Published in final edited form as:

Genesis. 2009 August ; 47(8): 559–572. doi:10.1002/dvg.20534.

Analysis of epigenetic alterations to chromatin during development

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Abstract

Each cell within a multicellular organism has distinguishable characteristics established by its unique patterns of gene expression. This individual identity is determined by the expression of genes in a time and place-dependent manner. It is becoming increasingly clear that chromatin plays a fundamental role in the control of gene transcription in multicellular organisms. Understanding the regulation of chromatin, and how the distinct identity of a cell is passed to daughter cells during development, is paramount. Techniques with which to study chromatin have advanced rapidly over the past decade. Development of high throughput techniques and their proper applications has provided us essential tools to understand the regulation of epigenetic phenomena and its effect on gene expression. Understanding the changes that occur in chromatin during the course of development will not only contribute to our knowledge of normal gene expression, but will also add to our knowledge of how gene expression goes awry during disease. This review opens with an introduction to some of the key premises of epigenetic regulation of gene expression. A discussion of experimental techniques with which one can study epigenetic alterations to chromatin during development follows, emphasizing recent breakthroughs in this area. We then present examples of epigenetic mechanisms exploited in the control of developmental cell fate and regulation of tissue-specific gene expression. Finally, we discuss some of the frontiers and challenges in this area of research.

Introduction

Each cell within a multicellular organism has distinguishable characteristics established by its unique patterns of gene expression. The identity of each cell is safeguarded and passed on to daughter cells during development by mechanisms that do not alter encoding DNA sequences; these heritable processes are termed “epigenetic”. Chromatin is fundamental in epigenetic regulation and is used to organize and package meters of DNA, which must fit into a few microns of space, in a way that permits dynamic gene regulation. The primary repeat unit of chromatin is the nucleosome, which consists of a core octamer with two copies each of four core histone proteins, H2A, H2B, H3, and H4, encircled by 147 base pairs of DNA. Rapid advances in the field of epigenetics, a term formerly restricted to methylation of nucleic acids, are revealing how enzymatic modifications of DNA or histone proteins profoundly influence numerous processes in the organism, including heritable changes during development.

Chromatin structure is inhibitory to DNA as a substrate in cellular processes such as replication, repair, and transcription, all of which must occur robustly and with great fidelity. The means by which access to DNA assembled into chromatin is yielded discriminately and

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in a highly regulated manner, especially during rapid cell division, is a major focus of scientific research. This review begins with a brief introduction to some of the key themes of epigenetic regulation of gene expression. We follow with a discussion of experimental techniques, emphasizing recent breakthroughs in this area, which are pushing forward our understanding of the regulatory roles of chromatin and DNA methylation during development. We then present certain paradigms of epigenetic mechanisms, exploited in control of developmental cell fate and regulation of tissue-specific gene expression. Finally, we discuss some of the frontiers and challenges in this area of research.

Regulated Alteration of Chromatin Structure

Post-translational modifications of histones

Accessibility to chromatin by DNA-metabolizing proteins and transcription factors is dictated in part by modifications of histone proteins, especially on their amino termini (N-terminal tails). The four core histone proteins are primarily globular with the exception of the N-terminal tails, which protrude from the core of the nucleosomal DNA/protein structure (reviewed in Kouzarides, 2007; Luger, 2006; Luger and Richmond, 1998; Morales *et al.*, 2001). Specific amino acids within the N-terminal tails can be modified by enzymatic processing or addition of a broad range of moieties, including lysine acetylation, lysine or arginine methylation, serine or threonine phosphorylation, lysine ubiquitination, lysine sumoylation, ADP ribosylation of glutamic acid, deimination, and proline isomerization. Modification of histone tails may change parameters of structural compaction, create a defined platform for regulatory interactions with proteins, serve as a “zip code” in higher-order nuclear organization, or alter the kinetics of enzymatic interactions with chromatin during transcription, repair or replication.

In general, acetylation of histones may induce relaxed chromatin and is considered an activating mark, while deacetylation compacts chromatin and is correlated with repression of gene expression. This modulation of chromatin relaxation is in part biophysical, as lysine is positively charged and interacts with negatively charged DNA to form a compact structure. Acetylation of lysine residues removes the positive charge and reduces interactions between histone proteins and DNA. This idea is supported by correlation between functional readout and acetylation state: histones at an actively transcribed gene (discussed in more detail below) are highly acetylated, while histones at a repressed gene, within the same cells, are deacetylated (Hebbes *et al.*, 1988). Further, DNaseI-sensitive regions of chromatin, which are actively transcribed (Weintraub and Groudine, 1976), are highly acetylated (Hebbes *et al.*, 1994). However, whether changing the charge of only lysine residues in histone tails is sufficient to substantially alter the conformation of DNA has been questioned (Peterson and Laniel, 2004).

Modified histone residues interact with specific, unique structural domains in a wide variety of proteins. These proteins therefore become Readers or Detectors of histone post-translational modifications (PTMs), and may also be Interpreters of PTMs to dictate access to chromatin. Specifically, bromodomains, found in many chromatin-associated proteins, bind to acetylated lysines. Similarly, methylated lysines are recognized by chromo, tudor, and MBT domains, as well as PHD domains (reviewed in Bottomley, 2004; Kouzarides, 2007). Post-translational modification of histones, and the subsequent binding of proteins to modified residues, may induce conformational changes in DNA, alter nucleosome positioning, and recruit downstream signaling proteins to regulate cellular processes.

The signaling effects of methylated histones are substantially more complex than acetylation, due to the fact that lysines can be mono, di, or tri-methylated, and arginines can be monomethylated or di-methylated, symmetrically or asymmetrically. Therefore,

methylation offers greater breadth in regulatory possibilities and may be either an activating or repressing mark, although arginine methylation is generally associated with activation. The methylation state of specific residues within histone tails is correlated with actively transcribed or repressed genes, as well as with particular stages of transcription, *e.g.* initiation, elongation and termination. Levels of trimethylated histone H3 at lysine 4 (H3K4me3) are highest at the core promoter and transcription initiation region of active genes. Procession of RNA polymerase II through elongation is marked by methylation of H3K36 (H3K36me) and H3K4 dimethylation (H3K4me2), followed by monomethylation of H3K4 at the terminus of transcription. Acetylated H3K9 (H3K9ac) and H4ac appear along broader regions of active genes, concentrated at the 5' upstream and coding region, in opposition to H3K9me, which is generally associated with transcription repression (reviewed in Saunders *et al.*, 2006).

