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AN RNA STORY, FROM CHROMATIN TO PROTEIN

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An RNA Story, from chromatin to protein

Thesis for Doctoral Degree (Ph.D.)

By

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"Macte nova virtute, puer, sic itur ad astra."

Publius Vergilius Maro

Popular science summary of the thesis

You can think of our DNA as a giant recipe book. One that you must treasure and keep as pristine as you can, safe from all the bubbling and splashing of the kitchen. Each recipe, what we call a gene, contains the blueprint for a component crucial to our cells' survival. As the DNA is secured away in the nucleus, the cell needs to copy these recipes on a more temporary molecule, called RNA, to bring the information elsewhere – think about it like post-it note. Once outside the nucleus, these RNA molecules direct the synthesis of proteins and are therefore called messenger RNAs.

Or, at least, that's what we thought for a long time. Turns out that these transient molecules have a broader role than information delivery. Just like paper, RNA can fold into a myriad of different tools that the cell uses for controlling all the processes that keep us alive. This non-messenger RNAs are so versatile that they are responsible of most of the difference between humans and worms, and their significance is also underscored by their prevalence, as that they make up most of the RNA within each of our cells.

In a broader context, RNA seems particularly adept at organizing the space inside cells. It achieves this by interacting with proteins and other RNAs to form concentrated droplets where all the components necessary for a specific process can gather, increasing efficiency. This phenomenon, known as phase-separation in technical terms, seems to occur wherever the concentration of RNA change over a certain threshold and is not dissimilar to what happens when we add oil to water. The process is particularly significant when the cell is stressed, by for instance a sudden increase in temperature, and results in the formation of temporary stress-related structures, known as stress granules. These not only protect certain essential molecules from stress-induced damage, but also orchestrate the adaptations needed to survive the new environmental conditions.

Nevertheless, the significance of phase-separation is not limited to stress responses; rather, phase-separation appears be an underlying mechanism to various cellular processes. For instance, as we describe in the first paper of this thesis, different kinds of RNA are constantly sticking onto or around the DNA and help its spatial organization within the nucleus, ensuring that certain regions of it are accessible. Furthermore, during the initial steps of gene transcription, the RNA produced helps in forming a bubble of components around it that keeps the process going. Similar, but much bigger, condensates form in the nucleus around RNA molecules that need further processing after transcription.

To fine-tune all these functions, parts of the RNA molecule can be modified – just as we would add notes and further instruction to a recipe. We only started appreciating the importance of these modifications a few years ago, and many scientists are trying to

decipher what they mean and how they are added to the different RNAs. “Many scientists” includes us: most of the work in this thesis is indeed focused on RNA modifications.

We recently discovered that dyskerin, an enzyme responsible for RNA modification, act on messenger RNAs more extensively than we thought. This modification seems to reduce the speed at which messenger RNA is read during protein production. While we are not sure yet why cells would need to slow down the production of certain proteins – or certain regions of them – we think that this might help ensure that the final product is correctly assembled. Moreover, when dyskerin is removed (and therefore does not modify RNA anymore), messenger RNAs accumulate much more into stress granules after a heat shock than they normally do. Thereafter, cells struggle to resume normal protein production, highlighting the importance for messenger RNA modification for proper stress response.

Much like the flavors in a gourmet dish, the functions of RNA blend and interconnect. As scientists, we find ourselves tasked with piecing together this intricate recipe using only basic instructions. Though challenging, the rewards are invaluable: uncovering the minute inner working of cells offers vital insight for combating the many diseases that afflict humanity.

Abstract

RNA functions expand well over just coding for protein effectors. Non-coding RNAs oversee translation, RNA processing, and spatial organization of the cellular space, therefore participating in the regulation of almost any cellular process. To add a further layer of complexity, both coding and non-coding RNAs are extensively modified. These modifications determine RNA stability and localization, often by changing the way the single RNA molecules interact with RNA-binding proteins.

In **paper I**, we describe how RNA contributes to maintaining an open chromatin structure by neutralizing the positive charge on the histone-tails. This effect, which seems to depend only on the charge of the RNA molecule, is not mediated by newly transcribed RNAs, but rather on RNA species that are stable in time, and possibly coincide with LINE-1 containing transcripts.

In **paper II**, we explore the role of dyskerin, the only RNA-guided pseudouridine synthase expressed by human cells, in mediating co-transcriptional modification of mRNAs, which in turn regulates their translation. Dyskerin travels along RNA polymerase II over transcribed genes, and binds – possibly modifying – thousands of mRNAs. After a short-term depletion of dyskerin, pseudouridylation levels on mRNAs drop dramatically, and translation levels show an overall increase. We show that this effect depends on the enzymatic function of dyskerin and that pseudouridine directly inhibits translation *in vitro*. Conversely, we find that prolonged removal of dyskerin results in an overall drop in translational rates, and that this is linked to rRNA processing defects caused by long-term depletion of dyskerin. Our results also reveal that mRNA pseudouridylation is reduced in cells from dyskeratosis congenita patients, where dyskerin is impaired, therefore offer novel insight on the molecular mechanism behind this syndrome.

In **paper III**, we investigate the role of mRNA pseudouridylation in the heat-shock response, focusing on the formation of stress granules. Pseudouridine levels increase after heat-shock, and removal of three different enzymes involved in mRNA pseudouridylation results in defective stress granule formation. Hypo-pseudouridylated mRNAs accumulate within stress granules, and translation recovery after stress is impaired.

Taken together, these results expand on the multi-faceted role of RNA and RNA modification in regulating multiple fundamental cellular processes. The insight they offer on the inner workings of human cells and the molecular mechanism behind dyskeratosis congenita will hopefully contribute to the identification of novel therapeutic targets for dyskeratosis congenita and other diseases.

List of scientific papers

I. Dueva R, Akopyan K, Pederiva C, **Trevisan D**, Dhanjal S, Lindqvist A, Farnebo M. Neutralization of the Positive Charges on Histone Tails by RNA Promotes an Open Chromatin Structure. Cell Chem Biol. 2019 Oct 17;26(10):1436-1449.e5.

II. Pederiva C*, **Trevisan DM***, Peirasmaki D, Chen S, Larsson O, Ule J, Baranello L, Agostini F, Farnebo M. Control of protein synthesis through mRNA pseudouridylation by dyskerin. Sci Adv. 2023 Jul 28; 9(30)

III. **Trevisan DM,** Q Zhou and Farnebo M. Pseudouridine synthases regulate mRNA localization to stress granules and translation re-initiation after stress. - *Manuscript*

* These authors contribute equally

Contents

List of abbreviations

CTCF	CCCTC-binding factor
DNA	Deoxyribonucleic acid
eIF2α	eukaryotic translation initiation factor 2 alpha
hnRNP	Heterogeneous nuclear ribonucleoprotein
LC-MS/MS	liquid chromatography with tandem mass spectrometry
m6A	N6-Methyladenosine
METTL3	Methyltransferase 3 enzyme
mRNA	Messenger RNA
PAR	Poly(ADP)-ribose
PKR	Protein kinase R
PUS	Pseudouridine synthase
RNA	Ribonucleic acid
rRNA	Ribosomal-RNA
scaRNA	Small Cajal-body associated RNA
snRNA	Small nuclear RNA
snoRNA	Small nucleolar RNA
SRRM2	Serine/Arginine Repetitive Matrix 2
tRNA	Transfer RNA
YTHDF	YTH domain family

1 Literature review

1.1 RNA properties and Functions

Although the chemical differences between RNA and DNA are limited, these two classes of nucleic acids differ from a structural standpoint. Whereas DNA always occur as a double helix, most RNA exists as a single-stranded. This means that an RNA chain more easily can adopt different conformations, just as polypeptide chains fold to build the final structure of a protein. This versatility allows RNA molecules to play crucial roles in gene expression, coding for proteins on one side and absolving structural and catalytic functions on the other.

Moreover, the single-stranded nature of RNA adds a further level of complexity to its interaction with other molecules. The phosphate backbone carries a negative charge that can interact with positively charged amino acids, such as the ones in the histone tails[1]; while the exposed nitrogenous bases allow for sequence-specific interactions, making RNA an excellent guide molecule for specific cellular processes, such as RNA modification.

Within cells, several types of RNA molecules carry out distinct functions. Most famously, messenger RNAs (mRNA) carry genetic information from DNA to the ribosome for protein synthesis. Furthermore, a plethora of non-coding RNAs directs many of the processes fundamental for the survival of the cell, such as chromatin organization, DNA damage repair, splicing, and translation[2]. Many of these functions are carried on through the binding of specific RNA-binding proteins and the formation of phase-separated compartments within the nucleus and the cytoplasm of the cell.

1.1.1 Non-coding RNAs

Only a fraction of RNA molecules encodes proteins, and the final product of most genes is the RNA itself. These non-coding RNAs, like proteins, are active components of a variety of biological processes, providing enzymatic activity, structural support, and regulation. Indeed, accumulating evidence suggests that what makes us so different from *Caenorhabditis elegans,* is not the protein-coding fraction of our genome, but rather what is transcribed from the non-coding regions[3].

Some non-coding RNAs can be considered infrastructural or housekeeping. These have generally been known for a longer time and comprise transfer RNAs (tRNA), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and small Cajal-body associated RNAs (scaRNAs). These RNAs are involved in translation and the processing of other RNAs: for instance, around 200 snoRNA species guide the co-transcriptional modification of rRNAs essential for their maturation[4]; while snRNAs recognize splicing sites and guide the excision of introns from pre-mRNAs.

In addition to the housekeeping non-coding RNAs, other non-coding RNAs have been more recently identified. These are classified by their length into small or long non-coding RNAs.

