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The role of important non-coding RNAs and related protein components in translation and transcription

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Doctor of Philosophy
by*

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Μελέτη του ρόλου σημαντικών μη κωδικών μορίων RNA και σχετιζόμενων πρωτεϊνών στη μεταγραφή και τη μετάφραση

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Abstract

Abstract

In less than a decade since the sequencing of the human genome, it has become clear that over 85% of the genome encodes RNA transcripts that do not translate into proteins. These transcripts are also known as non-coding RNAs (ncRNAs) and were previously divided into housekeeping and regulatory ncRNAs. Among the most prominent housekeeping examples of ncRNAs that we now know that exhibit essential regulatory roles are the transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), RNase P RNA, and telomerase RNA (Zhang et al., 2019). In addition, the ncRNA transcriptome has been recognized as a major regulome that includes microRNAs (miRNAs), long non-coding RNAs (lncRNAs), Piwi RNAs (piRNAs), tRNA-derived fragments (tRFs), vault RNAs etc., and controls crucial metabolic processes in the cell (Morris and Mattick, 2014; Schimmel, 2018; Lorenzi et al., 2021). Moreover, they have emerged as important biomarkers of homeostasis at the epigenetic, transcriptional, post-transcriptional and translational level (Schimmel, 2018; Slack and Chinnaiyan, 2019). Upon transcription, all ncRNAs undergo numerous maturation steps which are further mediated by important enzymes, ribonucleoprotein complexes or scaffold proteins of known or putative function, generally known as RNA binding proteins (RBPs). In many cases, the interactions between protein factors and ncRNAs can determine not only the function and the fate of an RNA molecule, but also can affect transcription and translation rates, overall. Elucidation of the role of ncRNAs-interacting proteins is critical to understand the hardcore biology and molecular functions of ncRNAs, to dissect the contribution of proteins in additional molecular functions and also to reveal the aspects of protein and ncRNAs in several human diseases and malignancies.

Main objective of the present thesis was the study of the role of important RNA-binding protein subunits which participate in biogenesis of the tRNA molecules but could also additionally carry out alternative tasks in non-canonical functions. In the first part of the thesis, the role of La protein overexpression in cancer was examined as a global gatekeeper and abundance regulator of Pol III ncRNAs. La overexpression in lung adenocarcinoma cells lead to a significant increase in cell proliferation and motility. Next Generation sequencing (NGS) analysis showed the

participation of La in translation and RNA metabolism. La accumulation affected the expression of mTORC2 complex and important tRNA maturation and translation initiation factors. In addition, upregulation of the tumorigenic tRF-1001 and its parent tRNA^{Ser}_{UGA} was also observed. Although the conserved LAM-RRM1 module is responsible for the RNA binding capacity of La, biochemical analysis showed that LAM motif alone can bind both oligo(U) and a pre-tRNA ligands. The biochemical, supported also by existing NMR-derived structural data, supports the La involvement in binding of diverse RNAs, that could account for a modulatory role of La in carcinogenesis.

In the second part of the thesis, the CRISPR/Cas9 genome editing tool was used to knock-out each individual protein subunit of RNase P holoenzyme in HeLa cancer cell line. Given the current knowledge on moonlighting roles of the 10 RBPs that form the RNase P holoenzyme in human cells, the screening analysis showed that POP1 and RPP30 protein subunits are essential for cell viability while RPP25 is dispensable. The RPP25 deficient cells showed an increased proliferation and migration rate. In a next step, Next Generation sequencing (NGS) analysis showed that ablation of RPP25 regulates genes with significant role in cancer and steroid metabolism but also it is observed a deregulation of expression levels of long-intergenic RNAs and other non-coding RNAs that participate in cancer. Moreover, small RNA-seq showed that specific miRNAs, tRNAs and tRFs are deregulated enhancing the cancer progression. Subsequent, global analysis of translation rates confirmed the elevated translational levels. Finally, the edited cell lines were used for in vivo characterization of RPP25-GFP tagged localization using fluorescence microscopy to reveal possible alternative localizations of RPP25 that could imply alternative functions in RNA-mediated phase separation.

Taken together, the current thesis attempted to provide a comprehensive picture of assembly, coordination, and heterogeneity of prominent RNA-binding proteins like La and RPP25, under various conditions and to identify pathways that regulate gene expression at the transcription and translation level, that could be further exploited for therapy.

Περίληψη

Σε λιγότερο από μια δεκαετία από την αλληλούχιση του ανθρώπινου γονιδιώματος, κατέστη σαφές ότι περίπου το 85% του γονιδιώματος κωδικοποιεί RNA μόρια τα οποία δεν μεταφράζονται σε πρωτεΐνες. Τα μεταγγραφα αυτά είναι γνωστά ως μη-κωδικά μόρια RNA (ncRNAs) και αντιπροσωπεύουν ένα δυναμικό μεταγράψωμα το οποίο περιλαμβάνει RNA που διατηρούν τη κυτταρική λειτουργία (housekeeping) όπως το μεταφορικό RNA (tRNA), το ριβοσωμικό RNA (rRNA), τα μικρά πυρηνικά RNAs (snRNAs), τα μικρά πυρηνικά RNAs (snoRNAs), το RNA της ριβονουκλεάσης P και το RNA της τελομεράσης (Zhang et al., 2019). Επιπλέον, το μεταγράψωμα αυτό περιλαμβάνει και ρυθμιστικά μόρια όπως τα microRNAs (miRNAs), τα μεγάλου μήκους μη κωδικά RNAs (long ncRNAs), τα PiWi RNAs (piRNAs), τα νέα θραύσματα των tRNAs (tRNA- derived RNA fragments; tRFs), τα vault RNAs και άλλα ncRNAs τα οποία συμμετέχουν και ελέγχουν διάφορες μεταβολικές διεργασίες στα κύτταρα (Morris and Mattick, 2014- Schimmel, 2018- Lorenzi et al., 2021). Όλα αυτά τα μη-κωδικά μόρια RNA έχουν αναδειχθεί ως σημαντικοί βιοδείκτες σε επιγενετικό, μεταγραφικό, μετα-μεταγραφικό και μεταφραστικό επίπεδο (Schimmel, 2018; Slack and Chinnaiyan, 2019). Ωστόσο, κατά τη διάρκεια της μεταγραφής τους υφίστανται διάφορα στάδια ωρίμανσης, τα οποία διαμεσολαβούνται από σημαντικά ένζυμα, ριβονουκλεοπρωτεϊνικά σύμπλοκα ή ακόμα και από πρωτεΐνες-ικριώμα (scaffold proteins) γνωστές ως RNA-binding proteins (RBPs). Οι αλληλεπιδράσεις μεταξύ μορίων RNA και πρωτεϊνών καθορίζουν όχι μόνο τη λειτουργία και τη τύχη του RNA αλλά και τους ρυθμούς της μεταγραφής και μετάφρασης του κυττάρου. Η διερεύνηση του ρόλου αυτών των αλληλεπιδράσεων είναι ζωτικής σημασίας για την κατανόηση των μοριακών μηχανισμών που διέπουν τόσο τα ncRNAs όσο και τις αντίστοιχες πρωτεΐνες, καθώς επίσης για την αποσαφήνιση του πιθανού ρόλου τους στην εμφάνιση διαφόρων ασθενειών και νεοπλασιών.

Σκοπός της παρούσας διατριβής ήταν η μελέτη του ρόλου σημαντικών πρωτεϊνών που δεσμεύουν RNA μόρια και συμμετέχουν τόσο στη βιογένεση των tRNA μορίων, αλλά και σε επιπρόσθετες σημαντικές κυτταρικές λειτουργίες. Στο πρώτο μέρος της διατριβής μελετήθηκε ο ρόλος της υπερέκφρασης της πρωτεΐνης La (Lupus antigen) στον καρκίνο. Η υπερέκφραση της La πραγματοποιήθηκε σε κύτταρα αδενοκαρκινώματος πνεύμονα και οδήγησε σε σημαντική

αύξηση του πολλαπλασιασμού και της μετανάστευσης των κυττάρων ενώ πειράματα αλληλουχισής νέας γενιάς (NGS) έδειξαν τη συμμετοχή της La στη μετάφραση και στο μεταβολισμό του RNA. Επιπρόσθετα, η συσσώρευση της La επηρέασε την έκφραση του συμπλόκου mTORC2 καθώς και σημαντικών παραγόντων που συμμετέχουν στην ωρίμανση του tRNA αλλά και στην έναρξη της πρωτεϊνοσύνθεσης. Επιπλέον, παρατηρήθηκε αύξηση της έκφρασης του tRF-1001 και του tRNASerUGA. Τέλος, μελετήθηκε η ικανότητα δέσμευσης του μοτίβου LAM, το οποίο έχει βρεθεί ότι παρουσία του RRM1 (LAM-RRM1) είναι υπεύθυνο για τη δέσμευση του RNA. Η βιοχημική μελέτη έδειξε ότι το μοτίβο LAM μόνο του, μπορεί να δεσμεύσει τόσο oligo(U) RNA όσο και πρόδρομα μετάγραφα tRNAs κάτι που επιβεβαιώνεται και από τα υπάρχοντα δομικά δεδομένα (NMR). Η ιδιότητα αυτή του μοτίβου LAM θα μπορούσε να εξηγήσει τη συμμετοχή της La στη δέσμευση μιας ποικιλίας RNAs και το ρυθμιστικό ρόλο της στην καρκινογένεση.

Στο δεύτερο μέρος της παρούσας διατριβής, χρησιμοποιήθηκε το εργαλείο γονιδιωματικής επεξεργασίας CRISPR/Cas9 για την απαλοιφή κάθε επιμέρους πρωτεϊνικής υπομονάδας του ολοενζύμου RNase P στη καρκινική κυτταρική σειρά HeLa. Τα αποτελέσματα έδειξαν ότι οι πρωτεϊνικές υπομονάδες POP1 και RPP30 είναι απαραίτητες για τη βιωσιμότητα των κυττάρων, σε αντίθεση με τη πρωτεϊνική υπομονάδα RPP25. Τα κύτταρα που δεν παρήγαγαν τη πρωτεΐνη RPP25 έδειξαν αυξημένο ρυθμό πολλαπλασιασμού και της μετανάστευσης τους. Πειράματα αλληλουχισής νέας γενιάς (NGS) στο μεταγράφων των κυττάρων έδειξαν ότι η RPP25 συμμετέχει στη ρύθμιση της έκφρασης γονιδίων που σχετίζονται με το μεταβολισμό των στεροειδών, ενώ επίσης παρατηρήθηκε απορρύθμιση των επιπέδων έκφρασης διαφόρων μη κωδικών RNAs που συμμετέχουν στον καρκίνο, συμπεριλαμβανομένων και των long-intergenic RNAs. Επιπλέον, αλληλουχισή νέας γενιάς των μικρών RNAs έδειξε ότι συγκεκριμένα miRNAs, tRNAs και tRFs απορρυθμίστηκαν ενισχύοντας τα παραπάνω αποτελέσματα για τη συμμετοχή της RPP25 στον καρκίνο. Στη συνέχεια, απουσία της RPP25 παρατηρήθηκαν αυξημένα επίπεδα μετάφρασης. Τέλος, η ανασυνδυασμένη RPP25 σε σύντηξη με την πράσινη φθορίζουσα πρωτεΐνη (GFP) εκφράστηκε στην επεξεργασμένη κυτταρική σειρά για μελέτη του υποκυττάριου εντοπισμού της και τη διερεύνηση της πιθανής συμμετοχής της στο διαχωρισμό φάσεων με τη μεσολάβηση RNA (RNA-phase separation).

Συνοψίζοντας, στόχος της παρούσας διατριβής ήταν να αναδείξει μια ολοκληρωμένη εικόνα της συναρμολόγησης, του συντονισμού και της ετερογένειας σημαντικών RBPs, όπως η La και η RPP25, υπό διαφορετικές συνθήκες και να προσδιορίσει μονοπάτια που ρυθμίζουν τη γονιδιακή έκφραση σε επίπεδο μεταγραφής και μετάφρασης, τα οποία θα μπορούσαν να αξιοποιηθούν περαιτέρω για θεραπευτικούς σκοπούς.

1. Introduction

1.1. Noncoding RNAs (ncRNAs) and RNA polymerase III transcriptome

The unprecedented discovery of catalytic RNA more than 30 years ago, (ribozymes, Nobel Prize in Chemistry 1989) consolidated the "RNA world" theory, changed our view of living systems and revolutionized modern biology (Weinberg et al., 2019). Since then, the advancement of the next generation sequencing methodologies allowed a complete view on hundreds of genomes. Today, the first draft of the human genome reported more than 20 years ago marked a new era in biomedical research leading to the discovery that almost 80% of the genome is pervasively transcribed, but with less than 2% corresponding to mRNAs coding for proteins (Ezkurdia et al., 2014; Salzberg, 2018). The non-coding RNA (ncRNA) transcriptome as we know it today is classified into two major classes: 1) the long-ncRNAs (lncRNAs) which include RNAs >200 nucleotides in length while 2) the short ncRNAs include RNAs <200 nucleotides. The short ncRNAs include transfer RNAs (tRNAs) which are passive carriers of amino acids involved in mRNA translation, ribosomal RNAs (rRNAs) which participate in assembly of the ribosomes, small nuclear RNAs (snRNAs) involved in splicing, small nucleolar RNAs (snoRNAs) involved in the modification of rRNAs, microRNAs (miRNAs) involved in gene silencing and piwi-interacting RNA (piRNAs) with main function to suppress the activity of transposons (Figure 1.1) (Amin et al., 2019; Genzor et al., 2021; Lorenzi et al., 2021).

Although, in the past the ncRNAs are considered as "dark matter", today, we known that they assigned roles as major modulators of gene expression both on post-transcriptional and transcriptional levels, they affect chromatin remodeling, cell cycle, or even more they have catalytic functions (Zhang et al., 2019). Interestingly, the most well-studied ncRNAs are miRNAs (<25nt) that are mainly transcribed by RNA polymerase II (RNA Pol II) to primary molecules which produce mature miRNAs with the help of ribonucleases (Drosha-Dicer) (Winkle et al., 2021). However, the RNA Pol II also transcribes the long noncoding RNAs (>200nt), some of which derived from mRNA splicing and although are essentially uncharacterized, it has been found that could participate to regulate the chromatin function, the translation of cytoplasmic mRNAs or interfere in signaling pathways (Statello et al., 2021).

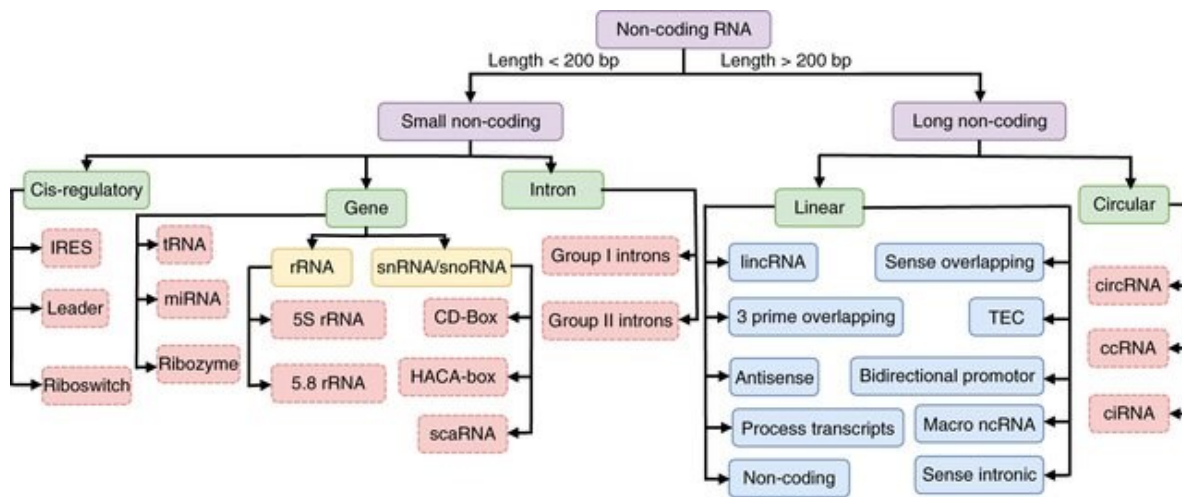


Figure 1.1: A classification of ncRNA molecules. The ncRNAs are classified according to their length (purple boxes) and then according to their structure/functionality (green boxes) for long and small non-coding types. The ncRNAs in pink boxes represent RNAs that are classified by deep learning in the literature while the yellow boxes are further subcategories. The ncRNAs in blue light box have not been well-studied or classified as non-coding in the literature. (TEC: to be experimentally confirmed). Adapted from Amin et al., 2020.

Interestingly, the ncRNAs that are transcribed by RNA pol II represent only a 5% of the total ncRNA transcriptome while more than 80% of ncRNA transcriptome are transcribed by RNA polymerase III (RNA pol III). The most abundant ncRNAs are synthesized by RNA Pol III are the tRNA molecules (more than 500 tRNA genes in the human genome) which also produce tRNA-derived fragments (tRFs), a new class of small regulatory ncRNAs with suggested role similar to miRNAs. However, the RNA pol III transcriptome also includes several miRNAs, 5S rRNA, U6 snRNA (pre-mRNA splicing), RPPH1 and RMRP which are the catalytic RNA subunits of the RNase P and RNase MRP ribonucleoprotein (RNP) complexes, respectively, responsible for processing of tRNA and rRNA (Dieci et al., 2007; Canella et al., 2010). Additionally, YRNAs (with involvement in replication initiation and translation regulation and in autoimmunity), 7SK RNA (regulation of RNA pol II transcription elongation), 7SL RNA (RNA scaffold of the signal recognition particle) and vault RNAs (vtRNAs) (putative role in the formation of speckles, inflammation, autophagy, and drug resistance) are also transcribed by Pol III (Figure 1.2; (Walter and Blobel, 1982; Egloff et al., 2018; Guglas et al., 2020; Gallo et al., 2022). Moreover, almost all RNA Pol III-synthesized ncRNAs are involved in autophagy, apoptosis, differentiation, stress response, inflammation, or viral

infection, pro-oncogenic signaling, autoimmunity, drug-resistance, and many other disease-related mechanisms, acting through specific RNA-RNA and/or RNA-protein interactions (Figure 1.2) (Yeganeh and Hernandez, 2020). Of note, these interactions are also involved in a compartmentalization inside nucleoplasm (like Cajal bodies) but also in cytoplasm (like stress granules) termed as RNA-phase separation (Tian et al., 2020). Eukaryotic cells form specific speckles without the need of a membrane allowing the processing of specific cellular processes within the cell (Liao and Regev, 2021). Although the exact role of RNA-phase separation, has not yet been well-defined, the formation of these granules has been associated with post-transcriptional modification, RNA splicing under stress conditions.

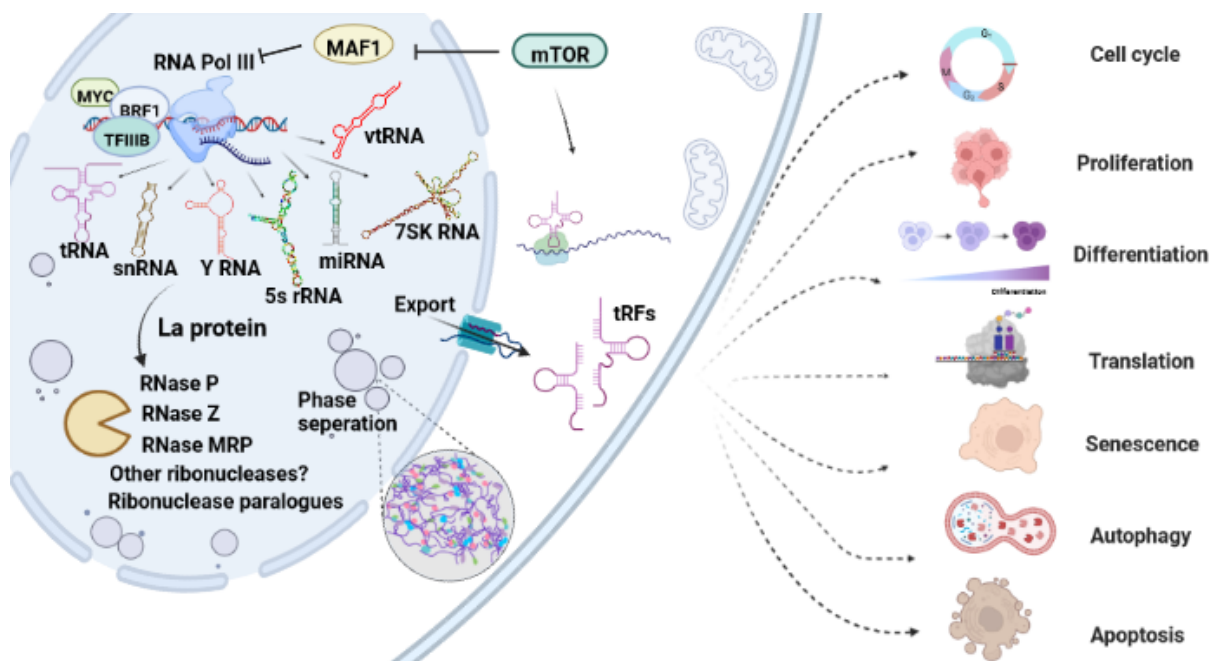


Figure 1.2. The RNA polymerase III transcriptome and its contribution in cellular pathways.

More importantly, the biogenesis and function of ncRNAs are defined by important RNA-binding proteins (RBPs; like La protein) and individual Ribonucleases or Ribonucleoprotein complexes (RNPs; like RNase P, RNase Z, Dicer, RNase MRP) whose characterization is critical to dissect the whole spectrum of molecular functions of ncRNAs (Hopper and Nostramo, 2019).

1.2. A general overview of the eukaryotic transfer RNAs (tRNAs)

1.2.1. Structure of tRNAs

Transfer RNA molecules (tRNAs) comprise almost 15% of cellular RNAs, are considered as the most abundant RNA species inside the cell and represent the adaptor molecules between the RNA and protein world (Figure 1.3; Palazzo and Lee, 2015). tRNAs match the anticodon triplet with the specific codon triplet of mRNA carrying the cognate amino acids for the synthesis of polypeptides. They are short RNA molecules consisted of 60-95 nucleotides in length and are considered as well-folded RNAs as they adopt a conserved characteristic cloverleaf secondary structure and an L-shaped three-dimensional structure (Figure 1.4). The cloverleaf structure consists of four different stems and loops. Typically, each arm contains the helical stems which is formed from Watson–Crick base pairs (bp) and an unpaired bases at the external part to form a loop. These features include the acceptor stem (blue), the D-arm (green), the anticodon arm (red), the T-arm (yellow), and a loop of variable length, the variable loop (purple) (Figure 1.4).

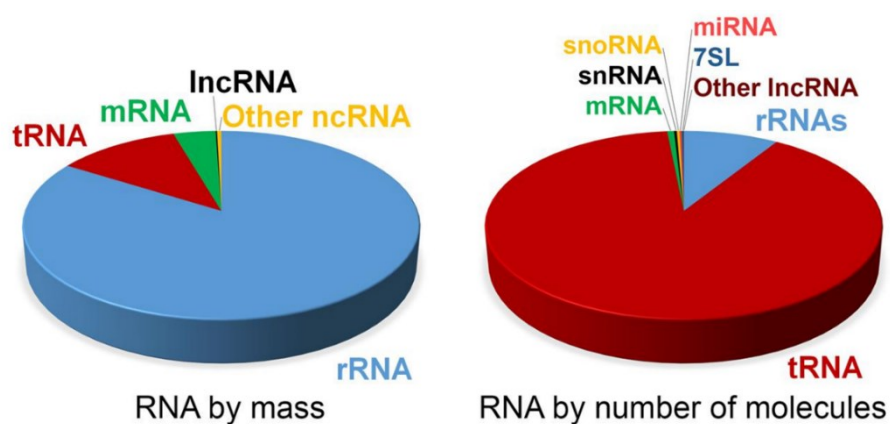


Figure 1.3. Quantification of ncRNAs by number of mass or number of molecules. The tRNAs correspond the largest amount of ncRNA pool. Adapted from Palazzo et al., 2015.

The acceptor stem of tRNAs consists of 7 base pairs (with the exceptions of tRNA^{His} and tRNA^{Sec} which contain 8 and 9 bp respectively), followed by the conserved 3' end which is single stranded and contains the discriminator base and the universal 3' CCA which in some prokaryotes is genome encoded while in eukaryotes is added post-transcriptionally by the specific 3'CCA nucleotidyl-transferase (Betat and Mörl, 2015). The conserved 3' terminal adenosine is

responsible for carrying the cognate amino acid. The D-stem is usually consisted by 4 bp ending in a loop called D-loop that often contains dihydrouridine (D) while the T-stem is consisted by 5 bp ending in a loop called T-loop that often contains a conserved pseudouridine (Ψ) at position 55. In the anticodon loop, the three outermost nucleotides represent the anticodon and use to be at positions 34 to 36. The T-arm, the acceptor arm and the anticodon arm are highly conserved in size, while the D-arm and variable arm can vary (Zhang and Ferré-D'Amaré, 2016). In addition, the L-shaped three-dimensional structure of tRNA forms interactions between nucleotides. In the “elbow” conformation, nucleotides from the D- and T-loops forms a Watson-Crick base pair in order to consist the canonical L-shape of tRNAs (Figure 1.4) (Zhang and Ferré-D'Amaré, 2016).

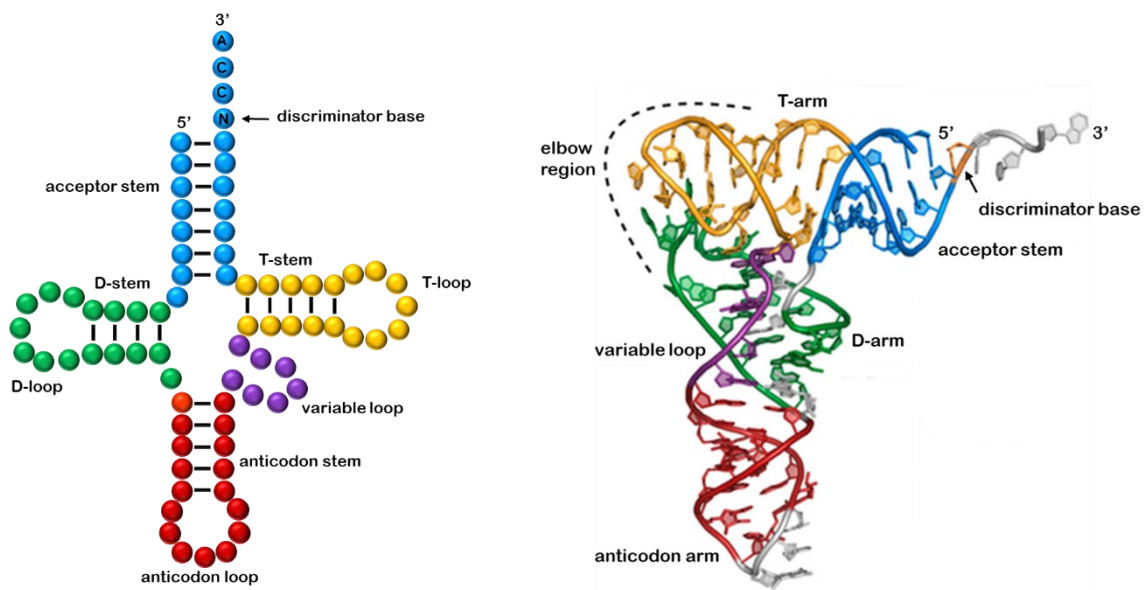


Figure 1.4. The structure of tRNA. A secondary (left) and a tertiary structure of tRNA (right).

