Coding RNAs with a non-coding function
Maintenance of open chromatin structure

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Introduction
Chromatin organization regulates access of the transcription, repair and replication machineries to the DNA at the level of the nucleosome, the folding of the nucleosome chain as well as its higher order architecture in the nucleus. With respect to the structural principles that govern the arrangement of the nucleosome chain during interphase, two types of models have been proposed. In the nuclear matrix model, a proteinaceous fiber network acts as the nuclear equivalent to the cytoskeleton to organize chromatin. In an alternative view, key determinants of genome structure during interphase are intra- and inter-chromosomal linkages that are tightly interconnected with the gene expression state of a given locus. This dynamic genome scaffold is established by (i) ‘transcription factories’ of RNA polymerase II that might be important for RNA splice-splicing, (ii) the nucleolus as a RNA polymerase I transcription organelle, (iii) gene clusters, in which gene dense regions are separated from gene poor regions and (iv) ‘active chromatin hubs’ between regulatory chromatin elements. This raises the question whether RNA itself is a structural organizer of chromatin. Indeed, several studies have reported on the association of RNA with chromatin and a possible role of RNA in establishing a nuclear scaffold or other nuclear subcompartments. Furthermore, the cell’s nuclear architecture shows distinct differences with respect to the biological activity of the corresponding genomic regions as described in detail in a number of reviews. Thus, transcription and genome organization are tightly connected from a structural point of view, but also in terms of an interleaved and modular transcriptome.

Results
RNA is required to maintain an open chromatin state. To investigate whether RNA is involved in chromatin organization, we microinjected different RNAs into the cell nucleus and compared their effects on chromatin distribution to proteinase K.
RNase A treatment of human cell lines led to the same drastic chromatin compaction that was observed with mouse NIH 3T3 cells. This indicates that the requirement of RNA for maintaining an open chromatin state is a general feature of mammalian cells (Fig. 2).

In order to confirm that the observed collapse of chromatin structure was a direct effect of the degradation of structurally important RNAs, we conducted time course experiments in living cells. The chromatin distribution was imaged in HeLa cells just after microinjection of RNase A (Fig. 3) via a YFP-fused histone H2A marker. RNase A treatment did not induce histone dissociation from the DNA as apparent from the persistent co-localization of histone H2A-YFP fluorescence and DAPI signal. The effect of RNA digestion on chromatin organization started within 1 minute after microinjection and is clearly seen after 5 min on the fluorescence intensity profile (Fig. 3): The fluorescence intensity in the control-injected cells showed some variations that reflect the differences in chromatin compaction in HeLa cells that have been evaluated in detail previously in reference 29 and 30. This fluorescence intensity distribution became much more heterogeneous in the RNase A-injected cells and revealed the aggregation of chromatin domains on the micrometer length scale. A longer RNase A treatment was studied by fixing NIH 3T3 cells immediately, 20 to 50 min and 150 to 180 min after injection (Fig. S2). Shortly after microinjection, the chromatin distribution changed and the maximum effect was reached after 20 min incubation. In contrast, induction of apoptosis required about 90 min and occurred clearly after the chromatin reorganization event (Fig. S3). At 150 to...

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**Figure 1.** Interphase chromatin distributions after exposure to different enzymatic activities that target RNA, DNA or proteins. (A–E) Confocal laser scanning microscope (CLSM) images of NIH 3T3 mouse fibroblasts microinjected with the indicated enzymes or the corresponding volume of buffer for control samples. Buffer-injected control samples were indistinguishable from each other and only the RNase A control is shown. DNA (blue) was stained with DAPI and RNA via PI (red) or with Syto RNaseSelect for MNase digestion in (F), which was conducted with permeabilized cells. Scale bars, 10 μm.
Chromatin-interlinking RNAs (ciRNAs) are enriched in a soluble nuclear RNA fraction. To further characterize the RNAs responsible for maintaining the native chromatin distribution, we investigated the ability of different RNA fractions to rescue the chromatin collapse after RNase A treatment in permeabilized HeLa cells. This is an alternative method to expose chromatin to RNase A, and yielded results very similar to the RNase A microinjection experiments. The assay consisted of RNase A treatment and subsequent addition of RNase inhibitor and different amounts of various types of RNA to evaluate their propensity to rescue the initial chromatin distribution. It is noted that the dissolution of the nucleoli could not be reversed in this manner under the conditions tested. The RNase A-induced changes of chromatin organization and their reversal could be clearly identified visually by following the changes in the fluorescence intensity distribution of the DAPI signal. In addition, these differences were also quantitated via computing the fractal dimension of the nuclear DAPI staining.

Single-stranded RNAs are essential for chromatin structure organization. Some features of the RNAs involved in the structural maintenance of chromatin could be inferred from comparing the effect of RNase A to other microinjected RNases. RNase A preferentially cleaves single-stranded RNA, RNase III and RNase V1 degrade double-stranded RNA and RNase H cleaves RNA in a DNA-RNA hybrid (Table S1). In addition, we tested cleavage of short RNA oligoribonucleotides (<7 nt) by the Orn oligoribonuclease. None of these RNases could reproduce the chromatin distribution pattern observed after RNase A injection (Fig. 1A and Table S1). With RNase H, the chromatin organization hardly changed (Fig. 1E), indicating that DNA-RNA hybrids are less important in higher-order chromatin organization. We conclude that the activity of RNA to maintain an open chromatin conformation involves single-stranded RNA linkages.