Small non-coding RNAs play central roles in the regulation of gene expression and are in turn divided in several sub-classes, of which the most studied are micro RNAs (miRNAs), involved in degradation of target mRNAs as part of the RNA-induced silencing complex (RISC)[5]. Long non-coding RNAs, on the other hand, are more than 200 nucleotides in length and often show mRNA-like properties – such as transcription by RNA polymerase II, 5′-cap and polyadenylation at the 3′ end[6]. These transcripts can also be involved in the regulation of gene expression[7], but they do not necessarily require sequence complementarity to their targets. Rather, their function is related to their specific structure, partially explaining their poor sequence conservation[8]. Due to their structural plasticity, long non-coding RNAs can form dynamic three-dimensional complexes and function as scaffold molecules[9]. As scaffolds, long non-coding RNAs associate with protein-complexes involved in histone or DNA modification[6] and can nucleate the assembly of sub-nuclear structures[10].

1.1.1.1 Non-coding RNAs involved in translation

The reactions that result in the synthesis of proteins are collectively termed translation and include the deciphering of the information encoded in the mRNA into a series of amino acids, and the formation of the peptide bond. Key steps in translation are performed by two families of non-coding RNAs: rRNAs and tRNAs.

The ribosome is a large complex composed of RNA by two-thirds (**– rRNA and tRNA are responsible for the translation process.**
Figure 1, left). There are four eukaryotic rRNAs, and a single copy of each is part of each ribosome: three out of the four rRNAs (18S, 5.8S and 28S) are cleaved out from the 45S precursor rRNA, while the fourth (5S RNA) is synthesized separately. Almost 80%[11] of the RNA present in actively dividing cells is rRNA and, within the ribosome, the rRNAs are responsible for the formation of the peptide bond, the central reaction of protein synthesis.

Amino acids do not directly read the codons in an mRNA molecule. Rather, the decoding step of translation is performed by tRNAs, a set of small RNA molecules that can bind to both the codon and the amino acid, and thus function as adaptors. tRNAs show a strikingly complex three-dimensional L-shaped structure: on one end, we find the anticodon, that decodes the information contained in mRNAs, while the other end of the molecule is loaded with an amino acid (**Figure 1**, middle). The specific interaction between the codon and the anticodon translates the sequence of nucleotides in mRNA into the chain of amino acids that constitutes a protein (**Figure 1**, right). This recognition step is uniquely critical, as factors that change the binding between the codon and the anticodon can

greatly influence the fate of the protein that is being synthesized. For instance, the stop codons that signal the end of the polypeptide chain are not recognized by any tRNA, but rather by the eukaryotic translation termination factor 1 (eRF1), that triggers translation termination. The isomerization of the uridine in the stop codons into pseudouridine can result in nonsense suppression, rather than translation termination[12].

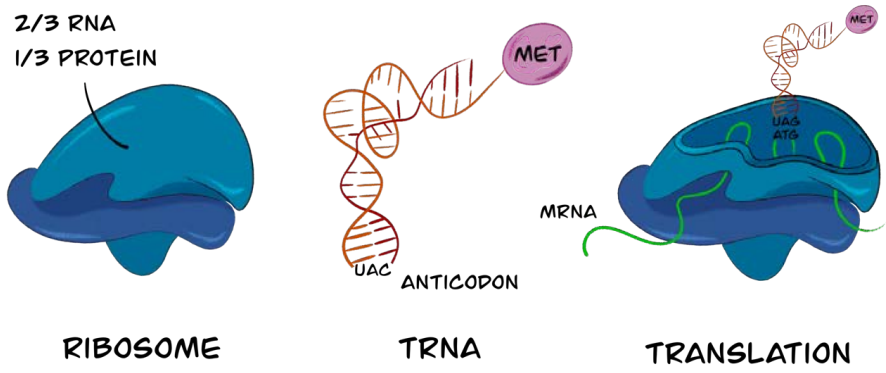

Figure 1 – rRNA and tRNA are responsible for the translation process.
Schematic representations of the ribosome (left), a tRNA loaded with a methionine on the end opposite to the anticodon (middle), and the decoding step of translation (right).

1.1.1.2 Non-coding RNAs involved in RNA modification

Most eukaryotic RNAs are covalently modified after transcription. These modifications are carried out by RNA-modifying enzymes, and while not all, some of them use guide RNAs that specify the site of the modification.

For instance, extensive chemical modifications occur in the 45S rRNA before the mature 18S, 5.8S, and 28S rRNAs are excised from it. These include about 100 methylations of the 2′-OH positions on the ribose backbone (2′-O-Methylation) and 100 isomerizations of uridines into pseudouridine. rRNA is modified in this manner co-transcriptionally[4], in the nucleolus, and under the guidance of snoRNAs. snRNAs are instead modified after transcription is completed[13] and under the guidance of scaRNAs. The localization of scaRNAs in Cajal bodies is determined by a specific localization signal within the scaRNA sequence, that is recognized and bound by the protein WRAP53β[14,15], an essential component of Cajal bodies[16].

The sno- and scaRNAs that guide the modification of rRNA and snRNAs are generally divided in two structural classes: C/D and H/ACA box RNAs. These two groups take their names from different sequence motifs they contain, which regulate the formation of specific secondary structures and in turn the recognition and binding by the enzymatic components of the modification complexes.

C/D box sno- and scaRNAs function as guide RNAs for 2'-O-methylation and share a conserved folding into a single kink-turn structure. These C/D guide RNAs interact with a set of four proteins: fibrillarin, NOP56, NOP58 and NHP2L1 – forming C/D-box sno/scaRNPs (**Figure 2**). The guide RNA recognizes its targets by sequence-complementarity and fibrillarin methylates it in specific positions depending on the distance from the box D conserved sequence, rather than on nature of the modified nucleoside[17].

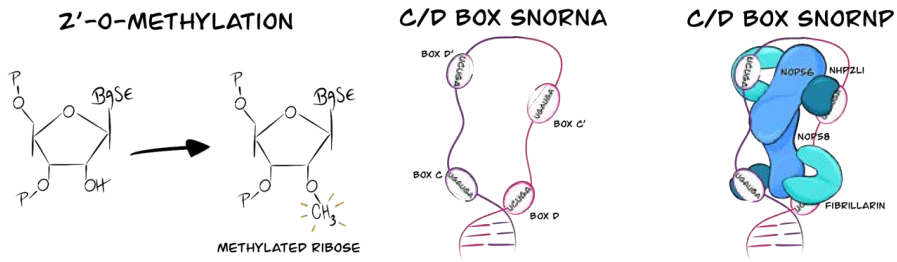

Figure 2 – C/D box snoRNPs introduce 2'-O-Methylation on RNA
Schematic representation of a 2'-O-Methylated nucleoside (left), a C/D box snoRNA (middle), and a C/D box snoRNP (right).

Similarly, H/ACA guide RNAs contain a unique structural motif that includes two hairpins linked by a hinge and followed by a short tail[18]. They interact with a second group of proteins forming the H/ACA complex, which is isomerizes uridines into pseudouridines. In this context, the enzymatic component is dyskerin, also known as DKC1. Aside dyskerin, three other proteins are part of the H/ACA complex: NHP2, NOP10 and GAR1. A complete set of proteins binds to each of the two hairpins in the H/ACA structure, which are in turn kept very close to each other by the association between the two dyskerin molecules[19] (**Figure 3**).

Dyskerin, NHP2 and NOP10 are initially recruited to the H/ACA RNA during its transcription[20], but the fully mature H/ACA complex is formed only when NAF1, a maturation factor that probably keeps dyskerin in a non-active conformation, is replaced by GAR1[21]. When dyskerin binds to the target RNA, a thumb-like loop closes over it to keep it in place, and GAR1 helps this movement[22]. NHP2 and NOP10, instead, ensure that the catalytic site of dyskerin is in the right conformation[22]. On top of guiding the modification of rRNA and snRNA, the H/ACA complex is also involved in the stabilization of TERC, the RNA component of the telomerase holoenzyme[23]. This last function seems to explain the most evident symptoms of dyskeratosis congenita, the systemic syndrome caused by the mutations of dyskerin, including skin and immune defects as well as progeria. However, some of the symptoms of dyskeratosis congenita appear in mice before telomere shortening becomes evident, suggesting that the loss of the enzymatic function of dyskerin might also have a role in the development of the disease[24].

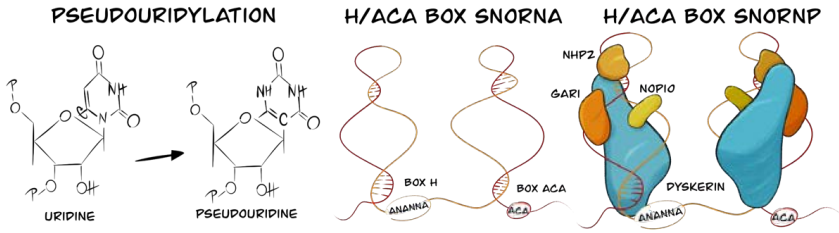

Figure 3 – H/ACA box snoRNPs introduce pseudouridine on RNA
Schematic representation of pseudouridine (left), a box H/ACA guide RNA (middle), and an H/ACA box snoRNP (right).

1.1.1.3 Chromatin-associated RNA

RNA can associate with chromatin and form "RNA-Clouds" over active gene promoters. These interactions are carried out via one of two modalities: *cis*-interactions, through which RNA acts in proximity of its site of synthesis; and *trans*-interactions, that require the release of the RNA molecule from its transcription site and the translocation to a second genomic locus, where the RNA accomplishes its regulatory role.

Considerable effort has been put into the development of techniques for unbiased detection of chromatin-associated RNAs and it led to the identification of several classes of RNA residing within or closely associated to chromatin[25]. For instance, nascent RNA can interact with chromatin at transcription sites, and has been shown to regulate transcription by hybridizing back to its template DNA and forming an R-loop. These structures can regulate gene expression by recruiting transcription regulators to promoters[26].

