Are genes switched on when they kiss?

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Chromatin loops are pervasive and permit the tight compaction of DNA within the confined space of the nucleus. Looping enables distal genes and DNA elements to engage in chromosomal contact, to form multigene complexes. Advances in biochemical and imaging techniques reveal that loop-mediated contact is strongly correlated with transcription of interacting DNA. However, these approaches only provide a snapshot of events and therefore are unable to reveal the dynamics of multigene complex assembly. This highlights the necessity to develop single cell-based assays that provide single molecule resolution, and are able to functionally interrogate the role of chromosomal contact on gene regulation. To this end, high-resolution single cell imaging regimes, combined with genome editing approaches, are proving to be pivotal to advancing our understanding of loop-mediated dynamics.

Introduction

In each mammalian cell, chromosomal DNA is compacted by extensive looping and reduced in size ~500 000 fold to be housed inside each diploid nucleus. Due to this tight compaction, regions of looped chromatin are permitted to interact or “kiss.” Chromosomal contact is strongly correlated with the transcriptional activity of interacting DNA elements. Therefore, the physical contact between looped chromatin appears to be fundamental to gene regulation. However, whether the “kissing” of chromatin is a direct cause, or consequence, of transcription is a long-standing question in molecular biology.

By identifying sites where chromatin interacts, the population-based chromosome conformation capture (3C) and derivative (4C, 5C, Hi-C, etc.) technologies (Table 1) allow us to “peer” into the highly interconnected environment of the nucleus and to characterize the global interactome (see ref. 8 for review). 3C-based techniques involve crosslinking of adjacent chromatin, followed by shearing of DNA, by digestion or sonication. Proximal genomic regions are then ligated to each other, so interacting DNA elements can be recovered by sequencing or microarray analysis. In-depth analyses of chromatin interaction data sets reveal a large heterogeneity that infers significant cell-to-cell variability. Thus, to decrypt the granularity of these events, the physical juxtaposition of interacting loci is verified at a single-cell level by fluorescent in situ hybridization (FISH). This microscopy-based assay, which can target RNA or DNA (Table 1), reveals proximal FISH foci in a subset of the population. Collectively, 3C and FISH data suggest that a special pool of cells in the population possesses the correct chromatin organization to permit specific chromosomal interactions.

These observations raise several important questions. (1) Does every cell possess a unique organization of its chromosomes, or alternatively, can each cell across a population adopt a similar spatial organization of their genome? (2) If it is indeed only a small percentage of cells that possess the appropriate chromatin arrangement to permit chromosomal

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Nucleus
interaction between DNs

Site-specific induction of DSB or nicks

chromatin architecture while

we summarize some of the latest advances

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One-to-one: interaction between a pair of pre-selected loci

One-to-all: genome-wide interaction of one pre-selected locus

Many-to-many: all interactions between many pre-selected loci

All-to-all: genome-wide interaction between all loci

One-to-one: interaction mediated by a particular protein between a pair of pre-selected loci

All-to-all: genome-wide interaction mediated by a particular protein between all loci

Interaction between DNA loci using BAC probes

Interaction between DNA loci using PCR-generated short probes

Fluorescent labeling of whole chromosomes

Interaction between RNA loci (intronic probes can identify interaction, as well as transcription, between DNA loci)

Transcriptional status and location of many genes on a single chromosome

Site-specific induction of DSB or nicks

Table 1. Techniques for unraveling chromatin interactions

| Method | Scope/description | Reference |
|--------|-------------------|-----------|
| 3C     | One-to-one: interaction between a pair of pre-selected loci | 9         |
| 4C     | One-to-all: genome-wide interaction of one pre-selected locus | 11        |
| 5C     | Many-to-many: all interactions between many pre-selected loci | 60        |
| Hi-C   | All-to-all: genome-wide interaction between all loci | 25        |
| ChIP-loop | One-to-one: interaction mediated by a particular protein between a pair of pre-selected loci | 61        |
| ChIA-PET | All-to-all: genome-wide interaction mediated by a particular protein between all loci | 10        |
| DNA FISH | Interaction between DNA loci using BAC probes | 62        |
| HD FISH | Interaction between DNA loci using PCR-generated short probes | 63        |
| Chromosome painting | Fluorescent labeling of whole chromosomes | 64        |
| RNA FISH | Interaction between RNA loci (intronic probes can identify interaction, as well as transcription, between DNA loci) | 65        |
| iceFISH | Transcriptional status and location of many genes on a single chromosome | 66        |
| ZFN/TALEN/CRISPR | Site-specific induction of DSB or nicks | 35, 39, 67, 68 |

