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Tuning in to epigenetic cross-talk

Chemical modifications to DNA, histones and RNA make changes happen. Scientists are exploring ways to track these modifications and how they interact.

By Vivien Marx

Fast changes are an everyday matter for cells. Among the events that enable this flexibility are chemical modifications to DNA, to the histone proteins around which DNA is wrapped, which make up the nucleosome, and to RNA.

Histone tails protrude from the nucleosome and can be chemically 'decorated' by the addition of chemical groups, such as methyl groups. The DNA itself can be methylated, too, most frequently at CpG islands, which are stretches of the genome particularly rich in cytosine–guanine dinucleotides. To capture the dynamics of epigenetic changes, scientists have many approaches that they are now tooling to eavesdrop on the cross-talk between such changes, such as histone modifications acting on one another or DNA methylation–histone interactions. As they map such interactions, some classic divides melt away.

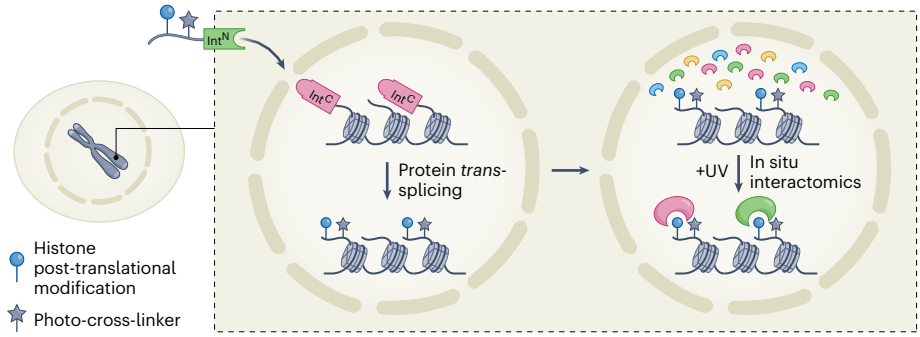
Chromatin structure and its histone proteins "were once thought of as static, non-participating structural elements," note C. David Allis, who recently passed away, and Brian Strahl, then both researchers at the University of Virginia[1]. But, they wrote, histones are actually integral and dynamic components of the machinery that regulates gene transcription. The 'histone code' describes how a diverse set of histone modifications can change chromatin structure and influence both histone–DNA and histone–histone contacts.

Princeton University researcher Tom Muir calls Allis a close friend with whom he collaborated for years. Not everyone agrees on the extent of a histone code, but Muir agrees that chromatin structure, including DNA and histone modifications, matters fundamentally for all DNA transactions. Allis helped to propel this area through "his own incredible research and by being a thought-leader and unwavering champion of the field." He drew many scientists to this field, says Muir.

The term "histone code" is one that Peter Laird, of the Van Andel Research Institute in Grand Rapids, Michigan, says he avoids. But histone modifications indeed inform the transcriptional machinery about which DNA locations are accessible and when and where modifications such as DNA methylation take place. "All of these mechanisms talk to each other; there's this cross-talk," says Laird.

Bait and trap

The Muir lab lays chemical traps[2,3] to capture the binding partners of histone-based post-translational modifications, or hPTMs. Their chemical proteomic approach involves photo-cross-linking followed by stable isotope labeling of amino acids (SILAC)-based mass spectrometry.



When laying chemical traps to capture epigenetic dynamics, the Muir lab uses inteins to assess proteins interacting with histone post-translational modifications.

They seek to avoid issues that arise with other methods. Chromatin immunoprecipitation mass spectrometry, or ChIP-MS, can fail because a given histone modification and a protein 'reading' it can hide the protein epitope from the antibody. When integrating photo-cross-linkable amino acids into the proteome, it's hard to assess interacting PTMs.

Muir and his team can assemble chemically modified chromatin in situ into which they insert chemically defined hPTMs and cross-linkers. Then, with mass spec, they assess the proteins interacting with this PTM. The team leverages protein splicing with inteins, which are protein segments that can ligate into new proteins. They express the histone fused to one intein fragment in cells and it is incorporated into the cells' chromatin. Next, nuclei are isolated from these cells and the scientists perform protein *trans*-splicing and crosslinking. The process brings the two intein protein partners together, and the modified histone ends up being assembled into the chromatin.