On the other hand, sno- and scaRNAs are consistently identified as chromatin associated RNAs[27]. While the specifics of their functioning in this context are still elusive, there are increasing reports of their roles in regulating key functions like DNA damage repair. Such regulation seems to happen by binding of the sno/scaRNA to certain enzymes and consequent inhibition of the enzyme's activity, tipping the balance towards certain pathway choices[28], or suppressing damage signaling altogether[29].

Lastly, repeat-containing RNAs are the most abundant family of chromatin-associated RNAs. Different species of repetitive RNAs seems to associate with different chromatin domains and promote either a closed, silenced, chromatin state by recruiting chromatin remodeling enzymes; or an open chromatin state, by dampening the interaction between the positively charged histone tails and the DNA backbone in a manner depending on their charge[1,30].

1.2 Chromatin organization and the role of RNA

Inside the nucleus, the DNA is hierarchically arranged in different levels of compaction, starting with the wrapping around histones, to the formation of chromosomes domains

and, finally, to chromosome territories. However, to allow replication and RNA transcription, the double-helix needs to be accessed constantly, and thus requires a complex concert of regulatory mechanisms that ensure that certain regions of the genome are accessible, while other remain compacted. These processes make our genome one of the most dynamic cellular structures.

The minimal packing unit of the eukaryotic genome is the nucleosome (**Figure 4**, top-left), which comprises about 150 base-pairs of DNA and the octamer of histones they are wrapped around. The nucleosomes are organized in polymorphic chromatin fibers that vary in diameter between 5 and 24nm[31] (**Figure 4**, top-middle). The degree of packing is generally determined by post-translational modification of the histone proteins by several histone-modifying enzymes. While the role of some of these modifications in promoting an open, euchromatic, structure or a more densely packed one (i.e. heterochromatic) are well described, many others seem to have a dual function that depends on the other modifications nearby[11].

Within each chromosome, heterochromatic and euchromatic domains show dynamic and cell-line specific spatial separation[32]. These domains self-organize following a model called of a "fractal globule" where the polymer collapses into a series of 'beads-on-a-string', that then fall onto each other progressively "until only a single globule-of-globules-of-globules remains"[32,33] (**Figure 4**, top-right). Long range interactions show that while heterochromatin may form the skeleton of a certain domain, euchromatin extends further out and interacts with other active regions[34,35]. This points toward a "practical" organization of the genome where chromatin domains with a similar grade of transcriptional activity and related functions cluster together thus allowing concerted regulation (**Figure 4**, bottom). Indeed, recent observations brought the attention to organizational domains formed by interaction between the initiation and termination sites of transcriptionally active genes. Several of these domains would then come together and form larger euchromatin or heterochromatin compartments. The mechanism of this is still under investigation, but possibly involves liquid-liquid phase separation driven by proteins able to interact with specific histone-tags, which can mediate sharp transitions between open and collapsed chromatin[31,36,37].

Furthermore, the position of each chromosome in the nucleus is spatially determined, with many regions showing consistent interaction with one another or with nuclear structures. These domains are therefore topologically, rather than functionally, associated. It seems that this effect depends on dynamic chromatin loop extrusion by proteins like the CCCTC-binding factor (CTCF) and the cohesin complex[38]. The driving force for this organizational effort might depend on the need of association between certain chromosome domains with specific nuclear bodies [39,40].

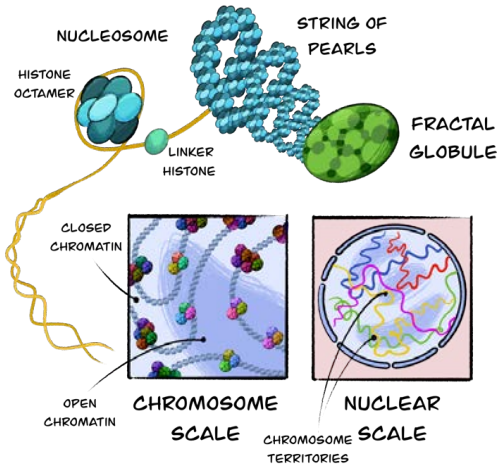

Figure 4 – The genome is organized in different levels of compaction.

Schematic representation of the various levels of chromatin organization, from the nucleosome (top-left) to a string of pearls (top-middle) and a fractal globule (top-right). Each chromosome occupies a defined territory within the nucleus (bottom-right). At a chromosome scale (bottom-middle), open and closed domains are physically separated, with each chromosome domain folded as a fractal globule.

The gene-regulating effects of these topologically associated domains remain a matter of debate: reportedly, the disruption of these domains can alter gene expression patterns, and therefore induce the aberrant expression of oncogenes[41]. Moreover, the disassembly of splicing speckles via depletion of Serine/Arginine Repetitive Matrix 2 (SRRM2) causes an overall compaction of chromatin[42]. However, the connection between topological domains and gene expression remains labile, as their genome-wide disruption seems to have relatively meager effects on overall gene expression, suggesting that these effects are probably locus specific[31].

1.2.1 Chromatin structure and organization by RNA

An effective way of reducing chromatin compaction is to remove positive charges on the histone tails. This is the proposed mechanism by which acetylation of lysines favors the opening of chromatin[43]. On the other hand, increasing the number of available positive charges can promote chromatin condensation[44]. As chromatin compaction is, at least partially, regulated by electrostatic interactions, the negative charge carried by RNA might play a crucial role in maintaining an open chromatin structure by dampening the charge on histone tails[1,45]. RNA is a particularly exciting candidate for this type of function, given its heterogeneous structure and ability to interact with both other nucleic acids and proteins. Not only RNA can bind to DNA at its site of transcription and forms R-loops[26,46]; but it can also recognize specific sequences of DNA by interacting with the major groove of the double-helix, forming triplex structures[47].

Several non-coding RNAs also have reported roles in scaffolding the recruitment of proteins involved in the maintenance of chromatin structure. This function seems to be majorly dependent on either of two mechanisms. On one end, RNA recruits chromatin remodeling enzymes[48] or bridges the connection between different genomic regions, by regulating the multimerization of chromatin binding proteins like CTCF[49]. On the other, RNAs can induce the opening of certain chromatin region by virtue of their charge or by recruitment non-DNA binding proteins, such as the scaffold attachment factor A (SAF-A), and promote their oligomerization[50] (**Figure 5**). Either way, these functions seem to

depend on repetitive elements present in the RNAs mediating them[51], suggesting that what was once considered the remnants of viruses not able to replicate anymore, or transcripts from "junk DNA", plays a key role in regulating the architecture of the nucleus.

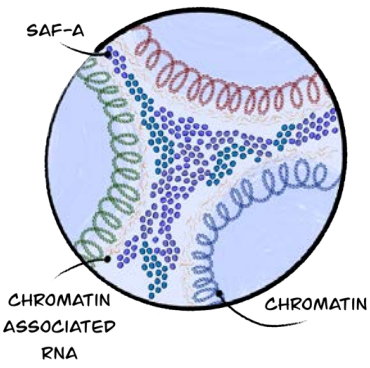

Figure 5 – SAF-A/RNA complexes associate with chromatin to promote an open chromatin structure.
SAF-A interacts with chromatin associated RNAs and regulates chromatin structure.

In other words, RNA, either alone or in association with RNA-binding proteins, contributes in several ways to maintain chromatin organization. *Cis* and *trans* regulatory functions act in concert to fine-tune the regulation of different loci[46]. In this scenario, changing the local concentration of RNA, or RNA-protein complexes, could constitute an additional and fast way for a cell to sway the balance between open and closed chromatin states and thus to regulate transcription. Altogether, this paints a vision of the nucleus as a compact structure, where highly dynamic membrane-less condensates formed around RNA organize the genome in a functional manner.

1.3 Regulation of gene expression by RNA modification

An additional, fascinating, aspect of RNA biology is its modification. Just like proteins are modified after translation, RNA can be modified post-transcriptionally in nearly 200 different ways[52]. The functions of most of these modifications are not detailed yet and seem to vary depending on the RNA species and its cellular context, but the conservation of their presence suggests that they may play a pivotal role in regulating the flow of genetic information. Consistently, mutations of the enzymes involved in RNA modification are often linked to disease, including cancer, genetic birth defects, and neurological disorders[53]. Moreover, RNA modifications seem to be dynamically regulated in response to various kinds of stress[54,55], suggesting a function not dissimilar to the one of protein post-translational modifications in regulating response to stimuli. Of particular interest in this context is the role that RNA modifications play in the innate immune response. Un-modified RNA is recognized as non-self by multiple protein systems, such as the antiviral innate immune response receptor (RIG-I) or the protein kinase R (PKR)[56,57], and activates the cell response to the infection. Thus, the widespread presence of RNA modification in all classes of RNA might have emerged initially as a defense mechanism against pathogens.

Most RNA modifications are more abundant on non-coding RNAs, particularly rRNA and tRNA, than on mRNAs[58]. This probably reflects the variegated functions of these non-coding RNA species, which often depend on their structure and their binding to proteins. Indeed, many RNA modifications are required for or prevent the binding of specific RNA-

binding proteins, the so-called "readers" of the modification, either by altering the chemical properties of the binding site, or by disrupting reader-recognized secondary structures[59] (**Figure 6**, left and middle). However, in recent years it has become evident that mRNAs are also modified[60]. Here, RNA modifications can alter many aspects of the mRNA life cycle, from its stability to its translation. From a stability perspective, mRNA modification seems to mainly act through reader proteins, which either protect the mRNA from degradation or direct it to decay. The involvement of mRNA modification in translation is, on the other hand, more variegated: while these modifications can also act as binding sites for specific reader proteins, including translation initiation factors[60]; the modification of the coding sequence of mRNAs has the potential for directly altering the binding to the ribosome or the pairing with the anticodons in tRNA, regulating the speed of translation[61] (**Figure 6**, right).