Regulatory outcomes of histone PTMs

Specific histone modifications characterize the broad classifications of chromatin: Euchromatin is either actively expressed or potentially inducible, and is associated with higher levels of methylated H3K4, H3K36, and H3K79. Heterochromatin is not actively transcribed and exists amid high levels of methylated H3K9, H3K27, and H4K20. Complexity is further increased by regulatory context. For example, while methylated H3K36 is associated with actively transcribed genes and elongation, it can also mediate recruitment of histone deacetylases to effect gene repression (reviewed in Berger, 2007; Kouzarides, 2007). Therefore, the “bar code” of histone modifications must be interpreted in a gene-specific and temporal manner.

The Writers of this bar code include a host of histone-modifying enzymes: histone acetyltransferases, methyltransferases, histone deacetylases, and histone demethylase enzymes (reviewed in Allis *et al.*, 2007; Kouzarides, 2007). These enzymes are often essential for normal embryonic development, and deletion of genes encoding chromatin and DNA modifying enzymes leads to a variety of phenotypes. Deletion of many histone modifying enzymes, including histone deacetylase *Hdac1* (Lagger *et al.*, 2002), is embryonic lethal. The majority of mutations in, or deletions of, histone-modifying enzymes have deleterious effects after implantation, suggesting important roles of these enzymes in regulation and control of specific gene expression at a specific time rather than global effects on transcription.

Developmentally significant roles of histone modifying enzymes include enzymatic modification of both histone and non-histone substrates, as well as mechanisms that do not rely on catalytic function. For example, the first identified lysine demethylase, KDM1 (LSD1), plays a critical role during development without a global increase in H3K4me2. Rather, its deletion is embryonic lethal due in part to its regulatory, post-translational modification of a non-histone target, DNA methyltransferase 1 (DNMT1), and profound effect on global DNA methylation (Wang *et al.*, 2009; Wang *et al.*, 2007).

Deletion of different enzymes that modify the same residues may lead to completely different phenotypes, indicating that not only modification by the enzyme but the enzyme itself has a critical role in development. For example, deletion of the methyltransferase genes *Suv39h1* and *Suv39h2* together results in lymphoma development and chromosome segregation defects (Peters *et al.*, 2001). However, deletion of other H3K9 methyltransferases such as *G9a* or *Eset* is embryonic lethal (Dodge *et al.*, 2004; Tachibana *et al.*, 2002). Deletion of the acetyltransferase *Kat2a* (*Gcn5*) is embryonic lethal due to widespread apoptosis (Xu *et al.*, 2000; Yamauchi *et al.*, 2000). However, a mutation in *Gcn5*, which abolishes its acetyltransferase activity but maintains overall protein integrity, supports embryonic development to a later time point and displays different phenotypic

outcomes compared to genomic deletion of *Kat2a (Gcn5)* (Bu *et al.*, 2007). For recent reviews on this topic, see (Lin and Dent, 2006; Surani *et al.*, 2007).

Chromatin Remodeling Complexes

ATP-dependent chromatin remodeling complexes play a major role in regulated alterations of chromatin structure, and therefore have significant impact on regulation during development. All of the members of a large super-family of chromatin remodeling proteins contain a highly conserved ATPase subunit, which can be traced back to the founding members of this family: the SWI2/SNF2 proteins. This larger grouping of chromatin modifiers can be further divided into smaller subfamilies based on their sequence similarities and roles in cellular processes (Eisen *et al.*, 1995). The best-studied and well-characterized families are the SWI/SNF, imitation switch (ISWI), INO80, and Mi-2/CHD families. These proteins function as large complexes, and can remodel chromatin in a number of ways, including nucleosome eviction and repositioning, to either activate or repress transcription (reviewed in Cairns, 2005; Johnson *et al.*, 2005).

Numerous studies in both invertebrate and vertebrate animals have demonstrated a requirement for specific components of chromatin remodeling complexes in development of various organ systems and/or viability in general. Individual deletion of most SWI/SNF family members is embryonic lethal, which makes determination of their exact roles in cellular processes and development difficult. Many groups have turned to tissue or organ-specific deletions, which have shown a requirement for multiple chromatin remodelers in differentiation of a variety of tissue types (reviewed in de la Serna *et al.*, 2006).

Studies of myeloid lineage differentiation were the first to show that ATP-dependent chromatin remodelers regulate mammalian loci (Kowenz-Leutz and Leutz, 1999). During differentiation, the SWI/SNF ATPase BRM binds and is recruited by transcriptional regulators of myeloid-specific genes. However, BRM is not required for regulation of every myeloid-specific gene, illustrating selectivity and specificity in gene regulation. Additional insights into the roles of chromatin remodelers during differentiation and development come from studies of erythropoiesis, T-cell development, adipogenesis and myogenesis. Work in myogenesis was the first to demonstrate that muscle-specific gene expression is dependent on a SWI/SNF chromatin remodeling protein (de la Serna *et al.*, 2001). Importantly, this work demonstrates that failure to induce a myogenic phenotype is due to lack of chromatin remodeling at muscle-specific gene loci.

The diversity of phenotypes caused by mutations of different members of the SWI/SNF superfamily illustrates the complexity and importance of chromatin regulation during development. For more detailed reviews on the roles of specific chromatin remodeling proteins during differentiation and development, see (Brown *et al.*, 2007; de la Serna *et al.*, 2006; Kwon and Wagner, 2007).

DNA Methylation

One of the earliest identified forms of epigenetic regulation of gene expression is DNA methylation (Holliday and Pugh, 1975; Riggs, 1975). In eukaryotes, methylation occurs on cytosine residues, almost exclusively at dinucleotide CpG. Since CpG sequences are frequently clustered in proximal promoters of genes in higher eukaryotes, modification at these sequences can have profound effects on transcription. DNA methylation is associated with inhibition of transcription and a repressed chromatin state (reviewed by Miranda and Jones, 2007; Prokhortchouk and Defossez, 2008), as well as X chromosome inactivation (reviewed in Chang *et al.*, 2006; Hellman and Chess, 2007) and maintenance of imprinted genes (Li *et al.*, 1993). DNA methylation can cause gene silencing by interference with

recognition and binding of positively acting transcription factors or by promotion of interaction and recruitment of negative factors.

