Perturbing Chromatin Structure to Understand Mechanisms of Gene Expression

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The study of nuclear structure falls between the fields of cell biology and molecular biology and draws on techniques from both fields. In recent years, many exciting advances have been made in these areas, including single-molecule and super-resolution imaging and the development of chromosome conformation capture (3C)-based technologies, which have brought the advent of genome-wide analysis of chromatin structure and contacts. However, many questions remain as to the function of nuclear structures, in particular their influence on transcription. Here we describe studies that have directly manipulated nuclear architecture at various levels and thus have clarified the causal influence of structure on transcription. We will also highlight open questions in the field, most notably regarding our understanding of the dynamics and variability in nuclear structure and its influence on gene expression.

Studies on diverse eukaryotic systems have revealed an influence of chromatin structural organization on gene expression (Bickmore 2013; Bickmore and van Steensel 2013; Nora et al. 2013; Deng and Blobel 2014; Sexton and Cavalli 2015). However, various levels of nuclear structure exert distinct degrees of influence on gene expression in different organisms, cell types, and gene loci. We will give examples of nuclear structures, with an emphasis on mammalian systems, and describe approaches that have been taken to manipulate them to elucidate their effects on gene expression. Finally, we will discuss the study of nuclear structural dynamics and will suggest much needed investigations into the functional relationship between nuclear structure and gene expression in populations and in single cells.

NUCLEAR RADIAL POSITIONING

Most broadly, association with the nuclear periphery has been associated with gene silencing, whereas transcribing genes are often found in the nuclear interior (Kosak et al. 2002; Bickmore 2013; Gibcus and Dekker 2013). Gene-rich chromosomes such as chromosome 19 in human cells tend to localize to the nuclear interior, whereas gene-poor chromosomes such as chromosome 18 tend to appear at the nuclear periphery (Cremer and Cremer 2010; Hübner and Spector 2010). In yeast, activated genes tend to localize to the interior or to nuclear pores, whereas silenced genes are targeted to the lamina (Brown et al. 2008; Ahmed et al. 2010). Lamina-associated domains (LADs) in mammalian cells tend to be correlated with the repressive chromatin marks H3K27me3 and H3K9me3, as well as gene-poor regions (Guellen et al. 2008; Bickmore and van Steensel 2013). In yeast, specific sequence “zip codes” can be sufficient to target

genes to the periphery, suggesting a regulated process (Ahmed et al. 2010; Brickner et al. 2012). In mammalian cells, sequence-specific targeting to the lamina has also been described (Zullo et al. 2012). At the mammalian β -globin locus, a number of transcription factors, as well as the locus control region, a powerful distal enhancer, have been shown to be required both for transcriptional activation in erythroid cells as well as repositioning away from the nuclear periphery (Bulger and Groudine 2011; Krivega and Dean 2012). Intriguingly, nuclear localization may even play a role in viral infections: A recent study observed that genomic regions localizing to the nuclear pore in human cells are more likely to experience HIV integration during infection (Marini et al. 2015). All such observations suggest that localization to the nuclear periphery could play an evolutionarily conserved role in transcriptional silencing.

However, examples exist of cell types with alternative modes of heterochromatin organization. In retinal rod cells of nocturnal mammals, chromatin organization is reversed with heterochromatin localizing to the nuclear center and euchromatin at the periphery (Solovei et al. 2009). It is thought that this organization alters the refractive properties of nuclei to aid the transmission of photons under low light conditions. Another example in which nuclear compartmentalization follows distinct rules involves olfactory sensory neurons, which monoallelically express only one of more than a thousand olfactory receptor (OR) genes in any given cell (Clowney et al. 2012). The inactive OR genes cluster around heterochromatin toward the nuclear center. During cell differentiation, this clustering correlates with loss of lamin B receptor expression. Forced expression of the lamin B receptor leads decompaction of OR heterochromatic foci and coexpression of numerous OR genes, albeit at low levels, supporting a causal role of

nuclear architecture in OR gene regulation. These observations suggest that the effects of nuclear localization might be determined by the local chromatin landscape rather than the nuclear position per se.

