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Abstract
DNA in higher eukaryotes is packaged with histone proteins to form nucleosomes which are further assembled into higher-order chromatin fibres to protect and regulate access to the genetic information. Chromatin folding above the nucleosomal level is controversial with differing views proposing unfolded irregular structures through to highly organised chromatin fibres. Using a combination of techniques including sedimentation studies, electron microscopy and super-resolution imaging views are converging to indicate that local chromatin is organised into a fibre, peppered with numerous discontinuities and points of flexibility. Reconciliation of different views suggests that in vitro and in vivo data are generally consistent but questions remain on how chromatin packaging is altered by cellular processes such as transcription, enzymatic chromatin remodelling and elusive DNA supercoiling.

Introduction
In higher eukaryotes DNA is wrapped around histone proteins (H2A, H2B, H3 and H4) to form nucleosomes and assembled into higher-order chromatin fibres [1,2]. Nucleosomes, the building blocks of chromatin, were first described 45 years ago [1,2] but still there are large gaps in our understanding of how these components form chromatin. This is not through lack of imagination or effort but rather chromatin is a massive macromolecular complex comprised of protein and nucleic acid (DNA and RNA) that makes it very intractable to study. Fortunately, new techniques are constantly being developed enabling us to better appreciate chromatin fibre structure.

Chromatin fibre folding
Many molecular elements important for genome regulation are conserved. In prokaryotes DNA is packaged with nucleoid associated proteins (NAPs), the most abundant being a histone-like U (HU) protein that resembles histone H2B [3,4]. HU is present in approximately 30,000 dimers per cell and is the most conserved NAP across bacterial species. It shows little sequence specificity, but has a preference for super-coiled DNA [5] and promotes compaction by forming loops through linking DNA segments, in comparison eukaryotes compact their DNA by wrapping it around positively charged histone proteins.

Despite much effort the folding of the higher eukaryotic chromatin fibre remains contentious; opposing views range from an irregular 10-nm fibre [6] to a more structured 30-nm fibre [7]. Basically, chromatin folding is relatively easy to imagine: positively charged histone proteins shield the negatively charged DNA to facilitate compaction [8]. In vitro, chromatin undoubtedly adopts a regular 30-nm wide fibre that can be readily visualised by electron microscopy. This level of folding which is salt dependent requires linker histones to neutralise the extensive negative charge in the stretches of DNA between nucleosomes [9,10]. It is the globular domain of the linker histone that locates the protein close to the nucleosome dyad [11] thereby positioning the long (100 amino acids or 15 nm) unstructured C-terminal tail of the molecule, rich in lysine and arginine residues, allowing it to drape along the chromatin fibre to induce packaging. Linker histone depletion causes chromatin unfolding, a transition that can be monitored by various physical techniques including sedimentation and electron microscopy; the process can be reversed by simply adding back purified linker histone protein (Fig 1A) [12] whilst a peptide encompassing only the globular and C-terminal domains of the linker histone appears to be as effective as the complete H1 molecule in inducing higher-order-chromatin structure [13]. Linker histone tails are highly disordered and recently in vitro studies have shown the H1 tails may form phase separated droplets (or coacervates) containing higher-order assemblies of peptide and DNA. Interestingly, phosphorylation of H1, which occurs in a cell cycle dependent manner and is functionally linked to mitosis, significantly affects formation of these structures [14]. These results suggest that linker histones can be considered as liquid-like “glue” within the fibre [15]. However, it needs to be remembered that by FRAP linker histones are highly dynamic [16,17], so are not stable components of chromatin. Consistently new structural studies of chromatin fibres with bound linker histones adopt different conformations reflecting the structural plasticity of chromatin [18,19].

The globular domain of the linker histone consists of a three helix histone fold motif, a structure very similar to that found in a wide range of chromatin binding proteins including pioneer transcription factors [20]. It is argued that these chromatin binding proteins can compete with and help to dissociate linker histones releasing their disordered tails from the fibre and, in a local context, opening chromatin [21]. Recent studies show that many chromatin binding proteins have extensive disordered domains [22]; examples include RNA polymerase [23]

and the mediator complex [24]. Consequently, it is tempting to speculate that on one hand the H1-carboxy tail will participate in extensive interactions shielding charges on the chromatin fibre to compact chromatin whilst on the other it will compete with the assembly of disordered transcription factor interactions. How is this process regulated? Although linker histone tail phosphorylation affects protein-protein interactions [14], RNA-protein binding can also form membrane-less liquid organelles through complex coacervation in a phosphorylation dependent manner [25]. Is it therefore possible that RNA generated through transcription could affect formation of these structures [26], or decrease linker histone binding locally disrupting chromatin fibre structure (Fig 1B)?

