MODEL-BASED REASONING: A COMMONALITY BETWEEN SCIENTIFIC

INVESTIGATION AND SCIENCE EDUCATION

By

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To the wonderful and best children ever, Kristian, Christopher, Krista and Kristina and my supportive husband, Timothy

and

To all my family members and friends whose support have been invaluable

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LIST OF ABBREVIATIONS

BM	Basement Membranes
BMP	Bone morphogenetic proteins
CR	Cysteine-Rich
CTAB	Cetyl Trimethylammonium Bromide
ECM	Extracellular Matrix
ECP	Eosinophil Cationic Protein
EDN	Eosinophil Derived Neurotoxin
EPO	Eosinophil Peroxidase
GBM	Glomerular Basement Membrane
GP	Goodpasture
H_2O_2	Hydrogen Peroxide
HEK	Human Embryonic Kidney
HIS	Histidine
HYL	Hydroxylysine
IG	Immunoglobulin
KDa	Kilodalton
MET	Methionine
LPO	Lactoperoxidase
LRR	Leucine Rich Repeat
MBP	Major Basic Protein
MPO	Myeloperoxidase
NC1	Noncollagenous globular domain 1
PCR	Polymerase Chain Reaction
PHG	Phloroglucinol
PMSF	Phenylmethanesulfonyl Fluoride
PXD	Peroxidase
PXDN	Peroxidasin
RPM	Revolutions Per Minute
SCN-	Thiocyanate
S=N	Sulfilimine Bond
SP	Signal Peptide
TGF-B	Tissue growth factor beta
TPO	Thyroid Peroxidase
VPO	Vascular Peroxidase
vWFC	Von Willebrand Factor C

CHAPTER I

OVERVIEW

An Interdisciplinary Graduate Program (IGP) is one that combines two different disciplines for a path of study designed to meet the career goal of an individual student. For my program of study, I combined the disciplines of biological sciences and educational learning sciences. I completed the course requirements for both disciplines and performed research in both areas of study. My committee members were evenly distributed of both disciplines (3 Education; 3 Science) providing me with proper guidance on how to navigate through this untraveled path. I had the pleasure of being a pioneer at Vanderbilt University for an IGP that combined biomedical science with education sciences.

This interdisciplinary path was necessary for two reasons. First, I entered the biological sciences department knowing that I wanted to perform scientific research to broaden my understanding of biological phenomenon, while I contributed answers to unknown questions in such a complex field. Second, in my heart, there was always a part of me that wanted to become a professor and teach biological sciences to K-12 students. Combining these two passions, I reached out to the graduate school to design an interdisciplinary path for my graduate studies.

For my educational science component, detailed in Chapter I, I investigated the understanding of elementary students about the process of decomposition within the context of assigned instruction. The instruction was specifically designed to bring

decomposition into their first-hand experience via a modeling approach. We found that the students became increasingly aware of decomposition as a process rather than an end-state and achieved significant growth in their understanding. This culminated in my first author paper that was published in the *Journal of Research in Science Education*. For my biomedical science component, detailed in Chapter III, my research was focused on peroxidasin, an enzyme that is important in tissue biogenesis. I identified peroxidasin as the only animal-heme peroxidase that catalyzes the formation of sulfilimine bonds within basement membranes. Subsequently, I determined which domains of peroxidasin are necessary for catalytic activity. This body of work led to my co-authorship on an article published in *Nature Chemical Biology in 2012,* and to a first-author paper that I am currently writing for submission to a biochemical journal.

This interdisciplinary plan of study helped me to gain a deeper understanding of biological science by conducting research and formal coursework and a deeper understanding of how students learn science through my opportunity as a graduate research assistant in the Peabody Science Education department. To complement my understanding of science education, I served an Assistant Director of the Aspirnaut Summer Science Academy in 2009, participated in the professional development teacher workshops, and co-constructed and taught of science lessons in a local elementary classroom. The latter experience revealed to me the need for a partnership between scientists and classroom teachers to help translate modern biology to elementary students.

CHAPTER II

EDUCATIONAL SCIENCE

YOUNG CHILDREN'S THINKING ABOUT DECOMPOSITION: EARLY MODELING
ENTREES TO COMPLEX IDEAS IN SCIENCE

Introduction

Although primary grades science instruction almost always includes the

study of nature, the typical focus is on the growth of organisms and the conditions

that support their life. Educational treatments of life cycles almost always conclude

with the death of the organism, even though processes of decay are at least as

consequential for the health and balance of our world. It is possible that educators

skirt this issue because they are unwilling to discuss death with young children;

moreover, because so much of the process of decomposition is not evident to the

unassisted eye, educators may legitimately be unsure how best to proceed with

youngsters whose knowledge seems so firmly anchored in things they can directly

see. Perhaps for these reasons, there is little research on children's thinking about

decomposition.

In the slim literature that can be found, the general focus is on what children

do *not* know, rather than on resources that they bring to further learning. Reading

these studies lends the impression that children from ages 5 to 16 have little to no

understanding about what happens during the process of decomposition (Leach et

al., 1996). Most studies found that more than half of the child participants believe

that matter from dead animals and plants simply disappears (Hellden, 1992; Sequiera & Freitas, 1986). When children are asked explicitly about rotting or decay, they report that decay is a state that simply happens to materials and that does not require an explanation (Smith & Anderson, 1986). Decay, therefore, is conceived as an index of the endpoint of life and not as a process (Driver et al., 1994). These teleological forms of reasoning are commonly observed even in older people (Hartley, 2011). In the previous research, of the very few children between the ages of 14-16 years old who understood that microbes were involved in the process of decomposition (Hellden; 1992), most believed that materials rot on their own and subsequently, microbes finish off the partially "self-rotted" matter (Cetin, 2007; Smith & Anderson, 1986). Because children generally do not have a well-developed theory of matter, it may not be surprising that they do not know that the material constituents of living organisms are neither created nor destroyed (Gayford, 1986).

Here we describe the development of very young children's thinking about decomposition in the context of instruction specifically designed to bring this process into their first-hand experience via a modeling approach. The instruction and associated study were part of a multi-year project on the development of elementary students' concepts related to ecosystems, change, and variability, concepts that we considered foundational (especially in their interrelationships) to developing a strong understanding of evolution as students moved into high school (Lehrer & Schauble, 2012 a & b). A hallmark of the instructional approach was to support students in developing, adapting, and/or revising models of processes in the world as a way of developing a better understanding of them. Accordingly, the

larger project follows students across elementary grades, tracking both the forms and processes of modeling that support the development of student conceptual knowledge in the life sciences. The research reported here focuses on first-graders, who had not previously participated in instruction related to this project.

The instruction about decomposition had two major goals. First, we aimed to help students build a more nuanced understanding of decomposition as a process. Identifying an appropriate level of understanding for very young students seemed important, given the role of decomposition in later taught, critical topics such as carbon cycling and climate change. Briefly, we sought to problematize students' views of soil (which they initially conceived as an inert substance, associated with contamination), of decomposers (although primarily, those visible to the unassisted eye), and of the process of decay (which, consistent with previous literature, they tended to think of as spoilage, particularly applicable to foods; as in previous research, they initially described decay as an inevitable conclusion to life that does not need further explanation). Our second goal was to explore young children's potential to work with (including developing) models of processes and objects in the natural world as tools for studying the world "out there." Modeling is a defining characteristic of science but is by no means an obvious epistemological gambit. People in general and children, in particular, do not necessarily find it self-evident why one would want to conduct investigations that involve representations of the natural world, rather than simply looking at the world itself (Bazerman, 1988; Windschitl, Thompson, & Braaten, 2008). Our ongoing research program (Lehrer & Schauble, 2012 a &b) focuses on learning about both the challenges and potential in

modeling approaches with students, including young students, and this paper contributes to that line of investigation.

Method

In the first-grade classroom where the research was conducted, the regular classroom teacher, with the first author's assistance, conducted science lessons on decomposition at least once a week during the course of a school semester (total number of sessions was about 18). The teacher had 13 years experience teaching at the time of the study, but had not previously taught decomposition in her class. Nor was she familiar with employing modeling approaches to science investigation. The instruction was designed in consultation with the second author, who had previously taught ideas about decomposition to students in middle school. However, in advance of this study, we did not know which ideas and modifications of previously developed instruction might be accessible to students as young as first-graders.

Participants

All the students in the class, who were six or seven years old at the time of the study, participated in the classroom activities and discussions. The class included 10 boys and 13 girls and was situated in a school proximal to a public housing project; 22 of the students received parent permission to participate in the study. Most of the students had been raised in this thoroughly urban environment, and the school population included a large proportion of students eligible for free and reduced lunch.

Procedure

Pre- and post- instruction written assessments were conducted with the 22 participating students. In addition, to supplement the written work, we identified a focus group of six students, chosen by the teacher to represent a wide range of student performance, as assessed by regular class assignments and tests. More intensive information, in the form of daily notebook entries and repeated individuals interviews, was recorded from these six focus students throughout the study.

During the previous autumn, students in the class had gone outdoors with their teacher and collected a large sample of fallen leaves. The class spent time that autumn comparing and contrasting the leaves' color, shapes, and sizes. The current study was initiated early in the following February, when the teacher reminded students of their leaf collection, which remained indoors and was at the time sitting in a pile on the floor in a back corner of the classroom. She next asked a guiding question that set the context for the decomposition study and, in addition, served as a pre-instructional assessment of students' thinking about decomposition. Specifically, students were asked to consider what happens to autumn leaves after they fall from deciduous trees. The teacher reminded students that many leaves fall every year. Given this, she asked, why isn't the entire world simply covered with leaves?

Students talked with each other about this question in groups of four, and following these initial discussions, each student individually wrote an answer to the teacher's initiating question, illustrated by a drawing. We refer to this work as the

initial assessment of student knowledge, and a parallel task (which we call the *final*

assessment) was repeated at the end of instruction. In both cases, in addition to

these written assessments, the first author conducted additional individual follow-

up interviews with the six focus students, to further probe their answers. These

interviews were conducted individually and were video recorded. The interviews

contained additional questions about students' conceptions of "dirt" (the children's

word for soils) and animals that live in "dirt."

Following the initial assessment, the teacher and first author implemented

six phases of instruction, varying in duration from a single class period to several

weeks. Figure 1 displays a timeline of this instructional sequence. As the figure

shows, the phases are numbered sequentially, by order of their initiation, but as

some of the phases were ongoing, there was considerable overlap among the

phases. We next briefly overview these phases, but further details on each, along

with information about student thinking, are featured in the Results section.

Phase 1. Each student brought a plastic bag filled with soil from a location of

choice near his or her home. Students examined and described the samples,

compared and contrasted their contents, and conducted individual soil profiles.

Phase 2. Students initiated ongoing observations, drawings, and textual

notebook entries to describe changes over time in two examples of decay: a ripening

and then rotting banana (one banana kept on each table group of four students), and

three Halloween pumpkins that were set outside the classroom window to decay.

Phase 3. The children made a visit to an outdoor compost bin and

investigated the contents. The school custodian explained how and why the bin was

installed and the components included in the bin. He then turned the materials in the bin as students watched and extracted samples of material for students to hold and observe.

Phase 4. Students inspected and compared soils from their home samples (first investigated during phase 1) to samples taken from the compost bin.

Phase 5. Children developed and observed changes in classroom models intended to represent the process of leaf decay. These models included lettuce leaves to represent autumn leaves and other components considered necessary to represent elements that might affect decay (different soil types, moisture levels, presence or absence of decomposing organisms, temperature, sunlight, etc.).

Phase 6. In the classroom, students initiated a study of earthworms and other (visible) decomposers that live in the soil. This work included magnified observations of the critters, enactments of the way they move, and reading of related trade books to learn about their structures.

As Figure 1 shows, these phases overlapped and varied considerably in duration.
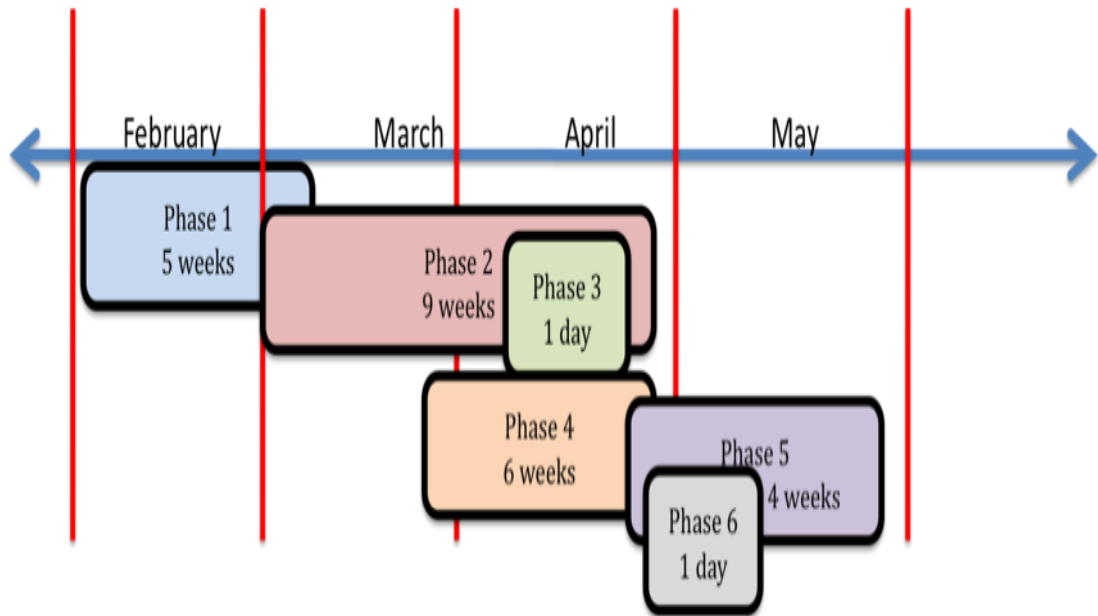
Figure 1: Timeline of Instructional Phases

Results

Initial Assessments of Student Knowledge

Working in groups of four, students first discussed and subsequently wrote

individual responses to the teacher's initial guiding question ("What happens to the

autumn leaves after they fall onto the ground? Why don't we see more and more of

them year by year until the entire world is covered with leaves?"). Most of these

responses were accompanied by drawings. At this initial assessment, the majority of

student responses (N = 11) suggested that the leaves simply disappeared, with few

attempts to explain where (although one child suggested they might have gone "to

another planet"). Students said that the leaves "disappeared," "died," were "blown

away," or were taken away by trash collectors, all reasonable replies, given the

experiences of these urban children. One child simply responded that the leaves

change color, which is certainly true, but irrelevant to the question posed. The

remaining half of the students reported that the leaves decrease in volume over time

and seemed to understand that somehow, this should be accounted for. However,

not very surprisingly, these students proposed mechanical (rather than chemical)

processes of change. They pointed out that people and animals step on leaves, which

then break into smaller pieces. In these children's views, leaves get ground into

ever-smaller pieces, and over time, the pieces become so small that they become

difficult to see. Some of these students felt that rain plays a part in this process,

perhaps softening leaves up so that they fall apart more easily. Otherwise, however,

these accounts did not propose changes in the material make-up of leaves.

The interviews with the six focus children confirmed these ideas and also strengthened our initial conjecture that students conceive of soil as a homogeneous, lifeless substance that is generally to be avoided because of its contaminating qualities ("Dirt is ugly….and the dirt has things in it that are really dirty"). For example, one of the focus children pointed out that dirt "comes from different places, but all places got dirt." Another remarked simply, "Dirt is dirt." When asked whether all "dirt" is the same, two students proposed that dirt can sometimes be different colors, but this was attributed to moisture—that is, children had noticed that when it is moist, soil takes on a darker color. The other four insisted that all dirt is the same kind of stuff. Furthermore, students stated that soil provides home for critters (or at least, earthworms) and serves to hold up trees so that they do not fall down. Five of the six children felt that soil somehow helps plants grow, but they had no idea how. It was common for students to propose ideas about soil that seemed to be based on its perceived utility or importance for humans: "If we didn't have dirt, the earth would look like all water everywhere. We would have no grass or green or anywhere to live." Another protested that without soil, humans would have nowhere to walk. The initial phase of instruction was intended to help students develop a more nuanced idea of the variable properties of soil and of the inter-relationships of these qualities (such as texture, structure, and moisture) with the animals and plants that are found there.

Student Thinking During Instruction

After children completed the initial assessment, the first phase of instruction was launched. All phases of instruction featured both individual and small group work, whole class discussions, observations with microscopes and flexcams, and class read-alouds of nonfiction literature on related topics. During instruction students were regularly asked to record their observations and questions in their science notebooks, which served to further document student thinking. The teacher used a camera and easel paper to record student comments during class activities. She left these artifacts on the walls over the course of the semester so that students could refer to their earlier comments and discuss "changes in how we used to think and how we think now." Copies of these artifacts were collected during or at the end of instruction and served as further evidence of changes in student thinking. In addition, we collected classroom video, audio, and field notes to establish records of student thinking during classroom activity, whole group discussions, and occasional informal interviews. Each week the classroom teacher and first author met to discuss student progress and to plan for the upcoming week; these plans were usually preceded by discussions between the first two authors of the manuscript.

We next describe each of the phases of the instruction, providing examples that illustrate the forms of student thinking that we observed and (where appropriate), changes in thinking as the instruction progressed.

Phase 1. Observation and analysis of soil samples. The purpose of the first phase of instruction, which lasted about five weeks, was to encourage students to begin to think and talk about the properties of soil that vary, including its texture,

structure, and permeability. All students brought a sample of soil from a location near their homes, and they spent several class sessions carefully combing through the soil on paper plates and describing its visible qualities and components, including small pebbles, roots, insects, and arthropods. Students were asked to describe how the soil felt, smelled, and looked under magnification of hand lenses. As students in the class proposed descriptive words, the teacher posted them on a word wall so that they could serve as a ready reference for ongoing journaling. Over time, the word wall came to include words that referred to texture (*hard, soft, rough, stiff*), color (*dark, light, brown*), moisture level (*squishy, wet, dry*), things found in soil (*bugs, flowers, leaves, rocks, roots, grass*), and words referring to quantity and other relevant ideas (*shaped, high amount, low amount, labels, data*). This kind of instructional adaptation makes it possible for students who are minimal readers to begin to record and subsequently refer to scientific observations.

Once they looked at it closely under magnification, students were surprised at the amount of variation that they observed within a single baggie-sized sample. In particular, they were surprised at the evidence of life sustained within the soil. As one wrote, "There are leaves and baby fragle (sic) sticks in it and a few roots in it." Another observed, "It feels rough and it has rocks in it, and grass. It smells like toast."

Next, the teacher drew children's attention to comparisons among soils from different locations. She asked them to contrast a grayish, dry clay, with a dark brown, moist soil taken from a garden, and a light-colored clumpy soil. Students examined these three samples at a center and wrote about their comparisons in

their science notebooks, using the words from the word wall. This work, in turn, provoked the need to further expand their list of descriptors. Constraining the comparison to three choices that varied in extreme ways seemed to help the children focus more intently on variations in soil color, texture, and moisture.

Moving outdoors, the class used an auger to remove a core of soil about a foot deep from a location near the school. Students inserted their hands into the empty hole and discovered that the bottom was noticeably cooler than the soil near the top. Children wondered whether organisms that live in the soil (like earthworms) might prefer the cooler, moister environment below he ground. They described changes in the soil from the top to the bottom of its core, including temperature, moisture, and color.

Returning to their samples from home, students conducted soil profiles by placing half a cup of soil in a mason jar, adding water, shaking the jars, and then letting the soil settle. The teacher set a classroom timer so that students could observe and draw what they noticed every ten minutes for the next thirty minutes. Students noticed that the soil layers were differentiating ("The middle is getting lighter and lighter"), that different samples showed different numbers and widths of layers, and that some of the materials within the soil were becoming more visible as they were suspended in the water. Students compared soils in each others' baggies to the soil profiles in the jar, trying to account for the different layers and colors that they saw. The teacher introduced soil components such as clay, sand, and humus, and explained that although soil may look "all the same," in fact, it is made up of materials that look and feel different.

As this part of the instruction came to a close, students were now aware that soils are not all the same; that soils differ in color, smell, moisture, granularity, and texture; and that soils often contain evidence of organisms that live there (bits of roots, twigs, and leaf; intact insects and isopods, etc.). Moreover, soil taken from the same geographic location varies with depth. Children also began to think about soil and leaves, and some wondered whether soil might be "made of leaves."

Phase 2. Ongoing observation of decaying banana and pumpkins. During the second phase of instruction, which lasted from the first of March through the third week of April, children intermittently observed decomposing fruits and vegetables within and outside the classroom. During this phase of instruction, we intended to introduce students to the understanding that decay is a process that occurs over time, that it is associated with changes in color; smell, size, and texture; and that its rate can be affected by environmental factors such as temperature.

First, the teacher put a single banana on each table where four students sat. The easy accessibility of the banana to sight (as well as smell and touch via occasional poking) encouraged students to notice gradual changes in its appearance from day to day, and the children drew and wrote descriptions of change in their notebooks. In addition, the students observed changes in three pumpkins that they had originally used during mathematics class the previous fall for investigations in measurement. Initially, the pumpkins were cut open indoors so that students could study the seeds. Afterward, however, they were set outdoors, but within sight from a window. Although the pumpkins could be seen, they were beyond students'

immediate range of visibility (and touch), and therefore, students tended to look at them only when directed to by the teacher.

Students' notebooks include detailed descriptions of changes in their tabletop bananas, along with carefully labeled drawings. For example, beginning descriptions included: "The banana is bright and yellow and green and big. And the stem is littl." "It is pointy at the top. It is yellowish green at the top. It is a moon shape, and it has a little black line."   Over time, the entries began to read: "The banana has little brown and yellow parts and it smells." "First the banana was yellow. Then the banana got brown and squishy. And made a hole were you can see. "The banana has bugs in it, and it has white on it, too." The descriptions noted changes in shape, smell, texture, color, and size.

Figure 2: Notebook series of descriptions of banana decay

As the bananas were rotting, students occasionally looked out the window at the pumpkins, but when the teacher brought students outdoors for a closer look after several weeks, they were shocked at the transformation. As Figure 2 shows, the pumpkins had entirely lost their original shape.

Figure 3: Classroom pumpkin after decaying outdoors for several weeks

Students speculated that the inside of the pumpkin had disappeared or "gone into the ground." When the first author asked students what happened to the pumpkins, children replied, "...it went into the ground. Then it will turn into dirt because the bugs will eat it." Other students raised the possibility that changes in the pumpkin were due to the fact that "It got water on it." These initial proposals about environmental effects were explored later, during the fifth phase of instruction, when students constructed models of decomposition that included factors that might affect its rate.

Phase 3. Visit to the compost bin. During a single day in mid-April, students visited the school's compost bin, located in the backyard beyond the school and maintained by the school custodian, an experienced gardener. Mr. B, the custodian opened the "earth machine," explained that this is where he brings uneaten fruit and vegetables from the cafeteria, leaves, and other yard waste to decompose and turn into humus. The bin was opened and the contents turned, and students inspected the contents. As in the previous work with their soil from home, students were asked to look carefully, to smell, and to feel—they noticed that the contents were moist and warm. The student who wrote the notebook entry in Figure 3 summed up the question most on students' minds at this point: "I wonder what is the stuff in dirt?"

① It came from my uncle Earth Machine.

dirts

② It fleif hard.
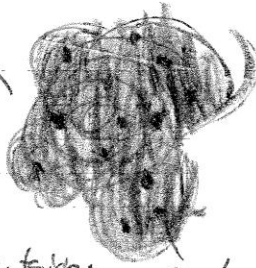
③ It smelled like outside.

Bugs

④ It looked like dirt.

⑤ It has baby bugs stich's and grass.

⑥ I wonder if dirt made dirty and what is the stuff in dirt.

Figure 4: First Grader's notebook entry about the compost bin.

