EPIGENETICS

TIMELINE

The molecular hallmarks of epigenetic control

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Abstract | Over the past 20 years, breakthrough discoveries of chromatin-modifying enzymes and associated mechanisms that alter chromatin in response to physiological or pathological signals have transformed our knowledge of epigenetics from a collection of curious biological phenomena to a functionally dissected research field. Here, we provide a personal perspective on the development of epigenetics, from its historical origins to what we define as 'the modern era of epigenetic research'. We primarily highlight key molecular mechanisms of and conceptual advances in epigenetic control that have changed our understanding of normal and perturbed development.

In 1942, Waddington coined the term 'epigenetics', which he defined as changes in phenotype without changes in genotype, to explain aspects of development for which there was little mechanistic understanding[1,2]. Almost three-quarters of a century later, we know that epigenetic mechanisms transduce the inheritance of gene expression patterns without altering the underlying DNA sequence but by adapting chromatin, which is the physiological form of our genetic information. Epigenetic mechanisms work in addition to the DNA template to stabilize gene expression programmes and thereby canalize cell-type identities. This importance of epigenetic control has long been recognized, but the enzymatic definition of distinct chromatin states that stimulate or repress gene activity was lacking.

Technological advances, such as chromatin immunoprecipitation followed by next-generation sequencing (ChIP–seq) and variations thereof, have enabled the analysis of the epigenome at or near base-pair resolution and allowed 'epigenomic profiling' in both normal and abnormal cells and tissues. In some cases, epigenomic profiling has served to better define critical DNA control elements, such as gene enhancers and promoters. When combined with DNA sequence analyses, insights into disease processes have been gained. Most of the known epigenetic modifications of chromatin are reversible, offering considerable promise for therapies drawing upon the adaptive nature of epigenetic control. Epigenetics has been and will continue to be one of the most innovative research areas in modern biology and medicine.

Here, we review the development of epigenetics from its historical origins to the 'modern era of epigenetic research', which we define as the past twenty years from 1996 to 2016. We describe seminal discoveries that culminated in the enzymatic definition of chromatin states that are representative of gene activity (euchromatin) and gene repression (heterochromatin), as well as mechanistic insights into the role of epigenetics in chromatin stability, gene regulation, transcriptional silencing and the reversibility of both histone modifications and DNA methylation. We provide an overview of how these mechanistic insights, in turn, have enabled a better understanding of cell-type identities by genome-wide chromatin profiling and have opened novel avenues for research into reprogramming, the response of chromatin to the environment, epigenetic therapy to improve human health and chromatin inheritance. We describe many — but by no means all — breakthrough discoveries and mainly highlight important mechanistic and conceptual advances. Seminal primary papers are cited, but for in-depth discussions and additional references the reader is at times referred to the textbook *Epigenetics*[3] or other timely reviews.

Foundation of epigenetics

Pioneering work carried out between 1869 and 1928 by Miescher, Flemming, Kossel and Heitz defined nucleic acids, chromatin and histone proteins, which led to the cytological distinction between euchromatin and heterochromatin[4] (FIG. 1a). This was followed by ground-breaking studies by Muller[5] (in *Drosophila melanogaster*) and McClintock[6] (in maize) on position-effect variegation (PEV) and transposable elements, providing early hints of non-Mendelian inheritance. Descriptions of the phenomena of X-chromosome inactivation[7] and imprinting[8,9] subsequently led to the general concept that identical genetic material can be maintained in different 'on' versus 'off' states in the same nucleus, but its underlying mechanisms were poorly understood.

DNA methylation. Chemical modifications of DNA bases were detected as early as 1948 (REF. 10) and a role for DNA methylation, in particular for 5-methylcytosine (5mC), in gene regulation was proposed in the mid-1970s by Holliday and Pugh[11], among others. By 1980, the functional connection between DNA methylation and gene repression was established[12], as was the existence of CpG islands[13]. The first 'epigenetic drug', 5-azacytidine (also known as 2′-deoxy-5-azacytidine and later called decitabine), which blocks DNA methylation, was used to alter gene expression and phenotypes in fibroblast cell lines[14]. Soon thereafter, Feinberg and Vogelstein[15] reported global DNA hypomethylation in cancer and, a decade later, local DNA hypermethylation of tumour suppressor genes was described — findings that were collectively reviewed[16]. These insights gave a compelling reason to pursue the 'enzymology' of DNA methylation. The successful purification and cloning of the mouse DNA (cytosine-5)-methyltransferase 1 (DNMT1) enzyme[17,18] and the generation

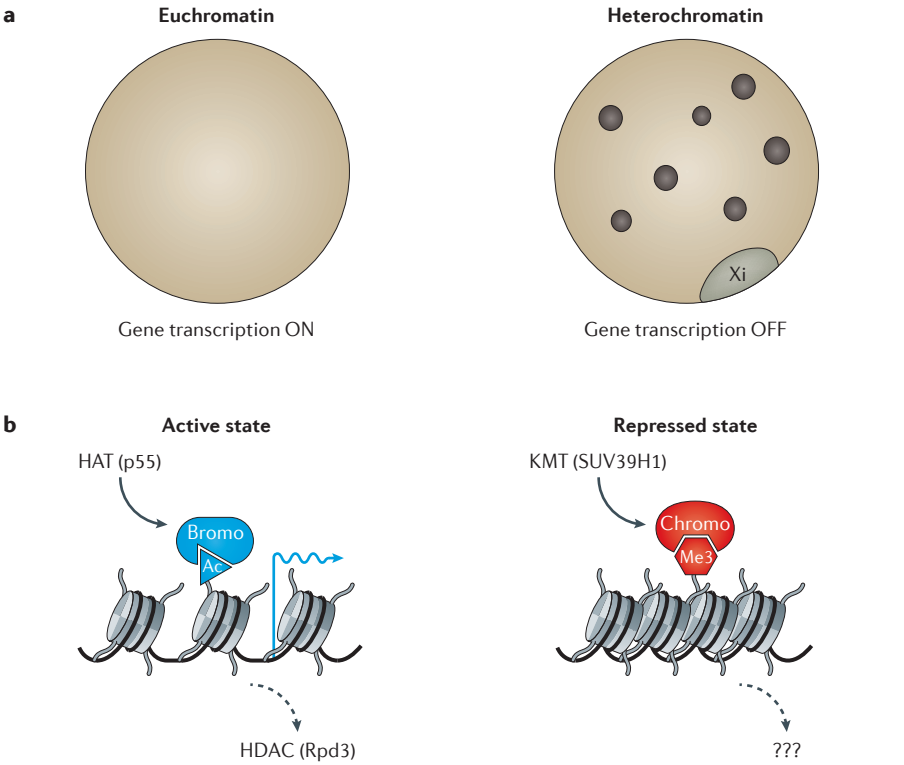


Figure 1 | **Euchromatin and heterochromatin. a** | Cytologically visible ground states of active (euchromatic) and repressed (heterochromatic) chromatin. Schematic representation of two interphase nuclei from female mouse somatic cells: the left nucleus displays broad and decondensed staining of unique DNA sequences and the right nucleus shows the characteristic heterochromatic foci (black dots) that are visualized by DAPI (4′,6-diamidino-2-phenylindole) staining of AT-rich repeat sequences. In addition, the densely staining Barr body (an inactive X chromosome (Xi)) at the nuclear periphery is indicated. In the early years, cytologists used various chromatin dyes and DNA-binding fluorochromes to discriminate euchromatic (decondensed and light staining) from heterochromatic (compact and dense staining) regions in eukaryotic chromatin. **b** | Enzymatic definition of chromatin states that stimulate gene activity (histone acetylation by p55 (also known as Gcn5)) or repress gene activity (histone methylation by Su(var)3–9 homologue 1 (SUV39H1)). In 1996, the nuclear histone acetyltransferase (HAT) p55 from *Tetrahymena thermophila* was described as a transcriptional co-activator that acetylates the histone H3 amino-terminal tail. The acetylated (Ac) lysine on H3 (H3K14ac) provides a docking site for bromodomain (Bromo)-containing accessory proteins that bind to and further stimulate nucleosome accessibility and transcriptional activity. Histone acetylation can be reversed by opposing histone deacetylases (HDACs), which often cause transcriptional repression. In 2000, the human histone lysine methyltransferase (KMT) SUV39H1 was described as an orthologue of a *Drosophila melanogaster* Su(var) position-effect variegation factor that methylates the histone H3 N-terminal tail. The trimethylated H3K9 (H3K9me3) provides a docking site for the chromodomain (Chromo)-containing heterochromatin protein 1 (HP1), which then impairs nucleosome accessibility and induces gene repression. The reversibility of histone lysine methylation was not known at that time.

and analysis of *Dnmt1*-mutant mice[19] proved important advances towards this goal. During the same time frame, the first DNA-methyl-binding protein, MeCP2 (methyl-CpG-binding protein 2), was identified[20]. DNA methylation and 5mC (considered the 'fifth base') had been firmly established as a crucial epigenetic mechanism in many, but not all, organisms.

