What Do You Mean, "Epigenetic"?

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ABSTRACT Interest in the field of epigenetics has increased rapidly over the last decade, with the term becoming more identifiable in biomedical research, scientific fields outside of the molecular sciences, such as ecology and physiology, and even mainstream culture. It has become increasingly clear, however, that different investigators ascribe different definitions to the term. Some employ epigenetics to explain changes in gene expression, others use it to refer to transgenerational effects and/or inherited expression states. This disagreement on a clear definition has made communication difficult, synthesis of epigenetic research across fields nearly impossible, and has in many ways biased methodologies and interpretations. This article discusses the history behind the multitude of definitions that have been employed since the conception of epigenetics, analyzes the components of these definitions, and offers solutions for clarifying the field and mitigating the problems that have arisen due to these definitional ambiguities.

KEYWORDS transgenerational; maternal effects; gene expression; epigenetic inheritance

INTEREST in epigenetics, as well as the usage of the term *epigenetic*, has increased significantly since the field was first conceived by Conrad Waddington in the early 1940s. In 2006, over 2500 articles related to epigenetics were published (Bird 2007), and in 2010, over 13,000 (Haig 2012). In 2013, however, this number rose to over 17,000, a striking 45 new publications every day, in addition to increases in scientific meetings and grant directives dedicated to the subject. Today, epigenetic concepts have spread into fields that do not routinely address genetics (at least explicitly), such as ecology (Bossdorf *et al.* 2008; Zucchi *et al.* 2013; Burris and Baccarelli 2014), physiology (Ho and Burggren 2010), and psychology (Ngun and Vilain 2014; Zhou *et al.* 2014). Despite its apparent popularity, the unfortunate fact is that the increased use of the term *epigenetics* is likely due more to inconsistencies in its definition than to a consensus of interest among scientists or a paradigm shift in the rules of inheritance. The term has taken on multiple meanings, describing vastly different phenomena. As a result, its usage oftentimes implies mechanistic connections between unrelated cases. The lack of a clear definition has led to confusion and misuse of the term, while also making research within the field of epigenetics difficult to synthesize and reconcile. There are many reasons why the etymology of epigenetics is so ambiguous, many of which relate to the scientific atmosphere in which the term was conceived; others are entirely philosophical. In this essay, we address these issues by providing a brief history of epigenetics (the term and the scientific field) and discussing various definitions, as well as the important differences between them. We will also address the challenges that exist, and will continue to exist, if these ambiguities are not addressed, and offer potential solutions for dealing with these challenges.

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History of the Term "Epigenetic"

To understand the meaning of the term *epigenetics*, one must understand the context in which it was derived. Conrad Waddington, who first defined the field in 1942(a), worked as an embryologist and developmental biologist. In 1947, he founded and led the first genetics department at the Institute of Edinburgh and would later found the Epigenetics Research Group in 1965 (Van Speybroeck 2002). Waddington had a strong appreciation for genetics and was an important advocate for uniting genetic principles with other fields of biology, such as cytology, embryology, and evolutionary biology; however, he was particularly interested in embryology and developmental genetics, specifically the mechanisms that controlled cellular differentiation. At the time, there were two prevailing views on development, both of which were derived from the 17th century: preformation, which asserted that all adult characters were present in the embryo and needed simply to grow or unfold, and epigenesis, which posited that new tissues were created from successive interactions between the constituents of the embryo (Waddington 1956; Van Speybroeck 2002). Waddington believed that both preformation and

epigenesis could be complementary, with preformation representing the static nature of the gene and epigenesis representing the dynamic nature of gene expression (Waddington 1956; Van Speybroeck 2002). It is through the combination of these concepts that he coined the term *epigenetics*, which he referred to as, "the branch of biology that studies the causal interactions between genes and their products which bring the phenotype into being" (Waddington 1942a; Dupont *et al.* 2009).

It is important to note that genetics was still a young field at this time, centered on Mendel's work on trait inheritance, with the *gene* being accepted as the unit of inheritance (Johannsen 1909); but, little was known about the biochemical nature of the gene or how it functioned. It wasn't until Beadle and Tatum (1941) published their work affirming the one-gene, one-enzyme concept that an understanding of gene function took discrete shape, and subsequent work on molecular biology defined gene structure. This gene-centric atmosphere, coupled with the emerging effort to understand gene regulation and expression, had a strong influence on the creation of epigenetics, both as a concept and a field of study (Jablonka and Lamb 2002).