DNA methylation is accomplished by three enzymes, DNMT1, DNMT3A, and DNMT3B. DNMT1 is considered a “maintenance” enzyme, meaning it acts on hemimethylated DNA after replication to restore double-stranded DNA to a fully methylated state. DNMT3A and DNMT3B are *de novo* methyltransferases, meaning they act on unmethylated DNA. The individual deletions of *Dnmt1* and *Dnmt3b*, and the combined deletion of *Dnmt3a* and *Dnmt3b*, causes embryonic lethality in the mouse, while deletion of *Dnmt3a* alone results in live pups that are small and die at approximately 4 weeks of age (Li *et al.*, 1992; Okano *et al.*, 1999). These experiments demonstrate the critical role of DNA methylation during development.

De novo DNA methylation plays a critical role in the development of certain cancers, *e.g.* renal cell carcinoma, colon carcinoma, lung cancer, and many others. Methylation of DNA contributes to cancer development in multiple ways: First, tumor suppressor genes may become aberrantly methylated and transcriptionally repressed. This occurs in approximately 10% of retinoblastoma cases, where the RB1 tumor suppressor gene is silenced by promoter hypermethylation. Second, regions of DNA that are normally methylated, such as repeated sequences and imprinted genes, may become demethylated (reviewed in Kurkjian *et al.*, 2008; Tycko, 2000). Therefore, the balance between active and repressed genes, epigenetically maintained by DNA methylation, is critical for cellular homeostasis and normal development.

Techniques to Study Chromatin During Development

Recent advancements in technology, an increased focus on the “epigenome”, and groundbreaking discoveries of DNA and histone modifying enzymes that target chromatin, have yielded an explosion of new data in this area. The accelerated pace of understanding how chromatin directly and indirectly influences gene regulation is facilitated by expansion and improvements of techniques for analysis of epigenetic mechanisms.

Some of the earliest methods used to study chromatin employed specific nucleases, including restriction enzymes, micrococcal nuclease (MNase) and DNase I, to probe the accessibility of chromatin structure. In 1976 it was discovered that a chick globin gene (discussed below) was more sensitive to DNase I in red blood cells that express globin compared to cells that do not express the protein (Weintraub and Groudine, 1976). Nuclease-sensitive regions of DNA that have reduced nucleosome occupancy were discovered in viral genomes in 1978 (Scott and Wigmore, 1978; Varshavsky *et al.*, 1978) and in cellular chromatin a year later (Wu *et al.*, 1979). Sites of hypersensitivity to DNase I are indications of a perturbed chromatin structure and are generally associated with binding of transcription factors. Further, restriction enzymes that are methylation-specific have been used for many years, in conjunction with Southern blotting and later PCR, to detect methylation of genomic DNA within restriction sites.

In the past few years, new techniques have been developed that couple direct assessment of chromatin modification state, interacting proteins, DNA methylation status, and nucleosomal occupancy with genome-wide analysis. The ever-increasing opportunities in epigenetic research, along with specific challenges offered by these technologies which include Chromatin Immunoprecipitation (ChIP), ChIP-PET, ChIP-chip, ChIP-Seq, Methylated DNA immunoprecipitation (Me-DIP), and DNA adenine methyltransferase identification (DamID), are discussed below and compared in Table 1.

Chromatin Immunoprecipitation (ChIP)

Today, one of the most frequently used techniques to study chromatin during developmental processes is chromatin immunoprecipitation (ChIP). First described in 1988 by Varshavsky and colleagues (Solomon *et al.*, 1988) to demonstrate histone H4 binding to the *Drosophila melanogaster* heat shock protein 70 (*Hsp70*) gene, this technique has shown an exponential growth in use. ChIP assays provide a “snapshot” of what is bound to chromatin and/or the modification status of chromatin in tissues or cells at a specific point in time. The ChIP approach is widely utilized in analysis of cultured cells; its usefulness in studies of development was greatly expanded by Farnham and colleagues, who established that ChIP could readily be adapted to solid tissues (Wells and Farnham, 2002). ChIP assays can be used to examine any target for which an antibody can be raised, including transcription factors binding to chromatin, histone modifications and histones themselves (reviewed in Massie and Mills, 2008).

There are two primary types of ChIP assay: conventional and native ChIP. With conventional ChIP (also referred to as xChIP), DNA-protein interactions are fixed by addition of a crosslinking agent, most commonly formaldehyde. Crosslinked protein/chromatin is fragmented into smaller sizes by sonication and/or incubation with MNase, to sizes within a range of approximately 200-800 base pairs in length. Fragmentation efficiency is critical for efficient immunoprecipitation, and may be empirically determined by analysis of fragment size: A small amount of chromatin lysate is removed, purified to remove proteins and RNA, and then separated by agarose gel electrophoresis to compare against DNA size standards. Specific proteins of interest, including histones themselves, may be immunoprecipitated from the lysate. Precipitated DNA is then purified by extractions and removal of proteins, followed by reversal of crosslinking, to leave behind only the DNA previously associated with the protein of interest (the antigen). ChIP-fractionated DNA is further analyzed by Southern blotting, semi-quantitative conventional PCR, quantitative real-time PCR or more recent genomewide methods (Figure 1), to assess the fraction of DNA enriched by antibody precipitation in comparison to input, genomic DNA (Massie and Mills, 2008;Orlando, 2000;Orlando *et al.*, 1997;Solomon *et al.*, 1988).

In a native ChIP (nChIP) assay, a crosslinking agent, which has the potential to induce artifacts or crosslink proteins that are not directly bound to DNA, is not utilized. Instead, chromatin is digested into smaller pieces with MNase, which will cut primarily in linker regions of chromatin but potentially any DNA that is not protected by nucleosomes. The nChIP is especially useful for the study of proteins tightly bound to DNA, such as histones, but is not a method of choice for weakly bound proteins or proteins of low abundance (DeAngelis *et al.*, 2008; O'Neill and Turner, 2003). For an overall review of ChIP methodology, see (Das *et al.*, 2004).

