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Review

Architectural hallmarks of the pluripotent genome

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ABSTRACT

Pluripotent stem cells (PSCs) have the ability to self-renew and are capable of generating all embryonic germ layers (Evans and Kaufman, 1981; Thomson et al., 1998). PSCs can be isolated from early embryos or may be induced via overexpression of pluripotency transcription factors in differentiated cells (Takahashi and Yamanaka, 2006). As PSCs hold great promise for regenerative medicine, the mechanisms underlying pluripotency and induction thereof are studied intensively. Pluripotency is characterized by a unique transcriptional program that is in part controlled by an exceptionally plastic regulatory chromatin landscape. In recent years, 3D genome configuration has emerged as an important regulator of transcriptional control and cellular identity (Taddei et al., 2004 [4]; Lanctot et al., 2007 [5]; Gibcus and Dekker, 2013; Misteli, 2009 [7]). Here we provide an overview of recent findings on the 3D genome organization in PSCs and discuss its putative functional role in regulation of the pluripotent state.

1. Introduction

Gene expression programs guide developmental decisions and underlie cell identity during all stages of development. Transcriptional activity is controlled by various factors including *trans*-acting chromatin and transcription factors, (distal) regulatory DNA elements, epigenetic decorations, and 3D chromatin organization [4–7]. As discussed in more detail below, the eukaryotic genome is stored in a compacted hierarchical fashion in the interphase cell nucleus (Fig. 1). Chromosomes occupy distinct nuclear sub-volumes that are called chromosome territories (CTs) [8]. Within a CT, along the linear chromosome axis, one can discern self-aggregating structural domains called topologically associated domains (TADs) [9–11]. These structural units serve as templates to accommodate physical contacts between genes and the cognate regulatory DNA elements that they encompass. At all levels of organization, genome architecture appears to be the result of a plethora of tissue-invariant and tissue-specific factors that compete for access to DNA to compact it or, oppositely, to expose sequences for reading, repairing and copying of the genetic code. Below, we first review current insight into the mechanisms that shape the genome and evaluate the functional implications of architecture at each topological level, starting at the sub-TAD level and gradually zooming out to higher-order genome structures. We next discuss the architectural specifics of the pluripotent 3D genome and elaborate on its significance for maintenance of the pluripotent state.

1.1. The dynamics and significance of enhancer–promoter contacts

Enhancers have emerged as important regulators of cell-specific gene expression patterns. Enhancers and other regulatory elements act on potentially distant target promoters via 3D chromatin contacts (Fig. 1), which can in some cases bridge distances of a megabase or more [12–15]. A forced enhancer–promoter loop was shown to be sufficient to induce recruitment of RNA Polymerase II and initiate transcription, even from a developmentally silenced gene although transcription elongation did not proceed at optimal rates [16,17]. The observation that loops persist when transcription is blocked [16,18,19] indicates that the process of transcription is not essential for maintenance of contacts, and that a different process is required to break up loops [20]. Taken together, current evidence suggests that enhancer–promoter loops form prior to and are required for efficient initiation of transcription. This is in contrast with elongation, the traversing of an RNA polymerase along the linear chromosome axis, which is likely not controlled at the 3D genome level.

The chromatin fiber behaves essentially like a polymer with certain flexibility when the effect of associated proteins is ignored. Chromatin loops are therefore likely to rely on random collisions between two sites and the further apart two sites sit on the linear chromosome, the less likely they are to autonomously contact each

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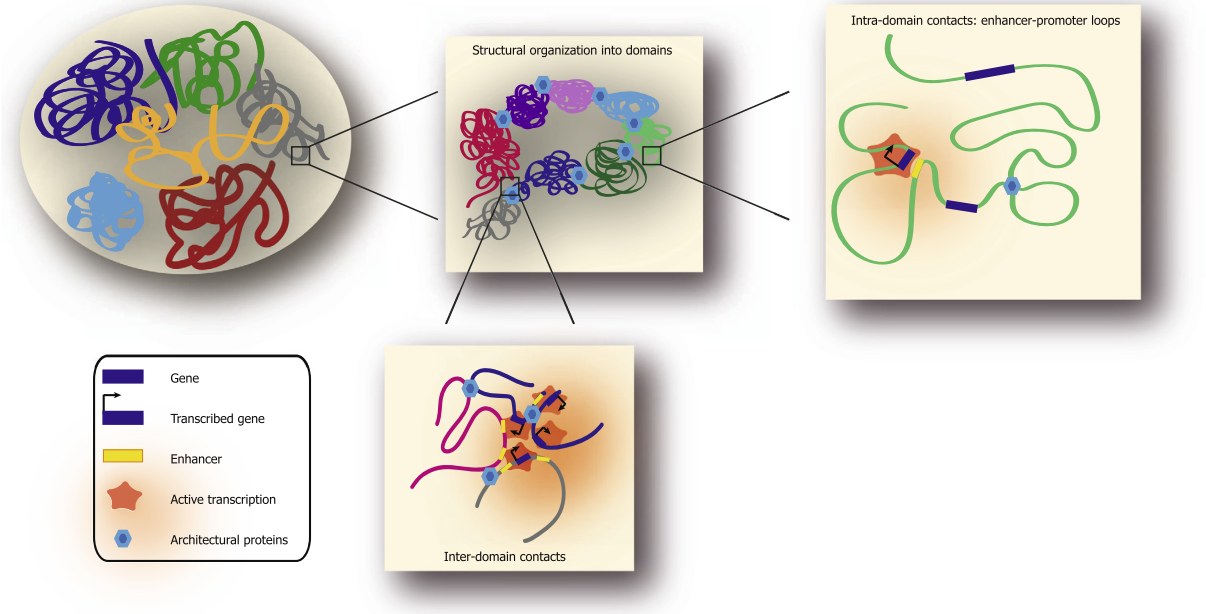

