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Mitotic Gene Bookmarking: An Epigenetic Program to Maintain Normal and Cancer Cell Phenotypes

Sayyed K. Zaidi¹, Jeffrey Nickerson², Anthony Imbalzano³, Jane B. Lian¹, Janet L. Stein¹, and Gary S. Stein^{1, #}

¹Department of Biochemistry and University of Vermont Cancer Centre, University of Vermont Burlington VT

²Department of Pediatrics, UMass Medical School, Worcester, Massachusetts, United States

³Graduate Program in Cell Biology and Department of Biochemistry and Molecular Pharmacology, UMass Medical School, Worcester, Massachusetts, United States

Summary

Reconfiguration of nuclear structure and function during mitosis presents a significant challenge to resume the next cell cycle in the progeny cells without compromising structural and functional identity of the cell. Equally important is the requirement for cancer cells to retain the transformed phenotype i.e., unrestricted proliferative potential, suppression of cell phenotype and activation of oncogenic pathways. Mitotic gene bookmarking—retention of key regulatory proteins that include sequence specific transcription factors, chromatin modifying factors and components of RNA Pol (RNAP) I and II regulatory machineries at gene loci on mitotic chromosomes—plays key roles in coordinate control of cell phenotype, growth and proliferation post-mitotically. There is growing recognition that three distinct protein types mechanistically play obligatory roles in mitotic gene bookmarking: 1) Retention of phenotypic transcription factors on mitotic chromosomes is essential to sustain lineage commitment; 2) Select chromatin modifiers and posttranslational histone modifications/variants retain competency of mitotic chromatin for gene reactivation as cells exit mitosis; 3) Functional components of RNAP I and II transcription complexes (e.g., UBF and TBP, respectively) are retained on genes poised for reactivation immediately following mitosis. Importantly, recent findings have identified oncogenes that are associated with target genes on mitotic chromosomes in cancer cells. We propose that mitotic gene bookmarking is an extensively utilized epigenetic mechanism for stringent control of proliferation and identity in normal cells and hypothesize that bookmarking plays a pivotal role in maintenance of tumor phenotypes, i.e., unrestricted proliferation and compromised control of differentiation.

Keywords

RUNX; epigenetic control; cell identity; lineage commitment; AML1-ETO

[#]**Corresponding Author:** Gary S. Stein, Department of Biochemistry & University of Vermont Cancer Centre, University of Vermont, Burlington VT 05405, Gary.Stein@med.uvm.edu.

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Introduction

Functional compartmentalization of regulatory proteins and nucleic acids in the interphase nuclear microenvironments is essential for physiological control of gene expression (1-6). This organization is disrupted in cancer, leading to deregulated transcriptional programming during the onset and progression of tumorigenesis (6–11). Mitosis is an essential cellular process that requires structural and functional remodeling of regulatory machinery in the nucleus (12-14). Disruption of this physiological process poses a serious challenge to cell phenotype and identity every cell cycle. Over the past two decades, mitotic gene bookmarking—retention of regulatory proteins and selective histone variants and modifications at gene loci that are poised for immediate reactivation post-mitotically—has emerged as a key epigenetic mechanism that plays a pivotal role in maintaining cell phenotype and identity through successive cell divisions. Studies by our group, and others, have shown that mitotic gene bookmarking is mediated by key regulatory proteins that include components of RNAP I & II machineries, chromatin modifying factors and sequence-specific transcription factors and coregulatory proteins as well as variants and selective modifications of nucleosomal histones (15-37). The functional outcome of mitotic gene bookmarking is the sustained normal cell phenotype across successive cell divisions. Evidence is accruing for role(s) of mitotic gene bookmarking in regulating stem cell plasticity and in the onset, progression and maintenance of the tumor phenotype. In this review, we present evolution of the concept and discuss recent findings that indicate mitotic gene bookmarking is a specific and broadly relevant epigenetic program for coordinate control of cell growth and identity through regulation of RNAP I & Pol II-mediated gene transcription and is obligatory to maintain normal and cancer phenotypes.

