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Chemical and biophysical methods to explore dynamic mechanisms of chromatin silencing

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Chromatin, the nucleoprotein complex organizing the genome, is central in regulating gene expression and genome organization. Chromatin conformational dynamics, controlled by histone post-translational modifications (PTM) and effector proteins, play a key role in this regulatory function. Recent developments in chemical biology, cell biology, and biophysics sparked important new studies, which probe direct causal connections between histone PTMs, chromatin effector proteins that write or read these modifications, and the involved functional chromatin states. In particular, the mechanisms of heterochromatin silencing have been explored in great detail in recent years. These studies revealed the highly dynamic nature of this chromatin state, its conformational heterogeneity, and different mechanisms of its formation. Here, we review how chemical biology and biophysics shaped our current understanding of the dynamic processes observed in heterochromatin and discuss the emerging technologies to detect chromatin organization directly in the cellular environment.

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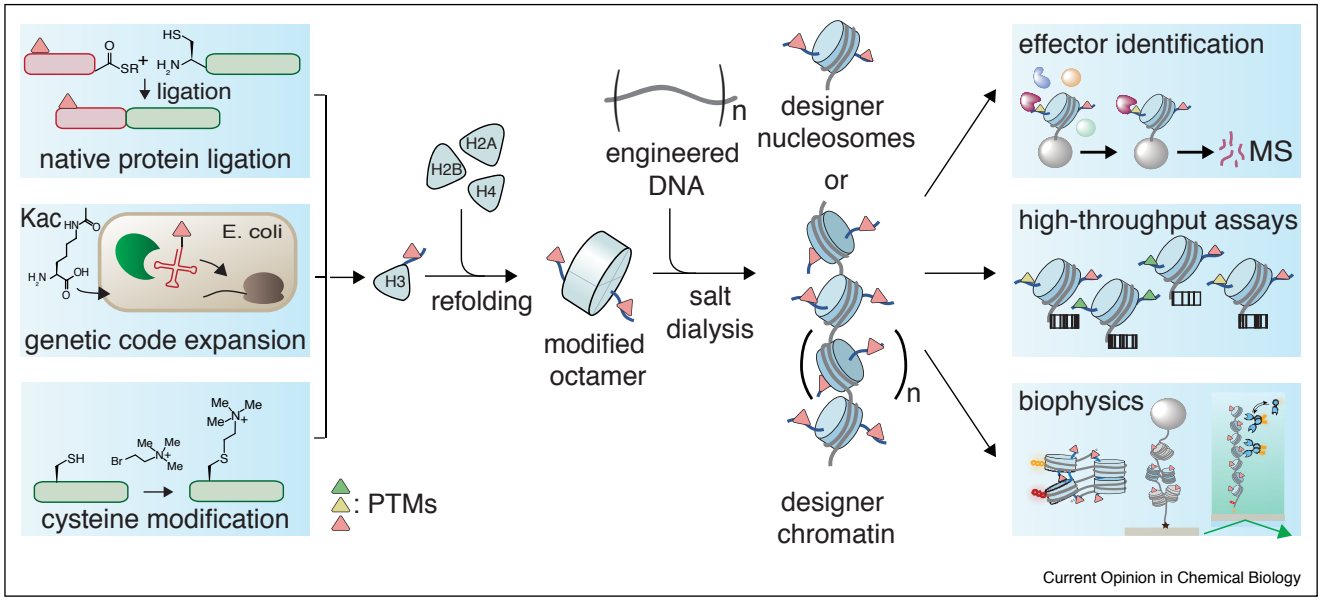
Introduction