Studies that manipulate radial positioning have shown that nuclear localization can be functionally important, but not in all cases. First, it is important to note that although many studies saw significant changes in nuclear localization, such shifts tend not to be absolute: there is usually a high degree of heterogeneity in nuclear positioning of a locus among a cell population (Taddei et al. 2006; Toebin et al. 2012). Activation by tethering of the viral-derived transcriptional activators VP16 or VP64 to endogenous loci both induces transcription and moves loci farther from the nuclear periphery on average (Chuang et al. 2006; Therizols et al. 2014). However, it appears that nuclear relocalization was due to the acidic charge of VP64 rather than its transcriptional activity. Indeed, tethering a different acidic peptide lacking transcription activating function also mediated interior localization, uncoupling localization from transcription (Therizols et al. 2014). An acidic activation domain was also sufficient to destabilize peripheral LAD positioning without affecting transcription (Kind et al. 2013). Also suggesting a nondeterministic role for nuclear positioning in an “activating neighborhood” was a study in yeast, which showed that inducing the *HXX1* gene caused its translocation to a nuclear pore, but that tethering to a pore was not sufficient for transcription, although it did potentiate induction (Taddei et al. 2006).

Experiments that force genes to move to the nuclear lamina have had similarly varied effects. Artificial tethering of some genes to the nuclear lamina can mediate silencing of those genes, both in yeast and in mammalian cells (Andrulis et al. 1998; Finlan et al. 2008; Reddy et al. 2008). Moreover, in both mammalian cells and worms, the related repressive marks H3K9me3 and H3K9me2, respectively, were sufficient and necessary to target genes to the lamina (Toebin et al. 2012; Kind et al. 2013). However, for certain genes nuclear lamina tethering was not sufficient to repress transcription (Finlan et al. 2008; Kumaran and Spector 2008). A potential solution to the variable effects of lamina targeting was suggested by a very elegant experiment by van Steensel and colleagues: Lamina-associated chromatin was fluorescently tagged, and then these initial LADs were examined after cells had undergone division (Kind et al. 2013). First, LADs were not strictly inherited but only partially, showing that peripheral localization is nondeterministic. Moreover, only 30% of the total LAD pool, as measured in bulk cells, localized to the nuclear periphery in any given cell. This suggests that peripheral association is correlated with but is not absolutely necessary for silencing. Perhaps silenced domains tend to passively diffuse to the nuclear periphery, where the relative paucity of transcription factors and polymerase reinforces their silencing.

The example of the varying roles of peripheral localization in silencing illustrates both the importance of functional assays for interpreting the role of nuclear structure in gene expression and the power of imaging ap-

proaches to clarify variability in structure between nuclei. The observation that the majority of structural patterns in the nucleus tend to be highly variable between cells suggests two models: (1) Nuclear structure may potentiate gene regulation while not being strictly required or (2) transient interactions with different genomic or nuclear elements could be sufficient to influence gene expression, as exemplified by the maintenance of nuclear localization patterns upon removal of the stimulus (Therizols et al. 2014). These are not necessarily mutually exclusive, but may both contribute to nuclear organization's influence on gene regulation.

