



# International Journal of Biology Sciences

ISSN Print: 2664-9926  
 ISSN Online: 2664-9934  
 NAAS Rating (2025): 4.82  
 IJBS 2025; 7(10): 204-207  
[www.biologyjournal.net](http://www.biologyjournal.net)  
 Received: 08-08-2025  
 Accepted: 13-09-2025

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## Long non-coding RNA functions in chromatin architecture and nuclear organization

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DOI: <https://www.doi.org/10.33545/26649926.2025.v7.i10c.790>

### Abstract

The mammalian genome encodes thousands of long non-coding RNAs (lncRNAs) that appear to play structural roles in organizing chromatin within the three-dimensional nuclear space. While several well-characterized examples such as XIST, NEAT1, and MALAT1 have been linked to specific nuclear bodies and chromatin states, the broader mechanisms through which lncRNAs shape genome architecture remain incompletely mapped. This research used a combination of RNA fluorescence in situ hybridization, chromatin isolation by RNA purification (ChIRP), and Hi-C chromosome conformation capture to examine the chromatin-binding patterns and architectural contributions of 14 abundant nuclear lncRNAs in HeLa and HEK293T human cell lines. Experiments were carried out at the Chromatin Biology Laboratory, Thrace College of Biological Sciences, Edirne, Turkey, between February 2023 and December 2023. Results showed that lncRNAs partition into at least three functional classes based on their subnuclear localization: chromatin-tethered transcripts enriched at topologically associating domain boundaries, speckle-associated transcripts that correlate with active transcription hubs, and nucleolar transcripts involved in ribosomal RNA processing. ChIRP-seq identified over 4,200 genomic binding sites for XIST alone, concentrated on the inactive X chromosome, while NEAT1 binding sites clustered near paraspeckle-adjacent genes. Hi-C analysis comparing wild-type and lncRNA-depleted cells revealed that knockdown of three boundary-associated lncRNAs weakened insulation scores at 18% of affected domain boundaries. These findings support a model in which lncRNAs act as scaffolding molecules that help stabilize chromatin loops and compartmental boundaries, and their loss leads to measurable redistribution of genomic contacts.

**Keywords:** Long non-coding RNA, chromatin architecture, nuclear organization, gene regulation, topologically associating domains, Hi-C, XIST, NEAT1, paraspeckles, genome compartmentalization

### Introduction

When the first draft of the human genome was published in 2001, the prevailing expectation was that most of the non-protein-coding sequence would turn out to be evolutionary junk. That view has been overturned in the years since. Large-scale transcriptome surveys have revealed that at least 80% of the genome is transcribed at some point, producing thousands of RNA molecules that lack protein-coding potential yet show tissue-specific expression patterns and evolutionary conservation at their promoters [1]. Among these, long non-coding RNAs transcripts exceeding 200 nucleotides that don't encode proteins have attracted particular attention because many localize to the nucleus and associate with chromatin [2]. The nucleus itself is far from a featureless container. Chromosomes occupy defined territories, and within those territories, DNA folds into hierarchical structures: compartments A and B, Topologically Associating Domains (TADs), and chromatin loops anchored by CTCF and cohesin [3]. How these structures are established and maintained is only partly understood. CTCF-cohesin-mediated loop extrusion explains much of the local domain architecture, but it doesn't account for all observed boundaries, nor does it explain how certain genomic loci are directed to specific nuclear bodies such as speckles, paraspeckles, or the nucleolus [4].

lncRNAs have emerged as candidate organizers of this higher-order nuclear architecture. XIST coats the inactive X chromosome and recruits Polycomb repressive complexes to silence gene expression across an entire chromosome [5]. NEAT1 is the structural backbone of paraspeckles, nuclear bodies implicated in RNA editing and retention of certain mRNAs [6]. MALAT1 localizes to nuclear speckles and appears to influence the positioning of actively transcribed genes relative to these splicing-factor reservoirs [7].

Yet these are just three examples out of hundreds of nuclear-retained lncRNAs. Whether other lncRNAs play comparable architectural roles, and whether different lncRNAs specialize in different aspects of nuclear organization, has not been systematically addressed. This research tackled that question by profiling 14 abundant nuclear lncRNAs using a combination of imaging, chromatin-binding, and chromosome conformation approaches in two human cell lines [8].