Visualising chromatin fibre folding in cells

A long-standing question asks how cellular chromatin compares to isolated or reconstituted chromatin fibres. We know from sedimentation studies that physiologically extracted mammalian heterochromatin has a more compact structure than bulk chromatin [27]. Similarly, chromatin fibres at transcriptionally active genes have a more disrupted organisation than fibres isolated from inactive genes and simple modelling of these fibres indicates varying levels of disruption (Fig 1B, 2A) [28]. It is difficult attributing precise values to disrupted chromatin fibres but on average it appears that bulk chromatin has 1 disruption per 11 nucleosomes, whilst active promoters have an additional large disruption [28]. But what do these fibres look like? In a study from 30-years ago native chicken erythrocyte chromatin was embedded, sectioned and analysed by electron microscopy (Fig 2B) [29]. The chromatin had a heterogenous structure consisting of clear chromatin fibres: some regions were relatively spindly whilst others showed thicker structures. When chromatin was depleted of linker histones before embedding it had a swollen structure with fluffy fibres, but the re-addition of one molecule H5 per nucleosome gave well defined fibres whilst two molecules produced chromatin fibres indistinguishable from “native” chromatin. Most telling from this study was that chromatin visualised within a swollen chicken erythrocyte nucleus had a very similar appearance to the purified chromatin. Images from this old study are reminiscent of recent chromatin images seen using a new approach developed by the O’Shea lab, termed ChromEMT [30]. In this method thin nuclear sections are fixed and labelled with DRAQ5 to catalyse the conversion of soluble DAB to an insoluble precipitate that can be visualised by electron-microscopy. The fibres visualised in both the Cattini et al [29] and Ou et al [30] studies are remarkably similar suggesting that indeed, chromatin inside cells does have a well folded organisation and isn’t a simple agglomeration of individual nucleosomes. To be clear though, this does not mean that chromatin in cells is organised into regular 30-nm fibres. Instead, chromatin should be considered as a disrupted fibre with a spectrum of structures from a regular organization to one with numerous discontinuities (Fig 1B), consistent with fibres analysed by ChromEMT [30], electron microscopy [29] and sucrose gradient sedimentation [31]. Although our evidence suggests that regular 30-nm fibres might be found in some regions of the nucleus such as satellite containing heterochromatin where individual nucleosomes are regularly positioned to fold into canonical fibres [7,27].

Within the chromatin field computational approaches such as all-atom, coarse-grained mesoscale and polymer models [32,33] often complement experimental studies helping to describe chromatin structure, across different length scales. Although atomistic models can retain all the characteristics of the chromatin fibre many assumptions have to be made and simulations are computationally expensive [34]. In contrast so called mesoscale models are well suited to analysing longer nucleosomal fragments and are useful for modelling the behaviour of chromatin fibres and individual components such as DNA linker lengths, histone modifications or histone tail dynamics [35]. Using this approach it is then possible to model larger scale structures such as the 55 kb HOXC gene cluster [36], and provide a framework for adding additional regulatory and structural features such as DNA methylation, transcription factor binding, long non-coding RNAs, and CTCF factors. As experimental data becomes more complex models for chromatin fibre structure are more of a necessity. For example, in mapping radiation induced cleavage sites across the genome, using an approach called RICC-seq, and interpreting data using di-nucleosome models [37,38] it was possible to show that chromatin marked by H3K27ac had a more disrupted chromatin fibre organisation. It is unlikely these disrupted fibres are caused by an individual factor but probably result from a combination of altered electrostatic interactions mediated by histone tails, transient loss of linker histone or fibre disruptions generated by passing polymerases or chromatin remodelling machines. Furthermore, recent cryo-EM data shows HP1 and polycomb binding to dinucleosome motifs in the chromatin New high resolution chromatin interaction techniques are starting to complement RICC-seq data and reveal further nucleosome structures [39]. Using this approach with molecular dynamics simulations suggest that yeast nucleosomes can adopt distinct folding motifs but how this will be translated to higher eukaryotes and more extensively folded fibres is unclear.

For examining the 3D structure of a genomic locus, polymer models of chromatin structure are most appropriate [40]. Using a forward or “mechanistic” model it is possible to identify the crucial factors for determining chromatin folding and, by relating simulations to experimental data, the models can be validated (Fig 3A). In using this approach to explore the properties of the complex Pax6 locus it was apparent that simple polymer models [41,42] (Pereira et al., bioRxiv doi: 10.1101/305359) were unable to recreate all of the experimental observations. However, from RICC-seq data and our previous analyses on chromatin fibre structure [28,31] it was evident that a simple homogenous chromatin polymer could not adequately represent