Fig. 1. Hierarchical levels of 3D genome organization. Interphase nuclei occupy distinct CTs (upper left). Within a CT, the chromosome is structurally organized into distinct TADs that are mostly demarcated by CTCF-associated TAD boundaries (upper middle). On a sub-TAD level regulatory elements such as enhancers sample the chromosome for compatible target genes that can be transcribed upon successful establishment of enhancer–promoter loops (upper right). Chromatin contacts between TADs that colocalize spatially have the ability to affect each other's transcription state (bottom).

other [21–26]. 3D contacts can stabilize when genome-associated proteins engage in protein-protein interactions [27]. Architectural proteins such as CTCF and cohesin [28–30] and general transcriptional co-activators such as Mediator and P300 [31,32], as well as more cell type-specific transcription factors [27,33,34] have been reported to be involved in loop formation and shaping of the genome. Enhancer–promoter contacts are frequently anchored by Mediator, cohesin, co-factor Nipbl, and (lineage-specific) transcription factors. These loop structures are proposed to be relatively dynamic during development, and are therefore considered important for regulation of key developmental genes [35–39].

Enhancer–promoter contacts correlate with but are not always sufficient to induce transcriptional activity; in some instances, they are believed to provide a spatial configuration that is poised for activation. Two distinct types of loops have been reported: pre-formed and *de novo* established loops, also referred to as permissive and instructive configurations, respectively [22]. The functional relevance of these differences in timing of loop formation is largely unknown and it is currently unclear whether these two configurations distinguish different categories of genes. Although the mechanisms that establish pre-formed loops require further investigation, they are speculated to facilitate rapid transcriptional activation: a permissive topology may optimally prime mammalian cells for a timely response to developmental stimuli [40–43]. Furthermore, pre-formed loops have been proposed to prevent bystander activation, via which unrelated neighboring genes can benefit from spurious contacts with unrelated regulatory DNA elements [11]. This contrasts with loops that are established de novo, presumably through the action of tissue- or lineage-specific transcription factors. These loops generally arise in a more tissue-specific manner at cell identity genes, which suggests a role in fate establishment [22,44].

Chromatin form generally precedes function and the 3D wiring of regulatory elements is assumed to coordinate cell type-specific expression patterns [16,45], appointing chromatin architecture as an integral feature of identity programming. Identity may be structurally safeguarded by the progressive formation of regulatory contacts required for later stages of lineage commitment, while 3D configurations required for earlier developmental stages are disrupted [46]. Recent work on the *Drosophila* genome revealed that only an estimated 6% of all identified enhancer–promoter interactions changes significantly during development. For the remaining 94% of loops, no dynamics in behavior were observed over time or between tissues, regardless of developmental transitions [47]. Based on these observations, it was proposed that enhancer-bound transcription factors assemble loops with target promoters, after which polymerase is recruited and maintained in a paused state. An additional cue, for example provided by recruited co-factors or looping of additional enhancers, may then trigger dispense of the paused state, allowing initiation and elongation of transcription [47]. High resolution Hi–C across a panel of human cell lines confirmed that many chromatin loops are conserved between cell types as well as during evolution, as evidenced from a comparison to Hi–C data generated in a mouse cell line. However, hundreds of tissue-specific loops between genes and enhancers were uncovered that corresponded almost exclusively with a highly increased transcriptional output of the contacted gene [44]. Thus, permissive and instructive configurations seem to co-exist in the genome to coordinate the faithful execution of cell-type specific transcriptional programs.

1.2. The functional importance of structural domains

The linear genome segregates into unit-like structural domains (Fig. 1) that are fairly conserved during differentiation and between mammalian species [9,10,44]. Initial Hi–C experiments revealed TADs with an estimated size of 1 Mb [9,11]. In the aforementioned more recent study, Hi–C experiments with increased sequencing depth and improved resolution allowed the appreciation of domain sizes ranging from 40 kb to 3 Mb, with a median of 185 kb [44]. TAD boundaries are enriched for CTCF-binding sites, housekeeping genes, short interspersed repeat elements, and tRNA

genes [9,11,36,44]. CTCF sites at boundaries often contact each other in nuclear space. This anchoring of dispersed sequences puts constraints on the flexibility of the intervening sequences, forcing them to sample the same search space and causing them to preferentially contact each other [44]. Hence, domains are believed to facilitate functional loops between genes and regulatory sequences contained within the domain [48,49], and indeed, most functionally relevant enhancer–promoter contacts occur within domains [9,44]. An interesting case was described for the HoxD locus, which is located near a TAD boundary. Subsets of genes within the locus were found to dynamically contact regulatory sequences either in the upstream or in the downstream TAD, in a spatiotemporal fashion [50]. It was recently shown that domain boundary disruption can lead to scattered regulatory activity that may trigger the de-regulation of surrounding genes [11,51]. Genome organization into contact domains thus seems to demarcate an 'arena' that can be probed by regulatory sequences for compatible genes, which limits their range of action and diminishes bystander effects.