1.2.2. Biogenesis of nuclear tRNAs

Transcription by Pol III

tRNA genes are transcribed by RNA polymerase III (17-subunit enzyme) assisted by a number of auxiliary factors that have been identified to facilitate the tRNA transcription initiation (Ramsay and Vannini, 2018; Ramsay et al., 2020). tRNA genes have two conserved internal elements, termed the box A and box B that correspond to D loop and T loop of tRNA, respectively.

These boxes promote transcription through the recruitment of a transcription factor TFIIIC (transcription factor III C), which is the first factor to associate with DNA, mediating in transcription complex assembly process (Figure 1.5). TFIIIC is a large six-subunits complex that promotes the binding of TFIIIB (transcription factor III B) upstream of the transcription start site (TSS) (Turowski and Tollervey, 2016). TFIIIB is composed of three subunits: Brf1 (B-related factor 1), Bdp1 (B double prime) and TBP (TATA-binding protein) (Kassavetis and Geiduschek, 2006). Brf1 is a modular protein that plays a major role in RNA polymerase III recruitment, the Bdp1 subunit of TFIIIB is specific to the RNA polymerase III machinery and is considered a histone-binding module while TBP is common to all eukaryotic RNAPs and binds to the TATA box *via* an induced-fit mechanism of protein-DNA recognition (Figure 1.5) (Akhtar and Veenstra, 2011; Girbig et al., 2022).

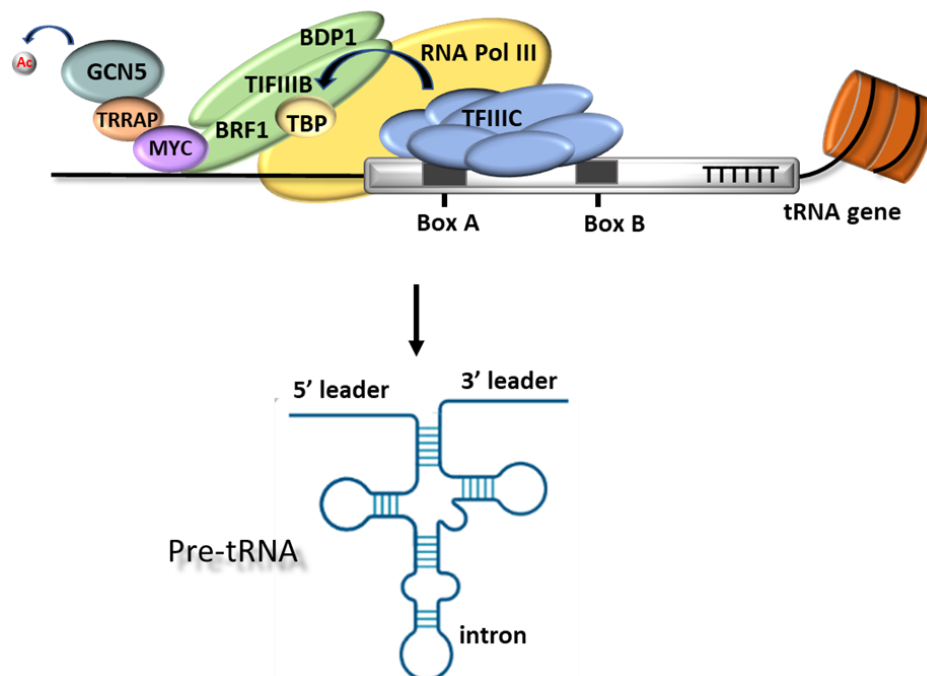


Figure 1.5. tRNA gene transcription. tRNAs are transcribed by RNA pol III through important transcription factors that mediate in this process. TFIIIC complex is bound to A- and B- box and leads to the recruitment of TFIIIB complex and RNA Pol III to start the transcription.

Apart from the above factors, GCN5 (General Control Non-repressed 5 protein), TRRAP (Transformation/Transcription Domain Associated Protein) and the protooncogene c-Myc can

also activate RNA polymerase III transcription (Kenneth et al., 2007). GCN5 is a histone acetyltransferase that acts as transcription activator, while TRRAP is an adaptor protein as it forms complexes the histone acetyltransferases. Among them c-Myc, a protooncogene, can recruit the GCN5 and TRRAP to activate the RNA polymerase III transcription. The transcription termination is promoted by poly-T termination signal that destabilizes the template-RNA polymerase III complex leading to a primary transcript, the precursor tRNA (pre-tRNA), which has extra sequences at their 5'- and 3'-ends and even has intron (Arimbasseri et al., 2013). However, pre-tRNAs have to undergo a series of processing steps to render mature and functional. These steps comprise cleavage of extra sequences found at their 5' and 3' ends, introns removal when present, addition of a CCA motif at their 3' end, base modifications. A concerted action of essential ribonucleases and proteins like RNase P, La protein, RNase Z are required for this process.

5' end maturation

The first maturation event includes the removal of 5' leader sequence by Ribonuclease P (RNase P). RNase P is an important and ubiquitous ribonucleoprotein that catalyze the cleavage of a single phosphodiester bond adjacent to the first nucleotide of the acceptor stem. Its endonucleolytic activity is attributed to RNA component which it has been isolated from organisms representing the three domains of life. From bacteria to mammals, RNase P is considered an ancient enzyme composed of one RNA and at least one protein subunit to a highly organized ribonucleoprotein complex (Shaukat et al., 2021).

3' end maturation

The 3' end formation of tRNA begins with RNA polymerase III termination generating a variable 3' oligo(U) tract, the 3'-trailer sequence. The 3'-trailer can be removed by more than one mechanism (Maraia and Lamichhane, 2011). The major pathway for 3' processing of nuclear tRNAs is mediated by Ribonuclease Z (RNase Z) with the presence of the La protein (Lupus antigen; Sjögren Syndrome antigen B; SSB) (Wolin and Cedervall, 2002). RNase Z is an endonuclease which functions by precisely cleavage after the discriminator base. In the human

genome, two forms of RNase Z are identified: the RNase Z^L (92.2 kDa), which is encoded by the gene ELAC2 and the RNase Z^S (40 kDa), which is encoded by the ELAC1 gene. The latest is present in the cytoplasm and recently than it is able to repair the 3' termini of tRNAs released from stalled ribosomes containing a 2'-3' cyclic phosphate (Yip et al., 2020). On the other hand, the RNase Z^L is both localized in nucleus and mitochondria and is responsible for the tRNA maturation in both compartments. The La protein is a ubiquitous eukaryotic RNA-binding protein which interact with pre-tRNAs through recognition of their UUU-3'OH trailers. La facilitates folding and maturation of pre-tRNAs as protects the 3' termini from exonucleolytic digestion. Alternatively, in the absence of La, 3'-trailer sequence is removed by non-specific exonuclease activity (Maraia, 2001; Wolin and Cedervall, 2002).

Intron Splicing

After the precisely removal of the 5' and 3' terminal sequences of pre-tRNAs, the splicing of introns is a critical step in pre-tRNA maturation. Introns are usually located within the anticodon stem-loop of tRNAs and especially, are positioned between one base of 3' to the anticodon (Yoshihisa, 2014; Schmidt and Matera, 2020). tRNA splicing process is catalyzed by a protein complex called TSEN (tRNA splicing endonuclease). This complex was first identified in the yeast *S. cerevisiae*, and it is comprised by four subunits, the TSEN2 and TSEN34 which consist of the catalytic subunits and also two structural members the TSEN54 and TSEN15 (Trotta et al., 1997). However, in human, except for all the four TSEN homologs of yeast proteins, also co-purified a polynucleotide kinase CLP1 (polynucleotide 5'-hydroxyl-kinase polynucleotide kinase 1), the role of which remains elusive (Paushkin et al., 2004; Hayne et al., 2020). The intron excision occurs in the cytoplasm in yeast or in the nucleoplasm in human and followed by enzymatic splicing in two steps. The first one is an endonucleolytic cleavage at the 5' and 3' splice sites that generates an intron and two exons followed by ligation of the two remaining exons (Paushkin et al., 2004; Schmidt and Matera, 2020). In human, the ligation step is performed RNA ligase is the HSPC117 (hematopoietic stem/progenitor cell 117) (Popow et al., 2011). Interestingly, it has been reported that the eukaryotic splicing machinery recognizes the splicing sites of introns by a ruler-

mechanism in which the structural members of complex bind to specific features of pre-tRNA, measure the distance between the body of tRNA and the splice sites thus allowing the cleavage by the catalytic subunits (Xue et al., 2006).

CCA-addition

The CCA sequence is added post-transcriptional by enzymes called transfer RNA nucleotidyltransferases or CCA-adding enzymes (Betat and Mörl, 2015). The CCA addition to the processed 3' end is prerequisite for tRNA aminoacylation with the terminal adenosine forms the binding site of the appropriate amino acid. The CCA-adding enzymes catalyze the polymerization of CCA without using an oligonucleotide template (Xiong and Steitz, 2004)

Post-transcriptional modifications

Unlike all other transcripts, tRNA is the most extensively post-transcriptionally modified molecule in the cell. On average a eukaryotic tRNA contains 13 modifications per molecule (Zhang et al., 2022). Post-transcriptional modifications in many nucleotides throughout tRNA sequence represent a necessary step before a tRNA becomes completely functional. The complexity of such modifications ranges from the most common chemical modifications like methylations, sulfurations, pseudouridylations, as well as more complex modified moieties, like queuosines (Q) (Kouvela et al., 2021). The post-transcriptional modifications are essential to ensure the proper folding and stability of tRNA but also for tRNA recognition by aminoacyl-tRNA synthetases and modifying enzymes. In addition, tRNA modifications affect interactions between tRNAs and the ribosomes, mRNAs and factors that participate in this procedure. The complete set of nucleotides and modifications is called "tRNA identity elements set" (Giege et al., 1998)

Aminoacylation

A functional tRNA is an aminoacyl-tRNA which should be acylated with the cognate amino acid by an aminoacyl-tRNA synthetase (aaR). Aminoacylation is an ATP-dependent process that attaches the amino acid to the CCA sequence of 3' end of tRNAs. The aaRs recognize unique structural elements of tRNA: the anticodon stem loop and the acceptor stem but also the

discriminator base (Berg and Brandl, 2021). The aaRs are a family of 20 enzymes that are divided into two classes based on the structure and their catalytic domains (Stathopoulos et al., 2001; Chaliotis et al., 2017). The aaRs are considered important enzymes that implement the correct assignment of amino acids to their codons and consequently are considered the gatekeepers of translational fidelity. After aminoacylation, a Ran: GTPase exportin, termed as exportin-T is responsible for translocation of tRNAs in the cytoplasm where they function in translation (Figure 1.6).

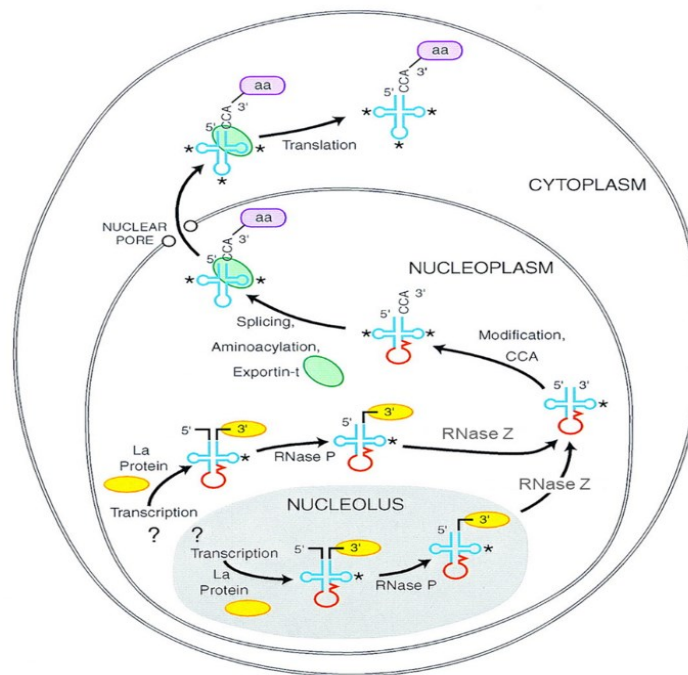


Figure 1.6. A tRNA biogenesis in a yeast cell. After transcription, the newly synthesized pre-tRNA, containing intron (red) and 5' and 3' extensions sequenced (black), is bound by the La protein (yellow oval). The 5' leader is removed by RNase P while the 3' trailer is removed by an endonuclease or exonuclease. The CCA- addition, splicing, aminoacylation and binding by exportin-t (green oval) occur at nucleoplasm. Certain nucleotide modifications are shown with asterisks.

1.2.3. Transcription by RNA Pol III and association with diseases

Transcription by Pol III demands the concerted action of multiple factors, which upon deregulation have been linked to various diseases. For example, mutations of *BDP1* or *BRF1* are associated with hereditary hearing loss and cerebellar–facial–dental syndrome, while mutations

of POLR3A or POLR1C are associated with leukodystrophy and Treacher Collins syndrome, respectively (Yeganeh and Hernandez, 2020). More importantly, aberrant expression of Pol III transcription factors but also the tRNAs has been correlated to cancer. Increased expression of *BRF1* has been found in hepatocellular carcinoma or in breast cancer acting as oncogene while in mouse embryonic fibroblasts BRF1 acts as tumor suppressor where knockdown of its expression leads to Ras-mediated malignancies (Yeganeh and Hernandez, 2020). In addition, several tRNAs are found upregulated in both tumors tissues and cancer cell lines indicating a possible role in tumorigenesis (Figure 1.7). In breast and lung tumors, it has been proposed that the increased levels of specific tRNAs can enhance translation of metastatic genes (Goodarzi et al., 2016; Skeparnias et al., 2020) while upregulation of initiator tRNA (tRNA^{iMet}) could enhance the translation of ECM components like collagen or integrins (Birch et al., 2016; Clarke et al., 2016). Moreover, in melanoma, it has been found that specific tRNAs like tRNA^{Arg}, tRNA^{Ala}, tRNA^{Asn}, tRNA^{Cys}, tRNA^{Lys}, tRNA^{Glu} and tRNA^{Thr} are upregulated through elevated expression of lncRNA TERT. More specific, TERT is bound to RNA pol III subunit RPC32 and enhance the recruitment of chromatin resulting in an increase transcription by RNA pol III (Khattar et al., 2016). Abnormally increased tRNAs abundance has also been reported in cervical cancer, in multiple myeloma and pancreatic cancer (Daly et al., 2005; Zhou et al., 2009; Wang et al., 2016).

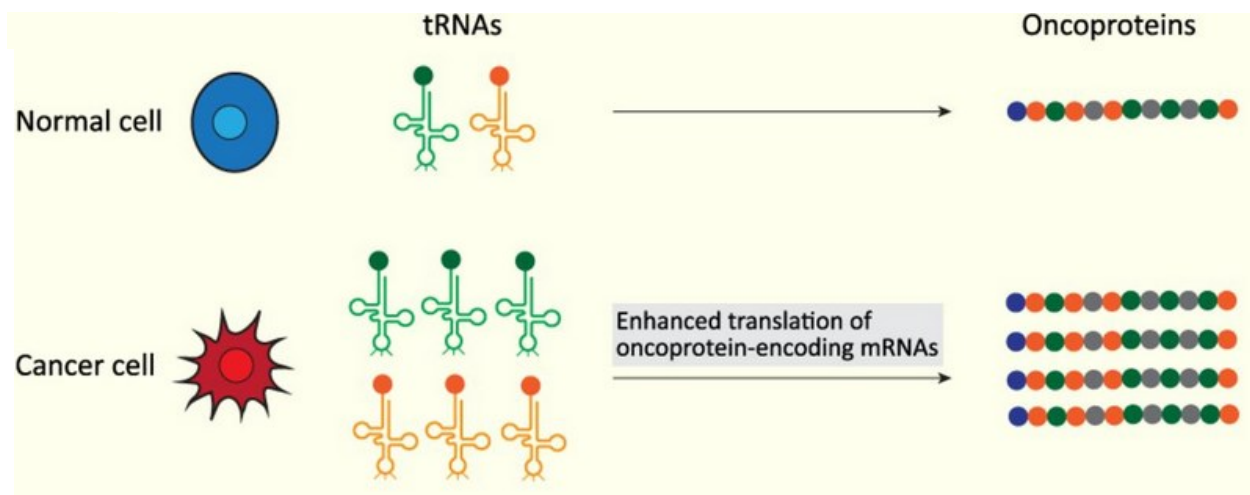


Figure 1.7. Model showing that overexpression of specific tRNAs could enhance the translation of oncogene in cancer. Adapted from Yeganeh et al., 2020.

1.2.4. Functions and emerging new roles of tRNAs

tRNAs were considered ‘housekeeping’ RNAs and represent adaptor molecules between the RNA and protein world, however, besides the canonical role, recent evidence suggested that tRNAs exhibit non-canonical functions that have been described in both prokaryotes and eukaryotes (Schimmel, 2018). According the amino-acylated status, the tRNAs have expanded in additional metabolic and cellular processes including the gene expression regulation at both the transcriptional and translational level, non-ribosomal peptide bond formation, bacterial cell wall biosynthesis, viral replication, cell stress response and antibiotic biosynthesis (Figure 1.8) (Raina and Ibba, 2014).

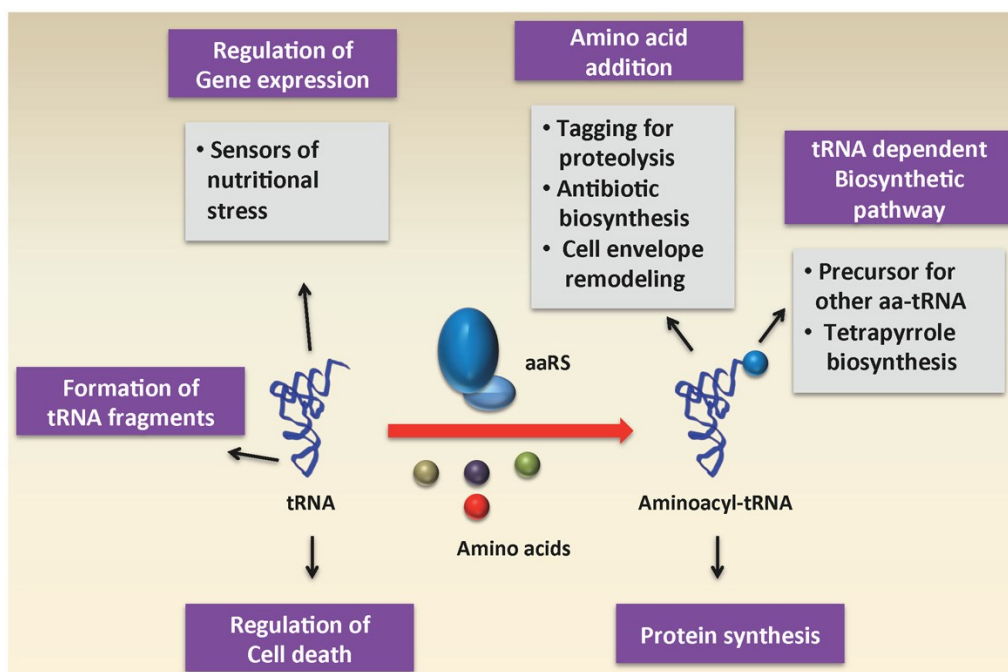


Figure 1.8. Emerging roles of tRNA molecules. tRNA participation in cellular processes regarding its aminoacylation status. Adapted from Raina and Ibba, 2014.

In eukaryotes, the discovery of tRNA-derived fragments (tRFs), a new class of abundant and stress-induced small noncoding RNAs, highlights the essential regulatory role of tRNAs in translation and beyond. Although, tRFs were first considered as degraded byproducts of tRNAs, later studies showed that tRFs are abundant in all species and are produced by precise

endonucleolytic cleavage of precursor or mature tRNAs. Although their existence was mentioned over the years, during the last two decades, the sequencing technologies characterized the tRFs and categorizing them depending on their length and the site of origin on the tRNA (Su et al., 2020). Therefore, there are tRF-1s, tRF-5s, tRF-3s, 5'-tRNA halves, 3' tRNA halves and i-tRFs (internal tRFs) (Figure 1.9). These distinct tRF classes have been linked to different biological functions which include global translation inhibition (attributed to 5' tRNA halves and tRF-5s), translation repression of specific mRNAs (attributed to tRF-3s), inhibition of specific gene transcription (attributed to tRF-5s), inhibition of viral RNA translation (attributed to tRF-1s), inhibition of apoptosis (attributed to 5' tRNA halves) and regulation of retrotransposon expression (attributed both to tRF-3s and tRF-5s). Stress-induced tRFs, for instance, have been found to interact with cytochrome c and inhibit the apoptosome formation, thus, fine-tuning the apoptotic pathway (Saikia et al., 2014).

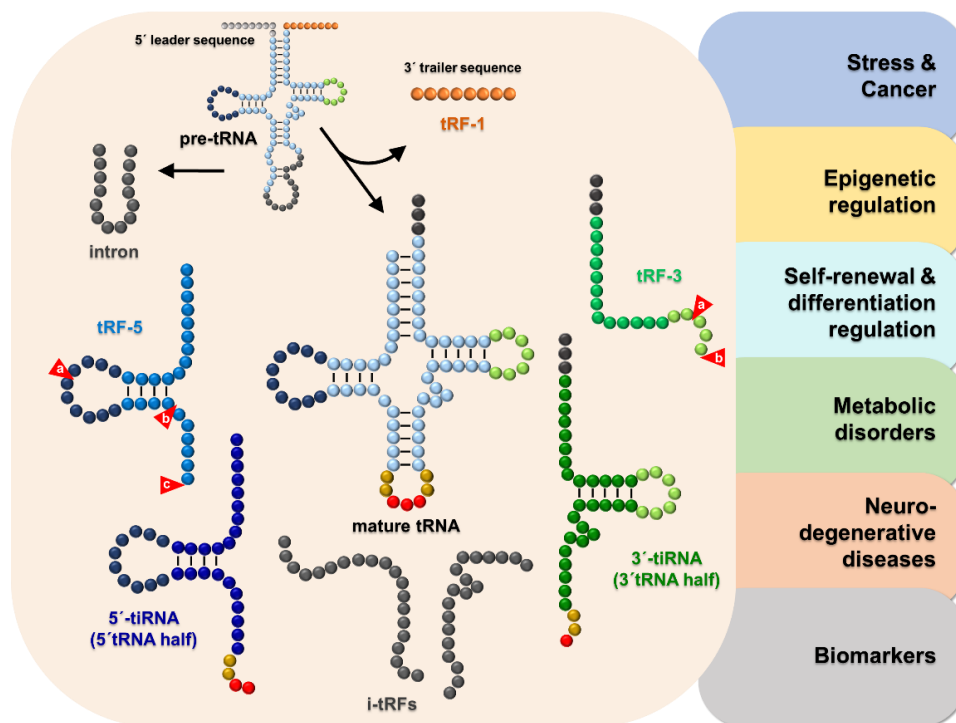


Figure 1.9. tRNA fragments are classified according to the origin and length (14-40 nt), from either pre-or mature tRNAs. The classes are tRF-5 (type a, b and c), tRF-3 (type a and b), tRF-1 (3 maturation products), i-tRFs (of intronic origin) and 5-or 3-tiRNAs (or tRNA halves; stress induced tRFs). tRFs have been implicated in cancer, stress, epigenetic regulation, self-renewal and differentiation, various metabolic disorders and neurodegenerative diseases or as emerging biomarkers. Adapted from Grafanaki et al., 2020.

1.3. La protein

1.3.1. An overview of La protein

La protein (Lupus antigen), also known as Sjögren Syndrome antigen B (SSB) or LARP3 (La related protein 3) was first isolated as one of the major antigens in patients suffering from specific rheumatic diseases like Sjögren's syndrome and systemic lupus erythematosus (SLE) (Mattioli and Reichlin, 1974; Alspaugh and Tan, 1975). While the exact role of La in autoimmune disorders remain elusive, it has been extensively studied as a ubiquitous RNA-binding protein which facilitates proper folding and maturation of nascent RNA pol III transcripts (Wolin and Cedervall, 2002). Although it was first described in human, homologs of La protein have been identified in almost all eukaryotes (with the exception of the *Plasmodium* genus), exhibiting highly conserved functions in RNA metabolism. Especially, it binds a plethora of RNA molecules including 5S rRNA, U6 small nuclear RNA (snRNA), 7SL RNA, Y RNAs as well as viral RNAs like VA RNA I, VA RNA II (adenovirus RNAs) or EBER 1 and EBER 2 RNA (Epstein-Barr virus) (Lerner et al., 1981; Francoeur and Mathews, 1982; Rinke and Steitz, 1982; Chambers et al., 1983; Hashimoto and Steitz, 1983). Approximately 20 million copies of La are found per cell and although it has been found that it is dispensable in yeast, in higher eukaryotes (i.e. *T. brucei*, *D. melanogaster*) including mammals, La is essential for viability (Blewett and Maraia, 2018).

La is an abundant phosphoprotein that is mainly localized in the nucleus of eukaryotic cell, however, La can shuttle to the nucleolus, where large rRNAs and initial processing steps of pre-tRNAs occur or it is also transiently exported to the cytoplasm to bind a subset of mRNAs during translation (Intine et al., 2003). Although La has been extensively studied as a chaperone of RNA pol III transcripts, increasing reports shown that La is associated with diverse RNAs without the UUU motifs and it participates in additional functions like in viral RNA stabilization, in miRNA biogenesis, and in promotion of IRES-dependent mRNA translation. The latest role associates La with cancer as it has been found that La is overexpressed in numerous malignancies including lung cancer, cervical, head and neck cancer as well as in chronic myelogenous leukemia (Sommer and Heise, 2021).

1.3.2. La-related proteins (LARPs)

La protein is considered as a genuine member of the La-related proteins (LARPs; La termed LARP3 within LARPs), an evolutionary and structurally conserved superfamily of eukaryotic RNA binding proteins, which exhibit important function in RNA metabolism. This superfamily is classified into five family members, the genuine La or LARP3 and four LARPs including LARP1, LARP4, LARP6 and LARP7 (Figure 1.10). All LARPs consisted of a common architectural feature, La motif (LAM) following by an RNA recognition Motif (RRM) or RRM-like motif, both these two motifs are known as 'La Module'. An evolutionary analysis concerning the presence of LAM-containing proteins, from sequenced genomes of 83 eukaryotic species shown that these proteins are absent from archaea and present in all eukaryotes (with exception of protists from the *Plasmodium* genus), suggesting that the LAM is an ancestral motif (Bousquet-Antonelli and Deragon, 2009).