TOPOLOGICALLY ASSOCIATING DOMAINS

The earliest image-driven approaches to nuclear structure encouraged studies of macroscale nuclear structures, such as the nuclear lamina, Polycomb bodies, and nucleolar-associated domains (Hübner and Spector 2010). In recent years, the application of formaldehyde cross-linking-based assays to measure proximity of genomic regions (e.g., 3C), and their application in a genome-wide manner (4C [circularized chromosome conformation capture], 5C [carbon copy chromosome conformation capture], and Hi-C [genome-wide chromosome conformation capture]) (Dostie et al. 2006; Splinter et al. 2011; Naumova et al. 2013), have allowed examination of more fine-grained structures across the genome, including the recently discovered topologically associating domains (TADs). These domains range in size from 100 to 1000 kb and have been observed in both *Drosophila* and mammals (Sexton et al. 2012; Nora et al. 2013; Dixon et al. 2015), although they are not evident in yeast, bacteria, or *Caenorhabditis elegans* (Umbarger et al. 2011; Crane et al. 2015). These domains correlate with many previously observed functional properties of metazoan cells: TAD boundaries tend to colocalize with boundaries of replication domains, lamina-associated domains, CTCF (CCCTC-binding factor) binding sites, and transitions between regions characterized by histone marks including H3K27me3, H3K4me3, and H3K9me3 (Dixon et al. 2012, 2015; Nora et al. 2012; Sexton et al. 2012; Kind et al. 2013; Phillips-Cremins et al. 2013; Pope et al. 2014). A majority of TAD borders in mammalian cells are bound by the insulator protein CTCF, which can form homodimers and may help mediate the structure and folding of TADs (Dixon et al. 2012; Sexton et al. 2012; Phillips-Cremins et al. 2013). Moreover, the majority of enhancer–promoter contacts occurs within TADs rather than between them, and genes within the same TAD tend to be more correlated in their expression than average (Dixon et al. 2012; Nora et al. 2012). TADs are relatively conserved between cell types and even between species (Dixon et al. 2012, 2015; Nora et al. 2012). Human genetic variation that influences TAD structure can in some cases be correlated with altered gene expression, suggesting a causal role of TADs in shaping transcription (Dixon et al. 2015). Supporting such a conclusion, an unbiased assay of enhancer activity showed that enhancer activity

is both distributed within a TAD and limited by TAD boundaries (Symmons et al. 2014). Given the above observations, TADs have been viewed as a fundamental structural unit of the metazoan genome.

The high degree of conservation between cell types and organisms suggests that TAD folding might be uniform between single cells of a population. Such a question is technically challenging to address. However, one pioneering study applied Hi-C to single cells and suggested that TAD contacts tend to be more consistent between single cells than larger *cis* contacts (Nagano et al. 2015). By placing DNA FISH probes either within or across TADs, intra-TAD contacts indeed were more common than inter-TAD contacts, though a high degree of variability was observed (Dixon et al. 2012; Nora et al. 2013). Computational modeling based on 5C data also suggests that TAD folding is dynamic (Giorgetti et al. 2014). Perhaps TADs overall may be more consistent than other nuclear contacts, but they are probably still quite labile over time.

These studies beg the question of how the structure of TADs is determined. In two studies, deletions of large boundary regions led to modest effects on cross-border contacts and gene expression (Nora et al. 2012; Andrey et al. 2013). Moreover, knockdown of CTCF altered but did not abrogate TAD structure (Zuin et al. 2014). Moreover, the sequence elements that determine TAD folding are not fully delineated. Targeted deletion of CTCF binding sites at TAD boundaries perturbed gene expression patterns supporting a role for CTCF in TAD delineation (Narendra et al. 2015). Surprisingly, in this case active chromatin dominantly spread into a previously silent domain, leading to aberrant gene activation. Moreover, in one recent report, deletions and inversions observed in human patients that cause limb malformations were due to the deletion of TAD boundaries, which allowed the inappropriate spreading of enhancer influence and inappropriate gene activation (Lupiañez et al. 2015). Intriguingly, the groups of Heard and Dekker used a mathematical model of chromatin conformation to predict TAD-internal regions that were essential for structure (Giorgetti et al. 2014). Indeed, large deletions of three putative “master” structural elements within an X-chromosome TAD did appear to largely dissolve TAD structure, suggesting that boundary elements may collaborate with internal elements to structure TADs. Therefore, TAD boundaries could be one mechanism to reduce enhancer promiscuity. The field now awaits imaging of TAD dynamics in real time in single cells. This would allow an understanding of the kinetics of TAD formation after mitosis and the consistency of TADs over time in single cells. If TAD imaging could be paired with the MS2 RNA system, it might allow understanding of the correlation between TAD dynamics and transcription (Darzacq et al. 2007).