1.3. The functional relevance of higher-order chromosomal structures

The relative positioning of interphase chromosomes [8] is established in early G1, during which chromosomes decondense and take up their preferred position. In general, smaller and gene-rich chromosomes tend to localize more centrally in the nucleus, while larger and gene-poor chromosomes inhabit more peripheral positions [52,53]. These spatial preferences touch upon an overall tendency of genomic domains with similar chromatin state, activity, and genomic content to unite spatially, while regions with opposing patterns are kept at bay [10,23,45,54]. In other words, 'birds of a feather flock together', a phenomenon that is also appreciable for repetitive regions such as the olfactory receptor gene clusters that cluster in nuclear space [54], the rDNA gene arrays that aggregate to form nucleoli, and the satellite repeats that form chromocenters [6,55]. The global spatial segregation of active and inactive chromatin is referred to as A/B compartmentalization [23,56]. Recently, the active (A) and inactive (B) compartments were divided further into six subcompartments with distinctive chromatin state, replication timing, and association with nuclear landmarks [44]. Nuclear subcompartments may form through self-organizing principles of 3D genome architecture [57] and may subsequently be stabilized by inter-TAD contacts [44].

While structural domains like TADs are clearly very important to guide regulatory activity to specific target genes, the relevance of higher-order levels of topology, describing the nuclear location of TADs in relation to each other, to the periphery or to nuclear substructures like chromocenters, is not so obvious. The sequestration of TADs to the repressive nuclear lamina has been proposed to be an important mechanism to establish or safeguard developmental gene repression in mammals, which is in agreement with the observation that lamina-associated-domains (LADs) are generally gene-poor and transcriptionally inactive [58] and that several genes detach from the lamina during lineage commitment to be activated later during development [59,60]. However, genes that reside in domains that lost their peripheral position in cells with a mutant form of lamin A remained inactive [61].

Although higher-order chromatin topology appears relatively stable throughout interphase [62], TADs adopt new nuclear positions in daughter cells after mitosis, irrespective of their previous radial position. Spatial switches typically concern complete domains or series thereof, which suggests that domains are the units of spatial dynamics [63]. Domain positioning after cell division follows probabilistic rules, implying that 3D surroundings and the inter-domain contactome of a given TAD will vary highly during successive cell cycles and between cells in a population [6,64–66]. Due to this strongly stochastic nature, the exact 3D surrounding of a given domain is unlikely to have a decisive impact on the stability of gene transcription or repression at the cell population level. Even when a regulatory element has the intrinsic capacity to regulate a gene in a distal domain situated elsewhere on the same or on another chromosome, the element will be constrained in its movements by the overall structural properties of its genomic context and will therefore not be able to actively search for this gene. Productive communication with the gene (Fig. 1) will only occur in the few cells that happen to have the corresponding domains placed in each other's proximity, with variegated expression as the predicted outcome [65–67]. This is indeed what was observed when an ectopically integrated orphan super-enhancer was found to upregulate a cognate target gene on a different chromosome: enhancer-dependent up-regulation occurred exclusively in the small subset of cells that happened to form the inter-chromosomal contact [65]. Variegated gene expression is a common phenomenon that is observed for many genes and that theoretically enables cells in a population to make autonomous cell fate decisions [68]. The highly variable nature of higher-order genome configurations across cells may therefore contribute to the ability of populations to adapt to stress and environmental changes.

In summary, enhancer–promoter loops form within tissue-invariant domains that represent the major structural and functional units within which gene expression is controlled. TADs position themselves following probabilistic rules in such a way that each domain may have different neighboring TADs with every cell division. A TAD's exact location is therefore not likely to be crucial, but may contribute to the faithful execution of transcription programs. With this in mind, we will now consider the 3D organization of the PSC genome and evaluate its relevance for stem cell identity.

1.4. Transcriptional characteristics of PSCs

PSCs provide a great system to explore the functional relevance of genome organization for cellular (stem cell) identity, as the 3D genome rearranges heavily during PSC differentiation and reprogramming [52,69–73]. Below, we will first describe several characteristic features of pluripotent chromatin and then discuss recent work that has expanded our understanding of the spatial organization of the pluripotent genome.

PSCs possess the dual capacity to generate all germ layers of the embryo proper and to self-renew infinitely [1,2]. Pluripotency is sustained by the coordinated action of a small number of transcription factors and chromatin regulators [74,75]. The core of the transcriptional circuitry contains "elite" factors Oct4, Sox2, and Nanog, which form auto-regulatory loops and control genes that help to maintain the pluripotent state [74–78], and contribute to the repression of key lineage genes [74]. A number of additional factors are believed to endorse pluripotency by maintaining appropriate levels of the elite factors. All factors in the circuitry are highly interconnected, mutually reinforcing, and extensively redundant, which confers robustness and flexibility to the system [74]. Besides the core circuitry, PSCs are characterized by a widespread low-level transcriptional activity [79,80] that encompasses exonic, intronic, and non-genic sequences, causing overall nascent RNA and mRNA levels to be two-fold higher than in differentiated cells [80].