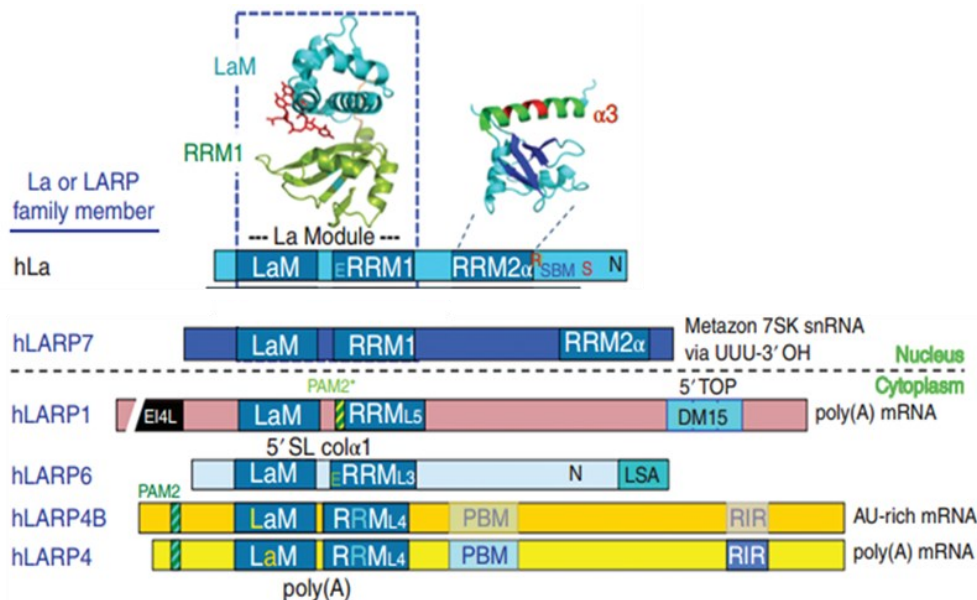


Figure 1.10 Schematic representation La-related protein superfamily (LARPs) in human. LAM and RRM motifs consist common structural elements of all proteins. The three-dimensional structures of La module and RRM2α are shown (PDBs 2VOD and 1OWX, respectively). N: nuclear localization sequence, E: nuclear export sequence, R: nuclear retention element, S: Serine-366, SBM: short basic motif, DM15: sequence responsible for binding to 7mGpppC-cap-5' TOP, RRM2α: RNA Recognition Motif 2, PAM2: poly(A)-binding protein interaction motif-2, LSA: LaM and S1-associated motif which contribute to mRNA recognition, PBM: poly(A)-binding protein interaction protein motif; RIR, RACK1-interaction region. EIML in LARP1 refers to eIF4-like region. Adapted from Maraia et al., 2017

Although LARPs have been not extensively characterized, they have several important modulatory cellular roles in transcription and/or translation and contribute to various diseases, including cancer (Stavraka and Blagden, 2015). The most closely related to genuine La is LARP7 which functions to specific transcript, the 7SK snRNA by binding, in a sequence specific manner to the UUU-3'OH end (Maraia et al., 2017; Hasler et al., 2021). Meanwhile, other LARPs (LARP1, LARP4, and LARP6) vary in both structure and function as they predominantly involved in various aspects of mRNA stability and translation. LARP1 and LARP4 are involved in mRNA stability while LARP6 associates with collagen mRNAs. Emerging evidence reveal that all LARPs dysregulated in cancer, however how they contribute to cancer pathobiology is still elusive.(Zhang and Stefanovic, 2016; Maraia et al., 2017; Egiz et al., 2019).

1.3.3. Architecture overview of genuine La in eukaryotes

Except for its characterization in *homo sapiens*, La has been also described in a variety of eukaryotic organisms including *M. Musculus*, *X. laevis*, *D. melanogaster*, *S. pombe*, *S. cerevisiae* και *T. brucei* (Scherly et al., 1993; Topfer et al., 1993; Yoo and Wolin, 1994; Lin-Marq and Clarkson, 1995; Van Horn et al., 1997; Westermann and Weber, 2000). In these organisms, two major evolutionary types of La structural organization exist, the N-terminal domain (NTD) which is highly conserved amongst eukaryotes and the C-terminal domain (CTD). In yeasts, La is smaller in size (32 kDa in *Saccharomyces cerevisiae*) and includes the characteristic N-terminal La motif (LAM; a winged-helix fold) followed by a short linker and an RNA recognition motif (RRM1), while in slime mold (*Dictyostelium discoideum*) as well as in human, La is longer and includes the conserved La module (La motif-RRM1) connected through an extended and very flexible linker with the remaining C-terminal half (Wolin and Cedervall, 2002; Apostolidi et al., 2014). The latter includes a second RRM motif (RRM2), a short stretch of basic amino acids (SBM) which is potentially responsible for the recognition of 5' triphosphate terminus of pre-tRNAs and two localization signal motifs, a nuclear retention element (NRE) and a nuclear localization signal (NLS) (Jacks et al., 2003) (Figure 1.11). The RRM2 motif has been found to be mediated in non-specific binding of structured RNAs, and recent NMR studies showed that it could be involved in

binding of La to a Hepatitis C IRES (Internal Ribosome Entry Site), an observation that has been verified also for other IRESs (Brown et al., 2016).

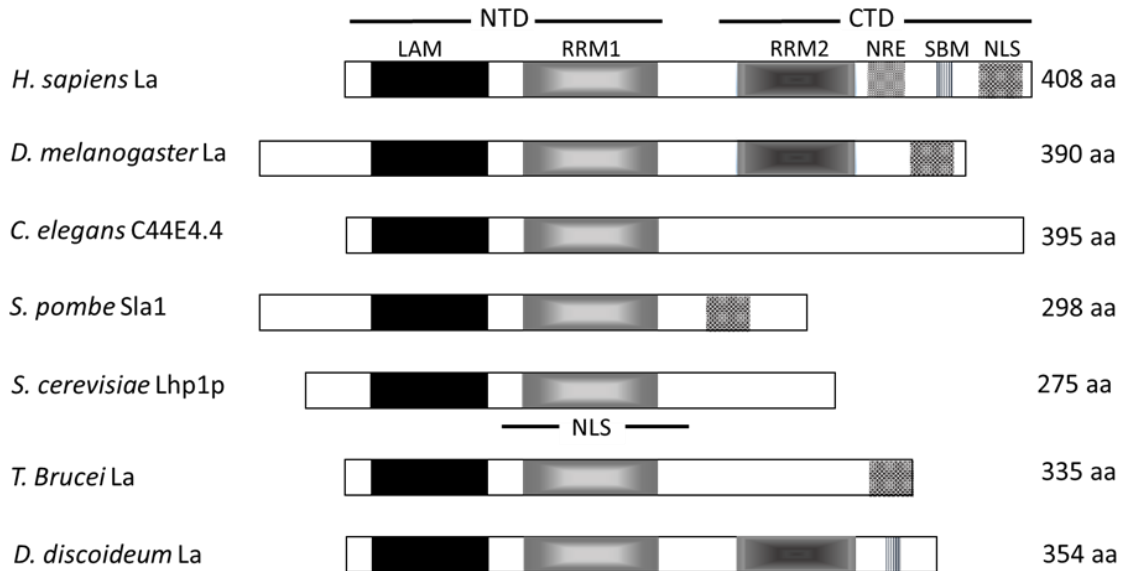


Figure 1.11. Schematic representation of genuine La orthologs. Homologs of La from human, *D. melanogaster*, *Caenorhabditis elegans*, *S.pombe*, *S.cerevisiae*, *T.brucei* and *D. discoideum*. All proteins contain a highly conserved NTD which includes the LAM and RRM1. The CTD is less conserved domain and is consisted of RRM2, a short stretch of basic amino acids (SBM), a nuclear retention element (NRE) and a nuclear localization signal (NLS). Adapted from Wolin & Cedervall, 2002.

The La and RRM1 motifs

The NTD structural domain of La includes the LAM and the RRM1 motif composed by 70 and 80 amino acids, respectively. The highly conserved domain, LAM, adopts a winged-helix fold containing three additional α -helix: $\alpha 1'$, $\alpha 2$ and $\alpha 4$ which form a hydrophobic pocket responsible for RNA binding. Within this hydrophobic cavity are mediated interactions (stacking interactions, H-bonds and electrostatic contacts) through the six residues: Q20, Y23, Y24, D33, F35 and F55 (numbering from human La) and the UUU-3'-OH ligand (Figure 1.12). It is noteworthy that the six residues are conserved in all LARPs with the exception of LARP4 and some plants in LARP6 members (Dong et al., 2004). Among these residues, the D33 determines a 3'-OH-dependent recognition mode and point mutations of this conserved residues results in a significant reduction in La's affinity for a 3'OH. The RRM1 consists one of the most common domains within eukaryotes

known to bind single-stranded RNA molecules. It adopts a typical $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ topology (β : beta-sheet, α : alpha-helix), followed by a short α_3 helix. More specific, the highly conserved and basic charged residues between β_2 and β_3 (loop 3) sheets have been shown to be responsible for interactions with pre-tRNAs (Alfano et al., 2004; Maris et al., 2005; Teplova et al., 2006; Bayfield et al., 2010; Naeeni et al., 2012).

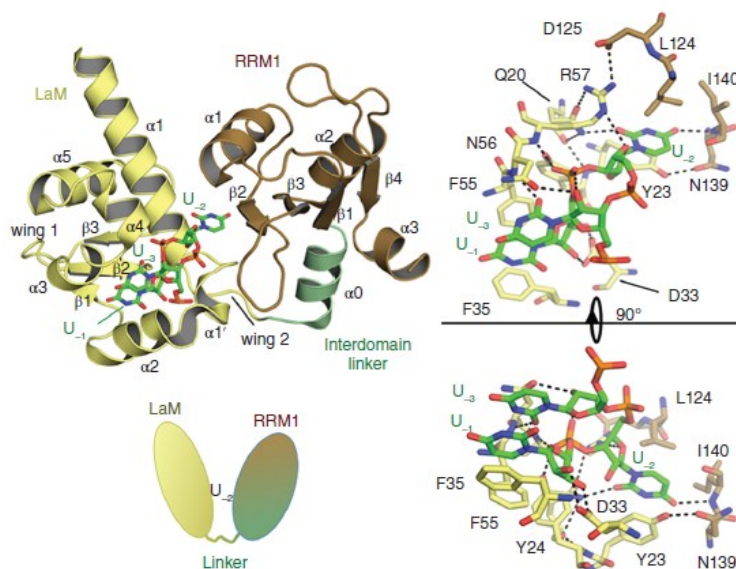


Figure 1.12. Structural organization of La Module (La motif and RRM1) of hLa in complex with UUU 3'OH RNA (PDB 2VOS) and a schematic V-shaped model. La motif (yellow color) acts synergistically with RRM1 (brown color) to bind the RNA which is shown as sticks (left) and a close-up view of hydrophobic cavity of LAM showing the residues that participates with RNA interaction (right) Adapted from Maraia et al., 2017.

LaM and RRM1 are linked with a flexible linker and move independently of each other in the absence of RNA while in the presence of RNA, the linker adopts a helical conformation assisting the correct positioning of both domains in order to act as a single functional group (Maraia et al., 2017). Actually, LAM:RRM1 folds around RNA polymerase III transcripts containing the UUU-3'OH feature, adopting a stable "V-shape" conformation (Figure 1.12). Structural characterization experiments of the LAM-RRM1:RNA complex was shown that LAM is responsible for the interactions with the terminal uridines of the RNA, while RRM1 participates in the binding through a non-specific interactions with the phosphate backbone of RNA (Teplova et al., 2006). More specific, a crystal structure of the hLa NTD with an 9nt-oligos RNA (UGCUGUUUUOH)

disproved the theory that the conserved RRM1 was responsible for the UUU-3'OH recognition and revealed that the hLa does not use RRM1 for the specific recognition of the UUU-3'OH sequence, but in order to create a cleft between the β -folded surface and the winged helix structure, into which the specific RNA will be inserted (Teplova et al., 2006). Interestingly, it has been proposed that LAM-RRM1 domain can bind mRNAs' poly(A) tails, enhancing mRNA stability or consolidate interactions with translation factors, an observation which seems also to be conserved among LARPs (Bayfield et al., 2010; Vinayak et al., 2018).

The RRM2 motif

On the other hand, the CTD structural domain of La protein varies among organisms and is less conserved. In mammals, this domain includes a second RRM, the RRM2 and some trafficking elements which are discussed below (Introduction 1.4.5). It has been proposed that RRM2 existed early during evolution, as it is found in lower eukaryotes like *D. discoideum* (Apostolidi et al., 2014; Argyriou et al., 2015). In human, RRM2 has a $\beta 1\alpha 1\beta 2\beta 3\alpha\beta 4\beta 4\alpha 3$ topology and contains an extra structural feature, a long $\alpha 3$ helix located over its β -sheet surface (Jacks et al., 2003). Although the function and role of the LAM and RRM1 module has been deciphered to some extent, the function of the RRM2 motif is still unclear. It has been proposed that RRM2 could be involved in binding of La to a Hepatitis C IRES (Martino et al., 2012, Brown et al., 2016). An additional study showed a possible role of $\alpha 3$ helix of RRM2 on the binding selectivity for various RNA ligands. Notably, a single-stranded RNA ligand had higher affinity and close interactions with the RRM2 domain while the $\alpha 3$ helix appeared destabilized. On the other hand, a hairpin RNA ligand displayed lower affinity and fewer interactions in the RRM2 and at the same time $\alpha 3$ helix changed its solvent accessibility, an observation similar to the behavior of RRM2- $\alpha 3$ helix of hLARP7 protein (Brown et al., 2016).

1.3.4. Functions of La protein and its ligands

Since its early discovery as autoantigen, La has been studied as the first protein to associate with the newly synthesized RNA polymerase III transcripts, contributing to their proper maturation of its ligands (Wolin and Cedervall, 2002). The best-characterized ligands of La protein are the pre-tRNA molecules. La recognizes the pre-tRNAs through binding of the UUU-3'OH

trailers, stabilizing them and preventing their degradation by exonucleases (Figure 1.13) (Rinke and Steitz, 1982; Stefano, 1984; Wolin and Cedervall, 2002). Experiments on *Xenopus laevis*, *S. cerevisiae* and HeLa cell extracts verify the exonucleolytic degradation of transcripts in the absence of La. The binding of La to the UUU-3'OH of RNA polymerase III transcripts, protect them from 3' to 5' decay by exonucleases such as Rex1p and the nuclear exosome (Copela et al., 2008). Thus, La is required for the normal maturation pathway of pre-tRNAs. However, apart from tRNAs, La also protects nascent RNAs that are transcribed by RNA polymerase II, from 3' exonucleases digestion. It was found that La binds both nascent spliceosomal (snRNAs) and snoRNAs (small nucleolar RNAs) such as U1, U2, U3, U4 and U5 (Kufel et al., 2000; Xue, 2000). In *S. cerevisiae* La is required for their assembly and its stabilization and during its absence, the expression levels of nascent transcripts are reduced, or shortened transcripts are produced. Moreover, in vertebrates, La has been reported bound to U1 (Madore et al., 1984).

In addition, La has been found that act as RNA chaperone through its conserved aromatic residues of $\beta 2$ - $\beta 3$ -loop3 region of RRM1 (Bayfield and Maraia, 2009; Kuehnert et al., 2015). A later study showed that both RRM1 and RRM2 is responsible for chaperone function. Also, the chaperone activity has also been identified in all LARPs, although the structural basis of this activity remains unclear (Naeeni et al., 2012).

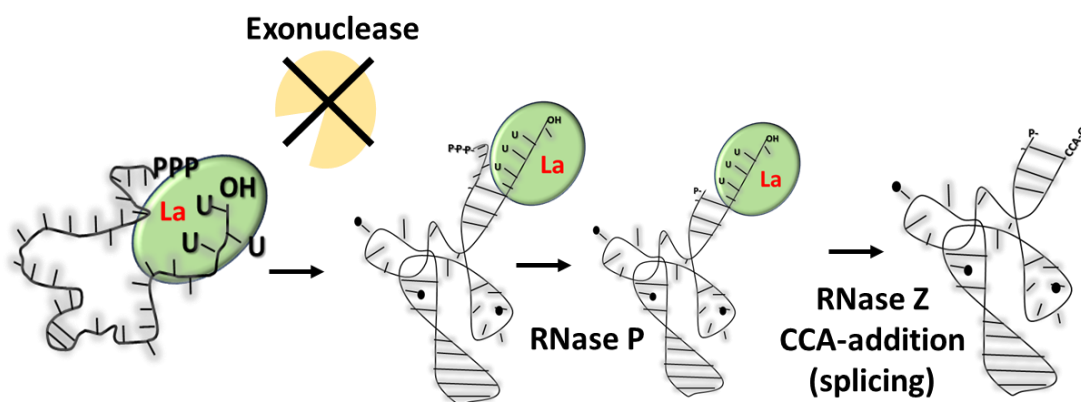


Figure 1.13. Schematic illustration of tRNA maturation in the presence of La protein. La is required for the maturation and correct folding of pre-tRNAs.

Beyond the folding and maturation of RNA pol III transcripts, recent studies reveal that La also recognizes non-UUU-3'OH RNA molecules and it participates in additional functions like viral RNA stabilization, miRNA biogenesis and in promotion of IRES-dependent mRNA translation (Stefano, 1984; Bayfield and Maraia, 2009; Martino et al., 2012; Liang et al., 2013; Vinayak et al., 2018). Interestingly, La was found in HEK293 cells to bind specific miRNA molecules like pri-let-7 miRNA *via* its LAM, followed by association with DGCR8, thus promoting miRNA processing through the regulation of the Drosha-DGCR8 complex formation (Zheng et al., 2017). Moreover, it has been recently found that La functions as gatekeeper to ensure the correct folding of tRNAs and protect the miRNA pathway from potentially tRNA fragments (Figure 1.14) (Hasler and Meister, 2016; Hasler et al., 2016). In the absence of La, some pre-tRNAs form stem-loop structures due to misfolding, which are recognized by exportin-5. In turn, they are exported to the cytoplasm, and they are loaded into the RISC complex, interfering with the miRNA pathway. Thus, La uses the chaperone function to ensure the correct selection of RNAs in the correct pathways (Figure 1.14) (Hasler et al., 2016).

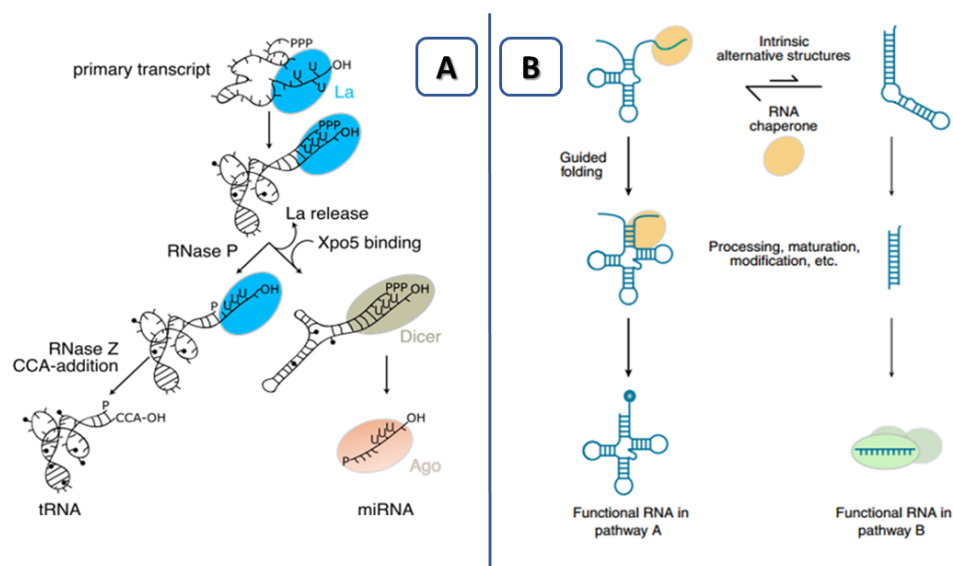


Figure 1.14: (a) La acts as a 'gatekeeper' for ensuring the correct processing of pre-tRNAs (left). Some pre-tRNA fragments can escape and be processed by Dicer into miRNAs (right). (b) Model of RNA chaperone function for correct pathway selection. Some transcripts could fold into alternative structures. The misfolded RNA can then be processed and channeled into pathways with potentially harmful consequences. RNA chaperones help RNAs to adopt the correct structure and ensuring the correct pathway. Adapted from Hasler et al., 2016

In addition, La has been identified in viral infections where it acts as an ITAF (internal trans-acting factor) for specific mRNA viral or cellular. *In vivo* experiment for hepatitis C virus (HCV) mRNA showed that La binds to viral IRES promoting the IRES-mediated translation. More specific, LAM and the RRM1 interact with the single-stranded region of the IRES element, while the RRM2 recognizes and binds the stem loop of the IRES. Similarly, La has the ability to bind additional viral RNA molecules, including the adenovirus -VAI and VAII- RNAs, poliovirus, EpsteinBarr virus- EBER1 and EBER2-RNAs, Vesicular Stomatitis Virus (VSV), Respiratory Syncytial Virus (RSV), parainfluenza or Rinderpest viruses, deadly rabies virus, dengue fever virus (DENV), Human Immunodeficiency Virus (HIV), Hepatitis B virus (HBV) and Hepatitis C virus (HCV) (Lerner et al., 1981; Kurilla and Keene, 1983; Kurilla et al., 1984; Chang et al., 1994; Ali et al., 2000; Maraia et al., 2017). Recently, a study revealed the participation of La and the tRFs in regulation of viral gene expression. More specific, La binds to tRF-1, is required for their production and increases their levels upon ectopic expression. These trailer-derived tRNA fragments compete with viral IRES for La-binding, thus inhibiting viral RNA translation (Cho et al., 2019).

Moreover, La also binds to cellular mRNAs containing IRES, or 5' TOP motif (5' terminal oligopyrimidines) or poly-A tails. For example, it binds directly in uOFRs of mdm2 (mouse double minute 2) enhancing its translation (Trotta et al., 2003). Additionally, the XIAP (X-linked inhibitor of apoptosis protein), NRF2 (nuclear factor erythroid-2 related factor 2), RRBP1 (ribosome binding protein 1), BiP (binding immunoglobulin protein), CCND1 (Cyclin D1), Laminin B1, and BCL2 (an apoptotic regulator) are some of the IRES-containing mRNAs bound with La (Holcik and Korneluk, 2000; Kim, 2001; Sommer et al., 2011a; Petz et al., 2012b; Zhang et al., 2012; Gao et al., 2016; Heise et al., 2016, 2; Govaere et al., 2017). Of note, La recognizes the stem-loop structures of IRES and uses its chaperone activity to unwind and reconfigure these structures in order to attract ribosomes enhancing the IRES-mediated translation of these transcripts (Kuehnert et al., 2015). Even more interesting is the observation that La can act as mediator in mRNA stability or consolidator of interactions with translation factors and ribosomes and, it has been suggested that under different conditions, La exhibits either sequence-specific or non-specific RNA binding modes, depending on the participating motifs or domains (Bayfield et al., 2021, Vinayak et al., 2018).

1.3.5. Localization and post-translational modifications of La

Apart from its characteristic structural domains, La contains elements that control its intracellular distribution and localization and consist of a nuclear localization sequence (NLS), a nuclear retention element (NRE) (Intine et al., 2002; Bayfield et al., 2007) and a nucleolar localization signal (NoLS) which are responsible for its nuclear-cytoplasmic shuttling (Figure 1.15) (Horke et al., 2004; Intine et al., 2004). La also contains an unstructured region (between 328-344 residues) known as the short basic motif (SBM) whose function is unclear (Fan et al., 1997). *In vitro* and *in vivo* studies have proposed the participation of SBM in the RNA processing as well as in chaperone activity, however its structural basis remains obscure. Moreover, La has a potential caspase cleavage site at D374 which is cleaved by caspase 3 (Ayukawa et al., 2000) and also a potential dimerization domain (between 293 to 348 amino acids) that although it has not been well-established it is considered that is essential for enhancing translation (Craig et al., 1997).

Although, La is mainly found in the nucleus, its subcellular mobility and localization elsewhere seems to be modulated by post-translational modifications or induced upon cellular stress or viral infection. La is able to shuttle between the nucleoli or cytoplasm where it binds different RNA ligands or interacts with other proteins. It is known that La undergoes post-transcriptional modifications regulating its stability, its expression or its function. Some of the modifications include phosphorylation, sumoylation, ubiquitylation, methylation acetylation (Figure 1.15) (Sommer and Heise, 2021). La has multiple phosphorylation sites like Thr-302, Ser-325, Thr-362, Ser-366, and Thr-389 and some of them determine its subcellular localization. In mammalian La, the most abundant phosphorylation site (~80%) is that of S366 by casein kinase II (CKII) (Schwartz et al., 2004). La phosphoSer-366 isoform is localized predominantly in the nucleus of eukaryotic cell while, the non-phosphorylated La can shuttle to the nucleoli of HeLa cells, is associated with nucleolin and is probably involved in rRNA biogenesis and initial processing steps of pre-tRNAs (Horke et al., 2004).

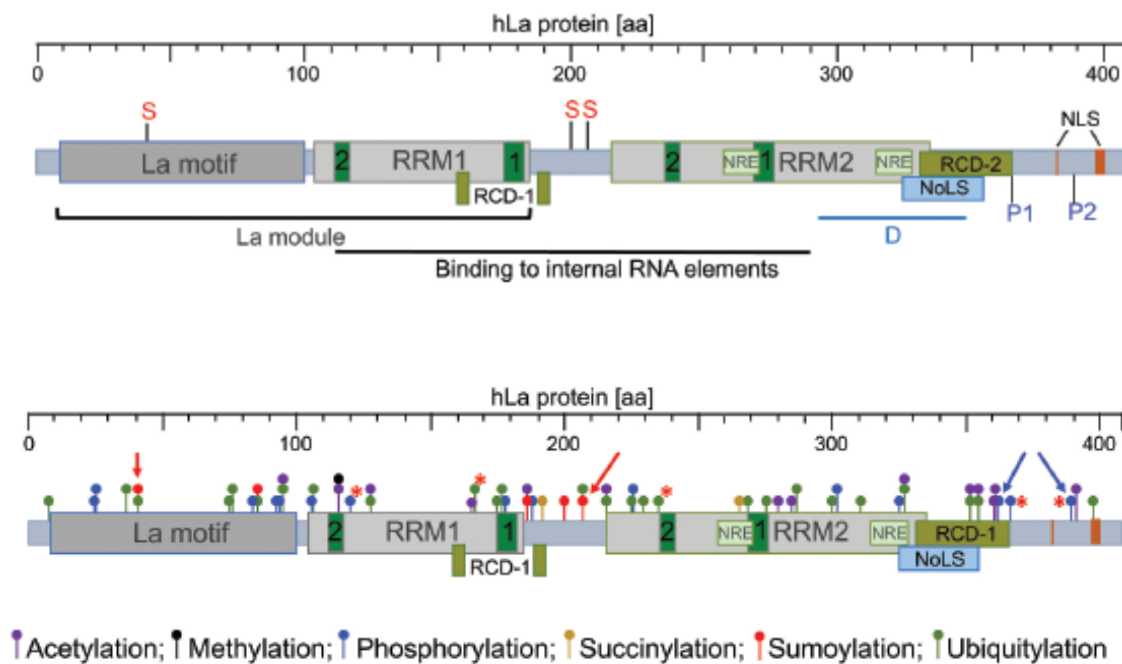


Figure 1.15. Schematic representation of architecture of La and its modification site. La is modulated by post-translational modifications like sumoylation S: SUMO-acceptor site, P1:CK2 phosphorylation site, P2: AKT phosphorylation site (up) and other modification like acetylation, methylation, ubiquitylation (down). Adapted from Sommer et al., 2021

In addition, non-phosphorylated La could also transiently shuttle to the cytoplasm to bind the 5'-terminal oligopyrimidine tracts (TOP) mRNAs during translation (Intine et al., 2004; Schwartz et al., 2004). Another important phosphorylation by AKT kinase refers to residues T389 and T301 in hLa and mLa, respectively. In human, this modification affect the binding of La to IRES element of cyclin D1 mRNA (Kuehnert et al., 2015), while in murine glial cells, the AKT-mediated phosphorylation of La could be linked to translation deregulation events. More specific, La shuttles to the cytoplasm where it is associated with polysomes and regulates the expression of specific mRNAs (Brenet et al., 2009). In addition, it has been found that the sumoylation of La could regulate its transport within the axonal compartment. Sumoylation of LAM, results in La export to sensory axons, while sumoylation within the linker between RRM1 and RRM2 motifs increases its binding to TOP mRNAs (van Niekerk et al., 2007). Although proteomic studies have

identified a number of post-transcriptional modifications in La, only the above have been well-characterized.

