ROBUST NEW TESTING REGIMEN FOR ANTIBODIES IN EPIGENETIC RESEARCH

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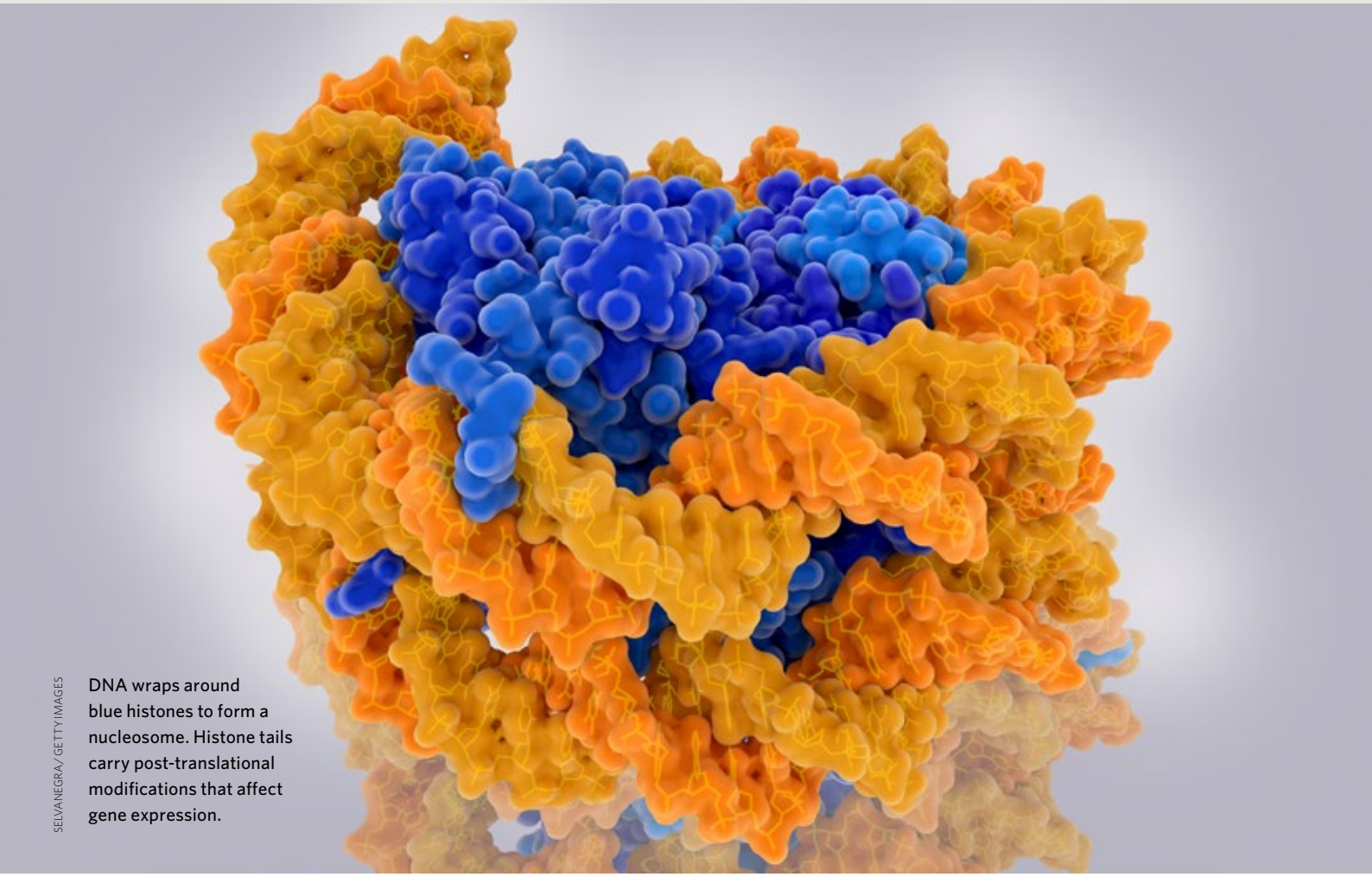

SELVANEGRA/GETTY IMAGES

DNA wraps around blue histones to form a nucleosome. Histone tails carry post-translational modifications that affect gene expression.