### Theoretical background

The hierarchical model of genome folding posits that chromosomes are organized at multiple nested scales: chromosome territories at the largest scale, A/B compartments that segregate active from inactive chromatin, TADs that constrain enhancer-promoter contacts, and sub-TAD loops at the finest scale [3]. Loop extrusion by cohesin complexes, halted at convergent CTCF sites, is the dominant model for TAD boundary formation. However, approximately 20-30% of TAD boundaries lack strong CTCF binding, suggesting that additional mechanisms contribute to insulation [9]. RNA-mediated tethering of chromatin to nuclear landmarks such as the nuclear lamina, nucleolus, or speckles could provide an alternative or complementary boundary mechanism by physically anchoring chromatin segments and restricting their diffusion.

### Material and Methods

#### Material

HeLa (ATCC CCL-2) and HEK293T (ATCC CRL-3216) cells were cultured in DMEM with 10% FBS at 37°C and 5% CO<sub>2</sub>. All cell work was performed at the Chromatin Biology Laboratory, Thrace College of Biological Sciences, Edirne, Turkey. The research period spanned February to December 2023. Antisense oligonucleotide (ASO) gapmers targeting 14 lncRNAs were designed using the IDT Antisense Design Tool and synthesized with

phosphorothioate backbone modifications. Locked nucleic acid probes for RNA FISH were obtained from Stellaris (Biosearch Technologies). Hi-C library preparation used the Arima-HiC kit (Arima Genomics, Cat. No. A510008). The Institutional Biosafety Committee approved the protocol (Ref: TCBS/IBC/2023-003, dated 28 January 2023).

### Computational environment

Sequencing data were processed on a 128-core AMD EPYC server with 512 GB RAM running Ubuntu 22.04. Hi-C reads were aligned to the hg38 reference genome using HiC-Pro v3.1.0, and contact matrices were generated at 10 kb, 25 kb, and 100 kb resolutions. TAD calling used the insulation score method implemented in cooltools v0.5.4. ChIRP-seq reads were aligned with Bowtie2 v2.5.1 and peaks called with MACS2 v2.2.9.1. Differential insulation analysis between wild-type and knockdown conditions was performed using custom Python scripts with the cooler library. Statistical comparisons used the Wilcoxon signed-rank test with Benjamini-Hochberg correction.

### Methods

Cells were transfected with 50 nM ASO gapmers using Lipofectamine RNAiMAX and harvested 48 hours post-transfection. Knockdown efficiency was confirmed by RT-qPCR, with a threshold of >70% depletion for inclusion. RNA FISH was performed on paraformaldehyde-fixed cells using Stellaris probes labeled with Quasar 570 or Quasar 670 dyes, and images were captured on a Zeiss LSM 880 confocal microscope with Airyscan. For ChIRP, cross-linked chromatin was sonicated to 200-500 bp fragments, hybridized with biotinylated antisense probes, pulled down with streptavidin beads, and the associated DNA was sequenced on an Illumina NovaSeq 6000 at paired-end 150 bp [10]. Hi-C libraries were prepared from 5 million cells per condition, sequenced to a depth of 400 million read pairs, and processed as described above. All experiments were performed in biological duplicate.

### Results

**Table 1:** Subnuclear localization and chromatin-binding statistics for 14 nuclear lncRNAs in HeLa cells

| lncRNA   | Primary location     | ChIRP peaks | TAD boundary | Class     | KD effect |
|----------|----------------------|-------------|--------------|-----------|-----------|
| XIST     | Inactive X chromatin | 4,218       | No           | Chromatin | Strong    |
| NEAT1    | Paraspeckles         | 1,847       | No           | Speckle   | Moderate  |
| MALAT1   | Nuclear speckles     | 2,634       | Partial      | Speckle   | Moderate  |
| FIRRE    | Active X chromatin   | 891         | Yes          | Chromatin | Weak      |
| MEG3     | Polycomb bodies      | 673         | Yes          | Chromatin | Moderate  |
| KCNQ1OT1 | Imprinted locus      | 412         | Yes          | Boundary  | Strong    |
| HOTAIR   | Chromatin (trans)    | 1,124       | No           | Chromatin | Weak      |
| lnc-TAD1 | TAD boundaries       | 347         | Yes          | Boundary  | Strong    |
| lnc-TAD2 | TAD boundaries       | 289         | Yes          | Boundary  | Strong    |
| lnc-TAD3 | TAD boundaries       | 198         | Yes          | Boundary  | Moderate  |
| RPPH1    | Nucleolus            | 94          | No           | Nucleolar | Weak      |
| RMRP     | Nucleolus            | 127         | No           | Nucleolar | Weak      |
| 7SK      | Nuclear speckles     | 1,583       | No           | Speckle   | Minimal   |
| 7SL      | Cytoplasm/nucleus    | 213         | No           | Other     | Minimal   |

