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Bivalent Epigenetic Control of Oncofetal Gene Expression in Cancer

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ABSTRACT Multiple mechanisms of epigenetic control that include DNA methylation, histone modification, noncoding RNAs, and mitotic gene bookmarking play pivotal roles in stringent gene regulation during lineage commitment and maintenance. Experimental evidence indicates that bivalent chromatin domains, i.e., genome regions that are marked by both H3K4me3 (activating) and H3K27me3 (repressive) histone modifications, are a key property of pluripotent stem cells. Bivalency of developmental genes during the G₁ phase of the pluripotent stem cell cycle contributes to cell fate decisions. Recently, some cancer types have been shown to exhibit partial recapitulation of bivalent chromatin modifications that are lost along with pluripotency, suggesting a mechanism by which cancer cells reacquire properties that are characteristic of undifferentiated, multipotent cells. This bivalent epigenetic control of oncofetal gene expression in cancer cells may offer novel insights into the onset and progression of cancer and may provide specific and selective options for diagnosis as well as for therapeutic intervention.

KEYWORDS bivalency, cancer, epigenetic control, nuclear structure, oncofetal gene expression

Epigenetic control of gene expression plays a pivotal role in physiological responsiveness and is often compromised during onset and progression of cancer. Epigenetic changes are heritable but do not involve changes in DNA sequences. Within a given cell, there are many distinct carriers of epigenetic information that are relayed to progeny upon cell division. Epigenetic mechanisms include methylation of CpG residues, modifications of nucleosomal histone proteins, regulation of gene transcription and protein translation by noncoding RNA molecules, and mitotic retention of transcription factors (1–8). From an architectural perspective, epigenetic control is engaged at multiple levels of nuclear organization from sequence-specific regulatory elements to chromatin remodeling at the nucleosomal level to large-scale inter- and intrachromosomal interactions (9–14). These epigenetic mechanisms function in a complex but coordinated manner to orchestrate cellular responses to extracellular signals.

The cellular epigenetic landscape is dynamically modified by a number of posttranslational modifications of nucleosomal histones (1, 3, 15, 16). These modifications function in concert—a phenomenon described as the histone code—to establish context-dependent chromatin landscapes that control access of transcription factors to gene regulatory regions (1, 3, 15, 16). This review focuses on the bivalent chromatin landscape defined by addition of three methyl moieties to lysine 4 and lysine 27 residues of histone H3 (referred to as histone 3 lysine 4 me3 [H3K4me3] and H3K27me3 throughout this article). Chromatin bivalency, i.e., the presence of both activating H3K4me3 and repressive H3K27me3 modifications at gene promoters, was first ob-

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FIG 1 Emerging role of bivalent chromatin in establishing pluripotent or cancer state of cells. The schematic shows the capacity of pluripotent stem cells to give rise to various tissue types during development. Bivalency appears to contribute to cell fate decisions during the pluripotent stem cell cycle. Studies are needed to establish how much of the bivalent chromatin landscape is recapitulated in induced pluripotent cells. It is increasingly evident that a subpopulation of cancer cells exhibits partial recapitulation of bivalent chromatin domains. This oncofetal epigenetic control of gene expression may be a key determinant for cancer onset and progression.

served in pluripotent stem cells (17–19). Emerging evidence indicates that bivalency poises genes for expression and is likely a key regulatory mechanism in *trans*-differentiation during lineage commitment as well as in dedifferentiation during onset and progression of cancer (Fig. 1) (20–24). We propose that partial recapitulation of the bivalent chromatin landscape of pluripotent cells in some cancer types—termed "oncofetal epigenetic control" in this review— can offer novel avenues for the development of specific and selective diagnostic and therapeutic approaches.

BIVALENT EPIGENETIC LANDSCAPE

Histone posttranslational modifications as indicators and regulators of gene transcription. A fundamental feature of any cell that undergoes significant phenotypic change is a permissive epigenetic environment that supports rapid changes in transcriptional output, which in turn contribute to diverse processes, including asymmetric cell division, cell fate/lineage commitment decisions, and developmentally regulated tissue formation (2, 25–27).

All four histone core proteins, histone 2A (H2A), H2B, H3, and H4, are subject to widespread covalent posttranslational modifications that include methylation, acetylation, phosphorylation, ribosylation, deimination, crotonylation, butyrylation, N-formylation, and proline isomerization (28–30). These modifications generally occur on lysine, arginine, serine, and threonine residues located at N-terminal "tails" of histone proteins, although several sites of modification in globular histone domains within the nucleosome core have also been identified (31). In general, the N-terminal regions of histone proteins contribute minimally to the overall structural integrity of the nucleosome (31, 32). Instead, these regions exert their regulatory activity through (i) regulation of higher-order chromatin structure by controlling interactions between nucleosomes, as well as between nucleosomes and DNA, and (ii) recruitment of regulatory proteins and complexes to specific genomic positions and combinations of posttranslational histone modifications.

