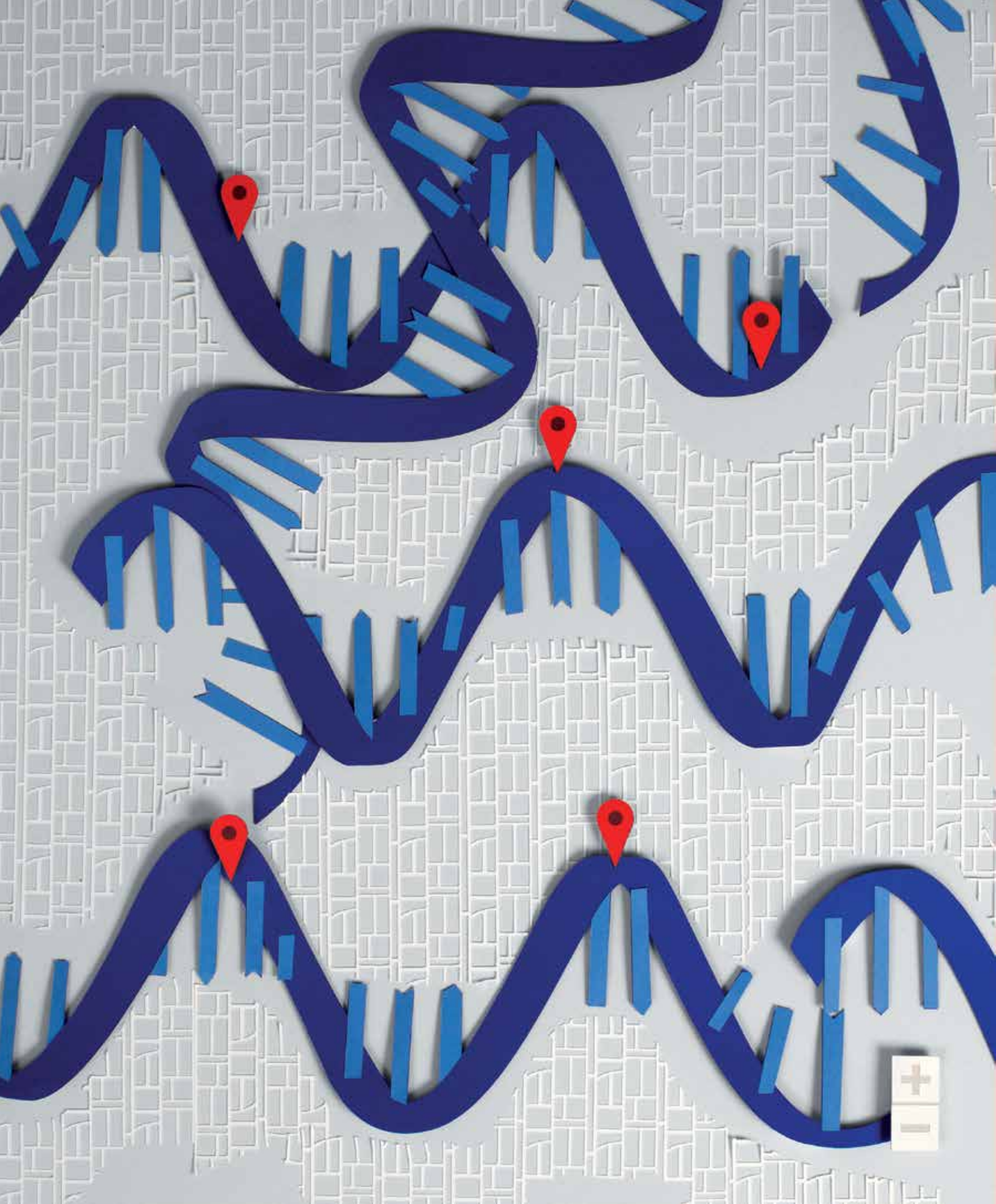

New Techniques Reveal How the Immune System Stays Nimble

Map Quest

By Nancy Averett

Denis Burkitt, an Irish surgeon with a glass eye and a missionary's zeal, traveled 10,000 dusty miles in a cantankerous Ford station wagon across equatorial Africa in the 1950s. His aim was to map the boundaries of an aggressive cancer that caused grapefruit-sized facial tumors forcing children's eyes to protrude, their cheeks to bulge, and their teeth to fall out.

