The Danger of Epigenetics Misconceptions (Epigenetics and Stuff...)

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The Danger of Epigenetics Misconceptions (Epigenetics and Stuff...)

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Abstract

Within the past two decades, the fields of chromatin structure and function and transcription regulation research started to fuse and overlap, as evidences mounted to support a very strong regulatory role in gene expression was associated with histone post-translational modifications, DNA methylation, as well as various chromatin-associated proteins (the pillars of the “Epigenetics” building). The fusion and convergence of these complementary fields is now often simply referred to as “Epigenetics”. During these same 20 years, numerous new research groups have started to recognize the importance of the chromatin composition, conformation, and its plasticity. However, as the field started to grow exponentially, its growth came with the spreading of several important misconceptions, which have unfortunately led to improper or hasty conclusions. The goal of this short “opinion” piece is to attempt to minimize future misinterpretations of experimental results and ensure that the right sets of experiment are used to reach the proper conclusion, at least as far as epigenetic mechanisms are concerned.

Key words: chromatin/ nucleosome/histone post-translational modifications/ChIP/epigenetics

Over the last few years, the number of publications referring to epigenetic events regulating the expression of multiple genes, cell differentiation, and other cellular events have skyrocketed. However, after being exposed to this large number of articles (to read and/or to review), which do contain scientific epigenetic statements and “facts”, it has become fairly obvious that a significant proportion of said publications are ignoring the wealth of information gathered over the last 25-35 years of investigation of chromatin and other epigenetic events.

As a reviewer and researcher involved in chromatin structure and function for an extended period of time, when reviewing such manuscripts, I try to indicate to the authors that they may benefit from a better awareness of older publications related to “obscure” topics, such as “Chromatin” by Ken van Holde (van Holde 1988) or “Chromatin: Structure and function” by Alan P. Wolffe (Wolffe 1998). Yes, the field of chromatin is that old, preceding even the use of the Internet as a source of information (not to mention the ubiquitous Wikipedia). Names of some of the early contributors to the field, such as Gary Felsenfeld, Morton Bradbury, Aaron Klug, Roger Kornberg, and many others (my apologies for not mentioning all the significant and noteworthy contributors to the field of chromatin, this letter would be too long), who have established a SOLID basis in this field of research on which to build, should not be ignored. Their work has established the foundation for the current wave of epigenetics research, and is still must-read material for the new contributors to the expanding field of epigenetics. All students in my laboratory are expected to acquire knowledge from this solid scientific foundation. In other words, one should learn how to stand and walk before attempting to run.

Examples of improper use of epigenetics information reaches even the companies that specialize in epigenetics, chromatin, and associated fields. I recently saw an advertisement for a company specializing in chromatin and epigenetics (not to be named, have fun finding it) using a

depiction of an array nucleosome with the DNA wrapped in a right-handed manner around the histone octamers. Correct me if I am wrong, but any decent textbook, review, or article would confirm that DNA is wrapped around the octamers of core histones in a left-handed manner (Finch et al. 1977; Luger et al. 1997). This may reduce my willingness to trust a company that does not even show chromatin the way it has been demonstrated to be structured.

So, this directly sends me to the main points that I would like to point out and summarize the most common issues related to incorrect information related to epigenetics and chromatin. I already mentioned the special nucleosomes with DNA wrapped in a right-handed manner. This, unfortunately, may only be a minor issue compared to some more serious misconceptions. The following sections are intended to address the main issues related to this problem.

Chromatin folding, structure and composition.

a. Chromatin folding and structure: The most common misconception that I have been exposed to relates to changes in histone composition (histone variants and histone post-translational modifications (PTM)) that are systematically equated with changes in chromatin compaction. Unfortunately, a simple western blot showing changes in histone variants or PTMs provides NO information about chromatin global or local three-dimensional structure. It can suggest that chromatin may be more likely in an “open” or “closed” conformation, but it does not allow for any quantification of chromatin folding, as is commonly implied. To stay on the topic: a change of signal in a western blot of any histone PTM does not, as a general rule, equate to changes in histone EXPRESSION (though it could, in which case you will have to rethink your experiment as histones H3 or H4 are commonly used as normalizer for change in gene expression by western blotting analysis. Lamin B1 can be considered as a good substitute for that purpose). This type of