ENHANCER–PROMOTER LOOPS

For a number of years, contacts between genes and enhancers have been theorized to control gene transcription (Krivega and Dean 2012; Levine et al. 2014). It is

thought that enhancers preferentially contact their target promoter to transfer, or increase the local concentration of, RNA Pol II and transcription factors (Bulger and Groudine 2011; Calo and Wysocka 2013). In many cases, it appears that enhancers activate promoters with some degree of selectivity (van Arensbergen et al. 2014); in fact, a recent study shows that in embryonic stem (ES) cells, only 66% of active promoters genome-wide contact the nearest enhancer, whereas others appear to be controlled by more distal elements (Schoenfelder et al. 2015). Therefore, the specific contacts between promoters and enhancers may be key structural elements in controlling transcription. Many studies have shown that deletion of proteins important for transcription of a locus abrogate looping and also gene expression (Drissen 2004; Vakoc et al. 2005; Song et al. 2007). However, affecting both parameters at once fails to answer the question of causation: Is looping required for enhancer activation, or is it simply a consequence of activation? Moreover, in typical loss of function experiments involving transcription factors, secondary effects on global gene expression and chromosome folding cannot be excluded. In several plasmid-based systems, which allowed specific rather than global manipulation of DNA topology, forced enhancer–promoter juxtaposition was sufficient for transcription (Mueller-Sturm et al. 1989; Petrascheck et al. 2005; Nolis et al. 2009). However, gene regulation in plasmids can be markedly different from native gene loci. Recent studies address these issues by showing that targeted tethering of Ldb1, a factor involved in enhancer–promoter looping, was sufficient for gene activation at the native, unaltered murine and human β -globin loci (Deng et al. 2012, 2014). This also indicates that at some loci chromosome looping might be the last and rate-determining step for transcription (Vernimmen et al. 2007). However, extensive enhancer–promoter contact structures can also exist before gene activation—for example, at the TH2 cytokine gene locus (Spilianakis and Flavell 2004). More recently, using the Hi-C method to examine chromosome conformation before and after TNF- α stimulation, it was found that many induced genes had preexisting enhancer–promoter interactions (Jin et al. 2014). It is possible that these different types of interaction dynamics reflect divergent and perhaps separable modes of enhancer function, such as “opening” chromatin or recruitment of basal transcription factors, versus stimulating transcription elongation (Bulger and Groudine 2011; Schwarzer and Spitz 2014). It is also possible that signal-responsive immediate-early promoter–enhancer pairs are present in poised loops, to enable rapid inductive transcriptional responses.

Studies using guided manipulation of enhancer–promoter contacts might be useful for select therapeutic applications. For example, forced enhancer contacts with the fetal type β -like globin gene (γ -globin) strongly activated its expression in primary adult human erythroblasts and commensurately lowered expression of the adult type β -globin gene (Deng et al. 2014). This approach might be useful in diseases in which elevated fetal globin gene expression is beneficial such as sickle cell

disease and some forms of β -thalassemia. In addition, dynamically altering contacts between enhancers and promoters or other regulatory regions might be exploited to study mechanisms of enhancer function.

Many questions remain to be answered to clarify the dynamics, sufficiency, and mechanism of enhancer–promoter looping. First, as mentioned above, enhancer–promoter contacts have been suggested to perform a variety of functions including RNA Pol II transfer and promoting elongation, and it is not clear which may be most important globally, or whether different enhancers might perform different functions (Bulger and Groudine 2011). Another level of complexity is raised by the observation from 3C-based studies that many promoters may contact multiple enhancers in the same cell type, either simultaneously or alternately (Sanyal et al. 2012; Kieffer-Kwon et al. 2013; Jin et al. 2014; Schoenfelder et al. 2015). Finally, there is little understanding of the dynamics of enhancer–promoter interactions. For example, is an enhancer required to continuously contact its target gene to enable transcription or is it simply required to set up the locus during development, leaving the gene to fire independently subsequent to activation? Alternatively, do looped contacts persist following gene inactivation and perhaps contribute to transcriptional memory? Because genes often contact multiple enhancers (Schoenfelder et al. 2015), do they all come together in a rosette around the promoter or does each contact the gene with specialized dynamics?

