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Nuclear architecture by RNA

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The dynamic organization of the cell nucleus into subcompartments with distinct biological activities represents an important determinant of cell function. Recent studies point to a crucial role of RNA as an architectural factor for shaping the genome and its nuclear environment. Here, we outline general principles by which RNA organizes functionally different nuclear subcompartments in mammalian cells. RNA is a structural component of mobile DNA-free nuclear bodies like paraspeckles or Cajal bodies, and is involved in establishing specific chromatin domains. The latter group comprises largely different structures that require RNA for the formation of active or repressive chromatin compartments with respect to gene expression as well as separating boundaries between these.

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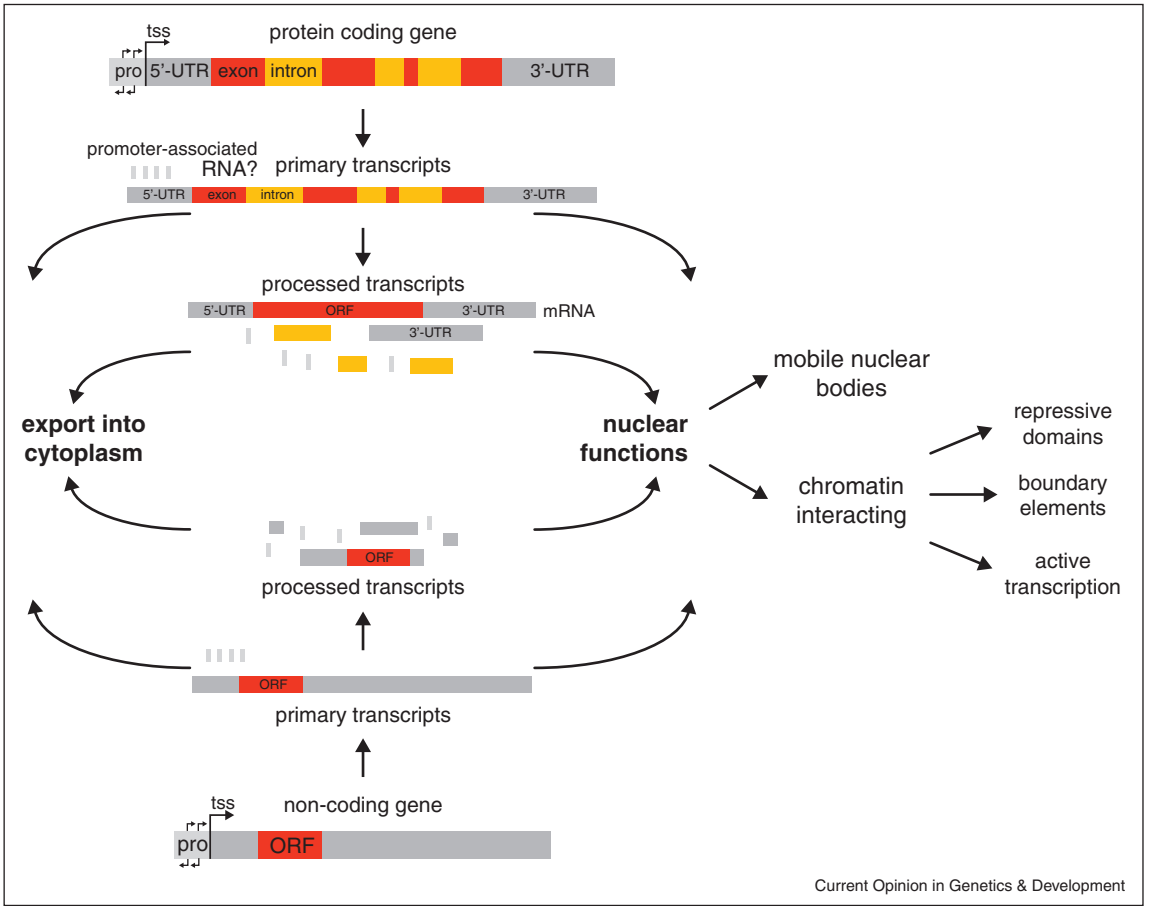
Introduction