ROBUST NEW TESTING REGIMEN FOR ANTIBODIES IN EPIGENETIC RESEARCH

Lack of access to appropriate antibodies to DETECT POST-TRANSLATIONAL MODIFICATIONS is a recognized problem for researchers. Now, reagents with improved specificity are available.

Genetic research has broadened greatly since the Human Genome Project. Although it is the genes that encode proteins, there are important instructions in the rest of the genome about how those genes are expressed, and everything is decorated with epigenetic marks that add complexity.

One particular type of epigenetic modification concerns histones, the spool-like proteins that package DNA. DNA winds around the histones, and eight histones form a nucleosome — the fundamental repeating unit of chromatin. Histones carry chemical additions, known as post-translational modifications (PTMs), that have diverse effects such as activating or repressing gene transcription. Correctly identifying the genomic location and abundance of histone PTMs is the first step to understanding their downstream functions.

Histone residues can carry several types of PTM, such as methylation, acylation and phosphorylation. A particular residue can be unmodified or carry multiple modifications: there can be up to three methyl groups at a specific lysine or arginine residue, for instance. This complexity makes distinguishing PTMs difficult, especially as many forms at the same residue can be closely related — and there are comparable modifications at different locations.

A standard way to study histone PTMs is chromatin immunoprecipitation (ChIP), but these experiments have suffered from poor reproducibility. In the last few years, it has become clear that there are major blind spots in many of the common tests employed to validate PTM-specific antibodies for their ChIP capability[1,2]. Thermo Fisher Scientific and EpiCypher have partnered to use EpiCypher's proprietary

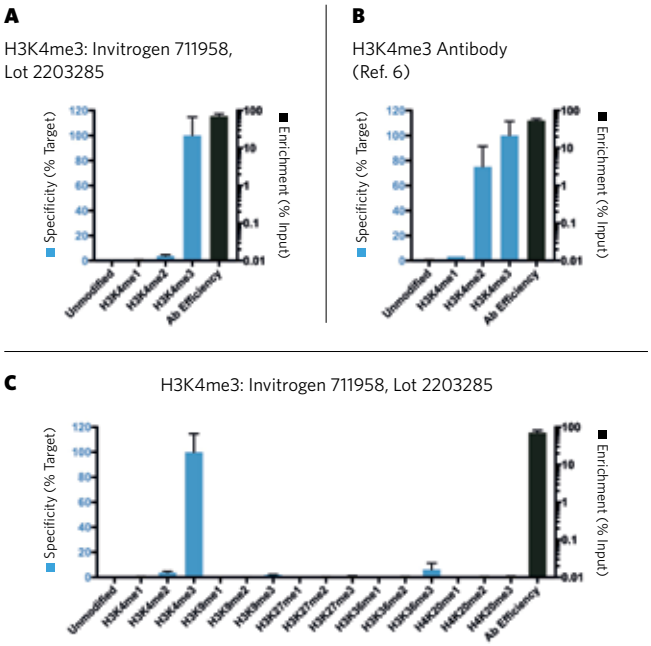

Figure 1. Comparison of two H3K4me3 antibodies using the SNAP-ChIP™ K-MetStat Panel.
Blue bars quantify the relative recovery of each unique lysine-methylated nucleosome using qPCR. The black bar represents the percentage of input target nucleosome recovered (antibody 'efficiency'). For the target PTM, the Invitrogen antibody (A) has much less cross-reactivity than the widely cited antibody (B). Further testing at other residues confirms its specificity (C).

technology, which accurately reflects histone biology *in situ*, to validate Invitrogen antibodies. This new technology has helped reveal the scale of the reproducibility problem, and is now being used to improve antibody validation up front, giving researchers confidence that they are using the right reagents for robust results.

The problem with peptides

Before being used in an experiment, antibodies should be validated in a functional assay that represents the intended application. Several approaches were tried for histone PTM antibodies, with peptide arrays the prevailing gold standard. Arrays challenge an antibody to distinguish its target modification among a large panel of similar but off-target modifications[3]. These arrays can carry hundreds of synthetic histone peptides, each bearing one or more distinct PTMs, to test the antibody against related marks, or in the context of neighbouring modifications.

However, peptide arrays ask the antibody to detect and differentiate histone PTMs in the context of linear peptides. As such, they are likely indicators of antibody specificity only under denaturing conditions, such as in immunoblotting. Histone PTMs in their native state on intact nucleosomes, as they are in ChIP experiments, have more complex configurations, which might contribute to off-target binding or reduced efficiency of target recognition.