To study a protein or modification of interest across a developmental continuum, cells or tissues are harvested at different time points, immunoprecipitated for the factor of interest, and analyzed for the DNA-binding site of interest. Our laboratory used this approach to determine a role for specific histone modifications, present at regulatory regions of the alpha-fetoprotein (*AFP*) gene, in dictating *AFP* expression levels over a time course of liver development. We compared developmentally staged liver tissue of wild type and p53-null mice by ChIP analyses of histone PTMs and transcription factor binding to establish a developmental role for p53 in recruitment of specific histone modifiers (Nguyen *et al.*, 2005; Tsai *et al.*, 2008). This approach has been applied to study many tissues and even whole embryos to track changes in transcription factor binding and chromatin modifications across a developmental time course (for example, Dacwag *et al.*, 2009; Davie *et al.*, 2007; Park *et al.*, 2005).

The key factor in all ChIP-based methodologies is a high-quality, highly specific antibody. Extensive controls must be used to validate the specificity of each antibody and the ChIP methodology. Negative controls such as non-specific IgG, readily purchased from a number of suppliers, and analysis of a distal region of DNA, expected to be negative for the protein under analysis, are needed. A positive control to assess the quality of the chromatin lysate and efficacy of antibody precipitation is an antibody that recognizes the C-terminal region of histone H3, which is relatively free of histone PTMs (Nguyen *et al.*, 2005).

The traditional ChIP protocol, as presented here, forms the basis of numerous, recently developed methods that greatly expand the power of chromatin analysis. Although these methods, described below, magnify the breadth of chromatin study to encompass genome-wide analyses, the limits of ChIP studies and the need for well-characterized antibodies and essential controls remain. These newer methods begin with the same steps as a traditional ChIP, but immunoprecipitated DNA is analyzed by genome-wide approaches in ChIP-chip, ChIP-PET, and ChIP-Seq (see Fig. 1). The amount of data offered by these methods requires considerable expertise in bioinformatics analysis to assess the quality of the data and decipher meaningful trends and correlations. As always, an important consideration in selection of an appropriate method of analysis is the amount of material required for each specific determination (see Table 1).

ChIP-chip

Traditional ChIP technology has a strong limitation in that each analysis is restricted to predetermined candidate target sequences, analyzed by PCR with specifically designed primers. The desire to overcome this bias towards a specific sequence of interest and identify genome-wide transcription factor binding sites or histone modifications, led to the development of ChIP-chip (Clark and Shen, 2006; Huebert *et al.*, 2006; Loden and van Steensel, 2005; Ren *et al.*, 2000; Shivaswamy and Iyer, 2007). ChIP-chip (also known as ChIP-on-chip) is a combination of ChIP and microarray (chips) of genomic DNA fragments. To date, genomic microarrays, consisting of sets of selected promoters, a random representation of CpG islands or portions of continuous genomic sequences, have been used to screen numerous ChIP samples (reviewed in Weinmann, 2004). For example, since its first use by Ren *et al.* in 2000 (Ren *et al.*, 2000), this technique has been exploited to map c-myc and p53 binding sites in the genome (Dang *et al.*, 2006; Smeenk *et al.*, 2008), to reveal Oct4, Nanog and Sox2 transcriptional networks (Boyer *et al.*, 2005) and identify polycomb target genes (Boyer *et al.*, 2006; Lee *et al.*, 2006a) in mouse embryonic stem cells.

The basic difference between ChIP-chip and ChIP-PCR is the method used to analyze the precipitated DNA (Fig. 1). For ChIP-chip, immunoprecipitated DNA, enriched by traditional ChIP methodology, is eluted after reversal of the crosslinks, purified and the ends repaired with DNA polymerase to generate blunt ends. A linker is ligated to each DNA fragment to enable PCR amplification of all immunoprecipitated DNA fragments, and a fluorescent label (usually Cy5) is incorporated during amplification by ligation-mediated polymerase chain reaction (LM-PCR). Similarly, the input DNA, a sample of genomic DNA that was not enriched by immunoprecipitation, is labeled with another fluorophore (usually Cy3). Both immunoprecipitation-enriched (ChIP DNA) and input DNA pools of labeled DNA fragments are mixed and hybridized onto a microarray imprinted with oligonucleotide probes, representative of the entirety or part of a genome, or oligomers tiled across regions of interest (such as a given promoter). A positive signal is established when the signal intensity of ChIP DNA significantly exceeds that of the input DNA on the array. These results are then subjected to statistical analysis and the significance of enrichment, due to the presence of a specific protein bound to the examined region, is determined. The scope of a ChIP-chip application depends, in part, on the availability of arrayed slides, as discussed in detail elsewhere (Massie and Mills, 2008).

A crucial step for ChIP-chip experiments is obtaining sufficient material (approximately 1-5 μ g) for DNA microarray hybridization. Immunoprecipitation of abundant proteins, such as histones, readily provides the required amount of bound DNA, whereas the yield offered by transcription factors with a limited number of genomic binding sites is much lower. Possible alternatives to increase the enriched DNA are to either increase the amount of starting material or pool individual ChIP DNA samples. Another widely used, alternative approach is to enrich the starting material for hybridization by amplification. PCR based methods may display unwanted bias towards certain DNA sequences, especially in mammalian systems where large amounts of repeat sequences occur. Linear amplification methods, based on T7 RNA polymerase transcription (Liu *et al.*, 2003), prevents this bias and have been used successfully for ChIP-chip analysis of nucleosomes and histone modifications (Dion *et al.*, 2007). Recently, a two step T7-amplification protocol, which can accurately and reproducibly amplify as little as 0.4ng of IP-enriched DNA for microarray analysis, was developed and will likely prove helpful in analysis of transcription factor-enriched DNA (van Bakel *et al.*, 2008). The ChIP-chip assay is reviewed in detail in (Lee *et al.*, 2006b).

ChIP-chip studies, as discussed here, can be applied to identify unknown transcription factor binding targets (Ren *et al.*, 2000), map genome-wide protein binding profiles (Iyer *et al.*, 2001), detect the distribution of histone modifications (Bernstein *et al.*, 2002), and to localize enzymes that catalyze modifications of histones (Reid *et al.*, 2000). As with every technique, ChIP-chip also has its limitations: a requirement for large number of cells (typically $\sim 1 \times 10^7$) and diminished or no representation of distal binding sites. Although the problem of starting material can be resolved by amplification strategies, the question of distal binding sites must be addressed with more recently developed technologies that combine ChIP with parallel sequencing (ChIP-seq).

