) refers to the number of individuals sampled (17

infants). **Heat map analysis allows for comparison of methylation patterns between individuals**. The color of each rectangle is significant; red represents a relative increase in average methylation (hypermethylation), and blue represents a relative decrease in average methylation (hypomethylation). The heat map is organized such that individuals from mothers with lower blood cadmium levels are found to the left side of the heat map and individuals from mothers with higher blood cadmium levels are found to the right side of the heat map.

6. Ask the students to work in pairs to complete their worksheet and interpret the heat map depicting cadmium-associated gene-specific changes in DNA methylation.

   [*Optional*] Time permitting, you may choose to have your students interact to a greater extent with the heat map by having them assign a score to designate the degree of methylation of each gene in each individual. As a class, decide how to score the shades of red and blue rectangles. For example, a gray rectangle would be given an arbitrary score of "0" and a bright red rectangle would be given a score of "3", a bright blue rectangle a score of "-3", etc. Assign student pairs to analyze one or two genes and then record their results at the front of the room. For each gene, have students calculate 1) the average "methylation score" for the 7 infants whose mothers had low blood Cadmium levels and 2) the average "methylation score" for the 10 infants whose mothers had high blood Cadmium levels. Students obtain positive averages for genes that are hypermethylated and negative averages for genes that are hypomethylated.

   This activity will lead to greater interaction with the heat map and an understanding of how the heat map was created. The different shades of red and blue rectangles correlate with quantitative methylation abundance data depicting different degrees of methylation; thus students are reverse-engineering the heat map when they conduct this exercise.

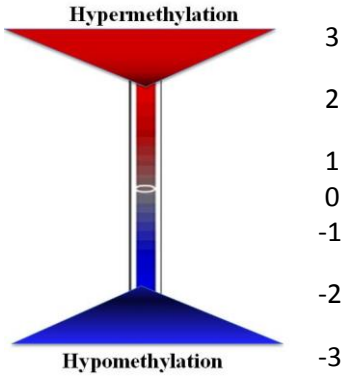

Figure 4. Arbitrary degrees of change from an average level of methylation ("0") for methylation scoring exercise.

7. To conclude this activity, review the answers to the questions posed on the student worksheet (see *Answer Key*). You may also wish to ask one or more of the following questions:
   - Did exposure to low levels of maternal cadmium *always* lead to hypomethylation of fetal genes? *No. Only 61 genes exhibited altered DNA methylation in association with cadmium exposure and of these 61 genes, individuals in the low exposure group showed variation in the extent of methylation.*
   - Did exposure to high levels of maternal cadmium *always* lead to hypermethylation of fetal genes? *No. Only 61 genes exhibited altered DNA methylation in association cadmium exposure and of these 61 genes, individuals in the high exposure group showed variation in the extent of methylation, though the majority of genes showed hypermethylation with increasing cadmium levels.*
   - Do changes in methylation for a particular gene mean that gene expression is altered? *No, changes in methylation may or may not lead to altered gene expression; whether gene expression is altered depends on the location of the hypo- or hyper- methylation events within the gene.*

- Is a change in methylation good or bad? *It depends on the role of the affected gene's product (e.g. protein) in the cell; methylation can lead to either positive (e.g., Agouti gene inactivation) or negative (e.g., tumor suppressor gene inactivation) phenotypic consequences.*

8. Your students may have questions about the infants born whose mother's had high blood cadmium levels. None of these babies were born with defects and all but one had a "normal" birth weight (greater than 2500g), although recent epidemiologic data from another study of 1800+ women suggests that cadmium exposure is correlated with an increased risk of having a lower birth weight baby. Long-term health impacts that could be associated with *in utero* exposure to cadmium will be monitored by tracking these children through the Children's Center at Duke University.
9. Time permitting, you may choose to have your students read the *Discussion* section of the featured scientific paper which places this research into the larger context of how scientists are examining the influence of prenatal exposures on adult-onset diseases with an emphasis on metal exposure.
10. To conclude this activity, let students know that the biological mechanism responsible for gene-specific DNA methylation as observed for cadmium is unknown and is an active area of research. In 2016, Dr. Fry and her research team hypothesized one potential mechanism that explains how gene-specific methylation might occur in response to exposure to an environmental contaminant. Their hypothesis, the *transcription factor occupancy theory*, states that the presence or absence of transcription factors at the promoter region of a gene may influence patterns of methylation by influencing the extent to which methyltransferase can access the promoter site in response to exposure to an environmental contaminant (Figure 5).