the underlying fibre structure and suggested, instead, that chromatin should be modelled as a heteromorphic fibre with varying flexibility (Fig 3B) [37]. This was achieved by adding additional points of plasticity to the fibre model to give a new highly predictive heteromorphic polymer (HiP-HoP) model for predicting chromatin folding (Fig 3C) [43]. A key advantage of using predictive models is that the spectrum of potential chromatin folding states can be explored and at the Pax6 locus it was apparent there was not one preferred structure but instead the locus could adopt one of many potential configurations (Fig 3D), however, it is not yet clear how these different states impact on gene transcription. Forward or "mechanistic" chromatin folding models can be further informed by additional experimental evidence, in particular 3C-like data generated using capture-3C or Hi-C. One approach developed by the Nicodemi lab [44] PRISMR (polymer-based recursive statistical inference method) evaluates the distance between the input Hi-C matrix and the contact matrix derived by polymer thermodynamics for the given model to develop an optimal model of a genomic region with the minimum number and type of required binding sites, to reproduce experimental Hi-C data. These simulations can then be used to assess the ensemble of 3D conformations and best interpret promoter and enhancer interactions and prioritise how they may function in gene regulation [45]. Further developments of chromatin polymer models can be used to explore different structural levels within the nucleus and to predict dynamic transitions. For example, by varying the concentration or interaction potential of protein binders chromatin appears to transition from an extended or globular state describing a phase separation driven folding mechanism. Extrapolating from these simulations can then be used to predict chromatin organisation in a chromosome or the extent of folded and unfolded domains within a locus [46].

Are we able to estimate a level of chromatin packaging from experimental data? Large-scale chromatin folding (0.1 to 1.5 Mb) follows a random walk model where the mean interphase distance squared is proportional to the distance along the chromatin fibre [47]. Using this approach we estimated that gene-poor regions were 3-times more compact than gene-rich regions [31] and that the HoxB locus was decompacted even further after gene activation [48]. It is difficult to speculate on the exact nature or level of chromatin folding (e.g. 10-nm, 30-nm fibre etc) but this data suggested there is at least a 30-fold difference in the levels of chromatin compaction, which would be consistent with large scale chromonema fibres that are occasionally seen in interphase cells [49]. At the time of these studies we were happy to accept the numbers, but in light of ChromEMT data it is difficult to imagine what these higher-order structures might look like as there is little evidence in the imaging data to support a structure above a disrupted 30-nm fibre. Instead it might suggest that large-scale chromatin fibres have similar local structures but exhibit different levels of "scrunching" or compression.

Protein determinants of local chromatin structure

It appears that generally chromatin fibres adopt structures incorporating a spectrum of disrupted foci (Fig 1B). These small disruptions constitute DNaseI hypersensitive sites that are often revealed by methods such as ATAC-seq (Fig 2A). Their positions, relative to the underlying sequence, often correspond to regulatory factor binding sites or locations where nucleosomes have been lost or are difficult to detect [50]. In contrast recent cryo-EM data of repressive proteins such as HP1 and PRC2 can bind to dinucleosome motifs, presumably stabilizing fibre structure [51,52]. If there were extensive differences in the overall levels of chromatin compaction across the genome it would be expected that this would be revealed as varying extents of generalised nuclease accessibility. In a recent study mammalian cells were digested with two different concentrations of micrococcal nuclease and purified single and di-nucleosomes were purified and sequenced. Surprisingly this data indicated that nucleosomes genome wide were released at similar rates, suggesting that, at this level of organisation, most chromatin fibres had similar levels of compaction [53]. This is consistent with our own work suggesting that euchromatic fibres isolated from the active X chromosome had similar structures to facultative heterochromatin isolated from an inactive X chromosomes [28], but the two were distinguished by clear disruptions near to the transcription start sites of active genes (Fig 2A).

In higher eukaryotes individual nucleosomes are positioned depending on the underlying DNA sequence and DNA binding proteins [54] whilst the average nucleosome repeat length varies in different tissues from 168 bp [55] to 240 bp [56]. Although nucleosome spacing is very apparent from bulk digests of chromatin most nucleosomes in metazoans are poorly positioned [57,58], although pronounced nucleosome phasing does occur adjacent to boundaries such as transcription start sites or tightly bound proteins such as CTCF [59]. Classic MNase-seq fails to reveal nucleosome spacing and chromatin regularity in regions with poorly positioned nucleosomes. To over-come this, nanopore sequencing, in a method called array-seq, was used to observe nucleosome arrays in Drosophila [60] and show that despite extensive nucleosome phasing at active genes nucleosome arrays are more irregular at active compared to inactive genes. Presumably the combined activity of polymerase and remodeling factors disturb individual nucleosomes' positioning and consequently disrupt chromatin fibre folding. In *Drosophila* about half of phased arrays are in the vicinity of promoters, whilst the other half do not correspond to well annotated features. Further examination identified an ATACG motif which binds a novel zinc-finger protein, Phaser. The function of this factor, other than its ability to position nucleosomes in vivo and in vitro, remains unknown [61]. Perhaps Phaser reflects an emergent class of proteins which by binding tightly at specific sites in chromatin can alter local chromatin structure for some unknown

purpose. In this context future nanopore sequencing-based nucleosome positioning studies should be of special interest.