UV light cross-links and traps the proteins bound to the modified histone tail, which are then characterized with SILAC-based proteomics. With this approach, the team can characterize the interactome of this synthetic hPTM, qualitatively and quantitatively, and thereby explore the physiological impact of established and new hPTMs. Some proteins may be depositing, or 'writing', epigenetic marks; detecting, or 'reading', them; or removing them, known as 'erasing'.

Along with David MacMillan's lab group at Princeton and others, Muir and his team continue this chemoproteomic stalking of protein–protein interactions with tailored labeling approaches related to histone modifications. They developed a proteome-scale method with photocatalytic proximity labeling. This can be used, for instance, to explore thousands of histone modifications found in the nuclei of cancer cells and assess the effect of cancer mutations and epigenetic drugs on chromatin interactomes. "We are very excited about the work," says Muir.

The team applies chemoproteomics with tandem mass tags and uses protein *trans*-splicing to install iridium-centered photocatalysts onto target proteins. Using blue LEDs in the presence of a biotin-diarizine probe, they characterize the interactome: proteins in a roughly 10-nm radius of the photocatalyst.

This project, says Muir, reveals that histone mutations in the acidic patch of the nucleosome can affect the levels of de novo methylation. Among other experiments, the researchers assessed a DOT1L methyltransferase inhibitor to explore how its substrate, H3K79, affects the chromatin microenvironment, in particular with a view to depleting H3K79 methylation at that location. Modification of H3K79—a lysine at position 79 of the histone named H3—plays a role in development and shapes cells differentiation. Methylation often represses gene transcription; in this instance, however, methylation sets transcription in motion.

Muir says that projects of this type work well with collaborators. Generally, they require expertise in synthetic chemistry, protein engineering, chromatin-related biology and biochemistry. In his group, this combination of expertise has been hard won over the years, he says, and "we are fortunate in my lab to have these bases pretty well covered." Trainees need to invest the time to gain familiarity with this scope of techniques "irrespective of whether you come at this as a biologist or a chemist."

Interactional flash-freeze

Using biochemical methods and cryoelectron microscopy, or cryo-EM, Evan Worden, as a postdoctoral fellow with Johns Hopkins University researcher Cynthia Wolberger and her team, determined how one histone modifier shapes a second modification and, in the process, the histone changes its conformation[4]. Worden has since started his own lab at the Van Andel Research Institute.

Cross-talk and conformational changes occur to make H3K79 more accessible for cellular processes. This histone methylation relies on ubiquitination of histone H2B lysine 120, and DOT1L methyltransferase methylates the histone. Worden finds it exciting how histones move and adapt to different effectors. He points out how, for instance, University of California San Francisco researcher Geeta Narlikar and her team use nuclear magnetic resonance methods to assess such events.

With a nucleosome made up of DNA and eight histone proteins, "there's hundreds of different modifications that can be added or subtracted," says Worden. And just about a hundred have been identified. "We're nowhere near or nearly close to understanding all of the intricacies of this substrate," he says. Ubiquitin, the subject of his PhD thesis, is a complex molecule, but once he took note of chromatin and histone modifications, "I thought, wow, this nucleosome is even more complicated."

In Worden's lab, biochemistry is partnered with cryo-EM. First comes biochemistry and obtaining the entire protein, and it can take months, sometimes a year or more, to get the biological systems to work, he says. He does not want trainee projects to just go from structure to structure, but rather wants the trainees to use structures to study biological questions.

Cryo-EM experiments oriented toward histone modifications show that many enzymes and proteins involved bind weakly or transiently. "You're going to capture that whole distribution of bound and unbound states," he says. The picture is complex: perhaps only a small percentage of the data will represent particles in a single state. And there may be a conformational continuum rather than one distinct state. Researchers can group particles and interpolate between captured states to assess how complexes might be moving.

Successful cryo-EM structures are those gained from methods that best biochemically isolate and stabilize a bound conformation, says Worden. This conformation might catch a 'reader' in the act of recognizing a specific methyl-lysine. With a mutated substrate,

lysine might be binding more strongly to the methyltransferase that 'writes' the methylation. A structure might be an 'eraser' in the process of removing methylation. Or a conformation can involve a variant that is di- or trimethylated.