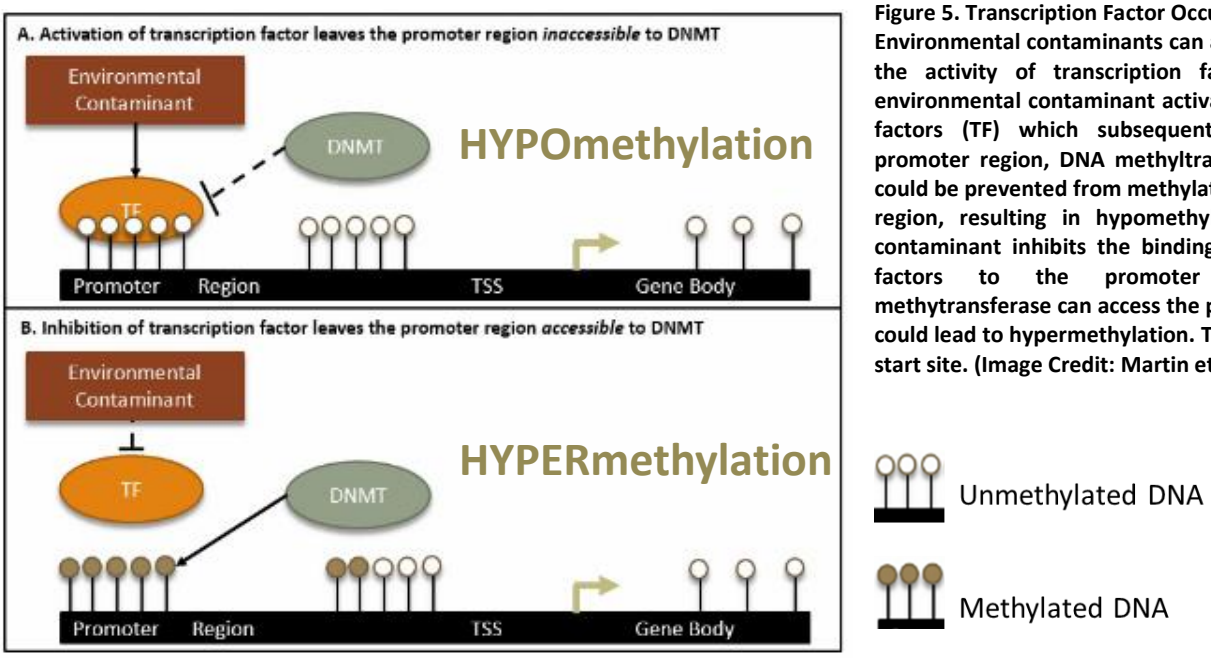

Figure 5. Transcription Factor Occupancy Theory
Environmental contaminants can activate or inhibit the activity of transcription factors. A. If an environmental contaminant activates transcription factors (TF) which subsequently bind to the promoter region, DNA methyltransferase (DNMT) could be prevented from methylating the promoter region, resulting in hypomethylation. B. If the contaminant inhibits the binding of transcription factors to the promoter region, DNA methytransferase can access the promoter and this could lead to hypermethylation. TSS = transcription start site. (Image Credit: Martin et al., 2016.)

In the context of the experimental findings presented here, the transcription factor occupancy theory provides one explanation for why some genes exhibited hypomethylation while other genes exhibited hypermethylation in response to cadmium exposure.

Resources

Featured Scientific Publication

Sanders AP, Smeester L, Rojas D, DeBussycher T, Wu MC, Wright FA, Zhou Y, Laine JE, Rager JE, Swamy GK, et al. *Cadmium exposure and the epigenome: Exposure-associated patterns of DNA methylation in leukocytes from mother-baby pairs*. Epigenetics. 2014 Feb; 9(2):212-21
http://www.ncbi.nlm.nih.gov/pubmed/24169490

Elucidating mechanisms of cadmium-induced toxicity and disease
http://sph.unc.edu/superfund-pages/research-projects/biomedical/elucidating-mechanisms-of-cadmium-induced-toxicity-and-disease/ (includes two minute video)

Towards Prenatal Biomonitoring in North Carolina: Assessing Arsenic, Cadmium, Mercury, and Lead Levels in Pregnant Women
http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0031354

Cadmium, ToxFaQ Sheet, ATSDR
http://www.atsdr.cdc.gov/toxfaqs/tf.asp?id=47&tid=15