The mammalian cell nucleus has a complex dynamic organization that integrates two apparently contradicting functions: On the one hand, the genome has to be protected from uncontrolled modifications that would compromise its function, and it has to be reliably replicated and segregated during cell division and meiosis. On the other hand, the genome has to be remarkably plastic to allow for the readout, processing, maintenance and transfer of the information encoded in the DNA sequence as needed for the cell to adopt different functional states. The switching of cellular programs is tightly linked to the regulation of gene expression, which in turn requires corresponding changes in chromatin organization. According to the current view, more than half of the mammalian genome can be transcribed although only 1.2% code for amino acids in proteins [1]. Thus, the nucleus has to provide an environment to promote or repress transcription essentially throughout the complete genome. Interestingly, many aspects of the underlying dynamic nuclear architecture involve RNA as an effector molecule with structural functions. These RNAs operate either by interacting directly or indirectly with chromatin or participate in establishing DNA-free nuclear subcompartments, in which certain genome-associated activities are concentrated.

The first studies on the association of RNA with chromatin date back to the 1960s. It was reported by Bonner and co-workers that RNA represents a significant fraction of chromatin [2]. The nature of the surprisingly short ~40 nt chromosomal RNAs identified in this early report and its mode of interaction with chromatin via a proposed covalent linkage to histones were controversially discussed [3,4]. Subsequent studies proposed a diverse set of architectural functions of RNA in the nucleus, including, for example, chromatin-associated RNAs as a structural component of heterochromatin [5], a role for RNA in eukaryotic chromosome structure via DNA–RNA linkages [4], or RNA as a component of a nuclear matrix [6,7,8]. However, during the 1960s to 1990s characterizing structural functions of RNA in the nucleus faced many technical difficulties with respect to systematically identifying the sequences of nuclear retained RNAs, and to associate them with a specific phenotype. As reviewed here, significant progress in both areas has been made in recent studies that provide insight into the principles of RNA-mediated nuclear architecture.

The structural functions of RNAs have been traditionally assigned to non-coding RNAs (ncRNA) that are typically classified into small (<30 nt) or long non-coding RNAs (>200 nt). However, this classification is not particularly instructive since both small and long non-coding RNAs comprise very heterogeneous classes with diverse and partly overlapping functions that are reviewed elsewhere in this issue [9,10]. Furthermore, the coding versus non-coding classification ignores the multifunctionality of RNA transcripts [11•,12] (Figure 1): (i) A number of so called non-coding RNAs have open reading frames (ORFs), and it is difficult to exclude that these may be translated at a certain development stage or in a specific tissue. Indeed, it has been reported that peptides were translated from rather short ORFs of some 'non-coding' RNAs [13]. (ii) Coding RNAs contain a significant amount of non-coding sequence elements in their introns and 5′-untranslated and 3′-untranslated regions (UTRs) that possess regulatory function. Interestingly, a recent report describes the separate expression of a large number of 3′-UTRs in human and mouse cells [14••]. These are likely to be generated by post-transcriptional cleavage of the