Considerable interest was expressed about the organisms that students observed living in the compost: "In the earth machine, I saw some worms and beetles and roly polys, too….spider, bug, slug, plant, and dirt." Most likely because many of the materials in the bins were foods, students speculated that the animals were eating the food. Students noted that, in contrast to the decaying bananas on their tables, there was relatively little odor from the material in the bin ("It smells normal," as one child pointed out). From Mr. B, the students learned that putting all those components into the bin and turning them, perhaps assisted in some way by the animals, resulted in the generation of soil. Just how, they were unsure. One of the students speculated in her notebook: "When Mr. B showed us the earth machean, I saw worms, appels, oranges, rollipollies, beatels, and then when Mr. B mixed up the stuff it made me feel like if you mixed it with worms and appels, oranges, rollypollys, beatels, soil, grass, leafs, you can make dirt." A second child wrote, "It looks brown and dark black. It has squished leaves in it. I wonder if the dirt grows by sun, water, soil?" By the end of the visit, most students had concluded that somehow, materials in the compost bin turned into soil. Some believed that animals within the bin were eating the contents and perhaps playing an (unspecified) role in their transformation. There was a good deal of conversation about how the animals might have gotten into the compost in the first place, a question resolved when Mr. B showed children that the bottom of the compost was in direct contact with the soil. Other students wondered why the material in the bin was so much darker in color, moister, and warmer than soil on the nearby ground. There were questions about the potential roles of sun and water, possibly provoked

by noting the qualities of the composting material. Back in the classroom, the teacher read aloud to the class selected excerpts from *What's Going on in the Compost Pile*, by Chappelle. The book introduced several key notions about compost, but it is unlikely that children understood them at much depth, and they probably assimilated most of this material to ideas they previously held. For example, the book stated that compost is decaying organic matter (defined as anything that comes from plants). However, it is unlikely that these first-graders necessarily equated fruits and vegetables with plants. The book further explained that insects, slugs, worms, air, and moisture all work together in compost to break down organic material, and that as organic matter decays, it becomes compost that fertilizes growing plants. We have no direct evidence of the interpretation children brought to the phrase "break down," especially as their initial ideas about decay emphasized simply breaking material into smaller and smaller pieces. The book contained a brief allusion to "microorganisms" and "bacteria," but the children probably had no idea what those terms referred to, so it makes sense that they focused more intently on organisms they could see in the compost. Although some children did suspect that moisture might play in role in creating compost, no one raised the possibility that air might play a role.

Phase 4: Comparison of soils from compost bin and home. Next, students compared the qualities of material from the compost bin with those of their home soils. The students' notebook entries reveal that they noticed differences in texture, smell, moisture, color, and contents. In a whole-class discussion that followed their

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initial comparison, the teacher posted students' ideas about the ways in which the samples were "same" and "different."

Same	Different
both had soil	his had bugs
both had rock	had sticks
little leaf pieces	our dirt was light
balls of dirt	M.B. - dirt wet and muddy.
color - black-brown	Ours - dry
squishy	Ours - had mulch
normal smell	M.B. - no mulch
felt rough - big lumps	
→ becomes small pieces when smushed	

Figure 5: Similarities and differences between compost and home soil samples.

In the children's eyes, the most important differences were the darker color of Mr. B's compost, the fact that it was moist to the touch, and especially, the number of visible organisms that it contained. The teacher posed the question, "What are those organisms doing in Mr. B's compost?" Someone recalled that Mr. B had told the class that the organisms they observed in the compost eat the organic material and that their waste produces that "nice dark stuff."

> Teacher: When we first looked inside, remember, it was lots of grass and fruit and leaves. Remember when Mr. B stirred it up and pulled it up, what did it look like?
>
> Student: It becomes mud.
>
> Teacher: Well, it becomes something that we use that Mr. B says is good for planting. What does it become?
>
> Student: Dirt.
>
> Teacher: Another name for dirt?
>
> Student: Soil.
>
> Teacher: ...becomes the soil. So you guys think the bugs and the worms eat the fruit and the leaves and poop it out and it turns into soil.

By this point in the instruction, most students held an idea about the process of composting that was overly simplified in many ways, yet more nuanced than their original thinking about decay. Their model of the process equated insect waste with compost and compost with soil. On the other hand, the mental model held by most of the children did now regard decay as a process and included the ideas that plant material gets transformed, that organisms play a role in that transformation, and

that soils vary in their color, moisture, content, and their capability to support the growth of living plant life.

Phase 5: Design and observation of models of leaf decomposition. Late in April the teacher posed a new question: "What do worms and bugs eat if we don't give them apples and oranges?" A student proposed, "They can eat the leaves?....Maybe they eat the leaves that fall?" This reply provided an opportunity for the teacher to remind the children of her original question, "What happens to the leaves that fall each year?" Students suggested looking up the answer to this question on the Internet. The teacher proposed instead that they observe change themselves, and further suggested that students could "pretend" that lettuce leaves take the place of "leaves that fall outside." (We proposed lettuce so that change would be accelerated and more visible to the children.) Suspecting that students might not necessarily accept lettuce leaves as stand-ins for autumn leaves, the teacher asked, "Are lettuce leaves like leaves from trees?" The children concurred that they were, "...because they come from plants." As the teacher prompted students to explain what happens when leaves fall from trees, students replied that they fall onto the ground. At this point, the teacher suggested that to model this situation, the class place some leaves in jars that contained the soil children brought from home. Other leaves were placed in jars that contained material from Mr. B's compost machine. The teacher and students agreed that they would observe carefully over time to see what happened to the lettuce leaves resting on these two kinds of substrates. "We will put dirt from different tables in the jars. We want to know what happens to the leaves over time. We will see whose dirt will cause change in the leaves, because we are not sure."

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Before proceeding, however, the teacher asked a further question: "What happens outside, though? We want to make sure this is like outside, going through the same thing that the dirt outside is going through. What happens when dirt is outside?" The children replied that in the outdoors, "dirt" is exposed to "sunlight and water." Asked how it might be possible to simulate those conditions with their jar models, the children volunteered that they could arrange for sunlight by placing the jars near a windowsill and to simulate rain, "…you can get some water from the sink and just put a little bit in to see what happens." The teacher asked, "Are we going to give one table (that is, the jar placed on one table) more water than the other table?" The students protested that this would be "unfair" and eventually agreed that each jar should receive two teaspoons of water.

An extended discussion followed about whether the jars should be lidded. Some students were concerned that if lids were put on, the critters inside the soil might be unable to breathe. Others worried that if the jars were left open, "But then the bugs will come out!" Eventually students agreed that even if the jars remained closed, there might still be sufficient air inside, so the procedure agreed upon was: "When you put the water in there, then you shut it back up. Then you just wait."

Eventually, the class negotiated two further conditions, a leaf that did not rest on soil and a leaf that did not rest on soil but was exposed to moisture by resting on a damp paper towel. At the conclusion of this discussion, the class had agreed on a comparison among four kinds of models: a jar model that included soil from home, a jar model with material from the compost bin, a Ziploc bag containing no soil, and a Ziploc bag with moist paper towels but no soil. In earlier work, we

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have described these kinds of representations as remnants, that is, fragments of the phenomena under study that are brought into classrooms in forms that make them amenable to closer investigation by students (often because they omit features that are not theoretically important). In this case, the jars contained attributes (soil, leaves, critters, moisture, sunlight) that are the same as or similar to those in the external world (with the exception of lettuce leaves to represent leaves on trees). However, the jars also omitted attributes (rocks and pebbles, twigs, gum wrappers) that children also saw outside. These models were placed on the tables where children sat (four children to a table), and students observed the leaves closely over the next four weeks. Periodically, leaves were removed from their jars and magnified with hand lenses or projected with a flex cam. Students recorded the changes that they observed, including changes in leaf shape, color, moisture, texture ("slimy"), and size. Children claimed after a few days that the leaf in the compost jar was "getting smaller faster" than the leaves in jars with their home soil. Ridges were observed on the edge of the compost leaf, and someone proposed, "The bugs are eating the leaves." One of the notebook entries stated, "I think the bugs caused a hole (in the leaf) because on it, I saw some teeth marks on it and also for the shape. The shape been, at first, the shape wasn't that crooked. It's turning crooked." Students also noticed that the leaves on the paper towel and in the jar without soil were not rotting as quickly as those that included soil. This led them to suspect that soil somehow was playing a role in decay. Recalling their previous observations at the compost pile and noting that the leaf in the compost model was decaying so much more quickly than the leaf in the jars that contained what they called "regular

dirt," students suspected that the critical factor was "bugs." As one recorded in his journal, "Table blue don't have much bite, and then Mr. B has more bite than us. Mr. B has more bugs than us, and Mr. B has dirt, and he has more bugs than us. The bugs poop is dirt and they eat the leaves."

Unlike many of the models pursued by scientists (which may be expressed computationally or via mathematical expressions or models of chance), remnants like the ones featured here have a rather low representational overhead, because they preserve similarity between the model and the target phenomena. Because they have this characteristic of similarity, remnants are often appropriate entrees to modeling for young students. Yet, their cognitive challenge is far from trivial. Even though they do not make rigorous representational demands, they still require children to construct and cognitively maintain the relationships between objects and relations in the model and those in the modeled world; to identify relevant attributes to include (such as moisture) and exclude (gum wrappers); and to agree on standard ways to observe and measure (how do we *know* that a leaf is smaller today than it was yesterday?). Although the first-graders were scaffolded in these decisions by the teacher, they debated energetically about qualities of the model (2 teaspoons of water), appropriate comparisons ("regular" versus compost soil, moist conditions versus dry, soil versus no soil), and interpretation of the outcomes.

Phase 6: Classroom study of decomposers. One day early in May, while students were still engaged in recording changes in the jar models, the teacher brought in some earthworms for closer study. Students examined the worms' behavior, structure, and environment, smelling, touching, and drawing them, and

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then simulating their movement by attempting to enact "earthworm crawling."

Students discussed decomposing material as food for the earthworms and proposed

a relationship: The more food in the soil, the more organisms in the soil. The teacher

read brief selections from *Earthworms*, by Holmes, a children's trade book that

described the lifecycle, structures, and behaviors of earthworms, including their

preferences for moisture and cool temperatures and for eating "rotting vegetables,

plants, leaves, and grass." These earthworm studies served to help connect

children's observations of decay in the jar studies with their studies of soil by

confirming students' suspicions that organisms in the soil are largely responsible for

decay. At one point the teacher suggested that there could also be "very small

organisms in the soil, even if we cannot see them," but this idea was not pursued

further.

Post-Instruction Assessment. As the academic year came to a close, we asked

the children once again to consider the teacher's initial question about autumn

leaves and again to write an answer. Figure 5 summarizes the changes in children's

responses from pre- to post-assessment. As the figure illustrates, at the close of

instruction, there were no more suggestions that leaves die, disappear, or somehow

travel to another planet. Many children continue to focus on mechanical change, that

is, that leaves break up into smaller pieces. In addition, however, it was clear that

students were now strongly influenced by the potential role of insects. Students

suggested that the leaves are eaten by "bugs," that bugs then "poop them out," and

that in some way, "poop turns into dirt."

The individual interviews with the six focus students confirmed these shifts and added some further insights about their thinking about the role of soil and organisms. Before instruction, students described dirt simply as "mud and water." In contrast, after instruction, students said dirt was made up of flowers, leaves, grass, oranges and juice (memories of the compost bin!), water, and soil. When asked where dirt comes from, students initially said they did not know or said it comes from the ground. After instruction, students said that "organisms poop it out" or that it comes from leaves. Constituents of dirt were initially considered to be mud

Student Response to Leaf Decomposition Question
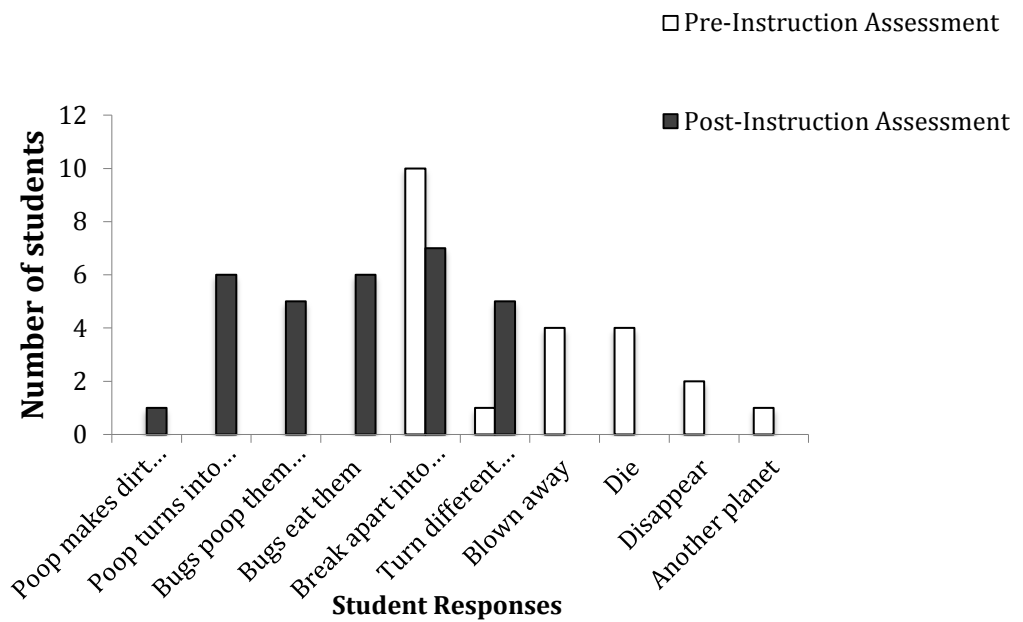
Figure 6: The number of responses of each type both pre- and post-instruction to the question, "What happens to leaves that fall each year?"

and water, seeds, and rock, and most students said dirt is homogeneous (the same kind of stuff). After instruction, students mentioned insects, insect waste, plants, rocks, water, and mud, and were adamant that there are many kinds of "dirt" with different constituents. The number of kinds of organisms one can find in soil increased from 3 at pre-instruction (roaches, ants, worms) to 7 at post instruction (worms, rolly pollies, roaches, beetles, ants, spiders, lady bugs). Instead of describing organisms as being at "home" in soil, students now focused instead on function: earthworms were described as "making more dirt" or "eating and finding food."

Discussion

Over several weeks of instruction, the first-grade children in this study made some modest progress in better understanding the decomposition of familiar organic materials (such as leaves and food). They became increasingly aware of decomposition as a process, rather than simply an end-state, and were able to identify changes that signaled decay, such as color, texture, shape, and smell, or the presence of mold. They discovered that different kinds of matter decay at different rates, and that environmental factors also seem to be associated with different rates of decomposition. With their lettuce leaf models, they concluded that temperature, moisture, and especially, the organisms living in soil, may be responsible for differences in the speed of decay. Their conceptions of soil shifted from a view of homogeneous, inert dirt to a view in which soils are highly variable in their make-up and loaded with life. Students were particularly interested in organisms, such as earthworms and isopods that fall into the soil and assist in the early stages of decomposition of organisms. In this instruction, we did not focus on the role of invisible decomposers, nor did we devote time to the mysteries of chemical change. It is possible that with additional instructional time, we might have found ways to open some of these thorny instructional issues with youngsters. Our purpose here, however, was restricted to setting the stage for learning more challenging biological ideas and modeling practices in subsequent education.

The relationship between the original target of query (autumn leaves) and the investigations undertaken by the children was representationally layered and required these young students to maintain several levels of systems intended to

stand in for other systems. For example, although to some students it remained literally a compost bin, the compost bin was also intended as a model of the process of decay, and over time, students did import elements from the bin (such as fruit, insects) into other contexts of decay. The simple lettuce leaf models that students worked with capitalized on resemblance between the items and relations in the models (i.e., lettuce, moisture, compost) and the objects being modeled in the world (i.e., tree leaves, rain, soil). As we have found in our earlier research (Lehrer, Carpenter, Schauble, & Putz, 2000), physical models like these, composed of remnants (actual materials taken from the target phenomena being studied), seem to be easiest for young or inexperienced children to access, and therefore provide an appropriate entrée to modeling. Children's drawings and narrative descriptions entailed a further increase in their growing representational capacity, and their notebook records of phases of decomposition, coordinated with time (days of decomposition) were yet an additional shift away from the phenomenon of interest and into the representational world itself. The teacher supported the descriptive qualities of these notebook entries, which at first were often embellished with hearts, flowers, and other decorative devices. Over time, as she encouraged students to compare and evaluate their illustrations, these embellishments began to drop away and the drawings began to show increasing realism and detail. In addition to the life sciences goals, therefore, a second important objective of this instruction was to assist students in developing a more sophisticated representational repertoire and applying these tools for understanding the world. These alternative ways of representing and depicting the world were critical tools for studying and

communicating about complex phenomena. They were important for the first-graders' growing understanding of decomposition and, moreover, are also central to the professional practice of science.

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CHAPTER III

BIOMEDICAL SCIENCES

IDENTIFICATION OF THE CRITICAL REGION OF PEROXIDASIN NECESSARY FOR SULFILIMINE BOND FORMATION IN COLLAGEN IV

The extracellular matrix (ECM) is the non-cellular, structural component surrounding and supporting cells that are found within all tissues and organs. It is composed of collagens, laminins, fibronectin, proteoglycans, elastin, and non-collagenous glycoproteins. ECM, which is constantly being remodeled, forms a three-dimensional network among the cells of different tissues in an organ-specific manner (Daley, 2008). It is a dynamic structure that provides essential physical scaffolding for the cellular constituents and interacts with cells to provide cellular cues that are needed for tissue development, adhesion, migration, proliferation, differentiation and homeostasis (Frantz, 2010). Basement membranes (BM) are a specialized form of ECM (Hynes, 2000). Basement membranes are in every tissue of the human body. Early in development, animals ranging from flies to humans direct the embryonic epithelium to orchestrate the organization of BM (Sherwood, 2006). BM separate cell monolayers from the underlying connective tissue, provide structural support to cells and influence and modify cellular behavior through signaling. The components of the BM alter BM-mediated cell signaling events and regulate cellular behavior in a tissue-unique manner (LeBlue, 2007).

Basement membrane integrity is of high importance. Basement membranes help maintain cellular compartments, where the cells don't cross BM. Only in special physiologic or disease situations do cells cross the membrane. When this process is compromised cancer cells are able to bypass the normal transmigration process and have lethal consequences, including metastasis (Christofori, 2006).

The four major components of the BM are type IV collagen, laminin, nidogen and perlecan. Type IV collagen and laminin individually self-assembly into suprastructures and both networks are crucial for BM stability (Poschl, 2004). Nidogen and perlecan bridge the laminin and type IV collagen networks, increase their stability and influence the structural integrity of the BM (LeBlue, 2007).

Collagen IV is part of a superfamily of ECM proteins. There are 28 different collagens that have been identified in vertebrates and they have very different biological functions. Of all the collagens, type IV collagen, an ancient protein, is the major constituent of the BM.

Type IV collagen protein networks provide structural integrity to epithelial and vascular tissues, serve as a scaffold for macromolecular assembly and interact with cell-surface receptors such as integrins to control cell adhesion, migration, proliferation and differentiation (Yurchenco, 2011). In mammals, six genes encode for six chains of type IV collagen and debited as alpha one through alpha six (Borza, 2001). Each alpha chain is composed of an N-terminal 7S domain, a triple helical collagenous domain, and a C-terminal noncollagenous globular (NC1) domain. Although there are 56 possible combinations for assembly, only three heterotrimers known as alpha 1-1-2, alpha 3-4-5 and alpha 5-5-6 assemble to form protomers in a

tissue specific manner (Khooshnoodi, 2006).   These protomers later organize into oligomers where the 150 residue, N terminal 7S domains associate into a 12-chain tetramer, and the 250 residue C terminal NC1 domains join together to form a hexamer (Figure 1; Khooshnoodi, 2008).
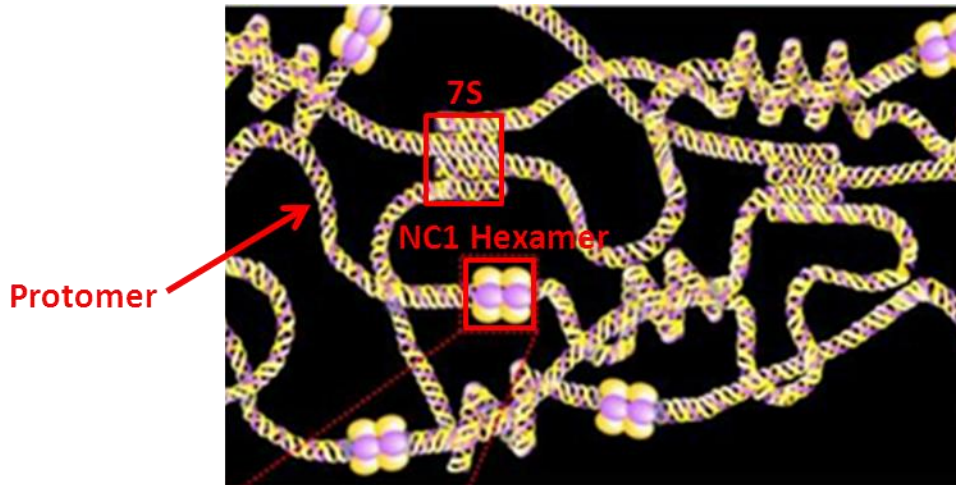
Figure 1. Assembly of protomers forming collagen IV network. Collagen IV protomers are heterotrimers composed of three alpha chains and are the building blocks of collagen IV. The 7S domain is assembled by tail to tail associations and the NC1 domain is assembled by head to head interactions that form the hexameric unit. The NC1 domain limits each alpha chain's ability to associate with other alpha chains at random and therefore the NCI domain endows specificity for protomer assembly**. *Adapted from Vanacore, 2009*

Collagen IV networks are very important and unique. Its genes, encoding the six different polypeptide chains, are expressed at different levels during embryonic development and this provides different tissues with specific collagen IV networks that have their own unique properties. The alpha 1 and alpha 2 chains are found in BM of all tissues, alpha 3-4-5 chains are found in glomerular basement membrane (GBM) of kidney, lung, testis, and eye, and the alpha 5 and 6 chains are found in BM of skin, smooth muscle, and kidney (Khooshnoodi, 2008).   These genes are important during embryonic development and provide different tissues with specific collagen IV networks.   Mice with genetic ablation of the alpha 1 and/or alpha 2 chains of collagen IV are embryonic lethal attesting to the critical nature of these ubiquitous chains in tissue development. Collagen IV networks are also important in signaling events in Drosophila development (Wang, 2008). Mutations in the alpha 3, alpha 4, and alpha 5 chains result in Alport's Syndrome characterized by glomerular basement membrane dysfunction and renal failure as well as ocular abnormalities and sensorineural deafness. The alpha 3 chain of collagen IV is the target for antibodies in Goodpasture's (GP) disease resulting in autoimmune damage to glomerular and alveolar basement membranes. These pathologic findings translate into life threatening, rapidly developing renal failure and/or pulmonary hemorrhage.