Chromatin, which organizes the eukaryotic DNA, controls all cellular processes requiring genome access. Thus, nature has evolved an elaborate network of processes that modulate the local and global structure, dynamics, and accessibility of chromatin from the molecular to the cellular scale and over timescales ranging from microseconds to hours [1]. Chromatin exhibits a hierarchical organization. Nucleosomes form the basic unit of chromatin and consist of about 147 bp of DNA wrapped around a core of histone proteins. Nucleosomes close in space have a propensity to interact through protein–protein interactions, resulting in higher-order organization into tetranucleosome units and helical fibers [2]. On larger scales, long-range inter-fiber interactions, which are mediated *via* a variety of architectural proteins, result in the formation of distinct chromatin domains (topologically associated domains, TADs) that often contain co-regulated genes [3]. The stability, dynamics, and function on the scale of individual nucleosomes to entire chromatin domains is regulated by the interplay of DNA binding factors, post-translational modifications (PTMs, or marks) of histones, DNA modifications as well as chromatin interacting proteins. Histone PTMs, in particular, exist in numerous combinations thereby contributing to differential gene regulation within the nucleus. PTMs, including lysine methylation, acetylation, or ubiquitylation, arginine methylation or serine phosphorylation, are found at both histone core domains and the unstructured histone tails protruding from nucleosomes [4]. Histone marks are key players not only in modulating interactions with chromatin effectors (writers, readers, erasers), but also in the dynamic regulation of chromatin structure and accessibility as a function of internal and external cellular signals. Dynamic combinatorial PTM patterns have thus been implicated in the regulation of gene transcription, DNA replication, and DNA damage repair [4]. Genomic techniques have resulted in a rapid expansion of our knowledge on the location of PTM patterns on chromatin. Conversely, the elucidation of the molecular mechanisms of function of PTM combinations and their dynamic interactions with chromatin factors remains a major challenge.

Chemical control of chromatin modification patterns

To perform experiments which allow to establish a causal link between a specific PTM pattern and its function, control over the modification state and architecture of chromatin is required. In recent years, chromatin research has thus strongly benefitted from the development of chemical biology approaches, which enable control over chromatin modification states *in vitro* and in cells. For detailed *in vitro* experiments, chemical methods, including the installation of PTM mimics *via* cysteine alkylation [5], protein synthesis *via* chemical ligation methods [6,7] or genetic code expansion approaches [8,9], have provided powerful tools for the synthesis of designer chromatin (Figure 1). Indeed, chemical methods enabled the synthetic incorporation of most known histone PTMs into nucleosomes [10], even with control over their asymmetric arrangement [11]. Combined with high-throughput methods, for example based on DNA-barcoding of

Figure 1



Methods to synthesize designer chromatin and selected applications.

modified nucleosome libraries [12], the interaction properties of effector proteins can now be elucidated on a large scale. Such a barcoding approach has recently enabled elucidation of PTM-dependent substrate specificities of classes of chromatin remodeling complexes [13], showcasing the power of high-throughput assays.

The establishment of mechanistic principles of gene regulation is a key application of chromatin engineering. Defined *in vitro* and cell biology systems enable highly detailed investigations of effector protein action and chromatin state establishment. In this review, we highlight recent progress in the elucidation of dynamic chromatin regulatory mechanisms by effector proteins. Because of space constraints, we focus our discussion on two classes of chromatin effectors: heterochromatin protein 1 (HP1) family members and polycomb complexes. Together, chemical and biophysical methods have provided new insight into the dynamics of chromatin state establishment. In the near future, we expect that such methods, combined with chemical biology, synthetic biology, and super resolution imaging approaches will reveal the mechanisms of dynamic chromatin organization and regulation in the cell.

Heterochromatin dynamics

A fundamental chromatin state associated with transcriptionally silent genomic regions is heterochromatin, which is demarcated by distinct histone PTMs, in particular di- or trimethylation of lysine 9 at histone 3 (H3K9me2/3) and lysine hypoacetylation. Moreover a family of key effector proteins, HP1 proteins, is critical for heterochromatin formation (in mammals, the family includes the subtypes HP1α, β or γ) [14]. Primarily found at centromeric and telomeric genomic regions as well as on the inactive X-chromosome in female mammals, heterochromatin forms compact chromatin domains that constitutively repress gene expression [15] through various mechanisms, including by reducing accessibility and through modulating the lifetime of mRNA [16]. All HP1 proteins contain a H3K9me2/3-specific reader domain (the HP1 chromodomain), which binds with low micromolar affinity to these histone PTM [17]. They further contain a dimerization domain [18], and can thus interact with chromatin in a multivalent fashion.

Detailed experiments using chemically defined chromatin substrates could shed light on the dynamic chromatin binding mechanisms of HP1 proteins. Chemical methods enabled the synthesis of H3 carrying a trimethyllysine analog at position 9 (obtained by cysteine alkylation [5], $H3K_C9me3$, Figure 1), which was then incorporated into nucleosomes and chromatin fibers of defined length. Binding experiments using the *Schizosaccharomyces pombe* HP1 subtype Swi6 revealed that chromatin association is greatly stabilized by multivalent interactions [19•]. This could be attributed to the ability of HP1 proteins to cross-bridge neighboring nucleosomes by engaging two H3K9me3-modified H3 tails at the same time. Moreover, detailed *in vitro* studies on the mammalian subtype HP1β, using reconstituted H3K9me3-modified chromatin fibers, showed that HP1 can also bridge individual chromatin fibers, thus forming long-range interactions [20•]. As HP1β proteins have also intrinsic DNA binding

ability [21], both direct DNA interactions and nucleosome binding contribute to this effect, which then results in the formation of compact chromatin compartments [20•].