The nucleosome. Studies by many groups led to the widely accepted model of nucleosomal organization of chromatin[21], which was first articulated in a provocative theory — the chromatin subunit model — put forward in 1974 (REF. 22) and visualized by the landmark X-ray crystal structure of the histone octamer–DNA particle in 1997 (REF. 23). As was shown, the basic unit of the chromatin fibre is the nucleosome core particle, which is composed of two copies each of four histone proteins (a histone octamer) that package 147 bp of DNA.

Histone modifications. In the mid-1960s, pioneering work of Allfrey[24] on histone modifications, in particular histone acetylation, led to the hypothesis that acetylation is closely linked to gene activity[25]. Many studies followed, including studies by Grunstein and others on histone-tail mutations in *Saccharomyces cerevisiae* that perturb gene silencing at telomeres and mating-type loci; this seminal work provided early functional evidence, including the first characterization of silent information regulator proteins[26,27]. Development of modification- or site-specific antibodies (for example, histone 4 lysine 16 acetylation (H4K16ac)) by Turner and others documented non-random patterns of histone acetylation, such as hypoacetylation of the inactive X chromosome in female mammals[28] or the silent mating type genes in yeast[29], as well as hyperacetylation of the twofold upregulated X chromosome in *D. melanogaster* males[30] or expressed β-globin genes in chicken red blood cells[31].

These major discoveries made a compelling argument that histone modifications, in addition to DNA methylation, carry information that can distinguish euchromatin from heterochromatin. Powerful genetic screens in flies[32,33,34], yeast[35,36] and plants[33,37] had identified other key factors for chromatin-dependent gene regulation, such as heterochromatin protein 1 (HP1), Suppressor of variegation 3–9 (Su(var)3–9), Enhancer of zeste (E(z)), Polycomb, Trithorax, cryptic loci regulator 4 (Clr4) and DECREASED DNA METHYLATION 1 (DDM1). However, the molecular function of these chromatin factors and how chromatin can 'switch' between euchromatic and heterochromatic states remained unknown.

Enzymatic definition of chromatin states

Gene activity and euchromatin. In 1996, using the ciliated protozoan model, *Tetrahymena thermophila*, Allis and colleagues[38] combined biochemical approaches with an in-gel assay to purify and clone the first gene encoding a transcription-associated histone acetyltransferase (HAT) from macronuclei — the active nucleus in this organism. Strikingly, this ciliate HAT (p55) was an orthologue of the previously described transcriptional coactivator Gcn5 from budding yeast, providing a direct link between histone acetylation and gene activity, and the yeast Gcn5 enzyme was shown to also exhibit HAT activity[38] (FIGS 1b,2). Interestingly, the ciliate enzyme contained active site residues found in other acetyltransferases (for example, cytoplasmic Hat1 in yeast)[39,40] and a highly conserved bromodomain, which was suggested by

Allis and colleagues[38] to direct chromatin recruitment in ways that remained unclear at that time. Conclusive evidence that targeted histone acetylation by Gcn5 leads to gene activation was provided in subsequent studies by Allis and his team[41]. Other HATs were identified, including TATA-box binding protein associated factor TFIID subunit 1 (TAF1; also known as TAF(II)250)[42], p300/CBP-associated factor (PCAF)[43] and CBP/p300 (CREB-binding protein and p300)[44,45], thus confirming and extending this paradigm to mammalian cells[46].

Approximately one month after the publication of the p55–Gcn5 HAT results, Schreiber and colleagues[47] reported the purification and cloning of the first histone deacetylase (HDAC), which they had identified by an affinity matrix using the HDAC inhibitor (HDACi) trapoxin. Remarkably, the mammalian trapoxin-bound protein was found to be an orthologue of the budding yeast transcriptional co-repressor Rpd3 (FIGS 1b,2). This landmark finding established that histone deacetylation is linked to transcriptional repression. Taken together, the 1996 HAT and HDAC work provided a compelling one–two punch that histone acetylation and deacetylation are directly coupled to 'on' and 'off' states of gene regulation, as had been first hypothesized by Allfrey[24].

The pioneering discoveries of HAT and HDAC led to increased interest in other proteins that might also exhibit these catalytic activities[46,48]. In 2000, Guarente and colleagues[49] demonstrated that a critical protein required for gene silencing in yeast, Sir2, was a NAD-requiring HDAC (FIG. 2). Subsequently, seven Sir2-like enzymes were identified in mammalian cells, which are now known as the Sirtuin protein family. Besides having distinct cofactor and catalytic requirements to the other HDACs, the Sir2-related HDACs triggered much research interest for their functions in metabolism and ageing, which are still under intense investigation today[50].

Despite remarkable progress, how histone acetylation functions to bring about an active chromatin state remained unknown. One long-held view was that histone acetylation regulated chromatin structure and gene activity by neutralizing the basic charge in histones, weakening interactions with DNA (*cis* effects). In 1999, Zhou and colleagues[51] documented the bromodomain from PCAF as an acetyl-lysine binding module for docking onto acetylated histones. This was the first histone-modification-binding domain to be described, suggesting a novel mechanism (*trans* effects) for the binding of bromodomain-containing factors to acetylated targets in chromatin (FIGS 1b,2). To date, a multitude of chromatin-binding modules have been described, many in atomic resolution with their cognate modified-histone ligands[46,52].

Gene repression and heterochromatin. The discovery of the first histone lysine methyltransferase (KMT) combined insights from dominant *D. melanogaster* PEV modifier factors containing an evolutionarily conserved SET domain, identified by Reuter[53], with cloning and characterization of mammalian orthologues by Jenuwein[54]. The SET domain is present in Su(var)3–9, E(z) and Trithorax proteins, all of which had been implicated in epigenetic regulation without evidence of enzymatic activity. Catalytic activity of the SET domain had been predicted by Jenuwein[55]; however, refined bioinformatic interrogation was needed to reveal a distant relationship of the SET domain with plant methyltransferases. Together with the exposed modulation of histone H3 phosphorylation by Su(var)3–9 homologue 1 (SUV39H1)[56], this insight suggested a crucial experiment: to test recombinant SUV39H1 for KMT activity

Glossary

Binary switches
The modification of adjacent or nearby histone residues affecting recognition and binding by reader proteins.

Cellular reprogramming
Conversion of a differentiated cell to an embryonic state.

Charge effects
The effect of post-translational histone modifications on altering the electrostatic interaction with DNA.

Enhancer of zeste
(E(z)). Originally identified in genetic screens for homeotic transformations in *Drosophila melanogaster* and later shown to encode a histone H3 lysine 27 (H3K27) methylating enzyme.

Erasers
Enzymes that remove histone modifications from chromatin.

Euchromatin
Light-staining, decondensed and transcriptionally accessible regions of the genome.

Heterochromatin
Dark-staining, condensed and gene-poor regions of the genome.

Histone cassettes
Short sequences in histone proteins with clustered histone modifications that direct the biological readout in a combinatorial fashion.

Imprinting
A chromatin state defined by whether the gene or genetic locus is inherited from the male or the female germ line.

Mating-type loci
Genetic elements in yeast containing mating-type information (a or α) that is activated by recombination from heterochromatic copies of one of the two mating-type alleles.

Multivalency
A property in which several histone modifications work together to increase the binding of reader proteins or the stability of a nucleosomal arrangement.