At that time, many, including Waddington, were interested in the process of gene control and expression. Experimental embryologists, such as Wilhelm Roux (1888), Hans Spemann (1967), Viktor Hamburger (1960), and the developmental geneticist Ernst Hadorn (1955) studied mutations by inducing changes in development through experimentation with chemicals or excision. Waddington, on the other hand, was more interested in the cellular processes that brought about these changes, rather than the stimuli that created them. One of Waddington's most important contributions was his acknowledgment of, and emphasis on, the flexible relationship between genotype and phenotype (Waddington 1942a,b, 1957), and this was an idea that many of his contemporaries, such as Nanney (1958a), Huxley (1956), Ephrussi (1953, 1958), and Lederberg (1958) (see below), were also interested in. Today, Waddington's views on epigenetics are most closely associated with phenotypic plasticity, which is the ability of a gene to produce multiple phenotypes, but he also coined the term *canalization* to refer to the inherent stability of certain phenoytpes (particularly developmental traits) across different genotypes and environments (Waddington 1942b; Siegal and Bergman 2002). Together, his concepts of plasticity and canalization suggest a general decoupling of genotype and phenotype and imply that regulatory processes must exist between the two. This realization was fundamental to Waddington's concept of epigenetics.

In 1958, 16 years after Waddington first coined the term, David Nanney published a paper in which he used the term epigenetics to distinguish between different types of cellular control systems. He proposed that genetic components were responsible for maintaining and perpetuating a library of genes, expressed and unexpressed, through a template replicating mechanism. He then deemed epigenetic components as auxiliary mechanisms that controlled the expression of specific genes (Nanney 1958a; Haig 2004, 2012). Most importantly, in addition to discussing variability in expression patterns, Nanney (1958a) emphasized the fact that expression states could persist through cell division. Although some have claimed that Nanney's usage of the term epigenetic was developed independently of Waddington's definition (he initially used the term *paragenetic*) (Haig 2004), considerable overlap can be found in their contemporary writings on genotype–phenotype relationships (Nanney *et al.* 1955, 1958a,b; Waddington 1939, 1942a,b), gene expression (Nanney *et al.* 1955, 1958a,b; Waddington 1939, 1942a.b), and the respective roles of the nucleus and the cytoplasm in gene regulation (Nanney 1953, 1957, 1958a; Waddington 1939, 1956). It is clear, however, that Nanney's contemplation of the stability of cellular expression states was an important addition to Waddington's ideas, which had significant impacts on the future direction of epigenetics. For a more detailed treatment of this history please refer to Haig (2004, 2012) and Holliday (1994).

Definitions of Epigenetics

It was largely through a shared interest in development and cellular differentiation that Waddington, Nanney, and others came to use the term *epigenetic*; however, the focus of those within the field did vary, with some, such as Waddington, being more concerned with gene regulation and genotype–phenotype interactions, and others, such as Nanney and Lederberg, being more interested in the stability of expression states and cellular inheritance. As stated by Haig (2004), interest in these different aspects of epigenetics led to a division within the field that can be directly linked to the definitional identity crisis that exists today.

Throughout the 1980s and 1990s, the definition of epigenetic moved farther away from developmental processes and became more generalized. For example, one definition from 1982 describes epigenetics as "pertaining to the interaction of genetic factors and the developmental processes through which the genotype is expressed in the phenotype" (Lincoln *et al.* 1982). This definition does include the term *developmental*, but its meaning seems to relate more to the development of the phenotype than to an ontological meaning. Although only slightly different from Waddington's original definition, this definition and others during this time broadened the meaning of epigenetics in important ways. It made the term more available and applicable to other fields by emphasizing the importance of genetic and nongenetic factors in controlling gene expression, while downplaying (although not ignoring) the connection to development (Medawar and Medawar 1983; Hall 1992; Jablonka and Lamb 2002).

Concurrently, research being done in the 1970s and 1980s on the relationship between DNA methylation, cellular differentiation, and gene expression (Holliday and Pugh 1975; Riggs 1975; Jones and Taylor 1980; Bird *et al.* 1985) became more closely associated with epigenetics. The work of Robin Holliday and others, on cellular memory and DNA methylation, particularly the finding that DNA methylation had strong

effects on gene expression and that these effects persisted through mitosis, corresponded to Nanney's (1958a,b) writings on the stability of expression states. This prompted Holliday to redefine epigenetics in a way that was more specific and squarely focused on the inheritance of expression states (while Nanney discussed epigenetic inheritance, his definition of epigenetics did not include a specific component on heritabililty). Holliday (1994) offered two definitions of epigenetics, both of which were admittedly insufficient when taken separately but comprehensive in covering all currently acknowledged epigenetic processes when taken together. The first definition posed that epigenetics was "the study of the changes in gene expression, which occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression." The second stated that epigenetics was "nuclear inheritance, which is not based on differences in DNA sequence." Wu and Morris (2001) streamlined Holliday's definition to state "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail change in DNA sequence."