Contact, how do these cells establish optimal chromatin organization? (3) What are the general principles governing loop-mediated transcriptional regulation? In order to accurately resolve these fundamental questions we need to move from indirect approaches conducted on millions of cells, to single cell functional assays conducted systematically across large populations of cells. This requires the ability to develop assays capable of discretely interfering with chromatin architecture while simultaneously monitoring the position of multiple DNA elements, as well as their associated transcription. Moreover, the ability to perform these assays in live cells will be essential to uncover the complexities and dynamic nature of such events. Here we summarize some of the latest advances in biochemical and microscopic assays that are profoundly altering how we understand gene regulation in the 3D space of the nucleus.

Higher Order Genome Organization Mediates Reproducible Chromatin Interactions

Chromosomal contact between genes and their enhancer elements are the best studied of all genomic interactions. Genome-wide chromatin interactome studies reveal that enhancer-promoter interactions are pervasive and may occur in cis even over large genomic distances, or in trans across different chromosomes. One of the most well-characterized examples is the enhancer-promoter model between the locus control region (LCR), and the promoter of the β-globin gene. By physically contacting the promoter, the LCR has been shown to induce the expression of the β-globin gene. Other similar enhancer-promoter interactions have also been described, such as looping between distal regulators and the α-globin locus, the CFTR locus, the c-MYC locus, and TNFα-responsive genes. Interestingly, recent studies demonstrate that enhancer-promoter contacts are not dynamic, but rather are formed prior to gene activation. Although it is unclear whether this is a general feature of chromatin organization these studies indicate that, once formed, specific chromosomal interactions may be highly stable. Yet, at a fundamental level, it is still poorly understood how these distal elements are able to locate their “interacting” partners in the highly crowded environment of the nucleus.

In situ hybridization assays that “paint” entire chromosomes reveal that chromosomal DNA is not haphazardly intertwined in the interphase nucleus, but rather divided into distinct territories (Fig. 1A). Moreover, the juxtaposition of neighboring chromosome territories is non-random, with smaller chromosomes preferentially occupying the nuclear center, while, larger chromosomes are more commonly located near the nuclear periphery. Territories are not insulated from each other, and regions of chromatin have been observed to “roam” into neighboring territories to engage in chromosomal contact. Although, these inter-chromosomal contacts are rare, “intermingling” between pairs of loci is highly correlated with translocation frequency. This reveals that the 3D positioning of chromosome territories in the nucleus is a key contributor to genomic structure and function.

At a population level, 3C-based approaches are able to interrogate chromatin organization to a resolution of ~5 Kb. Analysis of Hi-C data sets have uncovered that chromatin is roughly divided into two types of multi-Mb compartments, which can span the same, or different chromosomes. Gene-rich and transcriptionally active loci preferentially associate in compartment A and are spatially segregated from gene-poor and transcriptionally silent loci located in compartment B. These compartments are further organized into smaller chromosomal domains, referred to as topologically associating domains (TADs), which range in size from several hundred Kb to ~3 Mb (Fig. 1A). By constraining the DNA topology, TADs
may enhance the probability of loop-mediated interactions occurring. Hence, chromosomal contact, which may occur in cis over many Mb or in trans, would most likely result from the proximal localization of specific TADs on the same or a neighboring chromosome. Thus, this hierarchical mode of chromatin organization may facilitate specific chromosomal interactions, such as those that occur between distal regulatory elements and their target genes.

Do All Cells Possess the Same Genome Connectivity?

Within a TAD, or at the interface between TADs, chromosomal looping may also bring co-regulated genes into close proximity to permit chromosomal contact. These interactions are proposed to occur at discrete foci of active, hyper-phosphorylated RNA Pol II that have been referred to as multigene complexes or transcription factories. Numerous studies have demonstrated that loop-mediated contact between co-regulated genes coincides with alterations in the transcription of interacting genes, including those encoding globins, interleukins, Hox genes, and estrogen-responsive genes as well as TNFα- and TGFβ-responsive genes. Over 95% of genes engaged in promoter-mediated chromosomal contact in multigene complexes are associated with transcriptional activity. Therefore, chromosomal contact between co-regulated genes in multigene complexes appears to be a critical component of transcriptional regulation.