Importantly, dynamic experiments in cells, based on fluorescence recovery after photobleaching (FRAP), demonstrated that even within compact heterochromatin domains, individual HP1 molecules exchange rapidly showing short residence times in the millisecond (ms) to seconds time regime [22–24] (Figure 2a). Indeed, a combination of NMR and surface plasmon resonance (SPR) experiments showed that HP1β binding to synthetic mono-nucleosomes containing $H3K_C9me3$ is highly dynamic with exchange time constants on the ms timescale [25]. To reveal the mechanism of multivalent chromatin recognition by HP1 within chromatin fibers, different methods are required. Single-molecule fluorescence imaging enables a direct observation of HP1 binding to defined nucleosome arrays (containing semi-synthetic H3K9me3 prepared by protein ligation methods), under equilibrium conditions [26••] (Figure 2b). Such experiments directly showed that mammalian HP1α can engage chromatin in either a monovalent or a bivalent interaction mode (Figure 2c). When bound with only one chromodomain, HP1 proteins dissociate rapidly with dwell times in the hundreds of ms. In contrast, bivalent binding stabilizes its interaction and prolongs residence times to seconds. Moreover, individual HP1α molecules do not remain bound on a single histone tail, but can 'hop' to neighboring nucleosomes, thereby further contributing to a dynamic chromatin state. Importantly, variations in the intrinsic DNA and H3K9me3 binding ability between the mammalian HP1 subtypes α, β and γ results in distinct variations of their residence time on chromatin [27]. The ability to bind DNA is thereby important to ensure a rapid on-rate and to retain transiently dissociated HP1 molecules. In the cell, HP1α can be phosphorylated at its N-terminus and within the hinge region connecting the chromodomain to the dimerization domain [28]. Phosphorylation increases its specific H3K9me3 affinity (which prolongs the chromatin residence time) and concomitantly reduces its ability to interact with DNA [27]. The cell can thus regulate HP1 dynamics using PTMs on both the chromatin fiber and on the effector protein itself. As HP1 proteins carry a large number of PTMs [29], many of which are not yet characterized, the degree of regulation is still not fully understood.