The NC1 domain of the collagen IV alpha 3 chain is the target for autoantibodies in GP disease. Early work demonstrated that a covalent cross-link reinforcing the NC1 hexamer prevented binding of GP autoantibodies to their target. Thus, insight into disease pathogenesis attracted attention to the chemical nature of

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this cross-link. For many years, the cross-link was believed to be a disulfide bond. But, a landmark discovery in our lab in 2009 revealed the cross-link was actually a novel sulfilimine bond between the methionine residue 93 and the hydroxylysine residue 211 at the interface of adjoining protomers (Figure 2; Vanacore, 2009).
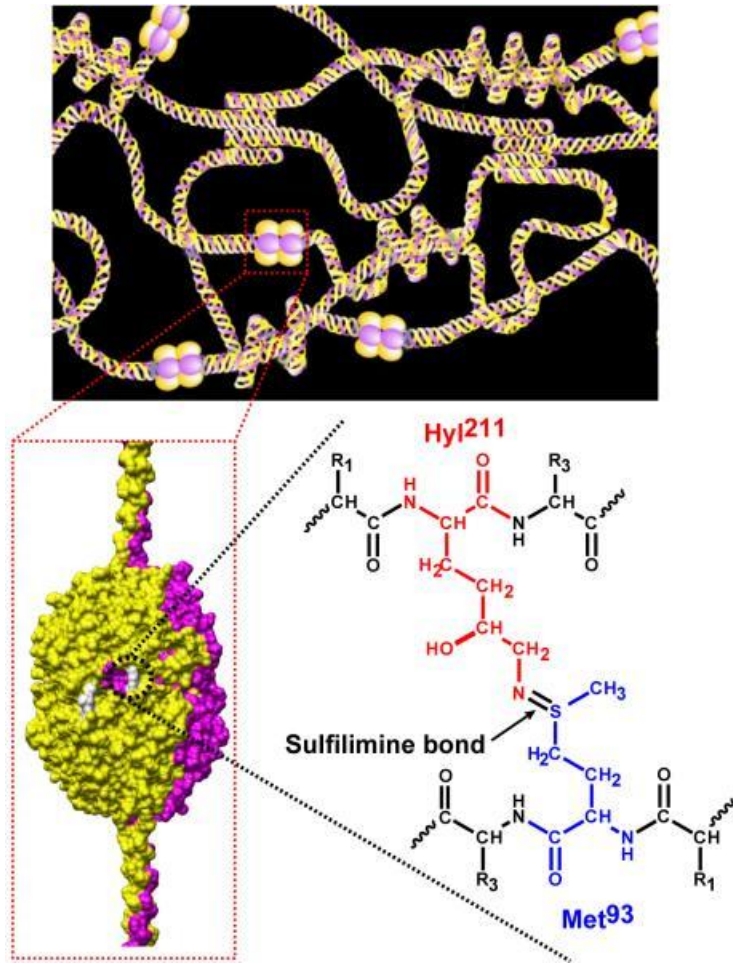
Figure 2. Sulfilimine bond within the collagen IV network. Diagram shows the sulfilimine bond located at the interaction site of the NC1 domains of triple-helical protomers. The space filling model below (yellow and pink) highlights that the sulfilimine bond is between the methionine sulfur 93 and the hydroxylysine nitrogen 211. *Modified from Vanacore, 2009*

Based on genomic sequence analysis and biochemical characterization, our lab subsequently demonstrated that the sulfilimine bond is evolutionarily conserved essentially throughout the animal kingdom. Since the sulfilimine bond plays such an intricate role in tissue development and human disease, it was necessary that the mechanism for the bond formation be elucidated. Therefore, our group began studying the possible mechanisms for mediating the sulfilimine bond formation. When I joined our lab, an internally generated body of work demonstrated that a peroxidase residing within the BM catalyzed sulfilimine bond formation in collagen IV.

Peroxidases are heme containing enzymes found in bacteria, fungi, plants, and animals. There are two superfamilies of peroxidases. One superfamily includes the proteins of fungal, plant and bacterial origin and the other of superfamily includes vertebrate enzymes that belong to the peroxidase-cyclooxygenase superfamily. The peroxidase-cyclooxygenase superfamily evolved independently from the plant, fungal and bacterial superfamily (Fiedler, 2000). Plant peroxidases are about 300 amino acids long with non-covalently bound heme. They are induced by UV light and stress, such as pathogen attack, wounding , heat or cold, while mammalian peroxidases are about 650 amino acids long and the heme is covalently bound (O'Brien 2000). H_2O_2 is known to react with ferric states of heme-containing peroxidases and catalases in a two-electron redox reaction that results in an "oxene", strong oxidizing intermediate and water (Vlasits, 2010). Vertebrate peroxidases use H_2O_2 to catalyze the oxidation of a variety of substrates and this plays an important role in innate immunity, synthesis of thyroid hormone and

extracellular matrix, as well as in the pathogenesis of a number of inflammatory diseases such as atherosclerosis (Dunford, 1999; Cheng, 2008).

Vertebrate peroxidases include eosinophil peroxidase (EPO), myeloperoxidase (MPO), lactoperoxidase (LPO), and thyroid peroxidase (TPO). MPO, EPO, and LPO are well-characterized peroxidases that are involved in the innate immune system and central to human oxidative defense. MPO and EPO are released into the phagocytic vacuole and the plasma, whereas LPO is secreted into milk, saliva and tears (O, Brien, 2000). TPO is involved in thyroid hormone biosynthesis. These peroxidases originated from the ancient peroxidase, peroxidasin, found in both invertebrates and vertebrates (Soudi, 2012). Vertebrate peroxidases are known to oxidize chloride, bromide, thiocyanate, or iodide to form hypohalous acids. MPO oxidizes chloride, bromide, and thiocyanate;EPO oxidizes bromide and thiocyanate; LPO oxidizes thiocyanate; and all animal peroxidases including TPO oxidize iodide (O'Brien, 2000).

Lactoperoxidase, LPO, is a heme-containing glycoprotein of 78kDa found in large quantity in secretory fluids such as milk, saliva, tears, and lung fluid (Ihalin, 2006). In the presence of H_2O_2, LPO oxidizes thiocyanate (SCN-) more efficiently than other peroxidases, and has therefore been used with these cofactors as an alternative sterilization method to pasteurization for raw milk (Campbell, 2012).

Thyroid peroxidase, TPO, is a 103kDa glycosylated protein localized on the apical membrane of thyroid follicular cells (Ruf, 2006). It is a membrane bound enzyme which is involved in the biosynthesis of thyroid hormones (Kimura, 1989) and has been linked to autoimmune thyroid diseases (Nazar, 2012). TPO catalyzes

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covalent incorporation of oxidized iodine into tyrosines in thyroglobin and coupling of iodinated tyrosines to form thyroid hormones (Ris-Staplers,2010).

Myeloperoxidase, MPO, the most widely studied peroxidase, is a 144kDa protein linked to innate immunity, oxidative stress, and inflammation (Hansson, 2005). It is a heme enzyme released by activated neutrophils from intracellular granules (Davies, 2011). The initial product of MPO-H_2O_2 chloride system is hypochlorous acid, which is an important mediator in host defense. Leukocytes from patients with inherited MPO defects have impaired fungicidal activity, predisposing them to disseminated candidiasis (Salmon, 1970). Patients with total or incomplete loss of MPO still phagocytose foreign material normally, but they have a greater risk of chronic infections (Nauseef, 1988).

Although the production of hypochlorous acid production is good for host defense, there can be negative consequence of its production, such as its generation of reactive intermediates (chlorine, chloramines, and hydroxyl radicals) that can lead to oxidative damage of host lipids and proteins (Karakas, 2005). These toxic agents can be released outside of the cell and can attack normal tissues and eventually contribute to the pathology of disease (Klebanoff, 2005). For example, within the cardiovascular system, MPO has been shown to contribute to vascular dysfunction. It also promotes lipid peroxidation and scavenges nitric oxide and MPO products to activate matrix metalloproteinases. MPO also promotes tissue remodeling that can result in atrial fibrillation and atherosclerosis (Rudolf, 2010).

Eosinophils, in their normal state, are highly granular proinflammatory leukocytes found in the body's host defense against parasites. In diseased states,

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they play a role in different inflammatory conditions such as bronchial asthma and gastroenteric diseases (Rothenburg, 2006). A hallmark of many allergic diseases is a predominance of eosinophils within inflammatory leukocytic infiltrates. (Jacobsen, 2007). They exert many of their inflammatory effects in allergic disorders by the degranulation and release of intracellular mediators, including the cationic granule proteins major basic protein (MBP), eosinophil peroxidase (EPO) , eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) (Walsh, 2011). EPO is a 77kDa, two subunit protein shares 70% amino acid homology with the neutrophil MPO and primarily generates hypobromous acid to damage parasites and other microbes (Ten, 1989; O'Brien, 2000).

Peroxidasin (PXDN), also known as VPO1, is a multi-domain, hybrid, protein that includes an enzymatic peroxidase domain with extracellular protein-protein interacting domains. PXDN is found in vertebrates and invertebrates, unlike the other peroxidases (EPO, LPO, MPO, and TPO) which are found only in vertebrates. The peroxidase domain of PXDN has 42% sequence identity to human MPO, 41% identity to human EPO, 39% to human LPO, and 41% to human TPO (Cheng, 2008). The 165 kDa homo-trimeric, protein was first identified in *Drosophila* and was hypothesized to function in the formation of ECM during embryogenesis (Nelson, 1994) Peroxidasin has also been recently linked to the development of the anterior chamber of the eye. Homozygous mutations in peroxidasin caused congenital cataract, corneal opacity and developmental glaucoma in two consanguineous Pakistani families (Khan, 2011), but the mechanism has not been elucidated.

While a small body of literature existed on peroxidasin, its physiological role remained ill-defined. Our lab identified the first function for peroxidasin as the enzyme that catalyzes the formation of the sulfilimine bond in collagen IV networks of basement membranes. We further found that peroxidasin generated hypohalous acids as reactive intermediates to form sulfilimine bonds. Absence of peroxidasin in *Drosophila* resulted in loss collagen IV, basement membrane, and tissue integrity pointing to a critical role for peroxidasin in tissue genesis. (Figure 3; Bhave, 2012).
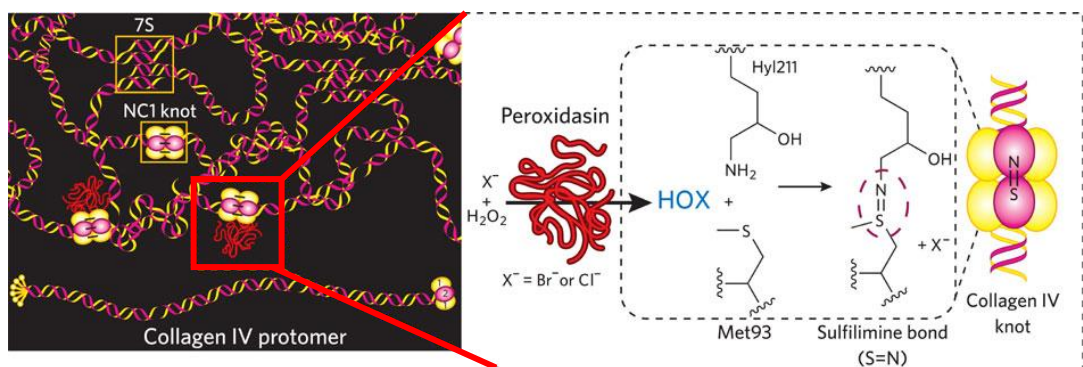
Figure 3. Proposed mechanism of peroxidasin catalyzed sulfilimine bond formation using hypohalous acids. Peroxidasin (red twisted ribbon), rests on the NCI dimer and introduces crosslinks (black lines) that stabilize the collagen IV network (within the red highlighted square). The view expanded from the red box (rectangle dotted box) shows peroxidasin using hydrogen peroxide (H202) to oxidize bromide (Br-) or chloride (Cl-) to produce hypohalous acids which crosslink the sulfur atom of methionine and the nitrogen atom of hydroxylysine to form the sulfilimine bond (S=N within pink bubble). *Adapted from Weiss, 2012*

PXDN is made of several domains and linking regions that include a hydrophobic signal peptide from amino acid 1-23, a leucine-rich repeat (LRR)domain from amino acids 57-184, an immunoglobulin (Ig) domain from amino acids 238-651, a peroxidase domain from amino acids 747-1352, a von Willebrand Factor C domain (vWFC) from amino acids 1415-1470 (Nelson, 1994). In comparison to the other known animal peroxidases, PXDN uniquely possesses domains that possibly participate in protein complexes and protein-protein interactions. (Figure 4).

The noncatalytic domains of PXDN are found in other proteins and are involved in many cellular functions. The LRR domain is normally known to occur in protein-protein interactions such as cell adhesion, signal transduction, extracellular matrix assembly, platelet aggregation, neuronal development, RNA processing and immune response (Bella, 2008). The Ig domain is well known to be involved in cell adhesion and pattern recognition (Soudi, 2012). The vWF domain is also called the chordin-like, cysteine-rich (CR) repeat and contains ten cysteines (Leary,2004). VWF is a multimeric blood glycoprotein that binds and stabilizes clotting factor VII and mediates platelet adhesion and is found in over 500 extracellular matrix proteins. The vWFC is well known for regulating bone morphogenetic proteins (BMPs), transforming the tissue growth factor beta (TGF-Beta), and oligomerization of proteins (Sadler, 2009).
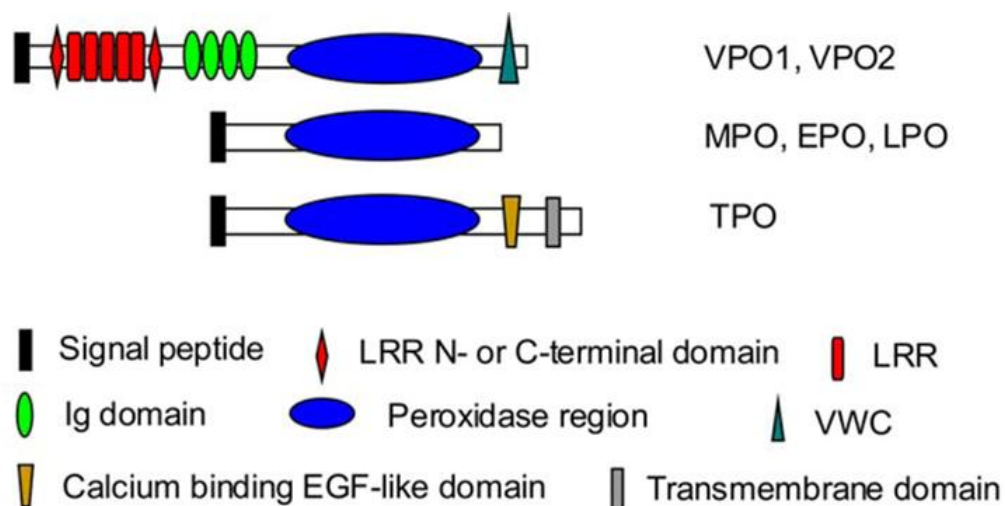
Figure 4. Predicted domain structure of animal heme peroxidases. VPO1 (PXDN) and VPO2 are closely related peroxidase homologs. Shown along with them on this diagram are vertebrate peroxidases MPO, EPO, LPO and TPO. All the peroxidases shown have the peroxidase domain. VPO1 and VPO2 also contain leucine rich repeats (LRR), immunoglobulin (Ig), and von willebrand factor C (VWC) domains, making them the largest animal heme peroxidases. *Adapted from Cheng, 2008*

The domains (LRR, Ig, and vWFC) of PXDN, not found in other peroxidases, have certain functions when found in other proteins but their function within the peroxidasin protein is unknown. Although these proteins may be involved in protein-protein interactions, there is a possibility that they may have added functions within this basement membrane bound enzyme. Peroxidasin is in basement membranes found in all tissues and function to cross-link collagen IV and provide basement membrane stability and support tissue integrity. Therefore, it is necessary to understand the structure-function relationships of peroxidasin involved in allowing it to execute its critical function. This then began the specific aim of my research, which was to **determine the structural elements of peroxidasin required for sulfilimine bond formation and basement membrane incorporation.** To this end, I constructed mutant peroxidasin truncation variants that strategically eliminated different domains of peroxidasin to determine the region of peroxidasin critical for sulfilimine bond formation. Here I show that the peroxidase domain of peroxidasin is sufficient for sulfilimine bond formation, and these results are optimized, to the level of wildtype peroxidase or higher, by the addition of the Ig domain.

MATERIALS AND METHODS

Cloning strategies

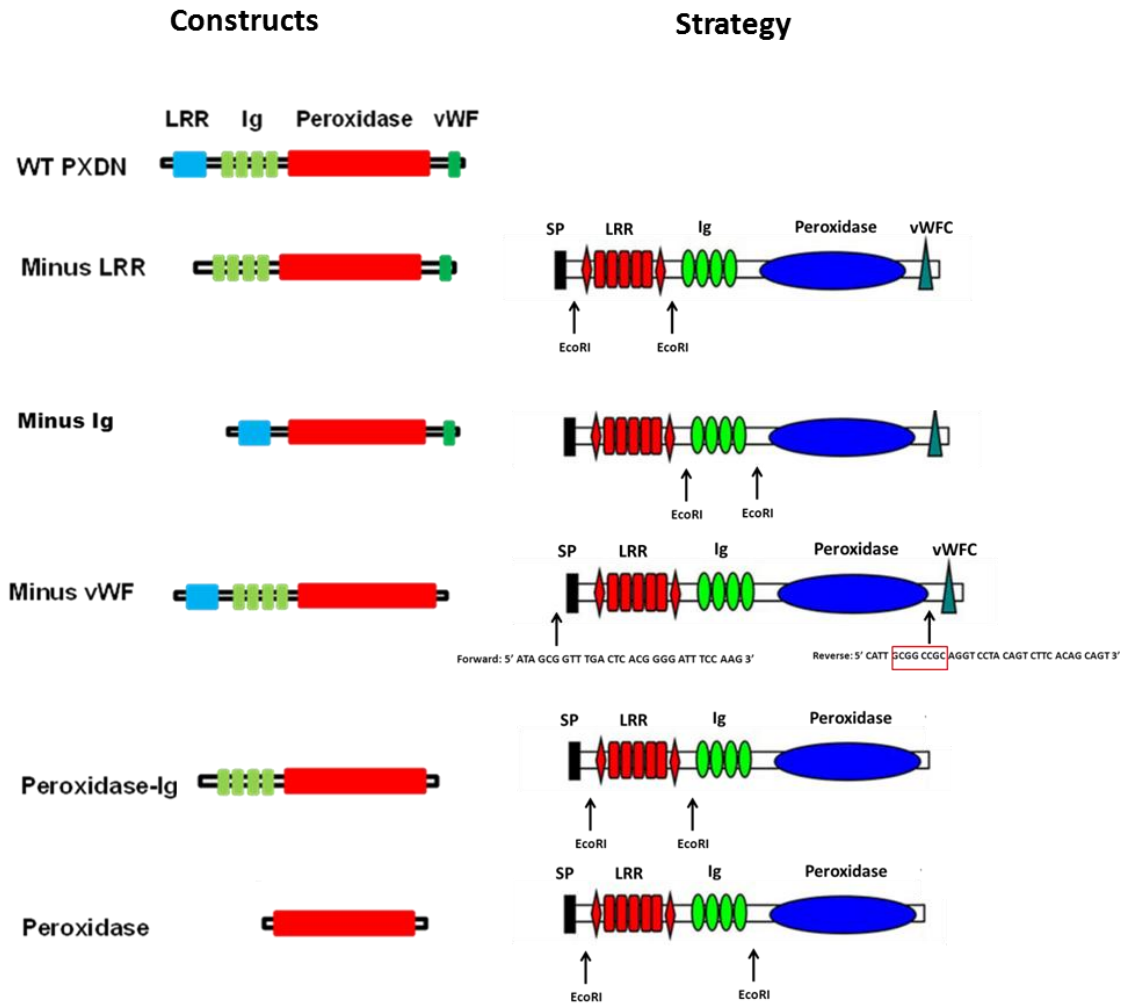
Figure 5. Peroxidasin and truncated peroxidasin variants domain structure nomenclature. Peroxidasin is a multi-domain protein with leucine rich repeats (LRR), immunoglobulin (Ig), and von willebrand factor (vWF) protein-protein interaction domains along with the enzymatic peroxidase domain. Various constructs where created with the strategic elimination of different domains of peroxidasin.

In order to create these constructs, different cloning strategies were used (Figure 5). They involved directly inserting restriction enzyme sites (EcoRI) at desired locations using QuikChange mutagenesis (Figure 6) and PCR (polymerase chain reaction). The wildtype peroxidasin had been previously cloned into a pCDNA 3.1 V5 His Tag TOPO vector flanked with Kpn1 and Not1 restriction enzyme sites. Therefore, in order to introduce other pieces of DNA into that site, their DNA must be flanked with Not1 and Kpn1 sites (sticky ends) for ligation. The pCDNA 3.1 vector that contained the wildtype peroxidasin was digested with EcoR1 and Not1 restriction enzymes and the wildtype peroxidasin was removed, maintaining the Kpn1 and Not1 sticky ends for later insertion. This construct was then used as empty vector and for inserting other pieces of DNA.

The first construct that was created was the Minus vWFC. To create the minus vWFC, I used wildtype peroxidasin within the pCDNA 3.1 vector as a template. I designed a forward primer that begin upstream of the vector, before the signal peptide of the peroxidase gene located within the vector. The forward primer would begin in the vector in order to amplify the beginning of the peroxidasin DNA, without removing any nucleotides before the start codon of wildtype peroxidasin. The primers for all constructs are shown in Table 1. As for the design of the reverse primers, I introduced a Not1 site after the peroxidase domain sequence and before the vWFC domain in the wildtype peroxidasin sequence. I then placed four extra nucleotides in front of the primer sequence, in order to make an optimum primer that would easily anneal to the template. Using the pcr conditions described in the materials and methods section (the same conditions were used for all the

constructs), the vector containing the wildtype peroxidain was then amplified to exclude the vWFC domain and include a Not 1 site after the peroxidase domain. This pcr product was then double-digested with Kpn1 and Not1 and ligated to the empty vector, creating the minus vWFC construct. All constructs were sequenced and checked for lack of mutations before use.

In order to create the minus LRR construct, two EcoR1 sites were inserted (each EcoR1 site was added one pcr reaction at a time) into wildtype peroxidasin before and after the LRR domain using primers in Table 1. To create the minus Ig construct, wildtype peroxidasin was used as a template for insertion of two EcoR1 after the LRR domain and before the peroxidase domain of peroxidasin. To create the Peroxidase Ig construct, the minus vWFC construct was used as a template for EcoRI insertion before and after the LRR domain. Finally the peroxidase construct was bioengineered by using the minus vWFC as a template and inserting two EcoRI sites before the LRR domain and after the Ig domain of peroxidasin. All PCR products were dpn1 digested for one hour at 37 degrees to remove the methylated parental template.