The addition of heritability to Waddington's original definition by Holliday was a significant change. While Waddington's definition does not preclude the inheritance of expression states [indeed Waddington (1942a) did briefly discuss heritability in his paper "The Epigenotype"], this aspect was not a fundamental part of his concept of epigenetics. Despite the more thorough discussion of heritable expression states by Nanney and others, this was the first definition to make heritability a necessary part of epigenetics.

The implications of Holliday's redefinition were significant. The field soon became a residence for perplexing phenomena that didn't fit squarely into other genetic fields and, in many regards, the inability to explain these phenomena by simple genetic explanations became a defining element of epigenetics. Prior to understanding RNA-based regulatory mechanisms, and still in early stages of understanding DNA methylation and histone modifications, the decoupling of genotype and phenotype exemplified by epigenetics provided an attractive refuge because it offered metaphorical language to describe the disconnect between a gene and its phenotypic properties. This included occasions where the expression of a gene varied depending on its location (such as position effect variegation in *Drosophila* or yeast), history (imprinting), or other circumstances (*e.g.*, the establishment of centromeres, telomere healing prior to sequence addition). The thrill and charisma of a "new" genetics initiated a virtually unparalleled wave of interest in epigenetics over a very short amount of time (Cold Spring Harbor Symposium on Quantitative Biology 2004; Haig 2012).

The Problem

It is not difficult to find articles in the current scientific literature that use *epigenetic* to mean any one of the definitions above, or others entirely. It is futile to argue over the correctness of any one definition; however, it is important to acknowledge that the lack of a universal definition has produced significant ambiguity across biological fields. As previously acknowledged by Haig (2004) and others (Bird 2007; Haig 2012; Mann 2014), what we have today is a pronounced dichotomy within the field of epigenetics. Waddington's *epigenetics* describes the interplay of genetic and cytoplasmic elements that produce emergent phenotypes (Van Speybroeck 2002; Jamniczky *et al.* 2010), and those in the biological sciences interested in gene-by-environment interactions and phenotypic plasticity use the term in this sense. As a result, Waddington's definition is largely used to describe the expression of environmentally mediated phenotypes, particularly in the fields of ecology (Rollo 1994; Pigliucci 2007; Bossdorf *et al.* 2008) and physiology (Jablonka 2004; Aguilera *et al.* 2010; Ho and Burggren 2010). Those in the field of genetics concerned with DNA methylation, chromatin activity states, chromosomal imprinting, centromere function, etc., predominantly use Holliday's notion of epigenetics. They are interested in how expression patterns persist across different cells (mitosis) and generations (meiosis). The phenomena being described by these two groups, and more importantly the mechanisms underlying them, are vastly different, yet they both use the same term: *epigenetic*.

This ambiguity has made even the simple task of identifying epigenetic phenomena difficult and also constrains more advanced pursuits to determine how epigenetic processes occur. After all, how can scientists effectively study a process when they cannot even agree on how to define it? With the usage of the term *epigenetic* increasing exponentially across scientific and mainstream literature, one must wonder: for all the interest and attention epigenetics is receiving, why don't we have a clearer understanding of it?

The primary challenge is reconciling Waddington's epigenetics with Holliday's epigenetics, because while both exist, they may not necessarily be related to each other. Is there room within one field to entertain both definitions? Moreover, do the phenomena underlying each have any business being categorized together, particularly when their connection is based more on history and semantics than deliberation? Answering these questions is important for streamlining the field, facilitating more effective interchanges between researchers, and developing clearer research objectives.

The second challenge lies in addressing the methodological problems that have accumulated within the field of epigenetics over time, due to the absence of a clear definition. The principles that provide the foundation of any biological field exist to direct research and achieve objectives within that field; however, without this clear foundation, our desire to understand epigenetics has dictated our experimental approaches, colored our mechanistic interpretations, and allowed us to gloss over inadequacies. Rather than building from clear first principles, the field of epigenetics continues to be a catchall for puzzling genetic phenomena from which categorizations and justifications were developed *a posteriori*. Working backward to reevaluate

the first principles of epigenetics will help put the field on a stronger track and will hopefully allow research to flourish.