Heterochromatin structure is less sensitive to RNA digestion than euchromatin. An investigation of the heterochromatin markers histone H3 trimethylation at lysine 9 (H3K9me3), the proteins HP1α and Suv39h1 revealed that the integrity of pericentric heterochromatin was partially maintained after RNase A treatment (Fig. 4). In mouse cells, H3K9me3, Suv39h1 and HP1α accumulate in distinct DAPI dense foci of pericentric heterochromatin referred to as chromocenters. These can be easily distinguished from the less dense euchromatic regions in the fluorescence microscopy analysis. This was exploited in the experiments shown in Figure 4 to evaluate differences between eu- and heterochromatin in response to RNase A treatment (Fig. 4A) in comparison to the effect of DNase I (Fig. 4C) and MNase (Fig. 4D). The results show that RNase A digestion had a strongly reduced effect on heterochromatin structure as compared to euchromatin. As reported previously, HP1α became dispersed, which is indicative of the participation of an RNA component in its interaction with the chromocenters. In contrast, two other markers of pericentric heterochromatin in mouse cells, Suv39h1 and H3K9me3, remained associated at the chromocenters upon microinjection of RNase A. The H3K9me3 modification is stably attached to pericentric heterochromatin and a good marker for preservation of its compaction state. It became more dispersed in the nucleus only after DNA digestion by DNase I and MNase (Fig. 4C and D). Thus, apart from the RNA-dependent binding of HP1α, the heterochromatic chromocenters were mostly resistant towards RNase A treatment. This indicates that the structural requirements of chromatin for RNA were more pronounced in euchromatin than in heterochromatin regions.

Chromatin-interlinking RNAs (ciRNAs) are enriched in a soluble nuclear RNA fraction. To further characterize the RNAs responsible for maintaining the native chromatin distribution, we investigated the ability of different RNA fractions to rescue the chromatin collapse after RNase A treatment in permeabilized HeLa cells. This is an alternative method to expose chromatin to RNase A, and yielded results very similar to the RNase A microinjection experiments. The assay consisted of RNase A treatment and subsequent addition of RNase inhibitor and different amounts of various types of RNA to evaluate their propensity to rescue the initial chromatin distribution (Fig. 5A). It is noted that the dissolution of the nucleoli could not be reversed in this manner under the conditions tested. The RNase A-induced changes of chromatin organization and their reversal could be clearly identified visually by following the changes in the fluorescence intensity distribution of the DAPI signal. In addition, these differences were also quantitated via computing the fractal dimension of the nuclear DAPI staining.
ciRNAs are nuclear-retained RNAP II transcripts. To determine sequence features of ciRNAs, we compared the F2 RNA, soluble nuclear extract, nuclear RNA, chromatin and total RNA fractions by deep sequencing for two independent preparations. While the overall amount of intergenic RNAs, RNAP II primary transcripts and spliced RNAs was similar (Table 1; Materials and Methods), a higher abundance of spliced transcripts was observed in the F2 RNA fraction. A more detailed analysis revealed that the F2 RNA fraction was significantly enriched in spliced transcripts with long 3'-UTR sequences (Tables 1 and S2 and Fig. 7B and C). The most enriched clusters in the F2 RNA fraction were compared to those in the total RNA fraction as described in Materials and Methods. In the F2 fraction 56 ± 5% of these clusters were overlapping with 3'-UTR regions longer than approximately 800 nt, whereas these represented only 23 ± 12% in the total RNA sample (Table S2). These findings support the model of a structural role of a whole class of RNAs as opposed to a few specific transcripts, that have functional sequence elements encoded in the 3'-UTR.33

The F2 RNA fraction was depleted of polyadenylated transcripts, which could hinder nuclear export.34 This conclusion is based on (i) the low abundance of polyadenylated RNAs in this fraction when isolating them by oligo T affinity purification, (ii) the persistence of a reconstituting activity in the F2 RNA fraction after depletion of polyadenylated RNAs (Fig. 7A), and (iii) an amount of A>20/T>20 sequence reads that was only about half of that of the total RNA sample.

RNA maintain the structural integrity of RNAP II transcription factories. We compared the effect of RNase A with the inhibition of RNAP II by α-amanitin for 4 h. This process was monitored via 5-bromouridine 5'-triphosphate (BrUTP) labeling of nascent RNA. In both HeLa and NIH 3T3 cells, the native

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**Figure 3.** Chromatin aggregation kinetics after RNase A microinjection in living cells. Chromatin reorganization and aggregation in HeLa cells as monitored via H2A-YFP fluorescence occurred immediately after RNase A microinjection. This is readily apparent from the changes of the fluorescence intensity profile before (blue line) versus 5 min after (red line) microinjection. No such changes were observed in the control cells upon buffer microinjection. Scale bars, 10 μm.
transcription factories by cleaving their DNA component by DNase I or MNase treatment, the initially punctuated RNAP II distribution was transformed into much larger aggregates (Fig. 8C). Notably, a similar pattern was obtained after RNase A microinjection as well as after α-amanitin treatment (Fig. 8C). Upon addition of the F2 RNA fraction at a concentration of 10 ng/μl the initial dotted distribution of RNAP II was restored (Fig. 9). This correlation points to a possible function of ciRNAs as a structural component of transcription factories.