Methylation of histone 3 lysine 4 (H3K4) is a highly evolutionarily conserved modification that has been linked to a number of biological processes, including DNA repair, meiotic recombination (33), heritability of transcriptional states through cell divisions (34), and, most notably, marking of transcriptionally active genes near the transcription start site (TSS) (35–38). In addition to the specific role of trimethylated H3K4 (H3K4me3) within promoter regions, other H3K4 modifications are more widespread; monomethylation (H3K4me1), dimethylation (H3K4me2), and H3K4 acetylation signals outside promoter regions have been identified as markers of functional regulatory elements, such as enhancers (37–39). The enzyme complexes that methylate H3K4 are highly conserved among eukaryotes, and mammals have six nonredundant complexes that regulate H3K4 methylation [Su(var)3 to Su(var)9, enhancer of zeste, Trithorax 1A (SET1A) and SET1B, and <u>mixed-lineage leukemia 1 (MLL1)</u> to MLL4] (40). Deletion(s) or disruption(s) of SET family genes through translocations (and subsequent loss of H3K4 methylation) leads to cancer in vertebrates (40) and life span changes in invertebrate models (41) (see "Formation and Regulation of Bivalent Chromatin Domains" below for a detailed discussion of these enzymes).

Although there is strong evidence that the presence of H3K4me3 is indicative of active gene expression, it is clear that the H3K4me3 modification on its own is not sufficient to regulate gene activation (42). Alteration of endogenous *Drosophila* histone H3.2 genes to carry a nonmethylatable residue in place of Lys4 (H3.2K4A) resulted in only minor effects on global transcriptional activity (43). Similarly, deletion of histone-lysine *N*-methyltransferase 2F (KMT2F) and KMT2G cofactor CFP1 (CXXC finger protein 1) resulted in minimal changes in transcript levels (44, 45), and deletion of *SET1* in *Saccharomyces cerevisiae* had little impact on global transcription patterns (46) and, in some cases, resulted in a net upregulation of genes (47, 48). In addition, induced deposition of CFP1-dependent H3K4me3 at engineered, nonmethylated CpG islands was not sufficient to drive transcription in mouse fibroblasts (49). Taken together, these points suggest that the majority of H3K4me3 modified regulatory sites are not required for gene activation and that additional contextual cues are needed to regulate the initiation and maintenance of transcription (50).

Two marks are better than one: epigenetic bivalent domains provide transcriptional plasticity. Trimethylated H3K27 (H3K27me3) is normally associated with regions of the genome that are transcriptionally repressed by polycomb repressor complex 2 (PRC2). In embryonic stem cells (ESCs), H3K27me3 colocalizes with H3K4me3 modifications primarily near the TSS of developmentally regulated genes (17, 51) to form distinct bivalent domains. These bivalent domains act to restrict the expression of developmental genes during self-renewal and poise developmentally important, lineagespecific transcriptional regulators for expression in response to differentiation cues (25, 52). One of the hallmarks of bivalent domains is that they are frequently associated with CpG islands (52). Several studies have shown that CpG domains, whether methylated or unmethylated, may be sufficient to recruit PRC2 to methylate H3K27 (24, 53, 54). The association of H3K27me3 with CpG islands would indicate a role for H3K27me3 as a marker for DNA methylation. Although embryonic stem cell DNA is largely devoid of methylation, DNA in promoters marked with H3K27me3 was more likely to become methylated during differentiation than that in promoters lacking H3K27me3 (55). However, some PRC2-mediated, tissue-specific bivalency was observed independently of CpG islands (26). Disruption of H3K27 methylation by replacement of wild-type (WT) nucleosomes with methylation-deficient H3K27R mutant nucleosomes resulted in developmental aberrations due to a loss of Hox gene repression, similarly to the effects observed with PRC2 loss of function or deletion of the functional catalytic subunit of PRC2: enhancer of zeste homolog 2 (EZH2) (56–58). It has been suggested that silencing of lineage-specific genes is mediated by PRC2 complexes and H3K27me3 in early development and is maintained by the recruitment of DNMT1 and DNA methylation at bivalent sites to compensate for the reduced expression of PRC2 components that is common in differentiated tissues (59, 60) (see "Formation and Regulation of Bivalent Chromatin Domains" below for a detailed discussion of how the PRC2 complex deposits H3K27me3).

Given the important role for bivalently marked promoters in stem cells, it has been suggested that these bivalent domains are linked to oncogenesis. Cancer cell lines have fewer bivalent domains than normal cells, with higher variation and lower stability of the cell lines (20). Although there is a reduction in the number of bivalent sites in cancer through loss of H3K27me3, DNA at bivalent promoters was observed to be hyper-

methylated (20, 61, 62), whereas in normal cells, those promoters tend to have lower levels of methylation, which is accompanied by increased gene expression (63, 64). In the context of cancer, bivalency seems to be crucial for keeping cells in a differentiated state as loss of bivalent control at the developmentally regulated *HOX* genes plays an important role in both oncogenesis and tumor suppression (22, 65).

The role of acetylation in promoting bivalent chromatin has been largely understudied. Dysregulation or aberrant deposition of histone acetylation is a common trait of several malignant tumors and has been a successful target for therapeutic intervention (66, 67). Many of the histone residues that are methylated (e.g., H3K4, H3K9, and H3K27) can alternatively be modified by acetylation, generally promoting gene activation and antagonizing gene repression (68–74). Specifically, H3K4 acetylation may play a role in establishing or maintaining bivalent domains in certain cell types. It has been demonstrated that deacetylation of H3K4ac by histone deacetylase 3 (HDAC3) is an important regulator of marker gene expression in hypoxia-induced epithelium-tomesenchyme transition (EMT) (75). In breast cancer subtypes, H3K4ac was associated with estrogen-responsive and cancer-related genes at promoters that were also marked with H3K4me3 and H3K27me3 (76), suggesting that H3K4ac may be able to functionally replace H3K4me3 at certain promoters.