Transcription-dependent changes in chromatin structure

Previously, the visualisation of nucleosomes by super-resolution microscopy suggested they formed little clusters or nests in the nucleus, although it was difficult to determine what individual structures were being visualised [62]. Building on this idea and to address the dynamic properties of chromatin the Maeshima lab have used live-cell super-resolution imaging to visualise individual nucleosomes [63]. In this approach cells stably expressing a histone H2B tagged with a photoactivatable mCherry were imaged using oblique illumination microscopy and small numbers of H2B-mCherry molecules were stochastically activated enabling individual nucleosomes across the nucleus to be analysed. Many previous techniques have monitored chromatin mobility in terms of mean squared displacement (MSD) of a locus but here chromatin mobility was presented as a heatmap across the nucleus showing that the more peripheral, less transcriptionally active, regions had slower nucleosome mobility than the more transcriptionally active areas of the nucleus.

Taken at face value this data would indicate that transcriptionally active regions of the nucleus are generally more mobile than inactive regions which is consistent with studies at specific genome loci [64,65]. Surprisingly though when transcription is inhibited using drugs or by RNA polymerase degradation chromatin became more mobile [66]. This apparent conflict might arise as the levels of chromatin organisation being examined are different – live cell imaging is focussing on the mobility of individual nucleosomes whilst studies at gene loci are looking at much larger genomic segments. It is also possible that we need to consider the local chromatin environment setup by the transcription machinery; the polymerase does not bind in isolation but instead interacts with a huge number of other proteins, for some genes it has been suggested that up to 50-70 proteins need to bind. Presumably many of these proteins are not binding simultaneous, but still a huge number of proteins are crowded into a relatively small space on the chromatin fibre. To put this into context a nucleosome is approximately 100 kDa in size. If each of these transcriptional regulators is only 50 kDa it would imply that a protein complex could at least be a megadalton, dwarfing the size of the nucleosome. Furthermore, many chromatin binding proteins have disordered protein domains [22] which are reported to form phase-separated structures [24,67,68]. These specialised microenvironments (or factories) might alter local dynamic properties of chromatin and influence transcription.

Few studies have been able to visualise changes in chromatin fibre structure upon transcription. A notable exception are the classic studies by Daneholt on balbiani ring genes in Chironomus where there is a clear compaction of the chromatin fibre after transcription inhibition (Fig 4A) [69,70]. The underlying mechanism is unknown but the process of transcription will alter DNA supercoiling as RNA polymerase introduces negative and positive supercoils within the locus (Fig 4B) [5]. In cells the level of supercoiling is balanced by topoisomerase activity [71] but localized hot spots may accumulate and facilitate transcription factor binding [72,73]. However, if levels of supercoiling increase free DNA will buckle introducing writhe. This can be tested in vitro by attaching one end of DNA to a solid surface and the other rotated via a magnetic bead. Twisting of the fibre introduces writhe which in turn facilitates the binding of a bipartite transcription factor (Fig 4C) [74]. It is not clear whether this process will work in the context of chromatin but altered supercoiling could influence local topology and consequently protein factor binding; an area for future research.

Conclusions and perspective

Using a combination of molecular techniques such as sucrose gradient sedimentation [28], micro-C [39,75], ChromEMT [30], and live cell imaging [63] we are building up a picture of the dynamic packaging of interphase chromatin. Cumulative evidence suggests that cellular chromatin fibres are heterogenous – instead of imagining a regularly packaged 30-nm fibre, chromatin fibres are disrupted (Fig 1B). Specific genomic regions such as heterochromatin may have more regular chromatin fibres but otherwise irregular nucleosome positioning and the action of polymerases or remodelling machines will continuously disrupt chromatin architecture. As analyses become more complex we will have to rely on more computational approaches and in particular make better use of molecular dynamic simulations to model chromatin structure (Fig 3). We also need to better investigate the dynamic properties of chromatin to understand how structure changes in response to nuclear processes such as transcription or enhancer/promoter interactions but ultimately, we would like to have a high-resolution structure for each promoter or regulatory element and understand how they are remodelled through the cell cycle or upon gene activation.

Conflict of interest statement

Nothing declared

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References and recommended reading

14** Using a combination of biophysical approaches they investigate the binding of an H1 peptide to a model DNA sequence and show that complexes containing disordered histone tail peptides form phase-separated droplets in a phosphorylation dependent manner

19** The authors show that a crystal structure of a 6-nucleosome array bound to H1 has a two-start helix and adopts an extended conformation in solution whilst subtle changes in ionic conditions reveal structural plasticity of the fibre

25* Using in vitro approaches they show that RNAs with cationic peptides form liquid-like organelles through complex coacervation and this process can be regulated by peptide phosphorylation.

30** Approaches to examine chromatin fibre structure in cells are limited. The authors have developed a method (ChromEMT) for staining chromatin in thin cellular sections and show that cellular chromatin can adopt heterogenous structures, paving the way for future experiments to understand how chromatin might be altered in response to transcription.

36* The authors have developed their mesoscale model of chromatin structure incorporating data on nucleosome positioning, nucleosome-free regions (NFRs), acetylation islands, and LH binding and used this to model 55 kb of the HOXC locus.