Discussion

In the present study we report on the drastic genome reorganization that occurs upon digestion of single-stranded RNAs in the nucleus. By combining fluorescence microscopy and microinjection experiments with high-throughput RNA sequencing, a novel structural function of RNA for interphase chromatin organization was characterized and assigned to a class of transcripts termed ciRNAs.

The conformational change observed upon depleting these RNAs could be rescued with a purified nuclear RNA fraction (F2). According to the sequence analysis of this fraction, ciRNAs
originate from mostly long (>500 nt) RNAP II transcripts that are subject to splicing. They have reduced polyadenylation, which could impede their nuclear export.34 Their long 3'-UTRs may contain sequence elements that are needed for interlinking chromatin domains. This is in line with the view that 3'-UTRs contain a number of interaction motifs that are encoded in their sequence or secondary structure.33 As inferred from the size distribution of the purified F2 fraction (Fig. 6C) in comparison with the corresponding genes and 3'-UTRs (Fig. 7C), it is concluded that a significant part of the RNAs in our preparation are not full-length transcripts with the complete 3'-UTR. This might be due to endogenous RNA processing or cleavage during the purification. In this context it is noteworthy that a recent report describes the separate expression of a large number of 3'-UTRs in human and mouse cells.54 These are likely to be generated by post-transcriptional cleavage of the corresponding protein-coding sequences. The ciRNA-containing fraction was extracted from the nuclei with a low salt buffer. Accordingly, it represents a fraction of nuclear RNA that is different from the RNA components reported as part of the nuclear matrix since these remain stably attached to the nuclear pellet during buffer extraction.7,20 Similarly, they do not remain associated with purified chromatin fragments as reported for yet another fraction of nuclear RNAs21,24 that were also included in our analysis.

The ciRNAs are instrumental to prevent a collapse of interphase euchromatin and serve to maintain its open and transcriptionally active state. This is concluded from our finding that RNase A micro-injection had a strongly reduced effect on pericentric...
heterochromatin foci in the mouse NIH 3T3 cell line as compared to bona fide euchromatin regions. As reported previously in experiments with permeabilized cells, HP1α partially dissociated from these regions when RNase A was added, but the DAPI staining indicated that the structural integrity of the chromocenters was mostly retained (Fig. 4). In contrast to the loss of the H3K9me2 histone methylation reported in the previous study, we did not observe a significant influence on the presence of H3K9 trimethylation. Both H3K9me2 and H3K9me3 are characteristic features of pericentric heterochromatin, and the observed differences might be related to the experimental conditions (e.g., permeabilization versus microinjection). Our results lead to the conclusion that heterochromatin is mostly resistant to RNase A treatment apart from a partial dissociation of HP1 since three markers of pericentric heterochromatin in mouse, namely intense DAPI staining, H3K9me3 and Suv39h1 persisted. In contrast, the more decondensed euchromatin regions were severely affected and collapsed. An additional line of evidence that relates ciRNAs to active transcription sites comes from the disruption and re-establishment of RNAP II transcription factories upon depletion of RNA with RNase A and subsequent addition of ciRNAs (Figs. 8 and 9).

The change in the observed chromatin compaction pattern induced by RNase A bears some striking similarities to that observed upon ATP depletion or by incubating the cells in a higher ionic strength medium. While ATP depletion could have a similar direct effect as the inhibition of RNAP II by α-amanitin, the chromatin compaction induced via changes of the salt concentration has been explained with a general macromolecular crowding effect. Furthermore, an increased degree of histone acetylation leads to μm-scale rearrangements of the chromatin compaction state as well. Thus, similar
Figure 7. Analysis of RNA fractions in rescue experiments and by deep sequencing. (A) Percentage of cells in which addition of the indicated RNAs (to a concentration of 10 ng/μl unless noted otherwise) rescued the initial homogeneous chromatin after RNase A treatment. Samples of the F2 RNA fraction included the depletion of polyadenylated RNA ("poly A-depleted F2") or addition together with α-amanitin (F2 + α-amanitin). RNAs analyzed by high throughput RNA sequencing are marked with a red star; "sol." stands for soluble. Error bars are the standard deviation for a Poisson distribution. (B) Distribution of the reads from the F2 (black) and total (red) RNA fractions within the B4GALT1 gene (green) as an exemplary genomic region (exons, large green rectangles; introns, thin green line). (C) Cumulative probability distribution of 3’ UTR length in the different RNA fractions. A comparison of the distributions reveals an enrichment of the F2 RNA fraction in long 3’ UTR. The maximal difference to the other distributions is at about 1,200 nt length with a p-value < 0.005 according to a Kolmogorov-Smirnov test. Error bars are smaller than the size of the symbols.
structural changes of the nuclear chromatin density distribution can be induced by very different triggers. The normal variations in chromatin compaction throughout the nucleus that are apparent in the CLSM images of human or mouse cell lines have been characterized at higher resolution by electron microscopy imaging and structured illumination microscopy. This led to a model in which more dense chromatin domains are surrounded by a loosely packed biologically active perichromatin compartment (PC) that extends into regions with a largely reduced DNA concentration. We speculate that the above-mentioned large-scale structural changes preferentially affect the “open” and biologically active PC enriched in transcription sites that surround domains with higher chromatin density. As a result of RNA degradation, increased osmolarity in the medium, ATP depletion or reduced histone acetylation, chromatin that extends into this regions could collapse and associate with the more densely packed chromatin domains.