Figure 1

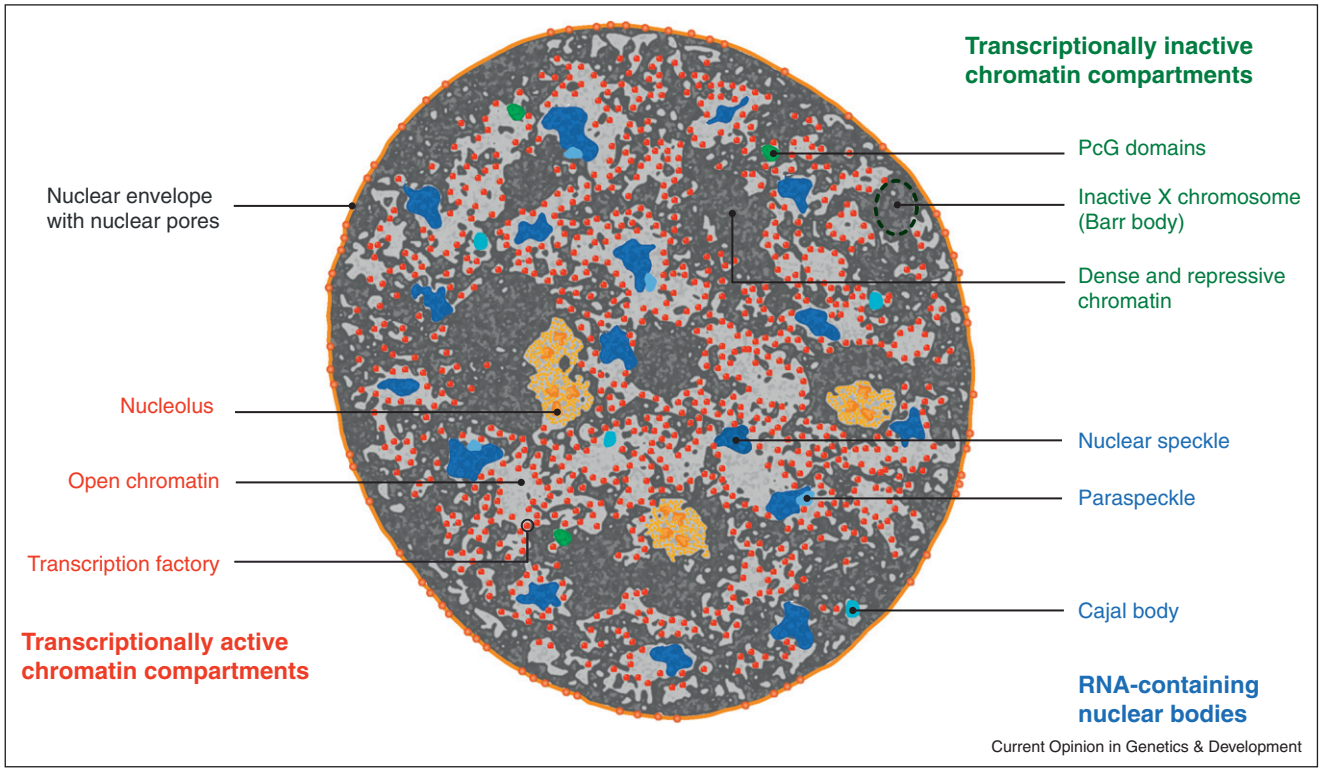


Multifunctional RNA transcripts and their role in nuclear architecture. RNAs retained in the nucleus comprise both coding and non-coding primary and processed transcripts. The different types of sequences are heterogeneous in length and seem to be associated with a variety of nuclear functions that cannot be linked directly to their transcriptional origin. Therefore, a classification of RNA transcripts with nuclear architectural activity according to their association with chromatin and their relation to the gene expression status is used here.

corresponding protein-coding sequences. (iii) While the initial primary transcript may be 'long', it might be processed or a mapping analysis might reveal that the part relevant for the activity under investigation is significantly shorter as, for example, demonstrated for the RNA-directed DNA methylation of ribosomal RNA (rRNA) genes [15••]. (iv) A structural function of coding RNA transcripts in maintaining an open chromatin structure was reported [16••].

Accordingly, another classification is applied here to discuss the architectural role of RNA in the nucleus (Figures 1 and 2). The obvious first distinction is made between RNAs that are instantaneously exported into the cytoplasm versus those that remain at least transiently in the nucleus to exert a structural function. These nuclear-retained RNAs are either a component of mobile nuclear bodies that are devoid of DNA or associate with chromatin to establish domains with specific activities. The latter group is further divided according to their gene expression status into repressive and active chromatin compartments. Such a large-scale bipartite organization of the genome has been demonstrated in a number of studies, for example, a microscopy-based three-dimensional analysis of gene clusters [17], chromosome conformation capture (3C) based genome-wide interaction analysis [18], or the timing differences between early and late replicating DNA loci [19]. The spatially segregated open and closed chromatin compartments are not simply the result of active transcription. Nuclear architecture actively contributes to determining specific gene-expression programs, and thus shapes cellular functions [20]. These considerations lead us to define three subclasses of RNAs that determine large scale chromatin states in mammalian cells: (i) RNAs that form repressive chromatin compartments, (ii) RNAs that organize boundary elements between regions that differ in their gene expression status, and (iii) RNAs that