Labs have been able to capture the locations of epigenetic marks, and learn whether they activate or silence gene transcription and whether they co-localize with other proteins; but what is somewhat of a "dark secret of epigenetics," says Worden, is understanding a mark's impact downstream. "What are the protein factors that are linking the mark and its position with its actual downstream function?"

A team at Hong Kong University set out to decode a histone modification by looking at methylation 'readers' of H3K79. Using a photoaffinity probe and cryo-EM, they found that a protein called menin is a reader[5]. What Worden likes about this work is that labs have accumulated much evidence on how H3K79 methylation shapes gene expression, during normal development and also in cancer, but what's remained unclear is how the cell interprets this methylation. "There was this huge, blank space in the middle between what we know about the modification and where it is and what we know that it's doing," he says. By identifying the protein that binds and recognizes this methylated lysine, the Hong Kong team filled in this blank space.

The Worden lab group collaborates with the lab of Van Andel's Peter Jones, with its long history in epigenetics, especially DNA methylation. Together they are exploring DNA methyltransferases, also with a view to structure. Beyond this project, the Worden team works on bacterial histones looking at host–pathogen cross-talk. Classic textbooks may state that only eukaryotes have histones, but some viruses do, too, and bacteria have histone-like proteins that bind to DNA and proteins that interact with human chromatin. There's a melting pot of different features shared between domains of life, he says. Cross-talk between a bacterial pathogen and eukaryotic cell chromatin takes place during infection when proteins interact with and modify human chromatin. This "very interesting push and pull and interaction between bacteria and hosts" is rather underappreciated, he says. At least eight bacterial human pathogens have proteins that can modify human histones and some can modify human DNA, too.

Getting to high throughput

Early in his career, Laird felt that DNA methylation seemed "very boring—descriptive and correlative, but not very interesting as a driving mechanism." Then, as a postdoctoral fellow in the lab of Rudolf Jaenisch at the Whitehead Institute for Biomedical Research, he learned of work characterizing the difference between cancer cells and healthy cells in terms of the distribution of DNA methylation content[6,7].

En Li, also in the Jaenisch lab, then managed to knock out the DNA methyltransferase gene *Dnmt1* in mice[8] and showed that the knockout was embryonically lethal, meaning that this is an important gene, says Laird. Laird built on this work to assess DNA methylation in a mouse model of cancer and was surprised by its big impact. When he and his collaborators were able to prevent polyp development in a mouse predisposed to intestinal polyps, he realized how much methylation matters in tumor formation and decided to devote his career to DNA methylation in human cells.

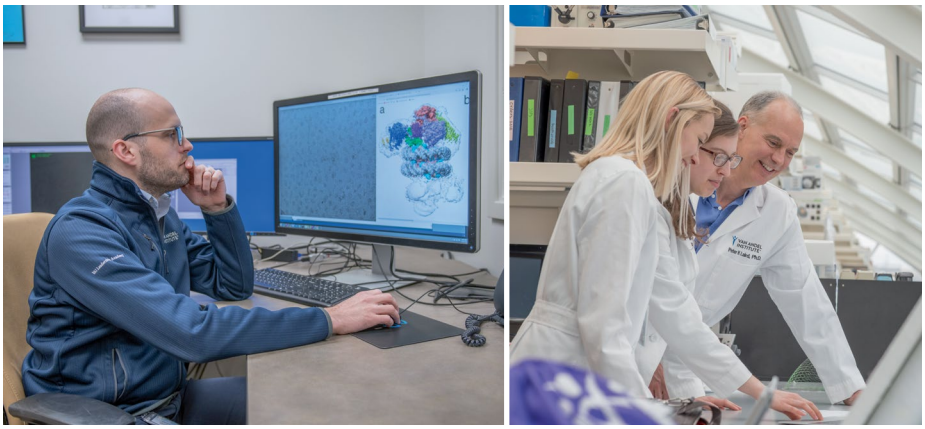
Much of his work has been on high-throughput analysis of DNA methylation, including highly sensitive detection also in cancer. Early in his career, scientists were focused on gene expression arrays and the Human Genome Project, and most "totally ignored DNA methylation as a field," says Laird. Funding for work in this area was tough to find. He and others began a pilot project that became part of The Cancer Genome Atlas.