Ruminations on Important Terms: Dependence, DNA Sequence, and Heritability

Understanding why some genes are turned on or off is certainly less mysterious now than when the field of epigenetics was born, largely because of the identification of regulatory gene–gene and gene–protein interactions. These findings go a long way to explain the changes in gene expression that Waddington termed epigenetics, but the real difficulty is in satisfying Holliday's addendum of heritability. These regulatory components are all encoded by DNA; however, Holliday's conceptualization of epigenetics requires that the status of gene expression, not just the components needed for gene expression, be heritable. Also, this phenomenon requires an additional mode of inheritance that is not dependent on DNA sequence. To fully comprehend Holliday's definition, we must first make sure that all of the elements are accurately defined. This requires not only taking a critical look at how Holliday's description defines the terms *dependence*, *DNA sequence*, and *heritability*, but also the range of possible meanings.

Dependence

The term *dependence* carries several potential meanings. In a strict sense, any molecule that cannot exist in the absence of DNA could be considered to be dependent on DNA. Therefore, any molecule or process that relies on DNA for its creation, perpetuation, and/or activation is dependent, and this would include any molecule that requires DNA as a substrate. From this perspective, anything from DNA methyltransferases (DMNTs), which are expressed by specific *DMNT* genes, to histones, which use DNA as a substrate during modification, would be considered dependent on DNA.

It is likely, however, that Holliday and others would argue that this is not the meaning they had in mind when they made this distinction. Instead, they refer to dependence in a stricter sense as the relationship between the location of a *particular chromosomal locus*, *the specific base pair DNA sequence* within that locus, and a reliable *expression state* (Holliday 1994). For example, Holliday's argument is that the ability of the same DNA sequence to produce different expression profiles without a base pair change shows a lack of dependence on the primary sequence because something outside of the sequence must be controlling expression. This then requires that we understand what exactly is meant by *DNA sequence*.

DNA sequence

Many characteristics of DNA sequence are often overlooked and underappreciated. Most geneticists are primarily concerned with euchromatic regions containing sequences that make up genes and encode proteins. This isn't too surprising, given that these are the portions of DNA responsible for producing the majority of proteins vital to cell survival and function. Repetitive sequences, including those found in the heterochromatin, are often viewed as less important and commonly referred to as *junk DNA* (Ohno 1972; Brosius and Gould 1992; Kapranov and Laurent 2012; Graur *et al.* 2013). The ambivalence toward repetitive sequences likely stems from the fact that their function is poorly understood, and that the tools for investigating them are undeveloped. The bias toward protein-coding regions and the difficulty in working with repetitive sequences has shaped, and perhaps limited, our understanding of the role gene sequence plays in gene expression; however, there is evidence that other aspects of DNA, aside from the base pair sequence within gene regions, are important for gene expression.

One example is that the expression of a gene can be dependent on other sequences lying outside of the coding region (*cis*- and *trans*-regulatory elements or repetitive sequences). This makes it difficult to understand, and therefore reject, a relationship between gene expression and primary sequence because the expression of one gene may be dependent on the primary sequence of another section of DNA (see Figure 1). These problems are solved by expanding the definition of a gene to include regulatory elements and a rigorous requirement to map the genetic locus of regulatory changes. The former is easily accomplished (but often suffers from ambiguity and difficulties in precisely determining the boundaries of a gene), while the latter is rarely pursued in epigenetics literature.

A second, often overlooked characteristic of DNA sequence is location, which can impact gene expression in both coding and noncoding regions. Position-effect variegation (PEV) demonstrates that moving a gene sequence to a different location within the genome can affect its expression (Gowen and Gay 1934; Spofford 1976; Karpen 1994), and in these cases nondependence is still upheld by most epigeneticists as long as no changes occur in the transposed sequence. But why is the location of a gene sequence viewed as unimportant? To those who use transgenesis, a common practice in biology, it is abundantly clear that the location of an inserted transgene has significant effects on its expression (Al-Shawi *et al.* 1990; Wilson *et al.* 1990). In fact, Waddington explicitly promoted the idea of incorporating gene position and arrangement as an element of the genotype due to its important effects on expression (Waddington 1939).