Polycomb
Originally identified in genetic screens for homeotic transformations in *Drosophila melanogaster* and later shown to encode a chromodomain-containing methylated histone H3 lysine 27 (H3K27me)-binding factor.

Position-effect variegation
(PEV). Stochastic and variegated expression of a gene due to juxtaposition to heterochromatic domains.

Readers
Proteins that recognize and bind chromatin through histone modification recognition domains.

SET domain
A 120-amino-acid signature domain for histone lysine methyltransferases (KMTs) that is conserved in Suppressor of variegation 3–9, Enhancer of zeste and Trithorax.

Silent information regulator proteins
A complex of *trans*-acting silencing proteins involved in establishing and maintaining heterochromatin in budding yeast.

Suppressor of variegation 3–9
(Su(var)3–9). Originally identified in genetic screens for position effect variegation in *Drosophila melanogaster* and later shown to encode a histone H3 lysine 9 (H3K9) methylating enzyme.

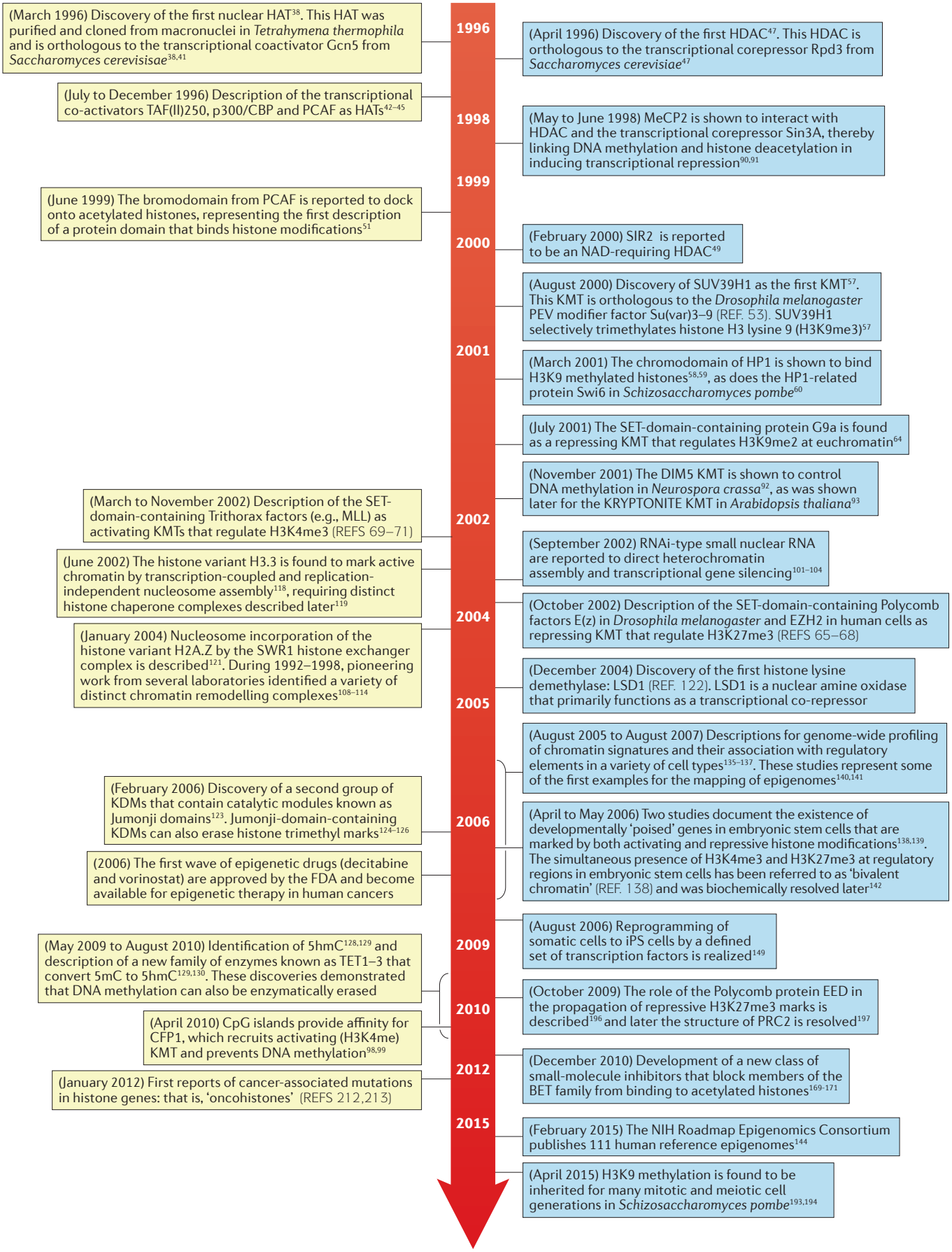
Topologically associated domains
(TADs). Large genomic regions promoting regulatory interactions by forming higher-order chromatin structures separated by boundary regions.

Transgenerational inheritance
Transmission of epigenetic information that is passed on to gametes without alteration of the DNA sequence.

Trithorax
Originally identified in genetic screens for homeotic transformations in *Drosophila melanogaster* and later shown to encode a histone H3 lysine 4 (H3K4) methylating enzyme.

Writers
Enzymes that add histone modifications to chromatin.

X-chromosome inactivation
A process in which one of the two X chromosomes is randomly inactivated in female mammalian cells early in development.



on histone substrates[57]. This experiment revealed robust catalytic activity of the SET domain of recombinant SUV39H1 to methylate histone H3 *in vitro*. Follow-up collaborative studies between the Jenuwein and Allis laboratories[57] revealed that SUV39H1 selectively methylates histone H3 on lysine 9 (H3K9me3); this is a rewarding example of epigenetic 'magic', as the genetic classification of the *Su(var)3–9* gene by Reuter in *D. melanogaster* apparently 'predicted' the enzymatic substrate- and site-specificity (H3K9 methylation) of the encoded enzyme. In 2000, Jenuwein published the discovery of SUV39H1 as the first KMT[57] (FIGS 1b,2).

Soon after the SUV39H1 KMT discovery, the chromodomain of HP1 was shown to bind methylated H3K9 (REFS 58,59), as did the HP1-related protein Swi6 in *Schizosaccharomyces pombe*[60] (FIGS 1b,2). Taken together, these findings established a biochemical explanation for the formation and propagation of heterochromatin that had been lacking since 1928 (REF. 4) and identified the enzyme class for histone lysine methylation that had been elusive since the 1960s (REF. 61). Underscoring its importance, the SUV39H1–HP1–H3K9 histone methylation system for gene repression and heterochromatin assembly is now known to be more conserved than DNA methylation and is present in unicellular organisms (for example, *S. pombe*), plants and invertebrates (for example, *D. melanogaster*), as well as complex multicellular organisms (mammals and humans)[32,36,37,62].

The SET domain of SUV39H1 provided a signature catalytic domain, and numerous SET-domain-containing proteins were then examined as potential KMTs. Approximately 50 genes encoding SET-domain-containing proteins are found in the mammalian genome, and many of these proteins have been studied extensively in the field[63]. Histone lysine methylation can either be repressive, such as SUV39H1-mediated H3K9me3 (FIG. 1b), or activating, such as H3K4 methylation. Other groups identified silenced or active chromatin states that are developmentally controlled (for example, H3K9me2 by G9a[64], H3K27me3 by Polycomb and Enhancer of zeste homologue 2 (EZH2)[65–68], and H3K4me3 by Trithorax and mixed-lineage leukaemia (MLL)[69–73] (FIG. 2)), as well as the repressive chromatin structure of the inactive X chromosome in mammals[74–78]. Even non-SET-domain-containing KMTs have been described that methylate non-tail sites (for example, DOT1L, which methylates H3K79 (REF. 79)). In addition to histone lysine methylation, histone arginine methylation has been associated with gene regulation, as the co-activator CARM1 (coactivator-associated arginine methyltransferase 1)[80] or the protein arginine *N*-methyltransferase 1 (PRMT1)[81] can mediate hormone-dependent transcriptional stimulation via H3R17 (REF. 82) or H4R3 (REF. 81) methylation.

Clearly, histone modifications are critically important for chromatin-dependent gene regulation. However, whether histone lysine methylation, like histone acetylation, was enzymatically reversible (that is, the discovery of lysine demethylases) was a remaining key question that would require more time and insights from others in the field.