In search of true bivalency. Despite the emerging body of literature suggesting a fundamental role(s) of bivalency in gene regulation, key questions remain. For example, a significant amount of effort has gone into identifying chromatin regions that are truly bivalent; however, technological limitations present challenges for definitively establishing bivalency. Each nucleosome contains two copies of the core histone proteins H2A, H2B, H3, and H4; thus, there could be comodification (H3K4me3/H3K27me3) of one or both histone copies in the nucleosome. Several studies have shown, however, that the majority of nucleosomes demonstrate asymmetric modification, with a H3K4me3 mark on one H3 tail and a H3K27me3 on the other ("sister") tail (29, 77, 78). Although symmetric modifications have been shown to be symmetrically distributed (79, 80). Further complicating the issue of identifying bivalent chromatin domains, traditional chromatin immunoprecipitation sequencing (ChIP-seq)-based techniques are limited by the contribution of mosaic signals from a mixed population of cells (such as tumors) and of allelic differences within the same cell.

However, recent studies using sequential or combinatorial ChIP strategies have identified chromatin regions that are occupied by H3K4me3/H3K27me3 as well as other combinations of posttranslational modifications on the same nucleosome (81, 82). Currently, these studies are limited in scope because sequential combinatorial ChIP requires much more material (and is thus not always possible to carry out in human tumor samples), as well as technical understanding and the ability to analyze the resulting data sets (thus restricting these approaches to use by a few highly experienced researchers). Recently, there has been a significant technological advance in the ability to examine bivalency at the single-molecule/single-nucleosome level. Shema et al. showed definitively bivalent nucleosomes purified from pluripotent stem cells (77). Although outside the native context of the cell nucleus, a combination of this approach with fluorescence-activated cell sorter analysis to purify various subpopulations of tumors should establish the extent to which bivalency is truly recapitulated in cancer.

FORMATION AND REGULATION OF BIVALENT CHROMATIN DOMAINS

The H3K4me3 and H3K27me3 modifications are catalyzed by different histone lysine methyltransferases (KMTs) that are individually associated with the Trithorax group (TrxG) and polycomb group (PcG) complexes, respectively. While the precise mechanism(s) that targets these complexes to specific chromatin elements for deposition of bivalent marks is not clear, much progress has been made in understanding the regulation of the methytransferases that mediate bivalent epigenetic modifications. Here, we briefly review the proteins that catalyze the deposition of bivalent chromatin with a focus on the factors that regulate these modifications.

Forming the "active" component of bivalency: H3K4 methylation and KMT2 histone lysine methyltransferases. In mammals, methylation of histone H3 at lysine 4 is catalyzed by members of the histone-lysine N-methyltransferase 2 family (KMT2; also known as mixed-lineage leukemia [MLL]). The KMT2 enzymes are highly specific and catalyze the methyl transfer from S-adenosyl-L-methionine (SAM) to the ε -amino group of lysine 4 of histone H3, creating mono-, di-, and trimethylated H3K4me1/2/3 modifications. There are seven different KMT2 family members, six of which have been demonstrated to catalyze H3K4 methylation (KMT2A-2D and 2F-2G). Each KMT2 member has a characteristic catalytic SET domain (83-86). KMT2 enzymes form large multisubunit complexes that associate with distinctive sets of interacting proteins (87-92). Catalytic KMT2 complexes share a SET domain-mediated interaction with a group of proteins called the WRAD complex, which is composed of the following: WD repeat protein 5 (WDR5); retinoblastoma binding protein 5 (RBBP5); absent, small, homeotic discs 2-Like (ASH2L); and DumPY: shorter than wild-type 30 (DPY30) (93, 94). Depletion of individual WRAD subunits reduces KMT2 methyltransferase activity, indicating that the SET domain requires WRAD for methyltransferase activity (95-97). Interaction of KMT2 enzymes with WRAD is mediated via a WIN (WDR5 interacting) motif adjacent to the SET domain (98-102).

While different KMT2 members are important in regulating ESC pluripotency and early ESC differentiation, recent studies have clarified nonredundant functional roles of individual KMT2 proteins in mediating bivalent chromatin. Studies show that KMT2F and KMT2G (SETD1A and SETD1B, respectively) are likely responsible for H3K4me3 deposition globally at active promoters in ESCs (45). In contrast, KMT2B (MLL2) has been shown to serve as the principal H3K4 methyltransferase that marks bivalent promoters with H3K4me3 in mouse ESCs (103, 104). However, identification of the molecular mechanism(s) that guides methylation selectivity by the KMT2 enzymes remains elusive.

As discussed in the previous section, bivalent domains are frequently associated with CpG islands (105, 106). This observation may, in part, be explained by the finding that KMT2 proteins contain a DNA binding domain comprised of CXXC or zinc finger CXXC (ZF-CXXC) motifs that recognize hypomethylated CpGs and may play a role in targeting the DNA methylation complex(es) to bivalent domains (107). Furthermore, KMT2F and KMT2G are present in a complex with a CFP1 subunit which also includes a DNA binding domain that binds selectively to unmethylated CpGs (91). As bivalent domains are predominantly associated with CpG islands in ESCs, the CXXC portion of KMT2 proteins may come into play. Interestingly, CFP1 deficiency results in a drastic loss of H3K4 methylation levels at the promoters of highly expressed genes in ESCs but not at bivalent gene promoters (45). These findings together indicate that additional mechanisms are operative in directly linking DNA methylation with the enzymology of H3K4 trimethylation.