Molecular cloning strategy of truncated peroxidasin variants

1. Mutant strand synthesis:
   Perform thermal cycling to:
   -Denature peroxidasin-containing
    plasmid (parental DNA)
   -Anneal mutagenic primers
    containing desired mutation(Pink cross)

2. *DpnI* digestion of template:
   Digest parental methylated   DNA
   with *Dpn1*

3. Transformation:
   Transform mutated peroxidasin-plasmid
   into competent cells for nick repair
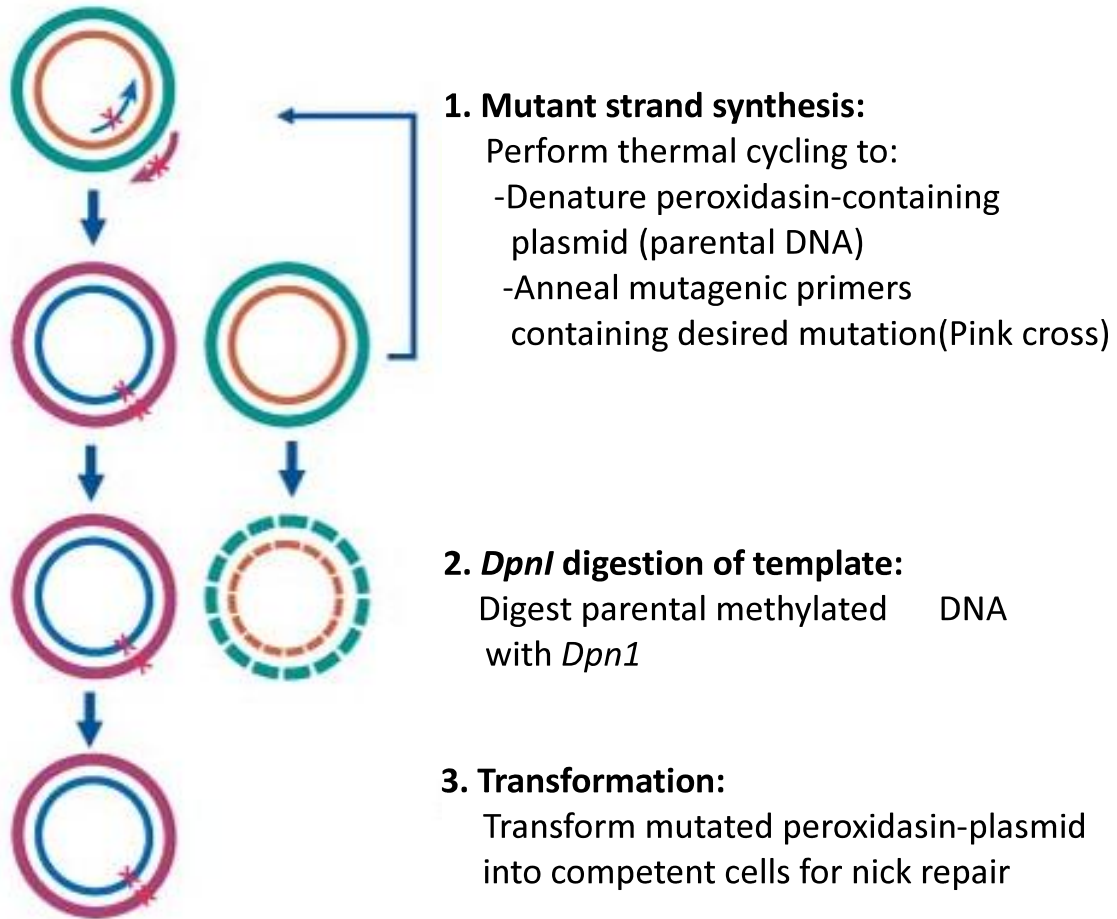
Figure 6. Quikchange site-directed mutagenesis cloning strategy for truncated peroxidasin constructs. Peroxidasin gene that had been cloned into the pCDNA 3.1V5-His Topo plasmid contained the target site for mutagenesis. The peroxidasin-containing plasmid (orange and green circles) was denatured at 95degrees for 1 minute for 30 cycles. The oligonucleotide primers (listed below) that contained the desired mutation (pink cross) were annealed to the plasmid at 55° C degrees for 1 minute for 30 cycles. Using the non-strand displacing action of *Pfu* Turbo DNA polymerase, the primers were extended at 48° C for 4 minutes for 30 cycles and were incorporated into the plasmid, resulting in the nicked circular strands. A final extension of 10 minutes was done at 68° C. Later, the methylated, non-mutated parental DNA template was digested with DpnI. The nicked, double-stranded DNA was then transformed into competent cells, where the nicks in the DNA were repaired. The mutated plasmids (constructs) were then amplified and used for future experiments.

PRIMERS USED TO INTRODUCE MUTATIONS (restriction enzyme sites) WITHIN PEROXIDASIN

Minus Vwfc Primers:
Forward: 5' ATA GCG GTT TGA CTC ACG GGG ATT TCC AAG 3'
Reverse: 5' CATT *GCGG CCGC* AGGT CCTA CAGT CTTC ACAG CAGT 3'

Minus LRR Primers:
1ST SET OF PRIMERS
Forward: 5' GGGTGTCCGAGCGAATTCCGCTGCCTGTGC 3'
Reverse: 5' GCACAGGCAGCGGAATTCGCTCGGACACCC 5'

2ND SET OF PRIMERS TO INTRODUCE THE ECORI SITE AFTER THE LRR REGION:
Forward: 5' CAGGGACGCTCAGAATTCGTGGCAACCATC 3'
Reverse: 5' GATGGTTGCCACGAATTCTGAGCGTCCCTG 3'

Minus Ig Primers:
1st SET OF PRIMERS
Forward: 5'CAGGGACGCTCAGAATTCGTGGCAACCATC 3'
Reverse: 5'GATGGTTGCCACGAATTCTGAGCGTCCCTG 3'

2ND SET OF PRIMERS
Forward: 5' CTCAGTGTGAATGAATTCGTTCCTGACGTC 3'
Reverse: 5' GACGTCAGGAACGAATTCATTCACACTGAG 3'

Peroxidase Ig Primers:
1st SET OF PRIMERS
Forward Primer: 5' GGGTGTCCGAGCGAATTCCGCTGCCTGTGC 3'
Reverse Primer: 5' GCACAGGCAGCGGAATTCGCTCGGACACCC 3'

2ND set of primers
Forward Primer: 5' ATCACCCCGGAAGAATTCGAGCTGAACTGT 3'
Reverse Primer: 5' ACAGTTCAGCTCGAATTCTTCCGGGGTGAT 3'

Peroxidase Only Primers:
1ST SET OF PRIMERS
Forward: 5' GGG TGT CCG AGC GAA TTC CGC TGC CTG TGC 3'
Reverse: 5' GCA CAG GCA GCG GAA TTC GCT CGG ACA CCC 5'

2ND SET OF PRIMERS
Forward: 5' CTC AGT GTG AAT GAA TTC GTT CCT GAC GTC 3'
Reverse: 5' GAC GTC AGG AAC GAA TTC ATT CAC ACT GAG 3'

Table 1. Primers designed for cloning the different constructs.

Matrix Production from PFHR-9 Cell Line

PFHR-9 mouse endodermal cells were grown to confluency for basement membrane deposition at 37 degrees with 10% CO2 in DMEM +10% FBS. Confluent 150mm dishes of Phfr9 were split into 6-100mm dishes. These cells were grown for 5 days in the presence of 50uM phloroglucinol to produce uncrosslinked collagen IV and 50ug/ml ascorbic acid to optimize collagen IV production.

After five days, the basement membrane is isolated. To attain this basement membrane free of the pfhr9 cells, the follow procedure was performed. First the media was removed from the cells for further analysis (peroxidase assay). Then hypotonic buffer (10mM Tris-Cl pH 7.5, 01mM CaCl$_2$, 0.1% bovine serum albumin) was placed on the cells for 10 minutes to make the cells swell. Then the hypotonic solution was gently removed. Next, to lyse and remove the PRHR-9 cells, hypotonic buffer with 0.5% Triton X-100 detergent was placed on the cells twice for 5 minutes and hypotonic buffer with 0.1% deoxycholate added to lyse the cells. The solutions were gently removed. Afterwards, we wanted to inactivate any endogenous crosslinking activity within the matrix. So we treated the matrix with 4M guanidine-Cl and 50mM Tris-Cl pH 7.5 for 15 minutes and then gently washed 5 times with 1X PBS. After removal of these solutions, these monomeric matrix containing plates were then stored at -80 degrees for later use.

"Overlay" of Peroxidasin Variant Expressing Cells on Uncross-linked Collagen IV Networks.

HEK 293T cells were grown in media to confluency at 37degrees in 5% CO_2. $3X10^6$

HEK 293T cells were overlaid on top of the uncrosslinked monomeric matrix on the

100mm dishes. 24 hours later, the overlaid HEK293T cells were transiently

transfected with human peroxidasin coding sequence and the truncated peroxidasin

variants, mouse myeloperoxidase cDNA, mouse lactoperoxidase cDNA or empty

pCDNA-V5-His-TOPO vector, as a negative control, using lipofectamine LTX per

manufacturer instructions . These cells were grown in the presence of media plus

5uM hematin and 5mM sodium butyrate (Figure 7) since hematin is a heme cofactor

and butyrate enhances peroxidasin expression (Cheng, 2008). 48 hours after

transfection, the media was harvested, protease inhibitors (leupeptin, pepstatin,

phenylmethanesulfonyl fluoride (PMSF). Protein was precipitated with 40%

ammonium sulfate. The precipitated protein was collected by centrifugation at

10,000g for 15 minutes. The supernatant was discarded and the pellet was

resuspended in CTAB buffer (150mM NaCL, 50mM Tris-Cl, pH 7.5, 0.2% CTAB,

0.5mM PMSF, 10ug/ml leupeptin, 1ug/ml pepstatin). After the media was collected,

the plate containing cell lysate and matrix was then harvested with deoxycholate

lysis buffer with protease inhibitors (1%sodium deocxycholate, 10mM tris-Cl, pH 8,

1mM EDTA-Na pH 8, 0.5mM PMSF, 10ug/ml leupeptin, 1ug/ml aprotinin, 1ug/ml

pepstatin). These samples were sonicated and were placed on ice for 10minutes.

The samples were then centrifuged at 14,000 for 10 minutes and then separated

into 1.5ml eppendorf tubes that contained cell lysate as the supernatant in separate

tubes and the pellet containing the matrix was then placed in a separate tube for

further analysis.   These samples then probed for matrix activity, dimer formation,

and protein expression.

Plate PFHR-9 cells at high density

Grow to confluency in presence of ascorbic acid + phloroglucinol (daily for 5 days)

Isolate matrix on plate

guanidine inactivation of endogenous peroxidasin

Overlay

PXDN and truncated variants transfected HEK

Empty vector

PXDN transfected + PHG

Add heme and sodium butyrate

Incubate 2 days & analyze for collagen IV crosslinking/dimer formation

Figure 7. Schematic diagram of matrix production and overlay method.
PFHR9 cells were grown in the presence of phloroglucinol (PHG) to deposit uncrosslinked collagen IV networks. The cells were then removed, and the basement membrane was extracted with 4M guanidine to inactivate endogenous peroxidasin. HEK cells were plated then transiently transfected with PXDN, truncated peroxidasin variants or empty vector were plated on top of the PFHR9 basement membrane, which was then analyzed for collagen IV crosslinking.

After the Pfhr9 cells were homogenized through sonication in 1% deoxycholate, the pellet that contained the matrix was washed with 1M NaCl and 50mM tris-Cl pH 7.5. The collagen IV protomers may associate head to head. Collagenase digestion leaves behind only the non-collagenous, globular NCI domain either as a trimer or hexamer (Figure 8). The pellet was then collagenase digested with 50ug/ml bacterial collagenase and 50uM pholoroglucinol, to prevent further crosslinking activity, at 37degree waterbath overnight. The samples were then centrifuged at 14,000rpm for 15 minutes. The pellet was discarded and the collagen IV NCI containing supernatant was collected and stored. Aliquots of the sample were run on a 12% gel and coomassie stained to analyze collagen IV crosslinking, dimer formation, for the various constructs.

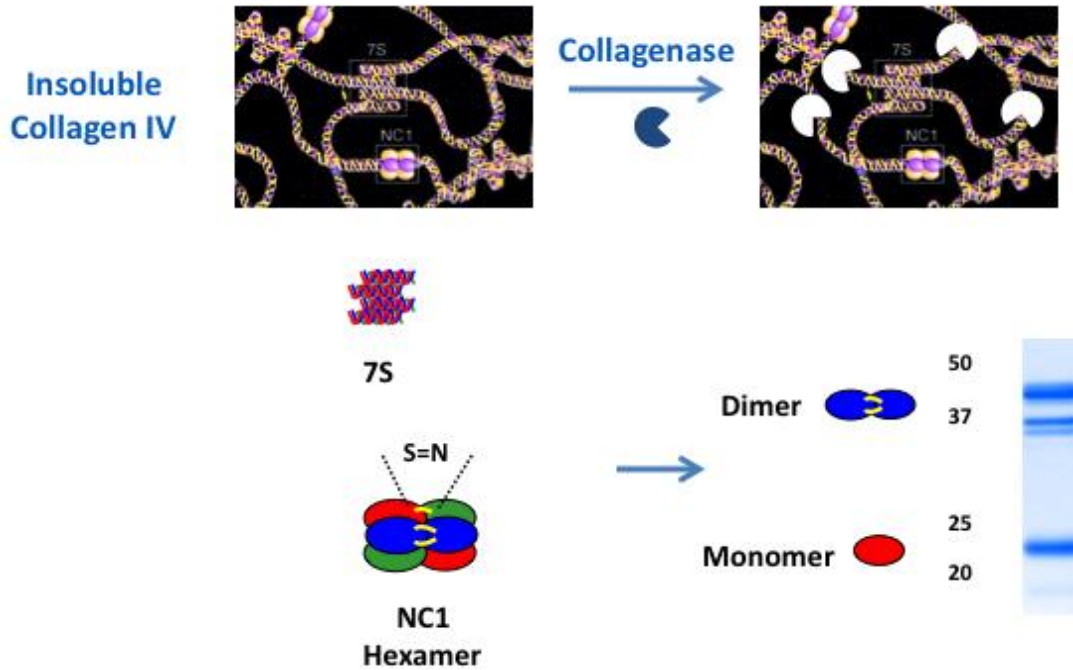**Figure 8. Collagen IV NCI hexamer collagenase digestion.** Collagen IV contains the 7S and crosslinked NC1 domain within its network. In order to attain the NC1 hexamer separate from the 7S domain, collagenase enzyme is used to digest the collagen. As shown in the coommasie stain, after overnight collagenase digestion at 37 degrees, the NC1 hexamer is then separated into dimer (crosslinked) and monomer (uncrosslinked) regions.

Peroxidase Activity Assay

Media from PXDN and PXDN truncated variants, MPO, LPO, EPO, and mock transfected cells were assayed for peroxidase activity using tetramethylbenzidine-based colormetric assay and developing buffer (0.4M sodium acetate, pH 5.4, 1%CTAB, 20mM tetramethylbenzidine, 50mM H202). 1ml of media was used for protein precipitation. Using 40% ammonium sulfate, proteins were precipitated and centrifuged at 14,000rpm for 15 minutes. Once the proteins were centrifuged to the bottom of the eppendorf tube, the supernatant was removed and the pellet containing proteins was resuspended in $1/100^{th}$ volume of CTAB buffer. $1/4^{th}$ of the sample was used added to developing buffer and measured at A_{650}. Activity was later expressed relative to peroxidasin (A_{650} of given peroxides were normalized to peroxidasin by dividing by the A_{650} for peroxidasin).

RESULTS

Peroxidasin uniquely crosslinks native collagen IV in networks

In vitro, all peroxidases (MPO and EPO) that make HOBr and HOCl can crosslink the basement membrane (Bhave, 2012), but whether they can crosslink the *in vivo*. We hypothesized that peroxidasin has the advantage because it resides in the basement membrane and the other peroxidases do not. To test this hypothesis, we performed "overlay" experiments with transiently transfected HEK293 cells using PXDN, MPO, LPO cDNAs and a empty vector as negative control. The results show that only peroxidasin crosslinked the matrix forming the sulfilimine bond. With respect to peroxidase activity, MPO demonstrated at least 30 fold greater activity than peroxidasin but unable to crosslink collagen IV (Figure 9). Subsequent results showed that EPO, another peroxidase, has high peroxidase activity but unable to crosslink the matrix (Figure 14). This establishes that peroxidasin is the only animal heme peroxidase that forms the sulfilimine bond within basement membrane. The results shown in figure 9, served as crucial evidence in the body of work that identified peroxidasin as the enzyme that catalyzes the formation of the sulfilimine bond. This discovery was reported in *Nature Chemical Biology, 2012*. Figure 9 is presented as Figure 5 in this publication. (See appendix A).
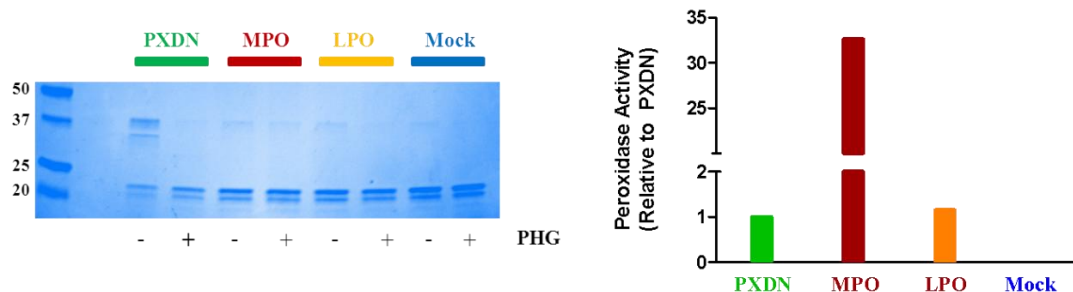
Figure 9. Collagen IV crosslink formation and peroxidase activity of peroxidases*. *(Left panel)* Coomassie blue-stained gel of collagen IV NCI hexamers isolated from uncrosslinked PFHR9 basement membrane overlaid with HEK293T cells that have been transiently transfected with human peroxidasin cDNA (PXDN), mouse myeloperoxidase cDNA (MPO), mouse lactoperixadase (LPO) or empty vector (mock). (*Right panel*)Media from PXDN, MPO, LPO, and mock transfected cells were assayed for peroxidase activity using tetramethylbenzidine-based colorimetric assay. Activity was expressed relative to peroxidasin (A_{650} of given peroxidase divided by A_{650} for peroxidasin).
*This figure appears as Figure 5 in *Nature Chemical Biology* article (Bhave, 2012), Appendix A.

Characterization of peroxidasin truncation variants.

To determine the domains needed for sulfilimine bond formation , as
described in methods, truncation peroxidasin constructs were created (Figure 5).
In creating these constructs, domains were removed and we want to be sure that the
DNA is entering the cell and expressing properly folded proteins that are secreted.
There was a possibility of improper folding or lack of secretion of the protein that
could have deviated from the expected results. Also, if the proteins were able to
enter the matrix, then they would have access to crosslinking the monomeric un-
crosslinked matrix, depending on the construct. If the proteins were not detectable
within the matrix, then we would not be able to conclude that the crosslinking or
lack of crosslinking activity being observed was due to the construct that was
transfected into the cells. The first issue was to determine whether these truncated
constructs were expressed, secreted and active extracellularly. So in order to
confirm the reliability of these constructs, the following experiments were done.
These constructs were transiently transfected into HEK293T cells on top of
monomeric, uncrosslinked matrix. Using western blotting analysis, the results show
that all the proteins are detectably being expressed and secreted at the correct
molecular weights in the cell lysates (Figure 10) and the media (11), except the
minus Ig. Results also show that full length peroxidasin is able to incorporate into
the matrix with high protein expression (Figure 12). In order for peroxidasin to
crosslink the matrix, it must have peroxidase activity. We checked for peroxidase
activity, as described earlier, and the results (Figure 13) show that all the
constructs, except for minus Ig, exhibit peroxidase activity often several folds

70

greater than WT PXDN. Minus Ig was anticipated to not have peroxidase activity

within the media because it was not able to be secreted into the media (Figure 11).
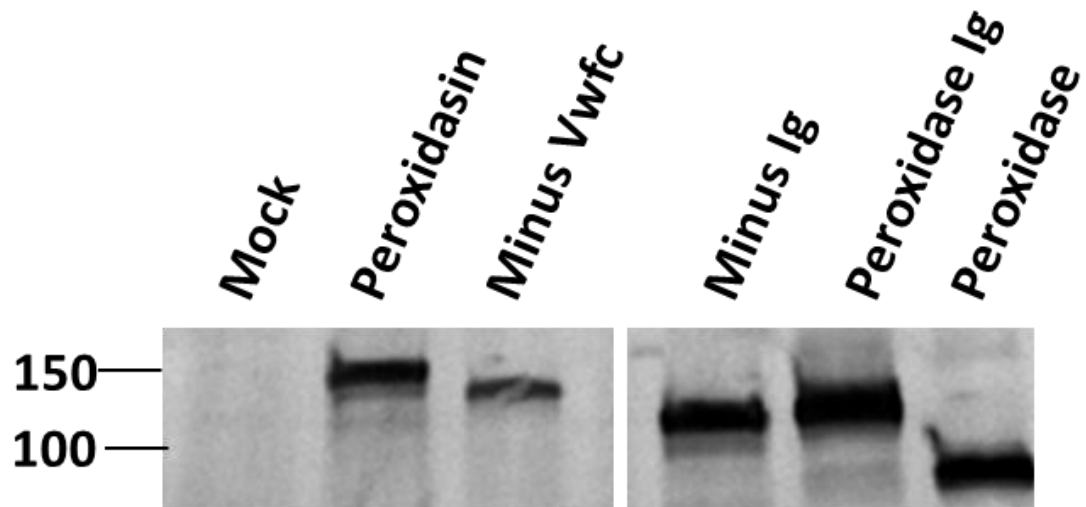
Figure 10. Protein expression levels of peroxidasin and truncated peroxidasin variants in the cell lysates. Western blot of cell lysate from overlay experiment. Proteins are at expected molecular weight. (Space within gel indicates removal of empty lane within the blot to bring the protein results closer on the picture).
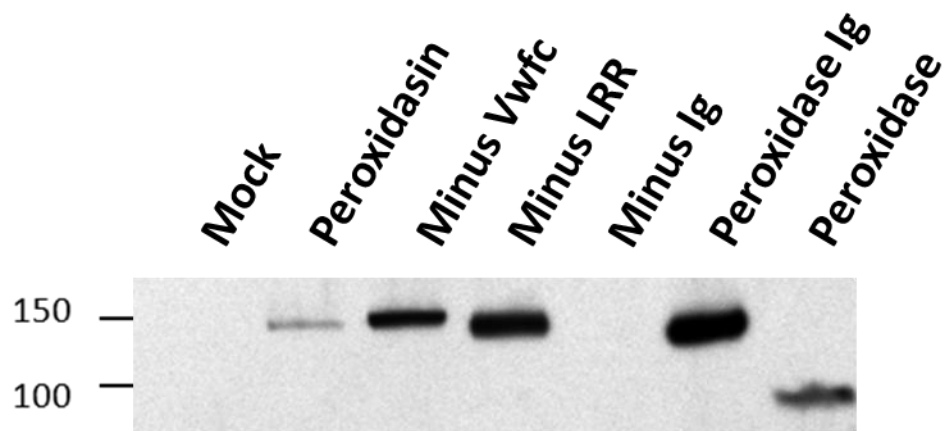
Figure 11. Peroxidasin and truncated peroxidasin variants are efficiently expressing protein in the media. Western blot with results from peroxidasin and truncated peroxidasin variants' media probed with antibody against peroxidasin. Results show protein was properly secreted into the media.
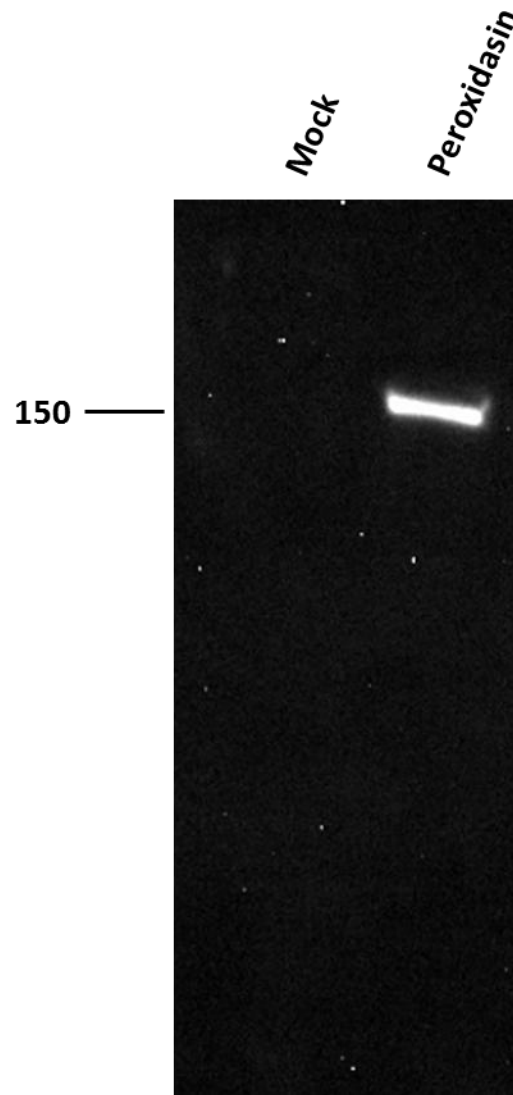
Figure 12. Protein expression levels of peroxidasin within the matrix. Matrix sample from the overlay experiment was probed with peroxidasin antibody for western blot detection of peroxidasin protein expression. Mock lane is empty vector.
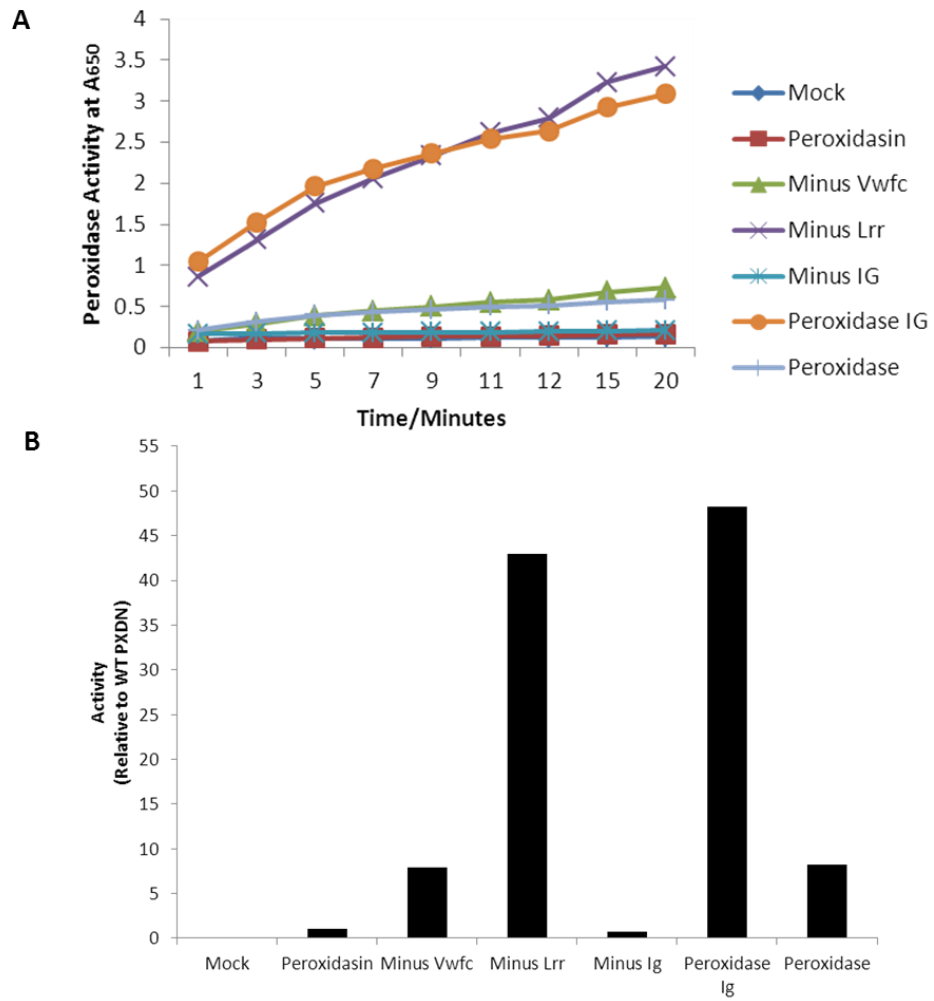
Figure 13. Peroxidase activity of peroxidasin and truncated peroxdasin variants. Media from PXDN and truncated PXDN variants, and mock transfected cells were assayed for peroxidase activity using a tetramethylbenzidine-based colormetric assay. The peroxidase activity at A_{650} and the peroxidase activity relative to peroxidasin for the proteins were plotted as shown below.