Moreover, shuttling of La to the cytoplasm has been observed after viral infection. Based on findings in poliovirus, it was suggested that La is cleaved by viral-encoded protease removing the NLS in the C-terminus. It is believed that cytoplasmic La binds to 5'-ppp termini and 3' U-rich tails of short noncoding leader transcripts facilitating the viral replication. Along the way, studies on La protein shown that La uses its chaperone activity to promote viral IRES-mediated translation (Maraia et al., 2017). Similarly, La protein becomes cytoplasmic during cellular stress like apoptosis of mammalian cells. During apoptosis, in human leukemic cells (HL-60), La is cleaved by caspase-3 at specific residue (D374) producing a 43-kDa truncated isoform without NLS, which is localized in the cytoplasm (Ayukawa et al., 2000). In addition, H₂O₂-induced oxidative stress or serum-starvation also have suggested the cytoplasmic distribution of La (discussed below).

1.3.6. La and cellular stress

To date, numerous studies have demonstrated the role of La under different cellular stress conditions or signals like apoptosis, oxidative stress, serum starvation or UV-irradiation. Early studies have suggested the nucleo-cytoplasmic shuttling of La after UV-irradiation in human keratinocytes where it co-localized with the epidermal growth factor (EGF) (Bachmann et al., 1990). Later studies indicated that La is transported into the cytoplasm participating in IRES-mediated translation. So far, the IRES-containing identified mRNAs include the X-linked inhibitor of apoptosis protein (XIAP), the nuclear factor erythroid-2 related factor 2 (Nrf2), the ribosome binding protein 1 (RRBP1), and the binding immunoglobulin protein (BiP) (Holcik and Korneluk, 2000; Kim, 2001; Zhang et al., 2012, 2; Gao et al., 2016). The X-linked inhibitor of apoptosis consists one of the regulators of apoptosis as it inhibits caspase activities. The XIAP was the first identified cellular mRNAs found to interact with La protein. Using *in vivo* and *in vitro* experiments, in HeLa cells, under serum deprivation and radiation-induced apoptosis, it has been found that La binds to 5' UTR of the XIAP IRES to enhance the IRES-mediated translation. Interestingly, that particular study characterized La as a subunit of XIAP RNP complex demonstrating that La can potentially modulate the expression levels of XIAP (Holcik and Korneluk, 2000).

In addition, in HeLa cells treated with H_2O_2 , La is accumulated in the cytoplasm and binds directly to the IRES of Nrf2, an important transcription factor that controls the expression of antioxidant genes. Immunofluorescence staining of HeLa cells displayed a rapid export of La to the cytoplasm (in 10 min) and La was found in isolated ribosomal fractions, an observation which suggests interaction of La with ribosomal proteins in response to oxidative stress (Zhang et al., 2012). Similarly, in the hepatocellular carcinoma cell line Huh7 after transient transfection with plasmid containing the HCV IRES and upon oxidative stress, La was also observed to translocate to the cytoplasm (Chan, 2016). Finally, although oxidative stress almost certainly induces La driven cap-independent translation of the HCV IRES further experimentation could reveal the exact mechanism through which La is recruited to IRES upon oxidative stress and viral infection.

Serum starvation is also a signal that affects cellular distribution of La. In both human embryonic kidney (HEK293T) and human hepatocellular carcinoma (Bel7402) cells after serum starvation (7.5% and 5% instead of the optimal 10%) the percentage of nuclear/cytoplasmic La changes according to the amount of FBS. More specifically, cytoplasmic La was significantly increased in cells growing at 5% FBS instead of 7.5%, suggesting that the reduction serum-starvation induces the translocation of the La protein from the nucleus to the cytoplasm. Moreover, it was shown that the serum starvation resulted in association of La with the RRB1, an endoplasmic reticulum (ER) membrane protein involved in ribosome binding and translocation of nascent proteins, particularly across the rough ER. Thus, it was proposed that La is a positive activator of the RRB1 IRES *in vivo*, under serum starvation conditions (Gao et al., 2016).

Regarding the ER membrane proteins, the BiP is also considered a ligand of La protein. BiP, also known as glucose-regulated protein 78 (GRP78), participates in unfolded protein response (UPR) through binding to misfolded or unfolded proteins that accumulate in the ER (Bravo et al., 2013). Since 2001, it has been mapped the binding site of La in BiP 5'-UTR between 115–207nt of IRES and it has been found that La enhances BiP IRES-dependent translation (Kim, 2001). Latter studies, in human salivary gland (HSG) cells, indicate that ER stress-induced apoptosis induces the cytoplasmic accumulation of La on the surface of apoptotic cells, in blebs budding from the membrane and on apoptotic bodies where it binds the BiP mRNA (Katsiogiannis et al., 2015). In addition, the association of La with BiP mRNA has also been reported during human

cytomegalovirus (HCMV) infection. Some viruses including the HCMV, uses the mechanisms of host cells in order to promote their own replication for example, virus use the translational machinery or ER chaperone activities of hosts. HCMV induces ER stress and modulate the UPR of infected cells, activating the BiP. As a results, HCMV-infected cells show enhanced BiP IRES activation and elevated levels of La, whereas, in La knockdown experiments both downregulation of BiP IRES activity and BiP protein levels was observed (Buchkovich et al., 2010).

1.3.7. Expression of La in cancer

An intriguing aspect of La protein is its aberrant expression in numerous cancers, including lung cancer, squamous cancer of the head and neck, cervical cancer, hepatocellular carcinoma (HCC) but also in chronic myelogenous leukemia (CML) and its expression is also induced by DNA-damaging drugs. In lung cancer, overexpression of La (>2-fold) has been correlated with poor survival rates among patients (Al-Ejeh et al., 2007). Although the exact role of this aberrant expression is under investigation several reports implicate La in the translation of selective mRNAs encoding tumor-promoting and anti-apoptotic factors, including BCL2, CCND1, Laminin B1, MDM2. As described above, La can facilitate the IRES-mediated translation initiation of specific transcripts to promote cell proliferation, migration and the invasiveness of cancer cells (Trotta et al., 2003; Sommer et al., 2011a; Petz et al., 2012b, 2012a; Heise et al., 2016, 2; Govaere et al., 2017).

In a BCR/ABL-expressing myeloid precursor cell line, it has been found that La increases the expression of MDM2, a p53-negative regulator, through binding on a conserved sequence of its 5' UTR (Trotta et al., 2003). Thus, it was suggested that La enhances the resistance of cells to apoptosis *via* indirectly modulation of the tumor suppressor p53 expression levels. Similar findings also observed in patients with JAK2-mutated myeloproliferative neoplasms (Nakatake et al., 2012). In addition, a possible involvement of La in carcinogenesis has been correlated through its phosphorylation at T389 by the AKT kinase, which is often affected in many cancers. It was suggested that La uses its RNA chaperone activity to directly bind the IRES structure of the CCND1 mRNA stimulating its IRES-dependent translation. In contrast, the chaperone function of La is inhibited by T389 phosphorylation by AKT1 suggesting that both the phosphorylation and the

ability of La to act as an RNA chaperone are critical to promote IRES driven translation of the CCND1 mRNA (Kuehnert et al., 2015). In addition, binding of La to proto-oncogene CCND1 has been also observed in cervical and prostate cancer cell lines (Sommer et al., 2011a,1). Moreover, aberrant expression of La was observed in squamous cancers of the head and neck where findings reveal that La drives migration and invasion (Sommer et al., 2011b). *In vitro* experiments showed that upon hLa depletion, cell proliferation, motility and invasiveness are reduced, followed by a downregulation of tumor promoting factors like β -catenin and matrix metalloproteinase 2 (MMP-2). Interestingly, further studies in head and neck squamous cell carcinoma indicate that depletion of La increases the sensitivity of cells to cisplatin and contributes to resistance by stimulating the translation of the anti-apoptotic factor Bcl2 (Heise et al., 2016). Finally, hLa has also been implicated in n hepatocellular cancer cells where it drives the epithelial to mesenchymal transition (EMT) through expression of the IRES-mediated target Laminin B1 (Petz et al., 2012b; Govaere et al., 2017).

1.4.RNase P (Ribonuclease P)

1.4.1. Function of RNase P and holoenzyme composition

RNase P is a multiple turnover enzyme responsible for the first and more important step of tRNA biogenesis, the correct formation of the 5' end of tRNAs. It hydrolyzes a phosphodiester bond in the pre-tRNA transcript by a single and accurate cleavage adjacent to the first base-pair of the acceptor stem, generating the 5'-mature tRNA and the 5' leader RNA fragment (Figure 1.16).

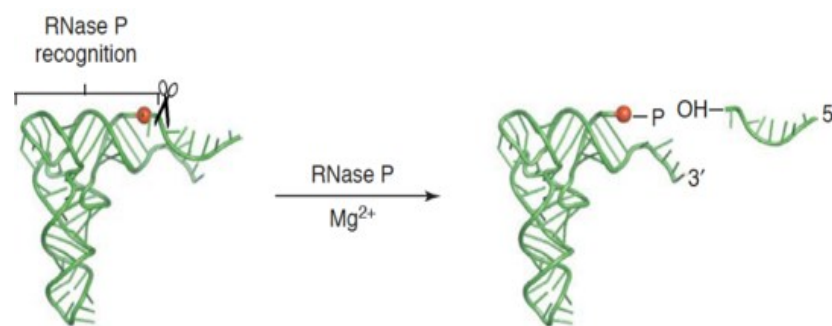


Figure 1.16. RNase P cleaves pre-tRNA generating a 5'-phosphate group and a 3'-OH.

This ribozyme has been characterized as “molecular ruler” as it relies on distance measurement for the tRNA recognition rather than a sequence-specific manner. More specifically, RNase P recognizes 12bp between the T-loop of pre-tRNA and the cleavage site, a universally conserved feature of all tRNAs (Wu et al., 2018). Moreover, for the catalysis, RNase P requires divalent metal ions on the active site, such as magnesium (Mg^{2+}), which play an important role in the non-specific charge neutralization, as well as in the enhancement of stabilization of the structure of pre-tRNA (Reiter et al., 2010).

Knowledge of detailed RNase P substrate specificity was enriched by analyses regarding the processing of additional substrates, apart from tRNAs. Thus, it has been found that RNase P has the ability to cleave multiple substrates like such as the 4.5S RNA (in *E. coli*) and long non-coding RNAs (lncRNAs), such as MALAT1 and MEN-b/NEAT1, which represent two striking examples of lncRNAs which contain a tRNA lookalike structure that is recognized and processed by RNase P (Peck-Miller and Altman, 1991; Sunwoo et al., 2008; Wilusz et al., 2008). Interestingly, the holoenzyme has been widely studied and isolated from organisms representing the three domains of life (Bacteria, Archaea, Eukarya) with the exception of *Nanoarchaeum equitans* due to the fact that in this organism the tRNA genes are transcribed without the 5' leader sequence (Randau et al., 2005). RNase P was first characterized almost 40 years ago in *E. coli*, and in bacteria consists of a single RNA subunit (M1 RNA, 350–400 nucleotides, 110–125 kDa) and a single protein (C5, 110 amino acids, 13 kDa) (Guerrier-Takada et al., 1983). However, as we move up the evolutionary ladder, RNase P exhibits a remarkable variability of its composition. The archaeal holoenzyme contains one RNA and five protein subunits while the human holoenzyme is more proteinaceous and includes an RNA (RPPH1) and ten protein subunits (Table 1.1) (Wu et al., 2018). During a long period of studies on RNase P, distinct 5' end repair activities have been also identified in organelles like chloroplasts and mitochondria. Intriguingly, it has been found that in these organelles RNase P activity is independent from a catalytic RNA subunit. In fact, for the 5' maturation of organellar tRNAs, a protein-only RNase P is responsible, an observation which raises questions on the evolutionary origin of this particular enzyme. As a result, two different RNase P holoenzymes can be detected in eukaryotes which act separately; one in the nucleus (nuclear RNase P) and a distinct one in mitochondria known as protein-only RNase P (mtRNase P).

which is composed by three protein subunits (MRPP1, MRPP2, MRPP3) (Holzmann et al., 2008; Walker and Engelke, 2008).

Table 1.1: RNase P subunits across the domains of life.

RNase P	Bacterial	Archaeal	Yeast (Nuclear)	Human (Nuclear)
RNA subunit	M1 RNA or P RNA (~400nt)	RPR	RPR1	RPPH1
Protein Subunits*	RnpA/ C5/ P protein (~13 kDa)			
		POP5 (~14 kDa)	POP5 (~19 kDa)	POP5 (~19 kDa)
		RPP29 (~15 kDa)	POP4 (~33 kDa)	RPP29 (~25 kDa)
		RPP30 (~25 kDa)	RPP1 (~32 kDa)	RPP30 (~29 kDa)
		RPP21 (~15 kDa)	RPR2 (~16 kDa)	RPP21 (~18 kDa)
		L7Ae/RPP38 (~13 kDa)	POP3 (~23 kDa)	RPP38 (~32 kDa)
			POP1 (~100 kDa)	POP1 (~115 kDa)
			POP6 (~18 kDa)	RPP20 (~16 kDa)
			POP7 (~16 kDa)	RPP25 (~21 kDa)
				RPP14 (~14 kDa)
				RPP40 (~35 kDa)
			POP8 (~15.5 kDa)	

* Proteins within the same row share sequence homology

1.4.2. Comparison of the evolution structure of the RNA subunits

Reports on RNase P over the years, have led to its characterization as a ribonucleoprotein complex with the RNA moiety being the catalytic subunit. Structural studies on RNase P RNAs (RPRs), from almost all organisms reveal similarities at the primary and the secondary structure, highlighting the presence of a universally conserved catalytic core in all enzymes. More specific, the RPRs is composed of two independently folding domains, the catalytic domain (C-domain) and the specificity domain (S-domain), which together cooperate to generate a functional tertiary structure (Figure 1.17). The C-domain is responsible for cleavage and recognizes the acceptor stem and the 3' CCA of pre-tRNA while the S-domain interacts with the T-loop of pre-tRNAs and is responsible for stabilization of substrate (Phan et al., 2021). Moreover, the RNA structural core includes five universally conserved regions (CR-I, CR-II, CR-III, CR-IV, CR-V), several coaxial stems (P; Paired region) and several internal loops (L). The CR-II and CR-III consists part of the S domain while in the CR-I, CR-IV belong to the C-domain. In addition, the coaxial stems of P2-P3, P1-P4

and P8-P9 represent conserved feature in all RPRs, with the P4 pseudoknot and surrounding joining regions to be the active site of the enzymes as the P4 helix is binding site of divalent metal ion (Figure 1.17) (Wu et al., 2018).

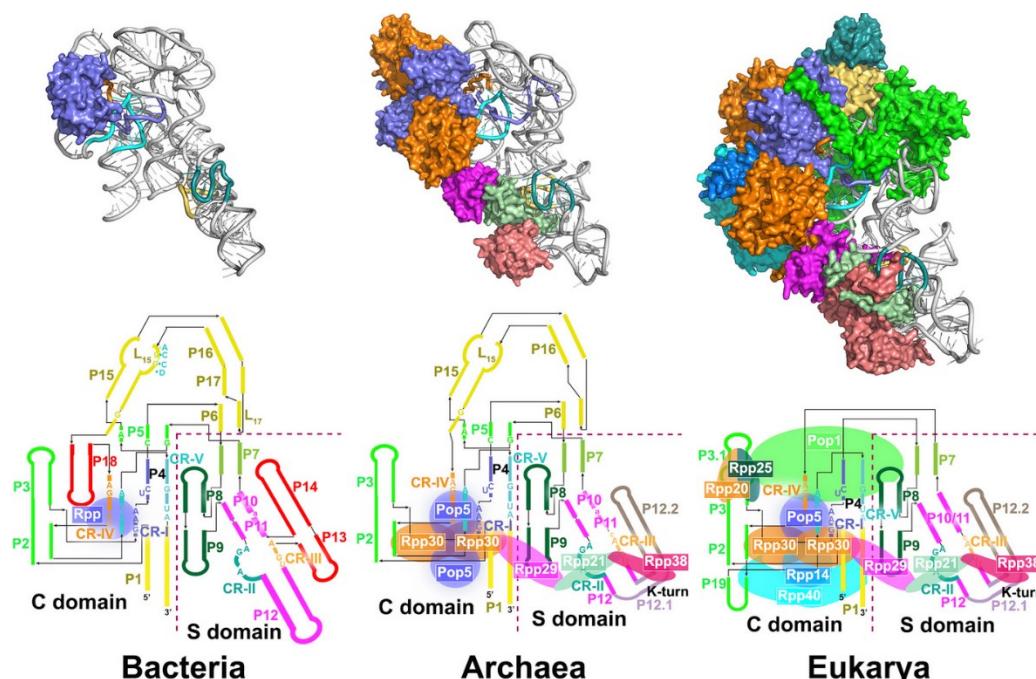


Figure 1.17. A structural evolutionary model of RNase P from bacteria to Eukarya (up) and secondary structure of RNase P RNAs in combination with protein subunits from all three kingdoms of life (down). Adapted from Wu et al., 2018.

During the evolution, from bacteria to archaea and eukaryotes, albeit the RPRs maintained their catalytic function, they lost some distinct RNA elements which are replaced by proteins, but also, they exhibited a remarkable slow turnover rate of cleavage. From bacteria to archaea, the ribozyme retained the majority of the RNA elements, especially in C- domain but lost some paired region (P13-P14) in the S- domain, which stabilizes the distance between the two tRNA-binding anchors in bacterial RNase Ps (Reiter et al., 2010; Wu et al., 2018; Wan et al., 2019; Phan et al., 2021). These regions were replaced by the RPP21 and RPP29 heterodimer. In addition, the P12 stem of bacterial RNA component evolved into a double-L-shaped structure with a K-turn that is recognized by the acquired protein RPP38 in archaeal RNase P. In contrast, from archaea to

eukaryotes, the C-domain has lost two major RNA elements. Firstly, the loop L15 that is essential for pre-tRNA recognition in bacterial RNase P and stems P5-P6-P15 of archaeal RNP, was compensated by a N-terminal highly basic motif of a POP1 protein and secondly, the P3 stem has more complex structure in eukaryotes which was interrupted by two internal single-stranded loops that are recognized by a co-evolved protein heterodimer RPP20-RPP25 (Wu et al., 2018) (Figure 1.17).

Finally, an important difference between bacterial and archaeal/eukaryotic RNase P is the RNA layers configuration. In bacteria, the RNA folds into a compact and double-layered configuration while an extended and single-layered conformation of RNA is present in archaea and eukaryotes as the second layer (which contains the P13, P14, P18, L14 and L18) has completely missed (Figure 1.18). The absent of this layer may explain the slower turnover rates of the RNA. Despite overall changes, the RNA-based catalysis of RNase Ps enzymes is ensured by RPPs in all domains of life, hold up the RNase P to be a relic of the RNA world (Phan et al., 2021).

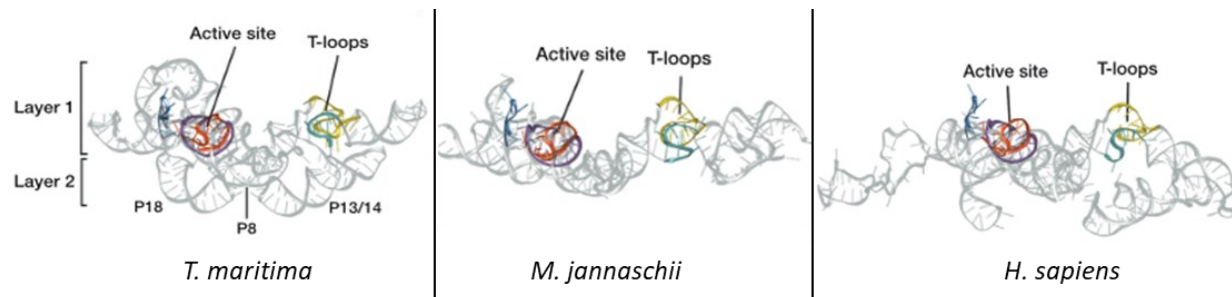


Figure 1.18: Structure of RNase P RNA subunit showing the RNA layers configuration difference between the different organisms across the three domains of life. Adapted from Phan et al., 2021.

1.4.3. Bacterial RNase P

In prokaryotes, RNase P is a complex of an RNA subunit (300-400nt) one small protein subunit (14kDa). The bacterial RNase P RNA subunit (RPR) was one of the first catalytic RNAs identified and the first found to act as a multiple turnover enzyme *in vivo* (Krasilnikov et al., 2003). The sequence and the secondary structure of RPRs can vary among prokaryotes thus they are classified into two major subtypes, the type A (ancestral) which consists of the most common type among bacteria, and the type B (from *Bacillus*) which are present in gram positive bacteria

(Phan et al., 2021). In some *Chloroflexi*, a hybrid of types A and B, named type C, has been found however, structural variation in this group has not been investigated. Comparative structural study on both major P RNA types reveal the existence of a common structural core within a somewhat different scaffold (Figure 1.19).

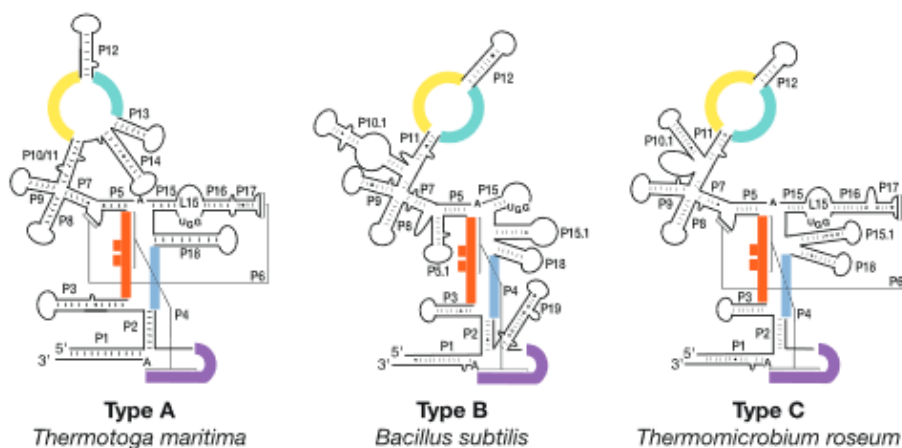


Figure 1.19: Secondary structure of bacterial RPRs subtypes among prokaryotes. Adapted from Phan et al., 2021.

The main differences between type A and type B P RNA are the presence of P16, P17 and P6 and the lack of P5.1, P10.1, P15.1 and P15.2 in type A. Although, some RNA elements are absent, both structures are quite similar in region involved in the catalytic function and in substrate recognition Figure 1.19) (Torres-Larios et al., 2005). Despite the variations presented in RPRs, the bacterial RNase P Protein (RPP) is highly conserved among prokaryotes (Tsai *et al.*, 2003). Although, it consists of 10% by mass of holoenzyme, is essential for *in vivo* catalysis (Guerrier-Takada et al., 1983). It has been also found that the sole protein plays important role either in substrate recognition, either in cleavage rate or in the stabilization of the tertiary structure of the RNA subunit (Jarrous and Gopalan, 2010). It should be noted that the crystal structure of holoenzyme, from a thermophile bacterium *T. maritima*, verifies important interactions between the protein component and the pre-tRNA as it has been found that the protein subunit can facilitate the processing of CCA-less tRNAs (Guerrier-Takada et al., 1984). Interestingly, almost 10 years ago, the publication of the bacterial crystal structure of RNase P, in complex with tRNA, highlighted the general catalytic mechanism of ribozyme as well as the binding mode of pre-tRNA

(Reiter et al., 2010). In the study, the pre-tRNA^{Phe} was used as the substrate for structure determination, however, only the mature tRNA was detected in the crystals due to it being cleaved by RNase P. Thus, to detect the interactions of the 5' leader the crystals were soaked with a short oligoribonucleotide. The structure showed that the 5' leader interacts with the protein component at the 5' end and nucleotides -1 to -3, at the 3' of the leader, interact with the P-RNA (Figure 1.20). Also, the protein interacts with the CR-IV and CR-V regions, the P15 stem, and the P2/P3 helix. Deciphering the interactions of the bacterial holoenzyme with tRNA, it should be noted that the crystal structure agree with previous biochemical studies, according to that the P RNA and tRNAs interact via stacking bases of the CR-II, CR-III and P11 helix with the T-loop and D-loop (G/U19 and C56, respectively), with an A-minor interaction between P4, the acceptor stem and finally *via* the formation of canonical base pairs at the 3' CCA with the L15 (Reiter et al., 2010).

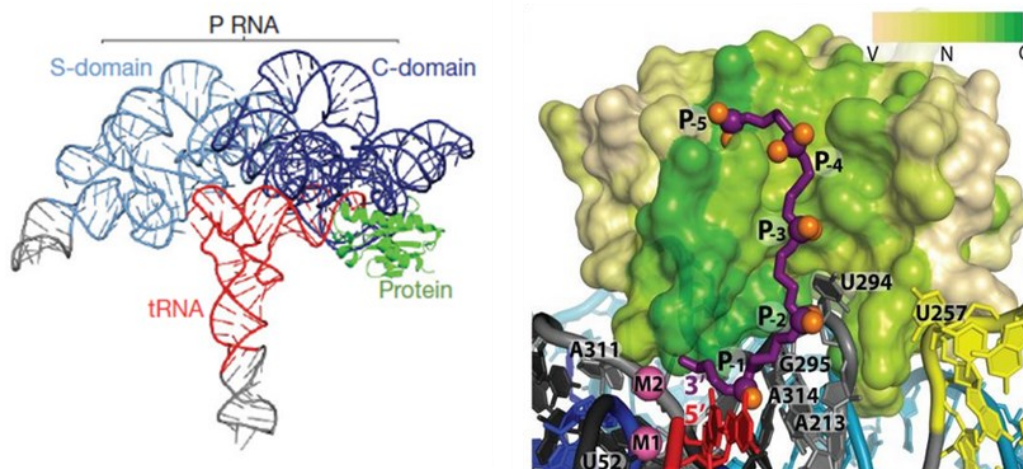


Figure 1.20: Crystal structure of bacterial RNase P holoenzyme in present of tRNA (left) and a surface representation of the protein component with 5' leader of pre-tRNA molecule (purple; right). Adapted from Reiter et al., 2010.