Pluripotency may refer to a spectrum of cellular states in vivo, yet two distinct pluripotent cell types can currently be isolated and maintained *in vitro*. Naive pluripotency describes the pre-implantation ground state of the embryo [1] and naive PSCs are considered fully unrestricted in their flexibility to form all lineages of the embryo proper [81]. PSCs derived from the epiblast

after blastocyst implantation are assumed to be primed for lineage commitment, a stage referred to as primed pluripotency [82,83]. Although naive and primed PSCs can be distinguished based on their developmental potential and their colony morphology, the pluripotency-specific core transcription circuitry is expressed in both stages [83].

The flexible and buffer-like nature of the pluripotency transcription circuitry is believed to rely partially on heterogeneity: undifferentiated PSCs have been reported to express *Nanog*, *Rex1*/*Zfp42*, *Stella*/*Dppa3*, *Esrrb*, *Klf4*, *Tbx3*, *Hex*/*Hhex*, and *Zscan4* in a heterogeneous manner [84]. Some of these genes are only expressed significantly in a small subset of cells in a PSC colony. For example, only ~5% of the cells in a colony express Zscan4, a finding that seems to correspond to its expression in embryos, where Zscan4 can only be detected at the 2-stell stage [85]. Expression of some of these factors may elevate a cell's propensity to differentiate into a certain lineage: PSCs that express Hex were found to have an increased tendency to contribute to extra-embryonic endoderm when compared to Hex-negative PSCs [86]. Expression heterogeneity can arise via transcription regulation, post-transcriptional events, cell cycle variation, or other phenomena. Using Nanog as an example, its heterogeneous expression levels were previously ascribed to allele switching [87], while a more recent study pointed out that protein levels derived from each allele are equal, regardless of transcription variability [88,89]. Culture conditions can strongly affect PSC gene expression levels and may boost discrepancies between studies [90]. Furthermore, the multiple subpopulations that comprise most stem cell colonies likely account for a part of the observed expression heterogeneity [91–93]. Although PSCs are generally assumed to hold a certain level of heterogeneity, the extent to which heterogeneity is inherent to PSCs was recently questioned; when mPSCs were supplied with a cocktail of inhibitors that enforce a truly naive state, lineage-primed subpopulations were suppressed and expression patterns started to show more similarity [94,95]. Human PSCs represent a primed pluripotent state in which levels of heterogeneity are higher than in naive mPSCs. Colonies of primed human PSCs grow in a hierarchical fashion that is characterized by a continuum of expression levels of early and late stem cell markers, combined with pluripotency and early differentiation genes [96,97]. Only a small subset of cells at the 'top' of the hierarchy in a primed PSC colony exhibits a selective capacity for efficient self-renewal. These cells showed a specific lack of lineage priming and were reported to express genes associated with cell-cell interactions [98].

1.5. The PSC chromatin signature

The transcriptionally promiscuous behavior of PSCs is supported by and at least partially due to a permissive overall chromatin state [80,99]. PSC genomes are relatively devoid of heterochromatin [80,100] and demonstrate exceptionally dynamic association of architectural chromatin proteins, high core histone turnover rates, and instable binding of linker histone H1 [101,102]. Together, these features presumably allow a profusion of chromatin modifiers and transcription factors to get access to DNA and trigger hyperactive transcription [80]. Concomitantly, this overall openness invokes a need for proper suppression of genes that pose the danger of differentiation. Polycomb repressive complex 2 (PRC2) is believed to play an important repressive role in PSCs, where it specifically associates with CpG-rich promoters of developmental regulators [103–106].

To exit the pluripotent state and embark upon differentiation, the core transcription circuitry needs to be dismantled. Several mechanisms and vulnerabilities are expected to be in place to allow this, and escape of pluripotency is usually rapid and efficient: as soon as Oct4 and Nanog levels drop below a hypothesized threshold level the circuitry collapses and pluripotency cannot be sustained [107]. For a PSC to differentiate and eventually adopt a certain cell fate, lineage-specific genes require activation, while pluripotency-related genes and gene clusters involved in other lineages require repression [108]. Bivalently decorated developmental promoters, co-marked with active H3K4me3 and repressive H3K27me3, reside in an alleged poised state that is hypothesized to facilitate efficient cell fate commitment when developmental cues are received [109,110]. Differentiation is accompanied by an overall tightening of the chromatin structure, which provides a more restrictive environment that dwindles widespread transcriptional promiscuity [102,111,112]. After establishment of cell identity, gene expression patterns are in general stably maintained and propagated to daughter generations [113]. Although differentiation was long believed to be a unidirectional path, pluripotency can be reinstated in differentiated cells by the ectopic overexpression of the 'Yamanaka' reprogramming factors Oct4, Sox2, Klf4, and c-Myc (OSKM) [3]. In the small subset of cells that typically reprogram successfully and transform into induced pluripotent stem cells (iPSCs), transcriptional patterns and chromatin state are reported to be much like regular PSCs [114,115].