Furthermore, as many RNA modifications happen co-transcriptionally, this functional coupling might extend in the other direction through the formation of phase-separated condensates around the transcription machinery[60,62]. This suggests that by regulating phase-separation at site of transcription co-transcriptional RNA modification could control transcription.

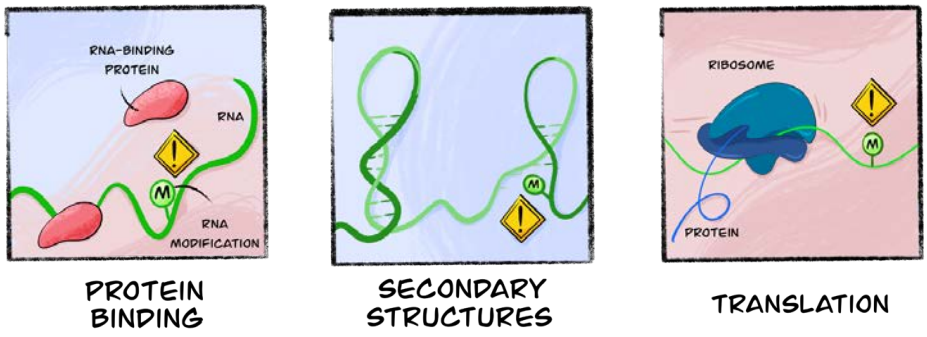

Figure 6 – RNA modifications regulate many aspects of the RNA life cycle.
RNA modifications often act by regulating the binding of RNA-binding proteins (left). By modifying the binding site of an RNA-binding protein the cell can favor or reduce the binding, therefore changing the fate of the specific RNA. Moreover, RNA modifications can alter the secondary structure of RNAs (middle). This is particularly relevant for non-coding RNAs, whose secondary structure plays important roles in determining their function. Lastly, modification of the coding sequence of mRNAs (right) can alter the translation efficiency by changing the binding between codon and anti-codon.

One of the most extensively studied RNA modifications is N6-methyladenosine (m6A). The addition of m6A is primarily carried out co-transcriptionally by the methyltransferase 3 enzyme (METTL3)[58,60] and, upon export to the cytoplasm, this modification is recognized by various reader proteins that mediate the downstream effects of the modification. m6A can be actively removed by eraser enzymes such as the Fat mass and obesity-associated protein (FTO) and AlkB homolog 5 (ALKBH5) RNA demethylases[58], suggesting a possible function in signal transduction in response to specific stimuli. Indeed, m6A levels change in response to stress[55] and METTL3 has recently be reported to translocate to sites of

DNA damage to locally methylate RNAs and recruit the repair-associated DNA polymerase κ[63].

m6A is one of the most prevalent modifications in eukaryotic mRNA: in humans, an average of 3 to 5 m6A modifications occur in each mRNA molecule (or about 0.2 to 0.6% of all adenosines)[64]. Within the mRNA sequence, m6A resides mainly in the coding sequence and the 3′ untranslated region (3′ UTR) of the molecule[58]. The level of this modification in mRNA is inversely correlated to transcriptional speed, with slower transcription rates resulting in more modified adenosines[65,66].

Function-wise, m6A modification has shown roles in many aspects of mRNA metabolism. Directly, m6A destabilizes hairpin helices and thus alters the secondary structure of mRNA; while through different reader proteins it regulates alternative splicing, translation, and decay of the modified mRNA. The binding to the YTH domain family 2 (YTHDF2) proteins, for instance, facilitates the degradation transcripts that contain m6A by recruiting the carbon catabolyte repression – Negative on TATA (CCR4-NOT) deadenylase[58,67]. Moreover, increased levels of m6A in mRNAs can impair their translation, suggesting an overall negative impact on gene expression[65].

1.3.1 Pseudouridine

Pseudouridine is the most abundant and the first discovered RNA modification[68]. It is the product of isomerization of a uridine by rotation of the base and substitution, with a carbon-carbon bond, of the nitrogen-carbon glycosidic bond to the ribose. This configuration grants increased rotational freedom and frees the N1-H as an additional hydrogen bond donor, expanding the opportunities for secondary structure formation, and ultimately increasing the stability of the modified RNAs[69]. Human cells can express 13 pseudouridine synthases, 12 of which recognize their target by directly relying on the RNA sequence and/or structural features[70]. The last pseudouridine synthase, dyskerin, recognizes its targets by a sno- or scaRNA guide that is bound to dyskerin prior to target recognition. Pseudouridine synthases also seem to mainly act co-transcriptionally, however direct recruitment to RNA polymerases is not necessary for their action. This does not exclude, however, that co-transcriptional action might facilitate the modification of certain targets, contributing to tissue-specific patterns of pseudouridylation[60].

There is no known eraser of pseudouridine, and possibly the only way to remove this modification is by degradation of the modified RNA. Nonetheless, the levels of pseudouridylation also change in response to stress[54,71], suggesting that pseudouridylation might act in concert with m6A in the stress signal transduction cascade. Not much is known of the readers of pseudouridine either[72]. However, this modification was recently reported to enhance the binding of methionine aminoacyl tRNA synthetase (MetRS) to tRNA[73]. Suggesting that the modalities of action are probably similar across different types of RNA modification.

Most of the functions described for pseudouridine are related to its effects on the secondary structures and stability of RNA. In tRNAs, indeed, the role of pseudouridine in preserving the three-dimensional conformation of the molecule has been extensively described[74]. This is further supported by the localization of pseudouridine residues in rRNA around the critical-for-function regions of the molecule, like the interface between the two ribosomal subunits, the decoding center, and the peptidyl transferase center[75].

Other than at the known sites in tRNAs, rRNAs and snRNAs, pseudouridines have also been recently found in mRNAs in comparable quantities to m6A[71]. Within mRNAs, distribute evenly between introns and exons[76]. The interest in mRNA pseudouridylation spiked in recent years, particularly due to its impact on the translation efficiency of synthetic mRNAs in living organisms. In this context, pseudouridine prevents binding of PKR to the RNA, therefore leading to increased protein production due to reduced immunogenicity. Moreover, pseudouridine prevents degradation by RNase L and so increases the stability of the synthetic. Both these properties were key for the success of the mRNA vaccines introduced during the SARS-CoV-2 pandemic[77].

Outside of the synthetic context, however, the effects of mRNA pseudouridylation have been more elusive. The study of mRNA pseudouridylation in living cells is particularly challenging because the same enzymes that seem to modify mRNA, also modify snRNAs, tRNAs and rRNAs[59]. Therefore, it is difficult to discern the specific effects of mRNA pseudouridylation at a cellular level, especially when looking at processes that require other pseudouridylated RNA species.

Nonetheless, several groups have now reported roles for mRNA pseudouridylation in regulating translation. After the initial reporter studies showing that pseudouridine enhanced protein production, several *in vitro* approaches instead argued that the modification slows translation[61]. These two opposing hypotheses, have been reconciled by the observation that while pseudouridine slows translation elongation, as suggested by the *in vitro* studies, it might also increase ribosome recruitment to mRNAs, favoring translation initiation by limiting phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2α) by PKR[78]. However, the biological relevance of the effects of pseudouridine on PKR activation by "self" mRNA remains to be explored. The initial work by Karikó and colleagues[79] indeed showed that the introduction of other modifications also increased expression of synthetic mRNAs, and therefore does not exclude that several mRNA modifications may act together to suppress PKR activation. In our hands, removal of dyskerin via siRNA treatment did not elicit phosphorylation of eIF2α[80], and conversely increased protein production until ribosomal defects arose; suggesting that the effects on elongation speed might prevail in living cells.

1.4 RNA and liquid-liquid phase separation

Life has evolved as a functionally specialized space in which subsets of molecules selectively segregate to improve chemical reaction efficiency[81]. However, multiple critical biochemical reactions must occur simultaneously within a cell. These frequently require many different steps, each catalyzed by a specific enzyme, and could not proceed efficiently if all the components were freely diffusing in the cellular space. Therefore, compartmentalization of the space within the plasma membrane is a critical need for survival.

Although the formation of internal membranes is the most visible of the adaptations that divide the cell in distinct sub-spaces, it is not the only one. Certain intracellular components can indeed separate into discrete liquid phases through condensation, like water droplets forming on the surface of a cold glass.

William Hardy and Edmund Wilson have proposed phase separation as an operational principle for the formation of membraneless cellular compartments over a century ago. In the last decade, however, there has been a resurgence of this idea as a model to describe how the internal organization of the cell is achieved[81]. Through liquid-liquid phase separation, a solution of molecules can spontaneously demix, concentrating specific molecules within liquid droplets[82]. Moreover, the formation and disassembly of these condensates rely on a variety of factors that cells can easily regulate, including protein concentration, post-translational modifications[82], making them an intriguing possibility for cell compartmentalization. Indeed, liquid-liquid phase separation is mediating the formation of many cellular bodies[82–84].

The formation of a network of multivalent interactions appears to be the primary driver of demixing[82,85]. From a protein standpoint, this depends on the presence of either arrays of modular protein domains that interact with specific ligands, or intrinsically disordered regions within the protein. Due to their lack of well-defined three-dimensional structure, disordered domains not only can interact with different ligands, but also reduce solubility, another parameter that affects the phase behavior of proteins[82]. Together with protein-protein interactions, protein-nucleic acid interactions have also proven critical for liquid-liquid phase separation within cells, and RNA is now appreciated as a key player in the formation of condensates.

Although less explored, this role of RNA has become evident in the last years. Many of the proteins in biomolecular condensates have RNA-binding domains and the presence of RNA favors their phase separation[84]. In accordance, raising the local concentration of RNA, as it happens for instance during transcription, triggers the liquid-liquid phase separation of proteins involved in RNA processing. Indeed, many nuclear bodies, such as nucleoli and Cajal bodies, form around newly transcribed RNA to facilitate its processing[84]. The ability of RNA to phase separate is determined by its molecular properties discussed above,

including its charge and structural flexibility[86]. Moreover, it is becoming evident that specific RNA modifications can affect the phase properties of RNA in a similar way as protein modifications do for proteins[87].