39* Using micro-C like approaches with molecular dynamic simulations the authors have analysed nucleosome-nucleosome interactions in yeast and propose that nucleosomes can adopt two distinct conformations that are compatible with both regular and irregular models of chromatin folding

43** The authors have developed a new polymer model for de novo predicting the chromatin structure of genomic loci. The model incorporates features of a heterogenous fibre structure, chromatin disruptions and loop extrusion. Interestingly the simulations can be used to examine the spectrum of configurations that a locus can adopt.

53* Using deep sequencing with MNase-seq the authors suggest that euchromatin and heterochromatin are similarly accessible to micrococcal nuclease and must therefore have similar structures.

60** To overcome the limitations in MNase-seq the authors have used emergent nanopore sequencing to investigate nucleosome array regularity across the Drosophila genome and show that silent promoters are more regular than those downstream of highly expressed genes.

63* Using super-resolution imaging this study examines chromatin domain structures and dynamics in living cells. Consistent with previous studies they report that heterochromatin-rich regions are less mobile that euchromatic regions of the nucleus.

74* The authors use an in vitro model system to show that negative supercoiling increases lac repressor induced looping under tension; similar mechanisms might occur in chromatin.

Figure legends

Figure 1. Chromatin fibre structures in higher eukaryotes. A. In higher eukaryotes extended nucleosomal chains are fully folded by linker histones in a reversible manner (top) and are readily visualised by electron microscopy (bottom). B. Sucrose gradient sedimentation studies indicate that chromatin fibres can adopt structures with varying levels of disruption [27].

Figure 2. Visualising cellular chromatin fibres. A. Higher eukaryotic chromatin fibres have regular disruptions. These are particularly pronounced around promoters or regulatory elements [28] and constitute DNaseI hypersensitive sites and are often revealed using techniques such as ATAC-seq. B. Native chromatin isolated from cells under physiological conditions has a disrupted organisation [29]. Linker histone depletion promotes fibre unfolding whilst adding back purified linker histones refolds the fibre. These fibres look reminiscent of fibres observed by ChromEMT [30] and together indicate that cellular chromatin is composed of folded fibres

and not an agglomeration of mononucleosomes. However, folding above a 30-nm like fibre is not readily apparent.

Figure 3. Polymer modelling of chromatin fibres can be used to predict folding. A. Chromatin can be modelled using a bead spring polymer and incorporating switching transcription factors and loop extrusion to generate potential structures [41,42]. B. Not all features of predicted chromatin folding are well supported by experimental data requiring the incorporation of a heteromorphic polymer into the chromatin fibre model to establish a new HiP-HoP model for chromatin folding [43]. C. HiP-HoP can be used to predict the 3D structure of complex genomic loci, such as *Pax6*. D. HiP-HoP provides information about different folding paths and configurations of chromatin folding.

Figure 4. Chromatin fibre remodelling during transcription. A. Visualising how chromatin structure changes during transcription is challenging. Old electron micrographs of balbiani ring genes indicate that transcriptionally active chromatin is rapidly refolded after transcription inhibition [70]. B. The process of transcription will positively supercoil DNA in front of the polymerase and negatively supercoil it behind [5,71]. C. In vitro model experiments using magnetic tweezers to analyse DNA attached to a solid support suggest that extensive twisting will promote chromatin/DNA to writhe and may facilitate transcription factor binding [74].