Accordingly, we propose here that ciRNAs maintain the organization of the PC as depicted in Figure 10. In this scheme, four types of mutually non-exclusive mechanisms are considered that would affect the integrity of the open chromatin/PC region. (i) ciRNAs represent a structural component of RNAP II transcription factories and stabilize these entities so that they can act as genome organizers as proposed previously in reference 10-12. (ii) ciRNAs are produced by RNAP II but assemble into chromatin cross-linkers that stabilize interactions between perichromatin fibrils independently of RNAP II. (iii) The PC topology is affected indirectly by ciRNAs via regulating the histone acetylation state or other chromatin-related modifications that change chromatin structure. (iv) Protein binding of ciRNAs could prevent chromatin association of a protein that would induce chromatin compaction.

All four mechanisms would be compatible with the experimental findings reported here. However, we favor the first hypothetical model depicted in Figure 10A, in which ciRNAs act as genome organizing cross-linkers of the PC by stabilizing transcription factories, due to the following considerations: (i) Several lines of evidence argue for a function of transcription factories as genome organizers. (ii) The disruption and re-establishment of RNAP II transcription factories upon depletion of RNA with RNase A and subsequent rescue with ciRNAs correlates with the changes in the chromatin compaction state (Figs. 8 and 9). (iii) The RNA sequence analysis in combination with the RNA rescue experiments suggests that spliced coding RNA transcripts with long 3’-UTRs are active in our assay rather than stably chromatin-bound (non-coding) RNAs. Thus, ciRNAs could directly act at the site of their production as a positive feedback signal to maintain the state of active transcription. In contrast mechanisms 2, 3 and 4 in Figure 10A would require additional signals for their targeting to transcriptionally active regions. (iv) The RNA mediated histone or DNA modifications according to mechanism 3 appear to be dependent on stably chromatin bound RNAs that are inactive in our assay. Additionally, the rapid collapse and rescue of the chromatin structure argues against recruitment of chromatin modifying enzymes. (v) An RNA-dependent sequestering of a protein involved in chromatin compaction as depicted...
This would serve to further delineate the nuclear topology of chromatin domains that require RNA for maintaining their structural integrity. Furthermore, applying the recent advances in fluorescence microscopy imaging of nuclear subcompartments to studies of the RNA-dependent organization of the genome would provide further details on the underlying nuclear architecture.

While the reports on RNA with a putative chromatin organizing function date back to the 1960s and 1970s, progress in the field has been hampered by the inability to identify the sequences of the involved RNAs, and to investigate the structural effects of RNA under conditions that preserve the native state of chromatin. Taking stock of nuclear RNAs by sequencing is now becoming

in mechanism 4 has so far not been reported. Only the opposite process, i.e., the RNA-dependent recruitment of a chromosomal protein has been described, e.g., for HP1 (Fig. 4), or the polycomb repressive complex PRC2.

Clearly, the models discussed above make a number of predictions that require further experiments to corroborate one or the other. For example, we anticipate that the comparative localization of different fluorescently labeled RNAs with respect to active RNAP II will be particularly informative to address the question whether ciRNAs are indeed a component of transcription factories. In addition, chromosome painting in concert with RNase A treatment would allow to identify the subfraction of the PC that is located in between chromosome territories.

- Figure 8. DNA and RNAP II distribution after α-amanitin, DNase I, MNase and RNase A. CLSM images of HeLa (A) and NIH 3T3 cells (B) treated as indicated. DNA was stained with DAPI (blue). BrUTP and RNAP II (red) were visualized by immunofluorescence. (C) IF staining of HeLa cells, injected (DNase I, RNase A), permeabilized (MNase) or treated with α-amanitin, using an anti-RNAP II antibody. Scale bars, 10 μm.
rescue experiments (see below), α-amanitin was added simultaneously with RNase inhibitor 5 min before addition of the RNA fraction.