During development, the regulatory landscape transforms severely; regulatory elements involved solely in regulating pluripotency genes are restricted or discharged, while novel regulatory sites need to be accessibilized, activated, and potentially propagated to turn on genes required for the predestined cell fate. While promoter chromatin states are highly stable and invariant between cell types [116,117], enhancer usage is largely unique, even in closely related cell types [118]. Rapid transitions of enhancer chromatin state allow dynamic poising, activation, and decommissioning of enhancers, which provides a system that allows fine-tuning of the expression patterns that underlie developmental decisions. Even when a gene remains expressed at stable levels throughout developmental stages, distinct enhancers may be responsible [119]. The Sox2 promoter, which participates in selective long-range contacts with distinct enhancers in PSCs and NPCs while transcription levels are comparable, nicely illustrates enhancer modulation [45,119,120]. A more genome-wide example of enhancer switching is provided by the transition from naive to primed pluripotency [121,122].

1.6. The pluripotent 3D genome

Although all general features of hierarchical genome organization are present in PSCs, several aspects of the 3D architecture of the PSC genome stand out in comparison to more differentiated genomes [102,123] and differentiation is therefore accompanied by topological re-organization at all structural scales [52,69]. Below, we evaluate our current understanding of the pluripotent 3D genome.

1.7. Higher-order chromosome folding in PSCs

One hallmark of the pluripotent 3D genome is its relatively non-settled higher-order structural organization. In particular, inactive genomic regions were found to exhibit little specific long-range contacts in PSCs [71], indicating that the B compartment is not (yet) strictly organized. Microscopy observations support this idea: chromocenters, the visually discernable inactive nuclear compartments that are composed of clustered pericentromeric regions, are more dispersed and randomly positioned in PSCs than in differentiated cells [52,124,125]. A recent study uncovered the chromosomal parts that preferentially co-localize with chromocenters, called pericentromere-associated domains (PADs). In somatic cells, PADs were found to represent inactive

regions, extensively overlapping with LADs. Thus, in committed cells, inactive regions have the preference to associate with the periphery or with chromocenters. In PSCs, such strict separation of active and inactive chromatin near chromocenters was not observed: PADs and LADs showed less overlap and PADs contained more often also transcriptionally active chromosomal regions [126]. This finding is in line with the above-mentioned unorganized state of the B compartment in PSCs, which may be a consequence of the overall malleable chromatin state and transcriptional landscape that is representative of the pluripotent state. The A/B compartments rearrange massively during early differentiation, initially even without major transcriptional changes [63]. Changes in radial position were reported for a number of key pluripotency loci that relocate towards the nuclear lamina during lineage commitment [59], but also for example for entire TADs on the future inactive X-chromosome, which make a similar move while the active X retains its centralized position in female nuclei [11].

1.8. Pluripotency factors contribute to the overall 3D genome

Within the A and B compartments categories of TADs with similar characteristics preferentially cluster together and a recent study allowed the identification of six spatial subcompartments, each occupied by genomic regions with a distinctive chromatin signature [44]. This clustering is also appreciable from preferential inter-TAD contacts; promoters form preferential long-range contacts with each other, not only in PSCs but also in other cell types, partially in a tissue-invariant manner [9,11,71]. Specifically in PSCs, promoters of pluripotency genes have a tendency to find each other in nuclear space, in *cis* as well as in *trans* [70,71,119]. Long-range co-localization of enhancers also occurs, but in a more tissue-specific manner [71]. Regions that contain a high density of binding sites for Oct4, Sox2, and Nanog (OSN) tend to cluster together over large distances in PSCs, and this preference is lost upon lineage commitment (Fig. 2a) [36,70,71,73,105]. This 3D configuration is dependent on the binding of pluripotency factors, as loss of Oct4, Nanog, or Klf4 led to a disruption of at least a part of the PSC-specific long-range contacts, while overall 3D genome topology was sustained [70,71,73]. Moreover, recruitment of Nanog to an ectopic site in the genome was demonstrated to be sufficient to induce 3D contacts with endogenous OSN binding sites, including other pluripotency genes, elsewhere on the chromosome [71]. A subset of the regions that participate in PSC-specific higher-order contacts is enriched for binding of PRC2 (Fig. 2b) [105], which matches with the Polycomb foci that have been observed previously in PSC nuclei [127,128]. Genetic ablation of PRC2 subunit Eed led to specific perturbation of these contacts, while overall 3D structure remained intact [105]. Although these PRC2-enriched clusters were found to segregate spatially OSN-bound sites, both clusters populate the active A compartment in PSCs [105]. Taken together, pluripotency factors and Polycomb group proteins contribute to the shape of the PSC genome. Similar contributions from tissue-specific transcription factors are also found to underlie the tissue-specific 3D configurations of various somatic cell types [34,43,65].