It is noteworthy to mention that several studies indicate that bacterial RNase P has been found to be targeted by antibiotics like puromycin, aminoglycosides or macrolides suggesting that it could serve as a molecular target for drugs against pathogens (Toumpeki et al., 2008).

1.4.4. Archaeal RNase P

Although bacterial RNase P holoenzyme consists of a single RNA subunit and one protein, archaeal RNase P is more complex ribonucleoprotein which is consisted of one RNA (RPR) and five protein subunits (POP5, RPP21, RPP29 RPP30 and L7Ae/RPP38) (Hall and Brown, 2002; Esakova and Krasilnikov, 2010; Jarrous and Gopalan, 2010). Based on secondary structure, three types of RPRs are found in archaea, the A-type (very similar to the bacterial RPR), the M-type and the P or T-type. Of these A-type and M-type exhibit endoribonuclease activity *in vitro*, with the difference that only the type A shows activity itself under high salt conditions while type M requires the presence of the protein (Figure 1.21). The type P, albeit is more extreme case, it shows itself *in vitro* activity. Among the three types of RNAs, we notice the small differences: the type A lacks P13, P14 and P18, the type M (from *Methanococcales*), expect from P13, P14 and P18, it also lacks P6, P16, P17 and P8 (is some cases) (Phan et al., 2021). On the other hand, the type P (from *Pyrobaculum aeophilum*) also refers as type T (from *Thermoproteaceae*) is very similar with the type A but lacks the S- domain (Figure 1.21). This type might be an intermediate form towards an organism lacking RNase P activity as is the case in *N. equitans*.

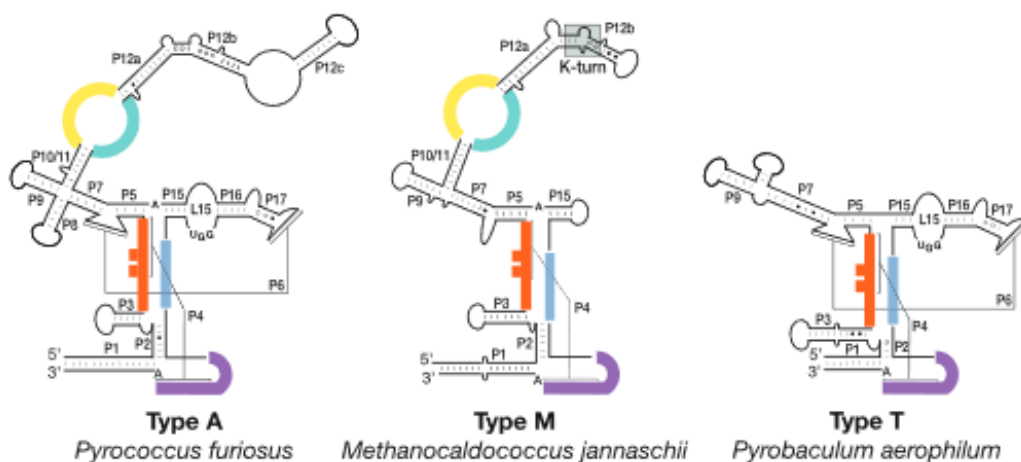


Figure 1.21: Secondary structure of archaeal RPRs subtypes. Adapted from Phan et al.,2021.

Although several studies had deciphered the archaeal RNase P, the complete picture of the holoenzyme has been described recently. The recently available Cryo-EM structure of the RNase P from *Methanocaldococcus jannaschii* completed the picture of the RNase P RNP repertoire.

Interestingly, the archaeal holoenzyme adopts a rigid dimeric conformation with two-fold symmetry, containing ten protein subunits and two catalytic RPRs (Wan et al., 2019). None of these proteins are homologous with the sole bacterial protein, however, the structure of POP5 protein resembles the structure of its bacterial counterpart.

In the RNP, the protein subunits function as two complexes one (POP5-RPP30) 2 heterotetramer and two RPP29-RPP21-L7Ae heterotrimers forming a long decamer. The structural analysis of the holoenzyme confirmed previous footprinting and kinetic studies that had showed that RPP21 and RPP29 (or Pop4) interact with S-domain and confer substrate affinity while the POP5 together with RPP30 bind to C-domain and enhance the catalytic activity (Figure 1.22 a and b). Moreover, the L7Ae/RPP38 protein binds the k turn motif in S-domain and it has been found that increases the optimal reaction temperature and k_{cat}/K_m (by ~360-fold) for pre-tRNA cleavage (Cho et al., 2010).

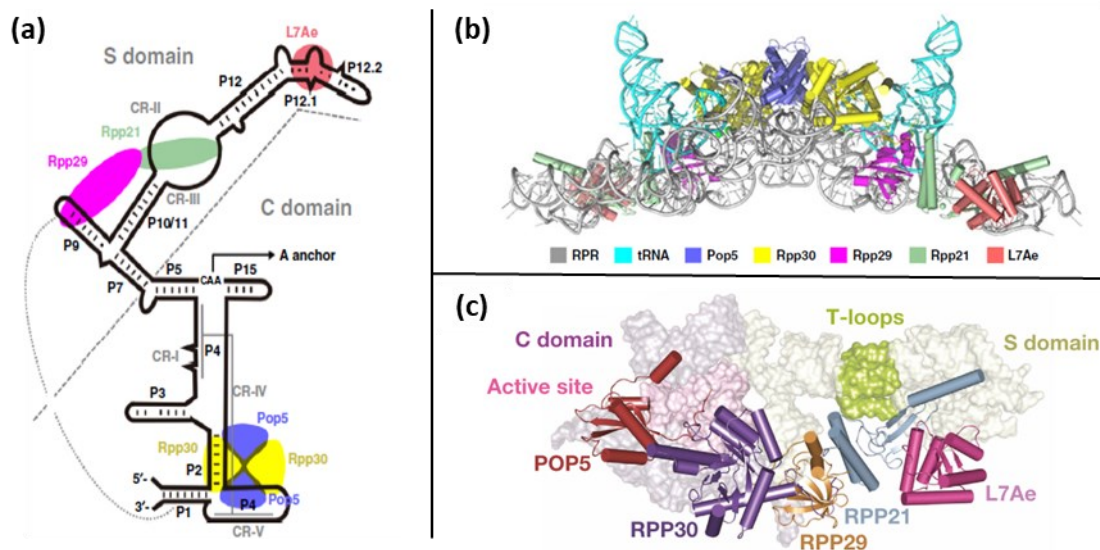


Figure 1.22: (a) Secondary structure of RPR from *Methanocaldococcus jannaschii* (b) the decamer conformation in complex with tRNA (c) protein subunits contact in archaeal RNase P. The RNA subunit is depicted in surface presentations while the proteins as cartoon. Adapted from Wan et al., 2019.

The archaeal RNase P-tRNA interactions presented in the Cryo-EM are very similar to that of bacteria as it still maintains the double RNA-based anchors for substrate binding with the difference that a complicated protein complex is necessary for the stabilization mechanism of the anchors. In fact, the three-nucleotide linker located between the P5 and P15 of the C-domain

interact with the acceptor stem of tRNA while the CR-II and CR-III of the S-domain directly interact with T and D loop of the pre-tRNA. In this mechanism, important role plays the protein POP5 which contributes to tRNA binding, stabilizing the CR-IV region similar to bacterial RPP-RPR CR-IV interaction and the RPP21 protein which directly interacts with the S domain that engaged T-loops and the pre-tRNA elbow (Figure 1.22c). It is noteworthy mentioned that, the equivalent sequence of the bacterial RPR that interacts with the CCA of the tRNA is dispensable in *M.jannaschii* possibly because this organism encodes CCA-less tRNAs and this tRNA recognition element was lost during evolution since the CCA trinucleotide is added post-transcriptionally after the 5' end processing has occurred. This observation is in agreement with the finding in the structure of human RNase P where it does not interact with the 3' end and also does not have a CCA recognition element on its RNA (Wan et al., 2019).

1.4.5. Eukaryotic RNase P

Nuclear RNase P has been isolated from both yeast and human but also has been detected from a wide range of eukaryote. In all eukaryotes, RNase P holoenzyme is more proteinaceous and includes one essential catalytic RNA subunit (smaller than its bacterial counterpart) and nine to eleven protein subunits. In yeast, RNase P is consisted of RNA component (RPR1 RNA) and nine additional protein subunits while in human, is consisted of RNA component (RPPH1 RNA) and eleven additional protein subunits (Shaukat et al., 2021). The RNA subunit from eukaryotes retains their catalytic core, however it is observed a variable P19 stem and the absence of P15 stem. Phylogenetic analysis showed that P15 stem present in most fungal lineages but not generally found in other eukaryotes thus, its absence results the stretching of the P19 hairpin. Similarly, it happens between the different types of bacterial and archaeal RNA subunit. On the other hand, in S- domain, it is present differences between the P8 and P12 stems. An additional P8 stem, termed as P8' or eP8 (eukaryal P8) exist in *S. cerevisiae* whose exact role has not determined yet. Moreover, except for yeast the majority of eukaryotes, has a larger P12 stem including the conserved structural motif within their P12 stems, the k-turn (Figure 1.23). Interestingly, reconstitution of yeast RNase P was performed by complexing RPR to RPP20-RPP25, followed by addition of POP1, RPP14-POP5-RPP30, and RPP29. In yeast, depletion of each protein

subunit, with exception of Pop3p/Ppp38, could lead to decrease in expression of mature RPR1 RNA possibly due to destabilization and impairment of maturation process of RNA (Xiao et al., 2002).

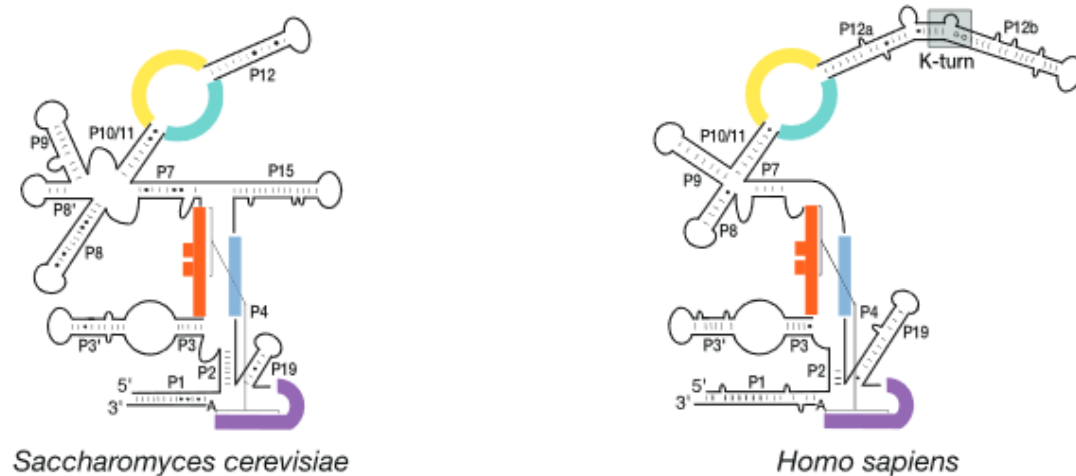


Figure 1.23: Secondary structure of eukaryotic RNase P RNA subunit from *S. cerevisiae* (left) and human (right). Adapted from Phan et al., 2021.

In human, RNase P is consisted of one catalytic RNA subunit (RPPH1) and several proteins subunits (RPP14, POP5, RPP20, RPP21, RPP25, RPP29, two copies of RPP30, RPP38, RPP40, and POP1). In the absence of protein components, the RPPH1 exhibits a weak endonucleolytic activity compared with its bacterial counterparts (Mann et al., 2003). Besides the essential catalytic RNA subunit which are transcribed by RNA polymerase II or III, two more putative pseudogenes have been detected to encode RNase P RNA subunits of smaller size (RPPH1-2P and RPPH1-3P). Similar genes were found differentially expressed in various murine tissues, raising more questions on the uniform composition of the holoenzyme in mammals (Li and Williams, 1995). The three murine paralogues exhibit >83% similarity with MmuRPPH1 while the human RPPH1-3P shows a 61,8% similarity with HsaRPPH1 indicating the possibility for binding RPPs and thus the formation of novel RNase P variants. RPPH1-2P shows 42,5% similarity so its RPP binding ability may be more limited (Shaukat et al., 2021).

Regarding the structure of human nuclear RNase P, a recent detailed Cryo-EM holoenzyme was published, in complex with tRNA^{Val} and highlighted the importance of protein subunits in the assembly and the function of holoenzyme (Wu et al., 2018). Interestingly, the structure revealed

a hand-shaped protein clamp tightly wrapped around the RPPH1. The RPPH1 is mainly located in one side and all the protein components on the other side. The protein moiety of RNase P consisted of protein three subcomplexes and a single POP1 polypeptide. The POP1 together with the heterodimer RPP20-RPP25 consist of the finger module of RNase P, the complex POP5-RPP14-(RPP30)₂-RPP40 characterized as the palm and the RPP21-RPP29-RPP38 form the wrist of the hand-shaped protein clamp (Figure 1.24).

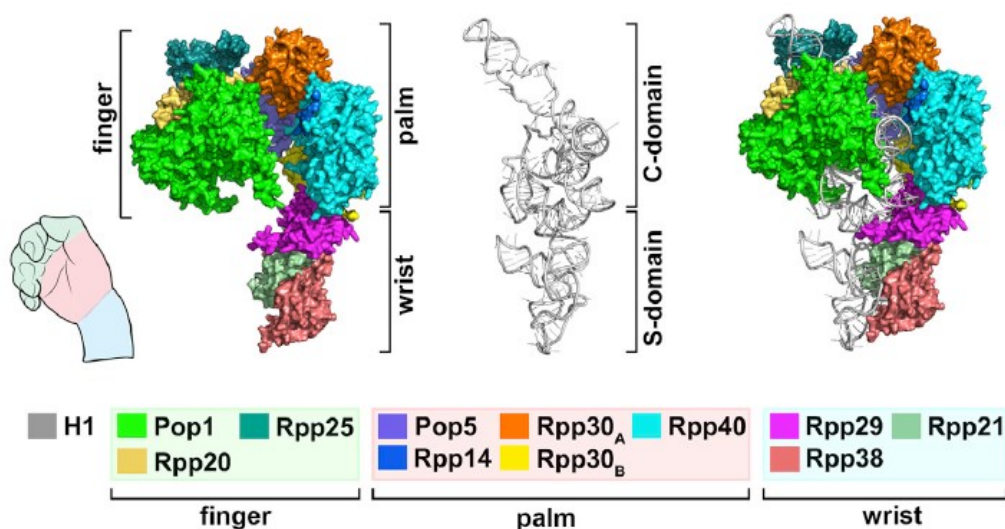


Figure 1.24: The cryo-EM model of human RNase P enzyme. Adapted from Wu et al., 2018.

All proteins act as scaffold to stabilize the RPPH1 in an optimal conformation for tRNA binding. More specific, the finger and palm module stabilize the C-domain of H1 RNA while the S-domain makes extensive interactions with wrist module. Importantly, the POP1 protein interacts with CR-IV and stems P1, P4, P7, P8 and P19 of H1 whereas RPP20 and RPP25 binds to P3 stem. The palm module, consisted by the heteropentamer, stabilizes the pseudoknot of H1 RNA. The POP5 and RPP14 mediate interactions with POP1 and RPP40 while the POP5 and the one copy from RPP30 (RPP30B) act synergistically with POP1 to enclose the pseudo-knot and stabilize the RNA catalytic center. In addition, the POP5 interacts and stabilize the 5' leader of pre-tRNAs similar to bacterial protein, although it is not considered homolog of the bacterial protein. In contrast, the wrist module mainly interacts with S-domain and the RPP21 and RPP38 together stabilize its

double-L-shaped structure conformation. Whereas the hRPP29 interacts with hRPP21 and hRPP38 and forms the bridge between the C domain and S domain of RPPH1 (Wu et al., 2018).

Of note, RNase P doesn't recognize specific RNA sequence patterns, but rather functions as a molecular ruler (Zhang and Ferré-Damaré, 2016) It anchors the tRNA elbow and the acceptor stem measuring the distance of 12bp which consists universally conserved feature from all tRNAs. In human, the recognition of tRNA elbow by RPPH1 is mediated *via* interactions of the T- and D-loops with the CR-II and CR-III of the S-domain and the anchoring of the acceptor stem through coordination of the active site (stem P4) and POP1 (Figure 1.25) (Wu et al., 2018)

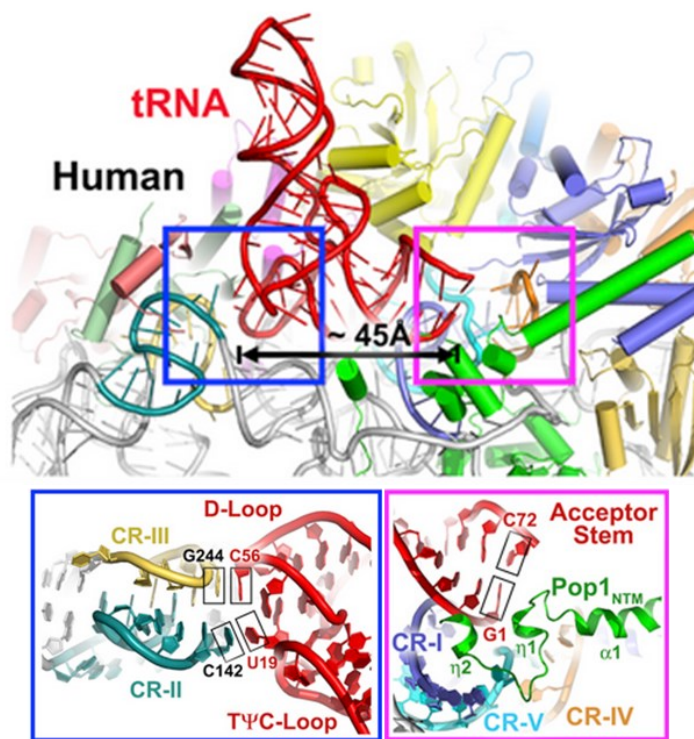


Figure 1.25: Model of tRNA recognition by human RNase P. Adapted from Wu et al., 2018.

1.4.6. RNase P enzyme from organelles

For many years, the nature of RNase P from organelles remained elusive with reports showing that it had very low RNA content. In some mitochondria and plastids, the RNase P are related to those of the prokaryotes (one catalytic RNA and one protein) from which these organelles originated. For example, the mtRNase P from *S. cerevisiae* is composed of an RNA subunit

(Rpm1r; 423 nt) which is encoded by the mitochondria genome and a single nuclear encoded protein subunit (Rpm2p; 105 kDa) (Stribinskis et al., 1996). However, some organisms additionally or alternatively encoded a Protein Only RNase P or PROteinaceous RNase P enzymes termed as PRORPs. PRORPs protein family possesses RNase P endonucleolytic activity and has been found in many plant organelles and also in human mitochondria. Studies on PRORPs from *A. thaliana* reveal the existence of three proteins, the PRORP1-3. PRORP1 act as single protein and exhibits RNase P activity in both mitochondria and chloroplasts of *A.thaliana* whereas PRORP2 and PRORP3 are active in the nucleus (Gutmann et al., 2012; Gobert et al., 2013; Pinker et al., 2013). Homologues to PRORPs were also exist in human mitochondria termed as MRPP3. However, MRPP3 cannot acts on its own and requires the presence of MRPP1 and MRPP2 for catalysis (Holzmann et al. 2008; Walker and Engelke 2008) (discussed below). Although, PRORPs were thought to be widespread among eukarya, later studies identified a protein-only RNase P enzyme initially in the hyperthermophilic bacterium *Aquifex aeolicus* and later in other prokaryotes. These enzymes called HARPs (Homologs of *Aquifex aeolicus* protein-only RNase P) and albeit more studies are needed to establish whether HARPs are indeed genuine RNase P enzymes, it has been found that HARP can remove the 5' leader from pre-tRNAs *in vitro* and successfully rescue the lethal deletion of RNase P RNP in *E. coli* and yeast (Nickel et al., 2017).

Human Mitochondrial RNase P (mt-RNase P)

Human mitochondrial RNase P, instead of being a typical ribonucleoprotein, is a protein-only enzyme consisting of three protein subunits the MRPP1, MRPP2 and MRPP3 with the latter being responsible for the catalytic activity (Holzmann et al., 2008). Interestingly, all proteins are nuclear encoded and beyond their participation in the enzyme composition, they exhibit additional roles. MRPP1 protein (also known as TRMT10C) is an RNA-methyltransferase, responsible for methylation (m¹A9 and m¹G9 methyl groups) of mitochondrial tRNAs (mtRNAs). MRPP1 depends on MRPP2 subunit binding to become functional (Lopez Sanchez et al., 2011; Vilardo et al., 2012). On the other hand, MRPP2 is a dehydrogenase which is involved to a wide variety of processes in mitochondria like isoleucine degradation, metabolism of sex hormones, β -oxidation of fatty acids, metabolism of neuroactive steroids (Esakova and Krasilnikov, 2010, 2; Vilardo and Rossmanith,

2015). Additionally, MRPP1 and MRPP2 regulate the expression of some mitochondrial long non-coding RNAs as well as the ribosome assembly resulting in defect of the translation of proteins that form the respiratory chain complexes. Mutations on these two protein components lead to severe multi-system disorders and mitochondrial diseases like maternally inherited essential hypertension, mitochondrial myopathy (Deutschmann et al., 2014; Sen et al., 2016; Reinhard et al., 2017; Karasik et al., 2021). Finally, MRPP3 is the catalytic subunit of mtRNase P and requires the binding of MRPP1/2 complex to process the 5' end of mt-pre-tRNA (Reinhard et al., 2017). Studies dedicated to characterizing the importance of these protein subunits, in mice and *Drosophila* showed that mutations or depletion of each individual subunit led to disruption of both 5' and 3' end processing of tRNAs and lethality (Saoji et al., 2021, 2022).

Although so far, the overall mtRNase P holoenzyme architecture had not been determined, a recent Cryo-EM study of human mitochondrial RNase P in complex with mitochondrial pre-tRNA-Tyr (7ONU in Protein Data Bank) reveals a novel composition of the holoenzyme (Figure 1.26).

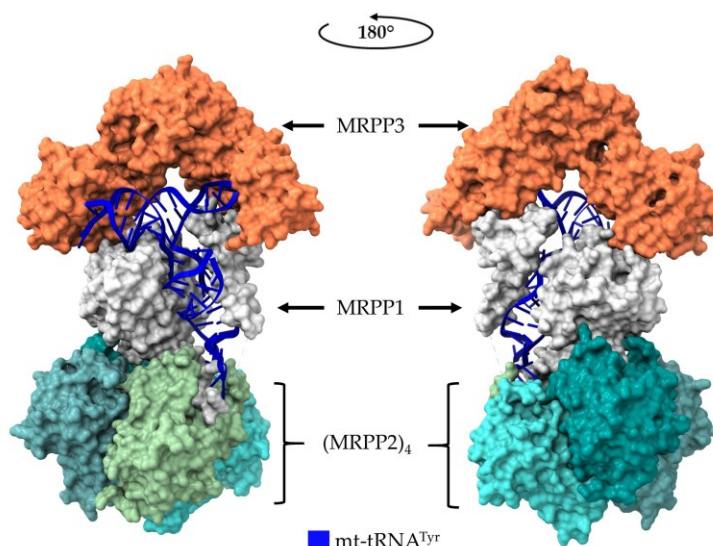


Figure 1.26 Cryo-EM structure of human mitochondrial RNase P in present with mt-tRNA^{Tyr} (PDB ID: 7ONU). Adapted from Bhatta et al., 2021.

The holoenzyme structure showed a hetero-hexamer complex, where the anticodon stem-loop of mt-tRNA is surrounded by a homo-tetrameric organization of MRPP2 which in turn interacts with a single MRPP1 protein subunit. The MRPP1 wraps around the tRNA molecule and make

extensive interactions with the catalytic subunit, MRPP3 which is located on top of the complex and binds the tRNA “elbow” and the acceptor stem of tRNA (Figure 1.26) (Bhatta, A., Dienemann, C., Cramer, P., Hillen, H.S., 2021). The structure reveals that mitochondrial RNase P conformation extends along the tRNA body, differently from their nuclear counterparts which recognize mainly the central spine of the tRNA (elbow and acceptor stem). This observation may indicate that mt-tRNAs require extensive protection, stabilization of their conformation and simultaneously post-transcriptional modifications.

1.4.7. A comparison of RNase P with RNase MRP and Telomerase

The eukaryotic nuclear RNase P has also evolved a closely related paralogue enzyme, the endoribonuclease RNase MRP (mitochondrial RNA processing). RNase MRP was first described by Chang and Clayton in 1987, as endoribonuclease that cleaves the primers for DNA replication in mitochondria, however later studies shown that this enzyme is mainly localized in nucleolus within gets involved in pre-rRNA processing by cleaving the internal transcribed spacer 1 (ITS1) at the A3 site (Schmitt and Clayton, 1993). Recently, it has been also found that participates in mRNA processing, either by cleaving in the 5'UTR of Cyclin B2 mRNA or is recruited to m6A containing mRNAs promoting indirectly their endoribonucleolytic cleavage, *via* the interactions with YTHDF2 (YTH N6-Methyladenosine RNA Binding Protein 2) and HRSP12 (2-iminobutanoate/2-iminopropanoate deaminase) (Gill et al., 2004). YTHDF2 and HRSP12 cooperatively interact with each other and recruit RNase P/MRP for cleavage. This cleavage destabilizes mRNAs and leads to their degradation by XRN1 and the cytoplasmic exosome (Park et al., 2019).

RNase MRP is absent from bacteria and archaea, and it has been proposed that during evolution, the RNase P RNA gene split into two leading to RNase MRP (Esakova and Krasilnikov, 2010). The RNA subunit of RNase MRP, termed as RMRP, folds into a structure similar to RPPH1 and is also responsible for RNA cleavage. Actually, RMRP is consisted of both C and S domain including the conserved regions CR-I, -IV -V and the P3 helix of C domain, however, the S- domain has not similarities between two enzymes that may explain their distinct substrate specificities (Figure 1.27) (López et al., 2009; Esakova and Krasilnikov, 2010; Perederina et al., 2010).

Mutations in the promoter of RMRP gene or within the RNA have been associated with many diseases including dwarfism and cartilage hyperplasia (Hermanns et al., 2006; Steinbusch et al., 2017; Vakkilainen et al., 2019).