Forming the "repressive" component of bivalency: H3K27 trimethylation and polycomb repressive complex. Trimethylation at H3K27 is catalyzed by PRC2 (19, 51, 105, 108–110), which is comprised of four core subunits: suppressor of zeste 12 protein homolog (SUZ12), embryonic ectoderm development (EED) protein, retinoblastoma binding protein 4 (RBBP4), and EZH1/EZH2 (EZH1/2). EZH1/2 is the catalytic subunit that serves as the histone lysine methyltransferase for H3K27 (reviewed in reference 111). In ESCs, genes with bivalent domains are targeted by pluripotency factors, including Oct4 and Nanog. Recruitment of PRC2 to these targets is directed by PRC2-associated protein Jarid2 or Mtf2 (110, 112–115). In addition to their role in establishing a bivalent chromatin landscape in pluripotent cells (19, 51, 108, 109), PRC2 and H3K27me3 are implicated in numerous physiological processes, including differentiation, tumorigenesis, senescence, and X-chromosome inactivation (53). Despite documentation of PRC2-mediated chromatin modification in ESCs, the determinants responsible for directing PRC2 occupancy at specific genomic elements remain a focus of active investigation.

There are also studies suggesting an interplay between PRC1 and PRC2 (116, 117). For example, a subset of genes repressed by PRC2 is also bound by the PRC1 polycomb complex (105). The recruitment and involvement of PRC1 at these loci may support gene silencing, at least in some biological contexts. The PRC1 complex catalyzes

ubiquitination of histone H2A on lysine 119 (H2AK119ub), a modification that appears to contribute to transcriptional repression through RNA polymerase II pausing as well as by facilitating recruitment of PRC2. While PRC1 and PRC2 reinforce each other's recruitment to genes for repression, it is noteworthy that PRC1-mediated repression during early mouse development does not require activity of RING1B, a ubiquitin ligase and a core component of PRC1 (118). Unlike H2A ubiquitination, H2B ubiquitination (H2Bub1) has been shown to occur cotranscriptionally, is positively linked with gene expression, and accurately reflects the advance of RNA polymerase II in live cells (119, 120). Whether there is interplay between PRC1-mediated ubiquitination of H2A at lysine 119 and H2Bub at bivalent chromatin domains for tighter epigenetic control of gene expression remains to be established. Similarly, neither PRC1 nor PRC2 is required for recruitment of histone variant H2A.Z, which colocalizes with PRC2, to developmental genes in mouse embryonic stem cells (121). Together, these findings indicate that additional studies are required to identify the precise mechanistic underpinnings of transcriptional regulation by the two complexes.

REGULATORY CONSEQUENCES OF BIVALENT CHROMATIN

Research into the biological roles of bivalent chromatin is in its infancy. Establishing a direct link between bivalency and cellular functions has been difficult due to limitations of traditional genetic approaches that can modify only one histone modification at a time. Advances in clustered regularly interspaced short palindromic repeat (CRISPR)-based genome editing may lead to definitively understanding the functional consequences of bivalency. Nonetheless, some progress has been made in examining the effects of bivalency in biological control and in cancer. Here, we review examples that suggest a link between bivalency and cellular processes, including both transient (e.g., cell cycle control, responsiveness to cell signaling) and long-term (e.g., lineage commitment) processes, in normal and cancer cells (17, 20–26, 76, 122–125).

Transient gene regulation for lineage commitment: pluripotent cell cycle progression. Pluripotent stem cells provide a unique model system to investigate the role(s) of bivalency in cell cycle and lineage commitment for several reasons. Pluripotent cells have (i) a largely bivalent chromatin landscape, (ii) unlimited proliferative potential, (iii) a shortened G_1 phase (126, 127), and (iv) the ability to differentiate into any cell type. Recent findings from our group and others indicate that bivalent chromatin marks undergo dynamic reassignment during the pluripotent stem cell cycle. For example, bivalently marked genes that are maximally upregulated upon differentiation of pluripotent cells are enriched in H3K4me3 during mitosis, and after differentiation, H3K4me3 on these genes becomes cell cycle independent. Deposition of H3K4me3 is mediated by cell cycle-dependent recruitment to bivalent gene promoters of chromatin modifiers that are involved in H3K4 methylation and demethylation (125). Dalton and colleagues have shown that CDK2-dependent phosphorylation of the KMT2B (MLL2) histone methyltransferase results in its recruitment to developmental genes in G₁ (128). Recruitment of KMT2B and deposition of H3K4me3 result in interactions between distal enhancers and proximal regulatory sites in promoter regions of developmental genes. These chromosomal interactions modify the higherorder architectural landscape and lead to brief activation of these genes that is restricted to G_1 (128). Transient activation of developmental genes explains why stem cell differentiation is often initiated during the G1 cell cycle phase and provides a role for bivalent chromatin in cell fate determination following mitosis.

Role of bivalency in long-term regulation of cell phenotype: maintaining hematopoietic lineages. Hematopoietic stem cells (HSCs) give rise to diverse cell types of both the myeloid and lymphoid lineages. The survival of this wide array of hematopoietic cells depends on a delicate balance between self-renewal and differentiation at each successive stage of lineage commitment. This balance is controlled in part by epigenetic regulation of gene expression.