PEPTIDE ARRAYS ARE LIKELY INDICATORS OF ANTIBODY SPECIFICITY ONLY UNDER DENATURING CONDITIONS.

It was clear that a better test was needed, one that more closely resembled the physiological reality of a ChIP experiment. In 2015, Alex Ruthenburg and colleagues at The University of Chicago used semi-synthetic nucleosomes to create such a tool[4]. In the approach a panel of fully defined nucleosomes, each with a specific histone PTM denoted by a DNA barcode, are added (or 'spiked') into a standard ChIP workflow for internal calibration[5]. The spike-in panel contains the target histone PTM as well as a variety of related modifications with the potential to cross-react, and is added before immunoprecipitation. After DNA isolation and downstream analysis (by qPCR or next-generation sequencing), the amounts of on- and off-target barcodes are compared to determine the antibody's specificity and efficiency.

The first nucleosome panel was put through its paces in a test of 52 commercial ChIP-grade antibodies, all described as being able to distinguish the three methylation states of the fourth residue (lysine, K) on histone H3. Each of these methyl forms (H3K4me1, -me2 and -me3) is thought to have a distinct biological function when it comes to chromatin regulation. The study compared antibody specificity, as determined using the nucleosome panel,

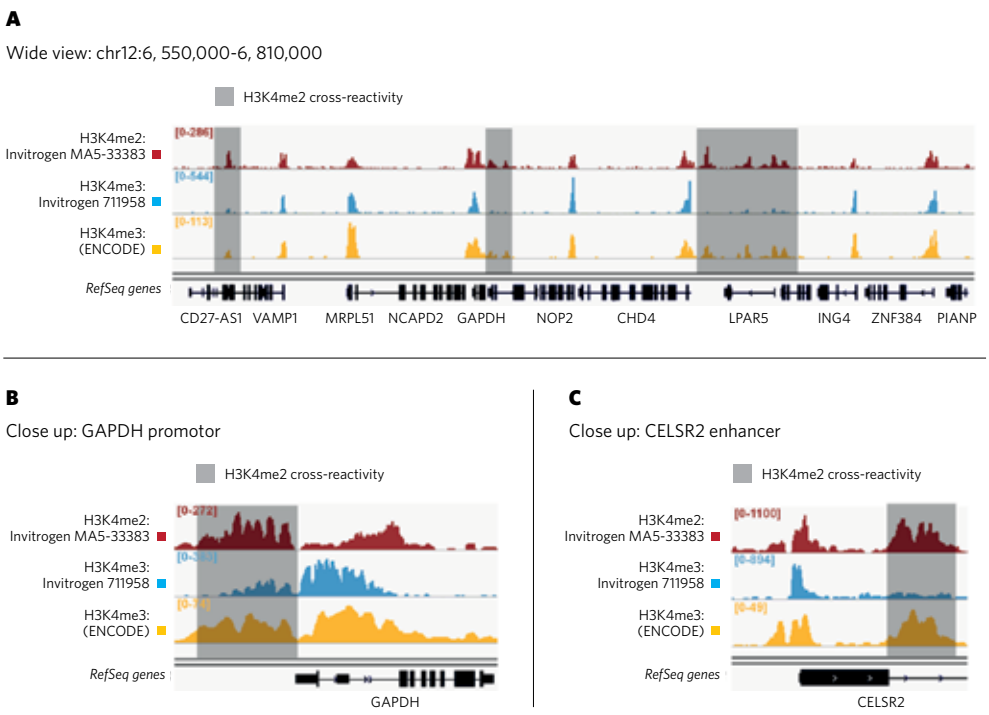

Figure 2. ChIP-seq results for H3K4me3.
Representative gene browser shots compare SNAP-ChIP verified Invitrogen antibodies for H3K4me3 and H3K4me2 with an H3K4me3 antibody from ENCODE (ref. 6). Peaks in the ENCODE antibody (orange) most closely resemble a combination of the H3K4me2 antibody (red) and the H3K4me3 antibody (blue) across a wide view (A), as well as up close in the GAPDH promoter (B) and at a putative enhancer region in CELSR2 (C). Gene browser tracks generated using the Integrative Genomics Viewer (Broad Institute).