Other limitations of ChIP-chip include a higher ratio of false positive targets and high labor and expenditure costs. These are major challenges in routine applications of ChIP-chip methodologies to chromatin analysis. Despite being a simple streamlined method, there are technical aspects of the procedure that are difficult to control experimentally, which could complicate analysis. These factors, including fixation, epitope accessibility, antibody specificity and, more likely, choice of microarray, may influence the outcomes in any examination of chromatin components and histone modifications by ChIP-chip. These deficiencies of ChIP-chip are reviewed in more detail by Hanlon *et al.* (Hanlon and Lieb, 2004).

Despite these challenges, the ChIP-chip technique is a method of choice to uncover relationships between regulatory factor dynamics and chromatin organization. ChIP-chip is a powerful tool to generate global maps of nucleosome occupancy and histone modifications. Comparison of genome-wide histone occupancy and modification to maps of binding by different ATP-dependent remodelers and covalent histone modifiers may help to decipher functional specialties of individual remodelers.

ChIP-PET

Difficulty in designing microarrays for ChIP-chip, with unique oligonucleotides homologous to a large number of regions in complex higher eukaryotic genomes, prompted development of a new approach, ChIP-PET (paired end tag). In ChIP-PET, short sequence 'tags' of immunoprecipitated DNA fragments are cloned into a plasmid library and then directly analyzed by sequencing (Loh *et al.*, 2006; Wei *et al.*, 2006). The ChIP-PET technique uses the efficiency of sequencing short tags, rather than entire inserts, to enhance information content and thereby increase accuracy of mapping to the genome. This technique was exploited by Wei *et al.* to generate a global map of p53 binding sites across the human genome (Wei *et al.*, 2006). They characterized over 65,000 unique p53 binding

elements with high specificity from a saturated sampling of over 500,000 PET sequences, uncovering nearly 100 previously unknown p53 target genes. Similarly, ChIP-PET was used to establish the transcription regulatory networks of Oct4 and Nanog in mouse ES cells (Loh *et al.*, 2006). In contrast to ChIP-chip, ChIP-PET proves to be a truly genome-wide approach because ChIP-enriched DNA is systematically cloned and sequenced. ChIP-PET also enables the prediction of novel DNA motifs that mediate protein-DNA interactions. Despite these advantages over ChIP-chip, ChIP-PET is very demanding in terms of time and costs. A vast amount of sequencing is required to achieve sufficient representation of immunoprecipitated fragments, which makes this technique expensive and labor-intensive relative to ChIP-chip approaches.

ChIP-Seq

In analysis of complex higher eukaryotic genomes, ChIP-chip data tend to have low resolution and are often quite noisy; two shortcomings that ChIP-seq promises to surmount (Johnson *et al.*, 2008). As a consequence, ChIP-chip is being rapidly displaced by ChIP-seq in genome-wide discovery of histone modifications and novel transcription factor binding sites. The ChIP-seq method directly offers whole-genome coverage and both low analytical complexity and sensitivity, which increase the depth of sequencing analysis. The major objective of ChIP-seq data analysis is to find the genomic regions enriched in a pool of IP-fractionated DNA fragments. Chromatin immunoprecipitation assays coupled with direct ultrahigh-throughput DNA sequencing (ChIP-seq) overcome the limitations of available ChIP methodologies for analysis of gigabase-size genomes of vertebrates with high resolution (Johnson *et al.*, 2007). The traditional ChIP technique was linked to robust Solexa 1G-sequencing and comprehensive statistical analysis to map in-vivo binding sites of neuron restrictive silencer factor (NRSF, also known as REST) in the human genome (Johnson *et al.*, 2007). In addition to uncovering transcription factor binding sites at likely regulatory sites, similar to ChIP or ChIP-chip of gene promoters, ChIP-seq supports discovery of binding motifs across a breadth of genetic landmarks, e.g. transcription start sites, enhancers, introns and exons, and conserved noncoding features.

ChIP-seq encompasses ultrahigh-throughput DNA sequencing of immune-enriched DNA and may be used to define “interactome” dynamics in response to specific signaling stimuli or genetic mutations (Johnson *et al.*, 2007). This method differs from other genome-wide ChIP methods, such as ChIP-chip or ChIP-PET, in design, feasibility, efficiency and cost. Unlike ChIP-PET, ChIP-Seq does not involve plasmid library construction. In comparison to ChIP-chip, ChIP-seq does not exhibit any of the difficulties of array hybridization chemistry such as T_m (temperature at which 50% of double stranded DNA or DNA-RNA hybrids are denatured), secondary structure formation and cross hybridization. Moreover, ChIP-seq is not restricted to species for which genome microarrays are available, but rather provides feasibility for analysis of any species with a sequenced genome. ChIP-seq relies on the power of new sequencing platforms like Solexa (Illumina) and 454 (Roche) to perform sequence census counting assays. The goal is to identify the nucleic acid content of IP-enriched DNA and map it to the genome. A typical ChIP-seq experiment yields approximately 40 million reads/short sequence tags (25 bp each) corresponding to the ends of the immunoprecipitated DNA fragments. These tags are then used to make direct digital measurements of the sequence content of the enriched DNA and then utilized to map onto the reference genome. Most of these tags uniquely map to the sense or antisense strand of the corresponding genome sequence. By careful statistical analysis the desired sensitivity can be achieved to detect rare molecular species. These types of sequencing tools do not need prior knowledge of genetic landmarks and therefore provide unrestricted amounts of vital information.

ChIP-seq has been widely used to map genome-wide profiles of STAT1 binding to DNA (Robertson *et al.*, 2007), as well as SRF, GABP and NRSF (Johnson *et al.*, 2007; Valouev *et al.*, 2008), RNA polymerase II and CTCF (Barski *et al.*, 2007) and histone modifications (Barski *et al.*, 2007). ChIP-seq offers several advantages over the ChIP-chip technique in terms of cost, labor, sample size, and evades the requirement to design and manufacture tiled microarrays. The data obtained offers sufficient sensitivity to detect sites of lower binding affinity and may permit detection of mutations within binding sites, which will be helpful in pinpointing changes in transcription factor binding and gene regulation in disease.