Through his travels, Dr. Burkitt discovered that the fatal malady was common in lush, forested regions and rolling savannas where mosquitoes and the malarial parasite they spread are endemic. During the next decade, he integrated his observations with those of others to realize that where DDT-spraying programs controlled mosquito populations—as on the island of Zanzibar, off the Tanzanian coast—both malaria and the cancer were almost nonexistent.

Sixty years later, African children are still dying from what's now known as Burkitt lymphoma—the cause of death for 3,000 children each year and the most common pediatric cancer in sub-Saharan Africa—and investigators are still trying to piece together its relationship to malaria.

Scientists are not without clues: Among Burkitt lymphoma patients, a particular chromosomal mutation—known for its potential to promote unregulated cell growth—is common. And that genetic glitch nestles within the immune system's B cells—the same cells that produce the antibodies needed to fight off pathogens, the same cells that malaria kicks into overdrive.

Many investigators suspect the malaria pathogen reprograms the immune system's antibody manufacturing process to produce the precancerous mutation. The details, though, remain a mystery. "The correlation has been reported for many years," says Columbia malaria expert David Fidock, PhD. "But no one had the faintest idea what connected the two."

This fall, Uttiya Basu, PhD, will embark on an investigation that might finally reveal the missing link. Like Dr. Burkitt, Dr. Basu boasts a detective's meticulous attention to detail. And like Dr. Burkitt, the microbiologist builds maps related to disease. But unlike Dr. Burkitt, whose maps spanned vast geographical regions, Dr. Basu will focus on microscopic areas deep within our cells, inhabited by long-ignored actors in the human genome known as noncoding RNA—the foremen of

the manufacturing sites—where he suspects antibody production sometimes goes bad. "If we can understand how malaria and Burkitt lymphoma are related," Dr. Basu says, "we could attack them simultaneously."

Dr. Burkitt moved to Africa in 1946. His dream of becoming a surgeon had been cut short by the loss of his right eye during a childhood scuffle, but he harbored another passion—missionary work. So he took a post at Uganda's Mulago Hospital. It was there in 1958 that he encountered a 5-year-old boy with a jaw tumor and extensive facial disfigurations. A few days later, while visiting a hospital in a nearby town, Dr. Burkitt saw another child with a similar tumor. When he returned to Mulago, he began digging through records; 29 other children had been admitted with comparable tumors.

Uttiya Basu, PhD

JÖRG MEYER

A few years later, Dr. Burkitt sent tumor samples to Michael Anthony Epstein, who thought a virus might be causing the children's jaw cancers. Dr. Epstein found virus particles for what is now known as Epstein-Barr virus (EBV) in some of the tumor cells, providing the first evidence that endemic Burkitt lymphoma cells are often infected by EBV. Nevertheless the role of this virus in the pathogenesis of Burkitt lymphoma remains controversial.

Whether from Epstein-Barr or malaria, pathogens only make us sick if they dupe our immune system. To detect such hazards, the immune system conducts constant surveillance—and it only works if we have the right antibodies. While genetic mutation can quickly go off the rails, the process serves a vital, adaptive function within the immune system. "We encounter more pathogens in our lifetime than the number of stars we can count," says Dr. Basu. "Each requires a perfect fit with an antibody to neutralize it, but we only have so many genes that can make antibodies."

To surmount that constraint, the immune system leverages a kind of guided mutation to shift DNA sequences within each antibody gene to create a rich diversity of antibodies.

Dr. Basu began studying those processes at Harvard in 2004 as a postdoc in the lab of immunologist Frederick Alt, PhD, a former member of the Columbia faculty. In Dr. Alt's lab, Dr. Basu homed in on activation-induced cytidine deaminase (AID), an enzyme whose presence during transcription seemed central to those guided mutation processes. "When I started working in Fred's lab, antibody diversity was new to me and it was very exciting," says Dr. Basu. "But in the last six to eight years, things have changed and how antibodies generate this diversity by genome rearrangement and mutagenesis has taken a new twist."

SENSE VS. ANTISENSE

For decades, biology's central dogma has been that DNA makes coding or messenger RNA (mRNA), mRNA makes proteins, and proteins drive our cellular functions. Since nonprotein coding RNA (ncRNA) does not appear in that litany, it has received relatively short shrift in the genomic medicine revolution. The disregard was once so pervasive, it even informs the molecule's alternative eponym—"antisense" RNA (sense RNA being, of course, mRNA).