Last year, Laird and his Van Andel colleague Hui Shen, along with teams at Children's Hospital of Philadelphia, the University of Pennsylvania and two companies, Illumina and FOXO Technologies, developed an array with which they were able to disentangle changes such as those related to aging and tumor development[9]. The Infinium Mouse Methylation BeadChip has nearly 300,000 CpG probes, with which one can query hundreds of thousands of CpG sites in parallel and perform DNA methylation analysis in mouse in high throughput.

The workflow involves automated sample processing and can get experimenters results from hundreds of samples within a few days and in a form, says the team, that is cheaper and easier to analyze than with whole-genome bisulfite sequencing. In bisulfite conversion, unmethylated cytosine is deaminated and converted into uracil, which enables detection of methylated cytosines. Whole-genome bisulfite genome sequencing delivers a genome-wide look at all CpGs that can be aligned to the genome. The process has lower throughput than the BeadChip, he says, but the high CpG coverage delivers deeper data on, for example, interactions between DNA methylation and histone modifications.

Single-cell and spatial thinking

Single-cell whole-genome bisulfite sequencing delivers high-resolution analysis of DNA methylation in samples, says Laird, but its coverage can be lower than that of bulk-based whole-genome bisulfite sequencing. He and others, for example the Salk Institute's Joseph Ecker, are keen on single-cell and spatial analysis, yet single-cell DNA methylation analysis remains challenging, says Laird. Published methods routinely get 5–10%, sometimes 20%, genome coverage per cell, which is mainly due to the way DNA is degraded during bisulfite conversion. Before sequencing



At the Van Andel Research Institute, Evan Worden (left) and his team use biochemistry and cryo-EM to assess histone modifications. Peter Laird and his group, here Nicole Vander Schaaf (middle), now on the faculty at Olivet Nazarene University and Jamie Endicott, now at Altos Labs, focus on high-throughput DNA methylation analysis. An increasing number of their questions are about DNA–histone cross-talk.

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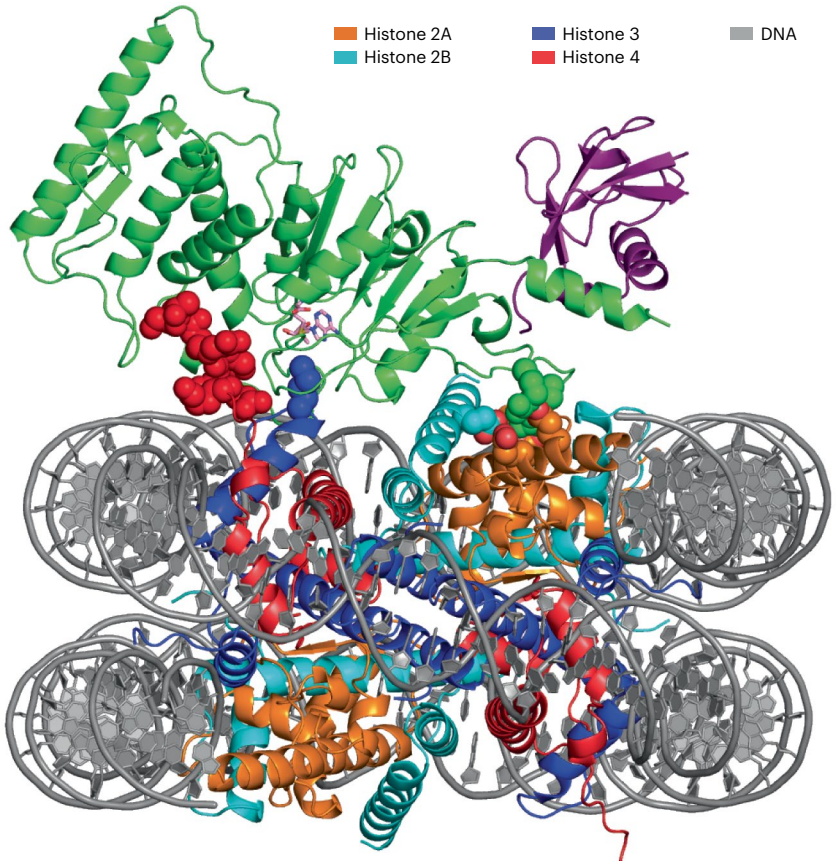