Disadvantages of all ChIP assays are their requirement for large amounts of starting material and a highly specific, validated antibody. Further, the possibility of artifacts, induced by the cross-linking process, exists. Utilizing multiple antibodies that recognize the same target protein or modification increases confidence in results obtained from ChIP experiments. The required experimental conditions may be quite specific for each cell or tissue type and/or protein of interest; therefore, time must be spent to establish protocols for each experiment.

MeDIP

Recently, the traditional ChIP assay was adapted to examine DNA methylation. In the methylated DNA immunoprecipitation (MeDIP) assay, an antibody that recognizes 5-methylcytidine (5mC) is utilized, and immunoprecipitated material is analyzed by PCR or on a genome-wide scale by microarray. The MeDIP assay was first used to determine methylation profiles in normal and transformed cells (Weber *et al.*, 2005). The authors found that DNA methylation profiles of male and female autosomal chromosomes are highly similar but the inactive X chromosome is globally hypomethylated. Further, they identified new genes that are aberrantly methylated in cancer cells (Weber *et al.*, 2005). MeDIP has recently been used to uncover genome-wide methylated DNA in both humans and plants (Weber *et al.*, 2007; Zilberman *et al.*, 2007). The assay could also be used to examine global methylation patterns across a developmental time course from animal tissues or embryonic stem cells during differentiation.

DamID

DNA adenine methyltransferase identification, or DamID may also be utilized in the study of chromatin interactions during development. First described in 2000 (van Steensel and Henikoff, 2000), this technique tethers the *Escherichia coli* DNA adenine methyltransferase (Dam) to a DNA/chromatin-binding protein. When this tethered chromatin protein binds to its consensus sequence, the Dam enzyme methylates adenines in the surrounding area. This DNA then has a signature that can be analyzed for methylation by methylation-specific restriction enzymes or antibodies. DamID is therefore utilized to identify regions of DNA where a potential interacting partner binds *in vivo*. The developers of DamID first verified the efficacy and accuracy of the assay by tethering Dam to the budding yeast gene Gal4 and introducing the fusion protein into *Drosophila melanogaster*. These flies were crossed with flies in which the Gal4 binding sequence had been introduced, and DNA methylation was verified at these known sites. The authors then utilized DamID to demonstrate binding sites of the endogenous *Drosophila* HP1 protein. These same authors later reported large-scale mapping of *in vivo* binding sites of three different chromatin proteins in flies (van Steensel *et al.*, 2001). The assay has also been adapted for cultured mammalian cells (Song *et al.*, 2004; Vogel *et al.*, 2006). For a recent review on DamID, and a comparison to ChIP, see (Orian, 2006).

Several advantages of DamID exist, including the fact that a specific antibody is not required. In addition, crosslinking agents, with their potential problems, are not necessary for this approach. Further, the Dam protein is small and can be fused to either end of the

protein of interest. Conversely, the Dam enzyme is very active; therefore, control experiments with the Dam enzyme alone must be performed, keeping the expression level of the fusion protein as low as possible. In addition, as is especially pertinent in developmental studies, adenine methylation will continue to accumulate over the lifespan of the organism, meaning that previous interactions may be detected with this technique (Southall and Brand, 2007).

Chromatin Regulation in the Pluripotent State

The ultimate example of developmental potential and tightly regulated control of cell fate is the pluripotent embryonic stem cell. Much recent work is focused on how stem cells maintain their pluripotency during self-renewal, and then respond to extrinsic and intrinsic signals by differentiation into specific cell types. The mechanisms by which this occurs are as of yet incompletely understood; however, many of the proposed models include a major role for chromatin in this regulatory paradigm.

Polycomb protein regulatory complexes

Embryonic stem cell plasticity and pluripotency appear to be determined in part by large protein complexes called Polycomb complexes, which play multiple roles in histone modification (Boyer *et al.*, 2006; Bracken *et al.*, 2006; Lee *et al.*, 2006a). Polycomb group (PcG) proteins are chromatin regulators that function as transcription repressors and were first identified in *Drosophila*, where they repress the homeotic genes (*Hox*) controlling segment identity in a developing embryo (Lewis, 1978; Pirrotta, 1998). Mutation or deletion of PcG gene(s) leads to a variety of developmental abnormalities in *Drosophila*. PcG complexes maintain *Hox* genes in a repressed state through epigenetic modifications of chromatin structure, and these functions are conserved in vertebrates (Akasaka *et al.*, 1996; Core *et al.*, 1997; del Mar Lorente *et al.*, 2000; Ringrose and Paro, 2004; Sparmann and van Lohuizen, 2006; van der Lugt *et al.*, 1994).

Several mammalian PcG members are implicated in control of cellular proliferation and neoplastic development (Sparmann and van Lohuizen, 2006). PcG proteins form three different types of complexes, known collectively as Polycomb Repressor Complexes (PRCs). These multi-protein complexes are PRC2, PRC1 and Pleiohomeotic (PHO) repressive complex (PHORC); each with a distinct role in repression of chromatin. PHORC binds to specific regions of DNA, followed by recruitment of PRC2 and PRC1. PRC2 catalyzes H3K27 methylation resulting in PRC2-mediated, epigenetic gene silencing. H3K27 methylation provides a binding surface for PRC1, which facilitates oligomerization, condensation of chromatin and inhibition of chromatin remodeling activity to maintain gene silencing (for a recent review, see Schuettengruber *et al.*, 2007). Together, PRCs are vital in maintenance of genes that antagonize pluripotency in a repressed state; thus, safeguarding stem cell identity during self-renewal.

Stem cell-specific challenges in epigenetic regulation

From this pluripotent state, embryonic stem cells differentiate in a lineage-specific manner and these demands are reflected in the global chromatin state of embryonic stem cells. Multiple laboratories report that chromatin of embryonic stem cells is generally more “open” and less condensed than differentiated cells. Differentiation is marked by an increase in a repressive histone modification, trimethylated H3K9 (H3K9me3), and a decrease in global H3 and H4 acetylation (for examples, see Keohane *et al.*, 1996; Lee *et al.*, 2004; Meshorer *et al.*, 2006).