Domain requirements for sulfilimine bond formation

Since I had characterized the constructs and they were secreted and enzymatically active, I then proceeded to look at sulfilimine bond formation.   In order to find out, we overlaid HEK293 cells on top of monomeric, uncrosslinked matrix, as described in methods, and analyzed for sulfilimine bond formation. Results show that the negative control, empty vector, and minus Ig are not able to crosslink the matrix, as expected (Figure 14). Peroxidasin and the truncated peroxidasin constructs minus vWFC, minus LRR, and peroxidase Ig were able to crosslink the matrix. Results demonstrate that addition of the Ig domain to the peroxidase domain of peroxidasin optimizes crosslinking activity and reconstitutes peroxidase activity higher than wildtype peroxidasin. Finally, there is a trend between wildtype peroxidasin and peroxidase, showing wildtype peroxidasin crosslinks higher than peroxidase.   Future experiments will be performed to confirm.
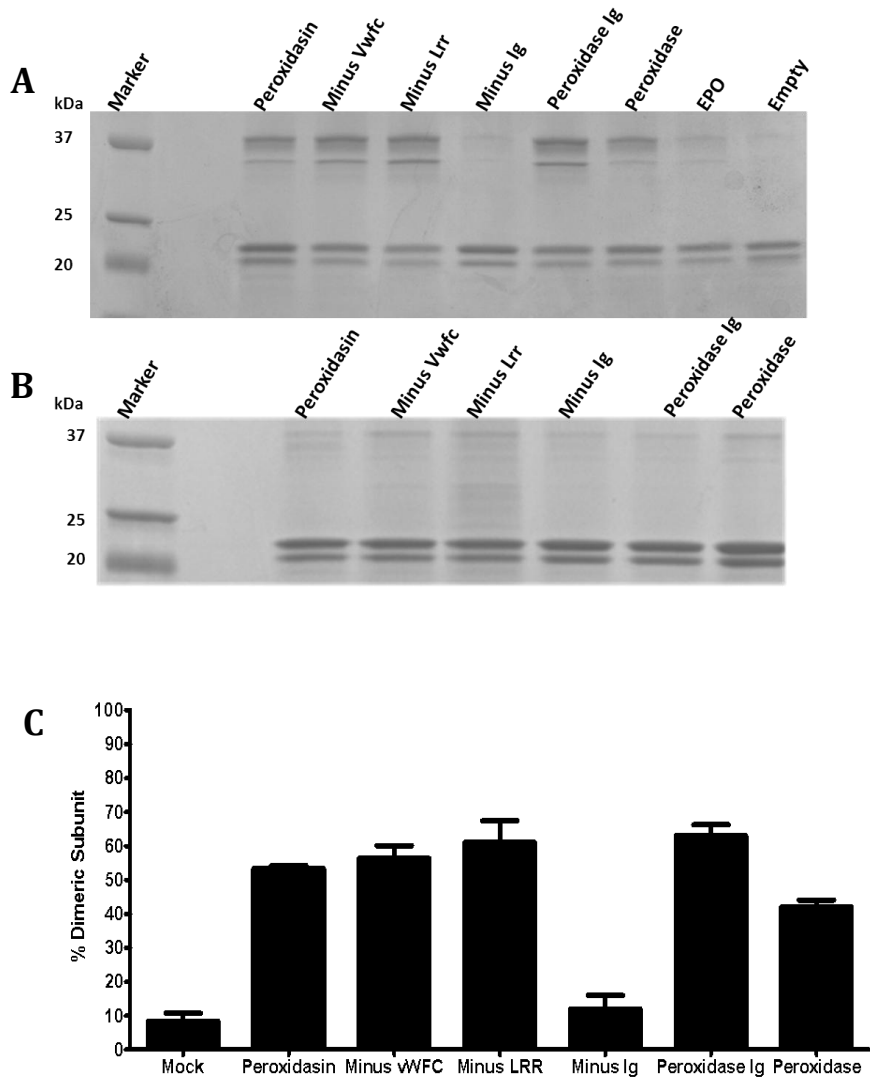
Figure 14. Dimerization/crosslinking of the matrix by peroxidasin and truncated peroxidasin variants. *(Panel A)* Coomassie stain. Peroxidasin and truncated constructs were transiently transfected in into HEK 293 cells in the overlay experiment and the samples were collagenase digested and run on a 12% gel and analyzed for crosslinking activity. *(Panel B)* Phloroglucinol (peroxidase inhibitor) treated samples from previous overlay experiment. Addition of the PHG inhibits the crosslinking activity of peroxidasin and the truncated variants, demonstrating the crosslinking activity is a due to the transfected peroxidases (Figure 15; panel B). *(Panel C)* Quantitated results from complied, repeated experiments of overlay experiments and dimerization.   Trend between wildtype peroxidasin and peroxidase (p=.10).

DISCUSSION

In summary, previous studies have been done on peroxidasin, since its discovery in *Drosophila* in 1994 by the Fesslers. For decades, there was no known *in vivo* function for human peroxidasin. Our lab first discovered that there was a sulfilimine bond, not a disulfide bond, holding the collagen IV hexamers together. This was the first time a sulfilimine bond had been identified in a biomolecule. A couple of years later, we discovered that peroxidasin is the enzyme which catalyzes bond formation using hypohalous acids (*Nature Chemical Biology, 2012*). My contribution to this discovery was that peroxidasin , among the animal heme enzymes, is the only one that can catalyze sulfilimine bond formation in basement membrane.

Subsequently, I identified subdomains of peroxidasin that are necessary for sulfilimine bond formation. This was accomplished by preparing deletion constructs, expressing them in cell culture, and measuring their capacity for crosslink. We showed that wildtype peroxidasin is expressed within the matrix, media, and cell lysates. All of the deletion constructs appeared to be expressed, secreted and enzymatically active with the exception of minus Ig. This construct is expressed, but is not found in media suggesting defective secretion. These results revealed that the peroxidase domain is sufficient for crosslink formation, but it requires the Ig domain for optimal activity. Therefore, this domain is a critical feature that distinguishes PXDN from EPO and MPO, conferring crosslink activity.

There is a trend (p=.10) towards peroxidase domain crosslinking not reaching full level of the wildtype peroxidasin. It suggests the Ig domain is needed

for complete reconstitution of wildtype peroxidasin crosslinking. The difference in crosslinking between the constructs seems small because it has a power n of 3. Future work is needed to confirm the trend with adequate power since the difference is relatively small.

In addressing the role of the domains in determining secretion and peroxidase enzymatic activity, there were large differences in truncated peroxidasin variants' secretion and activity in media. Any construct that lacked the LRR domain had robust secretion and high enzymatic activity in the media (minus LRR, Peroxidase Ig; Figure 13 and 14). The variants lacking the Ig domain, demonstrated poor secretion and enzymatic activity (minus Ig; Figure 13 and 14). LRR may be a negative regulator and Ig may be a positive regulator of enzymatic activity. Therefore, in wildtype peroxidasin, having both LRR and Ig may neutralize their regulation. The addition of Ig optimizes sulfilimine bond formation by possibly placing the peroxidasin closer to its substrate, collagen IV hexamer.

CONCLUSION

In 2009, the discovery of a new chemical bond in biology, was reported by Dr. Hudson and colleagues. The bond reinforced the quaternary structure of the collagen IV network. This work posed a key question: how does the bond form? My dissertation focused initially on identification of the enzyme that catalyzes bond formation. To this end, I identified peroxidasin as the only animal heme peroxidase that catalyzes bond formation (*Nature Chemical Biology*, Appendix). Subsequently, I sought to determine which domain of peroxidasin are required for crosslinking activity.   The finding revealed the Ig domain optimizes the crosslinking and completely reconstitutes crosslinking level to wildtype peroxidasin. In the future, I will focus on delineating the molecular requirements of peroxidasin to interact with collagen IV interaction. My preliminary work suggests that the peroxidase domain together with the Ig domain is required for binding to collagen IV.

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CHAPTER IV

OVERALL SUMMARY

With great pleasure, I declare that my interdisciplinary journey has as been one of true discovery, both in the generation of new knowledge about a biological system and in how students learn. Importantly, I gained an insight about modeling as an approach for biomedical investigations and for translating knowledge of biology to elementary students.

Scientists study complex phenomenon whereby the phenomenon is modeled in a simplified, yet systematic way to control variables in order to craft and answer important experimental questions. Modeling complex phenomenon within a controlled environment was commonly practiced in my overall dissertation work. Sometimes, it even seemed as if there were models within models.

For example, in the biomedical science aspect of my research, in which I characterized the enzyme called peroxidasin, there were modeling varieties that were highlights of the study. My objective was to elucidate aspects of the mechanism whereby peroxidasin forms sulfilimine bonds. These bonds help hold tissues together within the body, and so it was of great significance to understand the chemistry that underlies bond formation. The key question was what domain of peroxidasin is needed for bond formation. Indeed, we used a model cell culture system to answer this question. PFHR-9 murine endodermal cells produce large amounts of collagen IV, the predominant component of basement membranes that is

crosslinked by sulfilimine bonds. In our experiments, the PFHR-9 cells were grown

past confluency to accumulate a basement membrane-like matrix. This matrix is

similar to the basement membranes that underlie epithelial cells in animal tissues.

Thus, the PFHR-9 matrix is a good model for studying basement membrane

assembly and sulfilimine bond formation. The second model was the human

embryonic kidney (HEK) cell line that was used to produce engineered recombinant

domains of peroxidasin. Both models were instrumental in giving us an insight into

the mechanism of sulfilimine bond formation. The third model was the actual

enzyme, peroxidasin. This enzyme was expressed in HEK cells for study outside the

body, therefore representing another model to explore the chemical mechanism

outside of animal tissues. These examples demonstrate how modeling helped me as

a researcher to decipher biological mechanisms about how the enzyme peroxidasin

forms critical sulfilimine bonds in animal tissues.

In the other aspect of my dissertation work, I analyzed how elementary

students think about the process of organic decomposition based on models that

they created. In this study, students were asked a question about the fate of leaves

that fall each year. They were then asked to model the phenomenon in the

classroom. These elementary students were studying the complex process of

decomposition and needed a great entryway to start.

The best way of communicating the idea of what happens with the leaves

during the seasons seemed to be through student observation of the student hand-

made model systems.   There were models within the students' model systems to

aide in their observation and later conclusions. In order to help the students be

more conscious of the representations within the models on a regular basis, we used actual remnants of the phenomenon. For example, soil from their homes and the compost bins were models for the general outdoor soil in which decomposition occurs. The organisms, actual decomposers, from outdoors and in the compost bins were used to represent the decomposers within the ecosystem. Periodic water sprays were used to model the rain that occurs outdoors and finally the students used lettuce leaves to represent the outdoor leaves that fall each year. Through this in-depth modeling on the student level, the students were able to study the process of decomposition within an organized system within their classroom. I would also argue that the students were modeling the research scientists in laboratories by using inquiry-based methods and modeling to study a phenomenon and then making informed decisions based on their experimentations. The students also engaged in other scientific practices such as maintaining science notebooks of their observations and questions, having in-class discussions about their model systems, and having productive arguments about varying conditions to use in their experiments. These modeling practices helped these elementary students to investigate and understand scientific concepts.

My interdisciplinary path also provided an opportunity to experience the elementary classroom, from which I gained an appreciation for the educational challenges of both students and teachers. I learned that students are interested in science and how it works as early as elementary age and teachers do want to teach science on a level that students can understand, contrary to popular belief. I found that there is need for a bridge to help teachers translate modern biology to their students. Indeed, I served as that bridge.

As we went along, the teacher, students, and I learned a lot about how students learn and we made adjustments by redesigning lessons. We paced and rearranged the lessons to make certain aspects more salient and highlight areas that needed to be brought to the forefront to make it understandable by the students. This took the teacher's knowledge of how her students learned and it took the students voicing their opinions and helping with modeling their microenvironments. It took my knowledge as the scientist/researcher of knowing what direction we were trying to go with the research questions and the content knowledge to help fill in the spaces or territories in which the teacher would normally not have ventured due to fear of lack of understanding or authority on the subject. Progress came from every one's (scientist, teacher, and students) expertise coming together to get the work done and it was an effective way of getting students to learn scientific concepts on an elementary level. I conclude that a scientist as science classroom teacher and/or a partnership between scientists and classroom teachers is needed in the modern classroom in order to create lessons, curriculum, and standards that engage the students with hands-on level that involves reading, modeling and questioning/debating, activities that are beyond that of traditional memorization and recall for descriptive test questions.

APPENDIX A

YOUNG CHILDREN'S THINKING ABOUT DECOMPOSITION: EARLY MODELING

ENTREES TO COMPLEX IDEAS IN SCIENCE

Isi Ero-Tolliver, Deborah Lucas, Leona Schauble

Young Children's Thinking About Decomposition: Early Modeling Entrees to Complex Ideas in Science

Isi Ero-Tolliver · Deborah Lucas · Leona Schauble

Abstract This study was part of a multi-year project on the development of elementary students' modeling approaches to understanding the life sciences. Twenty-three first grade students conducted a series of coordinated observations and investigations on decomposition, a topic that is rarely addressed in the early grades. The instruction included in-class observations of different types of soil and soil profiling, visits to the school's compost bin, structured observations of decaying organic matter of various kinds, study of organisms that live in the soil, and models of environmental conditions that affect rates of decomposition. Both before and after instruction, students completed a written performance assessment that asked them to reason about the process of decomposition. Additional information was gathered through one-on-one interviews with six focus students who represented variability of performance across the class. During instruction, researchers collected video of classroom activity, student science journal entries, and charts and illustrations produced by the teacher. After instruction, the first-grade students showed a more nuanced understanding of the composition and variability of soils, the role of visible organisms in decomposition, and environmental factors that influence rates of decomposition. Through a variety of representational devices, including drawings, narrative records, and physical models, students came to regard decomposition as a process, rather than simply as an end state that does not require explanation.

Keywords Decomposition · Decomposers · Modeling · Organisms · Investigation · Compost · Matter

Introduction

Although primary grades science instruction almost always includes the study of nature, the typical focus is on the growth of organisms and the conditions that support their life.

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Educational treatments of life cycles almost always conclude with the death of the organism, even though processes of decay are at least as consequential for the health and balance of our world. It is possible that educators skirt this issue because they are unwilling to discuss death with young children; moreover, because so much of the process of decomposition is not evident to the unassisted eye, educators may legitimately be unsure how best to proceed with youngsters whose knowledge seems so firmly anchored in things they can directly see. Perhaps for these reasons, there is little research on children's thinking about decomposition.

In the slim literature that can be found, the general focus is on what children do *not* know, rather than on resources that they bring to further learning. Reading these studies lends the impression that children from ages 5 to 16 have little to no understanding about what happens during the process of decomposition (Leach et al. 1996). Most studies found that more than half of the child participants believe that matter from dead animals and plants simply disappears (Hellden 1992; Sequeira and Freitas 1986). When children are asked explicitly about rotting or decay, they report that decay is a state that simply happens to materials and that does not require an explanation (Smith and Anderson 1986). Decay, therefore, is conceived as an index of the endpoint of life and not as a process (Driver et al. 1994). These teleological forms of reasoning are commonly observed even in older people (Hartley 2011). In the previous research, of the very few children between the ages of 14–16 years old who understood that microbes were involved in the process of decomposition (Hellden 1992), most believed that materials rot on their own and subsequently, microbes finish off the partially "self-rotted" matter (Cetin 2007; Smith and Anderson 1986). Because children generally do not have a well-developed theory of matter, it may not be surprising that they do not know that the material constituents of living organisms are neither created nor destroyed (Gayford 1986).

Here we describe the development of very young children's thinking about decomposition in the context of instruction specifically designed to bring this process into their first-hand experience via a modeling approach. The instruction and associated study were part of a multi-year project on the development of elementary students' concepts related to ecosystems, change, and variability, concepts that we considered foundational (especially in their interrelationships) to developing a strong understanding of evolution as students moved into high school (Lehrer and Schauble 2012a, b). A hallmark of the instructional approach was to support students in developing, adapting, and/or revising models of processes in the world as a way of developing a better understanding of them. Accordingly, the larger project follows students across elementary grades, tracking both the forms and processes of modeling that support the development of student conceptual knowledge in the life sciences. The research reported here focuses on first-graders, who had not previously participated in instruction related to this project.

The instruction about decomposition had two major goals. First, we aimed to help students build a more nuanced understanding of decomposition as a process. Identifying an appropriate level of understanding for very young students seemed important, given the role of decomposition in later taught, critical topics such as carbon cycling and climate change. Briefly, we sought to problematize students' views of soil (which they initially conceived as an inert substance, associated with contamination), of decomposers (although primarily, those visible to the unassisted eye), and of the process of decay (which, consistent with previous literature, they tended to think of as spoilage, particularly applicable to foods; as in previous research, they initially described decay as an inevitable conclusion to life that does not need further explanation). Our second goal was to explore young children's potential to work with (including developing) models of processes and objects in the natural world as tools for studying the world "out there." Modeling is a defining characteristic of

science but is by no means an obvious epistemological gambit. People in general and children, in particular, do not necessarily find it self-evident why one would want to conduct investigations that involve representations of the natural world, rather than simply looking at the world itself (Bazerman 1988; Windschitl et al. 2008). Our ongoing research program (Lehrer and Schauble 2012a, b) focuses on learning about both the challenges and potential in modeling approaches with students, including young students, and this paper contributes to that line of investigation.

Method

In the first-grade classroom where the research was conducted, the regular classroom teacher, with the first author's assistance, conducted science lessons on decomposition at least once a week during the course of a school semester (total number of sessions was about 18). The teacher had 13 years experience teaching at the time of the study, but had not previously taught decomposition in her class. Nor was she familiar with employing modeling approaches to science investigation. The instruction was designed in consultation with the second author, who had previously taught ideas about decomposition to students in middle school. However, in advance of this study, we did not know which ideas and modifications of previously developed instruction might be accessible to students as young as first-graders.

Participants

All the students in the class, who were 6 or 7 years old at the time of the study, participated in the classroom activities and discussions. The class included 10 boys and 13 girls and was situated in a school proximal to a public housing project; 22 of the students received parent permission to participate in the study. Most of the students had been raised in this thoroughly urban environment, and the school population included a large proportion of students eligible for free and reduced lunch.

Procedure

Pre- and post-instruction written assessments were conducted with the 22 participating students. In addition, to supplement the written work, we identified a focus group of six students, chosen by the teacher to represent a wide range of student performance, as assessed by regular class assignments and tests. More intensive information, in the form of daily notebook entries and repeated individuals interviews, was recorded from these six focus students throughout the study.

During the previous autumn, students in the class had gone outdoors with their teacher and collected a large sample of fallen leaves. The class spent time that autumn comparing and contrasting the leaves' color, shapes, and sizes. The current study was initiated early in the following February, when the teacher reminded students of their leaf collection, which remained indoors and was at the time sitting in a pile on the floor in a back corner of the classroom. She next asked a guiding question that set the context for the decomposition study and, in addition, served as a pre-instructional assessment of students' thinking about decomposition. Specifically, students were asked to consider what happens to autumn leaves after they fall from deciduous trees. The teacher reminded students that many leaves fall every year. Given this, she asked, why isn't the entire world simply covered with leaves?

Students talked with each other about this question in groups of four, and following these initial discussions, each student individually wrote an answer to the teacher's initiating question, illustrated by a drawing. We refer to this work as the initial assessment of student knowledge, and a parallel task (which we call the final assessment) was repeated at the end of instruction. In both cases, in addition to these written assessments, the first author conducted additional individual follow-up interviews with the six focus students, to further probe their answers. These interviews were conducted individually and were video recorded. The interviews contained additional questions about students' conceptions of "dirt" (the children's word for soils) and animals that live in "dirt."

Following the initial assessment, the teacher and first author implemented six phases of instruction, varying in duration from a single class period to several weeks. Figure 1 displays a timeline of this instructional sequence. As the figure shows, the phases are numbered sequentially, by order of their initiation, but as some of the phases were ongoing, there was considerable overlap among the phases. We next briefly overview these phases, but further details on each, along with information about student thinking, are featured in the "Results" section.

Phase 1.   Each student brought a plastic bag filled with soil from a location of choice near his or her home. Students examined and described the samples, compared and contrasted their contents, and conducted individual soil profiles.

Phase 2.   Students initiated ongoing observations, drawings, and textual notebook entries to describe changes over time in two examples of decay: a ripening and then rotting banana (one banana kept on each table group of four students), and three Halloween pumpkins that were set outside the classroom window to decay.

Phase 3.   The children made a visit to an outdoor compost bin and investigated the contents. The school custodian explained how and why the bin was installed and the components included in the bin. He then turned the materials in the bin as students watched and extracted samples of material for students to hold and observe.

Phase 4.   Students inspected and compared soils from their home samples (first investigated during phase 1) to samples taken from the compost bin.