Martin, EM and Fry, RC. *A cross-study analysis of prenatal exposures to environmental contaminants and the epigenome: support for stress-responsive transcription factor occupancy as a mediator of gene-specific CpG methylation patterning.* Environmental Epigenetics. 2016 Jan; 2(1).
http://www.ncbi.nlm.nih.gov/pubmed/27066266

Featured Laboratory Techniques

Affymetrix Whole Genome Tiling Arrays (see figure outlining the technique)
https://www.bcm.edu/research/advanced-technology-core-labs/lab-listing/genomic-and-rna-profiling-core/services/affymetrix-whole-genome-tiling-arrays

Methylated-CpG island recovery assay (MIRA)
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3225287/figure/F1/ (Figure)
http://www.nature.com/labinvest/journal/v85/n9/full/3700311a.html

Additional Epigenetic Resources

DNA Wrap: Packaging Matters
http://erp.unc.edu/envhealth/epigenetics/

Epigenetic Influences and Disease
http://www.nature.com/scitable/topicpage/epigenetic-influences-and-disease-895

Epigenetics: Genome, Meet Your Environment
http://www.blc.arizona.edu/courses/schaffer/449/Epigenetics/Genome%20Meet%20Your%20Environment.htm

Dutch Hunger Winter

Persistent epigenetic differences associated with prenatal exposure to famine in humans
http://www.pnas.org/content/105/44/17046.long

Beyond DNA: Epigenetics
http://www.naturalhistorymag.com/features/142195/beyond-dna-epigenetics

Diethylstilbestrol (DES) Exposure

Commentary: Prenatal exposure to diethylstilbestrol (DES): a continuing story
http://ije.oxfordjournals.org/content/35/4/868.full

Acknowledgements

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This lesson was piloted and/or reviewed by following biology teachers:
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Robbie Carraghan, South Caldwell High School, Hudson, NC
Don Kirkpatrick, Marion High School, Marion, SC
Victoria W. Raymond, Northwood High School, Pittsboro, NC

Heat Map Analysis: Student Worksheet **(Answer Key)** Name:

UNC scientist Rebecca Fry, PhD, and her research team study children's health effects related to prenatal exposure to cadmium in North Carolina. Cadmium is a toxic metal that, like arsenic, poses a threat to children's health. Pregnant women can be exposed to cadmium through either smoking cigarettes or inhaling secondhand cigarette smoke, breathing contaminated air or by ingesting cadmium contaminated drinking water or food (e.g., shellfish, kidney or liver meats). **"There is evidence that maternal exposure to cadmium may leave marks on the baby's DNA that could be passed on to future generations,"** Fry explains. "We are looking at DNA collected from newborns' cord blood at birth to see if there is a relationship between their prenatal exposure to cadmium and epigenetic changes to their DNA."

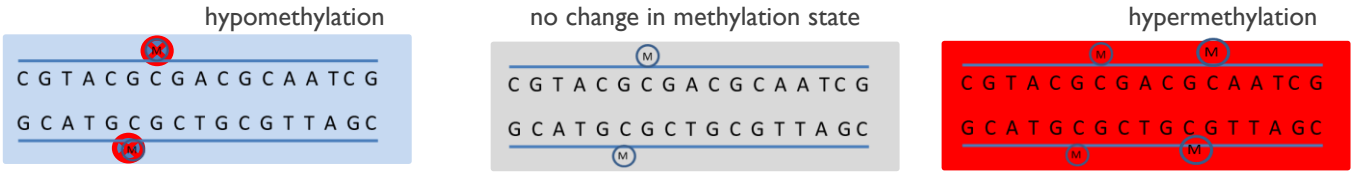
1. In your own words, state the central question guiding Dr. Fry's research. *Does maternal cadmium exposure alter DNA methylation patterns in newborns?*

Next, you will interpret a *visual representation* of the experimental data, referred to as a **heat map**, using the following questions as a guide as you view the heat map provided by your teacher.