The modern era of epigenetic research

It can be argued that the above studies, together with the development of new technologies such as genome-wide chromatin profiling, ushered in what we refer to as the 'modern era of epigenetic research', as evidenced by the multitude of publications in this field since the year 2000 (REF. 83). New meetings and initiatives supported a growing worldwide excitement over insights gained into the fundamental mechanisms that underlie epigenetic control. Chief examples include conferences specifically dedicated to epigenetics, by the Cold Spring Harbor Laboratory, the American Association for Cancer Research (AACR), the Gordon Research Conferences (GRC) organization, the Federation of American Societies for Experimental Biology (FASEB) and Keystone Symposia. In addition, several large consortia were launched, which connected many research groups in Europe (The Networks of Excellence 'The Epigenome' and 'EpiGeneSys'), the USA (US National Institutes of Health (NIH) Roadmap Epigenomics Project and ENCODE), Canada, Asia and worldwide (the International Human Epigenome Consortium (IHEC)).

Below, we describe major breakthrough discoveries that were brought forward between 2000 and 2016. These discoveries are chronologically ordered in FIG. 2, although we do not always present them instrict sequence but rather group them as coherent mechanistic and conceptual advances.

The histone code hypothesis and related theories. An ever-growing number of covalent histone modifications had suggested that the nucleosome carries epigenetic information[84]; however, it was unclear whether this information would be imparted by a *cis* or a *trans* mechanism. The bromodomain–acetyl-lysine binding discovery by Zhou and co-workers[51] in 1999 provided the first line of experimental evidence that led to the articulation of an influential hypothesis put forward one year later, known as the 'histone code hypothesis' (REF. 85). This theory proposed that combinatorial patterns of histone modifications specify distinct biological outcomes, in part by the recruitment of downstream effector proteins (called 'readers' to match the analogy of 'writers' for histone-modifying enzymes) or complexes in *trans*. The histone code hypothesis predicted that readers of other histone modifications would be identified. Indeed, many types of histone modification-binding modules have been recognized (for example, chromodomains, tudor domains and plant homeodomain (PHD) fingers), with structural insights explaining the binding specificity of respective ligands, leading to extensions of the histone code hypothesis, including its translation into a broader 'epigenetic code' (REF. 86). Other follow-up

◀ Figure 2 | **Timeline of major discoveries and advances in epigenetic research between 1996 and 2016.** The indicated discoveries and advances are detailed in the text and displayed as primarily 'activating mechanisms' (yellow boxes) on the left, or as primarily 'repressing mechanisms' (blue boxes) on the right. Notably, this is not a complete list. Months and years refer to the printed publication dates as indicated in PubMed (not online publication dates). 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; BET, bromodomain and extraterminal; CBP, CREB-binding protein; CFP1, CXXC-type zinc finger protein 1; DIM5, defective in methylation 5; EED, embryonic ectoderm development; E(z), Enhancer of zeste; EZH2, Enhancer of zeste homologue 2; FDA, US Food and Drug Administration; HAT, histone acetyltransferase; HDAC, histone deacetylase; HP1, heterochromatin protein 1; iPS cells, induced pluripotent stem cells; KDM, histone lysine demethylase; KMT, histone lysine methyltransferase; LSD1, lysine-specific histone demethylase 1; MeCP2, methyl-CpG-binding protein 2; MLL, mixed-lineage leukaemia; NIH, US National Institutes of Health; PCAF, p300/CBP-associated factor; PEV, position-effect variegation; PRC2, Polycomb repressive complex 2, RNAi, RNA-mediated interference; Su(var)3–9, suppressor of variegation 3–9; SUV39H1, Su(var)3–9 homologue 1; TAF(II)250, TATA-box binding protein associated factor TFIID subunit 1; TET 1–3, ten-eleven translocation 1–3.

extensions of these hypotheses included histone cassettes, binary switches[87] and the multivalency of effector–ligand binding reactions[88]. Although a healthy debate in the scientific community has questioned whether the covalent 'language' of histone modifications fulfils the requirements for being a true 'code' (REF. 89), the larger point is the unquestioned documentation that *trans* mechanisms of effector protein binding, in addition to *cis* mechanisms (charge effects), play a major part in histone and DNA modification readout.

Histone modifications and DNA methylation. The combinatorial nature of histone modifications also raised the question of whether histone modifications and DNA methylation were functionally linked. MeCP2 interacts with HDAC and the transcriptional co-repressor SIN3A to bring about transcriptional repression[90,91] (FIG. 2). Selker and colleagues[92], using a fungal model, *Neurospora crassa*, provided compelling evidence that histone H3K9 methylation (by the KMT DIM5 (defective in methylation 5)) is required for DNA methylation (FIG. 2). Subsequent studies supported these findings in plants[93], in which the H3K9 KMT KRYPTONITE controls DNA methylation. Here, a repressive pathway for the silencing of repeat elements uses H3K9me as a docking site for the DNMT chromomethylase. Multi-domain factors, such as ubiquitin-like PHD and RING finger domain containing protein 1 (UHRF1; also known as Np95) can bridge between H3K9 methylation and hemi-methylated DNA to stabilize DNMT1 (REF. 63). In a different way, the catalytically inactive DNMT3-like adaptor selectively binds through its ADD (ATRX, DNMT and DNMT3L) domain to unmodified H3K4 but is blocked by H3K4me3 chromatin[94]. The interdependence between histone modifications and DNA methylation for developmentally controlled gene expression[95,96] and for Polycomb-mediated silencing[97] has revealed a complex relationship. However, whether a distinct DNA sequence could direct the presence or absence of DNA methylation has remained unclear. Breakthrough findings in 2010 identified that CpG islands provide affinity for transcription factors, such as CXXC-type zinc finger protein 1 (CFP1), that recruit activating KMTs and prevent DNA methylation[98,99] (FIG. 2). Thus, CpG-rich DNA can target an active chromatin structure and protect it from *de novo* DNA methylation, even in the absence of ongoing transcription. Differences between low, medium and high levels of CpG DNA methylation can also explain whether distinct transcription factors gain access to their cognate binding sites[100].

Non-coding RNA and transcriptional gene silencing. Despite remarkable progress on histone and DNA modifications, little was known as to how these marks were added to specific genomic locations. A partial solution to this problem was provided by the discovery of small RNAs as potential 'templating' molecules for epigenetic machinery. In 2002, four groups, using fission yeast (Grewal[101] and Martienssen[102]) or *T. thermophila* (Allis[103] and Gorovsky[104]) models, reported the involvement of small RNAs in interacting with, and presumably directing, chromatin-modifying activities to genomic targets (FIG. 2). In contrast to double-stranded RNA (dsRNA) acting to inhibit gene expression by blocking the translation of messages in the cytoplasm, a process known as RNA-mediated interference (RNAi) or post-transcriptional gene silencing (PTGS), these small nuclear RNAs operate in a nuclear process known as 'transcriptional gene silencing' (TGS) (FIG. 3), guiding not only heterochromatin assembly and gene silencing in *S. pombe*[105] but also directing programmed DNA elimination in *T. thermophila*[106]. In both models, small RNAs were shown to interact with known components of the histone lysine methylation machinery, leading to provocative suggestions that these systems evolved to protect genomes from harmful DNA elements or viruses that might disrupt genomes if not properly silenced. In an extension of this model, non-coding RNA (ncRNA) transcription has generally been proposed as a genome-wide surveillance mechanism with roles in RNA quality control[107].

Although questions remain regarding the order of different molecular steps in these pathways, these findings underscore the notion that DNA, RNA and histone proteins, along with their modifications, act in a concerted fashion to bring about chromatin states that are important for dictating genomic functions. The TGS pathway represents a sequence-complementary mechanism for RNA-directed heterochromatinization, in which RNA signals back to DNA and establishes a repressed chromatin state that can be propagated across many cell divisions.

Nucleosome remodelling and histone variants. ATP-dependent chromatin remodelling complexes provided another important mechanism for altering histone–DNA contacts, promoting DNA accessibility and the exchange of new histones or transcription factors into and out of chromatin. During 1992–1998, elegant genetic and biochemical studies led to the identification of SWI/SNF[108–110], NURF (nucleosome-remodelling factor)[111] and other ATP-dependent nucleosome remodelling complexes[112–114], as well as giving early insights into their mechanism of action — an area of active epigenetic research to this day[34,115]. This is further stimulated by the high-frequency mutations of components of the human BAF (BRG1-associated factor) remodelling complex in cancer[116].