As discussed above, PRC2 plays a key role in deposition of the H3K27me3 mark and thus establishes bivalent chromatin domains. Interestingly, HSCs exhibit monometh-

ylation of, among other residues, H3K4 and H3K27 within enhancer regions of genes that are required for differentiation of HSCs to erythrocyte precursors. Furthermore, the majority of genes associated with trimethylation of these marks lose H3K4me3 after differentiation. These changes in the bivalent epigenetic landscape of HSCs are tightly associated with changes in gene expression (23), establishing a key role of bivalency in hematopoietic lineage commitment. It has also been shown that PRC1, the histone 2A (H2A) ubiquitin ligase, which functions as a transcriptional repressor, is required for maintenance of the self-renewing potential of HSCs.

Together, bivalent histone modifications play key roles in regulating the balance between HSC renewal and differentiation. They preserve the quiescent stem cell pool and support the development of the full repertoire of mature hematopoietic cells.

Mutations, loss of function, or mistargeting of key enzymes that mediate epigenetic modifications has been identified in both lymphoid and myeloid leukemias, further emphasizing the importance of epigenetic regulation in hematopoiesis. Knowledge of how these chromatin modifying enzymes and cofactors influence hematopoiesis presents opportunities for translational research (reviewed in "Bivalent Epigenetic Therapy" below).

Transient gene regulation in cancer: steroid signaling and bivalency. The steroid hormone estradiol functions primarily by binding to the steroid nuclear receptors ESR1 (ER α) and ESR2 (ER β), leading to mobilization of coregulators that form complexes altering chromatin structure in association with histone modifying enzymes (66, 67). While estradiol has biological roles in both normal proliferation and normal differentiation, providing the cellular plasticity needed for tissue remodeling throughout the reproductive years, unregulated estradiol exposure can result in constitutive activation of metabolic and cell cycle regulatory genes and increases the risk of breast cancer. There is strong clinical and epidemiological data linking estrogen exposure with an increased risk of developing breast cancer (68–74).

Epigenetic changes have been increasingly identified in the development of breast cancer. For example, the role of DNA methylation has been extensively studied and DNA hypermethylation is frequently associated with silencing of tumor suppressor genes, steroid receptor genes, and genes involved in cell cycle regulation (129–132). Additionally, alterations in patterns of histone modifications by several histone modifying enzymes, including KMT2B, KMT2F (H3K4), and EZH2 (H3K27me3), have been linked with cancer initiation and progression (133–136). Overexpression of the histone methyltransferase EZH2 is well documented in breast cancer, with increasing levels being associated with more-aggressive metastatic breast cancers (137–139). Overexpression of wild-type EZH2 has also been reported to serve as a transcriptional activator and/or repressor in ESR1-positive breast cancer by modulating estrogen signaling (140–144).

About 75% to 80% of breast cancers are ESR1 positive and these tumors generally exhibit increased levels of histone modifications compared to tumors in patients with a poorer prognosis (145). Epigenetic studies assessing the levels of H3K4me3 and H3K27me3 by our group, using three breast cancer cell lines, provide evidence of higher levels of H3K27me3 in two basal breast cancer cell lines (MCF10A and MDA-MB-231) with relatively unchanged H3K4me3 levels overall among the three cell lines (76). Importantly, analysis of these data reveals a bivalent epigenetic signature that is more prominent in the ESR1-positive MCF7 cell line and is similar to that identified in pluripotent stem cells (Fig. 2).

The concept of partial recapitulation of a stem cell-like epigenetic signature, i.e., oncofetal epigenetic control, in cancer cells is further reinforced by single-cell studies that show the presence of a quiescent subpopulation that exhibits high levels of pluripotency transcription factors Nanog and Oct4 in MCF7 cells (146–149). Additional studies are required to establish whether this oncofetal epigenetic signature is contributed by the cancer stem cell subpopulation and reflects the dedifferentiated nature of cancer cells and whether it could be used to identify specific and selective diagnostic and therapeutic targets (76).



FIG 2 Partial recapitulation of the oncofetal epigenetic landscape, i.e., reemergence of genes bivalently marked in stem cells, in cancer cells. Heat maps (left) and summarization profiles (right) of H3K4me3 (top) and H3K27me3 (bottom) enrichment in the H7-hESC cell line and in the MCF7 and MDA-231 cell lines in 1,172 H7-hESC bivalent genes are shown. The gene transcription start site (tss) and transcription termination site (tts) are indicated. Note the similarity between MCF7 and H7-hESC with respect to H3K27me3 near promoters and the lack of the same in MDA-MB-231. FE, fold enrichment. (The data in the figure are adapted and modified from reference 76 with permission.)

Bivalency for regulation of tumor phenotype: epithelium-to-mesenchyme transition in breast cancer. The epithelium-to-mesenchyme transition (EMT) is a cellular transformation by which epithelial cells lose their cellular polarity and cell-cell adhesion properties and become mesenchyme-like, with enhanced motility and invasive capacity (150, 151). Importantly, this change in the cell state is not permanent. Cancer cells that have gone through the EMT process in the primary tumor may later revert to the epithelial state through a mesenchyme-epithelium transition (152, 153). Therefore, the EMT program is coordinately regulated; multiple signaling pathways, as well as several epigenetic mechanisms, are involved in activation/repression of EMT (154, 155).