Mitotic Gene Bookmarking: Evolution of the Concept and Evidence for Specificity

A conceptual framework for mitotic gene bookmarking was initiated with the identification of a limited number of nuclease accessible sites on the condensed mitotic chromatin that persist through the cell cycle. In the 1990s, Levens and colleagues showed that the chromatin is conformationally distorted at transcription start sites (TSSs) in genes poised for reactivation following mitosis and proposed that a subset of factors remains bound to mitotic chromosomes, providing a molecular bookmark to restore chromatin conformation and gene expression post-mitotically (20,38). Consistent with this model, Wu and colleagues showed that the *hsp70i* gene promoter contains nuclease accessible sites that persist through mitosis. However, Wu and colleague also found that several sequence-specific transcription factors were displaced from the condensed mitotic chromatin (39). In 2003, our group identified the osteogenic master regulator RUNX2 as the first sequence-specific and phenotypic bookmark that remains associated with target genes on mitotic chromosomes (40). Subsequent studies identified mitotic retention of several tissue-restricted transcription factors, indicating that mitotic bookmarking is a key epigenetic mechanism for regulation of genes that coordinately control cell growth and lineage maintenance following mitosis (15,18,22,25,41). It was long thought that highly condensed mitotic chromosomes interfere with accessibility of gene regulatory factors, a concept that extended to accessibility of antibodies to detect

endogenous proteins on mitotic chromosomes by immunofluorescence microscopy (42,43). However, advances in genome-wide biochemical and cell biological approaches have supported unbiased examination of transcriptional and epigenetic states during mitosis. Recent studies by several groups demonstrate that mitotic gene bookmarking and downstream transcriptional events are a rule, and not the exception. The Tjian group has reported that the observation of sequence-specific regulatory protein displacement from mitotic chromosomes is an artifact of formaldehyde fixation (44). Using live cell microscopy, the authors show that the kinetics of formaldehyde fixation prevents detection of sequence-specific transcription factors that are retained on mitotic chromosomes, further strengthening the role of mitotic gene bookmarking as a physiologically relevant epigenetic mechanism. Studies from the Blobel group have established that nuclease accessibility of mitotic chromatin is a wide-spread phenomenon (45). Consistent with these observations, a recent report by Zaret and colleagues have shown that gene transcription occurs in waves throughout mitosis; genes required for essential cellular processes, e.g., proliferation and growth, are expressed from mid to late mitosis, while genes associated with cell identity and phenotype are reactivated immediately after mitosis (46). These findings, together with studies over the last two decades, indicate that mitotic gene bookmarking is a specific, selective, and wide-spread epigenetic program to sustain cell identity and retain options for plasticity.

Mitotic Gene Bookmarking as a Broadly Relevant Epigenetic Program for Cell Identity

Advances in genome-wide molecular, biochemical and cell biological approaches, as well as improved techniques for enrichment of pure mitotic cell populations without using chemical inhibitors, have allowed unbiased examination of transcriptional activity during mitosis under physiological conditions. Accruing evidence indicates that mitotic gene bookmarking is a broadly relevant and fundamental epigenetic program—with multiple converging and overlapping mechanisms—by which pluripotent cells exercise options for differentiation into different lineages and committed cells maintain identity through successive cell divisions. We discuss key mechanisms underlying mitotic gene bookmarking as an epigenetic program that are based on established and emerging evidence.

1. Mitotic retention of key components of RNA polymerase (RNAP) machineries support competency for a basal level of transcription throughout mitosis.

Mitosis is accompanied by striking biochemical changes, including a decline in nuclear RNA transcription (12,14,42). Mitotic repression of transcription was first noted over 60 years ago in studies analyzing incorporation of radio-labelled RNA precursors during the cell cycle (47). Initial studies examining mechanisms that repress transcription in mitotic cells reported that much of RNAP II elongation complexes were physically excluded from mitotic chromosomes, although some genes retained active RNAP II complexes (48). The authors suggested that limitations of pulse-labelling approaches may prevent detection of low levels of mitotic transcriptional activity. Recent approaches designed to detect subtle transcriptional changes have identified waves of transcriptional activity throughout mitosis. Using cell-permeable 5-ethynyluridine to pulse-label nascent transcripts, Zaret and