A third salient characteristic of DNA sequence is the copy number of nearby sequences. Studies have shown that repeat regions can play important regulatory roles (Lemos *et al.* 2008; Zhou *et al.* 2012) and that the proximity of coding regions to repeats (Dorer and Henikoff 1997), as well as the size of the repeating regions (Howe *et al.* 1995; Paredes *et al.* 2011; Sentmanat and Elgin 2012), can have unique effects on gene expression and chromatin structure. This also means that changes in repeat regions,

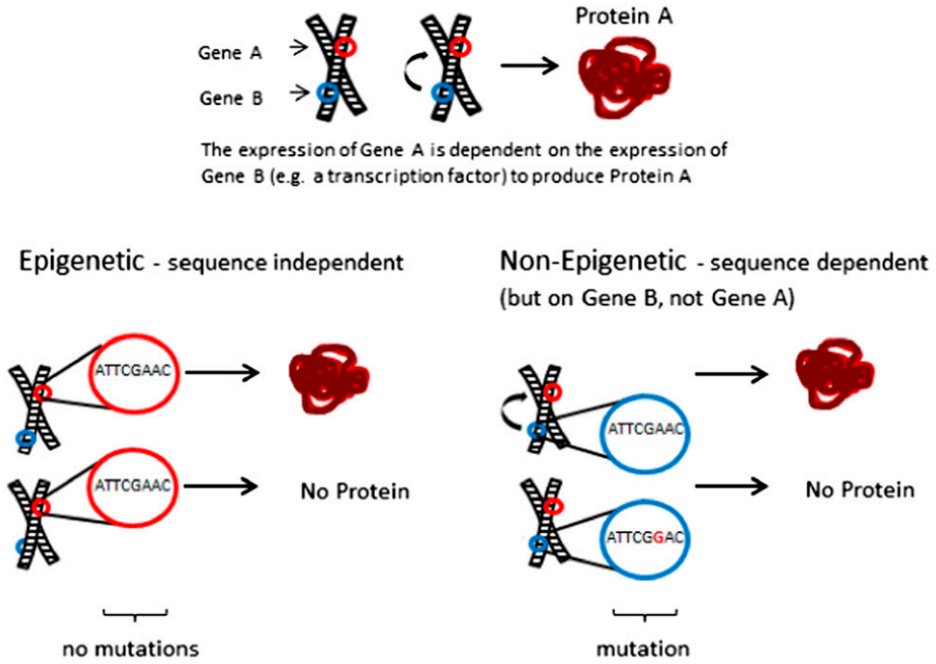

Figure 1 Imagine the expression of gene A is dependent on the expression of gene B (a transcription factor or si/piRNA perhaps). If we see variable expression in A, but no change in the sequence of gene A, we may conclude that this provides evidence for the expression of A being sequence independent and a product of epigenetics, as shown below. However, it is possible that sequence changes have occurred in gene B, producing transcriptional changes in A. This would make the expression of A *dependent* on the primary sequence of gene B but not the sequence of A itself. This makes the task of proving sequence independence difficult because you cannot simply look for sequence changes in the coding region of the gene in question, but must also be sure expressional changes aren't due to mutations elsewhere on the chromosome or other places in the genome.

which are notoriously difficult to detect, must also be ruled out to accurately show sequence independence.

Heritability

Perhaps the most important and definitive element found among definitions of epigenetics, is the *heritability* of expression states. With this addition one could argue that the definition of epigenetics was simultaneously expanded and constricted. On the one hand, incorporating heritability into the discussion forces us to consider epigenetics on a more conceptual level by thinking about the role of time and the relationship between the stimulus that causes an expressional change and the lasting or fleeting effects of that change. On the other hand, requiring that expressional changes persist through mitosis and/or meiosis in order for a phenotype to be considered epigenetic drastically reduces the number of observations that qualify. For these reasons, this aspect of Holliday's definition is the most controversial, particularly since it requires the acknowledgment of a new mode of inheritance.

From a semantics perspective, the inclusion of heritability also expands the meaning of the term itself, which has traditionally related to the transfer of only DNA. Using heritability to describe the transfer of non-DNA molecules, whether they are methyl groups, histones, or cytoplasmic compounds, broadens the concept of inheritance in an intriguing way. However, Holliday's definition doesn't actually delineate the difference between the inheritance of molecules and the transfer of molecules, nor does it state what kind of molecules can and cannot be inherited. Without this distinction it is very difficult to separate epigenetic phenomena from nonepigenetic phenomena, and also to investigate how such modes of inheritance may function.