Seminal studies, utilizing ChIP-seq, suggest a chromatin-based mechanism by which the embryonic stem cells maintain their pluripotency and address the challenges of lineage-specific differentiation. Specifically, stem cells contain regions of chromatin, termed bivalent domains, which simultaneously exhibit both “repressive” and “activating” marks of histone modification. This duality may allow developmental genes to be silenced in stem cells, yet poised for activation during differentiation of a specific cell lineage (Bernstein *et al.*, 2006). In this study, when profiles of genome-wide, histone modifications were compared between embryonic stem cells and other differentiated cell lines, such as primary mouse lung fibroblasts, mouse embryonic fibroblasts, myoblasts, and neuroblastoma cells, significantly fewer bivalent domains occurred in differentiated cells compared to ES cells (Bernstein *et al.*, 2006). Thus, the potentially conflicting presence of both activating and repressive modifications of chromatin in embryonic stem cells appears to restrict expression of lineage-specific genes while pluripotency reigns. Lineage-specific genes are maintained in a primed or poised state for expression, as appropriate during differentiation (Azuara *et al.*, 2006). Bivalent chromatin marks are also present in multipotent, hematopoietic stem cells/progenitor cells (HSCs/HPCs) (Cui *et al.*, 2009), which signify these marks as central to regulatory phenomena that safeguard “stemness”.

Numerous reports illustrate the defining role played by different epigenetic modifiers in regulation of a balance between pluripotency and differentiation (Keenen and de la Serna, 2009). Monoubiquitination of histones, known to play a critical role in regulation of chromatin structure during transcription elongation, acts in control of embryonic stem cell fate as well. The *Drosophila* gene *scrawny* (*scny*) encodes a ubiquitin-specific protease that deubiquitinates H2B and is required in germline, epithelial, and intestinal stem cells (Buszczak *et al.*, 2009). The data suggest that inhibiting H2B ubiquitination through *scny* represents a mechanism by which stem cells repress and avoid premature expression of key differentiation genes.

Determinations of regulatory mechanisms that establish and maintain the chromatin structure of a pluripotent stem cell, in comparison to alterations in chromatin structure during differentiation, illustrate the importance of epigenetic modifications and protein regulators (histone modifiers) during development. Recently, the presence of large H3K9me2-modified regions, specific to differentiated tissues and conserved between human and mouse, has been reported. These regions are termed large organized chromatin K9 modifications (LOCKs), which are substantially lost in cancer cells (Wen *et al.*, 2009). This correlation further cements the importance of epigenetic regulation in maintenance of homeostasis and differentiation. The number of reports demonstrating roles for chromatin and chromatin modifications in pluripotency, differentiation and development is likely to increase rapidly with the advent of new technologies to study chromatin. An illustration summarizing epigenetic characteristics of embryonic stem cells is shown in Figure 2.

Developmental Regulation of Tissue-Specific Gene Expression

The β -type globin genes

The β -globin locus offers one of the best-studied and well-understood examples of the regulatory relationship between chromatin and developmental expression of genes. Several features of this locus make it an excellent paradigm for the detailed examination of the relationship between chromatin and development, especially the fact that all vertebrates that utilize hemoglobin for oxygen transport express different globin genes at different stages of erythrocyte development. In humans, these genes are adjacent to one another and arranged 5' to 3' in a developmental progression of embryonic, fetal, and adult genes (reviewed by Orkin, 1995). Beginning in the early 1980s, it was observed that expression of the human β -globin genes is strongly influenced by the surrounding chromatin. First, evidence emerged

demonstrating that the switch from fetal to adult β -globin was associated with changes in chromatin structure that leads to loss of DNase hypersensitivity at the two fetal genes (Groudine *et al.*, 1983). Additional experiments (Forrester *et al.*, 1987; Forrester *et al.*, 1986; Tuan *et al.*, 1985) over the next few years revealed the presence of the “Locus Control Region” (LCR), a series of hypersensitive sites upstream of the β -globin genes, which have a major role in globin gene switching (reviewed by Stamatoyannopoulos, 2005).

Other observations regarding the chromatin surrounding the globin genes followed with the development of ChIP-based methodologies. In 1988, the first direct link between transcriptionally active chromatin (from chicken embryo erythrocytes) and core histone acetylation was provided (Hebbes *et al.*, 1988). The pattern of histone modifications at the globin locus in mammals are complex, and the details are beyond the scope of this review. Briefly, studies in mouse and human have shown that hyperacetylation of H3 and H4 exists at the LCR, which is always active, as well as the regions around the active genes at each appropriate developmental stage. Furthermore, dimethylation of histone H3 at lysine 4 generally follows the pattern of H3 and H4 acetylation (for more details, see reviews by Fu *et al.*, 2002; Kiefer *et al.*, 2008; Mahajan *et al.*, 2007). In addition to histone modifications, DNA modifications are present. An inverse correlation exists between methylation of CpGs found in the 5' regions of the β -globin locus and transcriptional activity (Kiefer *et al.*, 2008).

Another facet of the complex regulation of β -globin gene expression involves the recruitment of chromatin remodeling complexes to the β -globin locus, both to the promoter region (reviewed in Fu *et al.*, 2002) and to the LCR (Mahajan *et al.*, 2005). Each LCR element contains binding sites for both erythroid-specific, such as EKLF, and general transcription factors. These erythroid-specific factors can recruit ATP-dependent remodelers to the β -globin locus. For example, the SWI/SNF complex E-RC1 (EKLF co-activator-remodeling complex 1) was originally purified from mouse erythroleukemia cells, and includes the remodeling proteins BAF170, BAF57, BAF155, BRG1 and INI1 (reviewed in Fu *et al.*, 2002).

Lastly, alterations and modifications of chromatin structure are restricted to the β -globin locus in a tissue-specific manner by boundary elements. These DNA elements prevent spreading of structural changes effected by long-range regulatory elements, since silenced and active chromatin are often in close proximity (reviewed by Valenzuela and Kamakaka, 2006). At the β -globin locus in both mammals and chickens, the most 5' upstream, DNase I hypersensitive region in the LCR is a boundary element (Chung *et al.*, 1993; Li and Stamatoyannopoulos, 1994). This boundary element is constitutively DNase I hypersensitive, and insulates upstream DNA sequences from the powerful effects of the LCR.