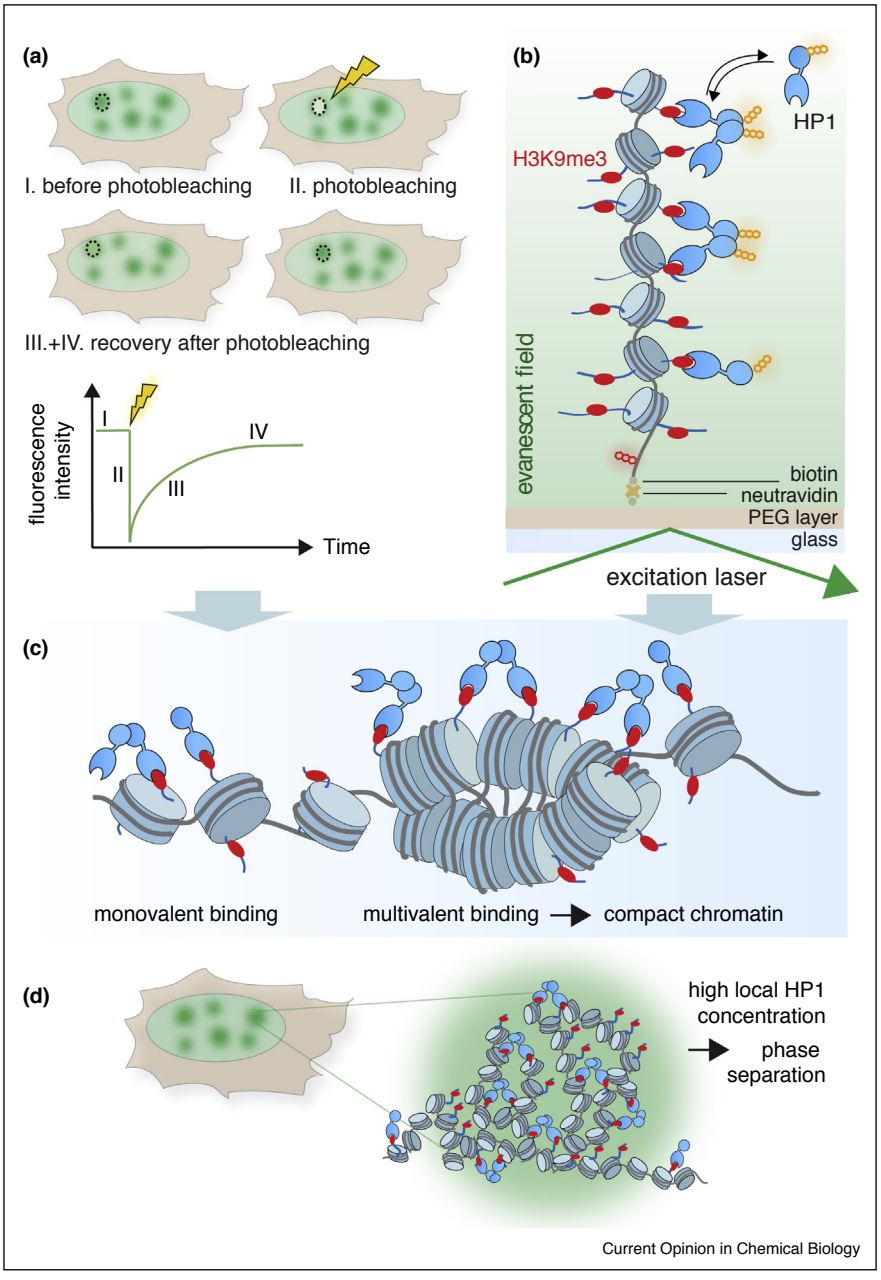
Finally, in the cell, multivalent chromatin interactions lead to local accumulation of HP1 into concentrated domains, which appear as defined foci. Biophysical studies have shown that HP1 has the propensity to form higher-order oligomers at high concentration [30,31••]. Such a self-association, together with multivalent chromatin interactions can trigger liquid–liquid phase separation behavior, which appears as a novel paradigm for chromatin domain formation [31••,32••] (Figure 2d). This intriguing concept can explain the organization of macroscopically stable and simultaneously highly dynamic domains. It will be interesting to see how widespread this phenomenon is for further chromatin effectors.

Chromatin structural dynamics

Transcriptional repression by HP1 proteins has been attributed to their ability to compact chromatin [33]. Indeed, cell biology observations [34] and electron microscopy studies of reconstituted chromatin, containing synthetic H3K9me3, revealed a compact state in the presence of HP1 [35]. From these studies, the local chromatin conformation could, however, not be resolved. Recently, great progress has been made to elucidate the local structure and dynamics in chromatin fibers, which together paint a detailed picture of heterochromatin organization.

X-ray crystallography studies [36,37] and cryo-EM investigations of chromatin fibers [38] (in the latter case containing 12 nucleosomes (12-mer fibers) and a linker histone H1 per nucleosome) revealed local chromatin structure as a helical fiber, with stacked tetranucleosome units as building blocks (Figure 3a). Intriguingly, recent X-ray structures of 6-mer chromatin fibers, showed alternative fiber structures in flat or a twisted state, depending on the solution's ionic strength [39•]. Importantly, PTMs at critical contact points between nucleosomes have been shown to disrupt fiber compaction. These include acetylation [40] and SUMOylation [41] of the H4 tail (which bridges neighboring nucleosomes), as well as ubiquitylation of a helix bundle at the H2B C-terminus [42] (which has to adopt a specific conformation [43]) and at the H2A N-terminus [44]. Together, these results as well as cross-linking studies [45,46] indicate that chromatin fibers can adopt multiple conformations and thus are expected to exhibit conformational dynamics. Force spectroscopy on reconstituted chromatin fibers of defined architectures detected stacking and un-stacking dynamics within and between tetranucleosome units, which were stabilized by H1 incorporation, but weakened by the histone chaperone FACT [47•] (Figure 3b). For a direct investigation of nucleosome–nucleosome interactions within chromatin fibers, single-molecule FRET (smFRET) methodologies provide high spatial and temporal resolution. In a tri-nucleosome system, smFRET detection revealed complex multi-stage nucleosome interaction dynamics on the microsecond to seconds level [48]. To observe dynamics in a full chromatin fiber, a convergent DNA ligation approach was developed that enabled the precise positioning of FRET dyes at defined locations within the fibers [49•]. Of note, an alternative method, employing DNA nicking followed by gap-filling using modified or labeled primers has since been developed, potentially