2. Notice that the map is organized into rectangles that form columns and rows:
   a. How many rectangles are in a vertical column? *61*
   b. What do these rectangles represent? *61 different genes*
   c. How many rectangles are in a horizontal row? *17*
   d. What do these rectangles represent? *17 individuals (infants) are being compared*

3. What is the significance of the blue and the red rectangles? *Red represents a relative increase in average methylation (hypermethylation) and blue represents a relative decrease in average methylation (hypomethylation).*

4. Each box below contains a DNA sequence with two methylated cytosines. By adding or crossing out methyl groups (M), depict hypermethylation, hypomethylation and no change in methylation state in the boxes below and then, using colored pens or pencils, indicate which box would appear red, blue and gray on a heat map by shading in the appropriate box:



methyl group(s) removed from DNA *no change in methyl groups* *methyl groups added to DNA*

5. What does this heat map reveal about infants exposed to **lower levels** of maternal cadmium? *Fry and her team concluded that there is evidence of differential methylation patterns among infants exposed to low levels cadmium in utero; some genes exhibited hypomethylation while others exhibited hypermethylation in association with exposure to cadmium. In general, the majority of genes exhibited hypomethylation as evident by the predominance of blue on the heat map.*

6. What does this heat map reveal about infants exposed to **higher levels** of maternal cadmium? *Fry and her team concluded that there is evidence of differential methylation patterns among infants exposed to high levels of cadmium in utero; some genes exhibited hypomethylation while others exhibited hypermethylation in association with exposure to high levels of maternal cadmium. In general, the majority of genes showed hypermethylation with increasing cadmium level.*

7. Do these data **prove** that cadmium causes changes to DNA methylation *in utero?* *No, these data only demonstrate an association between cadmium exposure and differential methylation.*

8. Where might Fry and her research team direct future research to expand upon these findings? *Next steps would be to determine the functional consequences of these epigenetic modifications and identify corresponding health effects, some of which could occur later in life. It is important to point out to students that an epigenetic modification, in this case methylation of DNA, will not necessarily result in a change in gene expression; in fact it is possible that only a very small fraction of differentially methylated genes will exhibit a functional change in gene expression. The location of methylation "marks" within the promoter/CpG islands will dictate whether or not a change in methylation status results in a functional change in gene expression. Much more research needs to be done to identify sites where methylation changes lead to functional changes.*

   You could extend this discussion by asking your students to design a follow-up experiment to identify which epigenetic modifications result in differential gene expression. RNA and protein from each mother-baby pair could be analyzed to determine if a change in methylation status resulted in a change in gene expression; unfortunately these data were not collected in this cadmium study. Dr. Fry and her team are also studying newborns exposed to arsenic in utero to assess the impact of arsenic exposure on the fetal epigenome, fetal mRNA and protein expression.

   Your students may also be curious about the identity/functions of the genes that exhibited differential methylation in response to cadmium exposure. Fry and her team determined that certain biological functions were "enriched" or over-represented among the differentially methylated genes. Genes that encode proteins that play a role in the regulation of transcription along with genes associated with programmed cell death or apoptosis appear to be most impacted by cadmium exposure.

Heat Map Analysis: Student Worksheet Name:

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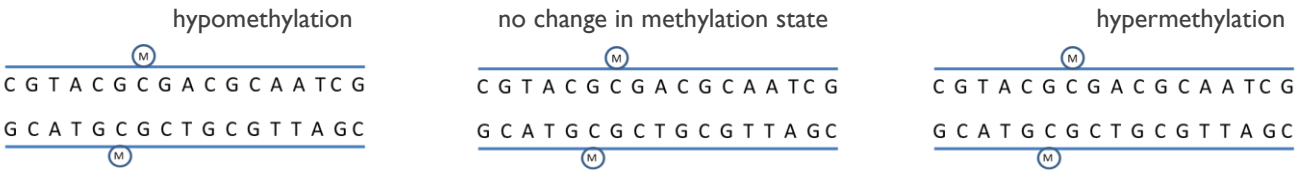
1. In your own words, state the central question guiding Dr. Fry's research.

Next, you will interpret a *visual representation* of the experimental data, referred to as a **heat map**, using the following questions as a guide as you view the heat map provided by your teacher.

2. Notice that the map is organized into rectangles that form columns and rows:
   a. How many rectangles are in a vertical column?
   b. What do these rectangles represent?
   c. How many rectangles are in a horizontal row?
   d. What do these rectangles represent?

3. What is the significance of the blue and the red rectangles?

4. Each box below contains a DNA sequence with two methylated cytosines. By adding or crossing out methyl groups (M), depict hypermethylation, hypomethylation and no change in methylation state in the boxes below and then, using colored pens or pencils, indicate which box would appear red, blue and gray on a heat map by shading in the appropriate box:



5. What does this heat map reveal about infants exposed to **lower levels** of maternal cadmium?

6. What does this heat map reveal about infants exposed to **higher levels** of maternal cadmium?

7. Do these data **prove** that cadmium causes changes to DNA methylation *in utero?*

8. Where might Fry and her research team direct future research to expand upon these findings?