1.9. Sub-TAD organization also depends on pluripotency factors

Pluripotency factors not only contribute to the overall folding of chromosomes, they also appear to establish many shorter-range chromatin loops that are unique to, and probably important for,

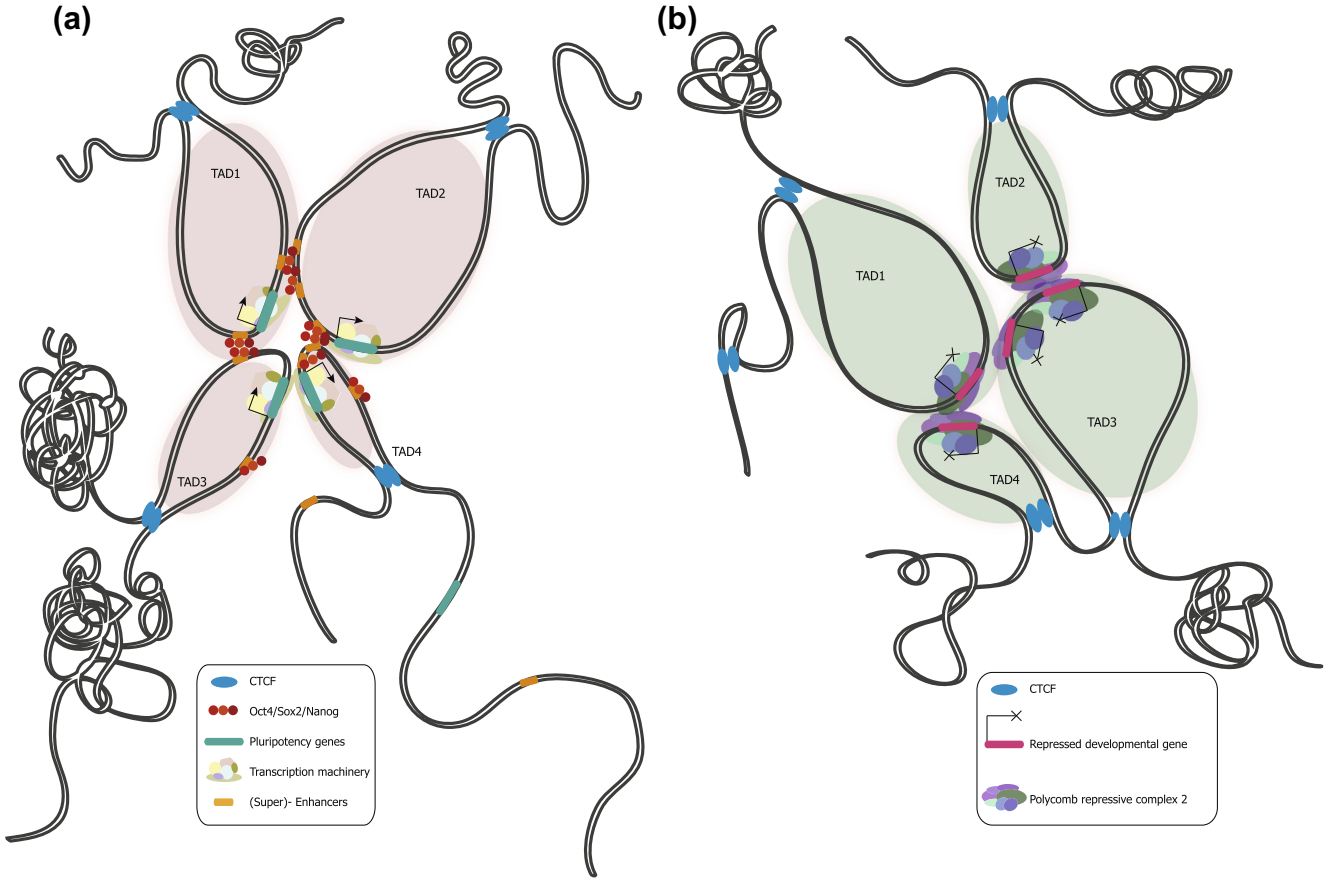

Fig. 2. PSC-specific higher-order genome folding clustered around OSN-enhancers and PRC2-enriched regions. (a) TAD-spanning higher-order contacts in PSC occur between Oct4, Sox2, and Nanog-bound enhancer clusters. (b) PSC-specific higher-order contacts also occur between regions enriched for binding of PRC2 repressive complex.

PSCs [31,36,129]. For example, Oct4 was shown to be involved in the local regulatory contacts of the murine Nanog locus in PSCs [130]. It has been argued that key identity factors such as Oct4, Sox2, and Nanog in PSCs recruit high levels of transcriptional machinery to certain genomic hotspots, called super-enhancers, which seem to control cell identity genes [131,132]. Many PSC-specific super-enhancers and their associated genes were recently found to reside in chromatin loops demarcated by CTCF and cohesin [51]. For several of these super-enhancer domains (SDs), boundary deletion by CTCF site excision was shown to thwart the insulating effect of the loop structure, which altered expression of genes inside and outside of the given SD. This indicates that the integrity of SD substructures is important for insulation-mediated control of local transcription. Intriguingly, in this study the SD chromatin structures appeared relatively conserved during differentiation [51], which is in line with the notion that CTCF and cohesin co-associated structures are rather tissue-invariant [36]. However, a different study showed that the distal super-enhancer involved in PSC-specific expression of Sox2 forms a chromatin loop that is highly tissue-specific [120] and also pluripotency genes Oct4 [31,36] and Nanog [99] were previously demonstrated to be engaged in enhancer–promoter contacts that are lost during differentiation [31,130]. Although this illustrates that the extent to which key pluripotency genes and super-enhancers adopt tissue-invariant or tissue-specific chromatin configurations remains to be elucidated in more detail, the latter stories clearly demonstrated the existence of PSC-specific regulatory chromatin loops that are often dependent on the binding of PSC-specific transcription factors.