Immunofluorescence and antibodies. The anti-lamin A antibody was a gift of Dr. Harald Herrmann-Lerdon (DKFZ, Heidelberg). The anti-trimethyl-histone 3 lysine 9 (H3K9me3), the anti-RNAP II (H5) and the anti-BrdU antibodies were purchased from Millipore, GeneTex Inc., and Becton Dickinson, respectively. After PFA fixation, the cells were permeabilized for 5 min in ice cold PBS containing 0.5% Triton and washed twice in PBS. For immunofluorescence using the H5 antibody, cells were first permeabilized 1 min in 0.25% Triton prior to PFA.

**Materials and Methods**

**Cell culture.** HeLa cells were grown in RPMI 1640 containing 10% FCS, 2 mM L-glutamine and 1% penicillin/streptomycin. NIH 3T3 mouse fibroblasts, wild type or expressing GFP-HP1α or Suv39h1-GFP, were cultured in Dulbecco’s MEM containing 10% FCS, 4.5 g/L glucose, 2 mM L-glutamine and 1% penicillin/streptomycin. MCF-7 were grown in Dulbecco’s MEM containing 10% FCS, 2 mM L-glutamine, 1% non-essential amino acids and 1% penicillin/streptomycin. All cell lines were incubated at 37°C at 5% CO₂. For expressing auto-fluorescent constructs (HeLa, H2A-YFP; NIH 3T3, GFP-HP1α or Suv39 h1-GFP; MCF-7, Bax-GFP) growth conditions were the same as those used for the corresponding parental cell lines. For inhibition of RNAP II, cells were grown 4 hrs in medium containing 50 μg/ml α-amanitin (Sigma). When used in the rescue experiments (see below), α-amanitin was added simultaneously with RNase inhibitor 5 min before addition of the RNA fraction.
of RNase A on the chromatin structure was not dependent on the buffer used in the injection mix. The buffer solutions tested were either water, 20 mM sodium phosphate buffer (pH 6.9), phosphate buffered saline (PBS), RNase H reaction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂), or DNase I reaction buffer (10 mM Tris-HCl, pH 7.5, 2.5 mM MgCl₂, 0.1 mM CaCl₂). RNase H, RNase III (10 U/µl or 100 U/µl and 1 U/µl respectively, Epicentre Biotechnologies) and RNase V1 (0.1 U/µl, Ambion) were provided in solution (50 U/µl in 100 mM Tris pH 8.5). RNA transcripts in HeLa cells were visualized via incorporation of BrUTP (Sigma) during RNA synthesis and subsequent labeling. BrUTP was transfectioned 30 to 60 min with FuGENE 6 (Roche) and visualized with an anti-BrdU/BrU antibody as described previously in reference 52.

**Enzymes and buffers.** RNase A was obtained from Qiagen or Roche and diluted in boiled water to 14 mg/ml (1 U/µl). The effect of RNase A on the chromatin structure was not dependent on the buffer used in the injection mix. The buffer solutions tested were either water, 20 mM sodium phosphate buffer (pH 6.9), phosphate buffered saline (PBS), RNase H reaction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂), or DNase I reaction buffer (10 mM Tris-HCl, pH 7.5, 2.5 mM MgCl₂, 0.1 mM CaCl₂). RNase H, RNase III (10 U/µl or 100 U/µl and 1 U/µl respectively, Epicentre Biotechnologies) and RNase V1 (0.1 U/µl, Ambion) were provided in solution. RNases were both certified as protease- and DNase-free and tested for the absence of such activities. DNase I was from Qiagen, and dissolved in boiled DNase I buffer to a concentration of 1.5 or 15 Kunitz units per µl. The MNase was provided in solution (50 U/µl, Fermentas) or lyophilized (Genaxxon Bioscience) and used at a stock concentration of 600 U/µl in MNase storage buffer (20 mM Tris-HCl, pH 7.6, 50 mM NaCl and 50% (v/v) glycerol). For the microinjection experiments and treatment of permeabilized cells MNase was applied in a reaction buffer containing 15 mM Hepes pH 8.0, 17 mM KCl, 4 mM NaCl and 10 mM CaCl₂. The Orn RNase was prepared as described in reference 53, and diluted to a concentration of 1 µM or 10 µM. Restriction enzymes Alu I, Dra I and Hinc II (cleaving every 250, 1,000 and 4,000 nucleotides on an average) were provided at 10 U/µl stocks (Fermentas). Proteinase K from Roche was diluted in PBS to concentrations of 10 µg/µl or 100 µg/µl.