Epigenetic changes are dynamic, and investigating them involves assessing interactions between changes, such as histone modifications acting on one another or DNA methylation–histone interactions.

library construction can begin, bisulfite must be removed. "DNA cleanup on very, very small quantities of DNA tends to lose parts of the genome that you never get back again in library construction."

He and his team aim to increase the coverage of single-cell whole-genome bisulfite sequencing. Preliminary results indicate that their method leads to over 50% coverage per cell, he says. Avoiding DNA loss will lead to more complex sequencing libraries, which helps, for instance, when spatially reconstructing epigenomic profiles in tumors.

A trend Laird finds noteworthy with the emergence of large methylome atlases is the way they expand and deepen a view of a sample. Groups went from studying individual CpG profiles to looking at more CpGs, albeit in small sample numbers. These days scientists advance both cell numbers and the number of CpGs scanned in each cell's genome at the same time.

Given the number of cells in the human body, and considering that around 50 million CpGs can be methylated or unmethylated, a back-of-the-envelope calculation that disregards strand-specific modification reveals that "the number of permutations that are possible is 2 to the power 50 million, approximately, which is larger than the number of atoms in the universe," says Laird.

Not all possible permutations will arise, not all are equally informative, but the numbers indicate how much information can be embedded in DNA methylation patterns. And methylation patterns likely vary from one person to the next.

The field has progressed, says Laird, "but I think we're just at the start of doing a very systematic, detailed profiling of this." Just as with The Cancer Genome Atlas, which amassed multiple data types from the same samples, he imagines what could be in epigenetics were it routinely possible to do the same with DNA methylation profiles, gene expression data, and chromatin accessibility and histone modification data. "Then we would have a better understanding of the biology of gene regulation and differentiation potential and how things are regulated."

"All of these mechanisms talk to each other; there's this cross-talk," says Peter Laird.

A number of groups have published DNA methylation atlases, for example for the mouse[10] and human[11] methylomes, and labs are working on, for instance, ways to capture methylation dynamics through development phases. In recently published work, Yale University researcher Rong Fan, Gonçalo Castelo-Branco at the Karolinska Institutet and members of their and other labs explore epigenetic co-profiling of mouse brain tissue[12]. They use published single-nucleus RNA sequencing, or snRNA-seq, data and an atlas of the mouse brain developed by snATAC-seq, a method to identify chromatin that is relatively accessible for transcription. By integrating the datasets, says co-lead-author Castelo-Branco, they projected the cell populations identified in the atlases onto the tissue sections to place them spatially.

With the co-profiling methods the team developed, they could map and integrate epigenomic and transcriptomic data from the same tissue section at nearly single-cell resolution. For this purpose, they developed ATAC-RNA-seq and spatial CUT&Tag-RNA-seq, which deliver genome-wide chromatin accessibility or histone modification data alongside the whole transcriptome, all from the same tissue section. ATAC-RNA-seq is a spatial assay for transposase-accessible chromatin that uses sequencing; CUT&Tag RNA-seq is a spatial assay of cleavage under targets and tagmentation with RNA sequencing. Their datasets can be browsed on the UCSC Cell and Genome Browser and via the AtlasXplore platform. Both resources and other single cell transcriptomic and epigenomic datasets are on Castelo-Branco's lab site.

For this work, they expanded on their previously developed method for spatially barcoding biomolecules in tissue, which they call DBiT-seq. DNA barcodes are delivered to a tissue slide surface with the help of a microfluidic chip designed with multiple channels. This process yields a mosaic of barcoded 'tissue pixels', each with its own distinct barcode combination. Castelo-Branco says he has set

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up DBiT-seq in his lab in Sweden, and Rong Fan has set up a company, AtlasXomics, that sells the components needed for DBiT-seq.