colleagues showed that mitotic cells maintain a steady state level of transcriptional activity, and that the genes involved in fundamental cellular functions, e.g., cell growth and proliferation, are among the first to be transcribed, while lineage specific genes are expressed to establish cell identity as cells exit mitosis (46). These findings are consistent with reports from our group, and others, that components of RNAP I machinery that include upstream binding factor 1 (UBF1) remain associated with ribosomal RNA genes during mitosis (49,50). A recent report from the Tjian group further shows that TBP, a key component of the RNAP II machinery, is also stably retained on mitotic chromosomes and facilitates recruitment of RNAP II to genes for transcription as cells progress through and exit mitosis (51). These studies support a mechanism for transcriptional memory through successive cell divisions that is defined by the retention of essential components of basal transcriptional machineries to support low levels of transcription throughout mitosis.

2. Modifications and variants of nucleosomal histones establish a transcription-permissive mitotic chromatin state

Low level, yet persistent, transcriptional activity during mitosis suggests that mitotic chromosomes are, at least partially, accessible to the necessary transcriptional regulatory complexes. However, the highly condensed nature of mitotic chromosomes has prevented a systematic, gene level analyses of chromosome accessibility during mitosis. Advances in approaches to examine three-dimensional genome organization have produced high resolution mapping of chromosome architecture in the interphase nucleus and during mitosis (12,52,53). Chromosome conformation capture assays have identified a linearly organized and longitudinally compressed array of consecutive chromatin loops that is shared by all chromosomes and is consistent across cell types (54). Despite a high degree of compaction, regions of mitotic chromosomes remain in an open conformation and accessible to regulatory proteins. These regions often contain mitotically bookmarked genes and share several properties that include: 1) nuclease accessibility; 2) enrichment of specific histone variants/modifications, and 3) association of the mitotic compaction protein cohesin with actively transcribed genes.

Several studies in the mid 1970s revealed that condensed mitotic chromosomes are amenable to nuclease digestion (55). Subsequent studies using ligation mediated polymerase chain reaction identified specific genes that are nuclease accessible, permanganate-sensitive (*i.e.*, contain single stranded (ss) DNA) and exhibit regulatory protein occupancy during mitosis. For example, the *Myc* gene is reactivated by ssDNA binding proteins (*e.g.* far upstream element binding protein (FBP)) immediately after cells divide. Consistent with a role for mitotic gene bookmarking in reactivating genes post-mitotically, the *Myc* Gene is nuclease accessible and permanganate-sensitive. Importantly, FBP remains associated with the gene during mitosis. Recent findings at higher resolution and genome-wide levels indicate larger chromatin domains of moderate nuclease accessibility remain open during mitosis, and the more dynamic changes in accessibility are locally dynamic. Furthermore, DNA hypomethylation marks a subset of promoters that retain accessibility during mitosis (45). These findings are consistent with a unique and selective behavior of bookmarked genes during mitosis, *i.e.*, nuclease sensitivity and accessibility to single-strand DNA binding proteins for rapid reactivation post-mitotically. Genome-wide studies are required to further

assess whether nuclease accessibility is a common feature of all mitotically bookmarked genes.

Emerging evidence indicate that the nucleosomes of bookmarked genes are enriched in histone variants, particularly in H2A.Z and H3.3. For example, nucleosomes at the myogenic gene *MyoD* are enriched in histone H3.3, and this enrichment supports persistent expression of the gene through 24 cell divisions (56). Similarly, nucleosomes immediately downstream of the transcription start sites (TSSs) contain H2A.Z and shift upstream to occupy TSSs during mitosis, thus reducing nucleosome-depleted regulatory regions (57). This change appears to be specific to active genes that are silenced during mitosis and rapidly reactivated post-mitotically. These authors report that, among other histone modifications, the activating histone mark—trimethylation of lysine 4 at histone H3 (H3K4me3)—is enriched at these promoters during mitosis, whereas other epigenetic markers of active chromatin are relinquished. More recent studies have identified H3K27ac, a histone mark that is enriched in the interphase enhancer regions, is also associated with mitotically bookmarked genes (26). Together, these findings point to a specialized chromatin landscape of mitotically bookmarked genes that is defined by selective incorporation of histone variants and retention of specific post-translational histone modifications and supports rapid recruitment of transcriptional regulatory machinery for gene reactivation immediately following cell division.