Within phase-separated compartments, RNA carries out fundamental functions that determine the structure, the dynamics, and the physical properties of aggregates. Depending on its concentration, RNA can trigger the formation or the disassembly of phase-separated structures[88]; while its sequence and secondary structure can function as a scaffold for the recruitment and assembly of proteins into condensates[88]. The typical example of this latter mechanism is the formation of paraspeckles around the long non-coding RNA Nuclear Paraspeckle Assembly Transcript 1 (NEAT1)[89] (**Figure 7**).

The biological implications of liquid-liquid phase separation are far-reaching. Biomolecular condensates regulate many cellular processes, including gene expression, signal transduction, and stress response[84]. These functions are achieved by affecting molecule concentration. For example, the processing of the histone mRNA is reduced if the necessary enzymes fail to concentrate within the histone locus body[90]. Moreover, the ability of minute changes in physical parameters to induce sharp phase transition can provide a way to assemble functional compartments in response to changes in their environment[91].

The first membraneless compartment observed was the nucleolus[84]: a huge biomolecular condensate that organizes around the nascent pre-rRNA[92], with more than 400 proteins and RNAs contributing to its formation. However, several other membraneless compartments have been observed and studied, both in the nucleus and the in the cytoplasm, including splicing speckles, that are the most prominent mRNA processing site in the nucleus[93], and stress granules, that form as a consequence of cellular stress and play a role in mRNA storage and protection[94] (**Figure 7**). Importantly, dysregulation of liquid-liquid phase separation and RNA dynamics are possibly at the basis of numerous diseases, including neurodegenerative disorders and cancer[95,96]. Moreover, several groups have reported that the binding of proteins to synthetic RNAs containing phosphorotioate modifications can trigger the formation of anomalous nuclear inclusion, termed PS bodies, that "ripen", becoming insoluble aggregates[97–100], in a manner similar to other condensates do in neurodegenerative pathologies[96]. These abnormal condensates seem to disturb many cellular processes by sequestering proteins[97,100], and their formation should be taken into consideration when evaluating the use of synthetic RNAs for therapeutic purposes.

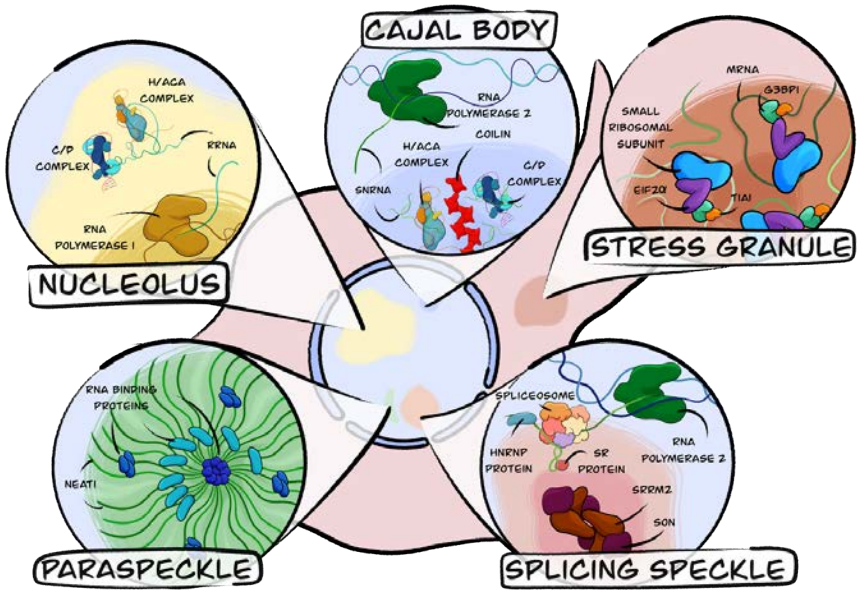

Figure 7 – Many phase-separated organelles are organized around RNA.
Nascent rRNA nucleates the accumulation of nucleolar factors around its transcription site, forming the nucleolus. snRNAs and mRNAs are transcribed in close proximity to their maturation sites: the Cajal body and the splicing speckle respectively. In response to proteotoxic stresses such as heat shock, the activation of the integrated stress response signaling pathway halts translation and leads to the formation of stress granules around mRNAs stalled during translation initiation. The function of paraspeckles is less defined, but their assembly strictly depends on the long non-coding RNA NEAT1.

1.4.1 Splicing Speckles

Splicing speckles (also called nuclear speckles) are membraneless RNA-protein granules (around 20 to 50 per nucleus) that are observed in the nucleus of eukaryotic cells[101]. They were first described by Santiago Ramón y Cajal, and while they were initially considered as passive storage sites for splicing factors, recent evidence indicates a more active role in pre-mRNA processing[102,103]. Splicing speckles are of irregular shape, however, when transcription or splicing are inhibited, they enlarge and become round, as unprocessed RNAs accumulate within them[101,104]. This, together with the observation that many of their components are continuously flowing in and out of them suggests that they might be phase-separated condensates[103].

Despite their dynamic properties, splicing speckles can be purified via biochemical fractionation. This way, the first attempts to systematically identify the components of these organelles[105,106] pointed at over 350 proteins, most of which are spliceosome-associated proteins and other pre-mRNA processing factors. In more recent years, hundreds of proteins have been reported as part of splicing speckles, either using enzyme-based proximity labelling or through microscopy. However, while some factors, such as SRRM2 and SON, are observed only in these structures, many others also localize in the nucleoplasm[103]. This suggests that specific sequence motifs might determine the strength of the localization of the single proteins in splicing speckles. Of particular

importance in this context seems to be the positive charge of amino acids like histidine and arginine[107,108].

Super-resolution microscopy, moreover, revealed that splicing speckles are sub-compartmentalized into an inner core and a periphery. The core contains the structural components SRRM2 and SON (**Figure 7**) that are essential for the formation of these organelles[104,109]; while the spliceosome factors localize in the periphery[104], in accordance with the observation that splicing largely happens in the outskirts of speckles, rather than in their inner regions[110].

Heterogeneous nuclear ribonucleoproteins (hnRNPs) and SR proteins have antagonistic effects on splicing[111], and proper splicing seems to depend on the binding of SR proteins to exons and of hnRNP proteins to introns[112]. This suggests a model in which the pre-mRNA sequence-dependent positioning favors its splicing: the exons, bound to SR proteins, would move into the periphery of the splicing speckle, while the introns would be prevented from entering by their binding to the hnRNP proteins. As a result, the exon-intron junctions would be located at the interface between the nucleoplasm and the speckle, where the spliceosomes are poised to splice[113].

1.4.1.1 Splicing speckles are hubs for mRNA processing

From a functional standpoint, splicing speckles are indeed involved in the removal of introns from pre-mRNAs. However, several groups have now reported that they associate with transcriptionally active regions of the genome[42,102,103,114]. This would suggest that splicing speckles are formed around highly transcribing genes in a similar way as nucleoli form around the rRNA genes[92].

Indeed, even though transcription only happens at the periphery of splicing speckles, these organelles contain various subunits of RNA polymerase II[115]. The phosphorylation state of the C-terminal tail of RNA polymerase II appears to control the condensate preference of the transcription complex: hypophosphorylated RNA polymerase II separates with Mediator during transcription initiation, while hyperphosphorylated RNA polymerase II is preferentially found in condensates formed by splicing factors[116]. On the other hand, known splicing factors, such as SC35, are required for transcriptional elongation[117], suggesting that association with speckles might have a role in promoting high levels of transcription.

Moreover, nascent transcripts transit through the more internal regions of the speckles before diffusing towards the nuclear pore complex, suggesting that the core of the speckle might carry out later stages of mRNA maturation that are required for export, such as quality control or mRNA modification. In accordance, pre-mRNAs that retain one or more introns (due to mutation, for instance) are retained within the speckle[110] and several mRNA modifying enzymes are found in these structures[80,118].

Transcription is only the first step in producing a mature RNA molecule, and the spatial proximity between the transcription compartments and splicing speckles would make these organelles perfect as "mRNA maturation centers", as it allows for rapid exchange of RNA maturation factors between the speckles and the transcription bubble.

1.4.2 Stress Granules

When eukaryotic cells are stressed, for example by a sudden increase in temperature (i.e. heat shock), they activate a complex signaling pathway known as the integrated stress response. The central event of this pathway is the phosphorylation of eIF2α on serine 51, which results in a global decrease in protein synthesis and the activation of selected genes that aid in cell recovery[119]. As translation arrests, the resulting ribosome-free mRNA accumulates in cytoplasmic ribonucleoprotein granules known as stress granules and processing bodies (P-bodies)[120]. While processing bodies contain components of the mRNA decay machinery such de-capping enzymes (Dcp1/Dcp2)[121]; stress granules form around mRNAs stalled in translation initiation and play a crucial role in their sequestration and triage[122]. After stress granules and processing bodies disassemble, the mRNA they contain can either return to translation or be degraded[120].

Stress granules mainly consist of mRNA and RNA-binding proteins (**Figure 7**), though their specific makeup can differ based on the nature of the triggering stress, implying different functions for different stresses[120]. However, many of the stress granule components are not essential for their assembly[123], and only cells lacking the paralogs Ras-GAP SH3 domain-binding protein (G3BP) 1 and 2 do not form stress granules at all[123,124]. In this context, the increasing free-RNA concentration that results from the phosphorylation of eIF2α triggers the opening of the G3BP1 structure, by displacing intramolecular interactions, which exposes its intrinsically disordered domains and thus initiates phase separation[123].