The subdivision of the genome into subchromosomal domains, or TADs, represents a highly conserved feature of chromosomal organization. This suggests that each cell across a population would possess a very similar arrangement of its chromosomes. Paradoxically, traditional in situ studies reveal a large degree of heterogeneity across a population of genotypically identical cells. For example, co-localization of FISH foci between the LCR and the β-globin gene is only observed in 5–10% of cells across a population. In these “jackpot cells,” LCR-mediated chromosomal interactions induce β-globin expression ~100-fold. This variability in β-globin gene transcript levels, or spatially variegated gene expression, may account for the stochastic effects of transcription observed across a population of identical cells. In a comparable manner to the LCR/β-globin chromosomal contact, interacting genes in multigene complexes are only
detected in a fraction of the population.\textsuperscript{4,6} Clearly, interrogating chromosomal interactions both at the level of a single cell and single molecule, is imperative to the interpretation of global interactome studies.

Recently, Nagano et al. adapted the Hi-C protocol to allow the simultaneous detection of thousands of contacts in the same nucleus.\textsuperscript{32} Interestingly, analyses of single cell Hi-C data reveals that intra-chromosomal contacts are highly consistent between cells. This provides strong evidence that, within a chromosome territory, the 3D compaction of chromatin is highly ordered. Conversely, inter-chromosomal contacts were highly variable between individual cells.\textsuperscript{32} Although the low sample number needs to be considered, this suggests that there may be considerable variation in the 3D organization of chromosomal territories across a population of cells. Clearly, these single cell interactome maps are critical to unravel the degree of variability in chromatin folding between individual cells. However, as these assays are performed in fixed cells, they are still unable to directly address how, or when, loop-mediated contact influences transcription of interacting genes.

**What are the Rules Governing Chromosomal Contact?**

Through physical contact, enhancers are proposed to promote transcription by increasing the concentration of transcriptional regulators at the promoter of their “interacting” partner.\textsuperscript{8,14} There are two opposing views of how enhancer-promoter regulation occurs: recent evidence shows that these interactions are preformed,\textsuperscript{18} whereas alternative evidence favors the view that these interactions are dynamically established.\textsuperscript{14,33} For example, through the looping out of ~50 Kb of intervening chromatin fiber, the LCR was shown to bring regulators to the promoter of the β-globin gene, to initiate its transcription.\textsuperscript{14} Similarly, upon induction by viral infection, NFκB is delivered to the promoter of inducible genes via inter-chromosomal contact.\textsuperscript{33} This raises an interesting question; does loop-mediated contact enhance the transcription of interacting genes in a multigene complex?

Chromatin interaction analysis with paired end tags (ChIA-PET) revealed that the GREB1 gene engages in multiple intrachromosomal interactions to form a multigene complex.\textsuperscript{6} Of the 4 interacting genes investigated, only GREB1 transcription is activated by the estrogen receptor-α (ERα).\textsuperscript{6} Interestingly, the global knockout of ERα disrupted not only GREB1 transcription but the transcription of other 3 non-ERα responsive genes (Fig. 2). Thus, in an analogous manner to enhancer-promoter interactions, this study suggests that the GREB1 gene loop may be a prerequisite for the transcriptional activation of other interacting members of the multigene complex.

TNFα, a major proinflammatory cytokine, is another stimulus that induces the coordinated assembly of coregulated genes in NFκB-dependent multigene complexes.\textsuperscript{30} SAMD4A, a ~221 Kb gene on chromosome 14, is rapidly switched on by TNFα in primary human umbilical vein endothelial cells (HUVEC).\textsuperscript{34} 4C analysis reveals that prior to stimulation with TNFα, SAMD4A seldom interacts with other genes.\textsuperscript{30} After activation by
TNFα, *SAMD4A* interacts with multiple coregulated genes to form a multigene complex.30 *TNFAIP2*, a gene located on the same chromosome but ~50 Mb downstream, and *SLC6A5* on chromosome 11, are two well-characterized interacting partners of *SAMD4A* (Fig. 3).30 siRNA approaches cannot be utilized to interrogate loop-mediated dynamics in the *SAMD4A*/*TNFAIP2*/*SLC6A5* multigene complex, as all three genes are activated by the same transcription factor (Fig. 3).4 This exposes the necessity to develop functional assays able to discretely alter gene loop topology, without the global ablation of transcriptional regulators.