Excellent examples of the utility of these remodelling complexes are provided with the incorporation of an underappreciated chromatin component — histone variants. Histone variants, differing by only a small number of amino acids from their major canonical histone counterparts, were thought to be too minor and too similar in amino acid sequences to have important functional consequences[117]. Focusing on an H3 variant (H3.3) in *D. melanogaster* tissue culture cells, the H3.3 variant was found to be deposited into chromatin independent of DNA replication, and shown to be targeted preferentially into actively transcribed chromatin[118] (FIG. 2). Soon thereafter, biochemical approaches documented an H3.3-selective chaperone (HIRA)[119], one that was distinct from that of the major H3 (H3.1 and H3.2) deposition system (chromatin assembly factor 1; CAF1)[120] operating during the S phase. Moreover, the rapid exchange of histone variants into and out of chromatin brought about by dedicated machinery that recognizes select histone variants[121] adds support to the notion that histone variants provide a major mechanism to add strategic variation to the chromatin fibre[115] (FIG. 2). Even centromeric chromatin has its own specialized H3 variant (known in mammals as centromere protein A (CENP-A)), with evidence accumulating that CENP-A marks centromeres with their own epigenetic identity[117]. Other non-H3 histone variants have also gained considerable attention: for example, H2A.X, which, when phosphorylated, strongly marks DNA double-strand breaks in chromatin; H2A.Z, a variant enriched at transcription start sites where it is anti-correlated with 5mC; and macroH2A, a long H2A isoform originally found to be enriched on the inactive X chromosome[117] (FIG. 3). Clearly, the puzzle of chromatin-mediated epigenetic regulation had many pieces, but histone variants suggested that even some of the smallest pieces were important ones.

All chromatin marks are reversible. 'Erasers' were only known for histone acetylation (deacetylases) and phosphorylation (phosphatases), two major histone modifications with well-documented turnover properties that provide dynamic responses to the transcriptional needs of the cell. By contrast, erasers of histone methylation were not known, making it likely to be a 'permanent' histone mark — that is, one that might not be enzymatically reversible. This was an attractive concept: if histone methyl marks were stable epigenetic marks, they might be potentially inheritable. Shi and colleagues[122] shattered this concept when, in 2004, they identified and characterized the first histone lysine demethylase (KDM), LSD1 (lysine-specific demethylase 1), a nuclear FAD-dependent amine oxidase homologue (FIG. 2). Still, the inability of LSD1 to demethylate trimethyl-lysine histones raised the intriguing possibility that lysine trimethylation could be a permanent epigenetic mark. However, soon thereafter, Zhang and co-workers[123] disproved this notion by introducing the field to a second class of lysine demethylases, Fe(II) and α-ketoglutarate-dependent dioxygenases with catalytic modules known as Jumonji domains (FIG. 2). This class of enzymes was capable of removing trimethyl-lysine marks in histones[124–126], lending support to the general view that all epigenetic marks were probably reversible. As with other erasers, KDMs exhibited remarkable substrate and site specificity, and KDM research exploded onto a rapidly growing list of epigenetic regulators with important catalytic and regulatory functions.

Historically, there was also considerable confusion regarding the fundamental issue of the reversibility of DNA methylation, in particular with regard to the loss of 5mC. Developmental biologists had long described waves of genome-wide DNA demethylation that occur in the germline and in early embryogenesis[127]; however, the process by which DNA methylation is erased long remained elusive, leading to studies suggesting passive (diluted through DNA replication) versus active (enzymatically driven) processes. A crucial piece of this puzzle was provided with the identification of 5-hydroxymethylcytosine (5hmC)[128,129]

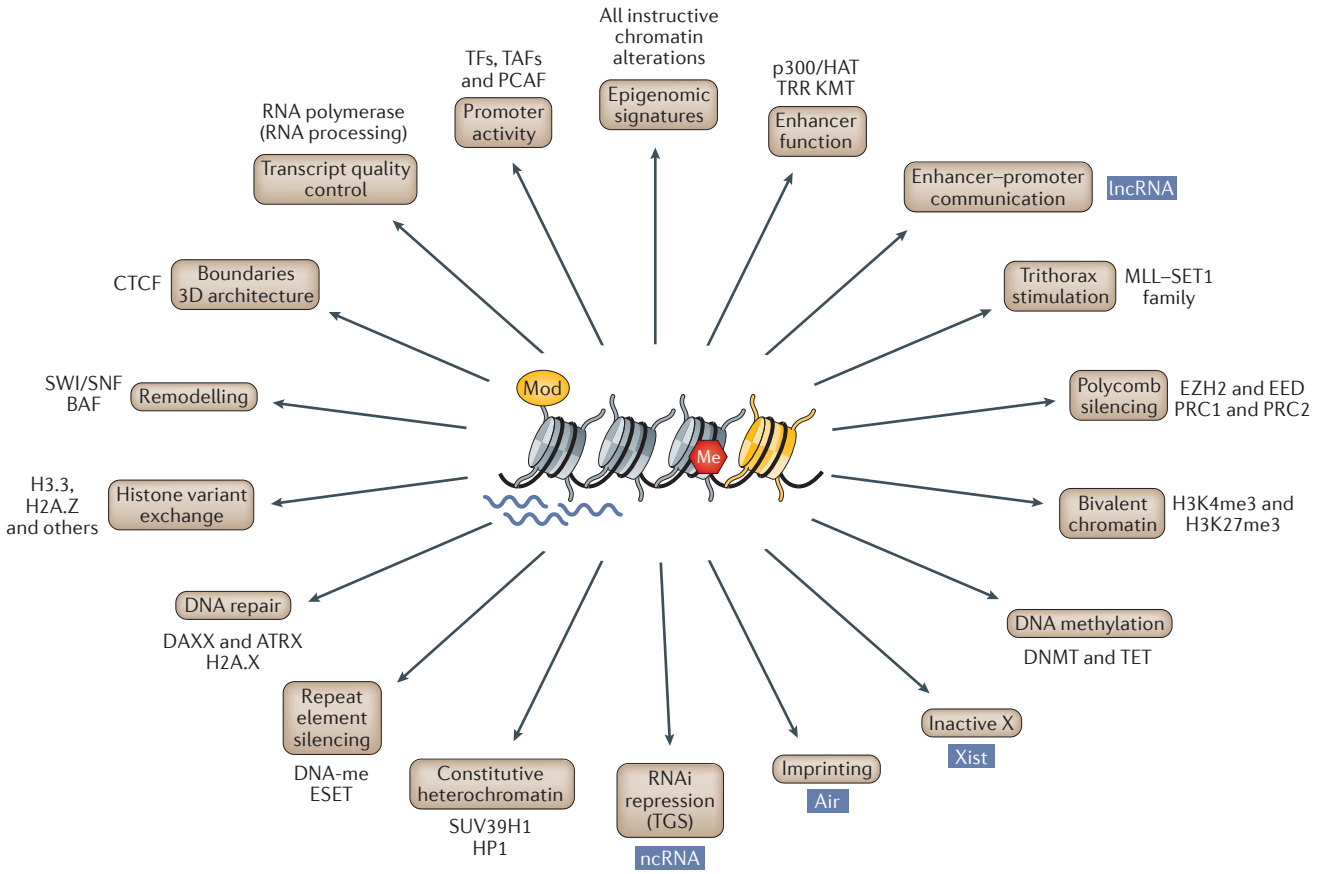


Figure 3 | **Key examples of chromatin contribution to epigenome function.** A chromatin template with four nucleosomes is depicted in the middle of the figure, together with chief mechanisms, such as histone modifications (Mod), DNA methylation (Me), histone variants and remodelling (yellow nucleosome) and non-coding RNA (ncRNA; wavy blue lines), that alter chromatin structure and function in an inter-dependent fashion. Distinct adaptations of this chromatin template have been associated with various functions of the epigenome (boxed examples). Also shown are some of the major chromatin factors that regulate these chromatin transitions. See text for details. Air, antisense insulin-like growth factor 2 receptor RNA; ATRX, α-thalassemia/mental retardation syndrome X-linked; BAF, BRG1-associated factor; DAXX, death-domain-associated protein; CTCF, CCCTC-binding factor; DNA-me, DNA methylation; DNMT, DNA (cytosine-5)-methyltransferase; EED, embryonic ectoderm development; ESET, ERG-associated protein with SET domain; EZH2, Enhancer of zeste homologue 2; H2A.X, histone H2 variant; H3K4me3, histone H3 lysine 4 trimethylation; HAT, histone acetyltransferase; HP1, heterochromatin protein 1; KMT, lysine methyltransferase; lncRNA, long non-coding RNA; MLL, mixed-lineage leukaemia; PCAF, p300/CBP-associated factor; PRC, Polycomb repressive complex; RNAi, RNA-mediated interference; SUV39H1, Su(var)3–9 homologue 1; TAFs, TATA-box binding protein associated factors; TET, ten-eleven translocation; TFs, transcription factors; TGS, transcriptional gene silencing; TRR, Trithorax related; Xist, X-inactive specific transcript.