DYNAMICS OF NUCLEAR STRUCTURES

A deeper understanding of the dynamics of nuclear structure will be required to clarify its effects on transcription. One fundamental question of structural dynamics is whether nuclear structure can be inherited across mitosis. During mitosis, chromosomes are condensed, territories are reorganized, most transcription factors are evicted from chromatin, and structures including TADs disappear (Naumova et al. 2013). Perhaps as a result of this global disruption, many structures are not recapitulated exactly after mitosis. LADs are largely redistributed (Kind et al. 2013) along with other structures such as nucleolar-associated domains (Hübner and Spector 2010). Certain parameters may be carried over between mitosis and might aid structural reconstitution to some extent: Certain transcription factors and histone modifications remain associated with mitotic chromatin to mediate gene reactivation via a “bookmarking” mechanism (Kadauke and Blobel 2013; Wang and Higgins 2013). At certain sites architectural proteins such as CTCF may remain bound and may even stably maintain certain chromatin loops (Burke et al. 2005). It will be intriguing to measure mitotic binding of architectural proteins like CTCF in a genome-wide manner, to examine whether TAD boundaries themselves are bookmarked.

It is also important to understand the lability of nuclear structures and contacts during the interphase to understand the impact of structure on transcription. Fixed-cell imaging of large populations of cells can uncover the spectrum of possible chromatin conformations and sug-

gest an inherent variability over time. For example, DNA fluorescence in situ hybridization (FISH) of TADs, LADs, and radial positioning of genes suggests that they can vary highly between cells under uniform conditions (Nora et al. 2012; Kind et al. 2013; Therizols et al. 2014). Fixed-cell imaging can also be powerfully used in genetic screens for factors involved in nuclear architecture: Two elegant examples include a screen for determinants of Polycomb bodies in murine cells and of LADs in *C. elegans* (Toebein et al. 2012; Gonzalez et al. 2014). In the future, an exciting possibility may be to use higher-throughput RNA FISH methods to allow more comprehensive connections to be made between nuclear architecture and transcription (Chen et al. 2015; Lee et al. 2015). However, ultimately to understand dynamics and stability of genomic organization, live-imaging approaches must also be used.

Such live-imaging studies tend to be technically demanding and low-throughput, but they provide invaluable insight. Few studies explicitly examining nuclear structure have been undertaken, but the techniques are under development. One elegant study performed by Misteli and colleagues induced simultaneous breakages in two chromosomes marked by tagged arrays and then visualized the joining events. This led to the observation that broken ends tend not to separate, or to increase their speed of movement, and that nuclear confinement dictates that neighboring breaks are much more likely to be rejoined (Roukos et al. 2013). Such techniques could be applied to intact loci in different developmental or genetic contexts to understand the function of nuclear structure. Along these lines, one study visualized a locus involved in rearrangement of the immunoglobulin locus in B cells and quantified the highly efficient process of finding its recombination target (Lucas et al. 2014). Excitingly, the CRISPR/Cas9 technology should now allow endogenous loci to be imaged without genetically altering them (Chen et al. 2013). Future studies could label two or more loci to address promoter–enhancer dynamics, or intra-TAD contact dynamics, by imaging in real time at native loci.

CONCLUSION

Studies of nuclear structure have revealed both cases in which structures appear strongly influential for gene expression and counterexamples of genes and chromatin marks that appear unaffected by structural alterations. Though intriguing trends have emerged, this suggests that there is further complexity to be understood before we will be able to draw predictive inferences about gene expression from knowledge of structure. Predictive power will be gained by further mechanistic studies, as well as by live-imaging approaches to help understand the dynamics and heterogeneity of different facets of nuclear structure.

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Cold Spring Harbor Symposia on Quantitative Biology

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Cold Spring Harb Symp Quant Biol 2015 80: 207-212 originally published online September 14, 2015

Access the most recent version at doi:[10.1101/sqb.2015.80.027359](https://doi.org/10.1101/sqb.2015.80.027359)

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