Site-specific DNA binding tools, including zinc fingers (ZF), transcription activator-like effectors (TALE) and the Type II CRISPR system (clustered regularly interspaced short palindromic repeats) are emerging as powerful ways to discretely interrogate gene regulation (Fig. 4A).35-38 Fusion of the FokI endonuclease to a ZF or TALE DNA binding region, or the CRISPR-associated protein Cas9, enables these tools to induce site-specific double strand breaks (DSB).37-39 Two features of FokI have made it an ideal enzyme to be used as a component of a site-specific nuclease. First, the well-characterized modular nature of the enzyme40-43 has allowed just its catalytic domain to be fused with customizable DNA binding proteins such as zinc fingers and TALEs. Second, since it acts as a dimer,44 two nucleases binding in opposite orientations on adjacent sequences on the genomic target are necessary to generate a DSB, essentially enhancing its specificity.

Typically, these site-specific nucleases are used in “cut and paste” experiments, whereby the cell’s own repair responses, nonhomologous end-joining (NHEJ) or homologous recombination (HR), are exploited to repair the DSB.45 Recently, we developed a novel single cell microscopy-based assay, to address the role of loop-mediated contact on transcription of interacting genes in the *SAMD4A*/*TNFAIP2*/*SLC6A5* multigene complex.46 In the assay, TALE nucleases (TALENs) were used to induce a DSB at the approximate site within each of these three gene loops that was established by 3C-technologies to engage in contact.4,46 We have also tested the ability of other site-specific nucleases to disrupt the *SAMD4A* gene loop (Fig. 4A). The DSB, or site of disruption, was detected by immunofluorescent staining of H2A.X pSer139, a factor of the DSB repair process.47 In parallel to the detection of the DSB, transcriptional activity of interacting genes in the multigene complex was determined using intronic RNA FISH. As introns are typically excised and degraded co-transcriptionally, intronic FISH foci represent the transcriptional start site (TSS).34 Consistent with previous studies,4 co-localization between the FISH foci of these three genes was only observed at a low percentage of all alleles across the population (~5%).46 This would suggest that this small fraction of the population represents a “special pool” of primed cells that permit loop-mediated contact.

Using the TALEN single cell assay, we observed that perturbing the *SAMD4A* gene loop did not alter transcription 5′ of the DSB (Fig. 4B section I). However,
Figure 4. Chromosomal contact permits transcription of coregulated genes. (A) Site-specific nucleases are powerful tools to discretely interrogate 3D gene regulation. Transcription activator-like effectors nucleases (TALENs) can be designed to target almost any DNA sequence, whereas zinc finger nuclease (ZFN) design is limited by the availability of triplet motifs, and CRISPR/Cas9 (RGEN) targeting requires the NGG or NAG protospacer adjacent motif (PAM). The disruption of gene loops can be visualized by immunofluorescent labeling of H2A.X pSer139. (B) TALEN-mediated disruption of sites of chromosomal contact reveals a hierarchical regulation between these three genes. (C) Repairing the disrupted SAMD4A gene loop restores transcription of interacting genes. Bar, 5 μm.
consistent with other studies, we observed silencing of \textit{SAMD4A} transcription 3' of the DSB (Fig. 4B section I). Intriguingly, despite occupying distal genomic locations, the transcription of \textit{TNFAIP2} and \textit{SLC6A5} were also significantly reduced (Fig. 4B section I). Further, the effect on co-transcription was hierarchical, with the disruption of \textit{TNFAIP2} altering \textit{SLC6A5} expression, but having no influence on \textit{SAMD4A} (Fig. 4B section II), whereas perturbing \textit{SLC6A5} did not influence \textit{SAMD4A} or \textit{TNFAIP2} transcription (Fig. 4B section III). This suggests a hierarchical assembly between these three genes, whereby \textit{TNFAIP2} “collects” RNA Pol II from \textit{SAMD4A}, which are then able to recruit \textit{SLC6A5} to the multigene complex (Fig. 5A). Is this ordered assembly of these co-regulated genes reliant on chromosomal contact? In our study, the TALEN-induced DSB is visualized by immunofluorescent labeling of Ser139 phosphorylated H2A.X. However, a large number of other proteins involved in the repair process are also recruited to the DSB site that may have several implications for loop-mediated contact. One possibility could be that chromosomal contacts between \textit{SAMD4A}, \textit{TNFAIP2} and/or \textit{SLC6A5} may still occur, but are “bridged” via the repair complex (Fig. 5B section I). This may occlude the ability of \textit{SAMD4A} to “deliver” Pol II to other interacting genes. DSB induction has been shown to enhance the mobility of damaged chromatin (see ref. 50 for review). Therefore, another explanation could be that, due to increased movement, the probability that the disrupted gene loop will be able to interact with other loci is significantly reduced (Fig. 5B section II). Alternatively, it is unknown how the DSB, and associated repair factors, influence gene topology. Therefore, we speculate that the DSB may prevent loop-mediated contact, by destabilizing, or “collapsing,” the disrupted loop (Fig. 5B section III). Nonetheless, in all of these scenarios, gene loop topology and chromosomal contact are strongly implicated in the regulation of multigene complex assembly. Therefore, our study provides the first direct evidence that chromatin contacts may be a cause, and not simply a consequence of transcription.