Many stress granule components, such as hnRNPA1, TAR DNA binding protein 43 kDa (TDP-43), Fused in Sarcoma (FUS), and T-cell intracellular antigen (TIA-1) mainly localize in the nucleus under normal conditions[125], but accumulate to the cytoplasm upon stress[126–128]. Interestingly, these proteins also contain Poly(ADP)-Ribose (PAR) binding domains. A recent study suggested that stress-induced PAR chains synthesized in the nucleus are exported to the cytoplasm. This would provide a scaffold for the recruitment of nuclear proteins to stress granules[129]. Additionally, several studies showed that the liquid-liquid phase separation of stress granule proteins is promoted by the presence of PAR[130–133], suggesting that PAR might not only facilitate proper localization of protein to these structures, but also play a central role in their formation.

As many studies report a change in mRNA modification after stress[134–137], it is not surprising that recent research suggests a role for RNA modifications stress granule formation and regulation. For instance, m6A-containing mRNAs tend to accumulate in stress granules,

and this modification seems important for proper recovery of translation[138]. On the other hand, m6A also seems to repel G3BP1[139], and thus the accumulation in stress granules of m6A-modified mRNAs is probably dependent on the YTHDF1/3 reader proteins. Moreover, the recruitment of these mRNAs to stress granules mildly facilitates stress granules assembly[134,140,141]. These observations provide interesting insight in the mechanism by which mRNA modification might regulate mRNA localization to stress granules and, in turn, stress granule assembly altogether.

1.4.2.1 Stress granules are hubs for mRNA quality control and triage

The investigation of the functional implications of stress granule formation is following a similar flow to the study of splicing speckle functions. Initially, the observation that impeding stress granule formation resulted in hypersensitivity to stress[142,143] and reduced mRNA stability[144] brought the scientific community to think that these condensates were formed to sequester and protect the mRNA during proteotoxic stress, thus preventing further translation from generating misfolded proteins[94].

These considerations are still valid. Storage and protection of mRNA are probably part of the function of stress granules. However, in recent years, it has become evident that these structures are more dynamic than initially thought. For instance, inclusion within stress granules is gene-specific and some stress response mRNAs, such as heat shock protein (HSP) 70 and 90, evade condensation and are thus translated more during stress[145–147]. Moreover, translational shutdown after stress does not require the formation of stress granules[145] and some stress response genes are actively translated within these structures[148], suggesting that stress granules actively promote the translation of stress-response genes over housekeeping ones. Additionally, both processing bodies and stress granules move within the cell in a cytoskeleton dependent manner[94] and may thus regulate localized translation of the mRNAs they contain, a process of known importance for many cellular functions[149].

It is becoming apparent that many mRNAs do not spend a lot of time in stress granules, but rather continuously shuttle between stress granules and processing bodies when the two structures come into contact[120]. As translocation to processing bodies is often associated with decay[150] and seem to depend on the interaction of the mRNA with specific RNA-binding proteins housed in the stress granules[151], it seems that these structures are functioning as a triage center for mRNAs, where the mRNA is bound by specific proteins to either be protected or directed to decay. Supporting this model, the inclusion of specific mRNAs in stress granules seems important for their ability to return to active translation after the stress is removed[138].

2 Research aims

This thesis focuses on the role of non-coding RNAs and RNA modification in regulating various fundamental cellular processes.

Paper I focuses on a novel, charge-dependent, role of RNA in maintaining an open chromatin structure.

Paper II investigates the role of dyskerin, an RNA-guided RNA pseudouridine synthase, in modifying mRNAs and regulating their translational level.

Lastly, **Paper III** proposes a potential role of mRNA pseudouridylation in regulating stress granule formation and, therefore, stress responses.

3 Methodological Considerations

3.1 Measuring the pseudouridine content of mRNAs

Studying the pseudouridine content of mRNAs is particularly challenging due to its low abundance. During the years, several methods have been developed to try and circumvent this issue[74].

The most sensitive method is **liquid chromatography with tandem mass spectrometry** (LC-MS/MS). Due to the precision nature of the instruments used, this method allows for detection of very small amounts of the modification and is completely independent from antibodies, that could generate non-specific signal. Therefore, mass spectrometry probably offers the strongest evidence of a change in the modification content of any RNA preparation[74]. However, given the much higher representation of the modification in other RNA species such as tRNAs and rRNAs, small contaminations in the sample can skew the results, generating biases that are hard to correct for: while the amount of rRNA and tRNA specific modifications, such as m6.6A and m2.2G respectively, can be quantified as well to account for potential contamination, there are no generally accepted guidelines to determine whether a sample is contaminated or not. To minimize the potential contamination, we improved our mRNA purification in two ways: we added a silica-based affinity purification step that removes small RNAs (below 200 base pairs) such as tRNAs; and then we performed 3 rounds of Poly(A)+ selection using Oligo(dT) magnetic beads. These steps lead to samples that were considered pure by the experts at the PROMEC facility (Norwegian University of Science and Technology) that performed the analysis.

Nonetheless, LC-MS/MS does not offer any insight on what mRNAs are differentially modified after a treatment, nor on which sites in the mRNA are modified. To gain insight on these aspects, next generation sequencing can come to aid. **Pseudo-seq**[136] was developed almost ten years ago by the laboratory of Wendy G. Gilbert at the Massachusetts Insitute of Technology (MIT), and uses carbodiimide N-cyclohexyl-N′-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMC) to chemically modify the N3 position of pseudouridine, forming adducts that are resistant to alkaline cleavage and are bulky enough to stop reverse transcription. This method provides information about modification of RNAs with nucleotide resolution. However, the multi-step nature of the process requires a strict statistical analysis to avoid false-positive calling. Therefore, while it is very good at detecting very frequent modifications, such as the ones performed by canonical PUS enzymes, it is less reliable in identifying sporadic modifications.

More recently, **BID-Seq** (Bisulfite-induced deletion sequencing)[152] emerged as an alternative to Pseudo-seq. This method also exploits chemical modification of pseudouridine and promises quantitative and transcriptome wide mapping of the

modification. However, the technique is unable to identify some well-known modification sites in rRNAs due to either low representation or closeness to other modification sites.

Given their limitations, we deemed a sequencing approach risky for our experimental setup: while dyskerin seemed responsible for a relative large proportion of pseudouridine in mRNA (this modification was reduced of about 22% in mRNA after siDyskerin measured using LC-MS/MS) the protein also seemed to bind diffusely over the mRNA molecules in our iCLIP data, indicating that it might not modify only specific sites. We therefore turned to RNA-immunoprecipitation, using an antibody against pseudouridine, followed by qPCR. This allowed for detection of pseudouridine in specific transcripts, including some of the mRNAs strongly bound by dyskerin. While this approach does not offer any insight on the localization of pseudouridine within the sequence, it increases the resolution of our assay, when compared to LC-MS/MS. To minimize biases introduced using an antibody, we first tested its specificity using *in vitro* transcribed RNAs, and showed that the antibody binds much more to pseudouridine-containing RNAs than to RNAs that do not contain any pseudouridine. Moreover, we performed at least part of our analysis on purified mRNAs, to avoid the potential bias due to binding to proteins and other RNA species.

3.2 Measuring translation

Similarly to the measurement of pseudouridine, the quantification of the translation rates of mRNAs can be approached by several techniques[153]. This includes assessment of the oligopeptides produced by ribosomes (protein perspective) or the association of mRNAs with ribosomes (mRNA perspective). For our investigations, we decided to look at translation by both sides, to obtain not only information about the general rates of translation, but also about which mRNAs are involved in these changes. We decided to focus our analysis on ongoing translation, rather than the steady-state level of proteins within cells, given that pseudouridine is known to affect translation directly, and not much is known about changes in levels of specific protein products.

When looking at ongoing translation, the general approach is to pulse-label nascent proteins via radioactive amino acids, non-canonical amino acids, or specific chemicals, and then quantifying the labelled products via mass spectrometry or western blotting. One such nascent protein label is puromycin, a molecule that resembles a tyrosyl-tRNA conjugate and is therefore added by the ribosome to the nascent polypeptide in a non-specific manner[154]. Once puromycin is added to the polypeptide, it can be detected using an antibody, making it especially suitable for analysis via western blot. Puromycin-labelling was particularly easy for us to set up as we already had this molecules in the lab, since it also is an antibiotic used for selection of transgenic cell lines. Specific biotinylated derivatives of puromycin are available and can be used to isolate nascent proteins for analysis via mass spectrometry. However, RNA being the focus of our work, we deemed further analysis from this point of view out of our scopes.

On the other hand, methods that focus on quantifying the association of specific mRNAs with ribosomes focus on the isolation of ribosomes from cell lysates and then analysis of the associated RNA fragments. The most widely used approach in this sense, polysome profiling, uses sedimentation in a sucrose gradient to isolate polysomes, and then assesses the mRNA presence in this fraction by either UV spectroscopy, next generation sequencing, or qPCR. As a complement to the puromycin assay and to gain further insight into mRNA translation changes after knockdown of dyskerin, we employed ribosomal profiling to assess both the general levels of translation in cells depleted of dyskerin, and whether hypo-pseudouridylated mRNAs were translated differently. We first analyzed the total RNA content of the polysomal fraction via UV spectroscopy, and thereafter isolated RNA from this fraction to assess the level of translation of specific mRNA targets of dyskerin via qPCR. This was particularly insightful, as it showed that the effects on translation of pseudouridine are transcript specific, rather than general, as not all the hypo-pseudouridylated mRNAs we analyzed showed altered levels of translation.

3.3 High-content imaging and image analysis

Many of the results included in **papers I and III** were obtained through microscopy. Although a powerful instrument, microscopic analysis is subject to severe potential biases, especially when the changes observed are not extreme. While in **paper I** the changes observed were drastic to the point of not requiring quantification, the results presented in **paper III** are not of the same "black and white" nature and required a more careful approach.