With limited computational and analytical tools and a dominant paradigm intent on DNA and mRNA, scientists ignored ncRNA. Instead, they focused on transcription, the production line on which our genes make proteins. The manufacturing process begins when an enzyme attaches to a gene and uncouples the gene's DNA from its double helix shape—imagine opening a zipper. Next, the enzyme slides along one strand of the zipper, adding complementary nucleotides to create a strand of mRNA (a process termed "transcription"). Finally, when the enzyme reaches the gene's end, transcription stops and the freshly manufactured mRNA detaches and floats away to be translated on the ribosome and form a protein.

Think of AID, the enzyme Dr. Basu was investigating at Harvard, as a special foreman who only intermittently visits

the transcription factory floor. In its absence, DNA and RNA nucleotides match cytosine to guanine and adenine to thymine. But in its presence, the pairing process "deaminates," linking cytosine to uracil and creating a U:G mismatch.

At Harvard, Dr. Basu discovered the "switch" that activates AID in the immune system. Nature published the finding in October 2005. Little did Dr. Basu realize, when that highly acclaimed paper was released, just how much more there was to learn. AID creates the good mutations needed for antibody diversification but also introduces genomic alterations that can lead to chromosomal translocations including those found in people with Burkitt lymphoma.

The key genetic lesion detectable in 100 percent of Burkitt lymphoma cells, the chromosomal translocation involving the c-MYC oncogene, was identified in 1982 by Riccardo Dalla-Favera, MD, then an investigator at the National Cancer Institute. Now director of Columbia's Institute for Cancer Genetics, Dr. Dalla-Favera has published dozens of papers on the oncogene and its role in Burkitt and other forms of non-Hodgkin's lymphoma. His work has yielded new insights into the pathogenesis of human B-cell lymphomas and, in particular, the genetic lesions and biological mechanisms responsible for the development of these diseases.

Since joining the Columbia faculty in 2009, Dr. Basu has focused on how the immune system's B cells regulate AID—what is the quality control mechanism that allows most of us to benefit from AID-activated antibody diversification, and how does the process go wrong to create mutations and translocations like those seen in c-MYC? As would be expected, Dr. Basu was motivated and influenced by Dr. Dalla-Favera's earlier studies. In a 2011 Cell paper, Dr. Basu and his lab showed that a large cellular complex called the RNA exosome recruits AID to modify DNA during transcription.

P&S researchers use an array of options—biomolecular assays, whole exome sequencing, whole transcriptome sequencing, and genetically engineered mouse models—to disclose the cellular pathways disrupted by the genetic mutations common to lymphoma.

Until Dr. Basu's discovery of the RNA exosome's role in guided mutation, scientists thought of the exosome's primary job as degrading ncRNA. Combined with his new insight into the role of the RNA exosome in antibody diversification, Dr. Basu and his team turned their attention to a new question: What is the relationship between noncoding RNA and AID?

The RNA exosome works so fast to degrade ncRNA that scientists cannot even see it happen. To investigate its role, Dr. Basu and his students engineered a knockout mouse with a nonfunctioning exosome. The knockout lets noncoding RNA pile up in the murine B cells, giving Dr. Basu and his team enough time to collect data on them. Data in hand, they

turned to quantitative computational scientist Raul Rabadan, PhD, and his postdoc, Jiguang Wang, PhD, to build a tool that would analyze those accumulated ncRNA.

To help the investigators visualize their data, Dr. Wang wrote algorithms to sort the various ncRNA and create a Google Maps-like browser. Users type in a gene name and the program spits back any ncRNA in that region. Color coding highlights ncRNA expression in both exosome-deficient mice and normal mice; by zooming in and out, scientists can get a local or global view of the genome. "Because we can actually map the ncRNA and study it globally, we can learn much more about it," says Dr. Basu. "There are long ncRNA, micro ncRNA, enhancer ncRNA, and promoter ncRNA—they come in many flavors."