Holliday's concept of *heritability* also produces several complications in practice. First, it can be surprisingly difficult to discern between changes in gene expression due to the inheritance of an expression state and those due to a real-time reaction to a stimulus. To show that an expression state is inherited, you first need to have a clear understanding of the cause (*i.e.*, stimulus). Knowing the relationship between a given stimulus and its expressional effect(s) is paramount to creating a timeline and conclusively showing that a barrier exists between the two for which inheritance in necessary. For example, this would entail that a parent cell or organism experienced a stimulus that caused a specific expression pattern and then that a similar expression pattern was also evident in the offspring without the offspring having ever experienced the initial stimulus.

While these connections are easy enough to conceptualize, they can be difficult to prove empirically, not only because gene expression can be capricious, but because in many cases the stimuli impacting a parent also may impact the germ cells residing in the parent, germ cells which will ultimately go on to produce daughter cells and/or offspring. If the germ cells respond to a stimulus experienced by the parent, no barrier exists between the stimulus and offspring because expression in the primordial cells of the future offspring are also directly affected. For example, in mammals, any stimuli impacting a pregnant female carrying daughters may impact the mother, the fetus, and the germ cells of the fetus, which will go on to produce offspring (Youngson and Whitelaw 2008; Daxinger and Whitelaw 2012; Dias and Ressler 2014). This means that any stimulus experienced by the mother may also result in direct exposure to two additional generations of potential offspring. In this scenario, one would have to show a similarity in expression between the mother and her great granddaughter to verify a possible epigenetic connection (Skinner 2007; Skinner *et al.* 2013). However, if the expression pattern of the original germ cell were apparent in the offspring, it would still

satisfy Holliday's definition, as persistence through mitosis would have had to occur (Holliday 1994). This has led to some clarifications in the identification of epigenetic phenomena, but those attempts have yet to clearly delineate Waddington's and Holliday's views (Youngson and Whitelaw 2008; Berger *et al.* 2009; Grossniklaus *et al.* 2013; Dias and Ressler 2014).

The primary difficulty lies in identifying the mechanism of inheritance. Do the compounds responsible for perpetuating an expression pattern have to be closely associated with DNA, as in methylation and chromatin modification, or do cytoplasmic compounds qualify? If so, should the transfer of cytoplasmic compounds really be considered inheritance? Waddington stressed the importance of cytoplasmic compounds and their effect on gene expression (Waddington 1935), yet maternal or transgenerational effects mediated by cytoplasmic transfer from mother to offspring would not be considered epigenetic under Holliday's definition because the expression pattern of the offspring is not independent and simply results from the transfer of cytoplasmic compounds, such as RNA, transcription factors, prions, etc. (Ptashne 2008; Jarosz *et al.* 2014). These issues make the contrast between Waddington's epigenetics and Holliday's epigenetics much more evident.

Possible Solutions

The ambiguity surrounding the field of epigenetics, as well as the historical basis for this definitional confusion, has been discussed by many over the last 15 years (Holliday 2002, 2006; Jablonka and Lamb 2002; Haig 2004; Bird 2007; Berger *et al.* 2009; Mann 2014). This has led to the development of several new definitions and terms to help clarify the issue. Bird (2007) proposed that epigenetics could be redefined as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states," a definition that he feels unified Holliday's requirement for heritability with Waddington's more general definition. Mann (2014) also advocated keeping a broad notion of epigenetics, but offered the term "memigenetic" to denote expression states that are heritable. Despite these suggestions, a strong working definition for epigenetics has yet to be adopted, and we believe that this largely results from (1) attempting to combine Waddington's and Holliday's definitions into one comprehensive term and (2) the absence of specific terms within the available definitions that identify the mechanistic components underlying epigenetic phenomenon.

We don't feel that it is possible to reconcile Waddington's focus on gene regulation with Holliday's more specific criteria within one field and still maintain the level of clarity needed to produce a useful definition. The efforts to preserve a relationship between these two conceptualizations have been impaired by the fact that there are just too many phenomena, with too few mechanistic connections, to categorize into one field. Also, among the definitions that do maintain the requirement of heritability, we feel that many lack the detail to be functionally useful in directing the testing of specific hypotheses, particularly as it relates to the location or site (cytoplasm or nucleus) of epigenetic phenomena. To mitigate these shortcomings, we advocate defining epigenetics as "the study of phenomena and mechanisms that cause chromosome-bound, heritable changes to gene expression that are not dependent on changes to DNA sequence."