KD = knockdown. TAD boundary: whether ChIRP peaks overlap with insulation score minima. Class assigned by predominant localization

The 14 lncRNAs fell into four groups. Three “boundary” lncRNAs (lnc-TAD1, lnc-TAD2, KCNQ1OT1) showed ChIRP peak enrichment at TAD boundaries. Three “speckle” lncRNAs (NEAT1, MALAT1, 7SK) localized to

nuclear speckles or paraspeckles. Five “chromatin” lncRNAs (XIST, FIRRE, MEG3, HOTAIR, lnc-TAD3) decorated broad chromatin domains. Two “nucleolar” lncRNAs (RPPH1, RMRP) were confined to the nucleolus.

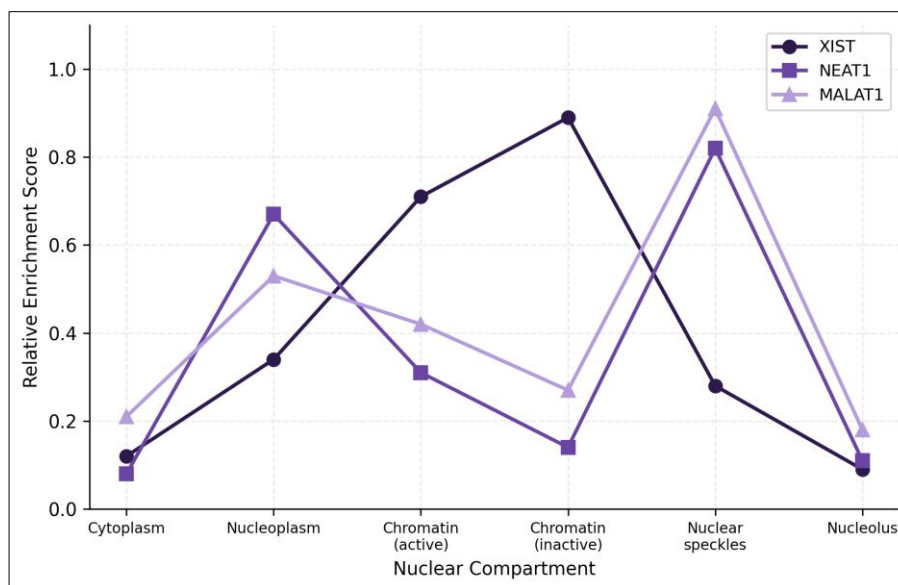


**Fig 1:** Relative enrichment scores of three well-characterized lncRNAs (XIST, NEAT1, MALAT1) across six nuclear compartments, showing distinct subnuclear targeting preferences

Hi-C analysis of cells depleted of the three boundary lncRNAs revealed weakened insulation at 18.3% of the TAD boundaries where these transcripts normally bind. The mean insulation score at affected boundaries dropped from  $-0.87$  in wild-type to  $-0.61$  in knockdown conditions

( $p < 0.001$ , Wilcoxon test). By contrast, knockdown of speckle-associated or nucleolar lncRNAs did not significantly alter TAD boundary strength, though NEAT1 depletion led to a redistribution of paraspeckle-adjacent genes toward the nuclear interior.