The E-cadherin (CDH1) gene provides a model system to examine the coordinated nature of epigenetic regulation during EMT. The CDH1 gene is downregulated during EMT by the binding of the Zeb, Snail, Slug, or Twist transcription factor to its gene promoter region (156). The repression of the CDH1 gene by CpG hypermethylation is commonly observed in several human cancers, including breast, bladder, liver, gastric, and prostate cancer (157–160). In breast cancer, hypermethylation on the CDH1 promoter is positively correlated with EMT (161). Expression of CDH1 is also regulated by histone modification. For example, hepatocyte nuclear factor 3 (HNF3) promotes CDH1 expression and inhibits metastasis by cooperating with the p300 acetyltransferase in cancer cell lines (156). In contrast, the CDH1 gene is also controlled through deacetylation. Snail homologue 1 (SNAI 1) binds to its promoter and recruits HDACs to deacetylate the promoter, thus suppressing the expression of the gene (162, 163). SNAI 1 also recruits LSD1 (lysine-specific histone demethylase 1) or SUV39H1 (suppressor of variegation 3-9 homolog 1, histone methyltransferase) to the CDH1 promoter, where the enzyme either removes methyl groups from H3K4 (resulting in H3K4me2 enrichment) or adds methyl groups to H3K9 (resulting in H3K9me3 enrichment), respectively (164, 165). EZH2, a component of the PRC2 complex, was also found to be associated with silencing of the E-cadherin gene by H3K27me3 during tumor progression (166). These findings establish a complex but precise regulatory network regulating CDH1 expression during EMT and suggest that this gene may frequently be in a bivalent configuration.

BIVALENT EPIGENETIC THERAPY

Mutations in proteins responsible for mediating epigenetic changes are widespread in cancer and present attractive opportunities for therapeutic targets (see Table 1 for examples). Therapies have been developed to target gain-of-function mutations in DNA methylases, as well as histone modifiers and readers, and to impact DNA methylation, histone modification, and reading and to epigenetically impact the tumor phenotype.

Drug	Target	Epigenetic class	Disease(s) treated
Azacitidine	DNA methyltransferase (DNMT1)	DNA methylation	AML
Decitadine guadecitadine	DNA methyltransferase (DNMT1)	DNA methylation	Recurrent fallopian tube carcinoma; recurrent ovarian carcinoma; recurrent primary peritoneal carcinoma; papillary thyroid carcinoma; follicular thyroid carcinoma; non- small-cell lung cancer; colorectal cancer; metastatic pancreatic adenocarcinoma
Vorinostat	Histone deacetylase	Histone modification	Cutaneous T-cell lymphoma; AML; glioma; prostate cancer
EPZ-5676 (pinometostat)	DOT1L (a histone methyltransferase)	Histone modification	MLL-rearranged leukemia
Tranylcypromine	LSD1 (a histone demethylase)	Histone modification	AML; refractory solid tumors
CPI-0610	BET family proteins	Histone reader	Lymphoma; multiple myeloma; acute leukemias
GSK525762	BET family proteins	Histone reader	NUT midline carcinoma
TEN-010	BET family proteins	Histone reader	AML; refractory solid tumors
AG-120	Mutant IDH1	Metabolic pathway	AML; glioma; cholangiocarcinoma; refractory solid tumors
Tazemetostat	EZH2 (a histone methyltransferase)	Histone modification (direct); chromatin remodeling complexes (indirect)	B-cell lymphoma; mesothelioma; synovial sarcoma; rhabdoid tumors; INI1-negative tumors

TABLE 1 Examples of the steadily growing repertoire of epigenetic therapies for a variety of malignancies that are currently being used or tested in clinical trials^a

^aAML, acute myeloid leukemia.

These include LSD1 inhibitors, EZH2 inhibitors, PRMT5 (protein arginine methyltransferase 5) inhibitors, DOT1L (disruptor of telomeric silencing 1-like) inhibitors, and BET (bromodomain and extraterminal) protein inhibitors (167). Loss-of-function mutations are more challenging to impact therapeutically; however, with a deep understanding of the pathways involved, drug development can focus on targeting synthetic lethal interactions. Synthetic lethality exploits the relationship between two genes wherein loss of one gene does not lead to cell death but loss of the two genes simultaneously does result in cell death. This strategy has been successfully applied to target loss-offunction mutations in chromatin remodeling complexes (for example, the synthetic lethal effect of an EZH2 inhibitor in SNF5-mutated rhabdoid tumors) and histone methyltransferases (168, 169).

Knowing that chromatin bivalency contributes in a significant way to oncogenesis, specific efforts are under way to target the enzymes that mediate construction of the bivalent signature in cancer. As described above, the members of the MLL (KMT) family of complexes are key mediators of H3K4 methylation. Oncogenic MLL translocations are frequently seen in acute leukemias and are particularly prevalent in a devastating form of infantile acute lymphoblastic leukemia (ALL). The majority of MLL translocations result in loss of the catalytic SET domain and occur with fusion partners capable of recruiting the H4K79 lysine methyltransferase DOT1L. Efforts to target the oncogenic MLL fusions have been largely focused on the development of DOT1L inhibitors, such as EPZ-5676, which is currently in clinical trials (170). It has additionally been found that H2B E3 ligase RNF20 is required for MLL-rearranged leukemogenesis, wherein there are increased levels of H3Bub in MLL target genes correlating with sites of H3K79me2, making RNF20 an attractive epigenetic target in MLL-rearranged leukemias (171). Interestingly, cancers characterized by an oncogenic MLL fusion must retain one wild-type MLL allele, as the normal H3K4 methyltransferase activity is essential for oncogenesis. Therefore, other efforts to target this epigenetic disease are focused on the function of WT MLL. There has been some investigation into using LSD1 inhibitors to restore the balance between MLL and LSD1 in H3K4 methylation (172). Other groups are using compounds that specifically inhibit the SET-WDR5 interaction in order to inhibit the WT MLL complex (42, 173).