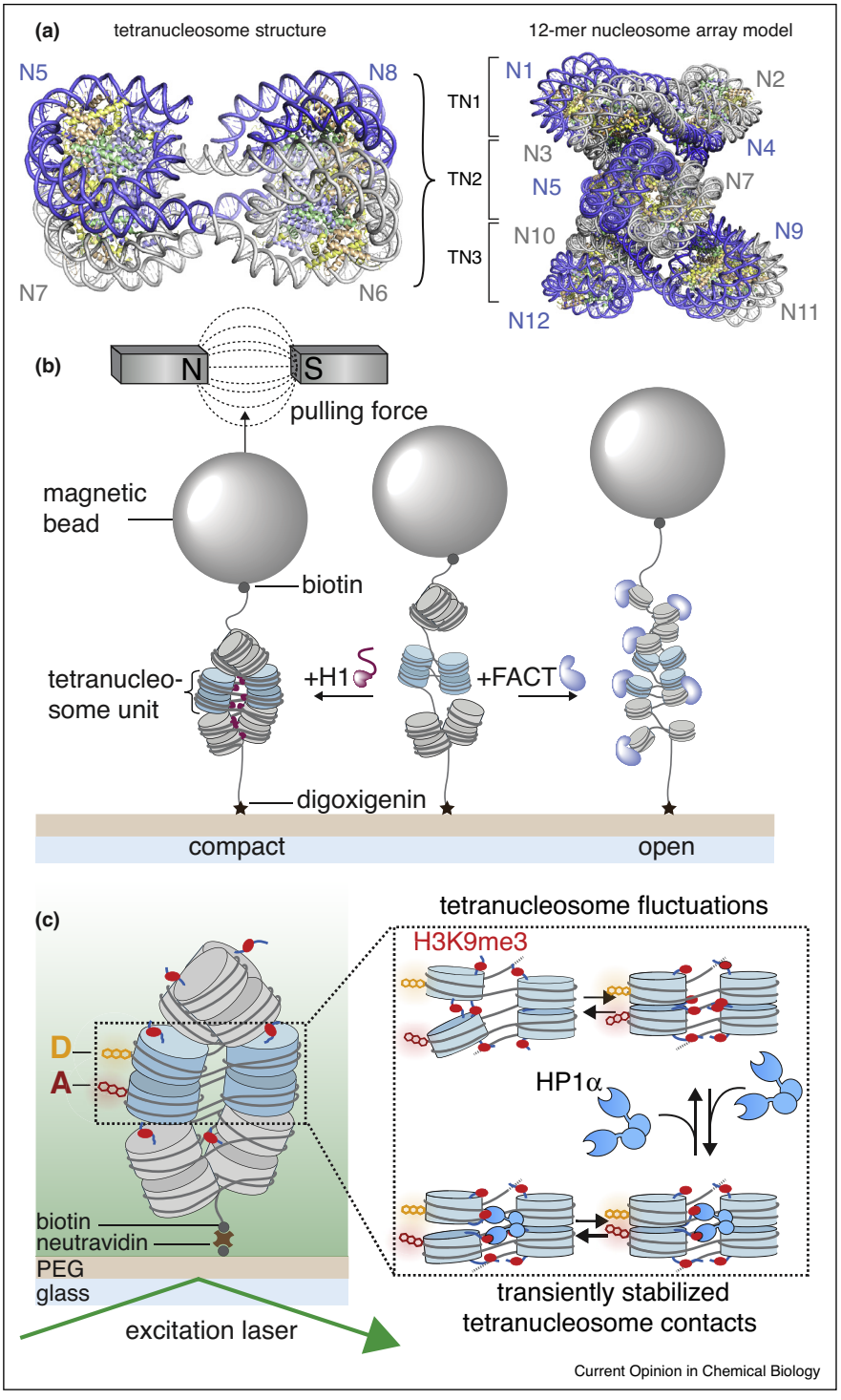
Figure 2



Dissecting HP1–chromatin interaction dynamics and heterochromatin domain formation. **(a)** HP1 interacts dynamically with chromatin as judged by fluorescent recovery after photobleaching (FRAP). Fluorescently labelled HP1 is expressed in the cells displaying heterochromatin foci. After the photobleaching event, the recovery of the fluorescence is recorded overtime providing information about HP1 binding kinetics. **(b)** Single-molecule total internal reflection fluorescence microscopy (smTIRFM) reveals the molecular interaction dynamics of HP1 proteins. Observing the co-localization of surface-immobilized, chemically modified chromatin fibers (red fluorophore) with diffusing, labelled HP1 protein (orange fluorophore) directly reveals the real-time interaction kinetics of HP1 with modified chromatin fibers. **(c)** A dynamic model of HP1 binding to H3K9me3 showing its effect on chromatin conformation. **(d)** Liquid–liquid phase separation induced by HP1–chromatin multivalent interactions as a mechanism for heterochromatin domain formation.