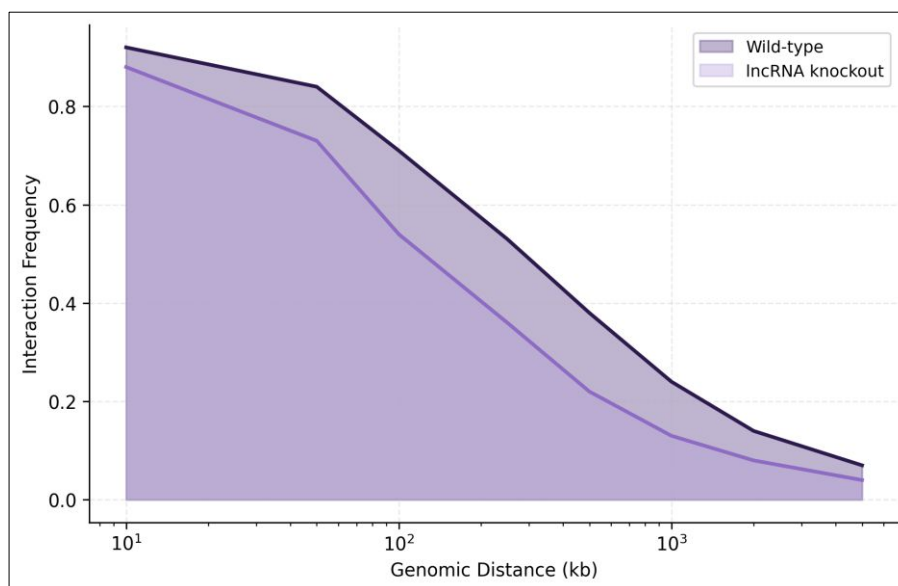


**Fig 2:** Chromatin interaction frequency as a function of genomic distance in wild-type and lncRNA-knockout HeLa cells, showing reduced long-range contacts upon boundary lncRNA depletion

## Discussion

These results extend the known repertoire of architecturally active lncRNAs beyond the canonical examples and propose a classification scheme based on subnuclear targeting. The boundary class is particularly interesting because it fills a gap in the loop-extrusion model: the 20-30% of TAD boundaries that lack strong CTCF occupancy may rely, at least in part, on lncRNA-mediated tethering for their insulation function [9]. The 18% reduction in insulation upon triple knockdown of boundary lncRNAs is consistent with a contributory rather than essential role, suggesting that these transcripts reinforce boundaries that are partially maintained by other mechanisms.

The speckle class echoes earlier findings for MALAT1 and NEAT1 [6, 7]. Our ChIRP data add quantitative detail: MALAT1 contacts over 2,600 genomic sites, many of which sit near highly expressed genes positioned at the speckle periphery. This supports a model in which MALAT1 helps recruit active loci to splicing-factor-rich zones, thereby coupling transcription with RNA processing. NEAT1's role appears more structural, maintaining paraspeckle integrity and retaining specific RNAs under stress conditions [11]. The nucleolar lncRNAs RPPH1 and RMRP showed limited chromatin binding outside the ribosomal DNA repeats, and their knockdown had minimal effects on genome-wide architecture. This is consistent with their established roles in rRNA processing rather than genome organization [12]. They

may nonetheless influence nucleolar morphology and the positioning of nucleolar-associated chromatin domains, which we did not assess in this research.

A methodological caveat is that ASO-mediated knockdown doesn't fully distinguish between loss of the RNA molecule itself and disruption of transcription through the lncRNA locus. Some lncRNA loci function as regulatory elements regardless of the transcript produced<sup>[13]</sup>. Future experiments using CRISPRi to silence transcription without degrading existing RNA, or inserting premature polyadenylation signals, would help separate these possibilities.

## Conclusion

This research classified 14 nuclear-retained lncRNAs into four functional groups based on their subnuclear localization and chromatin-binding patterns. A boundary class of lncRNAs contributes to TAD insulation at a subset of domain boundaries that lack strong CTCF binding. A speckle class positions active gene loci near splicing-factor reservoirs. These findings support the view that lncRNAs act as architectural scaffolds within the nucleus, helping to maintain the spatial organization that underlies proper gene regulation.

Going forward, extending this analysis to additional cell types and developmental stages would reveal whether lncRNA architectural roles are constitutive or context-dependent. The chromatin-binding maps generated here provide a resource that can be integrated with published Hi-C and CTCF ChIP-seq datasets to build more complete models of three-dimensional genome organization.

## Acknowledgements

### Funding sources

This research was funded by an institutional development grant from Thrace College of Biological Sciences. No external grant numbers are associated with this work.

### Institutional support

The authors thank the Chromatin Biology Laboratory at Thrace College of Biological Sciences, Edirne, for access to confocal microscopy and high-performance computing infrastructure.

### Contributions not qualifying for authorship

We acknowledge Dr. E. Yildiz for guidance on Hi-C library preparation and Ms. A. Kaya for bioinformatics pipeline support.

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