(FIG. 2). Importantly, studies led by Rao[129] and Zhang[130] identified a new family of enzymes known as TET1–3 (ten-eleven translocation 1–3) (FIG. 2) with the ability to convert 5mC to 5hmC in an oxidation-driven reaction that generates other intermediates (that is, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC)). Enzymatic excision of these modified bases by DNA glycosylases may follow, leading to a fully demethylated DNA template[131]. As with histone methylation, DNA methylation is proving to have a rich repertoire of writers, readers and erasers. Clearly, the complexity of this covalent modification 'language' of DNA[62] is increasing as much as that of histones.

Bivalent chromatin and epigenomic signatures. By 2005, histone marks such as acetylation, phosphorylation and methylation stood out as a group of intensely studied histone modifications. Sensitive approaches such as mass spectrometry continued to reveal a staggering number of histone modifications, although many — if not most — of these were less abundant than the major marks[132]. Correspondingly, modification-selective antibodies were being developed and often used to examine 'your favourite gene' using ChIP assays. Several foresighted laboratories took on a different and powerful approach, pioneering variations of genome-wide ChIP to dissect epigenetic landscapes more broadly in normal and abnormal settings. Early versions of this approach that compared H3K4me2 (as an 'on' mark) with H3K9me2 (as an 'off' mark) were both informative and striking, revealing a conspicuous anti-correlation between these marks along large chromosomal domains[133,134]. Extending these studies to embryonic stem (ES) cells using ChIP–seq proved especially informative, in part owing to the ability of ES cells to be coaxed into defined differentiation pathways[135,136]. Soon, consistent patterns of histone marks emerged. H3K4me3, for example, was associated with active promoter elements, whereas H3K27me3 was enriched within developmentally controlled repressive chromatin states[137] (FIGS 2,3). Instructive epigenomic 'signatures' were beginning to emerge, causing an interest in genome-wide approaches that remains today.

Yet, in 2006, this appealingly straightforward on–off logic for histone marks proved far too simple. Unexpectedly, landmark studies by Lander[138] and Fisher[139] documented the classes of developmentally 'poised' genes in ES cells that carry both activating and repressive marks. The pattern of an overlapping presence of H3K4me3 and H3K27me3 has been referred to as 'bivalent chromatin' (REF. 138) (FIGS 2,3). The discovery of bivalent signatures of poised genes was unexpected and important. It provided the first indication of an 'intermediate' state, wherein bivalently marked genes could be resolved during development into active or inactive states. Bivalent chromatin is not specific to ES cells; it is well documented in other cell types[140,141].

How bivalent chromatin is established and organized at a nucleosome level remained an important issue. Are opposing H3K4me3 and H3K27me3 marks on the same H3 tail, on distinct H3 tails within the same nucleosome or on neighbouring nucleosomes? Elegant studies spearheaded by Reinberg[142] showed that the H3K4me3 and H3K27me3 marks do not exist on the same H3 tail, leading to an asymmetric distribution within the nucleosome (FIG. 2). This arrangement has implications for the establishment and propagation of bivalent domains.

Not unexpectedly, this complexity serves, in part, to fine-tune the marking of other genomic *cis*-regulatory elements in chromatin. Cell-type-specific 'active' enhancers, for example, are often defined by a subset of epigenetic marks such as H3K4me1 and H3K27ac[143]. Thus, histone modifications, when profiled at a genome level, revealed reproducible patterns that allowed predictions to be made regarding what genetic elements are functional (referred to as epigenomic profiling). Chromatin alterations (a collection of core histone modifications and DNA methylation) were co-mapped with nucleosome position and transcription factor binding sites and integrated with the overall RNA output of the genome (FIG. 3). Instructive histone modification patterns — such as H3K4me1 and H3K27ac in enhancer regions, H3K4me3 in promoter regions, H3K36me3 in transcribed regions, H3K27me3 in Polycomb-mediated repressed regions and H3K9me3 in heterochromatin regions (FIG. 3) — have been used by the NIH Roadmap Epigenomics Consortium and the IHEC to profile reference epigenomes and to compare epigenomic signatures in normal versus diseased cell states[144] (FIG. 2). New advances in technology now allow the analysis of single-cell epigenomes with more precision and new insights into cell lineage commitment[145,146]. Single-cell transcriptomes have extended earlier findings to reveal that almost the entire genome is transcribed, giving rise to a range of ncRNAs with distinct regulatory functions[147], which probably contribute to epigenetic landscapes in important ways that remain under active investigation.

Development and disease

From the many breakthrough discoveries and conceptual advances detailed above, molecular hallmarks of epigenetic control emerged that are important for cell-type identity and cellular reprogramming (FIG. 4). Most importantly, these hallmarks respond to developmental and environmental changes and are potentially reversible by chemical inhibition of chromatin-modifying enzymes and modification reader proteins (FIG. 4). Many other aspects of epigenetic response — for example, during metabolic fluctuations of a varying diet, circadian rhythms, ageing and in manifesting phenotypic diversification from the same genomic template (such as imprinting and twin studies) — have recently been summarized[3] and are beyond the scope of this article. Here, we focus on the role of the molecular hallmarks of epigenetic control in development (for example, reprogramming) and in some key examples of human disease that are being treated or have been shown to be responsive to epigenetic therapy, such as cancer, inflammation and the immune response (FIG. 4).

An epigenetic barrier to reprogramming. Epigenetic control is crucial for cell-type identity and cellular reprogramming. Pioneering experiments by Weintraub and colleagues[148] showed that a *cis*-acting transcription factor, MyoD (myogenic differentiation) — a factor crucially important for muscle differentiation — could reprogramme fibroblastic cells. Twenty years later, this logic resurfaced when Yamanaka and colleagues[149] 'wound back the clock', providing seminal mechanistic insights into the classic nuclear reprogramming experiments of Gurdon, Briggs and others[150] (FIG. 2). Their ground-breaking studies demonstrated that a small cocktail of defined transcription factors (now known as 'Yamanaka factors'), when expressed in differentiated adult somatic fibroblasts, would induce pluripotency, giving rise to induced pluripotent stem cells (iPS cells). The potential for reprogramming somatic cells from adult tissues had exciting implications for regenerative medicine, even if the iPS cells process was inefficient and not ready for use in humans. To what extent

does the chromatin state impede the ability of somatic cells to be reprogrammed? Increased reprogramming efficiency has been brought about by blocking H3K9me3 KMT[151,152] or by stimulating Jumonji KDM and TET enzymes with vitamin C[153], suggesting that heterochromatin stands as a likely barrier contributing, at least in part, to the inefficiency of these reprogramming events (FIG. 4). In support of this notion, 'pioneer' transcription factors have been identified, which are able to bind to repressive chromatin regions to recruit co-factors and chromatin regulators that are capable of inducing downstream gene regulatory cascades that overcome repressive chromatin effects[154,155]. The general view that lineage plasticity (changes in cellular identity) has an epigenetic underpinning and may have a vital role in reprogramming is supported by alterations in expression levels of chromatin-modifying enzymes and fluctuations of co-factors that change epigenetic machinery, causing switches in cell fate[156,157].