Although selective nuclease accessibility, histone modifications and histone variants are associated with mitotically bookmarked genes, highly condensed nature of mitotic chromosomes and global reorganization of regulatory machinery during cell division suggests that additional mechanisms must exist to maintain structural integrity of bookmarked genes during mitosis. Genome-wide chromatin immunoprecipitation-sequencing studies have revealed that transcription factors are bound to genes in a highly clustered manner (58,59). At the chromatin architectural level, most clusters are formed around cohesin, a protein required for chromosome condensation during mitosis. Mechanistically, cohesin plays two key roles: during the S-phase of the cell cycle, it holds the replicating strands together at the TF cluster sites; during mitosis, it remains bound to the clusters when the transcription factors have been displaced from target genes (60). Functionally, loss of cohesin decreases both DNA accessibility and binding of TFs to clusters (58). These results provide a mechanistic explanation for nuclease accessibility of mitotic chromosomes and identify additional chromatin properties of mitotically bookmarked genes. Furthermore, these observations suggest that cohesin binding promotes re-establishment of TF clusters and reactivation of target genes after DNA replication as well as after mitosis, thus adding a structural component to the mechanism for maintaining cellular memory through cell divisions (58,59).

Consistent with accessible mitotic chromatin that is enriched in specific histone variants and posttranslational modifications, key chromatin remodeling proteins are retained on mitotic chromosomes. These include histone acetyl transferases (e.g., p300), DNA demethylases (e.g., DNMT1), ATP-dependent chromatin remodeling proteins (e.g., ISWI1), methyltransferases (e.g., MLL), and readers of histone modifications (e.g., BRD4) (17,32,40,61–64). While it remains to be established whether every bookmarked gene retains

all or some of the chromatin-modifying proteins, it is important to point out that many of these co-regulators interact with mitotically retained phenotypic proteins (e.g., RUNX) that occupy target genes in a sequence-specific manner and provide scaffolds for organization and assembly of regulatory complexes to coordinately control gene expression.

3. Phenotypic transcription factors provide specific and selective gene regulatory activity required for cell identity

As discussed above, components of RNAP machineries are retained on chromosomes during mitosis, and together with the mitotic retention of selective chromatin modifiers, create a regulatory environment that is permissive to steady state transcription during mitosis. Another level of specificity is provided by sequence-specific transcription factors that are often master regulators of their respective lineages. To date, more than 20 sequence-specific transcription factors have been reported to be retained on mitotic chromosomes (65). These include the osteogenic and hematopoietic RUNX transcription factors (40,66,67), the key erythroid regulator GATA1 (22,45), the muscle-restricted MyoD (41), and the liver-related FOXA1 transcription factor (18). Among the properties these regulatory proteins share are: 1) specific intranuclear localization in the interphase nucleus; 2) sequence-specific and dynamic interactions with the chromatin; 3) lineage-restricted physiological gene regulation; 4) interaction with multiple co-regulatory proteins that include transcriptional activators and repressors. (See more below.)

Regulatory Contributions of Mitotic Gene Bookmarking to Biological Control and Cancer

1. Coordination of RNAP I and II transcription.

The role for mitotic gene bookmarking in coordinating cell growth, proliferation and identity is illustrated by RUNX proteins, master regulators of osteogenesis, hematopoiesis, neurogenesis and gastrointestinal development (68). During mitosis, all RUNX proteins (RUNX1, 2 and 3) associate with RNA Pol I-transcribed ribosomal RNA genes and RNA Pol II-transcribed genes that are involved in control of the cell cycle, phenotype and differentiation (49,66,67). RUNX transcription factors are equally partitioned in progeny cells at the completion of cell division (40). The association of RUNX factors with ribosomal and cell cycle regulatory genes (e.g., the cell-cycle inhibitor p21) during mitosis bookmarks these genes for regulation during the early G1 phase of the cell cycle. In addition, RUNX bookmarking of differentiation-related genes that include Smads, downstream effectors of the transforming growth factor beta/bone morphogenetic protein signaling pathway, by RUNX proteins during mitosis provides a mechanistic basis for lineage-restricted transcriptional memory in progeny cells (69). Occupancy and regulation of RNA Pol I- and RNA Pol II-transcribed genes by RUNX proteins during interphase and mitosis enables coordination of cell proliferation, growth and differentiation by acting both at genetic and epigenetic levels.