**Microinjection.** Cells were grown on glass coverslips that were coated with matrigel (BD Biosciences). Each 10 µl injection mix contained (i) 2 µl propidium iodide (PI, 1 mg/ml, Invitrogen), which stains preferentially RNA but also DNA in case of low RNA concentrations, and was used as a re-localization marker to identify the injected cells, (ii) 1 µl of enzyme or storage buffer for the control injection and (iii) 7 µl of the corresponding reaction buffer. RNase A could be combined with all reaction buffers without affecting its activity. We noticed that an injection mix containing more than 5% glycerol would affect chromatin organization. Accordingly, the glycerol concentration was kept at ≤5% glycerol in all experiments. Microinjection was performed with a computer-assisted system (AIS2, CellBiology Trading). Injection pressure was set to 150 hPA, the injection time to 0.5 sec and the needle diameter at the output was about 300 nm. The injected volume depended on the injection pressure, injection time and capillary diameter and was 10–100 fl with the enzyme concentrations given in Table S1. We routinely injected 300 cells per sample within 20 min. During the injection process, cells were maintained in L-15 medium containing 10% FCS, 2 mM L-glutamin and 1% penicillin/streptomycin. Following the injection, cells were placed either back into cell culture medium in the incubator (37°C, 5% CO₂) for 20 min (protease K and RNase A samples) or 1 hour (all DNases, RNases except RNase A, and other samples) if not indicated otherwise. For fixation cells were incubated for 7 min in ice cold PBS, pH 6.8, containing 4% PFA. Fixed cells were washed two times in PBS, incubated for 5 min in a DAPI solution (Invitrogen) and mounted overnight with mowiol.

**RNA extraction.** RNA fractions were prepared according to the scheme in Figure 6. Total RNA was extracted from HeLa cells by guanidiniothiocyanat/phenol extraction with the Trifast kit (Peqlab). To purify nuclear and cytoplasmic RNA fractions 2 x 10⁷ HeLa cells were incubated 10 min on ice in 5 ml extraction buffer (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT) containing 5 µl Ribolock RNase inhibitor (Fermentas) and transferred to a pre-cooled tissue homogenizer. Cells were bounced on ice 10 times using a tight pestle. The homogenized cells were centrifuged at 1,200 rpm (300 g) for 5 min at 4°C. The pellet contained the nuclei and the supernatant the cytoplasmatic fraction. The cytoplasmic RNAs were phenol/chloroform extracted after protease K and DNase I treatment. The pelleted nuclei were purified by one more centrifugation step through a 12% sucrose cushion at 2,500 rpm (1,300 g) for 5 min at 4°C. The nuclear RNAs were prepared from the pelleted nuclei following the Trifast procedure. Homopolymeric poly(A) (500 to 1,500 nt in length), yeast tRNA and yeast rRNA were purchased from Amersham Biosciences, Ambion and Worthington, respectively. The soluble nuclear extract and nuclear pellet were prepared by incubating isolated nuclei in 3 ml DNase I buffer containing 3 µl Ribolock for 10 min. The nuclei were centrifuged at 2,500 rpm (1,300 g) for 4 min at 4°C. The supernatant contained the soluble nuclear RNA extract and the pellet the nuclear pellet RNA fraction. The nuclear pellet RNA was isolated with the Trifast procedure, and the RNA from the soluble nuclear extract was phenol/chloroform extracted after protease K and DNase I treatment. The F1 to F4 RNA fractions were obtained from the soluble nuclear extract by loading it on a 30 ml 5–30% sucrose gradient before protease K and DNase I treatment. The gradient was centrifuged at 16,000 rpm (31,400 g) for 16 hours at 4°C using a Beckman SW 32 Ti swinging-bucket rotor. The respective gradient fractions (Fig. 6B) were pooled and the RNA was phenol/chloroform extracted after protease K and DNase I treatment. The F2 RNA fraction was depleted from polyadenylated RNAs using the Oligotex mRNA kit from Qiagen. The chromatin fraction was prepared from isolated nuclei, after DNase I digestion (1 Kunitz unit in 3 ml DNase I buffer, 37°C, 10 min) followed by incubation in 0.1 mM EDTA (30 min, 4°C) and light
sonication. The sample was then centrifuged and separated into insoluble pellet and soluble chromatin. The soluble chromatin was loaded on a sucrose gradient as described above and fractions containing DNA fragments of 1,500 to 3,000 bp (equivalent to 8 to 15 nucleosomes with a 200 bp nucleosome repeat length) were pooled. RNA was extracted by phenol/chloroform after proteinase K and DNase I treatment.

RNA rescue experiments. Cells grown on 18 mm cover slips were permeabilized for 30 s in 0.1% Triton X-100 with PBS, washed with PBS and incubated for 10 min with 700 U/ml RNase A in PBS. Cells were washed in PBS and incubated 5 min in medium supplemented with 100 μl/ml RNase inhibitor (Ribolock, Fermentas) at 37°C, 5% CO2. For testing the various RNA fractions, cells were incubated for 15 min after RNA addition.

Confocal fluorescence microscopy. For confocal imaging, a Leica TCS SP5 confocal laser scanning microscope (CLSM) equipped with a HCX PL APO lambda blue 63x/1.4 NA oil immersion objective was used (Leica Microsystems CMS GmbH, Mannheim, Germany). A diode-pumped solid-state laser and an Argon ion laser were used for DAPI (λ = 405 nm), Alexa 488 or GFP (λ = 488 nm) and Alexa 568 (λ = 561 nm) excitation. For the multi-color analysis, sequential image acquisition was applied and emission detection ranges were adjusted to minimize cross-talk between the different signals. The detection pinhole had a diameter corresponding to one airy disk.