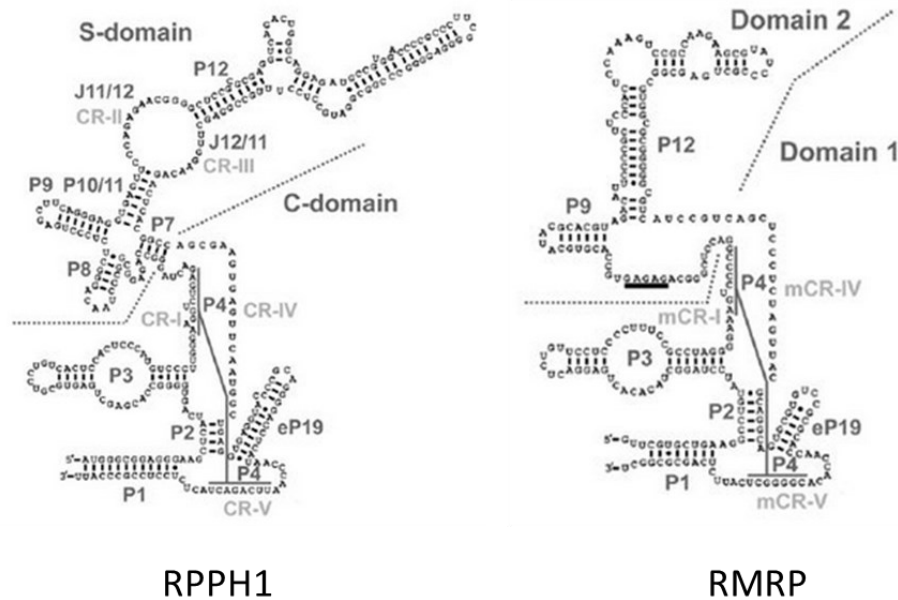


Figure 1.27. Secondary structure of RNA subunits of human RNase P (right) and RNase MRP (left). Adapted from Esakova et al., 2010.

Moreover, the two enzymes have a considerable overlap in protein subunit composition as they are shared at least seven common proteins. Interestingly, in human, RNase MRP shares nine out of ten protein subunits (except for RPP21) with RNase P (Esakova et al., 2008; Perederina et al., 2010; Shaikat et al., 2021). Of note, in RNase MRP, crystal structure of human RPP20-RPP25 proteins in complex with the RMRP showed their heterodimerization and binding to the P3 domain. The specific binding of RPP20 and RPP25 may stabilize the P3 stem (Yin et al., 2021).

Besides RNase MRP, yeast RNase P shares also three protein subunits with yeast telomerase (yeast homologs of RPP20, RPP25 and POP1). These subunits have been reported to bind at stem-loop region of telomerase RNA (known as TLC1 in yeast) with similar structure with P3 domain of RNase P, known as the CS2a/TeSS domain (Lemieux et al., 2016; Garcia and Zakian, 2020; Garcia et al., 2020). *In vitro* experiments shown that Pop proteins enhance the telomerase activity and play role in the stability and the transportation of RNA components from cytoplasm to nucleus,

however, the exact role of Pop proteins in telomerase remains unknown. Apparently, although the yeast telomerase seems to have a common evolutionary origin with RNase P, the human RNase P and telomerase RNPs do not share any composition similarities (Collins and Mitchell, 2002; Lemieux et al., 2016).

1.4.8. The protein subunits of RNase P and their roles beyond tRNA maturation

The diversity of RNase P enzymes indicates the transmission of structural and functional roles from the RNA subunit in bacteria to different proteins that emerged during evolution to form the archaeal and eukaryal RNase P. In fact, beyond the synthesis of the RNPs, some of the subunits can dissociate from the holoenzyme to carry out alternative tasks in cellular process like chromatin organization, transcription and translation regulation, either as individual components or as part of larger complexes (Table 1.2). The role of each protein subunit that contribute either individually either with other proteins or even with the RNA subunit is analysed below.

Table 1.2. RNase P RNP subunits and additional roles

Subunits	Additional roles	Subunits	Additional roles
RPP14	Exhibits 3'→5' exoribonucleases activity	RPP29	Promotes DNA repair through HDR, Nucleosome remodeling
POP5	Unknown	RPP30	Affects piRNAs transcription and tRF biogenesis
RPP20	ATPase activity, promotes transcription by RNA Pol I and III, binds to chromatin of rDNA genes, Th/To autoantigen	RPP38	Th/To autoantigen
RPP21	Promotes DNA repair through HDR	RPP40	Unknown
RPP25	Promotes transcription by RNA Pol I and III, binds to chromatin of rDNA genes, Th/To autoantigen	POP1	Transcription, Th/To autoantigen

RPP14

The *RPP14* gene is found in locus Ch3p14.3. This gene encodes transcripts which are bicistronic with a conserved downstream open reading frame producing both the hydroxyacyl-thioester dehydratase type 2 (HTD2) and the RPP14 protein subunit. hRPP14 is 14kDa protein and is detected in nucleoplasm and the dense fibrillar region of nucleoli (Jarrous et al., 1999a). Although RPP14 has not contain a nucleolar localization signal, it is suggested that is translocated into the nucleolus through a “piggyback” mechanism (Jarrous et al., 1999b). Homologue of RPP14 does not exist in archaeal and yeast however, based on structural studies the archaeal POP5 had evolved into two different eukaryal proteins (POP5 and RPP14 in human RNase P). The partners of RPP14 protein subunit have studied by two-hybrid system screening which reveal interactions with the OIP2/EXOSC8 (opacity-associated protein/Exosome component 8) and the LIMD1 (LIM domain-containing protein 1). hRPP14/OIP2 complex functions as exonuclease with 3'→5' exoribonuclease activity *in vitro* (Jiang and Altman, 2001). Although, OIP2 is not well characterized, it is considered as component of the RNA exosome complex, and both OIP2 and RPP14 contain an RNase PH domain which has an affinity for U- and AU-rich sequences (Jiang and Altman, 2001; Anderson et al., 2006). Moreover, RPP14 binds to LIMD1 which belongs to a DNA-binding family. LIMD1 is involved in different cellular processes including cytoskeletal organization, repression of gene transcription, cell differentiation, proliferation and migration and miRNA-pathway. However, further investigation should be conducted in order to understand the binding with RPP14 (Jiang and Altman, 2001; Bai et al., 2011).

POP5

The human POP5 protein subunit is encoded by POP5 gene which is mapped to chromosome 12q24.31. Similar to RPP14, hPOP5 is transported to the nucleus and nucleolus by a “piggyback” mechanism where it associates with the partially assembled RNase MRP/RNase P ribonucleoprotein complexes (van Eenennaam et al., 2001). Homologues of POP5 protein are widely distributed among organisms and very similar in size, with the exception of fungal POP5 that has an insertion of ~25 amino acids at the N-terminal end (Rosenblad et al., 2006). However,

beyond the participation of POP5 in holoenzyme, an alternative role has been not yet determined.

RPP20

In human, *POP7* gene is mapped to chromosome 7q22.1 and encodes the RPP20 protein also known as RPP2 or RPP20. Interestingly, RPP20 has been also found localized in nucleoli after heterodimerization with hRPP25 (Welting et al., 2006). Both RPP20 and RPP25 belong to the Alba-like superfamily (Acetylation lowers binding affinity), that has been characterized as multifunctional proteins as they participate in various processes like RNA stability and metabolism, genome organization, transcriptional and translational regulation (Goyal et al., 2016). However, only the RPP20 has been characterized as an ATPase in HeLa cells (Li and Altman, 2001). This activity is not required for RNase P function, but it is likely that RPP20 uses the ATP for other intracellular functions like chromatin remodeling (Li and Altman, 2001). Several studies showed that RPP20 binds on chromatin of active rDNA genes. ChIP analysis revealed that it binds on promoter regions of 18S and 28S rDNA (Reiner et al., 2008). The binding of RPP20 in rDNA targets is performed in a cell cycle-dependent manner and specific, RPP20 is recruited to rDNA at early G1 phase (Reiner et al., 2008). Moreover, *in vivo* experiments with a conditional lethal yeast strain of Rpp2, a yeast homolog of RPP20, showed an accumulation of pre-tRNA molecules and defects in the processing of 35S rRNA. Actually, in *S. cerevisiae*, Pop7 represents an essential protein (Stolc et al., 1998).

Regarding the interacting partners of RPP20, the potential interactors are the RPP25, the Hsp27 (heat shock protein 27), SMN (Survival Motor Neuron), and KIAA0065 (Zinc finger protein 33A) with the latter having a, so far, unknown role (Jiang and Altman, 2001; Hua and Zhou, 2004). RPP20 strongly interacts with RPP25 and together bind to P3 subdomain, a conserved feature found in three RNP complexes (RNase P/MRP/Telomerase) (Yin et al., 2021). The specific binding of both proteins was accompanied by the increased complexity of P3 domain. Structural studies in eukaryotic organisms, including yeast and human, showed that P3 stem has more complex structure, longer and unpaired internal loops, compared to prokaryotes which consisted of one helix. Thus, proteins were acquired to stabilize the P3 stem but also may play role to an accurate

holoenzyme assembly or could confer in substrate specificity. This hypothesis may be exemplified in yeast, where deletion or mutations in specific sites of the P3 domain could reflect the dispensability of the protein subunits. More specific deletion of P3 hairpin of RMRP, that is recognized by Po6 (RPP20 homologue), leads to reduced survival, while deletion or mutations at the end of P3 domain, that Pop7 (RPP25 homologue) binds, does not cause any defect (Lindahl et al., 2000; Ziehler et al., 2001; Li, 2004; Perederina and Krasilnikov, 2010, 3; Perederina et al., 2010). The integration of the RPP20 and RPP25 into the RNPs in eukaryotes and the dual affinity of the Alba-like family to bind RNA and DNA could unveil a key event in the evolution of the RNPs that participate, and the acquirement of new abilities and functions through binding to chromatin in the nucleus.

In addition, a yeast two hybrid screening as well as pull-down experiments showed a complex formation between RPP20 with Hsp27. Hsp27 is an ATP-independent chaperone which is induced by oxidative stress, during aging or in cancers (Alderson et al., 2019). Interestingly, during the isolation of RNase P holoenzyme from HeLa cells, Hsp27 is enriched in active fractions while it dissociates from the complex after increasing of the washing steps without RNase P lose its activity. However, although this chaperone is not necessary for RNase P, it can enhance its activity when is added to the RNase P reaction mixtures (Jiang and Altman, 2001). Moreover, co-immunoprecipitation experiments have shown that RPP20 interacts with SMN protein both in human and *Drosophila*. SMN proteins have been involved in neurodegenerative disorders, like spinal muscular atrophy (SMA) and also are involved in assembly of snRNP (small nuclear ribonucleoproteins) or play role in rRNA processing through interaction with fibrillarin, Sm or Sm-like (Lsm) proteins. Immunofluorescence experiments showed that in response to cellular stress like heat shock and UV irradiation, both proteins are co-localized in the cytoplasm in punctuated structures called stress granules (SGs). The exact role of both proteins accumulation in the granules is still elusive (Hua and Zhou, 2004).

RPP21

Human RPP21 is encoded by the *RPP21* gene which is mapped to the locus Ch6p22.1 and resides in the class I gene cluster of the major histocompatibility complex (MHC). hRPP21 is the

unique in RNase P holoenzyme as it has not been found in another holoenzymes (Jarrous et al., 2001). It has multiple subcellular localization as it has been mainly detected in the nucleoplasm but also is accumulated in nucleoli and Cajal bodies in RPP21-overexpressing cells (Jarrous et al., 2001). Knockdown experiments of RPP21 in HeLa cells showed significant decrease of 5S rRNA and 45S pre-rRNA synthesis (Reiner et al., 2008). Further studies demonstrated, the role of RPP21 independently from its participation in RNase P. Of note, it been found that RPP21 act synergistically with RPP29 participating in DNA damage response through homology-directed repair (HDR) (Abu-Zhayia et al., 2017). More specific, both proteins are recruited together to double stranded DNA breaks (DSBs) and through binding with poly ADP-ribose moieties and with RPPH1 could facilitate the homology directed repair (HDR) (Abu-Zhayia et al., 2017). Finally, it has been reported that RPP21 interacts with histone H3.3 at transcriptionally active sites, however its role in this case has not yet been determined (Newhart et al., 2016).

RPP25

In human the *RPP25* gene is mapped to chromosome 15q24.2. Similar to RPP20, RPP25 is a member of the Alba-like superfamily, but without ATPase activity and is mainly localized in the nucleus and nucleolus of eukaryotic cells (Welting et al., 2006). Functional analysis using RNAi experiments reveal that knockdown of RPP25 prevents the recruitment of RPC6 and the RPC7 (DNA-directed RNA polymerase III subunit 6 and 7) in the transcription initiation complex thus resulting in the inhibition of transcription for 5S rRNA, rRNAs and tRNAs (Reiner et al., 2008; Serruya et al., 2015). Moreover, similar to RPP20, RPP25 is also recruited to chromatin in a cell cycle-dependent manner as it binds on rDNA genetic loci at late G1/S phase. Moreover, the RPP25 protein has been reported as autoantibody component of the Th/To antigen complex, however, further studies are needed to evaluate its exact role.

Interestingly, in vertebrates exist a paralogue gene coding for RPP25-Like protein (RPP25L) which also known as C9orf23 or bA296L22.5. In human *RPP25L* gene is mapped to chromosome 9p13.3. The predicted RPP25L showed 43% identity to RPP25 protein in *homo sapiens*, however, a comparison of their structure reveals that the RPP25L (as is deposited in the AlphaFold-Protein Structure Database) has a conformation very similar to RPP25 (Figure 1.28) (Jumper et al., 2021;

Shaukat et al., 2021). Although, the role of RPP25L is still elusive, the observation of the structural similarity with RPP25 may implies that it serves as an alternative protein subunit of RNase P. Surprisingly, a recent study contributes to this hypothesis showing that RPP25L could be a facultative subunit of the RNase P/MRP complexes. Studying the paralog interactions in different cancer cell lines and through CRISPR-Cas9 depletion assay and rescue experiments, they observed that RPP25L could replace the function of RPP25 in the RNase P holoenzyme (Köferle et al., 2022).

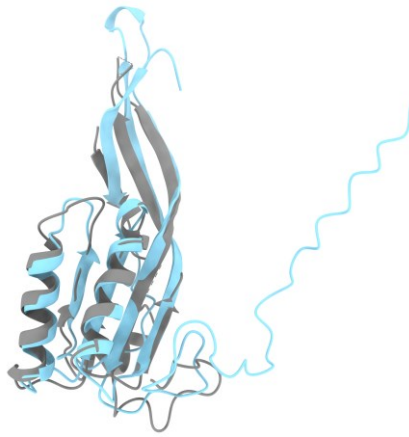


Figure 1.28: Structural prediction model after superposition of the RPP25 Cryo-EM structure (grey, PDB ID: 6ahu) with the RPP25-like (light blue, Uniprot ID: Q8N5L8). Adapted from Shaukat et al., 2021

RPP29

hRPP29 is encoded by the *RPP29* gene which is mapped to the locus 19q12. Localization studies showed that RPP29 protein is detected in the nucleolus (in dense fibrillar component - DFC) and also in Cajal bodies of the cells (Jarrous et al., 1999b). Similar to RPP21, silencing of RPP29 causes defects in pre-rRNA synthesis in HeLa cells (Reiter et al., 2010) while in U2OS cells, it has been reported that RPP29 directly affected the HDR and not the non-homologous end joining (NHEJ) as when cells are exposure to ionizing radiation (IR), it is observed an accumulation of damaged DNA and phosphorylation of markers that mediate in double-strand breaks (DSBs), like RPA2 (Replication Protein A2) (Abu-Zhayia et al., 2017). Remarkably, RPP29 except for its participation in DDR, it has been found that RPP29 also co-localizes with histone H3.3 through interactions with amino acid residues at the N-terminal of the histone, suggesting that it may

serve as potential epigenetic modulator. More specific, RPP29 inhibits the incorporation of H3.3 at euchromatic regions *via* regulation of the post-translational modifications of the N-terminus and also represses mRNA and protein expression. (Newhart et al., 2016; Shastrula et al., 2018). Moreover, it has been found that RPP29, together with fibrillarin and RPL23a are also detected in complex with histone H3.3, suggesting that these proteins may be members of the RPP29 interactome (Newhart et al., 2016).

RPP30

The RPP30 protein subunit is encoded by the *RPP30* gene which is located on 10q23.31 and its function has been thoroughly studied. So far, RPP30 is studied through knockout experiments in different organisms showing the importance of this protein. Since 1997, Rpp1 which is homologous of RPP30 in *Saccharomyces cerevisiae*, has been characterized as an essential protein subunit as its depletion induces simultaneously unprocessed pre-tRNAs at both 5' and 3' ends but also leads to defects in rRNA processing (Stolc and Altman, 1997). Of note, depletion of Rpp1 inhibits the cleavage of the three major site of 35S precursor rRNA: the A0 and A1 sites in the 5' external transcribed sequence (5' ETS), at A2 and A3 sites within the internal transcribed sequence 1 (ITS1) and at the E and C2 sites in the internal transcribed sequence 2 (ITS2). Another significant role has been pointed out in *A. thaliana* where the homologue of RPP30, GAF1 (Gametophyte Defective 1), participates in the process of gametogenesis. Homozygous *gaf1/gaf1* mutant has never been obtained while *gaf1+/-* mutants affects both male and female normal development of gametophytes. The female gametophyte division was impaired at early stages, while the male competence is reduced (Wang et al., 2012). At the same time, in *D. melanogaster*, RPP30 has been found to play a fundamental role for viability while mutations are also correlated with sterility (Jagut et al., 2013; Molla-Herman et al., 2015). These mutations have been found to activate DNA damage checkpoint through Chk2 pathway where PCNA was not associated with DNA in RPP30 mutant germline cells indicating a potent breakdown of the replication forks. A possible mechanism suggests that because of defective RNase P function leads to RNA Pol III stalling at tRNA genes and thus DNA polymerase collides with RNA Pol III at tRNA genes and leads to replication stress (Molla-Herman et al., 2015).

Notably, in *Drosophila*, RPP30 has been also correlated with piwi-RNAs that protects against transposons. In RPP30-mutated ovaries the piRNA molecules has been found to be significantly downregulated resulting in the enhance of transposable elements thus introducing further genomic instability and thus enabling the infertility of mutant flies (Molla-Herman et al., 2015). It is suggested that the observed decreased levels are due to defect in regulation of piRNA cluster transcription, and not in inadequate biogenesis of piRNAs. In concordance to above, piRNA downregulation is also observed in RPP30-mutated mice, mainly due to the loss of H3K9 trimethylation of the chromatin that is adjacent to tRNA genes. This can influence the production of piRNA clusters found nearby to tRNA gene clusters. Finally, apart from the alterations observed in RPP30 mutant ovaries regarding piRNAs, the pool of tRNA derived fragments is also affected. RPP30 mutations induce alterations in the production of tRFs, with an essential upregulation of tRF-5s, i-tRFs and tRF-3s while downregulation of tRF-1s was observed (Molla-Herman et al., 2020). This observation may be the result of the reduced maturation of the 3' trailer of pre-tRNAs, as it is well-known that 5' leader sequence removal by RNase P enhances the RNase Z activity at the 3' end (Hopper, 2003).

RPP38

In human, the RPP38 protein is produced by the gene which is located in the genetic locus 10p13. The subcellular localization of RPP38 is also dual as it is detected in nucleolus and the Cajal bodies (Jarrous et al., 1999b). Studies on knockdown of RPP38 reveal that RPP38 contributes to impaired enzyme activity of RNase P, while reducing the expression levels of RPP21, RPP25, RPP29, POP5 subunits and lamins A and C (Kovrigina et al., 2003; Reiner, 2006; Oshima et al., 2018). Furthermost, in patients suffering from systemic sclerosis scleroderma (SSc), the RPP38 has been identified along with POP1, RPP30 and RPP25 as the major components of Th/To autoantigen (Van Eenennaam et al., 2002). Latest data in molecular diagnostics have revealed that RPP38 subunit's epitope probably enhances the sensitivity in the anti-Th/To autoantibody detection analyses (Koenig et al., 2019).

RPP40

Similar to POP5, hRPP40 has not been found with a non-canonical function other than its function in the holoenzyme. In human, *RPP40* gene is mapped to chromosome 6p25.1 producing the RPP40 protein subunit which is recently acquired in the holoenzyme based on the structural studies. Although, its localization has not been experimental determined, it is hypothesized that it is located in the nucleoplasm and nucleoli of cells (Jarrous, 2002; Shaukat et al., 2021).

POP1

POP1 gene, located at chromosome 8q22.2, produces the largest protein, almost 115kDa, of RNase P holoenzyme. Although, POP1 is not widely studied, it has been found that in yeast is essential for viability and also its mutations lead to accumulation of unprocessed pre-rRNA and pre-tRNAs similar to RPP30 and RPP25. Subcellular localization experiments have identified the localization of POP1 in the nucleolus and nucleoplasm of cells, as expected for an RNase MRP and RNase P subunit (Lygerou et al., 1994, 1996). Further experimentation showed that POP1 has similar function, as chromatin remodeler, along with RPP29. hPOP1 is recruited in transcriptionally active site of histone H3.3 (Newhart et al., 2016). Although POP1 and histone H3.3, has not been found to interact each other, knockdown of POP1 showed lower transcriptional rate at a transgene array that recruits H3.3 histone (Newhart et al., 2016). In addition, it has been found that silencing of POP1 decrease the RNase P and MRP RNA levels while increases the viperin protein as its mRNA is a target for either RNase MRP or RNase P (Mattijssen et al., 2011; Sloan et al., 2013).

Moreover, in *Drosophila*, POP1 acts synergistically with RPP20, RPP25 and Pop4 (RPP29 homologue) in splicing of RPR intron. In arthropods, like *Drosophila*, the RNase P RNA subunit (RPR) are not transcribed by a distinct gene, instead of the RPR is produced as part of the 2nd intron during the transcription of the *ATPsynC* gene. After the transcription, the nascent RPR transcript is bound to the protein subunits POP1, RPP20, RPP25 and Pop4 in order to escape from alternative splicing and nucleolytic degradation as a typical fate of an intron. The subunits stabilize the RNA which further is trimmed to mature by the Rat1/Xrn2 exonuclease and the

nuclear RNA exosome (Figure 1.29). Importantly, silencing of each protein subunits decreases the RNA P activity and causes accumulation of pre-tRNA molecules, whereas knockdown of POP5, RPP14, RPP21 or RPP30 does not affect abundance of RPR (Palsule et al., 2019).

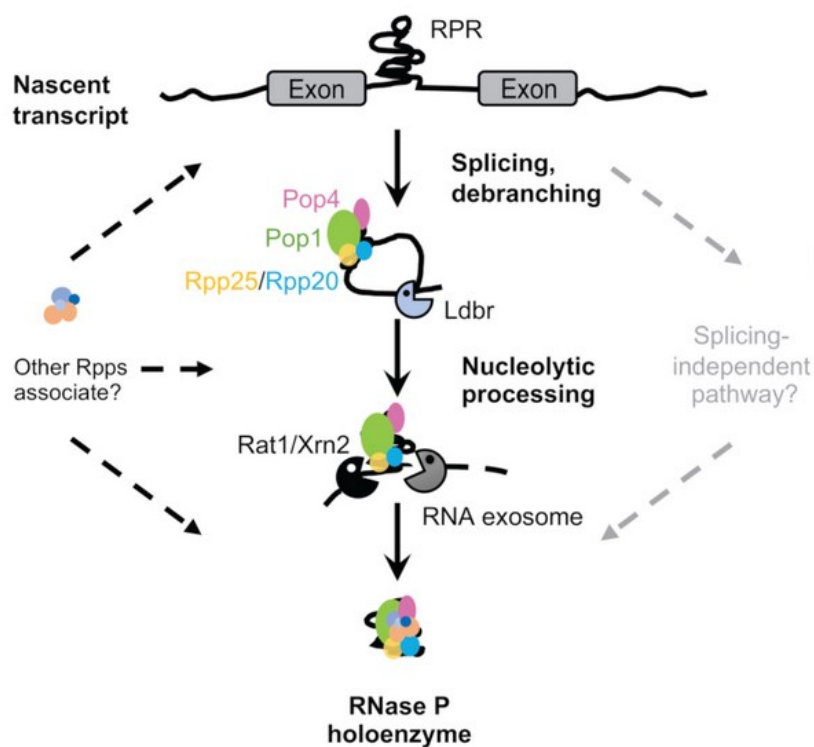


Figure 1.29: Proposed model for maturation of intronic RPR (RNA subunit of RNase P) in *Drosophila*. Adapted from Palsule et al., 2019.

Finally, POP1 has been identified as an autoantigen in patients suffering from connective tissue diseases (Lygerou et al., 1996), whereas recent reports emphasized the role of POP1 in anauxetic dysplasia 2 (ANXD2) showing that patients with mutations in the *POP1* gene, is characterized by extremely short stature (Elalaoui et al., 2016; Barraza-García et al., 2017; Abdulhadi-Atwan et al., 2020).

2. Aim of the study

The discovery of catalytic and regulatory ncRNAs complemented the contemporary RNA world theory and brought RNA biology in the spotlight. Thousands of ncRNAs, representing more than 80% of the genome, have been assigned roles as major modulators and biomarkers of homeostasis at the epigenetic, transcriptional, post-transcriptional and translational level. The in-depth understanding of noncoding RNA biology includes primarily regulation of transcription, maturation and folding into structured RNA molecules, as well as understanding how RNA-RNA or RNA-protein interactions dictate their role not only as important biomarkers, but also as guardians of genome integrity or functional components of almost all metabolic processes, signaling pathways and dynamic gene networks that modulate cell's fate. Until today, several key-events that mediate their biogenesis, correct folding and function remain open questions. So far, it has been proposed that these events are mediated by ribonucleases or RNP complexes. However, these complexes are mediated by proteins that beyond the synthesis of the RNPs, also participate in additional cellular processes such as transcription, DNA damage repair, replication and chromatin remodeling.

The aim of the present thesis was to study the role of RNA-binding proteins that mediate tRNA folding and maturation. Among them, La protein which is the responsible for the protection correct folding of all nascent RNA Pol III transcripts was studied. Aberrant expression of La has been studied in different cancer types, including lung cancer, suggesting a modulatory role of La in the regulation of RNA Pol III transcripts. In the present study, overexpression of La was performed in A549 NSCLC cell line and was analyzed to get insights on the role of La in cell viability, proliferation rate, cell cycle regulation, migration, translation rate, followed by NGS analysis to identify important gene networks that are affected. The analysis supplemented studies on the binding properties of La modules as independent RNA binding domains, that could account for several additional roles of La.

In addition to La, the role of the protein subunits of RNase P were studied for their ability to act as individual component in several cellular activities. Towards this goal, the CRISPR/Cas9 genome editing tool was chosen to knockout the genes corresponding to each protein subunit. Special focus for further analysis was given to RPP25 subunit which has only limited characterized so far. Moreover, a paralogue gene encoding for an RPP25-like protein suggests that both proteins might have synergistic roles, either as individual components, or as subunits of the RNase P holoenzyme. The approach of genome editing-based screening was followed for all ten protein subunits of RNase P and the RPP25 deficient cells were analysed for changes in cell viability, proliferation rates, cell cycle regulation, migration, metabolic activity and translation rates using polysome profiling and NGS analysis. Finally, the edited cell lines were used for *in vivo* characterization of RPP25-GFP tagged constructs to decipher alternative roles of RPP25 though alternative localization beyond nucleus and the possible involvement in phase separation was also investigated as part of the important interactions between RNA molecules and RNA binding proteins (RBPs).