We feel that this definition makes a strong distinction between gene regulation (Waddington's definition) and epigenetic inheritance (Holliday's definition), and also emphasizes that epigenetic phenomena must deal exclusively with chromosome-bound changes. By making these distinctions, we have efficiently separated expressional changes caused by cytoplasmic compounds, which are more closely tied to gene regulation, from those which occur on, or in close association to, the chromosome. Doing so makes the focus of the field much clearer and identifies epigenetic mechanisms more explicitly.

We feel that this definition touches on several important elements not encompassed by other definitions, yet commonly implied in most uses. To further explain the reasoning behind our definition, as well as its utility for improving epigenetic research, we would like to offer a *clarification* and a *test*.

The Clarification

In the battle between Waddington and Holliday's definitions, we have clearly chosen Holliday's conceptualization, and this has occurred for two reasons. First, although the usage of Waddington's general definition has increased within nongenetic fields, particularly ecology and physiology, to describe environmentally mediated phenotypes and trait plasticity, we feel that these topics fall more clearly under the heading of gene regulation. Second, the phenomena that pose the most serious challenges to traditional genetic theory, which dictates that identical sequences should behave identically, are genomic imprinting, X inactivation in mammals, centromere/telomere establishment and stability (McClintock 1939; Ahmad and Golic 1998; Barry *et al.* 2000; Maggert and Karpen 2001; Blasco 2007; Black and Cleveland 2011; Mendiburo *et al.* 2011), and perhaps others. Most of the work on these issues has and continues to occur in the field of genetics, and we believe that the epigenetics fits most appropriately within the realm of genetics, given this strong precedent of research. That being said, we do want to clarify some points regarding Holliday's definition and the current state of the field of epigenetics.

Holliday's addendum on heritable expression states arose as a *hypothesis* to explain the phenomena listed above; however, rather than this hypothesis being thoroughly tested, it quickly perpetuated several new ideas regarding potential mechanisms for inheritance (methylation, histone modifications, *etc*.) without strong empirical proof for the necessity of such mechanisms. Although Holliday's ideas on the perpetuation of expression states and cell memory are innovative and may very well prove to be accurate, we feel an important step in the process of developing these ideas has been overlooked. This is particularly true when the attempts to validate

these hypotheses have, as of yet, proved inconclusive. What can it mean to say that DNA methylation is repressive when activation of a gene removes methylation (*e.g.*, Bird 2002; Nagae *et al.* 2011; Hackett *et al.* 2012; Qian *et al.* 2012; Gan *et al.* 2013; Xie *et al.* 2013; Bestor *et al.* 2014)? The search for the mechanism of semiconservative histone modifications continues (Deal *et al.* 2010; Xu *et al.* 2010; Nakano *et al.* 2011; Tran *et al.* 2012; Whitehouse and Smith 2013) despite evidence that the modifications respond to expression state rather than control it (Kilpinen *et al.* 2013; Ptashne 2014; Teves *et al.* 2014). It's not that histone modification and DNA methylation are not correlated with gene expression differences—they are—but the possibility that they may be responsive rather than causal has not been disproved (Henikoff 2005; Ptashne 2013). We include causation in our definition to reflect these shortcomings, in acknowledgment of the inadequacies in sequencing repeat regions and the conceptualization of important terms (*DNA sequence* and *heritability*) discussed earlier, and as an attempt to spur research that focuses on these fundamental issues.

The definition of epigenetics proposed above contains the necessarily vague "gene expression" so as to not exclude *a priori* any units of inheritance, including protein-encioding genes, telomeres, centromeres, functional RNA gene products (such as the rRNA, miRNAs, pi/siRNAs, etc), origins of replication, G-quartets, genome instabilities, or anything else that can manifest a phenotype. Our explicit addition of "chromosome bound" encompasses the already- implied popular use of the term epigenetic, where local changes in gene expression are induced and inherited *at the specific gene being regulated*. This explicit statement added to Holliday's (1994) definitions, later merged by Wu and Morris (2001), assures two things. First, that epigenetics is not inferred from cytoplasmic or nucleoplasmic factors, *e.g.* perdurance of a proteinaceous transcription factor (Ptashne 2013). Second, that *heritable memory* (rather than "inheritance") is an explicit property of epigenetic gene regulation. The most heavily cited examples of epigenetic phenomena (*e.g.*, genomic imprinting) fulfill these criteria, and other cases that are more dubious (*e.g.*, stress-sensitivity in offspring of stressed pregnant mammal mothers) are excluded until better understood.