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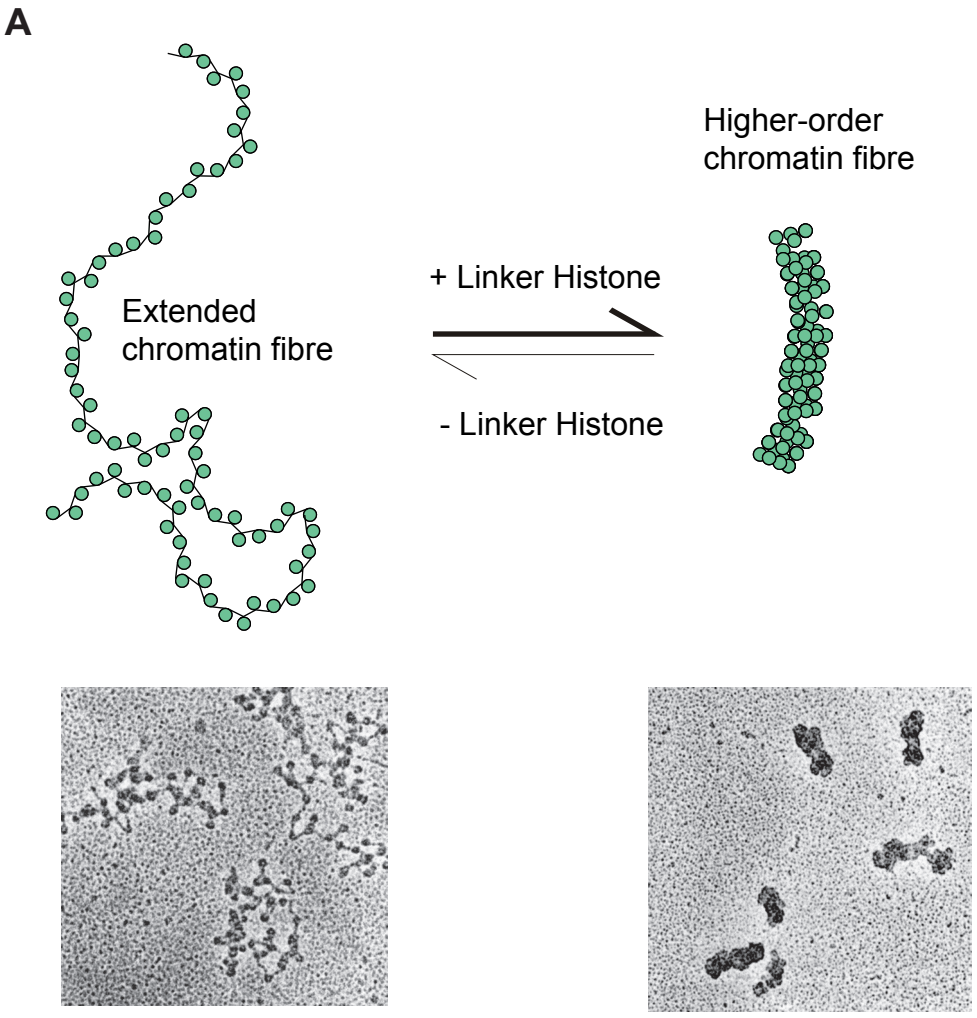