3. Materials and Methods

A. Materials

3.1. Chemicals

Table 3.1. Chemicals were used in present study

Materials	Supplier
Acetic acid	MERCK
Acrylamide	SIGMA - ALDRICH
Agar	SERVA
Agarose - Ultrapure	INVITROGEN
Ammonium persulfate	SERVA
Ammonium chloride	MERCK
Ampicillin (Na+ Salt)	APPLICHEM
Anatomix plus developer replenisher	Fuji Hunt
[γ -32P] ATP (6000 Ci/mmol)	IZOTOP
Bis-acrylamide	SERVA
Boric Acid	MERCK
β -mercaptethanol	SERVA
Bradford reagent	APPLICHEM
Bromophenol blue	SIGMA – ALDRICH
Calcium Chloride (CaCl ₂)	MERCK
Chloroform	MERCK
Coomasie G250	FLUKA
Cycloheximide (CHX)	APPLICHEM
DEPC	RESEARCH ORGANICS
DMEM	GIBCO
DMSO	SIGMA – ALDRICH
dNTPs	BIOLINE
DTT	NEB
EDTA	SERVA
Ethanol 99% denatured	APPLICHEM
Ethanol Absolute	MERCK
Fetal Bovine Serum – FBS	BIOSERA
Gel Red	BIOTIUM
Glycerol	DUCHEFA
IPTG	DUCHEFA
HEPES	SERVA
Hydrochloric acid	MERCK
Isopropanol	MERCK
KH ₂ PO ₄	MERCK
Kanamycin	APPLICHEM
Lipofectamine (2000)	INVITROGEN
Magnesium Chloride (MgCl ₂)	MERCK
Methanol	CHEM – LAB
Methylene-bisacrylamide	SERVA

MnCl ₂	SIGMA - ALDRICH
N, N methylene bis-acrylamide	SERVA
NaH ₂ PO ₄	MERCK
Na ₂ PO ₄ ·H ₂ O	MERCK
Na ₃ VO ₄	SIGMA
OPTIMEM	GIBCO
Peptone	MERCK
Phenol	MERCK
Phenol equilibrated, stabilized	APPLICHEM
Penicillin/Streptomycin	BIOSERA
Potassium Chloride (KCl)	MERCK
Puromycin	SIGMA
PVDF	MACHEREY NAGEL
RbCl ₂	SIGMA - ALDRICH
Resazurin	BIOTIUM
SDS	SERVA
Sodium Acetate trihydrate	MERCK
Sodium Chloride (NaCl)	SIGMA
TEMED	RESEARCH ORGANICS
Trizol reagent	THERMOFISCHER SCIENTIFIC
Tris ultrapure	APPLICHEM
Triton 100x	MERCK
Trypsin EDTA 10X	BIOSERA
Tryptic Soy broth	BIOLIFE
Tryptone	APPLICHEM
Tween-20	SIGMA - ALDRICH
Xylene cyanol	SERVA
Yeast Extract	SIGMA

3.2. Enzymes- Reagents-Kits

Table 3.2. Enzymes and other buffer were used in present study

Reagents	Supplier
BamHI	NEB
BbsI	NEB
BSA	NEB
HindIII	NEB
KpnI	NEB
NheI	NEB
XhoI	NEB
10x CutSmart	NEB

DNase I	NEB
10x DNase I buffer	NEB
DreamTaq DNA polymerase	Thermo Scientific
2X KAPA SYBR FAST qPCR Master Mix	KAPA Biosystems
Nucleospin plasmid DNA mini prep	MACHEREY NAGEL
Nucleospin Gel and PCR Clean-Up	MACHEREY NAGEL
NEB Buffer 1.1	NEB
NEB Buffer 2.1	NEB
PEI	Polysciences
PNK	NEB
Protein Ladder (10-250 kDa)	NEB
Proteinase Inhibitors	NEB
Recombinant RNase Inhibitors (RRI)	NEB
T4 DNA ligase	NEB

3.3. Buffers, Solutions and Media

Bradford (5X): Final volume 500ml: 100 mg Coomassie Brilliant Blue G-250, 47 ml Methanol (100%), 100 ml Phosphoric Acid (H₃PO₄) (85%).

Developer (Anatomix PLUS Developer Replenisher): 125ml A buffer, 12.5ml B buffer, 12.5ml C buffer, 350ml dH₂O.

DNA Loading Buffer/Dye (6X): Final volume 40ml: 100 mg Bromophenol Blue, 100 mg Xylene Cyanol FF, 6 g Ficoll.

Fixer (X-Fix Fixer & Replenisher): 112.5ml X-Fix A buffer, 12.5ml X-Fix B buffer, 375ml dH₂O

Laemmli buffer (4x): 250mM Tris-Cl pH 6.8, 40% glycerol, 8% SDS, 2.8 M β-mercaptethanol, 0.2% Bromophenol Blue.

LB - Lysogeny Broth: 10g/L Peptone, 5 g/L yeast extract, 10 g/L Sodium Chloride pH=7.4 (Adjustment with NaOH).

LB- agar: 10 g/L peptone, 5 g/L yeast extract, 10 g/L Sodium Chloride, 15 g/L agar.

Lysis Buffer: 20 mM Tris-HCl pH 7.5, 120mM KCl, 1mM EDTA pH 8, 2% NP-40, 1mM Dithiothreitol (DTT) and protease inhibitors - 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 mM PMSF- and 1 mM Na₃VO₄.

PBS 1X: Final volume 1L, adjust 100ml 10X PBS σε ~900ml ddH₂O. Adjust the pH to 7.4 value by slowly adding HCL.

PBS-Tween 0,1%: Final volume 50ml: Add 50ml 1X PBS and 0.05 ml Tween-20.

Phosphate-buffered saline (PBS) (10x): 137mM NaCl, 2.2 mM KCl, 10mM NaH₂PO₄, 2mM KH₂PO₄. pH=7.4 (Adjustment with HCl).

Transfer blotting buffer (1x): 2.5 mM Tris, 0.192 M glycine, 20% methanol.

Tris Buffered Saline (TBS) (10x): 24.2 g Tris-base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).

Tris- HCl pH 6.8 0.5M-0,4% SDS: Buffer for acrylamide gel.

Tris- HCl pH 8.8 1.5M-0,4% SDS: Buffer for acrylamide gel.

Tris/glycine/SDS buffer (1x): 25 mM Tris, 250 mM glycine, 0.1% SDS.

Tris-acetate-EDTA (TAE) (50x): 242 gr/L Tris, 57.1 ml/L acetic acid and 100ml/L EDTA 0.5M pH 8.

Wash Buffer for Western: 1X TBS or PBS, 0.1% Tween-20.

Stripping solution: 62.5 mM Tris-HCl 1M pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol

3.4. Bacteria strains

DH5α: E. coli strain used for general cloning and sub-cloning applications, [genotype: E. coli F- φ80lacZΔM15 Δ(lacZT A-argF) U169 deoR recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-] (INVITROGEN)

BL21 (DE3) Rosetta: This strain is designed to enhance the expression of eukaryotic proteins that contain codons rarely used in E. coli. It express T7 RNA polymerase that is under the control of a lacUV5 promoter and induced by IPTG. [genotype: E. coli F- ompT hsdSB (rB-, mB-) gal dcm pRARE2 (Camr)] (INVITROGEN)

Stbl3: *E. coli* strain used for cloning unstable inserts such as lentiviral DNA containing direct repeats [genotype: *F-mcrB mrr hsdS20(rB⁻, mB⁻) recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20(Str^R) xyl-5 λ⁻ leumtI-1*] (Invitrogen).

3.5. Bacterial Vector

pcDNA3.1: Eukaryotic expression vector containing 5428 bp. It is used for transient expression of genes. It contains a CMV promoter and an ampicillin/neomycin resistance gene for bacterial and eukaryotic expression respectively.

pcDNA3.1-GFP: This is the vector mentioned above which contains an enhanced green fluorescent protein (GFP) gene that is used for fusing EGFP to the C-terminus of a partner protein. The EGFP cloned into pcDNA3.1 vector using BamHI and EcoRI enzymes.

pET-20b(+): A 3716 bp bacterial vector that encodes a signal sequence (pelB) for expression of proteins in the periplasm. It is used for transient expression of genes. It contains a T7 promoter and an ampicillin resistance gene. It is designed for prokaryotic expression of proteins as fusion products with a C-terminal His•Tag sequence. The recombinant protein with hexahistidine binds to immobilized metal ions with high affinity and can be easily purified by chromatography using Ni-NTA beads (NOVAGEN).

pET-28b(+) (NOVAGEN): A 5369 bp bacterial vector. It is used for transient expression of genes. It contains a T7 promoter, kanamycin resistance gene and an IPTG regulated lactose operon. It is designed for prokaryotic expression of proteins as fusion products with N-terminally 6xHis-tagged proteins with a thrombin site. The recombinant protein with hexahistidine binds to immobilized metal ions with high affinity and can be easily purified by chromatography using Ni-NTA beads (NOVAGEN).

pUC57: The pUC57 vector, containing a codon optimized gene sequence of La for expression in *E. coli*, was purchased from GenScript and was used for cloning of La protein. (GENSCRIPT)

pSpCas9(BB)-2A-Puro (PX459) V2.0: This vector consists of 9200 bp which is used for genome editing in mammalian cells. It encodes the wild-type Cas9 nuclease (with a NLS sequence for nuclear localization) from *Streptococcus pyogenes* along with a sgRNA. The vector can be digested using BbsI enzyme, and a pair of 20bp oligos can be cloned into the vector before the sgRNA scaffold. The transcription of sgRNA is controlled by RNA Pol III U6 promoter. It carries an ampicillin/puromycin resistance gene for bacterial and eukaryotic expression respectively. PSpCas9 (BB) -2A-Puro (PX459) V2.0 is provided by Feng Zhang's lab (Figure 3.1).

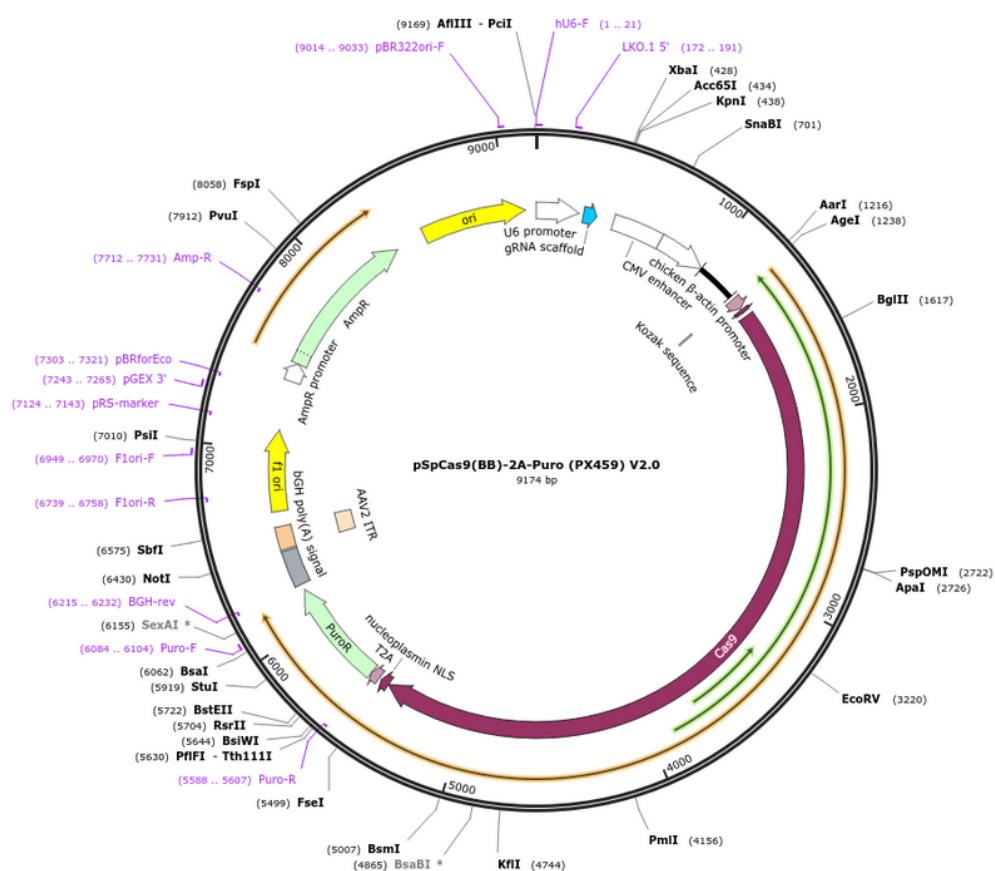


Figure 3.1. pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid vector used for CRISPR/Cas9 methods.

3.6. Plasmids Constructs

Table 3.3. All constructs that are used in this thesis

Vectors	Antibiotic resistance in bacteria	Enzyme (insertion)
pcDNA3.1 (empty vector)	Ampicillin	
pcDNA3.1+gfp	Ampicillin	BamHI-EcoRI
pcDNA3.1+La	Ampicillin	NheI-kpnI
pcDNA3.1-RPP25	Ampicillin	NheI-kpnI
pcDNA3.1-RPP25L	Ampicillin	NheI-HindIII
pET28	Kanamycin	-
pET28-RPP25	Kanamycin	NheI-kpnI
pET28-RPP25L	Kanamycin	NdeI-HindIII

3.7. Antibodies

Table 3.4. List of antibodies used for the study

Antibody	Catalog #	Supplier
β -Actin	4967	Cell Signaling
p-mTOR (S2448)	5536	Cell Signaling
mTOR	2983	Cell Signaling
eIF4E	2067	Cell Signaling
4E-BP1	9644	Cell Signaling
phospho-4E-BP1 (T70)	9455	Cell Signaling
Fibrillarin	2639	Cell Signaling
eIF3 p110	sc-74507	Santa Cruz Biotechnology
La	sc-80656	Santa Cruz Biotechnology
PCNA	sc-56	Santa Cruz Biotechnology
c-Myc	sc-40	Santa Cruz Biotechnology
puromycin	MABE343	EMD Millipore Corp
GAPDH	2118	Cell Signaling
POP1	12029-1-AP	Proteintech
RPP25	15461-1-AP	Proteintech

RPP20/Pop7	14964-1-AP	Proteintech
RPP38	sc-398112	Santa Cruz biotechnology
RPP40	sc-515079	Santa Cruz biotechnology
Coilin	14168	Cell signaling
Anti-Rabbit IgG HRP-linked		Santa Cruz biotechnology
Anti-Mouse IgG HRP-linked		Santa Cruz biotechnology

3.8. Primers

All primers were designed by using NCBI and were purchased by Macrogen.

Table 3.5. List of primers used for genes expression analysis and cloning

Gene	Forward	Reverse
ACTB	AGCGAGCATCCCCAAAGTT	GGGCACGAAGGCTCATCATT
ANG	TGGGCGTTTTGTTGTTGGTCTTC	CGTTTCTGAACCCCGCTGTGG
BDP1	AGTGCAGACAGATTTGGGGG	AGCCTTCATCCTTCATCATCTCC
CCNA2	CTGGTGGTCTGTGTTCTGTGA	TCTTGGATGCCAGTCTTACTC
CCNB1	TGTGATTGCTGCCATAATTCTAAGT	ACACAAAACCAAAATGAAAAGTGGC
CCND1	CAATGACCCCGCACGATTTTC	CATGGAGGGCGGATTGGAA
CCNE1	GCTGCCCTCTCCACATTATCA	ATAGCAGCACTTACAAAACAGTTCA
CDKN1A	TCTGACCCCAAACACCTTCCAG	GACACATGGGGAGCCGAGA
DICER	GTACGACTACCACAAGTACTTC	ATAGTACACCTGCCAGACTGT
EI4EBP	TCCTTGTGCCTCCACTGATG	CTAAGGAAGGGGTGGTTGCG
EIF1	TGTCCAAGGGATCGCTGATG	TTCAGTGAGCCACAAGCACT
EIF1A	CGCTACCCGGAAAGAAGTCA	TAGCCGTCCATTTCCCAACA
EIF2A	GGCCCCATTTATGATGTAGTTTGG	TCCGGGCACCAAGCAAAAATA
EIF2B1	AGGGCGAATCTCACCAGTGCC	CGTTGAGGTGGCAGAGGGCTT
EIF2S1	AATAGGCGCTTGACCCAC	TGTTCTCTCCAGGGTTGTCTG
EIF3A	GCAAAGGAGGGGTATACCAGT	TGTCAGTACGATCCTGAGTGTC
EIF3C	GCGCTGGCCTCAAGATTTTC	AGTTCCCTTCTTGCCTCGTG
EIF4A1	TATGACCTTCCCAACACAGG	CCTCAGATGAGGTCAGCAACA
EIF4B	AGGGGAAGACTATCTCCCTAACA	TCATCCGTTTCATCAGCCAG

EIF4E	AGGACTGAAACCACCCTCCAGCA	TCGGGGGATTAGGAGTAGGGGTGG
EIF4G1	TTTGAGACTCCCCTCCGAGT	CCTCGTCATACAGTGCGTCA
EIF5	GCTCCGTATCCAGCAGTGAG	CATTCGACGCCTTTGAGCTT
EIF5B	GGGTGTTGAAGTGCCATCAAA	CCAATCATCCAATCCAGCATCC
ELAC2	GCTCACCAGTTTCCGCTGTA	GACACAAACACAGCAGCCAG
HIF1A	TCAAAGTCGGACAGCCTCAC	TAGCTGCATGATCGTCTGGC
GTF3A	AGCAAGCTGAAACGACATGC	CCTTCTCTTGACAGCGACA
KAT2A/GCN5	TCACTTCCCCAAATTCTGTCC	ATGTCACCCATCACACGGAG
MAF1	ATGGCAGGAGACGACAAACA	ATGGCAGGAGACGACAAACA
MDM2	CAGCAGGAATCATCGGACTCA	AAGATCCGGATTGATGGCG
MMP2	TGATGGCATCGCTCAGATCC	GGCCTCGTATACCGCATCAA
mTOR	AGCTGCCAAGTGCCGAGCAT	AGCAGCCATCCGGGCCATCTT
MYC	TTTCCTGTTGCAAGGGTCTGA	AGAAGATGTCCCAGAAGGCAA
Outer Primer		GCGAGCACAGAATTAATACGACT
RICTOR	TTTTCGGGGATTCTGGATGC	GACAGCAAATGGGAAACCTCA
RNU6	GCTCGCTTCGGCAGCACATA	AACGCTTCACGAATTTGCGTGT
POP1	TGCCTTGGGGAGAGGCCAACA	GGTTCACACACTGGCAGCT
POP4/RPP29	GCGAATGACTCCGATGTCCAGCC	ATCTGTGGCTGCGTGTCTGGC
POP5	AGCTTGTGTGGTCAGCTCTCCCT	AAGTGGGCCTGAAGCCTGAGCA
POP7/RPP20	GGAAGCCGGGAAGGCGATTCA	TGTCATTGGGTCTCCGGGGCA
RPP14	TGCGCCTTGGGTAGCTTGTCT	GCGGCATCAACCTCCCCAAACA
RPP21	TCGTCTTGC GGCGACCACTATC	TGGGCTGTGTTTGGCAAGGGTT
RPP25	TGACCGCGCACTAGCTGTCCT	GCCAACCCTCACAAACCGGCA
RPP30	CTGAAAGTGACGCCAAGGCTGC	AAGTGA CTCTCATCAGCCCAGGACA
RPP38	ACCACGGATTCGCCATCGTGAG	GCTCCAGCGGATGATGTATGGGT
RPP40	CCGCGGCACTTACTGGTTTGC	TCCCATTGGTAGCAGGGCAACAG
RPPH1	AGCTTGGAACAGACTCACGG	AATGGGCGGAGGAGAGTAGT
RMRP	CTCTGTTCTCCCTTTCCG	CTCAGCGGGATACGCTTCTT
RPTOR	ATCCCACCGCTCACTCATC	TTGACGCTCACACTCACGAT
SSB	AGTCGTTGCTGTTGCTGTTG	AGGTAGGGGTTTGCTTGAG
TBP	GTGGGGAGCTGTGATGTGAA	GGGAGGCAAGGGTACATGAG

TP53	CTGCATTTTCACCCCACCCTT	CACACAGGTGGCAGCAAAGTT
tRF-1001	GAAGCGGGTGCTCTTATTTTAAAAA	
tRNA ^{Ser} UGA	CAGCGATGGCCGAGTGGTTAAG	CGCAGCGAGCAGGGTTCGAA
TRRAP	CAGCGACTGCGATTGCTTAC	GAGGTAGGGGTTGATGTGGC
VEGFA	TGCAGATTATGCGGATCAAACC	TGCATTACATTTGTTGTGCTGTAG
XPOT	TGTTTCATGCCCTGCTTCAT	TCAGCAAATCCCACTGGTCC
		GACAGATGGTACCCTG
hLaNheI-KpnI	GATAGCGGCTAGCATGGCGGAAAA CGGCGATAACG	ATCGCCCGCACCATTTTCG
		TATGTGGGATCCCTGATCGCC
hLaXhoI-	ACTAGCGGCTCGAGATGGCGGAAAA	CGCACCATTTTCGGTTTTCTGTTGC
BamHI	CGGCGATAACGAGAAGATGGCG	
NheI-	GTGACAGCTAGCATGGAGAACTTC	GACTCGGGTACCCGCCGT
KpnI_RPP25	CGTAAGGTG	CTGATCCTCGTCCG
NheI_hindIII -	CGGTACCGCTAGCATGGAG-	CAAGCTAAGCTTTACG-
RPP25L	CACTACCGGAAAGCTGG	ATCGGGTGCTCGAG
NheI_hindIII	CGGTACCGCTAGCATGGAGCAC-	CAAGCTGAAGCTTCG-
RPP25L_STOP	TACCGGAAAGCTGG	ATGGGGTCTCGAGC
NdeI_HindIII	CGGTACCCATATGGAGCA	CAAGCTGAAGCTTCG-
RPP25L	CTACCGGAAAGCTGG	ATGGGGTCTCGAGC
	GAATTCCTAATACGACTCACTATA	
tRNA ^{Gly} (CCA)	GCATGGGTGGTTCAGTGGTAG	TGGTGCATGGGCCGGAATC
	GAATTCCTAATACGACTCAC	
tRNA ^{Gly} (5'	TATAGTTGGCGTTTGCCATCGG	TGCATGGGCCGGAATC
leader)		
tRNA ^{Gly}	GAATTCCTAATACGACTCACTATAGC	
(3'trailer)	ATGGGTGGTTCAGTGGTAG	AAAATGGGAGGGCGTGCTGCATG
tRNA ^{Agr} _Dicty	AGCTTAATACGACTCACTATAGTTGAA	TTTAAAAAATATGTCATGGGCA
ssDNA (70nt)	ACTCGGGATCCTAAAACTCTCGTCTCTAGTATGCTGATTTAAAGCATATAGGGACGAGAGTTAGCTCGCG	

Table 3.6. List of target guides used for the study during CRISPR/Cas9 genome editing

Name	Sequence (5'→ 3')	Targeted exon
POP1_CRISPR_g1_F	CACCGATCCTGGAAC TTCACGACAG	Exon 3
POP1_CRISPR_g1_R	AAACCTGTCGTGAAGTTCCAGGATC	Exon 3
POP1_CRISPR_g2_F	CACCGAGGTCCCGAGGGCACGTCTC	Exon 3
POP1_CRISPR_g2_R	AAACGAGACGTGCCCTCGGGACCTC	Exon 3
POP5_CRISPR_g1_F	CACCGTGGCGATCGTGTCCCGTACG	Exon 2
POP5_CRISPR_g1_R	AAACCGTACGGGACACGATCGCCAC	Exon 2
POP5_CRISPR_g2_F	CACCGCGTACGGGACACGATCGCCA	Exon 2
POP5_CRISPR_g2_R	AAACTGGCGATCGTGTCCCGTACGC	Exon 2
RPP20_CRISPR_g1_F	CACCGTTCCTAAGGGTGTATTCCAC	Exon 2
RPP20_CRISPR_g1_R	AAACGTGGAATACACCCTTAGGAAC	Exon 2
RPP20_CRISPR_g2_F	CACCGTAAATGTCATTGGGTCTCCG	Exon 2
RPP20_CRISPR_g2_R	AAACCGGAGACCCAATGACATTAC	Exon 2
RPP25_CRISPR_g1_F	CACCGCCCGGCGCCAGGTCTGCGAA	Exon 1
RPP25_CRISPR_g1_R	AAACTTCGAGACCTGGCGCCGGGC	Exon 1
RPP25_CRISPR_g2_F	CACCGCGCCGGGCGCGGTGCACATG	Exon 1
RPP25_CRISPR_g2_R	AAACCATGTGCACCGCGCCCGGCGC	Exon 1
RPP40_CRISPR_g1_F	CACCGATGCCGGTGGCGCGACTTG	Exon 1
RPP40_CRISPR_g1_R	AAACCAAGTCGCGCCACCGGCATC	Exon 1
RPP40_CRISPR_g2_F	CACCGCAACCACAAGTCGCGCCAC	Exon 1
RPP40_CRISPR_g2_R	AAACGTGGCGCGACTTGTGGTTGC	Exon 1
RPP38_CRISPR_g1_F	CACCGCGCTCCAGCGGATGATGTAT	Exon 1
RPP38_CRISPR_g1_R	AAACATACATCATCCGCTGGAGCGC	Exon 1
RPP38_CRISPR_g2_F	CACCGCCTGTCCTCAAGCGTCTGT	Exon 1
RPP38_CRISPR_g2_R	AAACACAGACGCTTGAGGACAGGC	Exon 1
RPP14_CRISPR_g2_F	CACCGTCATGTAGTGGTACTCGGAA	Exon 2
RPP14_CRISPR_g2_R	AAACTTCCGAGTACCACTACATGAC	Exon 2
RPP14_CRISPR_g3_R	CACCGACTTTCATGTAGTGGTACT	Exon 2
RPP14_CRISPR_g3_R	AAACAGTACCACTACATGAAAGTC	Exon 2
RPP21_CRISPR_g1_F	CACCGCAAGACGAGCCGCTTCGCAA	Exon 2

RPP21_CRISPR_g1_R	AAACTTGCGAAGCGGCTCGTCTTGC	Exon 2
RPP21_CRISPR_g2_F	CACCGTCGCCAGCGCCTGGTTCTCG	Exon 2
RPP21_CRISPR_g2_R	AAACCGAGAACCAGGCGCTGGCGAC	Exon 2
RPP29_CRISPR_g1_F	CACCGTGTGGCGGGTGAAGTACTCC	Exon 3
RPP29_CRISPR_g1_R	AAACGGAGTACTTCACCCGCCACAC	Exon 3
RPP29_CRISPR_g2_F	CACCGCGGGCCGAGGCCTTCGTGA	Exon 3
RPP29_CRISPR_g2_R	AAACTCACGAAGGCCTCGGCCCGC	Exon 3
RPP30_CRISPR_g1_F	CACCGTTGCATCAAATTGAGGGCAC	Exon 7
RPP30_CRISPR_g1_R	AAACGTGCCCTCAATTTGATGCAAC	Exon 7
RPP30_CRISPR_g2_F	CACCGATTGTGGAGTCTTTGATAGC	Exon 7
RPP30_CRISPR_g2_R	AAACGCTATCAAAGACTCCACAATC	Exon 7

Table 3.7. List of primers used for the validation during CRISPR/Cas9 genome editing

Name		Primers for Validation	Size of amplified product	Tm used in pcr
POP1 Vd	F:	TGGTCAATGTTGCCACCCAA	414	64
	R	CAAAGTCCAGGCTAGAGGCG		
POP5 Vd	F:	ATTCACATCCGGTTTGCACG	680	63
	R	GGGCTTTCCCGGAGTACTTT		
RPP14 Vd	F:	AGCTGGGTGCTCATAAGGAAAAT	1000	64
	R:	CACAGCCGAAATAAGCAGCTG		
RPP20 Vd	F:	GGAAGGAAACAGGGACATCG	788	62
	R	GTTGCGGATCCGAGTCAGT		
RPP21 Vd	F:	GCCTTCCAGAGGCTCAACTT	623	62
	R	TGCAAGTGGCGTTAGGAACT		
RPP25 Vd	F:	GCCGAGAGCGCTGAGTAGAG	791	65
	R	GTCTGATCCTCGTCCGCAAC		
	F:	TCAGAAAAGCAGGGCCAGAG	544	64

RPP29 Vd	R:	CCAAGAAGGGTAAGCACCGC		
RPP30 Vd	F:	GCTTCCCCACAGCCAAGTTA	636	62
	R:	TCAACTCAGTGAGGACCATTG		
RPP38 Vd	F:	CAGGGCTCTGGTAACTTCGTT	851	62
	R:	ACAAGCGTATGCTCACCTAAT		
RPP40 Vd	F:	TCAGACATCCACCAAAGAGCG	793	64
	R:	GGTGAGATCCACAGGTACGC		

B. Methods

3.9. CRISPR/Cas9 genome editing protocol

3.9.1. Single-guide RNA (sgRNA or gRNA) oligo design

sgRNA oligos were designed with the CRISPR Guide RNA design tool in Benchling (<https://www.benchling.com/crispr/>). This tool allows to visualize, optimize, and annotate multiple gRNA sequences. It designs a 20nt guides directly upstream of the PAM (Protospacer adjacent motif) sequence of our intended target site. PAM type was set to 5' NGG as it is required for Cas9 cleavage. In a first step, the genomic region of interest (fully annotated) was identified, and the tool found which exons are present in all transcript variants of our targeted gene. Benchling's tool designed a number of sgRNAs based on latest algorithms to instantly assess off-target effects and on-target efficiency for guides (Hsu et al., 2013; Doench et al., 2014, 2016). The human genome (assembly homo sapiens GRCh38/hg38) was used as a reference genome. gRNA sequences with the higher off-target and on-target score were selected for each gene. We also exclude the possibility that all potential off-target sequences to be part of important genes in cell. Additionally, due to the fact that the U6 promoter that transcribes the sgRNAs prefers a G sequence as a starting nucleotide, we add one in guides that didn't begin with a 5'G. Oligos were designed according to the specific configuration that was necessary for cloning into pX459

(Figure 3.2). The forward and reverse strands pair with each other and additional 5'-CACC and 5'-AAAC overhangs added for subsequent ligation into plasmid.