LIKE SPACE EXPLORATION

The flavors Dr. Basu's lab was most interested in were those controlled by the exosome. But first they had to build their exosome-deficient knockout mouse, sequence its ncRNA, and hand off that information to Dr. Wang, a process that took nearly 12 months. In 2012, the data were finally ready. Now a research scientist at Regeneron Pharmaceuticals, Evangelos Pefanis'14 PhD, was a graduate student at the time. On one fateful February morning, Dr. Pefanis got to the lab early, opened his laptop, and began typing gene names into Dr. Wang's browser. Right away, he says, "I could see something interesting was going on." Moments later, when Dr. Basu walked in, Dr. Pefanis waved him over. "Hey, take a look at this gene," he said. "And take a look at this one and this one."

Dr. Basu stared at the screen as the graduate student clicked through the data. There they were, plain as day: strands of ncRNA spread across the genome, clustering around genes known to undergo bidirectional transcription, a unique regulatory mechanism that may play a role in switching genes on and off. There also were large quantities of ncRNA on the parts of antibody genes where DNA double-strand breaks happen to create the "good" mutations that diversify our antibody inventory. But there were also large quantities of ncRNA near five precancerous genes known to be erroneous targets of AID, including c-MYC, the proto-oncogene common among people with Burkitt lymphoma. The results, which were published in Nature in October 2014, were strong evidence: Antisense RNA plays a leading role in guiding mutations. The implications of the results were further discussed in a review article in Advances in Immunology published in May 2015.

"When you make a discovery like that," says Dr. Basu, "it's a bit like being the first to go to the moon, or Mars, or some other crazy planet."

Perhaps even more important than revealing the existence of that crazy planet, his team had assembled the tools to explore it. Now that they could poke around within the immune system's ncRNA, Dr. Basu's team created additional exosome-deficient mouse models to extend their reach.

In the new knockout mice, they found additional types of antisense RNA, including genetic sequences that activate gene transcription (called enhancers). These antisense RNA also tended to be adjacent to genes susceptible to both good and bad AID-induced mutations. They also found that without a functioning exosome quickly degrading the ncRNA as soon as its job was done, the presence of ncRNA set the stage for yet another type of mutation. When functioning optimally, Dr. Basu and his team concluded, the antisense RNA works like a matchmaker: It steps in to attract AID to various genes but then it better get lost; its mere presence can lead to inappropriate attraction of AID to genes, which will cause unwanted mutations.

Finally, because DNA exists in tertiary structures called chromosomes that are bunched and looped, Dr. Basu and his

New Celiac Risk Factor Identified

FOR PEOPLE WITH CELIAC DISEASE, ingesting gluten—proteins found in wheat, rye, and barley—triggers an autoimmune reaction characterized by severe gastrointestinal symptoms. An estimated 40 percent of the population has the gene variants associated with celiac disease, but only 1 percent of people with these genes will develop intestinal inflammation and damage after ingesting gluten.

Researchers from Columbia's Celiac Disease Center and the Department of Microbiology & Immunology—including Peter Green, MD, director of the Celiac Disease Center, and Sankar Ghosh, PhD, chair of microbiology & immunology—have identified a segment of RNA that, when suppressed, may contribute to celiac-associated intestinal inflammation. The findings point to a possible new risk factor for the disease.

In a series of experiments reported in Science, the team demonstrated that a long non-coding chain of RNA dampens the expression of celiac-associated genes. They then discovered that people with celiac disease had unusually low levels of this RNA in their intestines, suggesting that the reduced levels may contribute to the inflammation seen in celiac disease by turning off the normal regulatory pathway.

students wondered if some antisense RNA might influence that three-dimensional topography. The investigators knew that sometimes DNA loops shift and when they touch one another, the adjacency can alter gene expression. Might antisense RNA serve not only as a matchmaker among enzymes and genes, but also as a facilitator to the unique bunching and looping of DNA strands within a chromosome?

Indeed, when Dr. Basu's team started removing strands of antisense RNA depending on their location within the genome, they found that they could impede the capacity of a regulatory component of antibody genes to initiate guided mutation some 2.6 million nucleotides away from the material they had removed.

As they pondered these discoveries, which Cell published in May 2015, Dr. Basu and his students began to wonder: Could something other than a scientist—a pathogen or a virus, perhaps—also disrupt all of that carefully calibrated ncRNA machinery?