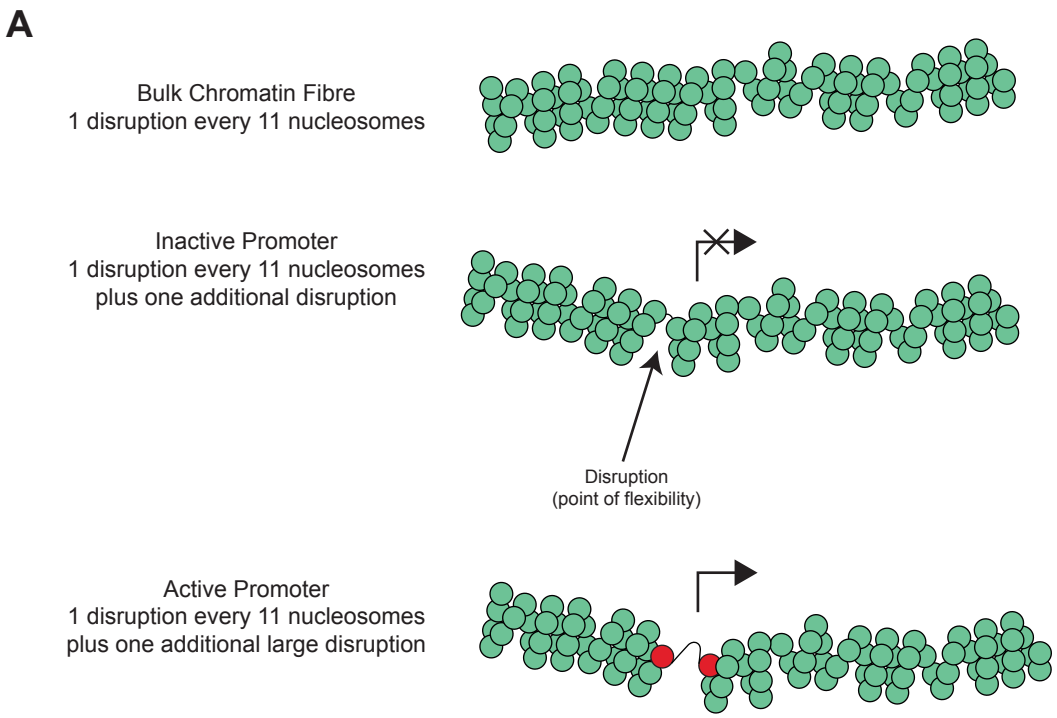
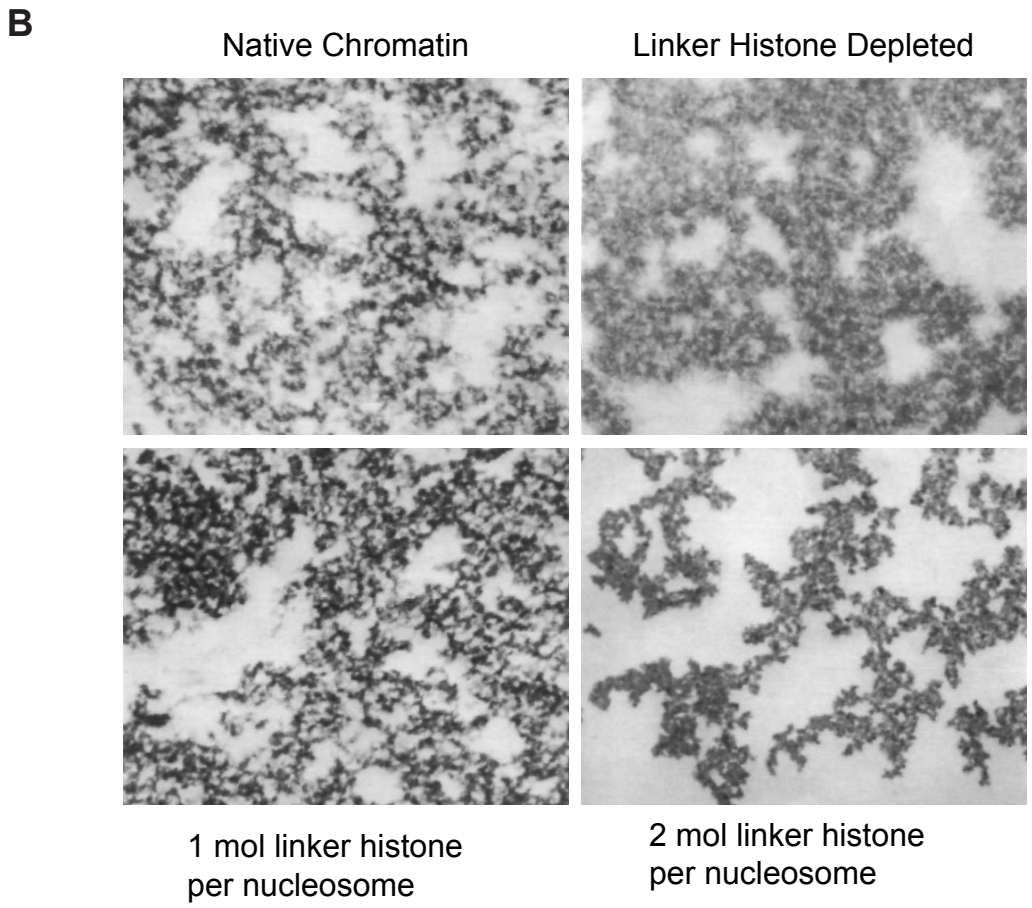