Phase 5.   Children developed and observed changes in classroom models intended to represent the process of leaf decay. These models included lettuce leaves to represent autumn leaves and other components considered necessary to represent
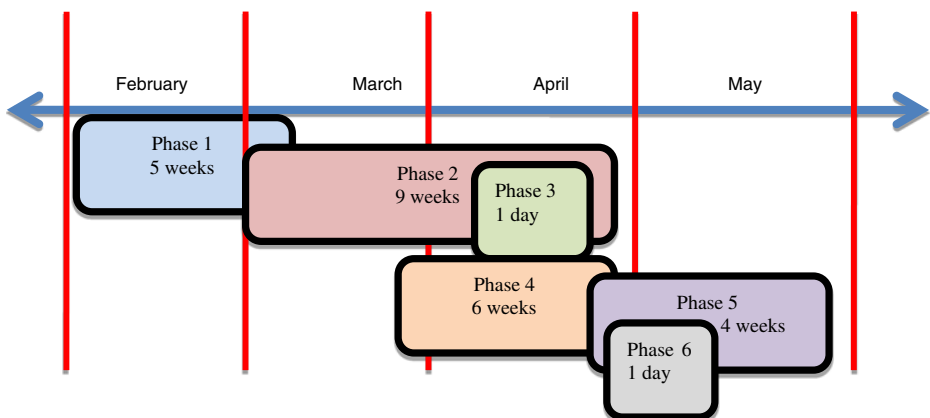

Fig. 1   Timeline of instructional phases

elements that might affect decay (different soil types, moisture levels, presence or absence of decomposing organisms, temperature, sunlight, etc.).

Phase 6.   In the classroom, students initiated a study of earthworms and other (visible) decomposers that live in the soil. This work included magnified observations of the critters, enactments of the way they move, and reading of related trade books to learn about their structures.

As Fig. 1 shows, these phases overlapped and varied considerably in duration.

Results

Initial Assessments of Student Knowledge

Working in groups of four, students first discussed and subsequently wrote individual responses to the teacher's initial guiding question ("What happens to the autumn leaves after they fall onto the ground? Why don't we see more and more of them year by year until the entire world is covered with leaves?"). Most of these responses were accompanied by drawings. At this initial assessment, the majority of student responses ($N=11$) suggested that the leaves simply disappeared, with few attempts to explain where (although one child suggested they might have gone "to another planet"). Students said that the leaves "disappeared," "died," were "blown away," or were taken away by trash collectors, all reasonable replies, given the experiences of these urban children. One child simply responded that the leaves change color, which is certainly true, but irrelevant to the question posed. The remaining half of the students reported that the leaves decrease in volume over time and seemed to understand that somehow, this should be accounted for. However, not very surprisingly, these students proposed mechanical (rather than chemical) processes of change. They pointed out that people and animals step on leaves, which then break into smaller pieces. In these children's views, leaves get ground into ever-smaller pieces, and over time, the pieces become so small that they become difficult to see. Some of these students felt that rain plays a part in this process, perhaps softening leaves up so that they fall apart more easily. Otherwise, however, these accounts did not propose changes in the material make-up of leaves.

The interviews with the six focus children confirmed these ideas and also strengthened our initial conjecture that students conceive of soil as a homogeneous, lifeless substance that is generally to be avoided because of its contaminating qualities ("Dirt is ugly….and the dirt has things in it that are really dirty"). For example, one of the focus children pointed out that dirt "comes from different places, but all places got dirt." Another remarked simply, "Dirt is dirt." When asked whether all "dirt" is the same, two students proposed that dirt can sometimes be different colors, but this was attributed to moisture—that is, children had noticed that when it is moist, soil takes on a darker color. The other four insisted that all dirt is the same kind of stuff. Furthermore, students stated that soil provides home for critters (or at least, earthworms) and serves to hold up trees so that they do not fall down. Five of the six children felt that soil somehow helps plants grow, but they had no idea how. It was common for students to propose ideas about soil that seemed to be based on its perceived utility or importance for humans: "If we didn't have dirt, the earth would look like all water everywhere. We would have no grass or green or anywhere to live." Another protested that without soil, humans would have nowhere to walk. The initial phase of instruction was intended to help students develop a more nuanced idea of the variable properties of soil and

of the inter-relationships of these qualities (such as texture, structure, and moisture) with the animals and plants that are found there.

Student Thinking During Instruction

After children completed the initial assessment, the first phase of instruction was launched. All phases of instruction featured both individual and small group work, whole class discussions, observations with microscopes and flexcams, and class read-alouds of nonfiction literature on related topics. During instruction students were regularly asked to record their observations and questions in their science notebooks, which served to further document student thinking. The teacher used a camera and easel paper to record student comments during class activities. She left these artifacts on the walls over the course of the semester so that students could refer to their earlier comments and discuss "changes in how we used to think and how we think now." Copies of these artifacts were collected during or at the end of instruction and served as further evidence of changes in student thinking. In addition, we collected classroom video, audio, and field notes to establish records of student thinking during class-room activity, whole group discussions, and occasional informal interviews. Each week the classroom teacher and first author met to discuss student progress and to plan for the upcoming week; these plans were usually preceded by discussions between the first two authors of the manuscript.

We next describe each of the phases of the instruction, providing examples that illustrate the forms of student thinking that we observed and (where appropriate), changes in thinking as the instruction progressed.

Phase 1. Observation and Analysis of Soil Samples The purpose of the first phase of instruction, which lasted about five weeks, was to encourage students to begin to think and talk about the properties of soil that vary, including its texture, structure, and perme-ability. All students brought a sample of soil from a location near their homes, and they spent several class sessions carefully combing through the soil on paper plates and describing its visible qualities and components, including small pebbles, roots, insects, and arthropods. Students were asked to describe how the soil felt, smelled, and looked under magnification of hand lenses. As students in the class proposed descriptive words, the teacher posted them on a word wall so that they could serve as a ready reference for ongoing journaling. Over time, the word wall came to include words that referred to texture (hard, soft, rough, stiff), color (dark, light, brown), moisture level (squishy, wet, dry), things found in soil (bugs, flowers, leaves, rocks, roots, grass), and words referring to quantity and other relevant ideas (shaped, high amount, low amount, labels, data). This kind of instructional adaptation makes it possible for students who are minimal readers to begin to record and subsequently refer to scientific observations.

Once they looked at it closely under magnification, students were surprised at the amount of variation that they observed within a single baggie-sized sample. In particular, they were surprised at the evidence of life sustained within the soil. As one wrote, "There are leaves and baby fragle (sic) sticks in it and a few roots in it." Another observed, "It feels rough and it has rocks in it, and grass. It smells like toast."

Next, the teacher drew children's attention to comparisons among soils from different locations. She asked them to contrast a grayish, dry clay, with a dark brown, moist soil taken from a garden, and a light-colored clumpy soil. Students examined these three samples at a center and wrote about their comparisons in their science notebooks, using the words from the word wall. This work, in turn, provoked the need to further expand their list of

descriptors. Constraining the comparison to three choices that varied in extreme ways seemed to help the children focus more intently on variations in soil color, texture, and moisture.

Moving outdoors, the class used an auger to remove a core of soil about a foot deep from a location near the school. Students inserted their hands into the empty hole and discovered that the bottom was noticeably cooler than the soil near the top. Children wondered whether organisms that live in the soil (like earthworms) might prefer the cooler, moister environment below the ground. They described changes in the soil from the top to the bottom of its core, including temperature, moisture, and color.

Returning to their samples from home, students conducted soil profiles by placing half a cup of soil in a mason jar, adding water, shaking the jars, and then letting the soil settle. The teacher set a classroom timer so that students could observe and draw what they noticed every ten minutes for the next thirty minutes. Students noticed that the soil layers were differentiating ("The middle is getting lighter and lighter"), that different samples showed different numbers and widths of layers, and that some of the materials within the soil were becoming more visible as they were suspended in the water. Students compared soils in each others' baggies to the soil profiles in the jar, trying to account for the different layers and colors that they saw. The teacher introduced soil components such as clay, sand, and humus, and explained that although soil may look "all the same," in fact, it is made up of materials that look and feel different.

As this part of the instruction came to a close, students were now aware that soils are not all the same; that soils differ in color, smell, moisture, granularity, and texture; and that soils often contain evidence of organisms that live there (bits of roots, twigs, and leaf; intact insects and isopods, etc.). Moreover, soil taken from the same geographic location varies with depth. Children also began to think about soil and leaves, and some wondered whether soil might be "made of leaves."

Phase 2. Ongoing Observation of Decaying Banana and Pumpkins During the second phase of instruction, which lasted from the first of March through the third week of April, children intermittently observed decomposing fruits and vegetables within and outside the classroom. During this phase of instruction, we intended to introduce students to the understanding that decay is a process that occurs over time, that it is associated with changes in color; smell, size, and texture; and that its rate can be affected by environmental factors such as temperature.

First, the teacher put a single banana on each table where four students sat. The easy accessibility of the banana to sight (as well as smell and touch via occasional poking) encouraged students to notice gradual changes in its appearance from day to day, and the children drew and wrote descriptions of change in their notebooks. In addition, the students observed changes in three pumpkins that they had originally used during mathematics class the previous fall for investigations in measurement. Initially, the pumpkins were cut open indoors so that students could study the seeds. Afterward, however, they were set outdoors, but within sight from a window. Although the pumpkins could be seen, they were beyond students' immediate range of visibility (and touch), and therefore, students tended to look at them only when directed to by the teacher.

Students' notebooks include detailed descriptions of changes in their tabletop bananas, along with carefully labeled drawings. For example, beginning descriptions included: "The banana is bright and yellow and green and big. And the stem is littl." "It is pointy at the top. It is yellowish green at the top. It is a moon shape, and it has a little black line." Over time, the entries began to read: "The banana has little brown and yellow parts and it smells." "First

the banana was yellow. Then the banana got brown and squishy. And made a hole were you can see." "The banana has bugs in it, and it has white on it, too." The descriptions noted changes in shape, smell, texture, color, and size (Fig. 2).

As the bananas were rotting, students occasionally looked out the window at the pumpkins, but when the teacher brought students outdoors for a closer look after several weeks, they were shocked at the transformation. As Fig. 3 shows, the pumpkins had entirely lost their original shape.

Students speculated that the inside of the pumpkin had disappeared or "gone into the ground." When the first author asked students what happened to the pumpkins, children replied, "…it went into the ground. Then it will turn into dirt because the bugs will eat it." Other students raised the possibility that changes in the pumpkin were due to the fact that "It got water on it." These initial proposals about environmental effects were explored later, during the fifth phase of instruction, when students constructed models of decomposition that included factors that might affect its rate.

Phase 3. Visit to the Compost Bin During a single day in mid-April, students visited the school's compost bin, located in the backyard beyond the school and maintained by the school custodian, an experienced gardener. Mr. B, the custodian opened the "earth machine," explained that this is where he brings uneaten fruit and vegetables from the cafeteria, leaves, and other yard waste to decompose and turn into humus. The bin was opened and the contents turned, and students inspected the contents. As in the previous work with their soil from home, students were asked to look carefully, to smell, and to feel—they noticed that the contents were moist and warm. The student who wrote the notebook entry in Fig. 4 summed up the question most on students' minds at this point: "I wonder what is the stuff in dirt?".

Considerable interest was expressed about the organisms that students observed living in the compost: "In the earth machine, I saw some worms and beetles and roly polys, too….spider, bug, slug, plant, and dirt." Most likely because many of the materials in the bins were foods, students speculated that the animals were eating the food. Students noted that, in contrast to the decaying bananas on their tables, there was relatively little odor from the material in the bin ("It smells normal," as one child pointed out). From Mr. B, the students learned that putting all those components into the bin and turning them, perhaps assisted in some way by the animals, resulted in the generation of soil. Just how, they were unsure. One of the students speculated in her notebook: "When Mr. B showed us the earth machean, I saw worms, appels, oranges, rollipollies, beatels, and then when Mr. B mixed up


Fig. 2 Notebook series of descriptions of banana decay

Fig. 3 Classroom pumpkin after decaying outdoors for several weeks

the stuff it made me feel like if you mixed it with worms and appels, oranges, rollypollys, beatels, soil, grass, leafs, you can make dirt." A second child wrote, "It looks brown and dark black. It has squished leaves in it. I wonder if the dirt grows by sun, water, soil?" By the end of the visit, most students had concluded that somehow, materials in the compost bin turned into soil. Some believed that animals within the bin were eating the contents and perhaps playing an (unspecified) role in their transformation. There was a good deal of conversation about how the animals might have gotten into the compost in the first place, a question resolved when Mr. B showed children that the bottom of the compost was in direct contact with the soil. Other students wondered why the material in the bin was so much darker in color, moister, and warmer than soil on the nearby ground. There were questions about the potential roles of sun and water, possibly provoked by noting the qualities of the composting material.

Fig. 4 First Grader's notebook entry about the compost bin
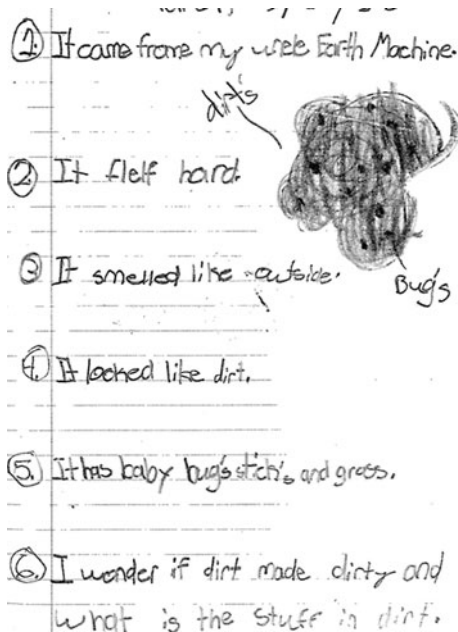
Back in the classroom, the teacher read aloud to the class selected excerpts from *What's Going on in the Compost Pile*, by Chappelle 2008. The book introduced several key notions about compost, but it is unlikely that children understood them at much depth, and they probably assimilated most of this material to ideas they previously held. For example, the book stated that compost is decaying organic matter (defined as anything that comes from plants). However, it is unlikely that these first-graders necessarily equated fruits and vegetables with plants. The book further explained that insects, slugs, worms, air, and moisture all work together in compost to break down organic material, and that as organic matter decays, it becomes compost that fertilizes growing plants. We have no direct evidence of the interpretation children brought to the phrase "break down," especially as their initial ideas about decay emphasized simply breaking material into smaller and smaller pieces. The book contained a brief allusion to "microorganisms" and "bacteria," but the children probably had no idea what those terms referred to, so it makes sense that they focused more intently on organisms they could see in the compost. Although some children did suspect that moisture might play in role in creating compost, no one raised the possibility that air might play a role.

Phase 4: Comparison of Soils from Compost Bin and Home Next, students compared the qualities of material from the compost bin with those of their home soils. The students' notebook entries reveal that they noticed differences in texture, smell, moisture, color, and contents (Fig. 5). In a whole-class discussion that followed their initial comparison, the teacher posted students' ideas about the ways in which the samples were "same" and "different."

In the children's eyes, the most important differences were the darker color of Mr. B's compost, the fact that it was moist to the touch, and especially, the number of visible organisms that it contained. The teacher posed the question, "What are those organisms doing in Mr. B's compost?" Someone recalled that Mr. B had told the class that the organisms they observed in the compost eat the organic material and that their waste produces that "nice dark stuff."

Teacher: When we first looked inside, remember, it was lots of grass and fruit and leaves. Remember when Mr. B stirred it up and pulled it up, what did it look like?
Student: It becomes mud.
Teacher: Well, it becomes something that we use that Mr. B says is good for planting.

Fig. 5 Similarities and differences between compost and home soil samples

What does it become?
Student: Dirt.
Teacher: Another name for dirt?
Student: Soil.
Teacher: …becomes the soil. So you guys think the bugs and the worms eat the fruit and the leaves and poop it out and it turns into soil.

By this point in the instruction, most students held an idea about the process of composting that was overly simplified in many ways, yet more nuanced than their original thinking about decay. Their model of the process equated insect waste with compost and compost with soil. On the other hand, the mental model held by most of the children did now regard decay as a process and included the ideas that plant material gets transformed, that organisms play a role in that transformation, and that soils vary in their color, moisture, content, and their capability to support the growth of living plant life.

Phase 5: Design and Observation of Models of Leaf Decomposition Late in April the teacher posed a new question: "What do worms and bugs eat if we don't give them apples and oranges?" A student proposed, "They can eat the leaves?....Maybe they eat the leaves that fall?" This reply provided an opportunity for the teacher to remind the children of her original question, "What happens to the leaves that fall each year?" Students suggested looking up the answer to this question on the Internet. The teacher proposed instead that they observe change themselves, and further suggested that students could "pretend" that lettuce leaves take the place of "leaves that fall outside." (We proposed lettuce so that change would be accelerated and more visible to the children.) Suspecting that students might not necessarily accept lettuce leaves as stand-ins for autumn leaves, the teacher asked, "Are lettuce leaves like leaves from trees?" The children concurred that they were, "…because they come from plants." As the teacher prompted students to explain what happens when leaves fall from trees, students replied that they fall onto the ground. At this point, the teacher suggested that to model this situation, the class place some leaves in jars that contained the soil children brought from home. Other leaves were placed in jars that contained material from Mr. B's compost machine. The teacher and students agreed that they would observe carefully over time to see what happened to the lettuce leaves resting on these two kinds of substrates. "We will put dirt from different tables in the jars. We want to know what happens to the leaves over time. We will see whose dirt will cause change in the leaves, because we are not sure." Before proceeding, however, the teacher asked a further question: "What happens outside, though? We want to make sure this is like outside, going through the same thing that the dirt outside is going through. What happens when dirt is outside?" The children replied that in the outdoors, "dirt" is exposed to "sunlight and water." Asked how it might be possible to simulate those conditions with their jar models, the children volunteered that they could arrange for sunlight by placing the jars near a windowsill and to simulate rain, "…you can get some water from the sink and just put a little bit in to see what happens." The teacher asked, "Are we going to give one table (that is, the jar placed on one table) more water than the other table?" The students protested that this would be "unfair" and eventually agreed that each jar should receive two teaspoons of water.

An extended discussion followed about whether the jars should be lidded. Some students were concerned that if lids were put on, the critters inside the soil might be unable to breathe. Others worried that if the jars were left open, "But then the bugs will come out!" Eventually students agreed that even if the jars remained closed, there might still be sufficient air inside,

so the procedure agreed upon was: "When you put the water in there, then you shut it back up. Then you just wait."

Eventually, the class negotiated two further conditions, a leaf that did not rest on soil and a leaf that did not rest on soil but was exposed to moisture by resting on a damp paper towel. At the conclusion of this discussion, the class had agreed on a comparison among four kinds of models: a jar model that included soil from home, a jar model with material from the compost bin, a Ziploc bag containing no soil, and a Ziploc bag with moist paper towels but no soil. In earlier work, we have described these kinds of representations as remnants, that is, fragments of the phenomena under study that are brought into classrooms in forms that make them amenable to closer investigation by students (often because they omit features that are not theoretically important). In this case, the jars contained attributes (soil, leaves, critters, moisture, sunlight) that are the same as or similar to those in the external world (with the exception of lettuce leaves to represent leaves on trees). However, the jars also omitted attributes (rocks and pebbles, twigs, gum wrappers) that children also saw outside. These models were placed on the tables where children sat (four children to a table), and students observed the leaves closely over the next four weeks. Periodically, leaves were removed from their jars and magnified with hand lenses or projected with a flex cam. Students recorded the changes that they observed, including changes in leaf shape, color, moisture, texture ("slimy"), and size. Children claimed after a few days that the leaf in the compost jar was "getting smaller faster" than the leaves in jars with their home soil. Ridges were observed on the edge of the compost leaf, and someone proposed, "The bugs are eating the leaves." One of the notebook entries stated, "I think the bugs caused a hole (in the leaf) because on it, I saw some teeth marks on it and also for the shape. The shape been, at first, the shape wasn't that crooked. It's turning crooked." Students also noticed that the leaves on the paper towel and in the jar without soil were not rotting as quickly as those that included soil. This led them to suspect that soil somehow was playing a role in decay. Recalling their previous observations at the compost pile and noting that the leaf in the compost model was decaying so much more quickly than the leaf in the jars that contained what they called "regular dirt," students suspected that the critical factor was "bugs." As one recorded in his journal, "Table blue don't have much bite, and then Mr. B has more bite than us. Mr. B has more bugs than us, and Mr. B has dirt, and he has more bugs than us. The bugs poop is dirt and they eat the leaves."

Unlike many of the models pursued by scientists (which may be expressed computationally or via mathematical expressions or models of chance), remnants like the ones featured here have a rather low representational overhead, because they preserve similarity between the model and the target phenomena. Because they have this characteristic of similarity, remnants are often appropriate entrees to modeling for young students. Yet, their cognitive challenge is far from trivial. Even though they do not make rigorous representational demands, they still require children to construct and cognitively maintain the relationships between objects and relations in the model and those in the modeled world; to identify relevant attributes to include (such as moisture) and exclude (gum wrappers); and to agree on standard ways to observe and measure (how do we *know* that a leaf is smaller today than it was yesterday?). Although the first-graders were scaffolded in these decisions by the teacher, they debated energetically about qualities of the model (2 teaspoons of water), appropriate comparisons ("regular" versus compost soil, moist conditions versus dry, soil versus no soil), and interpretation of the outcomes.

Phase 6: Classroom Study of Decomposers One day early in May, while students were still engaged in recording changes in the jar models, the teacher brought in some earthworms for

closer study. Students examined the worms' behavior, structure, and environment, smelling, touching, and drawing them, and then simulating their movement by attempting to enact "earthworm crawling." Students discussed decomposing material as food for the earthworms and proposed a relationship: The more food in the soil, the more organisms in the soil. The teacher read brief selections from *Earthworms*, by Holmes 1998, a children's trade book that described the lifecycle, structures, and behaviors of earthworms, including their preferences for moisture and cool temperatures and for eating "rotting vegetables, plants, leaves, and grass." These earthworm studies served to help connect children's observations of decay in the jar studies with their studies of soil by confirming students' suspicions that organisms in the soil are largely responsible for decay. At one point the teacher suggested that there could also be "very small organisms in the soil, even if we cannot see them," but this idea was not pursued further.

Post-Instruction Assessment As the academic year came to a close, we asked the children once again to consider the teacher's initial question about autumn leaves and again to write an answer. Figure 6 summarizes the changes in children's responses from pre- to post-assessment. As the figure illustrates, at the close of instruction, there were no more suggestions that leaves die, disappear, or somehow travel to another planet. Many children continue to focus on mechanical change, that is, that leaves break up into smaller pieces. In addition, however, it was clear that students were now strongly influenced by the potential role of insects. Students suggested that the leaves are eaten by "bugs," that bugs then "poop them out," and that in some way, "poop turns into dirt."

The individual interviews with the six focus students confirmed these shifts and added some further insights about their thinking about the role of soil and organisms. Before instruction, students described dirt simply as "mud and water." In contrast, after instruction, students said dirt was made up of flowers, leaves, grass, oranges and juice (memories of the compost bin!), water, and soil. When asked where dirt comes from, students initially said
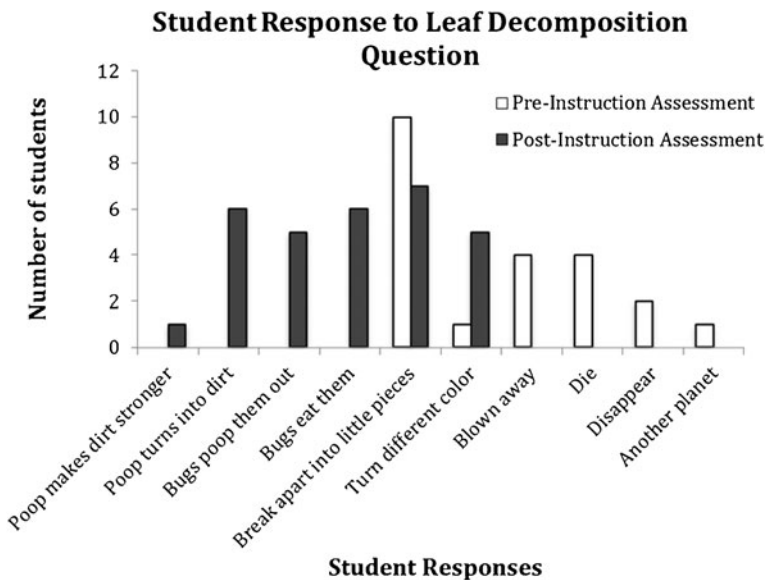

Fig. 6 The number of responses of each type both pre- and post-instruction to the question, "What happens to leaves that fall each year?"

they did not know or said it comes from the ground. After instruction, students said that "organisms poop it out" or that it comes from leaves. Constituents of dirt were initially considered to be mud and water, seeds, and rock, and most students said dirt is homogeneous (the same kind of stuff). After instruction, students mentioned insects, insect waste, plants, rocks, water, and mud, and were adamant that there are many kinds of "dirt" with different constituents. The number of kinds of organisms one can find in soil increased from 3 at pre-instruction (roaches, ants, worms) to 7 at post-instruction (worms, rolly pollies, roaches, beetles, ants, spiders, lady bugs). Instead of describing organisms as being at "home" in soil, students now focused instead on function: earthworms were described as "making more dirt" or "eating and finding food" (Fig. 6).