speeding up chromatin labeling [50]. Two complementary smFRET methods were then combined to directly measure internal dynamics in compact chromatin fibers with microseconds temporal and Å spatial resolution [49•]. These studies revealed that tetranucleosome units are in constant dynamic exchange with open states, and that chromatin fibers exhibit conformational heterogeneity on the single-molecule level (Figure 3c).

Figure 3



Structural dynamics of chromatin fibers. **(a)** Chromatin higher-order structure: Tetranucleosome structure (Ref. [36]), showing face-to-face stacking of next-neighbor nucleosomes; and the conformation of a 12-mer chromatin fiber (Ref. [38]), exhibiting discrete tetranucleosome units. Tetranucleosome (TN), nucleosome (N), donor fluorophore (D), acceptor fluorophore (A); **(b)** H1-mediated compaction and FACT-mediated decompaction of chromatin fibers as revealed by magnetic tweezers study [47•]; **(c)** The model of transient stabilization of tetranucleosome by HP1 according to the FRET-based single-molecule TIRF measurement [49•]. Donor fluorophore (D) and acceptor fluorophore (A) are placed at the specific positions on neighboring nucleosomes on the DNA allowing for detection of FRET fluctuations.

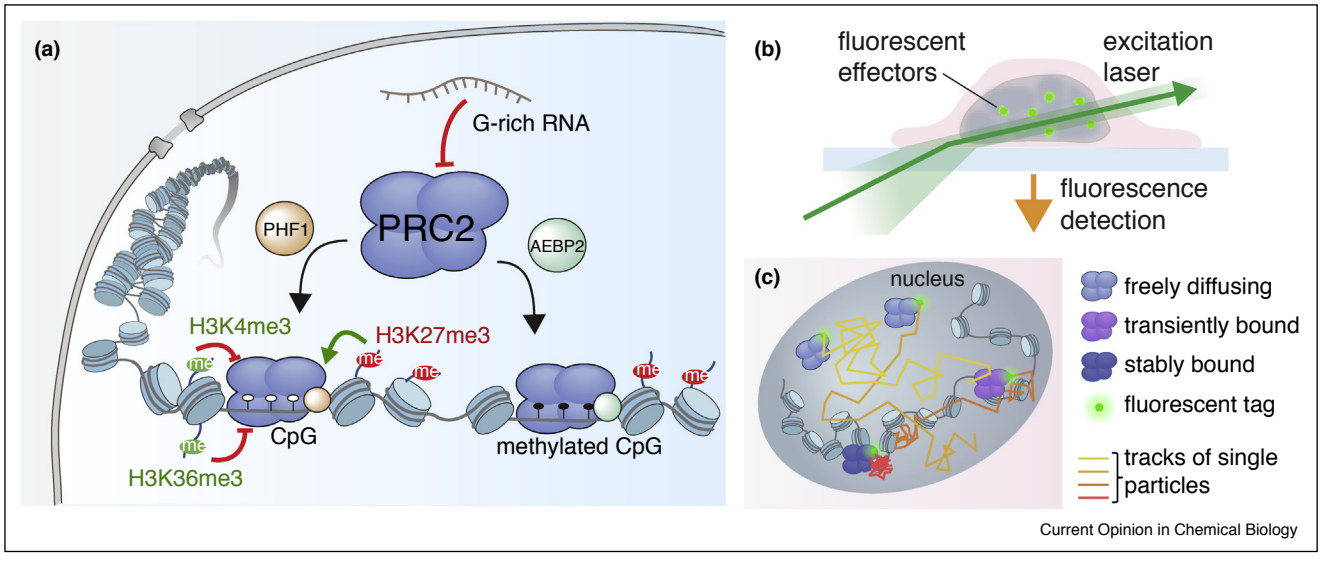
With a fundamental picture of the conformational and dynamic landscape of local chromatin organization, the effect of HP1 on fiber conformation could then be directly determined using smFRET on fluorescently labelled H3K9me3 containing chromatin fibers. These experiments revealed that HP1α stabilizes transient stacking interactions between nucleosomes, through the formation of inter-nucleosome cross-bridges. Nevertheless, the HP1α compacted state remains highly dynamic due to fast effector dissociation and rebinding (Figure 3c). Intriguingly, a recent cryo-EM study directly revealed such HP1-mediated inter-nucleosome cross-bridges [51]. Together, these studies revealed that heterochromatin adopts a compact, but highly dynamic and structurally heterogeneous state. Moreover, local chromatin structure is susceptible to PTMs and the effect of bound effector proteins. Using the developed systems, the conformational effects of further effectors can be investigated in the near future.