Cancer and epigenetic therapy. Most of the breakthrough discoveries mentioned above were not motivated by the need to have clear disease connections. Cancer research, for example, traditionally focused on genetic alterations (such as mutations, gene rearrangements and copy number variation) underlying tumorigenesis, leading to defined 'hallmark' capabilities exhibited by most cancers[158]. Early on, deviant epigenetic signatures (for example, DNA methylation) were found to have potential clinical importance in cancer, providing strong motivation to advance epigenetic therapies[16,48,159]. The application of chemical inhibitors of DNA methylation (DNMTi) by Jones and Baylin[159] to reactivate aberrantly silenced tumour suppressor genes, along with the use of HDACi, such as TSA (trichostatin A) and trapoxin developed by Yoshida[47], followed by the use of SAHA (suberoylanilide hydroxamic acid; also known as vorinostat) by Marks[160] in the clinic, paved the way for this exciting area of epigenetics[161] (FIG. 4).

In 2006, these concepts turned into reality as the first wave of US Food and Drug Administration (FDA)-approved epigenetic drugs (decitabine and vorinostat) became available for the treatment of human cancers (FIG. 2). Reversing epigenetic mistakes in people with promising clinical outcomes provided one of the most compelling arguments for the importance of epigenetics. A cottage industry of epigenetics-centred, reagent-based biotech companies was

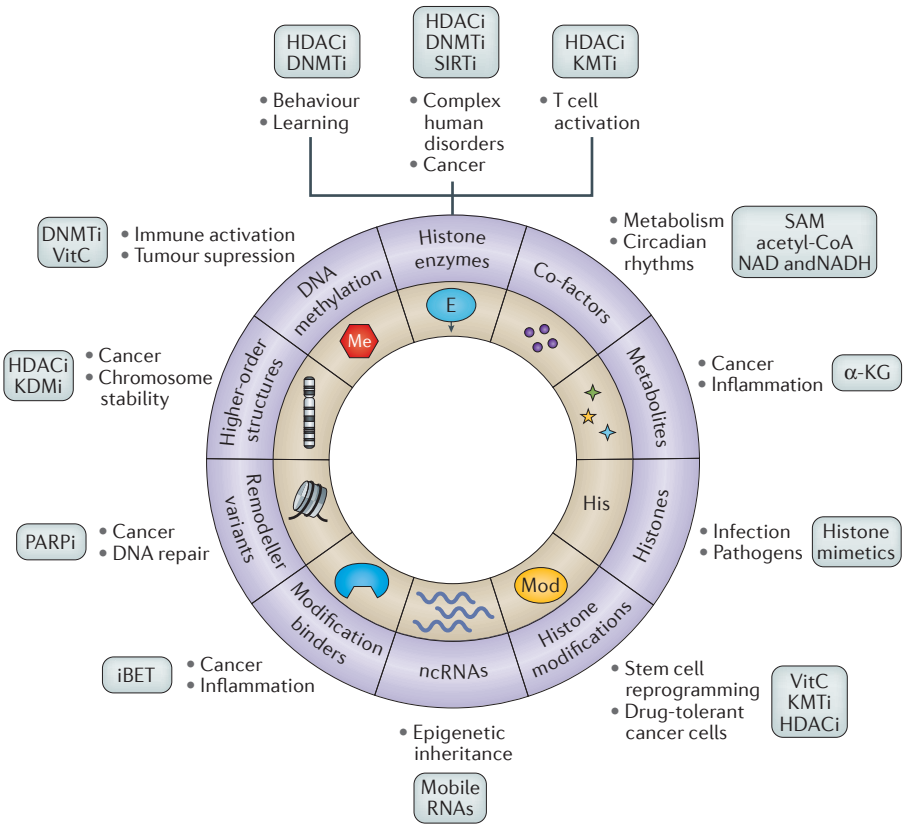


Figure 4 | **Molecular hallmarks of epigenetic control and examples for their medical relevance, together with possible therapeutic modulation.** Key mechanisms of epigenetic control and their associated co-factors are displayed in the circle. In contrast to 'hard' alterations of the DNA sequence (mutations), 'soft' adaptations of the chromatin template (modifications) are potentially all reversible. This distinction represents one of the key hallmarks of epigenetic control, providing a basis for 'epigenetic therapies'. Known examples for the relevance of epigenetic control in development and disease are indicated and are described in the text. Pharmacological intervention and possible reversal of dysregulated epigenetic control by small-molecule inhibitors (epigenetic therapy) (for example, histone deacetylase inhibitors (HDACi) or DNA methylation inhibitors (DNMTi)) or metabolic co-factors (for example, α-ketoglutarate (α-KG)) is shown by the small boxes. Although many of these treatments with epigenetic inhibitors have been shown to ameliorate disease in some clinical settings, some are still at an exploratory stage. Detailed information on the development and use of epigenetic inhibitors[48,159,168] and the function of metabolic co-factors[50,157] has recently been published. iBET, bromodomain and extraterminal inhibitor; KDMi, histone lysine demethylase inhibitor; KMTi, histone lysine methyltransferase inhibitor; ncRNAs, non-coding RNAs; PARPi, poly(ADP-ribose) polymerase inhibitor; SAM, *S*-adenosylmethionine; SIRTi, sirtuin inhibitor; VitC, vitamin C. Adapted with permission from REF. 158, Elsevier.

emerging. Even major pharmaceutical companies were getting into the action following the general notion that, unlike genetic alterations, mistakes made in epigenetic signatures would be reversible. Lessons learned from promising clinical outcomes with DNMTi and/or HDACi therapies provided a compelling argument that other classes of writers and erasers might also stand as worthwhile drug targets[162,163] (FIG. 4). Moreover, drug-tolerant cancer cells respond to combination therapy of HDACi and depletion of KDM, which ablates survival mechanisms and induces higher levels of DNA damage[164] (FIG. 4).

The general concept that cancer cells may have more fragile chromatin and higher 'epigenetic noise' (REF. 165) could explain why they are more susceptible to selective killing by treatment with epigenetic inhibitors combined with, for example, radiation therapy[166]. Although rapidly emerging literature has provided a wealth of links between epigenetics and other, non-cancer disorders[167] (FIG. 4), cancer stands as the most compelling disease that may respond to epigenetic therapy[159,168]. In 2012, certain types of cancer were even connected to 'driver' mutations in histones, referred to as 'oncohistones' (BOX 1; FIG. 2).

Box 1 | **Oncohistone mutations**

Histones are encoded in large multi-gene families, so cancer-associated mutations in histone genes were not anticipated. In close succession, however, results from the Jabado[212] and Baker[213] laboratories introduced 'oncohistones' to the scientific community in 2012. Many, but not all, of the heterozygous mutations were found in histone H3.3, the relatively minor H3 variant, suggesting some kind of 'dominant-acting' mechanism. An intriguing clue was provided by the observation that the mutations identified mapped at or near well-known sites of histone modification (for example, H3 lysine 27 (H3K27)). Oncohistone mutations act, in part, to inhibit the writers of these marks, such as Enhancer of zeste homologue 2 (EZH2), a subunit of Polycomb repressive complex 2 (PRC2), in the case of H3K27 methylation[214]. Other mechanisms are also possible and under active investigation. Moreover, when 'engineered' mutations (at select H3 lysine positions to mimic oncohistone mutations from patient tumours) are added back to human cells in *trans*, methylation at these sites is reduced, which is consistent with the prediction that this form of epigenetic dysfunction might be found in other cancer types[214]. In support of this, high-frequency histone mutations have been identified in other human cancers (for example, H3K36M mutations in chondroblastoma)[215]. Although all mutations in histone genes are classified as genetic alterations, the dominant involvement of these mutations in diverting the chromatin structure of cancer epigenomes makes it likely that additional mutations in histone-encoding genes will be uncovered at a rapid pace[216,217].