Image analysis. Changes of the nuclear chromatin distribution upon treatment with RNase A were evaluated by visual inspection. All cells on a given CLSM image from an optical section were classified into two categories: “intact chromatin” and “aggregated chromatin”. To confirm that this reflected true differences in the chromatin distribution among 125 cells classified as having an intact chromatin distribution and 137 of the aggregated chromatin category were subjected to an analysis of the chromatin distribution by texture analysis via computing its fractal dimension. The approach was similar to that used previously for evaluating chromatin decondensation due to histone acetylation. 29 The fractal dimension was calculated for a 5 x 5 μm square in the center of the nucleus using the ImageJ plugin MapFractalCount by Per Henden and Jens Bache-Wiig. It is based on an improved box counting method. 35 As shown in the histogram in Figure 5B the two groups appeared as two clearly distinct distributions in the fractal dimension analysis with an overlap of about 10%. This could be considered as the upper limit for the error associated with our classification and confirms that our visual analysis is able to reliably distinguish the differences in the chromatin compaction state.

RNA sequencing. For high-throughput sequencing, two independent preparations of each of the different RNA fractions were analyzed. For data set 1, RNAs were depleted of rRNAs using the human/mouse RiboMinus transcriptome isolation kit (Invitrogen) and for data set 2 the Ribo-Zero rRNA Removal Kit (Epicentre) was used. After rRNA depletion, RNAs were subjected to metal ion catalyzed cleavage to sizes between 60–200 nucleotides with the Ambion RNA fragmentation reagents. Libraries for Solexa sequencing were generated according to the standard protocol for mRNA (Illumina) that comprised first strand cDNA synthesis, second strand cDNA synthesis, end repair, addition of a single A base and adapter ligation. Adapter sequences were 5'-P-GAT CGG AAG AGC TAT TCG TAT GCC TTC TGG TTG-3' and 5'-ACA CTC TTT CCC TAC AGC ACG CCT TCC GTA TC'T-3' with C* denoting a phosphorothioated cytosine. Products were amplified by PCR using the primers 5'-ATT GAT ACG GCC GCG ACC GAG ATC TAT ACT CTT TCC GTA CAC GAC GCT CTT CCG ATC'T-3' and 5'-CAA GCA GAA GAC GGC ATA CGA GCT CTT CCG ATC'T-3'. PCR products were size excised from low melting agarose gels (200–400 bp range) and phenol extracted. Sequencing was performed on the Illumina GAIIx platform at the sequencing core facilities of the EMBL, DKFZ and BioQuant in Heidelberg, Germany.

RNA sequence analysis. Initial sequence analysis was with the Bioconductor (http://www.bioconductor.org) package for the R statistical programming language to assess the reads quality and to produce a reads coverage file. The integrative genomics viewer (http://www.broadinstitute.org/igv) was used to visualize the coverage file and the RefSeq genes (NCBI). Reads were aligned with Bowtie 36 on the GRCh37/hg19 (2009) assembly version of the human genome reporting unique hits without mismatches and with and without trimming of the 3' and 5' ends. For the different RNA fractions 23-26 million reads of 36 nt length were obtained for data set 1. Between 49 to 71% of these mapped to the reference genome sequence. Ribosomal RNAs, which represented about 20% of the reads, were excluded from the analysis. For data set 2, the number of reads was between 25 and 35 million out of which 63-79% were mapped to the human genome. The ribosomal RNAs excluded from the analysis represented less than 3% of the reads of the different RNA fractions except for the chromatin sample (16% rRNAs). Peak calling was done with MACS by building a peak model to shift every read according to half the starting fragment size 37 and eventually combining overlapping peaks. Clusters were then called based on a significant enrichment in reads as compared to the local read distribution around the summit of each peak. These clusters were annotated according to RNA databases for transcription start sites (TSS), cap-associated gene expression (CAGE), promoter-associated small RNAs (pasRNAs), 3'-untranslated region (3'-UTR), small nuclear RNAs (snRNAs), tRNAs, micro RNAs (miRNAs), repeat-associated RNAs (rasRNAs), 3'-untranslated region (3'-UTR) range) and phenol extracted. Sequencing was performed on the Illumina GAIIx platform at the sequencing core facilities of the EMBL, DKFZ and BioQuant in Heidelberg, Germany.

Features of the F2 RNA fraction and total RNA were analyzed with respect to their content of poly A and poly T tracts (≥ 20-mers). To determine the most enriched clusters of the F2 RNA and total RNA fractions, common clusters from the F2 RNA fraction and total RNA (2,185 clusters, data set 1 and 2,364 clusters,
data set 2) were compared based on their amount of reads. The distribution of the ratios of their reads number (for each common cluster: reads in F2 RNA fraction/reads in total RNA) was calculated. To determine the most depleted clusters of the F2 RNA fraction on one hand and the most enriched clusters of the F2 RNA fraction on the other hand, we selected the clusters corresponding to the 10% lowest and 10% highest ratios, respectively.