One of the main biases that microscopy analysis encounters is under-representation: it is often not feasible to image the whole slide, especially when employing a confocal microscope, due to time. Therefore, we incur the risk of representing only a subset of the cells, rather than the entire population. Moreover, although robust, the staining protocol is also subject to variation, which is particularly important to consider when evaluating signal intensity as a proxy for the localization of specific molecules in certain regions of the cell. Lastly, the analysis of the images is generally performed via software, and not manually. Although the pipeline of the analysis is designed with care, it will always contain a certain degree of error, that needs to be considered.

To circumvent these issues, it is important to ensure that an appropriate number of pictures are taken from each slide, and that enough repeats are performed to ensure that the downstream statistical analysis is robust to the confounding effect of potential biases. Ideally, involving other scientists in the process should be recommended, although not always possible. When the results are reproducible across different people, their strength increases. Lastly, as with any other experiment, the single approach is not enough to prove a hypothesis true and examining the scientific question via different techniques is pivotal to ensure reproducibility.

3.4 Ethical considerations

In **paper II** we utilized fibroblast and lymphoblast cells derived from patients with dyskeratosis congenita. The primary fibroblasts (GM01774/L37del, AG04646/A386T, and GM01786) are commercially available and were purchased through the Coriell Institute Cell Repository. The only information known about the patients included genotype and parental relationship to other donors (GM01786 is the mother of GM01774). Similarly, the Immortalized Lymphoblast cell lines were provided by S.A. Savage (NIH) in a deidentified manner, under NIH IRB-approved protocol, ClinicalTrials.gov, Identifier NCT00027274, as described in Niewisch et al.[155]. The data generated from the use of these cells had no role in determining patient management, nor disclosed any information about the subjects that would allow their identification.

All the experiments in paper II, as well as in **paper I** and **paper III** were performed on commercially available cell lines.

4 Results and discussion

4.1 Paper I

Neutralization of the positive charges on histone tails by RNA promotes an open chromatin structure

This first publication investigates a previously undescribed role of RNA in regulating chromatin structure through its charge. Upon treatment with RNases that specifically degrade single-stranded RNA (RNase A, RNase T1, or RNase I), DNA in cells becomes resistant to DNase I treatment and is condensed. Depletion of double-stranded RNA (via RNase III) or RNA-DNA Hybrids (via RNase H) did not show the same "DNase-protecting" effect.

Compacted, DNase-resistant, chromatin structures formed by removal of single-stranded RNA localized around nucleoli and in the nuclear periphery, known sites of heterochromatin, indicating that loss of single-stranded RNA converts pre-existing heterochromatin into a hyper-compacted state and/or that newly formed compacted chromatin moves to these sites. Interestingly, sensitivity to DNase I could be restored by the re-introduction of physiologically relevant levels of RNA as well as of bacterial tRNA, arguing against a sequence-specific involvement of the molecule.
Moreover, through the permeabilization required for the RNase treatment, the soluble components of the cell are removed, therefore suggesting that this process is passive, and does not require chromatin remodeling enzymes.

In an effort to identify the factors involved in this process, we turned to cell lysates. We found that removal of single-stranded RNA causes the precipitation of several proteins, including all the core histones. Histone solubility was, similarly to the effects on chromatin, restored by the addition of RNA from various sources, in a concentration-dependent manner, as well as by using other negatively charged polymers - like poly-L-glutamic acid (PGA), heparin, and long stretches (>70 nucleotides) of single-stranded DNA. High concentrations of salt (NaCl), but not 1-6-hexanediol, also restored histone solubility - suggesting that histone precipitation depends on electrostatic interactions. Indeed, removing the highly-charged the N-terminal tail of a YFP-tagged H2B variant, prevented its precipitation.

Moreover, via an *in vitro* histone transfer assay, we demonstrated that RNA *per se* facilitates the formation of nucleosomes in a concentration-dependent fashion, thus modulating histones directly. In this context, the effect of RNA on the formation of nucleosomes was determined by the ionic strength of the solution: at physiological salt concentration, RNA stimulated nucleosome assembly, while at lower concentrations seemed to have an inhibitory effect. Nonetheless, at low ionic strengths electrostatic

interactions are enhanced, and histones bind to RNA more than to DNA. It is therefore possible that at these conditions histones and RNA interact excessively and nucleosomes cannot form[156,157].

Taken together, our observations suggest that the negative charge of single-stranded RNA neutralizes the positive charge on histone tails, modulating their interaction with other histones and with DNA. This effect seems to depend on direct interaction between histones and RNA, as it is also evident solely in the presence of these specific molecules. Indeed, charge is an important determinant of histone solubility: the repulsion between the charged histone tails counterbalances the hydrophobic attraction between the histone fold domains. Consequently, dampening of the positive charge on the histone tails facilitates correct folding of histones[158]. Moreover, reducing the positive charge of histone tails is known to favor an open chromatin structure, as demonstrated by the effects of the acetylation of lysines [43]. Conversely, transient rises in the concentration of positive ions, such as Mg2+, seem to favor chromatin condensation [44].

The biological significance of this phenomenon is yet to be determined. One possibility is that high levels of RNA help maintain an open chromatin structure during transcription. On the other hand, inhibiting transcription does not generate any resistance to DNase I cleavage. This suggests that long-lived RNA stably associated with chromatin, rather than newly transcribed RNAs, are responsible for the effects that we observed.

We explored the hypothesis that LINE-1 repeats, which are known to stably associate with chromatin[159], could be responsible for this phenomenon. Indeed, we could recover 2-3% of cellular LINE1 using native RIP of YFP-tagged H2B, while other RNAs were pulled-down to a lesser extent. Considering that transcripts that contain LINE-1 repeats are resistant to TRIzol extraction[159], this interaction is probably more extensive than appreciated via this technique.

However, we still do not know whether other RNA species have a similar effect. This appears likely, given the sequence independence of the effect. To restore chromatin sensitivity to DNaseI DNA oligomers needed to be at least 70 nucleotides long, suggesting at least a length is a requirement for this effect. In addition, transcripts from intronic repeats are heavily modified. This modification regulates their interaction with many RNA-binding proteins, including the nuclear matrix protein SAFB[160]. This suggests that RNA modification is also contributing to the regulation of chromatin structure. Nonetheless, investigating what properties of an RNA molecule are important for this function is an exciting research frontier.

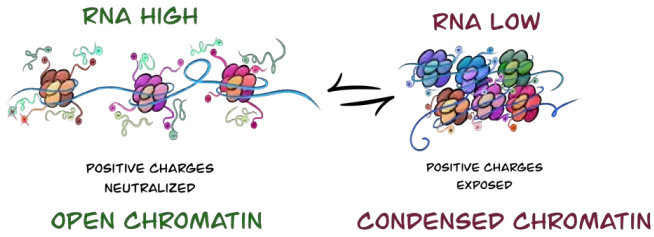

Figure 8 – Neutralization of the positive charges on histone tails promotes an open chromatin structure.
Representation of the model build on the findings of paper I. When RNA concentration is high, its negative charges shield the positive ones on the histone tails, favoring histone solubility and an open chromatin environment. Conversely, when RNA is removed, or its concentration is low (right), the positive charges on the histone tails are exposed, which reduces histone solubility and results in closely compacted chromatin.

4.2 Paper II

Control of protein synthesis through mRNA pseudouridylation by dyskerin

In this second publication, we investigated the role of the H/ACA complex and its enzymatic component dyskerin in the pseudouridylation of mRNAs in human cells, and the effects of this process on translation. Our results show that dyskerin likely modifies mRNAs during their transcription, and that its removal results in increased translation rates. This effect depends on the catalytical activity of dyskerin, and seemingly not on defects of other known targets of dyskerin, such as rRNAs. Altogether, our findings suggest a model in which dyskerin pseudouridylates mRNAs co-transcriptionally to control their translation and therefore protein expression.

An indication of the involvement of dyskerin in mRNA modification came from the initial observation that the H/ACA complex localizes to splicing speckles known to regulate processing of messenger molecules[161]. In line with this, we found that dyskerin binds to thousands of mRNAs, and that silencing of dyskerin globally reduces mRNA pseudouridylation. Moreover, dyskerin interacts with RNA-polymerase II in a manner that is partially dependent on RNA and travels together with the polymerase along transcribed genes throughout the genome, suggesting co-transcriptional modification.

Triptolide and RNase A treatments, which reduce the association of dyskerin with genes and RNA polymerase respectively, increase – rather than reduce – the amount of dyskerin in splicing speckles. Therefore, the localization of the H/ACA complex in these condensates is not essential for the modification of mRNAs.

Nonetheless, we cannot exclude that inclusion of dyskerin in splicing speckles enhances the efficiency of the modification: splicing can happen in the nucleoplasm, but highly transcribed genes localize around splicing speckles[101,103], and although it is yet unclear whether high levels of transcription are cause or consequence of this localization, it has been suggested that proximity to splicing speckles might increase transcription due to faster processing of the nascent mRNA[93]. Similarly, a reservoir of dyskerin in splicing speckles might increase its availability for interaction with RNA polymerase II, thus ensuring efficient pseudouridylation. As the determinants of the H/ACA complex localization to splicing speckles are yet to be described, we could not investigate this possibility.

The interaction of dyskerin with RNA polymerase II suggests that dyskerin might be involved in the transcription process. However, we did not observe major changes in the relative levels of mRNA with an RNA-sequencing approach. Although the levels might be altered across every mRNA with no change in the relative levels, measuring transcriptional speed via ethyl-uridine pulses did not show clear changes in either direction (data not shown), suggesting that dyskerin is not essential for the transcription process.

Dyskerin preferentially binds to the coding sequence of spliced mRNAs, suggesting that mRNAs are modified after splicing is completed and that the modification could have an effect on translation. In accordance, pseudouridine has known effects on translation: as described above, it increases translation initiation by reducing PKR activation and reduces translational elongation by preventing proper positioning of the tRNA in the peptidyl transferase center of the ribosome[61].