Figure 2



Scheme of nuclear architecture with subcompartments that involve RNA for their structural integrity. These comprise DNA-free mobile nuclear bodies (blue) and chromatin compartments. The latter are subdivided into transcriptionally active (red) and inactive (green) chromatin regions as well as the boundaries between these. The structural organization of the nucleus shown in the scheme represents what is seen by high-resolution fluorescence microscopy (e.g. ref. [69]). Note that the distribution of dense and open chromatin regions is strongly dependent on cell type and organism.

stabilize actively transcribed regions. In this context, it is noteworthy that the mode of RNA interactions with proteins and DNA displays a high structural variability. As summarized in ref. [21], it involves interaction via a DNA–RNA hybrid duplex, a DNA–DNA–RNA triplex, non-covalent interactions with chromosomal proteins, and possibly also covalent linkages as mentioned above [2,4]. A number of studies relate the function of chromatin interacting RNAs to targeting posttranslational histone and DNA modifications to specific genomic sites (e.g. [9,10,15••,21]). While these modifications can indirectly affect nuclear architecture [22], we focused here on the direct links between nuclear architecture and RNA.

RNA as an essential structural component of mobile nuclear bodies

The nucleus harbors mobile nuclear interchromatin subcompartments referred to as nuclear bodies, in which a diverse set of biological activities is enriched [23]. These can translocate within the nucleus in the absence of any specific interactions with the genome, and some of them contain RNAs as constituting component. The prototypic example is the paraspeckle. Paraspeckles play a role in regulation of gene expression via retaining certain messenger RNAs (mRNAs) and are built around the long noncoding Men ε/β (also known as Neat1) RNA [24,25,26]. Interestingly, it was demonstrated recently [27••] for a surprisingly large number of nuclear bodies that creating a nuclear accumulation of specific coding or ncRNA molecules by tethering them in multiple copies to DNA is sufficient to initiate their *de novo* formation: Paraspeckles formed via the Men ε/β ncRNA, SC35 domains (also termed splicing speckles or interchromatin granules) via spliced β-globin-MS2 pre-mRNA, Cajal bodies and histone locus bodies via histone pre-mRNA, and nuclear stress bodies via repetitive noncoding satellite III transcript. The role of Men ε/β ncRNA in paraspeckle assembly was dissected via induction of transcription and the direct visualization of the recruitment of paraspeckle proteins [28•]. Together, refs. [27••] and [28•] provide evidence that RNA transcripts can act as 'seeds' to recruit soluble RNA-binding nuclear body components from the nucleoplasm and induce the formation of distinct types of nuclear bodies [23]. In addition to the above-mentioned nuclear subcompartments, other

RNA-containing bodies have been identified based on the local enrichment of certain RNA sequences within the nucleus. One example is the Gomafu RNA, which is found in distinct nuclear bodies distributed throughout the nucleus of specific subsets of neurons [29]. Gomafu bodies may play a role in RNA splicing since the Gomafu RNA has tandem repeats of the SF1 splicing factor binding sequence UACUAAC [30]. Another novel nuclear body-like RNA domain contains GAA-repeat sequences of ~1.5 kb to ~4 kb in length [31•]. These foci are enriched in proteins identified previously also in nuclear matrix preparations, which suggests that they could exert a structural role for nuclear organization. To some extent the $(GAA)_{500\text{-}1300}$-containing nuclear foci appeared to associate with GAA·TTC-repeat-containing DNA regions. These sequences are known to have the propensity to form DNA–RNA triplexes [32], which might be a mode of interaction of these RNAs with the genome. It is currently unclear if the $(GAA)_{500–1300}$ foci represent mobile RNA-containing nuclear bodies like those mentioned above as opposed to chromatin-associated domains that are discussed in the following.