These observations beg a new question, what are the protein and/or sequence specific factors driving the hierarchy? Replacing the region of contact with a
GFP sequence was sufficient to restore the transcription of interacting genes (Fig. 4C). This suggests that, while the sequence with the first intron that is engaging in loop-mediated contact is not determining the hierarchy, the topological framework of the multigene complex is a requirement for 3D transcriptional regulation. A recent study revealed that the co-localization of these NFκB-regulated genes may be driven by promoter-specific sequence elements. Therefore, discrete repair experiments within the endogenous promoters of these genes may unveil whether promoter-specific sequence elements are involved.

What are the Dynamics of Loop-Mediated Chromosomal Contact?

Chromosomal interactions detected by 3C and FISH-based approaches represent a snapshot of chromatin organization across a heterogeneous population of cells. Therefore, live microscopy-based assays are indispensable to chromatin interactome studies. Historically, co-localized RNA or DNA FISH foci are used to validate chromosomal interactions that are detected by 3C-based approaches. Overlapping FISH foci between SAMD4A, TNFAIP2, and SLC6A5 are only observed at 5% of all alleles. However, when we disrupted the SAMD4A gene loop we observed a significant decrease in TNFAIP2 and SLC6A5 expression (at more than 5% of alleles). As the half-life of the SAMD4A intron is ~5 min, it is possible that the intronic FISH foci may not always represent the exact site of transcription. Therefore, we speculate that percentage co-localization as measured by intronic RNA FISH may not always be an accurate measure of “jackpot” or “primed” cells. Live-cell imaging reveals that the movement of individual loci is limited to a 0.5 μm area, which corresponds to the approximate size of a ~1 Mb TAD. Therefore, each locus constrained within a TAD may be able to interact with every loci located in the same TAD, or even proximal TADs, within the time frame of each cell division. Thus, live cell assays are critical to deciphering the principles governing loop-mediated gene regulation.

In fixed cells, light microscopy studies reveal that the co-localization of coregulated genes occurs at discrete foci of RNA Pol II. However, these microscopy approaches are unable to accurately discern the dynamics of RNA Pol II/gene loop assembly. Moreover, the resolution of conventional light microscopy is at best, ~200 nm in the x- and y-axes and ~500 nm in the z-axis. Novel super-resolution microscopy approaches such as photo-activated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM) and selective plane illumination microscopy (SPIM) are able to breach this limit, to achieve sub-diffractive resolution. These approaches are based on repetitive stochastic activation and deactivation of fluorescent proteins or probes, allowing the acquisition of super-resolution information and localization down to 20 nm. Recently, through the endogenous labeling of RNA Pol II, Cisse et al. used PALM to study RNA Pol II dynamics in live cells at subdiffractive resolution. By replacing the endogenous Pol II catalytic subunit (RPB1) with the photo-switchable fluorescent protein, Dendra2, they were able to show that the clustering of RNA Pol II precedes transcription. This study provides critical insight into the dynamics of multigene complex assembly. However, to what extent does the replacement of an endogenous protein subunit with GFP alter the normal functioning of RNA Pol II? Clearly, in order to resolve questions around the rules governing chromosomal contact and transcriptional regulation, requires the ability to perform functional assays at their endogenous locations, and as discretely as possible. To this end, high-resolution single cell imaging regimes combined with genome-editing approaches, including those involving transcriptional activation and/or repression and live imaging, will pave the way to a fuller understanding of 3D transcriptional regulation.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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