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Nucleosomes at Active Promoters: Unforgettable Loss

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A variety of chromatin features have been implicated in the regulation of gene expression, including nucleosome occupancy at promoters, histone modifications and variants, and DNA methylation. In this issue of *Cancer Cell*, Lin and colleagues use a powerful single-molecule approach to explore the relationship between nucleosome occupancy and gene expression in cancer cells. They show that nucleosome occupancy is mostly all-or-none at the multiple start sites of the *MLH1* CpG island. After demethylation by drug treatment, nucleosomes are permanently lost as transcription becomes reactivated. Thus, epigenetic maintenance of gene expression may require that promoters are maintained free of nucleosomes.

A growing body of evidence has revealed that chromatin changes correlate with differences in gene expression, leading to the widely held view that transcription factor binding at promoters acts through nucleosomes to activate or repress gene expression (Li et al., 2007). However, it is unclear how the various chromatin differences can lead to changes in the on-versus-off state of promoters. One idea is that histone modifications alter the accessibility of DNA by stabilizing interactions between chromatin-associated proteins and the histones that they bind to (Cosgrove et al., 2004). But then how do these histone tail interactions result in up- or downregulation of gene expression? A paradigm originating from studies of the yeast *PHO5* promoter is that nucleosomes are simply evicted from promoters, and the naked DNA that results allows for transcription factors to gain access to their binding sites and for the basal transcriptional machinery to assemble (Boeger et al., 2003; Reinke and Horz, 2003). Indeed, a distinguishing feature of active promoters in general is that they are depleted in nucleosomes relative to silent promoters (Mito et al., 2005).

A limitation of studies that have been used to map chromatin characteristics, such as histone modifications and variants, is that they provide data that are averaged from large numbers of individual DNA molecules. For example, ChIP-chip and real-time PCR assays can provide sensitive measurements of chromatin features and of nucleosome occupancy, but these are relative measurements that cannot distinguish between a change in the amount of a feature relative to a control and its absolute abundance (van Leeuwen and van Steensel, 2005). Therefore, it has remained possible that the reduction in nucleosome occupancy seen in such studies is not complete eviction of nucleosomes, but rather partial loss or even transient unwrapping. To address this uncertainty, Peter Jones' group introduced a single-molecule modification of the DNA methylation mapping technique for mapping chromatin accessibility (Fatemi et al., 2005; Kladde et al., 1996) (Figure 1A). The M.SssI DNA methyltransferase specifically methylates the cytosines of CG dinucleotide base pairs, making these bases resistant to deamination by treatment with sodium bisulfite. As a result, M.SssI methylation of nuclei followed by DNA extraction and bisulfite treatment results in DNA with CGs intact, but with all other cytosines converted to uracil. The uracil bases are replicated as if they were thymines, so that PCR amplification, cloning, and sequencing of a region using M.SssI- and bisulfite-treated DNA yields sequences from single molecules in which CGs that have been methylated by M.SssI are sequenced as CGs, but those that have escaped M.SssI methylation are sequenced as TGs. In this way, blocking of a CG from the action of M.SssI in nuclei can be quantified by sequencing a collection of PCR-generated clones,

just as bisulfite treatment of native DNA has been widely used to map sites of natural DNA methylation. Because nucleosomes will block M.SssI from the ~150 bp wrapped around the core particle, treatment of nuclei with M.SssI results in a high-resolution nucleosome occupancy map.