In the future, the hope is that by combining chromatin accessibility, histone modification and transcriptomic data, one could obtain a more comprehensive view of a tissue's gene regulation network. He and his colleagues are working in that direction, says Castelo-Branco. His recent lab member Marek Bartosovic has started his own lab at Stockholm University, and Yanxiang Deng, a former member of Fan's lab at Yale, has started a lab at the University of Pennsylvania.

Epi-editing

At this year's Third International Summit on Human Genome Editing, Angelo Lombardo described epigenetic editing techniques from his lab at the San Raffaele Telethon Institute for Gene Therapy (SR-Tiget) in Milan, Italy. SR-Tiget is a joint venture between The Telethon Foundation, a charity, and the San Raffaele Scientific Institute. Lombardo has a second affiliation with Vita-Salute hospital, which is affiliated with Vita-Salute San Raffaele University. He and his colleague Luigi Naldini have long explored both gene silencing and activation[13]. Naldini directs SR-Tiget, and they co-founded Epsilen Bio, now a subsidiary of Chroma Medicine, for which Lombardo consults.

In the Lombardo lab project, 'epi-editing' is done by repurposing a silencing machinery inherent to human embryos. The scientists identified components in this process and built a method to silence targeted genomic sites in somatic cells in vitro and, most recently, in vivo in mice. This approach is far from clinical application but is currently a useful research tool.

The platform uses components of what the embryo uses to permanently silence endogenous retroviruses, says Lombardo, for example Krüppel-associated box (KRAB)-containing zinc finger proteins. Other proteins are involved too, such as DNA methyltransferases. The 'epi-editors' the team built have a DNA-binding domain—which can be a zinc finger protein; a transcription-activator-like effector nucleases, or TALEN; or a deactivated Cas9—that does not cut but does bind DNA. To this, they append an epigenetic effector domain from a naturally occurring effector.

The KRAB domain binds and silences well, says Lombardo, but the modification is transient. Long-term silencing requires other proteins, including DNA methyltransferases and their cofactors. After achieving epigenetic silencing in vitro, more recently they did so in vivo in mice. They targeted the *PCSK9* gene in mouse liver to affect a protein involved in cholesterol metabolism. The system can perform multiplexed silencing and act on DNA and histones, says Lombardo. In principle, multiple genes in a cell can be targeted, and without the translocations associated with cleavage by classic gene-editing tools. Among other aspects, when they combined epigenetic editing with gene editing, the scientists tinkered with the guide RNA length to achieve this silencing effect. In their tests of different DNA-binding proteins, they found that zinc finger proteins outperformed deactivated Cas9.

The interplay between histone PTMs and DNA modifications "has a long runway with much more yet to be discovered," says Tom Muir.

Cellular cross-talk, lab cross-talk

To date, says Muir, exchange between those working on DNA modifications and histone modifications such as methylation has been limited, "but hopefully that will change." His group has collaborated with the Columbia University lab of Laura Landweber on the role of adenine methylation in the ciliate *Oxytrichia trifallax*. They synthesized complete, epigenetically defined *Oxytrichia* chromosomes and assessed the impact of DNA methylation on nucleosome positioning, says Muir.

Speaking more generally about the interaction between DNA methylation and histone PTMs, Muir says that "this interplay between histone PTMs and DNA modifications has a long runway with much more yet to be discovered."

Worden, whose lab focuses more on histone modifications, collaborates with Van Andel's Peter Jones, which focuses on DNA methylation. It's perhaps surprising, says Worden, to generally find little scientific cross-talk between researchers working on DNA methylation and those working on histone modifications, but perhaps links still need to be firmly established.

Castelo-Branco sees himself more as a histone modification person, but "we are getting more and more interested in DNA methylation." He collaborates with his Karolinska colleague Maja Jagodic in this area. Approaches such as the ones he and his colleagues developed to enable spatial transcriptome and epigenome profiling in parallel—and, more generally, the emerging multi-omic, epigenomic, single-cell and spatial technologies—will help foster connections and provide an integrated view of epigenetic information and its interplay. Says Castelo-Branco, "I think we are heading to very exciting times, where we will get much clearer insights on epigenetic mechanisms in the context of development and disease."

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