As described above, PRC2, and its catalytic subunit EZH2, are largely responsible for maintaining the "repressive" half of the bivalent signature. Overexpression of and gain-of-function mutations in EZH2 have been described in a variety of cancers, most

notably B-cell-derived hematopoietic malignancies (174). Efforts undertaken to inhibit EZH2 have resulted in the identification of tazemetostat and CPI-1205, small-molecule inhibitors of EZH2 which are currently being tested in clinical trials for treatment of a variety of PRC2-dependent malignancies, including B-cell lymphomas. These examples highlight the therapeutic potential of targeting the enzymology for bivalent chromatin.

BIVALENT CHROMATIN: NOVEL REGULATORY DIMENSIONS

Emerging literature on bivalent chromatin and its pivotal role in regulation of key biological processes indicates additional regulatory functions for bivalency in regulating cancer phenotypes. Here, we highlight two key areas where additional research will yield a mechanistic understanding of bivalency in establishing cancer phenotypes and may provide options for therapeutic intervention.

Oncofetal gene expression. Oncofetal genes have been defined as highly expressed and stringently regulated during embryonic development and inactivated in most adult tissues but aberrantly reexpressed in many cancer types (reviewed in references 175 and 176). Notable examples of oncofetal gene products include alpha fetoprotein (AFP), carcinoembryonic antigen (CEA), high mobility group AT-hook protein 2 (HMGA2), abnormal cell lineage protein 28 homologue (LIN-28), insulin-like growth factor 2 mRNA binding protein (IGF2BP), 5T4 oncofetal trophoblast glycoprotein, sal-like protein 4 (SALL4), receptor tyrosine kinase-like orphan receptor 1 (ROR1), forkhead box M1 (FOXM1), NODAL, and teratocarcinoma-derived growth factor 1 (TDGF1; also called "Cripto growth factor") (reviewed in references 177 to 186). Collectively, oncofetal proteins contribute to tumor phenotypes through regulation of stemness, proliferation, invasion, and metastasis. Decades of research have established a number of oncofetal proteins as effective biomarkers for cancer aggressiveness (reviewed in references 177 to 180, 183, and 185). Unfortunately, there has been limited success in using oncofetal proteins as therapeutic targets. Furthermore, the mechanistic underpinnings of their reexpression or regulation in cancer are not well understood (187-191).

Recent studies have provided some insights into the regulation of oncofetal genes in cancer. A number of oncofetal genes have been identified as downstream targets of *let-7*, a tumor suppressor microRNA that plays key roles in a variety of cancer types (192). Targets of *let-7* include the well-characterized LIN-28 and HMGA2 oncofetal genes and the IGF2BP1 to -3 family member genes (178, 184, 193, 194). Each of these genes is involved in growth at the organism level, as well as in regulation of metabolism during development (178, 184, 193, 194). In cancer, each of these genes is reactivated and contributes to the tumor phenotype. For example, the LIN28B gene is associated with neuroblastoma (195), the HMGA2 gene is expressed in aggressive ovarian cancer (196), and IGF2BP family member genes are reactivated in a number of cancers, including breast, cervical, and hepatic cancers (197). These findings indicate that a common mechanism is operative in posttranscriptional regulation of several oncofetal genes and suggest that a common mechanism may also regulate transcriptional reactivation of these genes across cancer types.

While upstream transcriptional regulators of some oncofetal genes are known (198–201), surprisingly little is known about epigenetic regulation of their expression. Studies examining cancer-associated reexpression of various oncofetal genes have mostly focused on DNA methylation. For example, the *AFP*, *CEA*, *SALL4*, and *Nodal* genes are all unmethylated during development, become methylated in adult tissues, and are unmethylated or hypomethylated in several cancer types (202–205). As we discuss above in detail, many aspects of epigenetic control, including chromatin bivalency, are reacquired at the onset and during progression of cancer. We propose the hypothesis that once oncofetal genes become unmethylated at the onset of cancer, they acquire bivalent chromatin marks, poising them for increased expression throughout the progression of cancer. This hypothesis has the potential to explain the correlation between increased levels of various oncofetal proteins and tumor aggressiveness. It is equally possible that genes encoding oncofetal proteins are either transiently marked

by bivalent histone modifications or not bivalently marked at all. Instead, their upstream regulators are bivalently marked, thus allowing the expression of these genes in cancer cells. Lack of a true cancer progression model also limits the identification of the transient nature of bivalency. Additional studies are needed to establish whether oncofetal genes acquire a bivalent chromatin landscape during tumorigenesis. Such a bivalent landscape of oncofetal genes will provide options for regulatory plasticity and may offer selective and specific therapeutic targets.

Cancer stem cells. The quiescent nature of cancer stem cells results in escape of this cell population from the conventional chemotherapeutic approaches that often target actively dividing cancer cells (206-208). It will be important to investigate whether cancer stem cells—the cell population that represents a dedifferentiated statereacquire bivalency in regulatory regions of oncogenes. There are some examples that point to such a mechanism. For example, the gene encoding E-cadherin permits dynamic regulation of gene expression during the EMT. Within the CD24-/CD44+ stem cellenriched populations of human breast cancer cells, E-cadherin expression has been silenced and its promoter contains bivalent H3K4me3 and H3K27me3 modification. However, in CD24⁺ cells, which express high levels of E-cadherin, only the H3K4me3 mark is present on the E-cadherin promoter (209). To allow this dynamic switching between cell states, the key EMT transcription factor ZEB1 is also marked with bivalent chromatin (210). Systematic genome-wide studies using a purified cancer stem cell population are needed to examine how much of the bivalent chromatin landscape of pluripotent stem cells is reestablished in cancer stem cells. Such studies may also reveal the key differences between the two cell types in control of proliferation. Epigenetic therapies can then be developed to target bivalent chromatin and may be more effective in eradicating cancer stem cells.