1.10. Establishing a pluripotent 3D genome during reprogramming

An interesting concept to consider is the order of and causal relationship between epigenetic events, configurational changes, and gene expression dynamics that take place during the reprogramming of somatic cells to iPSCs. Although this has not yet been studied in systematic detail, various recent observations provide a first indication of the role that genome folding might play in reprogramming.

Successfully reprogrammed iPSCs enter a self-sustaining pluripotent state as soon as the endogenous pluripotency transcription factor circuitry is revived. Several lines of evidence suggest that function follows form during reprogramming, with local architectural changes preceding the initiation of pluripotency expression programs. During early reprogramming, the OSKM Yamanaka factors appear to associate first to regulatory sequences located distally to endogenous pluripotency genes [115]. This initial association does not re-activate endogenous pluripotency genes, which is presumably due to a lack of the 'required' 3D chromatin conformation and long-range contacts in pre-iPSCs [72,105]. The observed enrichment of OSKM at endogenous pluripotency promoters during later stages of reprogramming was hypothesized to reflect newly emerged enhancer–promoter contacts [115]. These findings are in line with other studies in which transcriptional up-regulation of the endogenous Oct4 locus was observed exclusively in the subset of cells that had instigated the PSC-specific long-range contacts of the Oct4 enhancer [73,133]. Successful rearrangement of the locus was linked to association of Klf4, and the authors suggested a mechanism that involves the Klf4-assisted loading of cohesin onto the Oct4 enhancer to re-establish enhancer–promoter loops [133]. A comparable phenomenon was described for the MyoD locus: during early reprogramming, Oct4 was detected at the MyoD enhancer only, whereas both enhancer and promoter were occupied during later stages. Only at this later stage, the locus had recruited silencing marks, which correlated with repression of MyoD [134]. In summary, binding of ectopic transcription factors to distal sites during reprogramming seems to be an early event that precedes establishment of PSC-specific enhancer–promoter loops and transcriptional activation or repression of endogenous pluripotency loci. Successful re-wiring of the local chromatin conformation may therefore well be an important prerequisite to reinstate the pluripotent transcriptome.

The observation that the PSC-specific higher order chromosome structure is lost upon differentiation and at least partially reinstated during reprogramming [70,71,105] suggests a functional relevance of the overall shape of chromosome that may contribute to the establishment of important enhancer–promoter contacts. It has been hypothesized that genome architecture centered on 3D clusters of transcription factor binding sites may strengthen the robustness of pluripotency by allowing more efficient transcription of associated target genes [71]. Yet, how would 3D clusters of binding sites dispersed over different TADs mechanistically affect transcription? Below we will look into the potential relevance of this organization in terms of transcription regulation, and describe how these long-range clusters may support the pluripotent state.

1.11. Pluripotency factors shape the PSC genome into enhancer clusters

Transcription factors can search target binding sites via a diffuse trial-and-error mechanism that encompasses multiple rounds of non-specific binding events preceding stable association [135,136]. It has been proposed that initial pioneering binding of one factor may allow assisted loading of nearby others, which potentially leads to the hierarchical assembly of enhanceosomes at given genomic locations [135]. Single-molecule super-resolution fluorescence microscopy has recently allowed the appreciation of 3D enhancer clusters induced by Sox2 association to the genome [137]. Although these clusters partially overlapped with PolII-enriched regions, their relative stability contrasted considerably with the bursting behavior that characterizes PolII clusters [138]. Furthermore, it was surmised that Sox2-bound enhancer clusters incite diverging of target search modes of Sox2 into (i) a long-range 3D diffusion mode to find binding sites in other clusters and (ii) a local search mode to find sites within the same cluster, resulting in increased genome occupancy [137]. These findings support a model in which 3D clusters of regulatory elements belonging to different TADs can trigger a more efficient local regulatory mode that is characterized by a higher transcription factor-target occupancy mode and reduced diffusive behavior of transcription factors. This reinforces the postulation that the PSC-specific higher-order genome architecture, organized around spatial clusters of pluripotency factor binding sites (of which many represent enhancer regions), might serve to strengthen the pluripotency regulatory network by enabling a higher and more efficient level of transcriptional control. Such 3D clustering of binding sites for tissue-specific transcription factors appears to also take place in somatic cells and may similarly contribute to maintenance of cellular identity [43].