Repressive chromosome domains structured by RNA

Several studies point to an important role of RNA in the formation and maintenance of higher order chromatin domains that are repressive with respect to their transcriptional activity. As discussed in the following, this function can involve a direct structural role of the RNA itself or an indirect RNA-dependent recruitment of a chromatin-structure modifying protein.

Pericentric, centromeric and telomeric domains

The targeting of architectural chromosomal proteins by RNA has been reported for pericentric, centromeric and telomeric heterochromatin domains: (i) Heterochromatin protein 1 (HP1) is a factor involved in establishing and maintaining the repressive state of pericentric heterochromatin. Its localization to this region is guided by major satellite repeat transcripts that bind to HP1 upon post-translational modification of the protein with the small ubiquitin-like modifier (SUMO) [33,34••]. (ii) The binding of centromere protein C (CENP-C), a protein necessary to induce the formation of a functional centromere, is stabilized by single-stranded RNAs [35•,36,37]. (iii) The interaction of telomere repeat factor 2 (TRF2), origin recognition complex (ORC) protein and HP1 with telomere-repeat-encoding RNA (TERRA) has been proposed to stabilize the structure of telomeres and to facilitate heterochromatin formation [38•]. In these cases, the RNA-protein interaction involves one or more specific RNA-binding domains within the protein that recognize RNA sequence and secondary structure motifs. How the resulting protein–RNA complex interacts with its specific genomic target is currently an open question. It could involve formation of a DNA–RNA hybrid, a DNA–RNA triplex or additional interaction of RNA with chromosomal proteins.