STUDYING MICE WITH MALARIA TO HELP BURKITT LYMPHOMA PATIENTS

This fall, Dr. Basu and his students will begin infecting mice with the malaria parasite—the first step in generating a new map to show how the mosquito-borne pathogen affects the ncRNA landscape. Based on earlier work from their team and other groups, the researchers have a hunch about the location within the genetic code where the mutation common to Burkitt lymphoma patients originates. To test their hypothesis, they are zeroing in on differences in that region between normal mice and infected mice. They expect that the pathogen will cause increases in ncRNA—as well as the increased potential for structural mutations—near a genetic component common among people with Burkitt lymphoma. Finally, they will experiment with methods to prevent the mutation by injecting mice with antibodies that inhibit AID expression. Eventually, they hope to develop drugs to cause that suppression.

Even as they dig deeper into the mechanisms at play in Burkitt lymphoma, Dr. Basu and his collaborators continue to refine their understanding of the various mechanisms by which lymphocytes operate within the immune system to monitor progression of cancer and onset of many other diseases. In November 2015, Cell Reports published Dr. Basu's paper, co-authored with Jianbo Sun, PhD, Dr. Rabadan, Dr. Pefanis, Dr. Wang, and other scientists, on the use of transcription analyses to identify a biomarker for B cells with immune suppression functions. Unpublished studies from Dr. Basu's laboratory done in collaboration with Columbia neurosurgeon Adam Sonabend, MD, have yielded identification of a novel mechanism of immune system-mediated clearance of cancer of the human brain.

Meanwhile, Dr. Rabadan and his systems biology colleagues have delved deeper into the association of Epstein-Barr virus with some cases of Burkitt lymphoma. By using next-generation sequencing, they were able to classify the RNA mutations associated with each of the three clinical forms of Burkitt lymphoma—endemic, sporadic, and immunodeficiency-related. In October, the journal PLOS Pathogens published their analysis. "When studying the mutational profile of endemic Burkitt tumors," wrote Dr. Rabadan and his co-authors, "we find recurrent alterations in genes rarely mutated in sporadic Burkitt lymphomas."

As technology has advanced, P&S investigators, including Dr. Dalla-Favera, Dr. Rabadan, and Laura Pasqualucci, MD, have leveraged an array of options including biomolecular assays, whole exome sequencing, whole transcriptome sequencing, and genetically engineered mouse models to disclose the cellular pathways disrupted by the genetic mutations common to lymphoma and Burkitt lymphoma in particular. That work promises to reveal new therapeutic targets to boost treatment options for the condition, which remains incurable in approximately 30 percent of patients.

Such endeavors would have been hard for Dr. Burkitt to imagine. When the missionary doctor encountered the disfiguring lymphoma that now bears his name, cancer treatments were in their infancy and nothing could be done for his young patients. "This is the gloomy part of medicine," he told a colleague. Little did he know that his own persistence in describing the disease—one that took him around the edges of his adopted land—might culminate decades later in another epic journey, only this time in the tiniest of landscapes.

Dr. Basu believes the work has implications far beyond Burkitt lymphoma, with the potential to help clinicians fight a wide variety of diseases at the molecular level. He looks forward to growth in the field as fellow investigators map ncRNA in cell types throughout the body, including neuronal cells and cardiac muscle cells, and continue to innovate and refine techniques for investigating how ncRNA operates. Says Dr. Basu: "There's all kinds of different noncoding RNA species just waiting to be discovered." ❖

Who's Who

- **Uttiya Basu, PhD,** associate professor of microbiology & immunology
- **Riccardo Dalla-Favera, MD,** the Percy & Joanne Uris Professor of Clinical Medicine, professor of microbiology & immunology, pathology & cell biology, and genetics & development, and director of the Institute for Cancer Genetics
- **David Fidock, PhD,** professor of microbiology & immunology and of medical sciences (in medicine/division of infectious diseases)
- **Laura Pasqualucci, MD,** associate professor of pathology & cell biology (in the Institute for Cancer Genetics) at CUMC
- **Evangelos Pefanis, PhD,** scientist, Regeneron Pharmaceuticals
- **Raul Rabadan, PhD,** associate professor of systems biology and of biomedical informatics and director of the Center for Topology of Cancer Evolution and Heterogeneity
- **Adam Sonabend, MD,** assistant professor of neurological surgery
- **Jianbo Sun, PhD,** former associate research scientist in microbiology & immunology
- **Jiguang Wang, PhD,** associate research scientist in biomedical informatics