Gilbert, Figure 1





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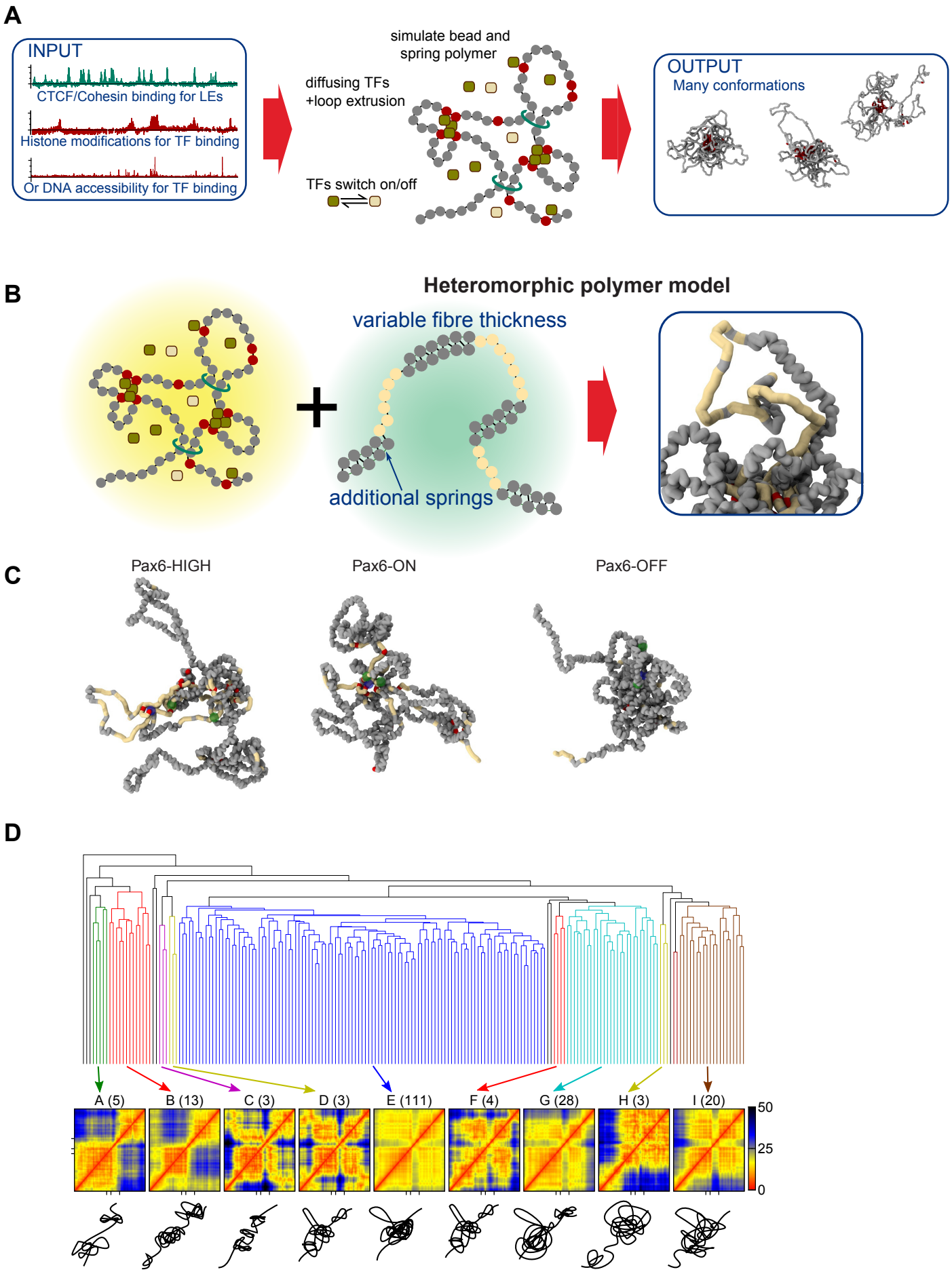
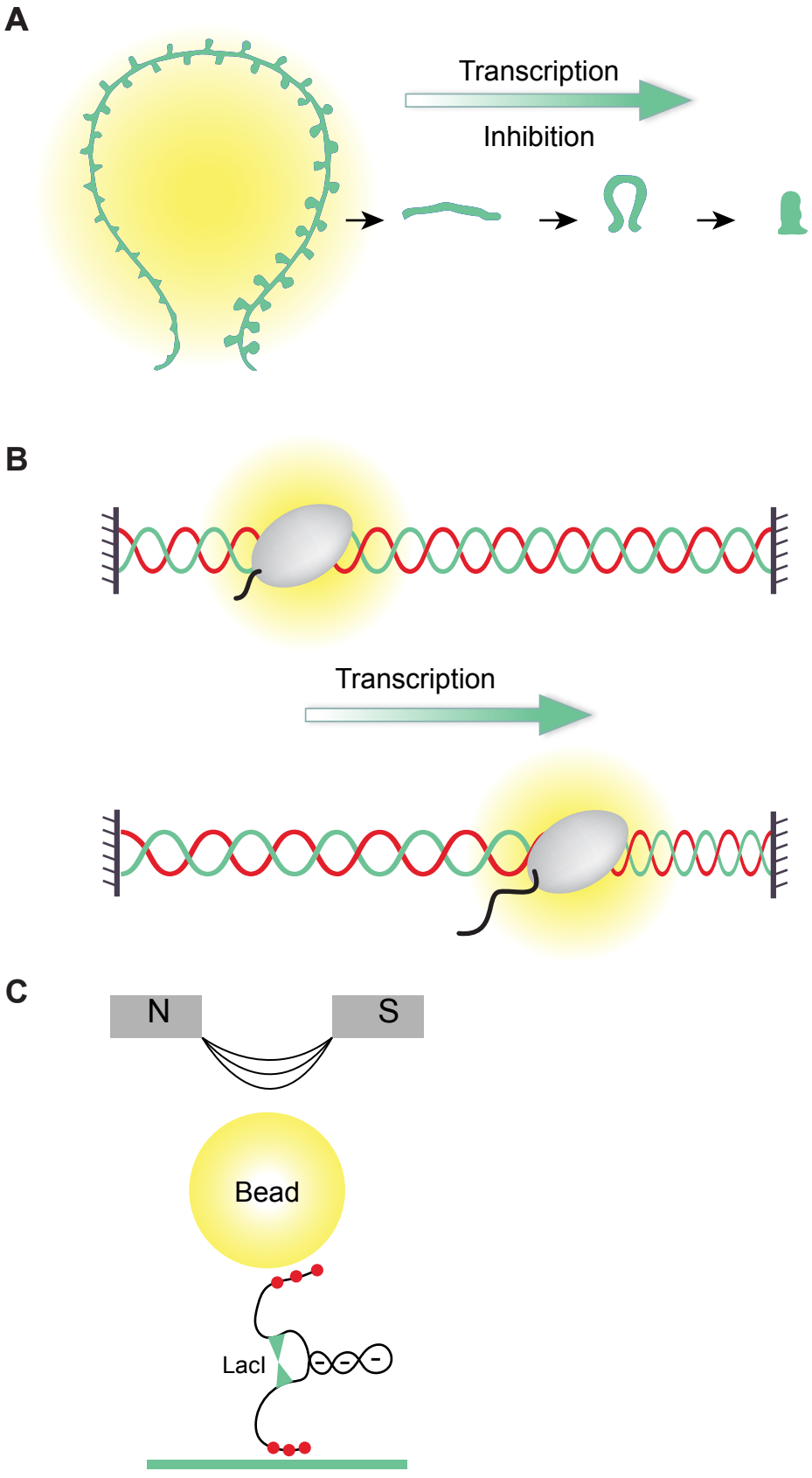


Gilbert, Figure 3



Gilbert, Figure 4