Dynamics of polycomb complexes

Polycomb group proteins provide a different cellular mechanism for gene silencing. In particular, polycomb repressive complex 2 (PRC2), a methyltransferase responsible for tri-methylating K27 in H3, is a critical effector involved in establishing a silent chromatin state [52]. The presence of H3K27me3, together with accumulation of PRC1-type complexes, then result in gene repression [15]. PRC2 has crucial roles during embryonic development and in stem cell renewal [52], and its mutations can result in cancer [53]. PRC2 is a multiprotein complex, consisting of structural proteins, including the methyltransferase domain EZH2 and the H3K27me3-specific reader domain EED, which exhibits a mid-micromolar affinity for the histone PTM [54]. This reader domain allows PRC2 to bind to its substrate, which also acts as an allosteric activator of EZH2 [55,56], thereby enabling a propagation of this PTM.

The mechanisms of chromatin recruitment and targeting of PRC2 have, however, been elusive. Synthetic chromatin combined with structural, biophysical, and biochemical assays could shed new light on dynamic PRC2 chromatin recruitment mechanisms [57••,58••]. In the first study [57••], PRC2-chromatin binding was tested employing a number of synthetic chromatin substrates, containing either H3K27me3, H3K4me3 (a PTM that antagonizes PRC2 activity) or H3K27M (an oncogenic PTM [59]). Intriguingly, PRC2 showed comparable binding affinities to all these substrates. Conversely, PRC2 directly bound linker DNA within chromatin fibers with nanomolar affinity. Structural studies of PRC2-bound dinucleosomes indeed revealed a CXC domain in EZH2 to be key for DNA binding [60]. Additional subunits or macromolecules, such as RNA, further regulate PRC2-chromatin interactions. Equilibrium binding studies showed that RNA motifs can directly bind PRC2 and thus prevent its chromatin engagement [57••]. Conversely, the subunit Adipocyte Enhancer-Binding Protein 2 (AEBP2) drives binding to methylated DNA sequences [57••], demonstrating that PRC2 recruitment can be regulated by altering the complex composition (Figure 4a).

Figure 4



Dynamic mode of PRC2 interaction with chromatin. **(a)** A model of PRC2 recruitment to chromatin, as a function of subunits, including PHF1 and AEBP2 and inhibited by RNA. On chromatin, PRC2 activity is allosterically activated by H3K27me3 and inhibited by H3K4me3/H3K36me3. **(b)** Schematic representation of highly inclined and laminated optical sheet (HILO) imaging to track single-particles in the living cells. **(c)** Schematic representation of the single-particle trajectories of fluorescently labelled PRC2, revealing their search process.

A second accessory protein, PHD Finger protein 1 (PHF1) (and its *Drosophila* homolog Polycomb-like (Pcl)), is critical for high PRC2 activity *in vivo* [61]. How is PRC2 sampling chromatin to find its binding sites, and how is PHF1 stimulating its activity? Single-molecule imaging of its search process using chemically defined chromatin fibers revealed two distinct interaction modes [58••]: short, probing interactions in the sub seconds time regime, as well as longer interactions (>2 s), which are associated with enzymatic activity [58••]. Interestingly, PHF1 was shown to prolong PRC2 residence time. A structural analysis of the *Drosophila* homolog indeed revealed a winged-helix DNA binding motif, responsible for this stabilization [58••]. Human PHF1 demonstrated a similar structural organization, and was found to specifically bind non-methylated CpG-rich sequences [62]. Together, through these studies the prolonged PRC2-PHF1 dwell times on chromatin could then be associated with increased enzymatic activity of methylation of H3K27, thereby linking enzyme binding dynamics to enzymatic function.