2. Maintenance of cell plasticity and lineage identity.

Mesenchymal stem cells (MSCs) have the capacity to differentiate into multiple lineages that include osteoblasts, adipocytes and myoblasts. Differentiation of MSCs into myoblasts requires the basic helix-loop-helix myogenic regulatory factors (including MyoD, Myf5, and MRF4) that bind to E-boxes in target gene promoters and play crucial roles in skeletal muscle development. Together with Mef2 proteins and E-box factors, these transcription factors are responsible for coordinating muscle-specific gene expression by negatively regulating proliferation and promoting differentiation. During the proliferative stage of mesenchymal stem cells, MyoD is localized to mitotic chromosomes and associates with ribosomal RNA genes and nucleolar-organizing regions (NORs)(41). The association of MyoD with the interphase nucleolus in early stages of myogenesis, and its replacement by myogenin in later stages, results in the down-regulation of ribosomal RNA genes, concomitant with initiation of the skeletal-muscle differentiation program. Consistent with these observations, adipocyte differentiation of MSCs recruits CCAAT/enhancer-binding proteins α and δ (C/EBP α and δ) to the C/EBP regulatory element in the C/EBP β gene promoter, upregulating the C/EBP β protein, which functions as a transcriptional activator of late-adipocyte genes. Studies from our lab demonstrate C/EBP transcription factors occupy ribosomal RNA genes during mitosis (41). As pre-adipocytes complete cell division, C/EBP proteins downregulate ribosomal RNA genes, consistent with their role in initiating adipocyte differentiation when ribosomal gene expression is decreased. The association of muscle and adipocyte-specific transcription factors with mitotic chromosomes in their respective lineages and the subsequent downregulation of ribosomal RNA genes in the interphase suggest that these phenotypic regulatory proteins mediate lineage commitment and maintenance through bookmarking of target gene loci. Pluripotent stem cells divide rapidly and exhibit an abbreviated G1 phase of the cell cycle (70). The pluripotent stem cell phenotype exhibits the capacity for unrestricted, but highly regulated proliferation, and plasticity to differentiate into any cell lineage, providing an optimal model to investigate mitotic bookmarking. Mitotically purified populations of undifferentiated stem cells retain the activating H3K4me3 mark on selective genes necessary for lineage commitment, thus poising them for expression (71). In the absence of extracellular differentiation cues, these genes reacquire the repressive H3K27me3 until the next cell division. In response to an extracellular differentiation signal, pluripotent cells exercise the option to commit to a defined lineage and the genes remain H3K4me3-marked. These findings are further corroborated by recent studies showing that in addition to bivalent chromatin marks—the activating H3K4me3 and the repressive H3K27me3—the enhancer-associated H3K27ac mark as well as pluripotent transcription factors SOX2 and KLF4 are retained during mitosis in pluripotent stem cells (26). A significant recent finding is the partial recapitulation of chromatin bivalency in early stage cancer cells. This “oncofetal epigenetic control” indicates that the bivalent chromatin plays a role in acquisition of tumor phenotype (72,73). It remains to be established whether some, all or none of the genes that reacquire the activating H3K4me3 and the repressive H3K27me3 histone marks in early stage cancer cells also relinquish the repressive H3K27me3 mark during mitosis to sustain plasticity of cancer cells. Together, these and other studies have identified mitotic gene bookmarking as a central epigenetic mechanism for lineage identity in committed cells and cell plasticity in pluripotent stem cells.