RNA sequence data have been submitted to the ArrayExpress database at www.ebi.ac.uk/arrayexpress under the accession number E-MTAB-582.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental material can be found at:

www.landesbioscience.com/journals/nucleus/article/17773

Note

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Supplementary Material

Coding RNAs with a non-coding function: maintenance of open chromatin structure
Maïwen Caudron-Herger, Katharina Müller-Ott, Jan-Philipp Mallm, Caroline Marth, Ute Schmidt, Katalin Fejes-Tóth, and Karsten Rippe

This file contains the following sections:
Supplementary Figures S1, S2 and S3
Supplementary Tables S1 and S2
Supplementary References
**Supplementary Figures**

**Figure S1.** Immunostaining of the nuclear envelope. CLSM immunofluorescence images with an anti-lamin A antibody (green) are shown together with DAPI counterstaining of the DNA (blue). Scale bars: 10 µm. (A) NIH 3T3 mouse fibroblast cells. The nuclear envelope remained intact after RNase A injection but some local folding was induced. DNase I injection had no effect on the lamin A immunostaining. (B) The nuclear envelope of HeLa cells was unaffected by RNase A microinjection. Scale bars: 10 µm.
Figure S2. Time course of RNase A-induced chromatin aggregation. NIH 3T3 mouse cells were injected as indicated and fixed within (A) 5 to 20 min, (B) 20 to 50 min, and (C) 150 to 180 min after injection. Note that in the control sample the cells remained stably adherent to the surface like the non-injected cell in panel C (arrow, absence of PI staining). In contrast RNase A injection induced a rounding up of the cells after about 20 min as shown in panel B, and only a few cells remained attached to the glass surface after 150 min. DNA was stained with DAPI, and RNA (red) with PI. Scale bars: 10 µm.
Figure S3. Time course of apoptosis induction after microinjecting RNase A into MCF-7 cells expressing Bax-GFP. Bax is a downstream effector of Bid in the caspase activation pathways via mitochondria, and can be activated also through other pathways 1-3. The cellular localization of Bax is a marker of induction of apoptosis. In the merged pictures, PI is in red color, Bax-GFP in green and the DAPI stained DNA is displayed in blue. Scale bars: 10 µm. (A) Cells fixed 20 min after RNase A injection. The MCF-7 cells expressing Bax-GFP showed the full collapse and reorganization of chromatin as observed before. Yet, both RNase A-injected cells as well as a non-injected cell (marked by an arrow) displayed a cytoplasmic distribution of Bax-GFP. Thus, apoptosis was not activated and Bax-GFP is localized in the cytosol or at the mitochondria in both injected and non-injected cells at this time point. (B) Cells fixed 90 min after RNase A microinjection. About half of the cells displayed a punctuated distribution of Bax-GFP, indicating the induction of the cellular events leading to apoptosis. In the RNase A sample, the apoptotic cells are identified by Bax translocation to the mitochondria, which became highly fragmented in the early stages of apoptosis. In the control cells, Bax-GFP was still localized in the cytosol or at the mitochondria.
Supplementary Tables

Table S1. Enzyme-specific effects of nucleic acid and protein digestion on chromatin structure.

<table>
<thead>
<tr>
<th>Enzyme (concentration) a</th>
<th>HeLa</th>
<th>3T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>proteinase K (100 µg/ml)</td>
<td>n. d.</td>
<td>+</td>
</tr>
<tr>
<td>RNase A (1 U/µl)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RNase H (100 U/µl)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RNase III (1 U/µl)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RNase V1 (0.1 U/µl)</td>
<td>-</td>
<td>n. d.</td>
</tr>
<tr>
<td>DNase I (15 Ku/µl)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MNase (0.6 U/µl) b</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Orn (10 µM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dra I (10 U/µl)</td>
<td>-</td>
<td>n. d.</td>
</tr>
<tr>
<td>Hinc II (10 U/µl)</td>
<td>-</td>
<td>n. d.</td>
</tr>
<tr>
<td>Alu I (10 U/µl)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The measured effects on the chromatin distribution were: +, large scale reorganization and aggregation of chromatin; -, no effect on chromatin structure was apparent; n. d., not determined.

a The microinjected volume was between 10 - 100 femtoliter. For all enzymes a control injection was done with the corresponding buffer that induced no change in the chromatin distribution.

b Enzymatic treatment via permeabilization of the cells and addition of MNase to the medium at the indicated concentration. The microinjection of MNase had no apparent effect on chromatin since sufficiently high enzyme activities could not be reached even when injecting concentrations of 60 U/µl.
Table S2. Length distribution of the 3’-UTR regions in the most enriched clusters of the F2 RNA fraction as compared to the total RNA fraction.

<table>
<thead>
<tr>
<th>3’-UTR regions</th>
<th>Data set 1</th>
<th>Data set 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total RNA (%)</td>
<td>F2 RNA fraction (%)</td>
</tr>
<tr>
<td>all</td>
<td>41</td>
<td>62</td>
</tr>
<tr>
<td>&gt; 800 nt</td>
<td>35</td>
<td>61</td>
</tr>
<tr>
<td>&gt; 2000 nt</td>
<td>18</td>
<td>41</td>
</tr>
</tbody>
</table>

Supplementary References