Polycomb group protein-chromatin compartments

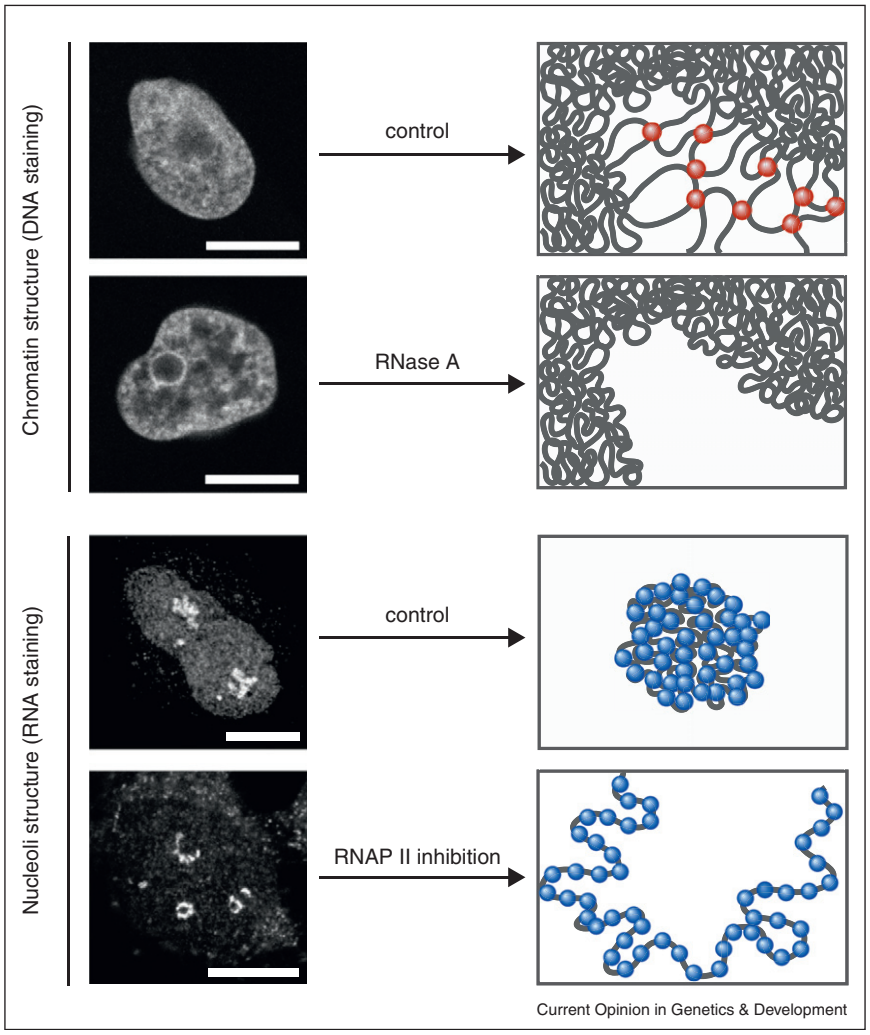
The polycomb group (PcG) proteins are enriched in distinct subnuclear foci called PcG bodies (Figure 2). In contrast to the mobile nuclear bodies discussed above they assemble around their target genomic regions. A local accumulation of a dense and repressive chromatin state is inferred from correlative light-electron microscopy analysis [39]. Recent evidence shows that the polycomb repressive complex 1 and 2 (PRC1 and PRC2) are guided by long ncRNAs to specific genomic domains to negatively regulate gene expression [40••,41•]. The RNA-induced targeting of PRC could affect chromatin organization in several aspects. First, the PRC-associated histone modification activities (e.g. trimethylation of histone H3 at lysine 27) could induce a repressive chromatin state via proteins that specifically recognize negative chromatin marks as discussed previously [40••,41•]. Second, it was shown for the *Drosophila* homologue that PRC1 itself compacts nucleosomal arrays *in vitro*, and thus could directly exert a repressive structural function [42]. Third, RNA-induced targeting of PRC complexes could modify the large-scale nuclear architecture of the genome and affect gene expression. Evidence that the latter mechanism is relevant comes from three recent studies. In flies, long-range interactions exist within the same chromosome arm between PcG-bound interaction domains separated by megabases of DNA [43]. Furthermore, Hox genes clusters are repressed PcG bodies, and this three-dimensional organization of PcG target genes stabilizes their epigenetic gene silencing [44]. Interestingly, a related regulatory mechanism exists in human cells that operates via modulation of the RNA-binding specificity of Polycomb 2 (Pc2) protein via (de)methylation of lysine residue 191 in response to growth signals [45••]. It was shown that methylated Pc2 binds preferentially to the TUG1 ncRNA and leads to the repression of growth-control genes via their targeting to PcG bodies, in which TUG1 provides an RNA scaffold. By contrast, unmethylated Pc2 binds to the MALALT1/NEAT2 ncRNA in SC35 nuclear domains. This switching of Pc2 RNA-binding specificity upon demethylation modifies nuclear organization and relocates growth-control genes to a nuclear environment that promotes their expression.

The inactive X chromosome or Barr body

A well-known example for a transcriptionally repressive chromatin compartment is the inactive X-chromosome in female mammalian cells. It is readily identified as a so-called Barr body on microscopy images using nucleic acid stains, and localizes to the nuclear or nucleolar periphery. The crucial role of the Xist ncRNA for determining chromatin structure and nuclear organization of the inactive X chromosome in mammals is well established and

reviewed in ref. [46]. Here, we refer only to the structural aspects of the Xist-mediated chromatin reorganization, which is tethered to the inactive X chromosome via the YY1 transcription factor that is capable of binding both RNA and DNA through different sequence motifs [47]. The inactive X chromosome was found to be organized into subchromosomal domains separated by interchromatin spaces with distinct compaction properties as revealed by transmission electron and fluorescence microscopy [48,49•]. The Xist-mediated silencing appears to be associated with a chromosomal organization into gene-rich domains depleted of long interspersed element (LINE-1) repeats and gene-poor regions with an increased association with LINE-1 sequences [50]. A 3C analysis showed that Xist RNA is needed for the characteristic higher order folding of the inactive X chromosome, and acts as a global structural organizer [51]. A role of PcG proteins in this function was proposed that involves mediating long-range intrachromosomal interactions. A link between Xist and PRC2 was demonstrated recently in a study that reported the simultaneous displacement of Xist and polycomb repressive complex PRC2 via a locked nucleic acids competitor [52]. Thus, PRC2 requires Xist to bind the X chromosome. Given the above mentioned structural functions of PRCs for the formation of repressive chromatin compartments it