In cells lacking dyskerin, we did not observe an increase in the phosphorylation of eIF2α, a downstream effect of PKR activation. Instead, we observed an increase in translation efficiency after loss of dyskerin, as measured by puromycin incorporation and polysomal profiling. Enhanced translation was observed both in human cells, and in a cell-free translation system[79]. The latter, which included translation of a synthetic GFP mRNA and where replacement of uridine into pseudouridine lead to higher GFP protein levels suggests that mRNA pseudouridylation is responsible for this effect. However, as dyskerin extensively modifies rRNAs too, the possibility that the altered translation depends on ribosomal defects remains.

Most of our experiments, however, were performed after 48h of dyskerin knockdown. This is shorter than the average half-life of a ribosome[162], and we accordingly did not observe significant defects in the processing of rRNA at this time point. However, longer depletion of dyskerin – which results in very clear rRNA processing defects – resulted in a reduction of translation rates. Therefore, we think that at 48h the ribosomal defects caused by the lack of dyskerin are not yet prevalent, thus allowing for the mRNA effects to become evident.
This is further supported by the measurement of translation in cells from patients who carry mutation in *DKC1*, the gene that encodes dyskerin: although mRNA pseudouridylation is defective in these cells, dyskeratosis congenita is characterized by ribosomal defects[163]. Accordingly, in fibroblast and lymphoblast from dyskeratosis congenita patients translational efficiency is decreased.

The complementarity between the H/ACA guide RNA and the target RNA regulates the efficiency of pseudouridylation[164] and, canonically, the substrates of dyskerin have been identified by perfect complementarity with a known sno- or scaRNA. Surprisingly, we found that dyskerin can also efficiently modify exogenous mRNAs, for which we do not expect a conserved guide RNA to be present in the cell, and moreover, the effect of dyskerin on these exogenous mRNAs alter their translation. This suggests that perfect complementarity is not required for pseudouridylation by dyskerin, as also suggested by others[164–166]. The ability of dyskerin to modify exogenous mRNAs opens interesting possibilities regarding its function: while modification of viral RNA has been shown to help evasion from the innate immune system during the early stages of infection[167], later on reducing the rate of translation of viral transcripts via pseudouridylation might help slow down the infection.

Biochemical studies have shown that pseudouridine can directly slow the progression of the ribosome through the codons that contain it[61]. However, the amount of pseudouridine in mRNAs is too low to justify the drastic effects we observed on translation after depletion of dyskerin: our and others quantification of mRNA pseudouridine suggests that only 0.1–0.2% of uridines in mRNA are modified[152,168]. This means that additional mechanisms likely also contribute to the effects of pseudouridine on translational. Pseudouridine seems to be enriched around RNA-binding protein binding sites[76] suggesting that reader proteins that are yet to be discovered are involved. The identification of these readers represents one of the major challenges in the field: the snoRNA dependency of the phenomenon makes it hard to identify a specific consensus sequence for pseudouridylation, as different snoRNAs have different target sequences[54]. Moreover, our conclusion that the modification of mRNAs does not require perfect complementarity with the guide RNA of dyskerin implies that the pseudouridylation sites might vary even within the single species of mRNA, making the computational identification of reader proteins particularly challenging.

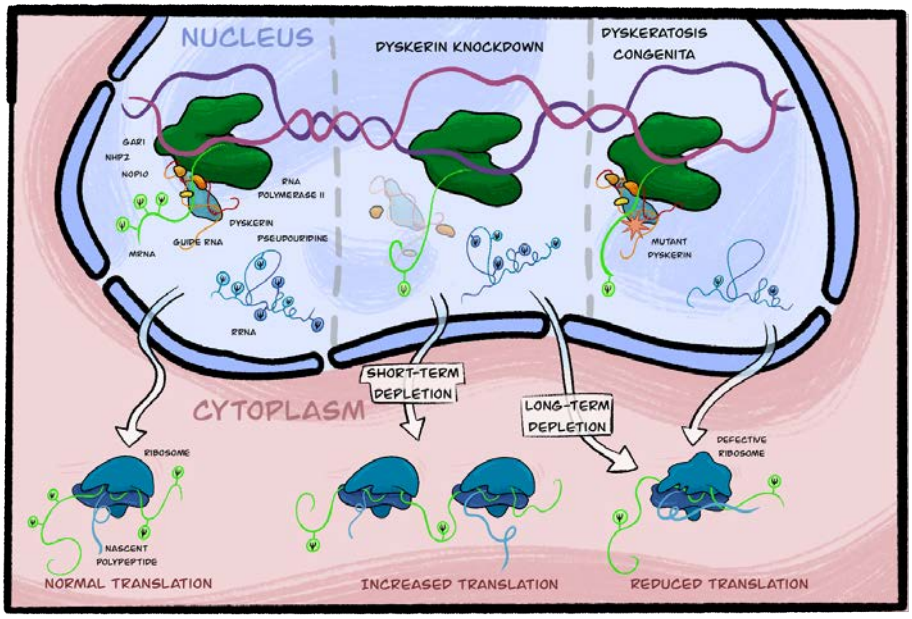

Figure 9 – mRNA pseudouridylation by dyskerin controls protein translation.
The model built on the findings included in paper II. In normal conditions, dyskerin modifies mRNAs at the site of transcription and translation proceeds as normal. However, when dyskerin is removed, the lack of mRNA pseudouridylation results in increased translation rates, which confirm the negative effect on translation that others have also described. This effect is only evident as long as the depletion of dyskerin is of relative short duration (48h). Long-term depletions with a prolonged siRNA treatment (96h) or mutations in *DKC1* (the gene that codifies for dyskerin) result instead in reduced translation as ribosomal defects arise.

4.3 Paper III

Pseudouridine synthases regulate mRNA localization to stress granules and translation re-initiation after stress

In this third study, we investigated the pseudouridylation of mRNA upon heat shock in human cells and its impact on stress granule formation. We confirmed that mRNA is pseudouridylated in response to heat shock via both LC-MS/MS and RNA-immunoprecipitation with a pseudouridine antibody. We cannot say, at this point, whether the increased pseudouridylation of mRNAs we observed in response to heat shock happens during transcription – as it seems to be the norm for mRNAs[80,169] – or whether it involves already mature, cytoplasmatic mRNAs. The short duration of the heat shock would suggest the latter but considering the relatively short half-life of mRNA[170], we cannot exclude the former. Furthermore, we have not yet confirmed that pseudouridine synthases explored in this study are responsible for this heat shock-induced pseudouridylation. We suspect their involvement given that their knockdown reduces the pseudouridine signal in stress granules, but further experiments involving mRNA pseudouridylation measurement after heat shock in combination with knockdown of these enzymes are in the works to confirm this.

Removal of selected mRNA pseudouridine synthases leads to the formation of less and bigger stress granules. We show that these effects likely do not depend on a structural role of these proteins within stress granules, as two of have not been detected within these aggregates, and prevention this location of the one that does locate there does not replicate the effects of its knockdown.

After initial seeding, stress granules fuse into bigger ones with denser cores[151]. In cells depleted of one of the PUS of interest in this study, we observed alterations in stress granules as early as 10 minutes into the heat shock, and these alterations did not resolve over time, but rather they worsened. Given these observations, we are tempted to suggest that in mRNA pseudouridine synthases-deficient cells, the seeding step of stress granules formation is impaired. However, we understand that the insight offered by our preliminary experimental setup is somewhat limited, and we cannot exclude that the disassembly or the dynamics of the stress granules in general, such as fusion and fission processes, are also affected. To address this, a live imaging approach would be more appropriate and is an interesting future perspective for this project.

Our observations thus far raise the intriguing possibility that the altered stress granules are a result of the reduced pseudouridylation state of the RNAs they contain. Knockdown of the mRNA pseudouridine synthases led to mRNA accumulation within stress granules and, in turn, in a reduction in translation during the recovery from heat shock. This elevation does not appear to be caused by an elevation of total mRNA, but could involve changes in the amounts of specific mRNA species recruited there. Investigating this,

possibly via next-generation sequencing, might provide insight on the mechanism by which pseudouridylation regulates mRNA recruitment to these condensates. Given the small percentage of uridines in mRNAs that are pseudouridylated[171] we are prone to think that the effect is not direct, but rather requires a reader protein within the condensates, in a mechanism like the one described for m6A and the YDTHDF proteins[140]. In this context, TIA1 and TIAR, which interact with uridine-rich sequences[172], become intriguing candidates, and their role is worth exploring further. Indeed, both proteins seem to control translation[173–175], a function that we and others have also related to the mRNA pseudouridine content[80,176].

We are still unsure about the causal relation in between the mRNA accumulation in stress granules and the reduction in translation rates at the end of the proteotoxic stress, but we are tempted to speculate that mRNA might not be efficiently released from the binding to the RNA-binding proteins within stress granules, either due to delayed disassembly or other factors, leading to reduced translation efficiency. However, we cannot directly exclude that ribosomal defects are driving the impairment in protein translation that we observe. Although rRNA processing defects are not observed at these early time points of enzyme depletion[80], the combined stress of PUS depletion and heat shock could exacerbate a modest defect in rRNA processing. For instance, various stress conditions increase internal ribosomal entry site (IRES) dependent translation[177], a process that seems to be regulated that rRNA pseudouridylation[178]. Therefore, further investigation in the ribosomal health after heat shock will be required to address this possibility.

Despite these considerations, the enhanced localization of mRNAs to stress granules suggests a direct involvement of mRNA modification in the formation and function of these structures. Considering the limited duration of the siRNA treatment we use here, which is shorter than the average lifespan of an rRNA molecule[162], it is unlikely that the effects observed reflect changes at the ribosomal level. Therefore, we propose a model in which pseudouridylation of mRNAs regulates their binding to different RNA binding proteins, thereby influencing translation and localization in phase-separated cell compartment.

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