Histone-modifying enzymes, both writers and erasers, have proved to be attractive drug targets in oncology. Work by the Bradner[169], Tarakhovskly[170] and Kouzarides[171] groups added readers to the list of novel epigenetic targets and therapies. Here, a select number of bromodomain-containing proteins, such as members of the BET (bromodomain and extra terminal) family, proved druggable by small molecules (for example, the inhibitors JQ1 or iBET) (FIG. 2) that bound to the acetyl-lysine binding pocket in ways that disrupted critical protein–histone interactions. In keeping with other small-molecule inhibitors of HATs and HDACs, downstream responses were remarkably non-random. For example, one of the better-studied bromodomain proteins that is targeted by these small molecules is BRD4, which, in turn, functions in a transcriptional elongation pathway that is critical for the expression of key tumour-promoting oncogenes, such as *MYC*, and pro-inflammatory genes, such as *NFKB*, in haematological cancers (FIG. 4). Other chromatin readers are also being investigated with great interest now that a clear precedence has been established with bromodomain inhibitors[172,173].

Immune defence. The immune system is particularly enriched for chromatin-mediated gene regulation in the specification of cell lineages, response to external signals and induction of cellular memory[174]. Cells of the haematopoietic lineages integrate signalling through chromatin alterations that regulate gene activity[175,176] and can result in 'memory' of an activated state, as recently shown for macrophages[177]. Inflammatory signals (for example, lipopolysaccharide) lead to transcriptional activation of pro-inflammatory genes (for example, *NFKB*) that often are already poised by RNA polymerase II (Pol II) occupancy[178], to enable a rapid response. Stalled RNA Pol II is blocked for elongation and requires the PCAF–HAT elongation complex — a feature of many genes[179]. The selective response of *NFKB* to iBET is thought to suppress inflammation by perturbing engagement of PCAF to pro-inflammatory genes[170].

Epigenetic control is also important for the activation of immune cells and allows strengthening of an immune response by pharmacological treatment. Unexpectedly, the EZH2 KMT has been shown to transduce T cell activation by methylating cytoplasmic actin[180], providing a clear example that many histone-modifying enzymes have non-histone substrates. Also, HDACi can maintain T cell activation by preventing activation-induced cell death[181] (FIG. 4). Pharmacological inhibition of the G9a KMT and the released gene repression results in the activation of interferon genes and leads to increased resistance towards pathogens[182] (FIG. 4). DNMTi affects not only tumour suppressor genes but also repetitive elements that respond to decreasing DNA methylation. Low-dose treatment of several cancer types with DNMTi activates endogenous retroviruses, leading to a dsRNA-mediated immune response that then targets the tumour cells[183,184] (FIG. 4). Breaking immune tolerance and strengthening the immune response are proving to be two major mechanisms to combat cancer cells. Because nearly all histone-modifying enzymes also target many non-histone nuclear and cytoplasmic proteins[185,186], a notion first described by Roeder and colleagues[187] for the acetylation of the tumour suppressor p53, careful use and analysis of small-molecule inhibitors for clinical studies is required. Specifically, the balance between attacking tumour cells and not weakening the defending immune cells through epigenetic therapy needs to be cautiously considered.

Epigenetic control involving post-translational modifications of non-histone proteins further extends the modulation of functional chromatin output. For example, distinct modification cassettes in histones[87], particularly of the ARKS/T-type, are also found in several non-histone proteins and allow post-translational modification and recognition by reader proteins. A short histone mimic within the G9a KMT was reportedly required to trigger its activity by automethylation, giving rise to the general concept of histone 'mimicry' (REF. 188). The non-structural protein 1 (NS1) of the influenza virus harbours an amino acid sequence that is very closely related to the histone H3 N-terminus and can signal H3K4 methylation. The H3K4-like methylation in NS1 will divert PCAF and attenuate the transcription of antiviral genes[189]. Thus, histone mimicry is used by pathogen-derived proteins to suppress cellular defences (FIG. 4). These provocative findings have been used to formally propose that certain histone peptide mimetics could be developed into novel epigenetic drugs.

Chromatin inheritance (memory). A central question in the active debate on epigenetic research has been whether histones and their modifications are true carriers of epigenetic information. Unlike DNA methylation, or other modifications to the nucleic acid template, mechanisms of histone inheritance have remained unresolved, in part owing to long-standing debates as to how histones (old versus new) are segregated at the replication fork[190]. Early hints that chromatin transitions may be inheritable were provided by Grewal and Klar[191] in *S. pombe* and Paro and co-workers[192] in *D. melanogaster*. Recently, the Moazed[193] and Allshire[194] groups demonstrated that H3K9 methylation can be transiently induced and inherited for many cell generations in the absence of *cis*-acting transcription factors or DNA sequences used to target it (FIG. 2). Importantly, this chromatin inheritance requires the depletion of antagonistic factors

that counteract H3K9 methylation. Similar findings by Strome and co-workers[195] have revealed Polycomb repressive complex 2 (PRC2)-mediated H3K27me3 chromatin inheritance in *Caenorhabditis elegans*. Taken together, these studies suggest that, at least in these models, histone proteins can transmit their message, although the exact molecular details are under active investigation. To that end, Reinberg, Gamblin and colleagues[196] have shown that propagation of the repressive histone mark H3K27me3 is brought about by positive allosteric regulation of the PRC2 KMT complex through its non-catalytic subunit EED (embryonic ectoderm development) (FIG. 2). These studies and novel structural work on PRC2 (REF. 197) are important as they provide biochemical evidence for feed-forward loops in chromatin that may well contribute to the inheritance of histone modifications.

Can results like these be extended to the inheritance of epigenetic factors across multiple generations, that is, to the general phenomenon known as transgenerational inheritance? Indeed, new studies in fruitflies and mice suggest that changes in diet and other environmental factors, in particular the paternal diet, can reprogramme the metabolism of offspring in a way that is propagated to future generations, leading to obesity in these generations[198,199]. As with the experiments in *S. pombe*, alterations in histone methylation seem to be involved. As histone marks can influence enzyme systems responsible for *de novo* DNA methylation as well as the expression of ncRNA, other more-conventional nucleic-acid-templated mechanisms are likely to also enter into the overall epigenetics inheritance equation. In plants, the 'masters of epigenetic control' — mobile RNA — have been shown to be carriers of epigenetic information[200], and small RNA sequences have been used to reprogramme fertilized mouse oocytes[201]. ncRNA and tRNA fragments have recently even been detected in sperm[202–204], which suggests that more than the DNA sequence can be inherited (FIG. 4).

Outlook

The past 20 years have witnessed unanticipated progress in dissecting the molecular mechanisms of epigenetic control, with far-reaching implications for a better understanding of normal development and the treatment of human disease. Here, we advocate for a more refined definition of epigenomic signatures, drawing upon advances in single-cell analyses[205,146], but stress the need to discriminate between the cause or consequence of epigenomic alterations. To this end, we anticipate the continued use of CRISPR–Cas9 genome editing technology to allow more comprehensive dissections of genetic and epigenetic control. Chromatin dynamics can no longer be thought of as a one- or two-dimensional problem, as long-range interactions in three-dimensional space — giving rise to topologically-associated domains (TADs) and other chromatin territories that structure and organize the genome — are well documented, as are new approaches to define them[206–208] (FIG. 3). Furthermore, the central importance of ncRNA in many aspects of epigenetic control extends well beyond RNAi-mediated TGS and microRNA-dependent PTGS, and reveals an ever-growing number of chromatin-associated RNAs (for example, long ncRNAs, enhancer RNAs and repeat RNAs) that initiate and stabilize distinct chromatin states, even among alleles sharing the same DNA sequence. In fact, RNA can be considered as one of the 'master molecules' of epigenetic control, and important functions of ncRNA have been detailed in recent reviews[147,209].

Exploratory research into more complex human disease (for example, metabolic and neurodegenerative diseases) and habitual functions (such as learning and memory) is also predicted to reveal responsiveness to combinatorial epigenetic therapies as more precise inhibitors are being developed (for example, HDACi, DNMTi, iBET and sirtuin inhibitors) (FIG. 4). Moreover, novel experimental systems are being used to start analysing the contribution of epigenetics to behaviour and phenotypic polymorphism in social insects[210]. In particular, for metabolic disorders and environment-driven adaptations, chromatin appears to be the physiological template to integrate changing inputs[50,157,211]. Given the progress made between 1996 and 2016, we anticipate that many more discoveries will continue to reveal how chromatin adaptations organize and expose the information that is stored in our genome.

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Competing interests statement
The authors declare no competing interests.