Figure 3



RNA-dependent organization of transcriptionally active domains. RNase A-microinjection experiments show a function of RNA in large scale chromatin organization and the maintenance of open chromatin regions [16••]. It has been proposed that this activity could involve RNA as a structural component of RNAP II transcription factories to stabilize their function as genome cross-linkers (red spheres) as depicted in the hypothetical model. The spatial conformation of the nucleosome chains associated with active transcription complexes is currently unknown. Interestingly, also nucleoli that may be considered as large RNAP I transcription compartments, seem to require RNA to maintain their structure. The specific inhibition of RNAP II results in the re-distribution of the RNAP I transcription sites (blue spheres) [16••,66]. This could reflect a structural role of RNA in maintaining nucleolus structure [16••,35•,66]. Scale bars, 10 μm.

appears likely that these contribute to the specific three-dimensional conformation adopted by the inactive X in mammals.

RNA-mediated boundary/insulator compartments

The CCCTC-binding factor (CTCF) transcription factor is considered to be the prototypic member of proteins that establish insulator or boundary elements to separate repressive and active chromatin compartments. It acts by promoting the formation of DNA loops, which is not only important for its insulator function but also for the clustering of active promoters [53,54]. Interestingly, chromatin interactions of CTCF are modulated by RNAs. It was reported that the LINoCR ncRNA is involved in a cascade of events that included epigenetic modifications and nucleosome repositioning and lead to CTCF eviction [55]. By contrast, the SRA ncRNA was proposed to stabilize the interaction of CTCF with cohesion at certain chromatin loci by binding both proteins simultaneously [56•]. Finally, transcription initiation RNAs, which are transcripts of 18 nt derived from sequences downstream and close to RNAP II transcription start sites, were found at genomic human and mouse CTCF binding sites [57]. It was concluded that these RNAs affect the local epigenetic modifications and with it CTCF binding. Thus, RNAs are involved in both the establishment and resolution of CTCF boundary elements between transcriptionally repressive and active RNAP II compartments [55,56•,57], and potentially also at ribosomal DNA within the nucleolus [58]. Interestingly, a genome-wide analysis revealed that in addition to CTCF also the YY1 DNA-binding factor was enriched at human chromatin insulators [59]. YY1 has the ability to bind both DNA and RNA [47], raising the possibility that RNAs are also involved in the association of YY1 at boundary elements.

RNA structures transcriptionally active chromatin compartments

The above mentioned spatial bipartite organization of the genome can be visualized as a chromatin distribution in which more dense repressive chromatin domains are surrounded by a loosely packed and transcriptionally more active perichromatin compartment [60] (Figure 2). In this model, perichromatin fibrils associate with RNA polymerase II (RNAP II) and extend into regions largely devoid of DNA. Several lines of evidence indicate that RNAP II operates in a coordinated manner within large complexes referred to as transcription factories [61]. These structures connect transcription and genome organization since they promote intra-chromosomal and inter-chromosomal linkages of actively described genome loci [60,62,63,64•].

Recently, it was demonstrated by RNase microinjection experiments that long nuclear-retained coding RNAs maintain higher order chromatin in an open configuration, whereas the structure of heterochromatin domains showed a reduced dependence on RNA [16••]. The associated RNAs were found to be enriched in sequences with long 3′-UTRs, and proposed to maintain the organization of transcriptionally active chromatin compartments by stabilizing RNAP II transcription factories (Figure 3). In addition, it has been observed that interference with RNAP II transcription resulted in the dissolution of nucleoli into so-called 'necklace' structures that were observed by electron microscopy [65] and fluorescence microscopy [66] (Figure 3). This suggests a putative role of some RNAP II transcripts in the architectural organization and maintenance of the nucleolus. This conclusion is supported by the finding that centromere satellite RNAs promote the accumulation and assembly of proteins at the nucleolus [35•].