analysis is designed to provide information about changes in histone post-translational modifications and not histone expression. As a standard procedure, one of the core histones is used to normalize the signal related to histone PTMs. If looking at changes in histone expression, then a different protein is needed to be able to normalize the signal and, by default, could not use core histones to normalize histone PTM signal. Similarly to the information provided by western blotting, a simple Chromatin Immuno-precipitation (ChIP) does NOT provide direct information about chromatin folding. It gives information about chromatin composition, and possibly, nucleosomes distribution over the probed region. If 3-D structural information is investigated to match one's gene of interest to euchromatin or heterochromatin (open vs. condensed chromatin), other techniques should be considered (confocal microscopy, Chromatin Conformation Capture (3C) or derived methods such as 4C (circular 3C) 5C (3C carbon copy), and Hi-C (for review see Dekker et al. 2013). Alternatively, biophysical methods such as analytical ultracentrifugation (Hansen et al. 1994) or Quantitative Analytical Gel electrophoresis (Georgel and Hansen, 2004) can be used to monitor the level of chromatin condensation, but only under specific conditions (purity and amounts of material available can be serious limiting factors when using such analytical methods).

b. Chromatin composition and associated functions: Histone acetylation, methylation, phosphorylation of specific amino acids are considered as marker for specific nuclear functions, such as transcription, DNA repair or recombination. For example, histone H3 di-methylated at Lysine 4 (H3K4me2) and histones H3 and H4 acetylation are usually considered as markers for genomic regions which are actively transcribed. As this is most commonly a correct assumption (especially for acetylation), a large number of exceptions to this “rule” have been described (general histone acetylation: Mehrota et al. 2014; H3K9 acetylation: Zsindley, N. et al. 2009, for

recent review, see Delcuve et al. 2012). In addition to having a potential role in gene repression, histone acetylation's role as a “cause” or a “consequence” of increased transcription remains controversial (for in depth analysis see Jin et al. 2011).

Histone modifications, by themselves, can also play a structural role in chromatin organization. Hyper-acetylation or phosphorylation of specific residue(s) can lead to significant conformational changes in chromatin (Davie and Hendzel 1994; Hayes and Hansen 2001; Hansen et al. 2010, Pepenella et al. 2014; Turner, B.M. 2014). These changes are not necessarily mediated by the recruitment of other chromatin-associated proteins. These structural changes can lead to short and/or long-distance interactions between specific chromatin loci that may play important roles in the regulation of cellular functions (Pepenella et al. 2014).

One more word of caution on histone modifying enzymes: The mere presence of histone modifying enzymes (“writers”: as described by Ruthenburg et al. 2007 and Bannister and Kouzarides 2011) at a specific locus does not necessarily equate to the presence of the cognate histone PTM. Nor does it *de facto* provide information on its function in that specific context and location. It merely indicates the presence of these enzymes and a potential for presence of associated modifications.

Histone bivalent marking also occurs. This occurs when histone PTMs associated with actively transcribed regions co-localize on specific DNA sequences with histone PTMs associated with repressed regions (Zhang et al. 2012). As expected, the “reader” (protein recognizing and binding to a specific PTM, Ruthenburg et al. 2007; Bannister and Kouzarides 2011) recognizing such bivalent marking(s) will likely be different from those binding to histones harboring a single PTM. Therefore, the choice of antibody(ies) used to detect and/or localize such monovalent vs. bivalent marking will be critical to provide the expected and accurate

information. Proper validation of the selected antibody(ies)' specificity is crucial to the interpretation of the results (for an extensive review on this topic, see Parseghian 2013). More specifically, in the case of histone tails harboring multiple PTMs, it is possible that a secondary PTM could mask the investigated epitope (epitope exclusion). It is also important to emphasize the fact that the interpretation of ChIP assays may be tricky. The presence of two histone PTMs identified by ChIP does not necessarily mean that both PTMs are on the same nucleosome, on the same tail and/or allele. Lee and Mahadevan nicely illustrated this point in a fairly recent review based on genome-wide analysis of PTMs locations published a few years ago (Lee and Mahadevan 2009). Practically, this may imply that sequential ChIP assays (also referred to as ChIP-Re-ChIP assay) may be needed to tease out the intricate epigenetic variations associated with the presence of multiple PTMs over specific loci.