The combination of LCR, chromatin modifications, boundary elements, chromatin remodeling enzymes and tissue- and stage-specific transcription factors, culminates in highly specific control of β -globin gene expression across development. How the globin genes are expressed only at certain stages of development and turned on and off sequentially has been widely studied, yet the exact mechanisms regulating globin “gene switching” remain incompletely understood and an active area of research.

The Albumin gene

The liver-specific albumin gene is initially activated by a chromatin-based mechanism of *de novo* binding by a “pioneer transcription factor” to silenced chromatin in the early mouse embryo. Seminal work by the Zaret laboratory led to a model of Forkhead transcription factor family member FOXA1 (for recent reviews on FoxA1, see Friedman and Kaestner, 2006; Kaestner *et al.*, 2000), and to a lesser extent GATA-4, binding to the albumin

enhancer present within highly compacted chromatin (Bossard and Zaret, 2000; Cirillo *et al.*, 2002; Gualdi *et al.*, 1996). The ability of FOXA1 to act in a pioneering fashion relies on its structural similarity to the winged helix domain of linker histones. Using *in vivo* DNase I footprinting and assays of *in vitro* reconstitution of nucleosome displacement, the Zaret laboratory developed a model where FOXA1 replaces linker histones, initiates a general opening of the albumin enhancer and enables binding of downstream activating proteins. Interestingly, the pioneering functions of FOXA1 are independent of any recruitment of chromatin remodeling complexes (reviewed in Cirillo and Barton, 2008). However, a recent report (Lupien *et al.*, 2008) examining FOXA1 recruitment to estrogen-regulated genes indicates that H3K4 dimethylation is correlated with FOXA1 recruitment to specific sites, and demonstrated that overexpression of the H3K4me1- and H3K4me2-specific demethylase KDM1 (LSD1) resulted in reduced FOXA1 recruitment. The authors conclude that the H3K4me2 mark is necessary to define the cell type-specific regions able to recruit FoxA1. Whether this also occurs at the albumin gene remains to be determined.

Other members of the forkhead family of proteins are now identified that serve a similar purpose (Cuesta *et al.*, 2007; Hatta and Cirillo, 2007). ChIP-chip analyses and bioinformatic interpretation, which finds chromatin-bound FOXA1 juxtaposed to estrogen receptor at a vast majority of estrogen-regulatory elements in breast cancer cells, suggests that pioneer activities by forkhead proteins may play a more global role in regulation of chromatin structure and expression (Carroll *et al.*, 2005; Lupien *et al.*, 2008). Use of the techniques described herein have led to a greater understanding of how chromatin plays a role in regulating Globin, Albumin and many other genes.

Discussion

Disruption of regulatory mechanisms that exert epigenetic control over expression and heritability is clearly linked to aberrant development and disease. Techniques to study chromatin and DNA methylation have advanced rapidly over the past decade. Development of high-throughput technologies provides essential tools to broaden analysis of epigenetic phenomena to a genome-wide scale. The new ChIP-based approaches provide exciting opportunities to address questions concerning epigenetic mechanisms and the identity of the genes directly regulated by an individual transcription factor.

Every technique has advantages and disadvantages that must be weighed to determine the best approach to answer the question at hand. With high-throughput, technologically advanced techniques come massive amounts of data. Therefore, one of the major issues to tackle is how to store, analyze, and correctly interpret the data generated by these new techniques. Many models and algorithms have been developed and reported over the past few years, each with its own advantages and disadvantages. For a few recent reports, see (Ji *et al.*, 2008; Ji *et al.*, 2006; Johnson *et al.*, 2006; Krebs *et al.*, 2008; Li *et al.*, 2008). In addition, it is becoming increasingly clear that, despite the push to develop more user-friendly and web-based programs, scientists with an expertise in bioinformatics to interpret and analyze the data will be in high demand.

Epigenetic changes and regulation are gaining more and more attention as the research community becomes increasingly aware of the role that chromatin plays in gene regulation, transcription, disease occurrence, and development. Understanding the changes that occur in chromatin during the course of development will not only contribute to our knowledge of normal gene expression, but will also add to our understanding of how gene expression goes awry during disease. For recent reviews on human disease and epigenetics, see (Hirst and Marra, 2009; Liu *et al.*, 2008). Understanding aberrant gene expression and its role in disease is a first step to developing new therapies and cures for human diseases, as well as

diseases that affect plants such as food crops. The expanding increase in use of the techniques described herein will surely contribute to the process.

Acknowledgments

Work in the Barton laboratory is supported by grants from the National Institutes of Health (GM081627 and DK070824). MEM is supported by the Training Program in Molecular Genetics of Cancer (T32 CA009299-29) and AKJ is a Laura and John Arnold Foundation Odyssey Fellow.

Contract grant sponsor: NIH, Contract grant number: GM081627, DK070824 and CA009299

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Figure 1.
Schematic representation of techniques used to analyze chromatin enriched by ChIP.

Figure 2.
Schematic representation of characteristics discussed in the text of chromatin in embryonic stem cells.

Table 1

Methods of chromatin and DNA methylation analysis

Technique	First used	Primary Question Addressed	Advantages	Limitations	Starting Material
DNase Sensitivity	1978	Accessibility of chromatin	Inexpensive	Limited to known regions of DNA	1-16 µg nucleosomes
ChIP	1988	Proteins bound to a specific region of DNA	Evidence of endogenous interaction at a specific gene	Requires a specific and validated antibody; crosslinking may induce artifacts	10 ⁶ -10 ⁷ cells
ChIP-chip	2000	Proteins bound to DNA on a genome-wide scale	Genome-wide analysis	Requires a specific and validated antibody; crosslinking may induce artifacts	1-5 µg DNA post-ChIP
ChIP-PET	2006	Proteins bound to DNA on a genome-wide scale	Genome-wide analysis	Requires a specific and validated antibody; crosslinking may induce artifacts; costly and labor intensive	1-2 µg DNA post-ChIP
ChIP-Seq	2007	Proteins bound to DNA on a genome-wide scale	Genome-wide analysis; high throughput	Requires a specific and validated antibody; crosslinking may induce artifacts	10-100 ng DNA post-ChIP
MeDIP	2005	Identify DNA methylation	Requires only one specific antibody	Does not address protein binding to DNA	4 µg DNA
DamID	2000	Identification of DNA-binding sites across the genome	Does not require antibody development	Dam-fusion proteins needed; DNA methylation is cumulative	Can be performed on a single fly