Discussion

Over several weeks of instruction, the first-grade children in this study made some modest progress in better understanding the decomposition of familiar organic materials (such as leaves and food). They became increasingly aware of decomposition as a process, rather than simply an end-state, and were able to identify changes that signaled decay, such as color, texture, shape, and smell, or the presence of mold. They discovered that different kinds of matter decay at different rates, and that environmental factors also seem to be associated with different rates of decomposition. With their lettuce leaf models, they concluded that temperature, moisture, and especially, the organisms living in soil, may be responsible for differences in the speed of decay. Their conceptions of soil shifted from a view of homogeneous, inert dirt to a view in which soils are highly variable in their make-up and loaded with life. Students were particularly interested in organisms, such as earthworms and isopods that fall into the soil and assist in the early stages of decomposition of organisms. In this instruction, we did not focus on the role of invisible decomposers, nor did we devote time to the mysteries of chemical change. It is possible that with additional instructional time, we might have found ways to open some of these thorny instructional issues with youngsters. Our purpose here, however, was restricted to setting the stage for learning more challenging biological ideas and modeling practices in subsequent education.

The relationship between the original target of query (autumn leaves) and the investigations undertaken by the children was representationally layered and required these young students to maintain several levels of systems intended to stand in for other systems. For example, although to some students it remained literally a compost bin, the compost bin was also intended as a model of the process of decay, and over time, students did import elements from the bin (such as fruit, insects) into other contexts of decay. The simple lettuce leaf models that students worked with capitalized on resemblance between the items and relations in the models (i.e., lettuce, moisture, compost) and the objects being modeled in the world (i.e., tree leaves, rain, soil). As we have found in our earlier research (Lehrer et al. 2000), physical models like these, composed of remnants (actual materials taken from the target phenomena being studied), seem to be easiest for young or inexperienced children to access, and therefore provide an appropriate entrée to modeling. Children's drawings and narrative descriptions entailed a further increase in their growing representational capacity, and their notebook records of phases of decomposition, coordinated with time (days of decomposition) were yet an additional shift away from the phenomenon of interest and into the representational world itself. The teacher supported the descriptive qualities of these notebook entries, which at first were often embellished with hearts, flowers, and other decorative devices. Over time, as she encouraged students to compare and evaluate their

illustrations, these embellishments began to drop away and the drawings began to show increasing realism and detail. In addition to the life sciences goals, therefore, a second important objective of this instruction was to assist students in developing a more sophisticated representational repertoire and applying these tools for understanding the world. These alternative ways of representing and depicting the world were critical tools for studying and communicating about complex phenomena. They were important for the first-graders' growing understanding of decomposition and, moreover, are also central to the professional practice of science.

Our study exemplifies the importance of beginning from resemblance and remnants of the target phenomenon because this is a great route to follow in most modeling studies with young students. Educators should begin by asking students to generate representations that do not require a lot of experience with specialized forms of inscription—remnants and drawings and other representations that look like the phenomena being represented are good places to start. Educators may find that they need to help children overcome their propensity to use drawings expressively, rather than depictively. Students learn to draw what they see when the features of drawings are compared and contrasted, to consider together how they "tell us" what we are looking at. Over time it is possible for educators to stretch these early forms of representation into those that are syntactically more complex, such as T-charts, tables, and simple graphs.

Appendix: Classroom Pre- and Post-written Assessment

Every year, in the autumn, all the leaves fall off of the trees to the ground below. You may have enjoyed raking the leaves into huge piles and jumping in them. Although you have only been around for 6 or 7 years, the leaves have been falling each of those years and many more. That's a lot of leaves!! Why aren't there mountains of leaves covering everything after all this time? In the space below, carefully explain what you think is happening so that a student in Mrs. Smith's class would understand. Draw and label a picture to help you explain what is happening to the leaves.

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APPENDIX B

PEROXIDASIN FORMS SULFILIMINE CHEMICAL BONDS USING HYPOHALOUS ACIDS IN

TISSUE GENESIS

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Peroxidasin forms sulfilimine chemical bonds using hypohalous acids in tissue genesis

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Collagen IV comprises the predominant protein network of basement membranes, a specialized extracellular matrix, which underlie epithelia and endothelia. These networks assemble through oligomerization and covalent crosslinking to endow mechanical strength and shape cell behavior through interactions with cell-surface receptors. A recently discovered sulfilimine (S=N) bond between a methionine sulfur and hydroxylysine nitrogen reinforces the collagen IV network. We demonstrate that peroxidasin, an enzyme found in basement membranes, catalyzes formation of the sulfilimine bond. _Drosophila_ peroxidasin mutants have disorganized collagen IV networks and torn visceral muscle basement membranes, pointing to a critical role for the enzyme in tissue biogenesis. Peroxidasin generates hypohalous acids as reaction intermediates, suggesting a paradoxically anabolic role for these usually destructive oxidants. This work highlights sulfilimine bond formation as what is to our knowledge the first known physiologic function for peroxidasin, a role for hypohalous oxidants in tissue biogenesis, and a possible role for peroxidasin in inflammatory diseases.

A basic organizational unit of animal tissues is a polarized epithelium attached to an underlying basement membrane, a specialized form of extracellular matrix[1]. The collagen IV protein network is the predominant constituent of basement membrane and provides structural integrity to epithelial and vascular tissues, serves as a scaffold for macromolecular assembly and interacts with cell-surface receptors such as integrins to control cell adhesion, migration, proliferation and differentiation[1,2]. The triple-helical protomer is the building block that self-assembles into collagen IV networks by oligomerization. The C-terminal trimeric NC1 domains of two protomers associate with each other to form a hexameric structure[3]. Notably, the C-terminal interface between two protomers is covalently crosslinked by a sulfilimine bond (S=N) between apposed lysine and methionine residues[4].

Collagen IV sequence homology suggests that the sulfilimine bond appears early in animal evolution at the divergence of Placozoa and Cnidaria, coinciding with the evolution of primordial basement membranes, and thus represents a potentially critical innovation for tissue biogenesis[4]. The sulfilimine bond also confers immune privilege to the collagen IV auto-antigen in human Goodpasture's disease, suggesting that its formation or cleavage participates in the pathogenesis of this autoimmune disease[5].

Given the critical role of the collagen IV sulfilimine bond in tissue development and human disease, we endeavored to delineate the molecular mechanism of bond formation. Here we show that peroxidasin catalyzes sulfilimine bonds directly within basement membranes using hypohalous acid intermediates. These findings represent what is to our knowledge the first known function for peroxidasin and highlight a biosynthetic role for conventionally toxic hypohalous oxidants.

RESULTS

A model to study collagen IV sulfilimine bond formation

To study sulfilimine bond formation, we used the PFHR-9 mouse endodermal cell line as an experimental system, as it produces biochemically tractable quantities of collagen IV (ref. 6). When grown past confluency, PFHR-9 cells progressively accumulated basement membrane, which we isolated to purify collagen IV NC1 hexamers after collagenase digestion. SDS dissociation of NC1 hexamers and gel electrophoresis revealed both crosslinked NC1 dimeric and uncrosslinked monomeric subunits (**Fig. 1a–c**). MS provided chemical evidence for a sulfilimine bond joining Met93 and hydroxylysine 211 (Hyl211) in adjacent protomers (**Fig. 1d**). We initially focused on known oxidative matrix-associated enzymes as possible mediators of sulfilimine bond formation in collagen IV. When small-molecule inhibitors were used during cellular deposition of basement membrane, structurally distinct peroxidase inhibitors including phloroglucinol (half-maximum inhibitory concentration (IC_{50}) = 0.5 μM)[7], methimazole (IC_{50} = 0.8 μM for thyroid peroxidase, 3 mM inhibits myeloperoxidase by 70%)[8,9] and 3-aminotriazole (near-complete inhibition of thyroid peroxidase at 2 mM and of myeloperoxidase at 10 mM)[10,11] universally prevented formation of collagen IV crosslinks. We initially examined iodide as a possible peroxidase substrate to form hypoiodous acid as a reactive intermediate (more details in Discussion). Unexpectedly, potassium iodide inhibited collagen IV crosslink formation, and therefore we used it as an inhibitor in subsequent experiments (**Fig. 2a**). Lysyl oxidase (β-aminopropionitrile; IC_{50} = 3–8 μM)[12] and transglutaminase inhibitors (putrescine; K_m 0.026–0.847 mM)[13] had no effect despite the use of concentrations exceeding published inhibitory constants
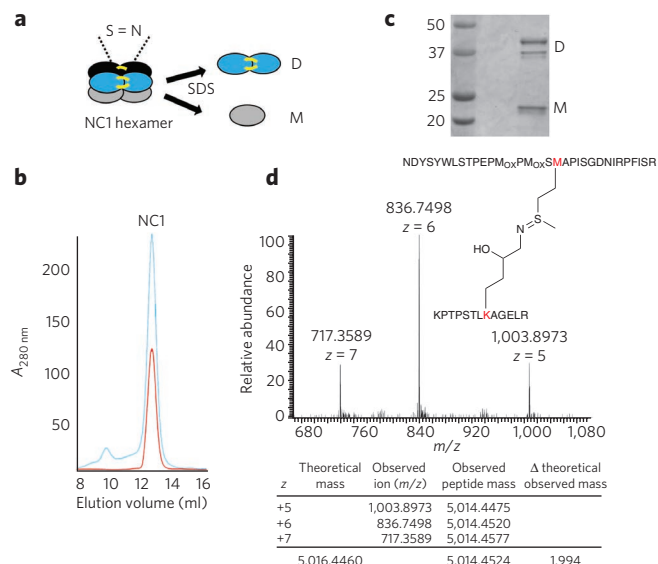
Figure 1 | PFHR-9 cells produce a basement membrane collagen IV network with sulfilimine crosslinks. (**a**) Schematic of collagen IV NC1 hexamer with sulfilimine crosslinks bridging the trimer-trimer interface. Upon addition of SDS, the hexamer dissociates into crosslinked dimeric subunits (D) and uncrosslinked monomeric subunits (M). (**b**) Gel filtration chromatography elution profile of PFHR-9 collagen IV NC1 hexamer (blue) and native, purified placental basement membrane NC1 hexamer (red) run successively. (**c**) SDS-PAGE of the purified NC1 hexamer with crosslinked dimeric (D) and uncrosslinked monomeric subunits (M). As seen in placental and Engelbreth-Holm-Swarm mouse tumor collagen IV, at least two and occasionally three dimeric subunit bands and one or two monomeric subunit bands were observed[47]. (**d**) MS of purified PFHR-9 NC1 hexamer revealed a tryptic peptide with a mean observed mass of 5,014.4524. The mass of the Met93-containing peptide added to the Hyl211-containing peptide provides a 'theoretical' mass of 5,016.4460. The difference between the theoretical and observed mass of 1.994 represents the loss of two hydrogens upon sulfilimine bond formation in collagen IV (ref. 4). M_{Ox}, methionine sulfoxide, a common oxidation product of methionine. Highlighted M and K residues represent Met93 and Hyl211, respectively, the sulfilimine-crosslinked residues of collagen IV.
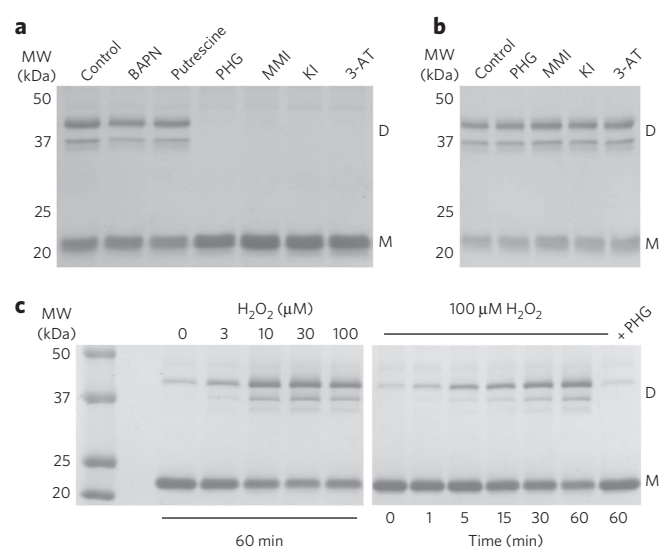

Figure 2 | A basement membrane peroxidase forms the collagen IV sulfilimine bond. (**a**) Coomassie blue–stained gel after SDS-PAGE of NC1 hexamers isolated from PFHR-9 cells grown in the presence of β-aminopropionitrile (BAPN; 500 μM), putrescine (2.5 mM), phloroglucinol (PHG; 50 μM), methimazole (MMI; 1 mM), potassium iodide (KI; 10 mM) or 3-aminotriazole (3-AT; 10 mM). Collagen IV NC1 hexamer from untreated cells (control) is shown for comparison. Gel is representative of five independent experiments. MW, molecular weight. (**b**) PFHR-9 basement membrane was allowed to form normally, isolated and treated with PHG (50 μM), MMI (1 mM), KI (10 mM) or 3-AT (10 mM) for 24 h at 37 °C. Collagen IV NC1 hexamer was isolated and underwent SDS-PAGE and Coomassie blue staining to visualize sulfilimine crosslink content. (**c**) Coomassie blue–stained gel after SDS-PAGE of NC1 hexamers after reacting uncrosslinked PFHR-9 basement membrane with H_2O_2 at varying concentrations for 1 h (left) or for varying durations with 100 μM H_2O_2 (right) in 1× PBS. The gel is representative of eight independent experiments. D represents NC1 crosslinked dimeric subunits, and M denotes uncrosslinked monomeric subunits. Full gel images are provided in **Supplementary Figure 13**.

(**Fig. 2a**). Peroxidase inhibitors did not perturb collagen IV assembly in this system, as NC1 hexamers formed quantitatively in the absence of sulfilimine crosslinks (**Supplementary Results**, **Supplementary Fig. 1**). Peroxidase inhibitors also did not break crosslinks after formation but specifically prevented bond formation (**Fig. 2b**). These findings suggest that a peroxidase, embedded within basement membrane, forms sulfilimine bonds in collagen IV. If so, an isolated basement membrane preparation should recapitulate this biochemical event *in vitro* with the addition of hydrogen peroxide (H_2O_2), a required substrate for peroxidases. PFHR-9 cells were grown in the presence of a peroxidase inhibitor (10 mM potassium iodide) to deposit a collagen IV network devoid of sulfilimine crosslinks. A basement membrane preparation was isolated and incubated without inhibitor in the absence or presence of H_2O_2. Sulfilimine bonds formed rapidly when peroxidase inhibitors were removed only in the presence of H_2O_2, pointing to a peroxidase residing within the basement membrane (**Fig. 2c** and **Supplementary Fig. 2**). Alternatively, H_2O_2 may chemically form sulfilimine crosslinks in collagen IV. To investigate this possibility, we extracted PFHR-9 basement membrane with 2 M guanidine to inactivate and/or extract the basement membrane peroxidase without affecting collagen IV. Indeed, guanidine pretreatment of the basement membrane eliminated crosslinking activity even in the presence of H_2O_2, consistent with the loss of an enzymatic activity rather than direct chemical oxidation by H_2O_2 (**Supplementary Fig. 3**).

Peroxidasin catalyzes formation of sulfilimine bonds

To rapidly identify candidates, we developed a new approach to covalently label and capture basement membrane–bound peroxidases. Inorganic azide (N_3^-) is a known suicide inhibitor of peroxidases. In the presence of azide and H_2O_2, peroxidases generate azidyl radicals that covalently attach to the peroxidase heme moiety to form an organic azide (R-N_3) and eliminate enzymatic activity ($K_i = 1.47$ mM, $k_{inact} = 0.69$ min^{-1} for horseradish peroxidase (HRP))[14]. PFHR-9 basement membrane was isolated and treated with azide and H_2O_2 to form an organic azide conjugate with matrix peroxidases. After basement membrane proteins were solubilized with SDS, azide-peroxidase conjugates were then biotinylated using alkyne biotin to react with the organic azide in a copper-catalyzed 'click' chemistry reaction[15]. Electrophoresed proteins were blotted with streptavidin-HRP to detect biotinylated proteins, revealing a single streptavidin-reactive band at about 160–200 kDa with reactivity increasing in a dose-dependent manner with azide concentration (**Supplementary Fig. 4**). Streptavidin agarose affinity chromatography was used to purify the azide-labeled peroxidase, revealing a single predominant band on Coomassie blue–stained protein gels at the same molecular weight as the band observed with streptavidin blotting (**Supplementary Fig. 4**). The stained protein band was excised and digested with trypsin. MS of the resulting peptides revealed peroxidasin as an azide-labeled peroxidase residing within PFHR-9 basement membrane (**Supplementary Table 1**).
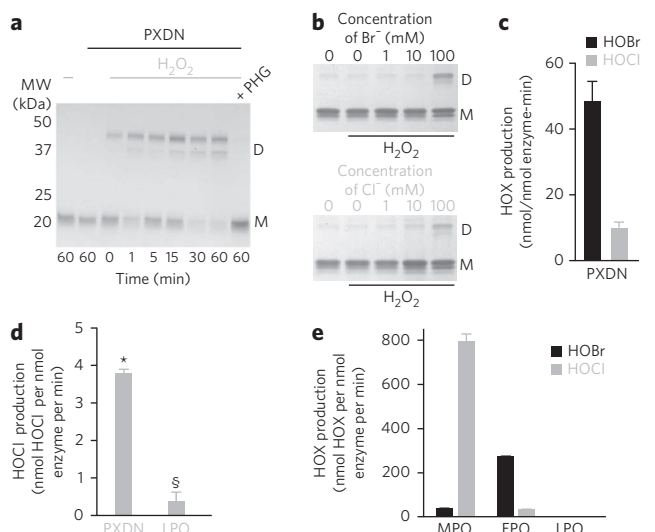
Figure 3 | Peroxidasin forms hypohalous acids and sulfilimine bonds in collagen IV. (**a**) SDS-PAGE of reactions consisting of 16 nM purified human peroxidasin (PXDN), 500 nM monomeric NC1 hexamer (3 µM potential crosslinks) and 10 µM H$_2$O$_2$ in 1× PBS. Control reactions without H$_2$O$_2$ or in the presence of the peroxidase inhibitor phloroglucinol (PHG; 50 µM) were also conducted. D represents crosslinked dimeric NC1 subunits, and M denotes uncrosslinked monomeric subunits. MW, molecular weight. (**b**) Coomassie blue–stained gel after SDS-PAGE of collagen IV NC1 hexamer is shown to illustrate relative amounts of sulfilimine-crosslinked dimeric (D) and uncrosslinked monomeric (M) subunits after incubation of uncrosslinked PFHR-9 basement membranes in varying buffer halide concentrations (Br$^-$ or Cl$^-$ as K$^+$ salt) with or without 1 mM H$_2$O$_2$. (**c**) PXDN-mediated hypohalous acid (HOX) production expressed as nmol hypohalous acid generated per nmol enzyme per min, measured in 1× PBS plus 100 µM NaBr. Values represent mean ± s.e.m. (n = 3). (**d**) HOCl production measured directly in 1× PBS without added Br$^-$. Values denote mean ± s.e.m. (n = 4). PXDN-mediated HOCl generation was significantly greater than that mediated by lactoperoxidase (LPO; *P < 0.05, unpaired two-tailed t-test), whereas LPO-mediated generation was not statistically different from zero (§ represents P = 0.32; one sample t-test). (**e**) HOX production, measured in nmol HOX generated per nmol enzyme per min for myeloperoxidase (MPO), eosinophil peroxidase (EPO) or LPO in 1× PBS plus 100 µM NaBr. Values represent mean ± s.e.m. (n = 3). Full gel images are shown in **Supplementary Figure 14**.

Recognizing the azide labeling technique as a screening tool with limitations, we next tested whether our identified candidate, peroxidasin, is truly capable of and responsible for the formation of sulfilimine crosslinks in collagen IV.

To determine whether peroxidasin is biochemically able to catalyze sulfilimine bond formation, we heterologously expressed and purified human peroxidasin (**Supplementary Fig. 5**). When reacted with purified NC1 hexamer, which was prepared without crosslinks, peroxidasin led to robust formation of crosslinked dimeric subunits at low enzyme/substrate ratios (<1:30) only in the presence of H$_2$O$_2$ (**Fig. 3a**). MS of the peroxidasin-reacted NC1 hexamer confirmed sulfilimine bond formation at levels near that of the native PFHR-9 hexamer (**Supplementary Fig. 6**). To determine whether the ability to catalyze bond formation is a universal property of animal peroxidasins, we reacted *Drosophila* peroxidasin with uncrosslinked collagen IV and found similar crosslinking activity (**Supplementary Fig. 7**). Taken together, peroxidasin crosslinks collagen IV NC1 hexamer *in vitro*.

Peroxidasin forms sulfilimine bonds via hypohalous acids
Animal heme peroxidases, such as peroxidasin, myeloperoxidase, eosinophil peroxidase and lactoperoxidase, catalyze oxidative
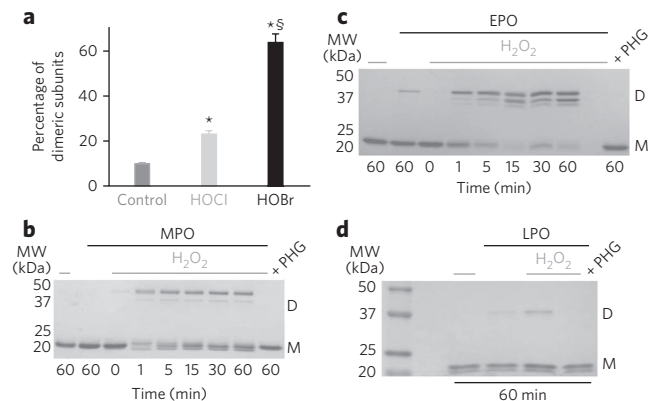

Figure 4 | Hypohalous acids form collagen IV sulfilimine bonds. (**a**) Five hundred nanomolar collagen IV NC1 hexamer (3 µM potential crosslinks) was incubated alone (control) or with 5 µM hypochlorous (HOCl) or hypobromous acid (HOBr) for 30 min at 37 °C. Percentage of dimeric subunit (mean ± s.e.m.) as quantified with densitometry of Coomassie blue–stained SDS-PAGE gels (**Supplementary Fig. 8**) increased significantly with HOCl and HOBr treatment (control: n = 10, HOCl: n = 9, HOBr: n = 6; analysis of variance with Tukey's *post hoc* comparison between groups; *P < 0.05 compared to control, and § represents P < 0.05 HOCl versus HOBr). (**b–d**) 16 nM myeloperoxidase (MPO) (**b**), eosinophil peroxidase (EPO) (**c**) or lactoperoxidase (LPO) (**d**) were reacted with 500 nM NC1 hexamer (3 µM potential crosslinks) for varying time points in 1× PBS with or without 10 µM H$_2$O$_2$. In the case of LPO, all reactions proceeded for 60 min. Collagen IV sulfilimine crosslink content was visualized after SDS-PAGE and Coomassie blue staining of the reactions. Each gel is representative of three independent experiments. Complete gel images are provided in **Supplementary Figure 15**. PHG, phloroglucinol; MW, molecular weight.

reactions using distinct halogenation and peroxidase cycles[16]. Both begin with hydrogen peroxide oxidation of the prosthetic heme iron to form an intermediate denoted compound I (ref. 16). Compound I may oxidize halides into their respective hypohalous acids (or related oxidants in equilibrium), which may directly or indirectly halogenate susceptible moieties. Alternatively, compound I undergoes sequential reduction to form single electron–free radicals of energetically favorable substrates in the peroxidase cycle. Both pathways eventually regenerate reduced, native enzyme[16]. To determine whether peroxidasin forms sulfilimine bonds using a halogenation cycle, we first tested whether peroxidasin crosslinks collagen IV in the absence of halides. When H$_2$O$_2$ was added to uncrosslinked basement membrane without halides, very few crosslinked collagen IV dimeric subunits formed until halide (Cl$^-$ or Br$^-$) concentrations approached 100 mM, suggesting the involvement of a peroxidase halogenation cycle (**Fig. 3b**). Peroxidasin is known to iodinate proteins, but little is known about its ability to oxidize other halides such as bromide and chloride[7]. Using taurine to trap hypohalous acids as stable taurine haloamines[11,17], peroxidasin formed hypobromous and hypochlorous acid at modest rates with a preference for bromide (**Fig. 3c,d**). Consistent with previous work, myeloperoxidase preferentially formed hypochlorous acid, eosinophil peroxidase primarily yielded hypobromous acid, and lactoperoxidase formed neither hypohalous acid (**Fig. 3e**)[16]. Taken together, peroxidasin produces hypohalous acids and requires halides (Cl$^-$ or Br$^-$) to form sulfilimine bonds, suggesting a link between the two activities.