Conclusions

The conformational flexibility of RNA is remarkable and makes it an ideally suited macromolecule to structure nuclear subcompartments. Single-stranded RNAs can recognize a specific DNA sequence via formation of DNA–RNA hybrids or associate to DNA duplexes in a DNA:RNA triplex. At the same time, variations in its sequence and/or secondary structure provide an essentially unlimited number of possibilities to form sites for the specific interaction with proteins. Thus, it is not surprising that RNAs can shape the nucleus in a variety of ways on different length scales ranging from single nucleotide (nt) and histone modifications to the organization of large nuclear subcompartments up to the size of a whole chromosome during X-inactivation. Since RNA is an essential factor for establishing transcription-repressed/active chromatin compartments, as well as the boundaries between these regions, its structural functions emerge as an additional regulatory layer of gene expression. Accordingly, the cell type dependent expression of these RNAs is expected to play an important role for establishing specific cell functions during differentiation. Recent findings demonstrate that transcription factors involved in controlling pluripotency and differentiation regulate the expression of large intergenic ncRNAs and thus support this view [41•].

To directly assess a causative role of RNA for nuclear architecture and chromatin organization various approaches were used: (i) RNAs were exogenously introduced to induce, for example, *de novo* nuclear body assembly [27••,28•] or DNA methylation [15••]. (ii) In the case of Xist, the comparison of inactive and active X chromosomes within the same cell allows it to identify the contribution of Xist to the inactive X structure [48,49•]. (iii) Knockdown by RNA interference can be used to identify the associated structural changes, as, for example, shown for the TUG1/MALAT1-dependent redistribution of growth-control gene promoters between PcG bodies and SC35 domains [45••]. (iv) RNase treatment

of cells either via microinjection [16••] or addition to permeabilized cells [33,34••] provides information on the structural role of RNA in the nucleus.

While these approaches clearly demonstrate structural RNA functions, the molecular mechanisms by which RNA changes nuclear architecture remain to be further elucidated in many instances. At the same time, the number of RNAs with putative functions in genome organization is increasing rapidly as genome-wide mapping of nuclear RNAs is becoming a routine task. Thus, methodological advancements that help to dissect the nuclear architecture phenotype of RNAs and/or provide insights into their mode of operation are highly valuable. In this respect, a number of technical advancements are noteworthy: (i) Labeling or tethering RNAs via a MS2 protein recognition sequence and related approaches in living cells has provided highly useful information in terms of their nuclear localization and mobility [67] as well as characterizing RNA found in nuclear bodies [27••,28•]. (ii) The labeling of RNA with fluorescent azides (or other functional groups) using 'click' chemistry represents a new approach for tagging nascent RNAs in the nucleus [68], but requires some further development to be applicable in a native cellular environment. (iii) Fluorescent labeling of RNAs in conjunction with recent advancement in fluorescence microscopy imaging of nuclear subcompartments [69,70] as well as emerging methods for a spatially resolved analysis of chromatin interactions [71] will provide further details on the architectural functions of RNA in the nucleus. (iv) RNA interference knockdown approaches, although highly informative [41•], may face difficulties owing to insufficient efficiencies as well as possible off-target effects. A true knockout of a nuclear RNA can be achieved via site-specific manipulation of the genome with zinc finger nucleases. This has been demonstrated recently for MALAT1 RNA and provided insight into the nuclear functions of this highly abundant transcript [72•]. (v) The biochemical separation of chromatin, nucleoplasm or nucleoli is well established. Accordingly, these nuclear fractions are readily accessible for investigations of their RNA content. Purifying other more dynamic supramolecular complexes or specific genomic regions is much more challenging. A particular impressive recent example in this respect is the successful isolation of transcription factories that allowed a characterization of their proteome [64•]. Taking advantage of the progress currently made in the areas mentioned above creates exciting opportunities to advance our understanding of the various RNA networks that operate in the nucleus to shape genome function via modulating nuclear architecture.

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