In this issue of *Cancer Cell*, Lin and colleagues (Lin et al., 2007) apply single-molecule nucleosome occupancy mapping to the *MLH1* "CpG island" that encompasses the bidirectional promoter for the *EPM2AIP1* and *MLH1* genes. *MLH1*, which encodes a DNA mismatch repair protein, has been shown to be hypermethylated and silenced at the CpG island present within its promoter region in a variety of cancers. The *EPM2AIP1*/*MLH1* promoter is bidirectional, and *MLH1* mRNA is transcribed from two nearby start sites. These three closely spaced transcriptional start sites (TSSs) span a 630 bp region that is sufficiently large to encompass multiple nucleosomes. In normal cells, all three genes are active, and traditional nucleosome mapping methods show that positioned nucleosomes lie immediately downstream of all three TSSs, but the TSSs themselves display low nucleosome occupancy. Single-molecule nucleosome occupancy mapping reveals that these TSSs are free of nucleosomes in the large majority of cases, because these cloned sequences have few if any unmethylated CGs, and the few exceptions show contiguous stretches of unmethylated CGs, consistent with full nucleosome protection (Figure 1B). Although limited stretches of consistent protection from M.SssI are seen at two positions in the otherwise accessible regions, protection is too short to be caused by nucleosomes, but rather suggests the presence of tightly bound DNA-binding proteins. Histones are known to be acetylated at active promoters, but the absence of nucleosomes at active TSSs means that acetylation occurs on adjacent nucleosomes, and indeed this is what was observed.

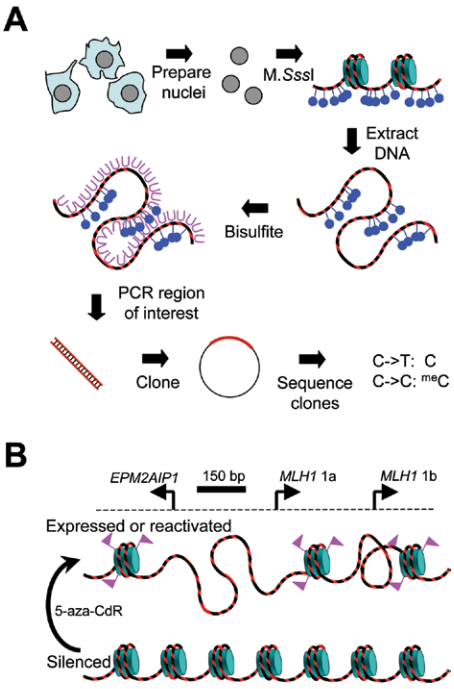

Figure 1. Single-Molecule Mapping of Nucleosome Occupancy

(A) Schematic diagram of the single-molecule methylation mapping method (Fatemi et al., 2005). M.SssI methylates cytosines of CG dinucleotides (blue lollipops) but is blocked by nucleosomes. After bisulfite treatment converts unmethylated cytosines to uracils (purple U's), the first round of PCR amplification puts in adenine across from uracil, converting a C/G base pair to a T/A base pair. Sequencing inserts from a few dozen clones identifies C→T changes as unmethylated cytosines, whereas cytosines are identified as 5-methylcytosine in the original DNA because they were not converted to uracils by bisulfite treatment.

(B) Transcriptional start sites at the bidirectional promoter within the *MLH1* CpG island (encoding *EPM2AIP1*, *MLH1* 1a, and *MLH1* 1b) are mostly nucleosome free when the genes are expressed, and adjacent nucleosomes are acetylated (magenta flags) (Lin et al., 2007). However, when the promoter is silenced in cancer, these sites are occupied by nucleosomes (green disks). Treatment with 5-aza-CdR results in nucleosome eviction and gene reactivation.