2. Conclusion and perspectives

The 3D PSC genome exhibits unique features at each hierarchical level of organization. At the inter-domain level, the genome folds around spatial clusters of high-density pluripotency factor binding sites (Fig. 2a). Sox2 was shown to be sufficient to induce spatial clusters of Sox2-bound enhancer regions [137], which fits with the observation that targeting of another pluripotency factor, Nanog, to an ectopic site in the PSC genome induced formation of new contacts with distal enhancer elements [71]. Spatial clustering of enhancers may optimize target search strategies of transcription

factors, which potentially allows more efficient transcriptional control of associated pluripotency genes. This structural organization might reinforce the maintenance of the pluripotent state and help to impose a threshold that needs to be overcome for a cell to embark upon differentiation. Upon dismantling of the pluripotency transcription factor circuitry, these PSC-specific clusters will most likely sunder rapidly, contributing to a fast dissolution of the pluripotent state.

These observations lead to several questions: are 3D enhancer clusters and the suggested enhanced regulatory mode specific for PSCs? When differentiation sets in, will other tissue-specific transcription factors adopt a similar role and anchor sites important for lineage maintenance in close spatial proximity, for auto- or co-regulatory events? Rearrangement of the regulatory landscape during development implies a shift of transcription factor occupancy throughout the genome. The reported enhancer switch between naive and primed pluripotent states insinuates that pluripotency transcription factors, which are expressed in both states, reposition from decommissioned naive-dominant enhancers onto newly activated seed enhancers [121,122]. Comparison of Oct4 binding profiles in naive and primed PSCs revealed that distinct regions are engaged in both states in vitro and Oct4 was found to operate in different regulatory protein cooperations in both states [121,122]. Together these stories provide insights into the means by which a common factor can convey cell type-specific effects, by teaming up with different binding partners at distinct target enhancers [121,122,139]. To improve our understanding of PSC-specific transcriptional control, variability within the protein-protein and protein-genome interactome can thus provide additional insights, especially when combined with knowledge on factors that serve a pioneering role in chromatin accessibility and 3D chromatin reorganization.

Besides the unique organization of the active compartment in PSCs, the inactive compartment in PSCs was shown to demonstrate little specific higher-order contacts [71]. Does this seemingly random or unorganized state have any functional relevance? Is the observed lack of significant contacts truly a reflection of disorganization, or does it represent an average of high levels of cell-to-cell variation in stable long-range contacts? As higher-order chromatin contacts can contribute to stochastic transcriptional regulation, the observed population heterogeneity of PSCs may in part be the consequence of a high degree of topological variation between the cells. As proposed previously [68], variegated regulation throughout a population can allow autonomous cell fate decisions. How does the pluripotent genome prevent unfavorable transcription when it resides in a state of openness that supports transcriptional susceptibility and higher-order variegated regulatory effects? In addition to improving efficient transcription of pluripotency genes, 3D enhancer clusters may counteract this by preventing further diffusion of factors into regions that should be kept silenced. Furthermore, other clusters in the PSC genome such as PRC2-bound regions or polycomb domains are proposed to strengthen repression of developmental regulators and differentiation-inducing genes [51,105]. In populations of differentiated cells, organization of the inactive compartment appears generally more defined. In combination with a chromatin state that is less prone to transcriptional promiscuity, the 'danger' of higher-order variegated gene expression leading to cell fate changes or identity deterioration may be progressively minimized along the lineage path.

2.1. Future perspectives and technical challenges

In order to improve our understanding of cell-to-cell variation in higher-order contacts and transcription, it would be desirable to be able to directly link chromatin organization and allelic transcriptional output in one assay. Furthermore, analyses in a live-cell setting or over a time course allow tracking of the dynamics and stochasticity of the relationship during a single cell cycle or through multiple divisions. Live-cell imaging of gene loci in single cells with single molecule detection methods and high-resolution live-cell imaging can complement such studies. To exceed the observational level, genome-editing approaches can be applied to enable disruption and forcing of loops, of which putative direct effects on transcription may provide novel insights into the functional relevance of the structure of interest.

While it is instrumental to develop reliable single-cell assays to study the individual members of a (pluripotent) culture, pluripotency may refer to an *in vivo* state of a population of cells, rather than individuals. It has been hypothesized that dynamic variation in a population of stem cells may allow rapid responses to a range of environmental cues [94]. Even when subsets of cells are transiently primed to differentiate into a certain lineage, or occur in a metastable transition state, overall population structure may be maintained robustly. This would imply that there is no 'unique' pluripotent state at a single cell level. A functionally pluripotent population of cells may be characterized by its highly entropic state, as the open chromatin state offers only weak regulatory constraints [94]. The concept of pluripotent capacity may rely on the interplay between the different individual cells in a pluripotent embryo, which might be reflected in the cell-to-cell variation observed *in vitro* and *in vivo*. To better understand how PSCs maintain their developmental capacity and self-renewing identity, pluripotency may need to be considered as a feature of a small cell population. Since massive *in vitro* expansion of the very small population of PSCs isolated from a blastocyst may skew the original balance of pluripotent stem cell states completely, it could be beneficial to focus more on actual blastocysts and directly analyze cells after isolation. Unfortunately, the cell number is very limited, and for comparisons between iPSCs and PSCs we likely still rely on PSC cultures.

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