3. Sustained tumor phenotype.

The tumor phenotype is characterized by deregulated differentiation program and unrestricted cell proliferation that, unlike in pluripotent stem cells, is not physiologically controlled. Recent studies suggest that mitotic gene bookmarking has an important role in the onset, progression, and perpetuation of disease. A key example is provided by the leukemic fusion protein AML1-ETO that blocks myeloid differentiation and enhances proliferative potential (74). The leukemic AML1-ETO mitotically bookmarks rRNA genes, as well as genes controlling cell proliferation and myeloid cell differentiation (66). Functionally, AML1-ETO upregulates rRNA and cell proliferation-related genes, but downregulates gene mediating myeloid cell differentiation, promoting the transformed phenotype. Another example of cancer-related mitotic gene bookmarking is the mixed lineage leukemia protein (MLL). MLL is a chromatin remodeling factor that is associated with leukemia and regulates transcription by recruiting chromatin modifying machinery to target genes. MLL mitotic retention favors rapid reactivation of target genes required for the onset and progression of MLL post-mitotically (17). In-depth and genome-wide studies are required to establish whether mitotic bookmarking of cancer-related genes is a shared activity of sequence-specific oncogenic proteins.

Concluding Remarks

It is increasingly evident that mitotic bookmarking is a central epigenetic mechanism to maintain cellular identity through cell divisions. Emerging evidence indicates that phenotypic transcription factors mitotically bookmark a subset of target genes and this bookmarking contributes to coordinate control of cell proliferation, growth and differentiation. Importantly, mitotic gene bookmarking by oncogenes in cancer cells may be necessary to maintain the tumor phenotype. Open-ended questions related to mitotic gene bookmarking that can provide mechanistic and clinically relevant insights into compromised epigenetic control in the onset and progression of cancer include: 1) Are mitotically bookmarked genes organized in shared nuclear microenvironments in G1 cells to facilitate coordinate control? And/or is mitotic gene bookmarking a mechanism to assemble coordinately regulated genes in shared nuclear regulatory microenvironments? 2) What is the role of co-regulatory proteins of transcription factor bookmarks in gene reactivation as cells exit mitosis? 3) To what extent are genes bookmarked that are not expressed immediately after mitosis but in subsequent cell cycle stages? (e.g., histone genes that are specifically upregulated in S-phase.) 4) What is the core regulatory network that is required to maintain cell identity? 5) Is mitotic bookmarking operative in cells that divide asymmetrically? 6) What is the contribution of mitotic bookmarking in tumorigenesis? It has been traditionally difficult to target transcription factors due to unfavorable pharmacokinetics and substantial off-target effects. From translational and clinical perspectives, mitotic bookmarking has the potential to provide preferential and selective therapeutic intervention.

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Figure 1: Mitotic gene bookmarking is a broadly relevant epigenetic program for cell identity. Studies over the past two decades have identified mitotic gene bookmarking as a broadly relevant epigenetic mechanism to coordinate cell proliferation, growth and identity in progeny cells. Several regulatory proteins and transcription factors involved in key cellular processes have been identified to bookmark target genes for post-mitotic reactivation/regulation. **(A)** A simplified representation of the differentiation potential of pluripotent stem cells. Only those lineages are shown for which a “master” phenotypic regulator has been identified to bookmark target genes in mitosis (labelled in red). In addition, recent findings demonstrate that key pluripotent transcription factors that include SOX2 and KLF4 mitotically bookmark genes in pluripotent stem cells. **(B)** Select signalling pathways, each involved in and essential for cell proliferation, growth and differentiation, are depicted. Dashed arrows represent multiple steps that are not shown for simplicity. For each pathway, downstream effectors that mitotically bookmark genes are shown in red (e.g., FOXL1, RBPJ, REX, and FISF2). **(C)** Accruing evidence has established key properties of chromatin of mitotically bookmark genes. Shown here are chromatin architectural proteins, cohesin

(blue ring) and CTCF (red triangles), histone variants H3.3 and FI2A.Z, as well as chromatin regulators that mediate histone acetylation (e.g., p300), deposit methyl moieties on nucleosomal histones (e.g., MLL complex), methylate DNA (e.g., DNMT1), and facilitate nucleosome remodelling (e.g., ISWI).

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