In addition, the multiple possible histone PTMs are, in and of themselves, not the end product of the epigenetic process associated with the highly dynamic nature of chromatin. They are mainly markers for chromatin "readers", proteins that will bind specifically to modified histones (for review, see Ruthenburg et al. 2007, Bannister and Kouzarides 2011). Therefore, a study that does not include the investigation of the presence of "readers" of specific histone PTMs at defined loci will be highly incomplete. The analysis will need to be modified to include the interactions between histones PTMs and their matching "readers" and associated proteins (Musselman et al. 2012). The complexity of the analysis of mechanisms directing interactions between "readers" and histone PTMs increased when researchers realized that neighboring PTMs influence the readers' recognition (McDonald et al. 2005; Ruthenburg et al. 2011; for review, see Musselman et al. 2012). The same core histone harboring bivalent PTMs, within a single nucleosome, may be recognized by multiple "readers", leading to different biological outcomes.

Before leaving the topic of histone PTMs: it is important to remind researchers that residues outside the histone N-terminal end of H3 and H4 (referred to as tail) can ALSO be post translationally modified and these PTMs can provide essential signals for a subset of specific “readers”. Note that most of the attention has been focused on histones H3 and H4 and their PTMs, but histones H2A and H2B are also modified and follow the same rules by matching histone PTMs to specific “readers”.

Is this complex enough? Of course not: of note here, the state of DNA methylation also plays an important role in the recruitment of the cognate “reader” at a specific locus.

DNA methylation

Here is a topic with a huge potential for a gross over-simplification regarding epigenetic regulations. Mapping methylated CpG over the regulatory elements of any gene will undeniably yield information that can be related to the state of expression of the gene of interest. However, once again, it is only the first step of the analysis. Without an understanding of the basic mechanism by which DNA methylation participates in the regulation of gene expression, the point made by researchers can be grossly missed. First reminder to be mentioned: Remember: all CpGs are not born equal. Location, location, location: CpG position and density matter (regulatory element, coding region or intergenic location; islands vs. isolated), as does the base next to the target cytosine. CpGs are not the only potential target for DNA methyl transferases. CpA, CpC, and CpT (globally referred to as CpH) can also be methylated, and their role and function in regulation of gene expression may not be equivalent to that of CpGs, as demonstrated by recent publications potentially linking CpH methylation to various nuclear functions as well as cell type specificity (Ziller et al. 2011; Guo et al. 2014). In addition to the density and the

neighboring deoxynucleotide, methylated Cs can be hydroxymethylated (Liyanage et al. 2014; Moen et al. 2015). The functions of methylated CpG (or CpH) and hydroxymethylated cytosines (spoiler alert) are not one and the same (for details see review by Plongthongkum et al. 2014 and Moen et al. 2015). From a technical point of view, one should also be aware of differences in specificity provided by various assays. For example, the Illumina Infinium HumanMethylation 450 BeadChip array, often used to investigate DNA methylation, does not discriminate between standard 5 methyl cytosine and 5 hydroxymethyl cytosine, leading to potential misinterpretations of the results related to epigenetic plasticity.

The second reminder is that, similarly to the concept of histone writers (enzymes adding specific PTMs to histones, Bannister and Kouzarides 2011), readers and eraser (enzymes removing specific PTMs from histones, Bannister and Kouzarides 2011), cytosines can be methylated by multiple DNA methyl-transferases (Lo and Weksberg 2014), recognized by various methyl-DNA binding proteins (for review, see Buck-Koehntop and Defossez 2013), and DNA de-methylases (Teif et al. 2014; for recent review, see Moore and Fan 2013).

So, to wrap up this summary and make a long story short: “Epigenetic Regulations” covers many complex variables. If one’s data set only consist of a western blot assessing histone PTMs under different sets of conditions, then conclusions must only be drawn based on the data available: you have potential global changes in histone PTMs under the different experimental conditions tested. This simple result provides no information on whether the “gene(s) of interest” is/are affected by the conditions in an epigenetic manner. One does NOT know whether the nucleosome distribution over that said gene(s) has been altered. One has no information on the chromatin folding state. One does NOT know whether specific transcription factors or chromatin remodelers, or chromatin-associated proteins have been recruited or removed from any

regulatory or coding sequences associated with the investigated gene. To get this type of information, a “few” more experiments will need to be performed. Similarly: just adding a simple ChIP experiment to your western blot will still fall short of getting you a meaningful epigenetic profile of the investigated gene(s). To support a hypothesis or complement a data set, follow established procedure (or develop novel ones). Epigenetic/chromatin researchers have compiled excellent guides to epigenetic or epigenomic research (for example: Zentner and Henikoff 2014). Researchers don’t have to systematically re-invent the wheel; it is sometimes better to follow the established procedures. The ensuing newly generated data set and analysis will provide valuable information to your field of research, and may not generate ire from the potential reviewers. **In conclusion: keep the great epigenetic science coming our way.**

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