The Test(s)

To make the strong claim of sequence independence, one must assure that there are no changes to any sequence in *cis* or in *trans* to the gene whose expression is being monitored. Ideally, one would sequence the entire genome, yet this is impractical on many grounds, not least of which are the large blocks of repetitive heterochromatin on most chromosomes, which modern molecular biology cannot assemble (and thus modern molecular biologists tend to ignore). Instead, careful (and laborious) work, such as that done by some (Brink 1956; Clark and Carbon 1985; Steiner and Clarke 1994; De Vanssay *et al.* 2012) showing frequent switching, should be considered strong evidence in the place of exhaustive sequencing. We must, however, always be concerned with the possibility of efficient inducible changes masquerading as "epigenetic" cases, *e.g.*, mating type switching in yeasts (Haber 1998), VDJ recombination (Blackwell and Alt 1989), repeat-sequence instability (Hawley and Marcus 1989), and induced mutation (McClintock 1983; Piacentini *et al.* 2014); after all, they do bear all of the hallmarks of epigenetic changes save one: we happen to know their mechanism. For that reason, it is critical to refrain from negative claims (that is, assertions of "no difference") as implied in "genetically identical chromosomes," when chromosomes have not been sequenced. Ideally, one should be able to make strong positive statements to conclude epigenetic gene regulation is at play.

One can experimentally test for sequence independence using a genetic approach. If we regard an expression state as a phenotype (and indeed Holliday's, and Wu and Morris's definitions clearly make mRNA production a phenotype), then it is a simple matter to map a phenotype to the location on the chromosome it stems from. In the example of A and B in Figure 1, if the stable expression state of A maps to the physical location of A on the chromosome, then we can have confidence that the expression state is a consequence of some feature (perhaps epigenetic) of A. Subsequent work showing lack of sequence dependency would confirm epigenetic regulation. If however, the status of A maps to the B locus, or to the heterochromatin, or even to the nucleoplasm, then there is no reason (and in fact no justification) to claim that A's expression state is epigenetic. It is likely instead controlled, through well-understood mechanisms, *e.g.*, by the presence of another factor (Ptashne 2013; Serra *et al.* 2014; Struhl 2014). In these cases, there is nothing meaningfully "dependent" about the "sequence" of A in terms of its regulation.

At an ideal extreme, identical reporter sequences should be placed in the same nucleus (through transgenesis or mating). If a regulatory change is epigenetic, then those sequences should (or could) behave differently, each independently maintaining a memory of their states. This idea is the intellectual foundation of the search for heritable histone modifications, DNA methylation, etc., yet is rarely directly tested. Strikingly, and underscoring our concern, in a few cases where data have been presented, the idea of allele-specific memory is either not tested or is directly refuted (Anway *et al.* 2005; Pembrey *et al.* 2006; Greer *et al.* 2011; Crews *et al.* 2012; Stern *et al.* 2012; Voutounou *et al.* 2012; Buescher *et al.* 2013; Padmanabhan *et al.* 2013; Wan *et al.* 2013; Gapp *et al.* 2014).

These conditions—nonsimilar behavior of identical sequences, mapping of the epigenetic state—are implied by most uses of the term epigenetic. Importantly, they are taken to imply a great deal about how gene expression works, suggesting that there is an entire layer of gene regulation that we are only now becoming aware of. Or is there? Before we rewrite the textbooks, divert funding initiatives, refocus our disease intervention strategies, or alter our view of neo-Darwinian biology, it is our obligation to attempt these simple tests to assure ourselves that we are not chasing a ghost.

Conclusions

The legacy of Waddington, and later Holliday and others, has enriched our understanding of chromatin structure, gene expression, and the environmental influence and nondeterministic capabilities of genes. However, without understanding the history of the term epigenetic, and the baggage that comes along with its different uses, we run real risks in biology. While gene expression, DNA methylation, regulatory RNAs, histone modifications, mitotic stability, and transgenerational inheritance are all correlated and intertwined, we must absolutely resist the temptation to equate them all mechanistically. We must utterly reject the notion that what we learn in one case (the mitotic inheritance of DNA methylation patterns at genomically imprinted control regions) are predictive of the properties of other cases (methylation causes inducible and meiotically heritable changes to mRNA transcription states) simply because they share the same ill-defined term, "epigenetics."

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