The *MLH1* promoter provides a suitable test case to ask what happens to nucleosome occupancy when genes are silenced in cancer and reactivated by drug treatment, because the genes are activated when 5-aza-CdR, a DNA methyltransferase inhibitor, is added. This test is complicated by the fact that silenced promoters are heavily CG methylated, and inhibition of endogenous DNA methyltransferases causes demethylation that can be mistaken for a nucleosome occupancy change. To avoid this problem, Lin et al. focused on cells that had been treated long enough to demethylate a substantial fraction of molecules and used PCR primers designed to selectively amplify only bisulfite-converted DNA in which CGs were replaced with UGs. The results were clear: 72 hr after drug treatment, about half of the promoter molecules were found to be bound by nucleosomes essentially throughout the promoter region, and most of remainder were completely free of nucleosomes at the three TSSs. By 44 days following drug treatment, the large majority of promoter molecules were nucleosome free at these sites. The fact that the fraction of promoter molecules with nucleosome-free regions corresponded to the level of gene expression in cells treated for 72 hr and 44 days strongly suggests that TSSs bound by nucleosomes are silent and those free of nucleosomes are active. Moreover, the fact that many of the demethylated promoter molecules were still bound by nucleosomes 72 hr after drug treatment suggests that DNA methylation is not directly responsible for reactivation, but rather leads to destabilization of promoter nucleosomes, whose loss results in reactivation.

Thus, as for the yeast *PHO5* promoter, which is activated by eviction of nucleosomes, a gene silenced in cancer and reactivated by drug treatment also appears to be regulated by nucleosome occupancy. Studies in a variety of model systems have delineated the multiple factors that can influence the stability of a nucleosome and therefore its propensity to be evicted from promoters. These include ATP-dependent remodeling complexes that likely provide the driving force for eviction, and histone chaperones, such as Asf1 (Anti-silencing function 1), which is involved in both assembly and disassembly of nucleosomes (Li et al., 2007). In addition to these direct effectors, many other factors can alter nucleosome stability, such as histone modifications of many

varieties (Cosgrove et al., 2004), the histone variants H3.3 and H2A.Z (Jin and Felsenfeld, 2007), and chromatin-associated proteins that bind to modified histones or to methylated DNA (Li et al., 2007). These effectors of nucleosome stability have often been referred to as "marks," leaving open the question of how marking can result in gene activation or repression and epigenetic maintenance of these states. But if these diverse chromatin modifiers act by simply increasing or decreasing nucleosome stability, then we may be much closer to a precise molecular understanding of epigenetic inheritance in development and disease.

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In Situ Carcinoma—Can We Predict which Patient Will Come Back with a Recurrence?

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The frequency of in situ carcinomas has been rising since the introduction of mammographic screening. The management of patients with preinvasive disease remains difficult due to our lack of ability to accurately predict which patients will recur and progress to invasive carcinoma. Although some factors, such as lesion size and extent of margin clearance, are strong predictors of recurrence, many patients are still under- or overtreated. In this issue of Cancer Cell, Gauthier and colleagues suggest that abrogated response to cell stress measured by analysis of p16 and the proliferation marker Ki67 accurately predicts recurrence in ductal carcinoma in situ.

Ductal carcinoma in situ (DCIS) is a heterogeneous disease, diagnosed with increasing frequency since the introduction of the mammographic screening program (Hofvind et al., 2007). A number of classification systems have been proposed, primarily based on nuclear morphology. The type of DCIS, lesion size, and most importantly, distance to excision margin have been shown to be strong predictors of recurrence (MacDonald et al., 2005). Approximately half of recurrences remain in situ, while half will be invasive. Patients with invasive carcinoma are at risk of metastases, and hence this represents a significant event for the patient.

Several studies have demonstrated that the risk of in situ and invasive recurrence is greater for high-grade compared to low-grade DCIS. This would suggest that more aggressive therapy is indicated for high-grade lesions, and although the best treatment for DCIS is still uncertain, there is little doubt that a margin ≥10 mm, endocrine therapy, and radiotherapy following excision reduces the risk of recurrence (MacDonald et al., 2005; Burstein et al.,2004). Nonetheless, it is clear that our ability to accurately predict which patient will recur is limited, leading to under- or overtreatment.

In the current issue of *Cancer Cell*, Gauthier et al. (2007) suggest that a simple panel of markers may solve that problem. DCIS with high P16+ and/or COX2+ and high Ki67+ confers a significant risk of subsequent in situ and invasive recurrence. What