If peroxidasin uses hypohalous acids as intermediates to form sulfilimine bonds, these intermediates should recapitulate the reaction when directly added to purified, uncrosslinked collagen IV NC1 hexamer. Indeed, reacting collagen IV with hypochlorous or hypobromous acid yielded crosslinked dimeric subunits (**Fig. 4a** and **Supplementary Figs. 8** and **9**). Alternatively, other peroxidases
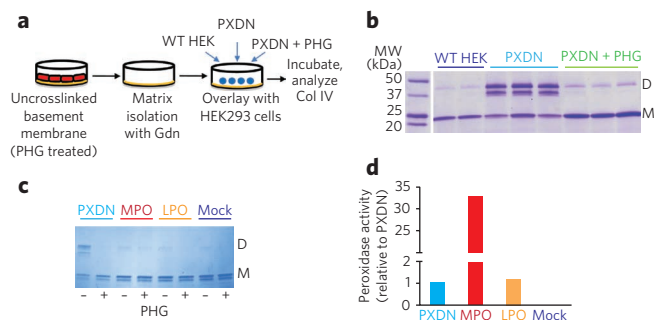
Figure 5 | Peroxidasin uniquely crosslinks native collagen IV networks. (**a**) Experimental design of 'overlay' experiments. PFHR-9 cells were grown in the presence of phloroglucinol (PHG; 50 µM) to deposit uncrosslinked collagen IV (Col IV) networks. The cells were then removed, and the basement membrane was extracted with 4 M guanidine (Gdn) to inactivate endogenous peroxidasin. Cells stably transfected with human peroxidasin (PXDN) or untransfected HEK293 cells (WT HEK) were plated on top of the PFHR9 basement membrane, which was subsequently analyzed for collagen IV crosslink content. (**b**) Collagen IV sulfilimine bond formation in the indicated experimental conditions as shown by stained SDS-PAGE gel. Two (WT HEK cells) or three (PXDN, with or without PHG) out of five independent experiments are shown. MW, molecular weight. (**c**) Coomassie blue–stained gel of collagen IV NC1 hexamers isolated from uncrosslinked PFHR-9 basement membrane overlaid with HEK293T cells transiently transfected with human peroxidasin cDNA, mouse myeloperoxidase cDNA (MPO), mouse lactoperoxidase cDNA (LPO) or empty vector (Mock). (**d**) Media from PXDN, MPO, LPO and mock-transfected cells were assayed for peroxidase activity using a tetramethylbenzidine-based colorimetric assay. Activity was expressed relative to peroxidasin (A_{650} of given peroxidase divided by A_{650} for peroxidasin). Full gel images are shown in **Supplementary Figure 16**.

should be able to catalyze sulfilimine bond formation when a halide is provided to form reactive hypohalous acids. Myeloperoxidase and eosinophil peroxidase formed sulfilimine crosslinks in collagen IV (**Fig. 4b,c**), whereas lactoperoxidase poorly catalyzed crosslink formation as it does not efficiently form hypochlorous or hypobromous acid (**Figs. 3e** and **4d**)[16].

Peroxidasin crosslinks collagen IV for tissue integrity

Though peroxidasin forms sulfilimine bonds *in vitro*, we tested whether peroxidasin catalyzes the formation of the sulfilimine bond within native insoluble collagen IV networks. HEK293 cells expressing human peroxidasin were plated on top of a PFHR-9–deposited basement membrane, which was produced in the presence of phloroglucinol to render a collagen IV network without sulfilimine crosslinks (**Fig. 5a**). Only overlaid cells expressing human peroxidasin formed dimeric crosslinked NC1 subunits, whereas wild-type HEK293 cells or peroxidasin-transfected cells in the continued presence of phloroglucinol failed to crosslink collagen IV (**Fig. 5b**). We hypothesized that peroxidasin, as a resident basement membrane protein[7], uniquely crosslinks collagen IV networks, whereas other peroxidases, though capable of bond formation in solution, will not form crosslinks within basement membranes. To test this hypothesis, HEK293 cells were plated on uncrosslinked PFHR-9 basement membrane and transiently transfected with peroxidasin, myeloperoxidase and lactoperoxidase cDNA or empty expression vector to determine whether peroxidasin specifically crosslinks collagen IV. Only peroxidasin formed sulfilimine bonds in collagen IV, even though myeloperoxidase enzymatic activity was at least 30-fold greater than peroxidasin (**Fig. 5c,d**). These data suggest that only peroxidasin, embedded within basement membranes, generates hypohalous acid in close proximity to its collagen IV substrate.
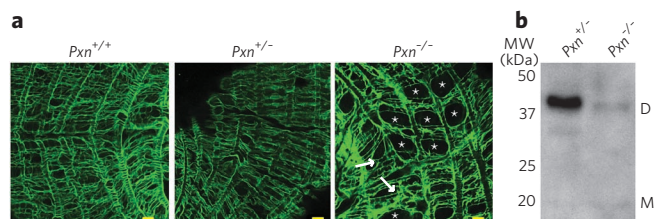

Figure 6 | Peroxidasin is critical for collagen IV and basement membrane integrity. (**a**) Confocal fluorescence microscopy images of *Drosophila* anterior midgut using a collagen IV GFP protein trap line (*viking*[G454]) to delineate collagen IV distribution. Representative sections from wild-type *Pxn*^{+/+}, heterozygote *Pxn*^{+/-} (*Pxn*[+/f07229]) and mutant *Pxn*^{-/-} (*Pxn*[f07229/f07229]) flies are shown. Distorted and torn collagen IV networks (arrows) with gross defects ('holes') in the circumferential muscle layer (asterisks) typified *Pxn*^{-/-} sections. Scale bars, 10 µm. (**b**) Immunoblot of collagenase-solubilized basement membrane isolated from *Drosophila* *Pxn*^{+/-} and *Pxn*^{-/-} larvae. *Pxn*^{-/-} mutants show grossly reduced collagen IV immunoreactivity at 20.4% that of the wild type, whereas *Pxn*^{+/-} flies maintained collagen IV NC1 content at 82% that of the wild type (**Supplementary Fig. 11**). *Pxn*^{-/-} mutants also show a shift in the percentage immunoreactivity, with 42% of total band density in the uncrosslinked form compared to <9% total band density in *Pxn*^{+/-} flies (**Supplementary Fig. 11**). MW, molecular weight.

Comparatively greater but spatially indiscriminate generation of hypohalous acid by myeloperoxidase artificially crosslinks soluble collagen IV NC1 hexamer but fails to crosslink insoluble, basement membrane collagen IV.

To further substantiate that peroxidasin functions to form sulfilimine bonds in collagen IV and to delineate the role of this function in basement membrane homeostasis, we turned to the *Drosophila* genetic model system, where peroxidasin was first discovered[7]. Using MS of purified *Drosophila* collagen IV NC1 hexamer, we first experimentally determined that the collagen IV sulfilimine bond is present in *Drosophila* larvae as sequence conservation of Met93 and Lys211 may not necessarily translate into a crosslink bridging these residues (**Supplementary Fig. 10**)[4]. With biochemical characterization of the collagen IV sulfilimine bond in hand, we examined basement membrane architecture in *Drosophila* larvae homozygous for a severely, hypomorphic peroxidasin (*Pxn*) allele (*Pxn*[f07229/f07229]; denoted as *Pxn*^{-/-}) before their demise as third instar larvae. With the collagen IV GFP protein trap line (*viking*[G454]), we visualized collagen IV networks within basement membranes of the longitudinal and circumferential midgut visceral muscles[18]. These networks appeared severely distorted and extensively torn in *Pxn*^{-/-} mutants when compared with heterozygous *Pxn*^{+/-} and wild-type *Pxn*^{+/+} larvae (**Fig. 6a**). Collagenase solubilization of larval basement membrane revealed that *Pxn*^{-/-} collagen IV NC1 content was about 20% that of the wild type (*Pxn*^{+/+}), based on immunoreactivity (**Fig. 6b**). Furthermore, *Pxn*^{-/-} mutants showed a shift toward uncrosslinked monomer subunits, with immunoreactivity rising to 42% of total band density compared to < 9% in *Pxn*^{+/-} larvae (**Fig. 6b**). Thus, peroxidasin forms sulfilimine bonds that crosslink collagen IV to reinforce basement membranes and maintain tissue integrity.

DISCUSSION

In this work, we demonstrate that peroxidasin catalyzes sulfilimine bond formation in collagen IV, the first known bond of its kind in a biomolecule[4]. Peroxidasin was initially discovered as a basement membrane constituent in *Drosophila*, but herein we establish its first bona fide function: namely, crosslinking collagen IV (ref. 7). Both the *Drosophila* mutant described in this work and *Caenorhabditis elegans* mutants of peroxidasin show defects in basement membrane

integrity similar to the effects of mutations in collagen IV itself[19,20]. Our data provide a molecular mechanism for this phenotypic similarity. Loss of peroxidasin function leads to fewer collagen IV crosslinks, destabilizes collagen IV and reduces its content within basement membranes. Mutations in human *PXDN* were recently discovered in a subset of individuals with inherited anterior segment dysgenesis and cataracts. Accounting for two peroxidasin homologs in humans[21], we hypothesize that partial loss of peroxidasin activity compromises the collagen IV network of anterior eye basement membranes and again recapitulates an ocular phenotype commonly observed in patients with partial loss of function in collagen IV (refs. 22–26). Taken together, peroxidasin, collagen IV and the sulfilimine crosslink form an important triad for basement membrane function and tissue biogenesis alongside laminin, nidogen and proteoglycan.

Though this work identifies what is to our knowledge the first function of peroxidasin, the formation of sulfilimine crosslinks in collagen IV may not be its only function. Peroxidasin is upregulated in response to transforming growth factor-β stimulation of fibroblasts and in renal interstitial fibrosis[27]. Collagen IV, a constituent primarily of basement membranes, is minimally present in fibroblast-generated extracellular matrix[3]. Thus, peroxidasin may form sulfilimine crosslinks in other matrix proteins or execute non-catalytic functions involving protein-protein interactions with cell-surface receptors and matrix proteins.

Peroxidasin generates hypohalous acids and requires halides to form sulfilimine crosslinks, whereas hypohalous acids produce sulfilimine bonds when directly applied to collagen IV NC1 hexamer. Similarly, hypohalous acids, including HOBr and HOCl, form an intramolecular sulfilimine bond to convert methionine into dehydromethionine[28,29]. We hypothesize that peroxidasin, embedded within basement membranes near its collagen IV substrate, locally generates hypohalous acids, which form an intermolecular sulfilimine bond across two collagen IV protomers in a reaction mechanism akin to the formation of dehydromethionine. Specifically, HOBr and HOCl react with the sulfur of Met93 to form a halosulfonium cation intermediate, which is then trapped by the Hyl211 amine to form a sulfilimine bond (**Supplementary Fig. 12**)[30]. Close proximity of the amine to the thioether creates a high effective amine concentration to prevent the halosulfonium cation from reacting with solvent water in a side reaction producing methionine sulfoxide. In collagen IV, the close apposition of Met93 and Hyl211 on separate NC1 trimers provides the required approximation of nitrogen and sulfur atoms to yield a sulfilimine bridging the NC1 trimer-trimer interface[29].

Although the parallel between the chemical synthesis and enzymatic catalysis of sulfilimine bonds suggests a mechanistic link, our data point to some differences. Iodine (I_2) or hypoiodous acid (HOI) also efficiently converts methionine to dehydromethionine[28,29,31], yet iodide paradoxically inhibits crosslink formation in collagen IV. Many possible mechanisms could explain this inhibition, including I^- quenching of reactive hypohalous acid intermediates[32], competition between I^- and H_2O_2 preventing compound I formation[33] or complex halide interactions at the peroxidasin catalytic site[33–35]. Future work will need to address the mechanism of iodide inhibition and formally test the proposed reaction scheme for sulfilimine bond formation (**Supplementary Fig. 12**).

Hypohalous acids typically conjure images of microbial destruction and unintended toxicity, but this work points to an unexpected, anabolic role for these highly reactive species. Peroxidasin is optimally suited to productively use hypohalous acids because its noncatalytic leucine-repeat-rich and immunoglobulin protein interaction domains presumably place peroxidasin in close proximity to its collagen IV substrate so that relatively modest amounts of hypohalous acids form sulfilimine crosslinks without pathologic 'collateral damage'. The use of hypohalous acids as anabolic

intermediates presumably depends on coupling peroxidasin oxidant generation with sulfilimine crosslink formation and possibly on local antioxidant mechanisms. Excessive peroxidasin activity either due to overexpression or increased H_2O_2 substrate availability may uncouple hypohalous acid generation from sulfilimine bond formation, allowing free hypohalous acid oxidants to accumulate and produce intended or unintended toxicity. Indeed, mosquito gut peroxidasin is upregulated after bacterial infection, and its knockdown reduces bacterial clearance and host survival[36]. Invertebrate peroxidasin may generate antimicrobial hypohalous acids as a primitive form of innate immunity analogous to vertebrate myeloperoxidase and eosinophil peroxidase[37].

Oxidative stress and reactive oxygen species have a central role in the pathogenesis of atherosclerosis, diabetes mellitus–associated complications and hypertensive vascular disease, which are the leading causes of morbidity and mortality in developed nations[38–40]. Human peroxidasin, also known as vascular peroxidase 1 (VPO1), is upregulated in cell culture models of hypertension and atherosclerosis and promotes smooth muscle proliferation and fibrosis, but the mechanistic connection between peroxidasin and downstream pathologic events is unknown[27,41–43]. As peroxidasin consumes H_2O_2 produced by cell-surface NADPH oxidases (NOX), enhanced NOX-generated H_2O_2 in pathologic states may promote peroxidasin-mediated matrix crosslinking and stabilization, eventually leading to tissue fibrosis[21,43]. Alternatively, 'uncoupled' peroxidasin activity may lead to hypohalous acid accumulation, promoting tissue injury. Indeed, myeloperoxidase has garnered considerable attention for hypochlorous acid–mediated oxidative modifications involved in the development of vascular inflammatory disorders such as atherosclerosis[44]. But unlike myeloperoxidase, whose deleterious actions require targeting to vessel wall, peroxidasin is omnipresent at the site of pathology within vascular basement membranes and therefore primed to generate deleterious oxidants and participate in disease pathogenesis[21,43,44]. Collectively, these results establish that peroxidasin forms collagen IV sulfilimine crosslinks, a post-translational modification critical for basement membrane integrity and tissue biogenesis, and draw attention to peroxidasin as an oxidant generator embedded within basement membranes readily capable of contributing to disease pathogenesis.

Note added in proof: Li et al.[45] recently showed that peroxidasin (VPO1) forms hypochlorous acid.

METHODS

Chemicals. Phloroglucinol, methimazole, potassium iodide and tetramethyl-benzidine were >99% pure, and β-aminopropionitrile, putrescine and 3-1,2,4-aminotriazole were >98%, >97% and ~95% pure, respectively. All chemicals were obtained from Sigma Chemical Co.

Collagen IV NC1 hexamer isolation. PFHR-9 cells were homogenized in 1% (w/v) deoxycholate with sonication, and the insoluble material isolated after centrifugation at 20,000*g* for 15 min. The pellet was then extracted with 1 M NaCl (or 2 M urea in some experiments) plus 50 mM Tris-Cl pH 7.5 and 10 mM Tris-Cl pH 7.5 and was digested in 50 mM Tris-Cl pH 7.5, 5 mM $CaCl_2$, 5 mM benzamidine, 25 mM 6-aminocaproic acid, 0.4 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mg ml^{-1} bacterial collagenase (Worthington). Collagenase-solubilized material was dialyzed against 50 mM Tris-Cl, pH 7.5. NC1 hexamers were purified using anion-exchange chromatography (DE52 Cellulose or Q Sepharose) followed by gel filtration chromatography.

***In vitro* basement membrane reactions.** PFHR-9 cells treated with potassium iodide (1–10 mM) to eliminate NC1 hexamer crosslinks were used for basement membrane isolation. To test halide dependency, we established halide-free conditions by washing extensively (at least five times) with 10 mM sodium phosphate pH 7.4. To try to extract or inactivate endogenous basement membrane peroxidase activity, we extracted the matrix preparation twice with 2 M guanidine-Cl, 50 mM Tris-Cl pH 7.5 and 10 mM EDTA-Na pH 8 followed by extensive washing with 1× PBS. Basement membrane was resuspended in the desired buffer with or without cofactors and inhibitors to examine *in vitro* NC1 crosslinking under various conditions. Basement membranes were collagenase solubilized to delineate collagen IV NC1 sulfilimine crosslink formation with SDS-PAGE and Coomassie blue staining.

5

Azide labeling and click chemistry biotinylation of labeled proteins. PFHR-9 membrane was isolated, washed extensively and resuspended in 1× PBS. Azide (0–10 mM) and 1 mM H_2O_2 were added and allowed to react for 1 h at 37 °C. The matrix was pelleted, washed extensively with 1× PBS and solubilized with 1× PBS plus 2% (w/v) SDS. Solubilized proteins were reacted with 100 µM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (Anaspec), 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (ThermoFisher Pierce), 1 mM cupric sulfate and 100 µM biotin alkyne (PEG_4 carboxamide-propargyl biotin; Life Technologies) for 1 h at 37 °C. Click chemistry reactions were quenched with 1 mM 3′-azido-3′-deoxythymidine (Sigma). For avidin-HRP detection, samples were electrophoresed under reducing conditions, transferred to nitrocellulose membranes and probed with streptavidin-HRP according to manufacturer instructions (ThermoFisher Pierce). To isolate biotinylated proteins, we precipitated click reaction products with two volumes of cold acetone to remove reactants, washed them with 70% (v/v) acetone and then resolubilized them in 1× PBS plus 2% SDS. Biotinylated proteins were captured with streptavidin-agarose beads (GE Life Sciences) and released with boiling for 15 min in SDS-PAGE sample buffer containing 50 mM dithiothreitol.

Purification of recombinant human peroxidasin. HEK293 cells stably transfected with the human peroxidasin coding sequence[27] were grown to confluency, and the medium was changed to serum-free DMEM/F12 plus 5 µM hematin plus 5 mM sodium butyrate. After 48–60 h, medium was harvested, protease inhibitors were added (0.5 mM PMSF, 1 µg ml⁻¹ leupeptin, 1 µg ml⁻¹ pepstatin and 10 mM EDTA-Na), and proteins were precipitated with 40% (w/v) ammonium sulfate (226 g l⁻¹). Precipitated protein was resuspended at ~1/50 of the original medium volume in 0.3 M sucrose, 0.1 M NaCl and 20 mM Tris-Cl pH 8.5; dialyzed against the same buffer; and chromatographed on a Mono-Q anion exchange column (GE Life Sciences). Enzymatically active fractions were pooled, precipitated to ~1/500 the original medium volume of 50 mM NaCl, 10 mM sodium phosphate pH 7.4 and 3 mM hexadecyltrimethylammonium chloride and were dialyzed against the same buffer. The dialyzed protein was further purified using ultracentrifugation on a 5–20% (w/v) sucrose gradient. Active fractions were pooled and concentrated to a final concentration of 0.25–0.5 mg ml⁻¹ of purified human peroxidasin.

HEK293 cell overlay on uncrosslinked collagen IV networks. PFHR-9 cells were grown in the presence of 50 µM phloroglucinol to produce noncrosslinked collagen IV. Basement membrane was isolated on plates using a modification of a previously published protocol[46]. To inactivate endogenous crosslinking activity, the basement membrane was treated with 4 M guanidine-Cl plus 50 mM Tris-Cl pH 7.5 for 15 min and then washed 5 times with 1× PBS. In the first set of experiments, HEK cells stably transfected with human peroxidasin were compared to wild-type HEK293 cells. In follow-up experiments, HEK293T cells were transiently transfected with human peroxidasin coding sequence[27], mouse myeloperoxidase cDNA (Origene), mouse lactoperoxidase cDNA (Origene) or empty vector (pCDNA-V5-His-TOPO without insert) using Lipofectamine LTX per manufacturer's instructions (Life Technologies). In both sets of experiments, cells were plated on PFHR-9 basement membrane in the presence of 5 µM hematin and 5 mM sodium butyrate. Plates were incubated for 24–48 h, and collagen IV was analyzed for NC1 crosslink formation.

Preparation of HOCl and HOBr solutions. Standard techniques were used to prepare HOCl and HOBr. Further details are provided in **Supplementary Methods**.

Measurement of hypohalous acid production by peroxidases. Hypohalous acids were trapped as stable taurine haloamines, which oxidize tetramethylbenzidine to yield a colorimetric measure of hypohalous acid concentration and production[17]. Further details are outlined in **Supplementary Methods**.

MS and identification of sulfilimine-crosslinked peptides. We used a modification of previously described methods[4]. Details are provided in **Supplementary Methods**.

Drosophila biochemistry and genetics. *Drosophila* collagen IV NC1 hexamer was essentially purified as described for PFHR-9 cells. Standard genetic techniques detailed in **Supplementary Methods** were used.

Statistical analysis. Statistical analysis was conducted using GraphPad Prism version 5.04 (GraphPad Software). Comparisons between two groups used two-tailed unpaired Student's *t*-tests, whereas multiple group comparisons were conducted using analysis of variance followed by Tukey's *post hoc* comparisons between specific groups.

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Acknowledgments

Author contributions

G.B. conducted, designed and analyzed data from the PFHR-9 cell culture experiments, purified collagen IV NC1 hexamers from *Drosophila* and conducted western blotting experiments on *Drosophila* mutants. C.F.C. conducted mechanistic experiments involving hypohalous acids and peroxidasin. R.M.V. conducted MS and analysis. L.I.F. prepared *Drosophila* materials, and C.K.-C. performed *Drosophila* genetics and confocal microscopy. I.A.E.-T. performed overlay experiments involving peroxidasin and other peroxidases. M.R. isolated collagen IV NC1 hexamers and sulfilimine-crosslinked peptides for further analysis. J.-S.K. isolated human peroxidasin expressing HEK293 stable cell lines, and V.P. established the PFHR-9 cell culture system for these studies. L.I.F. generated *Drosophila* mutant larvae, antibodies and protein reagents. L.I.F., J.H.F. and B.G.H. designed the study and wrote the paper along with G.B. All authors discussed the results and commented on the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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