Single-molecule tracking methods enable direct observations of effector protein–chromatin interactions in living cells (Figure 4b). Direct observation of individual PRC2 molecules was thus employed to reveal their nuclear search dynamics [63•] (Figure 4c). Consistent with the *in vitro* results, PRC2 is highly mobile, and the majority of molecules is freely diffusing through the cell, continuously sampling the chromatin landscape. In contrast, only a small fraction (<20%) remained chromatin bound. Pharmacological inhibition of the PRC2 complex member and H3K27me3-reader embryonic ectoderm development (EED) did not influence the dynamic behavior of the complexes, indicating that PRC2 targeting is achieved *via* DNA interactions and accessory proteins rather than histone PTMs. Intriguingly, another single-molecule tracking study could show that the oncogenic mutation H3K27M [59] results in a significant slowdown of PRC2 diffusion kinetics, as complexes are sequestered to non-native sites by the mutated histones [64]. Together, chemical chromatin synthesis, structural biology, biochemistry, and single-molecule methods *in vitro* and in cells were used to dissect the chromatin recognition mechanism of a key gene silencing complex. The mechanism of gene repression by polycomb complexes is, however, still not completely understood. Studying the interplay between local and large-scale chromatin structure and PcG protein accumulation might yield novel insights into the establishment of a silent chromatin state.

Emerging technologies to observe cellular chromatin organization

The novel tools discussed in the preceding chapters enabled in-depth investigations of the interplay between dynamic effector interactions and structural changes in the chromatin landscape. Novel methods based on genetic code expansion [65,66] split-inteins [67], transcription factor or Cas9-linked effector proteins [68,69] or epigenetic modulators [70] provide means to control the chromatin state site-specifically in living cells. As outlined before, single-particle tracking experiments of chromatin complexes such as PRC2 [63•,64], but also PRC1 [71], transcription factors [72] or the transcription machinery [73] have been powerful to elucidate dynamic chromatin recognition in cells. However, the underlying chromatin structure in a cellular context is still not well understood. Genomic approaches have enabled researchers to detect features of the local structure interphase chromatin [74–76], as well as long-range organizational principles [77], but do not provide direct structural information. Conversely, super resolution microscopy approaches show high potential for direct single-cell visualization of chromatin states. STORM (stochastic optical reconstruction microscopy) in combination with antibody-based labeling was used to reveal that chromatin in interphase cells is organized into individual clusters of nucleosomes [78]. The use of antibodies for labeling purposes, however, results in loss of spatial resolution. An alternative is presented by direct DNA labeling: After introducing the non-natural nucleotide 5-ethynyl-2′-deoxyuridine (EdU) into genomic DNA, click-chemistry enabled efficient DNA labeling of individual chromosomes [79]. Another option is presented by diffusible dyes activated by DNA binding [80]. Most developed are hybridization probes, which allow imaging of distinct chromatin regions. A recent study revealed the organization of active or polycomb-repressed chromatin domains at 20–50 nm resolution [81]. Repressed chromatin domains appeared highly condensed and prone to intermixing with other repressed regions. Sequentially labeling distinct genomic loci along a chromosome using hybridization probes further enabled tracing chromatin folding with a 30 kb genomic resolution in the nucleus [82]. These experiments revealed that chromatin is organized into distinct domains of globular conformation, overlapping with TADs, and sharing a similar chromatin state. Finally, electron microscopy methods to directly observe cellular chromatin organization are becoming more powerful. A recent study using a sensitizer dye-coupled with a contrast agent allowed the direct observation of negative-stained chromatin structure *in situ* by electron microscopy tomography (ChromEMT) [83]. This revealed a mostly disordered local structure, interspersed with short sequences of stacked nucleosomes. Direct cryo-electron microscopy tomography has also been successfully applied [84,85], revealing the conformation of individual nucleosomes in cells.

While dynamic structural methods are still lacking, there has been rapid progress in elucidating *in situ* chromatin structure. Combined synthetic and chemical biology methods and biophysical approaches are converging to enable the direct observation of chromatin function in cells.

Conclusions

The fields of epigenetics and chromatin structure and dynamics have undoubtedly advanced in recent years benefitting from a number of new methods. Synthetic chromatin provides a powerful platform for deciphering the crosstalk between histone PTMs at the single-molecule level at unprecedented detail. Simultaneously, a multitude of emerging *in vivo* tools is being developed to complement the *in vitro* data. Finally, super resolution imaging methods represent a powerful addition to the dynamic structural biology toolbox. They enable the real-time observation of single-molecules at work in live cells, and provide nanometer scale insight into complex cellular structures. Together, we thus believe that in the near future these developments will significantly expand the boundaries of knowledge about dynamic chromatin organization in cells, with widespread implications for basic science and biomedical applications.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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