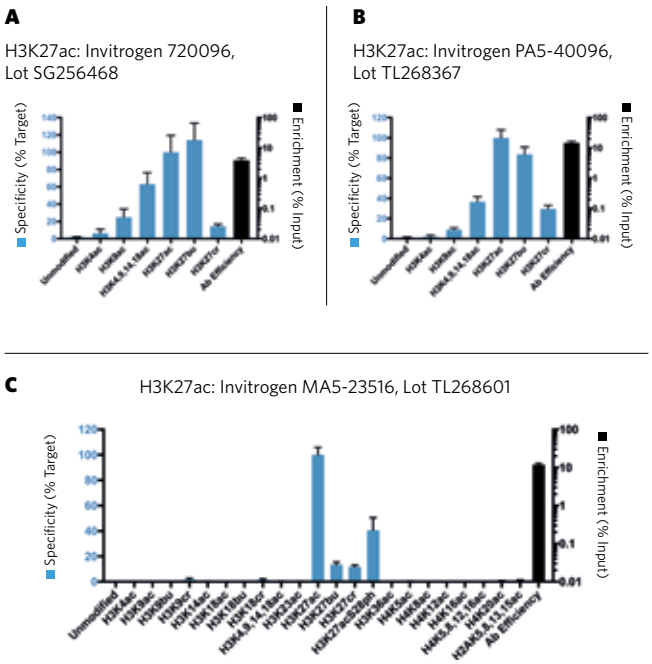

Figure 3. Comparison of three Invitrogen H3K27ac antibodies using the SNAP-ChIPTM K-AcylStat Panel. (A, B). Two H3K27ac antibodies show significant cross-reactivity and are no longer recommended for ChIP. (C) MA5-23516 demonstrates specific recognition of H3K27ac alone and, to a lesser extent, when the adjacent serine (S28) is phosphorylated.

with that determined by peptide array, using strict protocols developed by the ENCODE consortium (encodeproject.org). There was no correlation[6].

Creating superior PTM antibodies

A range of nucleosome panels (each containing a distinct PTM class) are now commercialized by EpiCypher as SNAP-ChIP™ (Sample Normalization and Antibody Profiling ChIP) Spike-In Controls. Invitrogen has a growing number of histone PTM antibodies whose specificities are verified using SNAP-ChIP spike-ins as part of the company's two-stage antibody specificity and functional validation testing.

ANTIBODY SPECIFICITY IS ESSENTIAL TO OBTAINING ROBUST REPRODUCIBLE DATA.

Figure 1 compares a recently developed Invitrogen H3K4me3 recombinant polyclonal antibody with one of the most commonly used antibodies (>750 citations, according to citeab.com) examined in the study6. The Invitrogen antibody shows high specificity (<5% off-target) and high efficiency (>70%) against the target PTM. However, the widely cited antibody, like many others tested against H3K4me3, has significant cross-reactivity with H3K4me26.

These results are reflected in ChIP-seq data. Figure 2 compares ChIP-seq results for SNAP-ChIP validated Invitrogen antibodies against H3K4me3 and H3K4me2 alongside ENCODE project data using the same widely cited H3K4me3 antibody. The ENCODE data indicate a combination of readouts from both H3K4me2 and H3K4me3, suggesting that the previously described association of H3K4me3 with enhancers could be due to undetected cross-reactivity to H3K4me2, an abundant PTM at these locations[6].

SNAP-ChIP panels can also examine other PTM types, such as lysine acetylation. H3K27ac is most often associated with active enhancers, though its role at these locations is unclear. Figure 3 compares the specificity of three Invitrogen H3K27ac antibodies and demonstrates their dramatically different capabilities. All three antibodies demonstrate expected enrichment/depletion at representative loci in ChIP, but SNAP-ChIP spike-in antibody verification reveals differences in specificity. Invitrogen recommends two of these antibodies for western blotting based on their specificity in peptide array, but not for ChIP, as their lack of specificity could contribute to misleading biological insights with ChIP.

Averting the reproducibility crisis

Antibody specificity is essential to obtaining robust reproducible data and drawing appropriate biological conclusions. Invitrogen and EpiCypher have partnered to create best-in-class 'SNAP-ChIP verified' antibodies for histone PTMs by using defined spike-in nucleosome controls to validate specificity and efficiency, directly in ChIP, of each manufactured lot of antibody. SNAP-ChIP spike-ins and allied technologies are now employed throughout the histone PTM antibody development process to screen and validate the highest-quality candidates and deliver lot-validated reagents for reliable ChIP studies. ■

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