OPTIONS AND OPPORTUNITIES

The complexity of gene regulation is reflected by the establishment and maintenance of bivalent chromatin through the interplay of various histone modifications, ranging from methylation of H3K4 and H3K27 residues to sequential addition of posttranslational modifications to or removal of posttranslational modifications from adjacent residues and/or histones, as well the coordination between these modifications and DNA methylation. Both redundancy and selectivity of enzymes and cofactors are required for deposition or removal of these marks and further highlight the intricate nature of bivalency. An additional level of control is presented by activation or repression of other genomic targets (mRNAs, long noncoding RNAs, and microRNAs) in a context-dependent manner that is dictated by extracellular signaling. Taking the findings together, this complexity provides options for cellular plasticity in a variety of biological or diseaserelated contexts.

However, several key issues remain unaddressed and require additional studies. (i) Experimental evidence discussed in this review provides a preliminary insight into the role of bivalency in cell fate decisions in pluripotent cells. Additional evidence is needed to establish whether bivalency is a prerequisite for lineage commitment and whether distinct gene subsets are bivalently marked prior to acquisition of distinct cell phenotypes. This knowledge will illuminate the significance of partial recapitulation of bivalency in some cancer types. (ii) In addition to H3K4me3/H3K27me3-mediated bivalency, other histone modifications may poise genes for rapid transcription. For example, trophoblast and endodermal stem cells demonstrate a pattern of bivalent H3K4me3/H3K9me3 modifications on lineage-specific genes that function independently of H3K4me3/H3K27me3 to regulate differentiation (211). Similarly, in mesenchymal stem cells, H3K4/H3K9me3 bivalent chromatin at several key adipogenic genes was demonstrated to keep lineage-specific genes poised, but repressed, until commitment to adipogenesis took place (21). It remains unclear how prevalent such "noncanonical bivalent domains" are and whether these are associated with gene regulation in specific cellular pathways. (iii) Partial recapitulation of pluripotent bivalency in some cancer types (termed "oncofetal epigenetic control" in this

review) is an exciting recent observation. Further research is required to establish whether the same set of genes reacquires bivalency across tumor types. (iv) Our group has noted that the numbers of bivalent genes differed within the same cancer type as well as across cancer types. This variability may reflect the hetero-geneous nature of tumor cell population and/or variability in cancer stem cell populations. It will be important to examine bivalency at the single-cell level in purified cancer stem cell populations. (v) Is oncofetal epigenetic control a consequence of or a prerequisite for cancer onset and progression? Despite the open-ended questions concerning these issues, bivalency is emerging as a key determinant of cellular plasticity, and its partial recapitulation in a number of cancer types offers new avenues for identification of biomarkers and for development of therapeutic approaches that are selective and specific.

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mal stem cell (MSC) commitment to osteogenic lineages and their involvement in malignant disease processes, such as metastatic niches and tumor microenvironments. The differentiation of MSCs as a normal process (such as bone development) or as an aberrant process due to association with neoplastic tissues is regulated by transcriptional regulators, early chromatin remodeling events, and epigenetic modifiers. These epigenetic processes have obvious implications for controlling MSC commitment, potentially leading to novel therapies to increase bone mass under chronic disease conditions or to interfere with unwanted differentiation in cancer-associated environments. B, Conway T, Kaspi A, Wu Z, Nikolskaya T, Merino VF, Lo PK, Liu XS, Nikolsky Y, Sukumar S, Haviv I, Polyak K. 2011. Epigenetic regulation of cell typespecific expression patterns in the human mammary epithelium. PLoS Genet 7:e1001369. https://doi.org/10.1371/journal.pgen.1001369.

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cused on understanding the mechanisms of breast cancer progression. Epithelium-to-mesenchyme transition (EMT) has been implicated as an essential process in breast cancer metastasis and chemoresistance. Understanding the epigenetic silencing of E-cadherin, a key mechanism activating EMT, can help the development of promising targets for the prevention of metastasis.

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Gary S. Stein trained in biology and pathology and is dedicated to basic, translational, and clinical cancer investigation. He pioneered discovery of mechanisms controlling proliferation and differentiation, emphasizing compromised genetic and epigenetic regulation in cancer. His focus is on mechanisms and biomarkers, including noncoding RNAs associated with prostate, leukemia, and breast cancer prevention, and early detection, treatment, and survivorship. A prior-



ity is characterizing genetic and epigenetic regulation mediating cell cycle control and the abbreviated pluripotent cell cycle in human embryonic stem cells, induced pluripotent cells, and cancer stem cells. He is investigating breast and prostate tumor metastasis to bone, including microRNA-mediated control, and is defining mechanisms that govern the combinatorial organization and assembly of regulatory machinery in nuclear microenvironments and epigenetic control of cell fate and lineage commitment. Dr. Stein is currently the chairperson of the Department of Biochemistry at the University of Vermont and the Director of the University of Vermont Cancer Center.