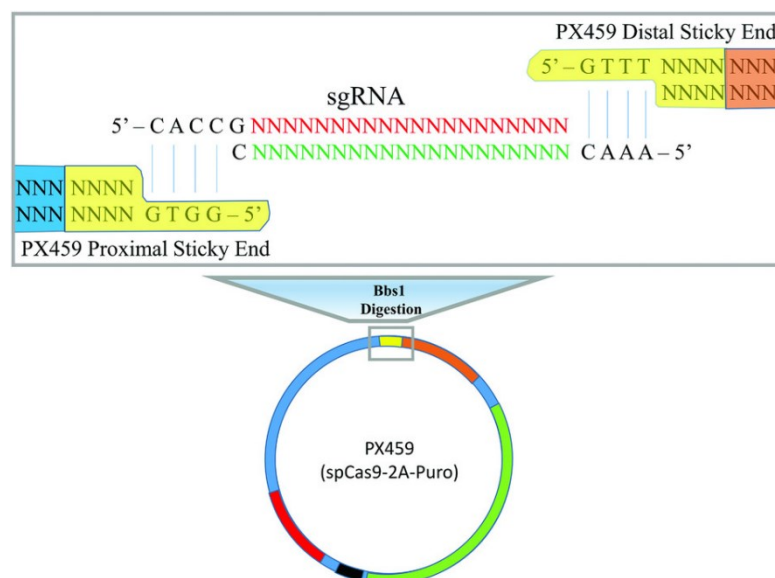


Figure 3.2. Cloning site of single guide RNA in pX459 vector

3.9.2. Single-guide RNA cloning protocol

To clone the sgRNAs into plasmids the following steps were performed:

i. Vector digestion

pX459 plasmid digestion	
plasmid	1µg
(10,000 units/ml) <i>BbsI</i>	10U
10x Cutsmart Buffer	1x
ddH ₂ O up to 20 µL	
Incubation for 1 hour at 37°C	

The digested plasmid run on an agarose gel (1%) to determine the relative sizes and were purified from gel using NucleoSpin® PCR clean-up & Gel extraction-MACHERY-NAGEL, according to the manufacturers' recommendations.

ii. Oligo annealing

The designed oligos were resuspended in ddH₂O (100μM). For phosphorylation and annealing of each pair of oligos, the following reaction was performed:

Reagents	Concentration
oligo 1 (100μM)	1 μL (100μM)
oligo 2 (100μM)	1 μL (100μM)
10X T4 Ligation Buffer (NEB)	1x
(10,000 units/ml) T4 PNK enzyme (NEB)	5U (0.5 μL)
ddH ₂ O up to 10 μL	

The temperature of annealing used in a thermocycler was according to the following: 37°C for 30 min and after 95°C for 5 min and then ramp down to 25°C at 5°C/min.

iii. Ligation

For ligation reaction the phosphorylated and annealed oligo duplex were diluted in 1/200 and the following reaction was performed:

Reagents	Concentration
Oligos (phosphorylated and annealed) (1/200)	1 μL
Digested plasmid	50ng
10x T4 Ligation Buffer (NEB)	1x
(400,000 units/ml) T4 DNA ligase enzyme (NEB)	400U
ddH ₂ O up to 10 μL	

The reaction was incubated O/N at 16°C. A control ligation reaction, containing only the vector and the ligation mix, was also performed, in order to determine the re-circularization frequency of the vector.

iv. Transformation

The transformation was performed using chemically competent Stbl3 cells (see paragraph 3.10- for chemical competent protocol) as follows:

- 50 μ L of competent cells (stored at -80°C) was gradually thawed on ice.
- 5 μ L of ligation reaction was added in each transformation and was incubated on ice for 20 minutes.
- Each reaction was heat-shocked at 42°C for 45 seconds and then immediately placed on ice for 2 minutes.
- 500 μ L of LB medium was added to each reaction and the cells were incubated at 37°C for 1 hour to recover.
- 300 μ L of cells were plated on LB-agar, containing the appropriate antibiotic (ampicillin) and the plates are incubated at 37°C for 16 hours.

v. Small-scale plasmid preparation (mini prep)

A 5 ml of LB culture, containing the appropriate antibiotic, was inoculated with a single bacterial colony, and was incubated overnight at 37°C , under shaking. A glycerol stock solution was prepared with 700 μ L of bacteria culture and 700 μ L of 50% glycerol solution, were mixed and then was freezed directly into -80°C . The glycerol allows to reduce the harmful effect of ice crystals which can damage bacterial cells thus allowing them to be stored safely.

The rest of bacteria culture used for plasmid isolation. The plasmids were purified using the NucleoSpin® Plasmid - MACHEREY-NAYGEL kit according to the manufacturer's instructions. The concentration of eluted DNA was determined with the MultiScan spectrophotometer (ThermoScientific). The plasmid was sequenced before the experimental procedure.

3.9.3. Transfection of vector in cell lines

A transient transfection was performed for CRISPR/Cas9 genome editing. These protocols are described below in paragraph 3.11.2 and 3.11.3. After transient transfection, the cells were

selected with 2 µg/ml puromycin antibiotic for two days. Of note, we used as positive control sgRNAs targeting AAVS1 (adeno-associated virus integration site 1). The AAVS1 locus is located in position 19q13.42 and is considered as epigenetically open for efficient cleavage, thus cutting or modification at this site results in no phenotypic changes (Yada et al., 2017).

3.9.4. Selection and Expansion of Clones

After transfection and antibiotic selections of cells, a single colony formation was performed with limiting dilution. More specific, 96-well plates were used for an array dilution method and an initial inoculation of 4000 cells (2×10^4 cells/ml) in first well (A1) was performed. As shown in Figure 3.3, 200 µL of cell was added in A1-well with proper concentration and then, 100 µL of medium was transferred from the first well to well B1 by gently mixing. The same procedure was repeated from B1 to C1 etc., until the H1 well. Complete culture medium was added to wells A–H in Column 1 to reach a final volume of 200 µL/well. Using a multichannel pipette, 100 µL of cell was transferred across the plate horizontally and across the rows as second dilution (Figure 3.3).

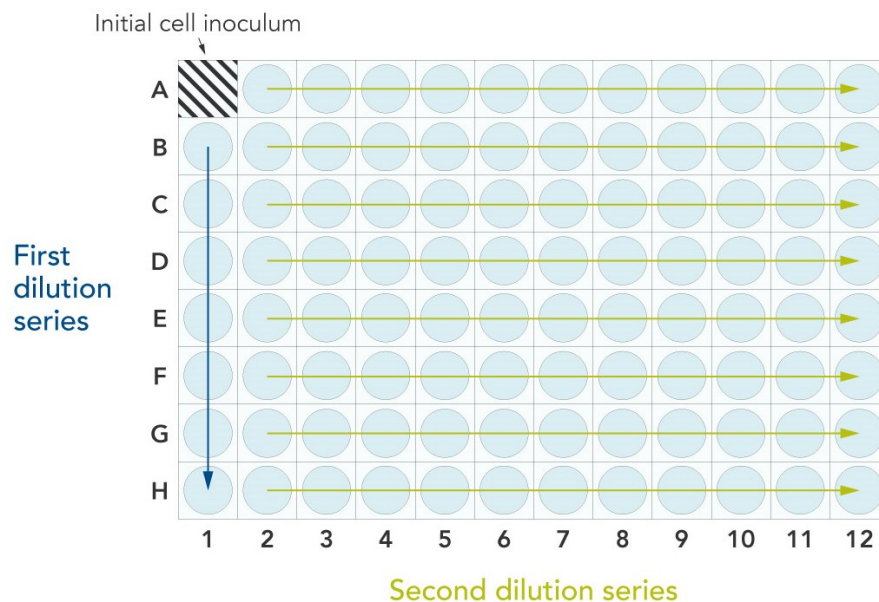


Figure 3.3: Plate setup for array dilution method.

A final volume of 200 µL complete culture media was added to all wells. The plates were incubated at 37°C in a CO₂ incubator. The cell growth was checked after 4–5 days, and wells

containing just 1 single colony were marked. The colonies were subcultured from the wells into larger wells. When cells reached at 90% of confluency, a number of cells used for storage according to the cryopreservation procedure (§3.11.1), whereas the rest of cells used for DNA extraction, as described below.

3.9.5. Verification of clones

i. DNA extraction

To verify the isolated clones, DNA extraction was performed with TRIzol (Invitrogen) following the manufacturer's protocol. Briefly, 500 μ L TRIzol reagent was added directly to the culture dish (10^8 cells) and was incubated for 5 minutes to lyse the cells and to permit the solubilization of all contents including the nucleoproteins complex. The lysate was transferred to a new tube and was added 100 μ L of chloroform used for lysis. The tubes were incubated for 2–3 minutes followed by a centrifugation at $12,000 \times g$ for 15 minutes at 4°C . After centrifugation the mixture separates into three phases, a lower red phenol-chloroform or organic phase which contains proteins, an interphase which contains DNA, and a colorless upper aqueous phase containing the RNA. To isolate DNA, the aqueous phase was removed, 150 μ L of 100% ethanol was added and was mixed by inverting the tube. The tubes were incubated for 3 minutes followed by a centrifugation at $7,000 \times g$ for 15 minutes at 4°C to pellet the DNA. The supernatant, which contains the proteins, was removed and the pellet was resuspended in 500 μ L of 0.1 M sodium citrate in 10% ethanol, pH 8.5 for 30 min by inverting the tube several times (the sodium citrate chelates the metal ions which activate the DNases and also the sodium ions will bind to and neutralize the (-) charge on the phospho backbone of DNA. This reduces hydrogen bonding sites for water, which then results in less solubility). The pellet of DNA was centrifuged for 5 minutes at $5000 \times g$ at 4°C and the supernatant was discarded. The pellet was washed with 500 μ L of 75% ethanol to remove some of the salt and then was centrifuged for 5 minutes at $5000 \times g$ at 4°C . The supernatant was discarded, and the DNA pellet was allowed to air dry for 5 min. The DNA was dissolved in 200 μ L of 8 mM NaOH. To remove insoluble materials, a centrifuge for 10 minutes at $12,000 \times g$ at 4°C was followed. The DNA was transferred to a new tube and their concentration was determined with the MultiScan spectrophotometer (Thermo Scientific). The

DNA solution was adjusted to pH 8.4 using HEPES (add 86 μ L of 0.1 M HEPES, free acid/ml of DNA solution) in order to amplified by PCR.

ii. Amplification of Targeted Region by PCR

The genomic DNA from each clone was amplified by PCR. Specific primers were designed in order to span the target region of predicted Cas9 cut site (table 3.7). The sgRNA is located inside the amplified pcr product which size is about ~400–1000 bp. All targeted amplicons were amplified by high-fidelity PCR (polymerase chain reaction) according to the manufacturer's protocol using the KAPA HiFi kit (Roche-KAPA BIOSYSTEMS):

Reagents	Final concentration
5x KAPA HiFi Fidelity buffer	1X
10 mM dNTPs mix	0,3 mM
10 μ M FW primer	0,3 μ M
10 μ M RV primer	0,3 μ M
Template DNA	50-100ng
KAPA Hifi DNA polymerase	0,5 U

The amplicons were analyzed by agarose gel electrophoresis. The % w/v concentration of the agarose gel prepared depends on the size of the DNA fragments to be analyzed. Each desired product was extracted from gel using NucleoSpin Gel and PCR Clean-up from Macherey-Nagel according to the manufacturer's protocol. To validate the sequence direct sequencing of PCR products was performed.

iii. Analysis of CRISPR Editing Results

For identifying CRISPR induced indels by direct Sanger sequencing, the CRISP-ID analysis tool was used (Dehairs et al., 2016). Moreover, as an alternative tool, the Inference of CRISPR Edits (ICE) was used (<https://ice.synthego.com/#/>). ICE compared chromatograms and calculated probable allele sequences and InDel distributions.

3.10. Basic cloning procedures

The steps followed for the construction of each plasmid during this study are briefly described below:

Polymerase chain reaction (PCR)

Specific primers were designed for each gene of interest and were amplified by high-fidelity PCR (polymerase chain reaction) according to the manufacturer's protocol using the KAPA HiFi kit (Roche-KAPA BIOSYSTEMS):

Reagents	Final concentration
5x KAPA HiFi Fidelity buffer	1X
10 mM dNTPs mix	0,3 mM
10 μ M FW primer	0,3 μ M
10 μ M RV primer	0,3 μ M
Template DNA	5ng (for plasmids) or
KAPA Hifi DNA polymerase	0,5 U

Each desired DNA fragments were analyzed by agarose gel electrophoresis. The % w/v concentration of the agarose gel prepared depends on the size of the DNA fragments to be analyzed. To visualize the DNA a GelRed® Nucleic Acid Gel Stain, 10,000X was used (Biotium). The desired PCR products were extracted from gel using NucleoSpin PCR Clean-up from Macherey-Nagel according to the manufacturer's protocol.

Digestions, purification and ligation of digested DNA fragments

PCR products and plasmids were digested using restriction enzymes from NEB (Table 3.2) according to manufacturer instructions. Insert and vector DNA (500ng) were digested for 1 h with 1 U of the corresponding restriction enzyme. The linearized DNA fragments were analyzed by agarose gel electrophoresis and were purified either by NucleoSpin PCR Clean-up kit or if necessary, by NucleoSpin Gel extraction kit. The purified vector and insert were enzymatically joined together through a ligase reaction. The ligation of insert and vector DNA in a molar ratio

1:5 was performed using T4 DNA Ligase from NEB according to the instructions of the manufacturer. The reaction was incubated O/N at 16°C. A control ligation reaction, containing only the vector and the ligation mix, was also performed, in order to determine the re-circularization frequency of the vector.

Transformation

An aliquot of 50 µL chemically competent (cc) *E. coli* cells (cc preparation described below) was thawed gently on ice. The ligation reaction (5 µL) was mixed with the cells and bacteria was incubated for 20 min on ice. After the incubation, the cells were heat shocked 42°C for 45sec and were immediately transferred on ice for 2 min. 500 µL of pre-warmed at 37°C liquid LB medium was added in tubes and the cells were incubated at 37°C for 1h under shaking conditions. After growing in LB medium, the transformed cells are plated on LB-agar, containing the appropriate antibiotic for the selection of transformed cells. The plates were incubated overnight (O/N) at 37°C.

Preparation of competent *E. coli* cells

E. coli cells (Dh5a, Rosetta or Stbl3) were used in order to prepare chemically competent cells. A fresh colony of desired strain were inoculated into LB medium for a starter culture. 200µL of the pre-culture were subsequently transferred into a larger culture (12mL) and were incubated to slowly growth at 30°C until OD₆₀₀ =0.4-0.6. After incubation, harvested cells were incubated with calcium chloride (CaCl₂) to make the cell membrane permeable. Cells were transferred on ice for 5min and then were centrifuged at 5.000 rpm for 5 min. The supernatant was discarded, the cells were resuspended into 2 mL of TFB1 buffer and were incubated on ice for 5 minutes. A centrifuge at 5.000 rpm for 5 minutes 4°C was followed. The supernatant was discarded, and cells were resuspended into 1 mL of TFB2 buffer and then were incubated on ice for 15 minutes. Aliquots of 50 µL of chemically competent cells were finally prepared.

TFB1: KCL: 30 mM, RbCl: 100 mM, CaCl₂: 10 mM, MnCl₂: 50 mM, Glycerol: 5% pH:5.8 (adjusted by acetic acid)

TFB2: MOPS: 10 mM, RbCl₂: 10 mM, CaCl₂: 75 mM, Glycerol: 15% pH:6.5 (adjusted by KOH)

Colony PCR

In order to identify the clones that bear the desired recombinant plasmid, a screening colony PCR was prepared. A number of colonies were transferred into tubes containing 50 μ L liquid LB. A DreamTaq DNA Polymerase (Thermo Scientific) were used according to the manufacturer's protocol. The PCR products were analyzed by agarose gel electrophoresis.

Reagents	Final concentration
10x dreamtaq buffer	1X
2mM each dNTPs mix	0,3 mM
10 μ M FW primer	0,3 μ M
10 μ M RV primer	0,3 μ M
Template DNA	5 μ L
Dreamtaq DNA polymerase	0,1 U

Small-scale plasmid preparation (mini prep)

The positive clones were transferred in 5 ml of LB culture containing the appropriate antibiotic and were incubated O/N at 37°C, under shaking. After incubation, a glycerol stock solution was prepared and 3ml of the culture was used for plasmid purification using the NucleoSpin® Plasmid - MACHEREY-NAYGEL kit according to the manufacturer's instructions.

DNA sequencing

To check accuracy of plasmids, all of them were sequenced before being used for the experimental procedure.

3.11. Maintenance of cell lines and cell culture

This study encompass a broad range of cancerous cell lines that are described below:

Cell line	Description	origin	Cell culture media	conditions
HeLa cells	Immortal human cells derived from a cervical carcinoma isolated. They were isolated from 31-year-old patient. They contain 76 to 80 total chromosomes. These cells proliferate abnormally rapidly as HeLa cells have an active version of telomerase during cell division, which copies telomeres over and over again.	ATCC	Dulbecco's modified Eagle's medium (DMEM)- high glucose stable glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS)	at 37°C in a 5% CO ₂
A549	Human lung adenocarcinoma cells. They are isolated from the lung tissue of a 58-year-old Caucasian male with lung cancer.	ATCC	Dulbecco's modified Eagle's medium (DMEM)- high glucose stable glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS)	at 37°C in a 5% CO ₂

3.11.1. Thawing, cryopreservation and passaging of cells

To thaw cells, cryovials were transferred from liquid nitrogen into a 37°C waterbath. Warm cell culture medium was added into vials and cells were transferred into 15 ml conical tube for centrifugation at 1000x g for 5 min. The supernatant was discarded carefully, cells were resuspended in 10 ml culture medium and placed into cell culture dishes (100mm).

For cryopreservation, the growth medium was removed, and cells were washed twice with 5ml PBS. 1x trypsin enzyme was added and plates was incubated for 10 min in incubator (37°C in a 5% CO₂). Trypsin is a protease which hydrolyzes protein at carboxyl side of the lysine or arginine residues and enables the cells to be detached from the plate. Two volume of pre-warmed medium was added to inactivate trypsin. The medium was gently dispersed by pipetting to unstick the cells. Cells were transferred into 15 ml tubes for centrifugation at 1000x g for 5 min.

The supernatant was discarded, and pellet of cells were resuspended in 1 ml freezing medium (90 % FBS, 10 % Dimethyl sulfoxide-DMSO). Cells were transferred in cryovials and placed into freezing container (Mr. Frosty) for storage at -80°C overnight. The freezing container is filled with isopropanol to allow the samples to achieve a slow cooling rate of -1°C/minute which is the optimal rate for cell preservation. For long-term storage, cryovials were transferred in liquid nitrogen.

To split cells, the above procedure was performed, when cells reach at 80-90% confluent with the difference that pellet of cells was resuspended in growth media and part of them were transferred to a new culture dishes or plate according to experimental procedure.

3.11.2. Transient transfection of cells

Cells were seeded on cell culture 6-well dishes and grown until they reached about 60% of confluency. Prior to transient transfection, the growth media was replaced with complete growth medium without P/S. The transfection was performed with Lipofectamine 2000 (Invitrogen) and the OPTI-MEM media is used. It contains insulin, transferrin, hypoxanthine, thymidine, and trace elements, which allow the reduction in serum supplementation by at least 50%. For each well of cells, 3 µL of Lipofectamine 2000 Reagent was added into the above diluted OptiMEM:DNA solution, was mix gently and was incubated for 30 minutes at room temperature to form DNA-Lipofectamine 2000 Reagent complexes. After incubation, 100 µL of the DNA- Lipofectamine 2000 Reagent complexes were added directly to each well containing cells and were mixed gently by rocking the plate back and forth. The cells were incubated at 37°C in a CO₂ incubator for 18-24 hours post-transfection before assaying for transgene expression.

3.12. Total RNA isolation and cDNA synthesis

Total RNA was extracted from cell cultures using Trizol (Invitrogen) according to the manufacturer's protocol. TRizol reagent was added directly to the culture dish and was incubated for 5 min to lyse the cells and to permit the solubilization of all contents. The lysate was transferred to a new tube and was added 200µL of chloroform used for lysis. The tubes were incubated for 2–3 min followed by a centrifugation at 12,000 × g for 15 minutes at 4°C. After

centrifugation the mixture separates into three phases, a lower red phenol-chloroform or organic phase which contains proteins, an interphase which contains DNA, and a colorless upper aqueous phase containing the RNA. The upper phase was transferred to a new tube and 500 μ L of isopropanol was added to precipitate the RNA. An incubation for 10 min and a centrifugation for 10 min at $12,000 \times g$ at 4°C was followed. The supernatant was discarded, and the pellet was resuspended in 1mL 75% ethanol. The samples then were centrifuged for 5 minutes at $7500 \times g$ at 4°C and the supernatant was discarded. The RNA pellet was allowed to air dry for 10 min and was dissolved in 35 μ L of RNase-free water. For small RNA isolation (<200nt), the mirVana Isolation Kit (Invitrogen) was used according to the manufacturer's instructions. In both cases, RNA samples were treated with DNase I (New England Biolabs) to ensure no contamination by genomic DNA according to the manufacturing protocol. A cleanup with phenol/chloroform extraction was performed and an ethanol precipitation was followed. tRNA-derived fragments (tRFs) (<40nt) were separated on a 10% UREA-PAGE. The quantity and yield of each RNA sample was evaluated by Multiskan Sky Microplate Spectrophotometer. Subsequently, the samples were used for cDNA synthesis using SuperScript™ II Reverse Transcriptase Kit (Invitrogen) following the manufacturer's protocol. Typically, 3 μ g of total RNA was used for cDNA reaction (20 μ L final volume), and the large fractions (>200nt) was reversed transcribed using oligo-dT primer while small RNAs (<200nt) were initially polyadenylated using E. coli Poly(A) Polymerase (New England Biolabs) and then reverse transcribed using an oligo-dT adapter primer.

3.13. Quantitative polymerase chain reaction (qRT-PCR)

50 ng cDNA was used as template to perform qPCR reactions using KAPA SYBR FAST qPCR Kit (Kapa biosystems) following the manufacturer's protocol. Three segment was constructed in thermal profile including one segment 3 min of initial denaturation at 95°C , second segment with forty cycles, followed by segment amplification step: 3s at 95°C for denaturation, 30s at respective annealing temperature and the third dissociation or melting segment followed by 1 min at 95°C , 30 sec at 55°C and 30 sec at 95°C . Reactions were set up in 96-well plates and performed on an MX3000P real-time thermocycler (Agilent). Reference genes used for

normalizing was ACTB and RNU6 to measure the mRNA and small-RNA expression, respectively. The Ct values were analyzed using the $2^{-\Delta\Delta CT}$ method.

3.14. SDS-PAGE and Western Blot analysis

For protein analysis, 100mm dishes with cells that had reached 80% confluence were twice washed with 1X PBS containing 1mM EDTA pH 8.0 and incubated on ice. The cells were scratched (with rubber policeman) on ice in 5 mL ice cold PBS-EDTA and transferred in a 15 mL falcon tube. The cells were collected by centrifugation (6500 rpm, 5 min at 4°C). Cell pellets were suspended in RIPA buffer [150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1% NP-40, 0,5% Triton X-100, 1 mM EDTA pH 8.0, 0.5% Sodium Deoxycholate, 1mM Dithiothreitol (DTT) and protease inhibitors (1 µg/ mL aprotinin, 1 µg/ mL pepstatin, 1 µg/ mL leupeptin, 1 mM PMSF και 1 mM Na₃VO₄)] and incubated for 20 min on ice. During the 20 min period the samples were suspended with vortex for 1 min every 5 min. After the incubation the samples was centrifuged at 13.000 rpm for 20 min at 4°C and the supernatant was transferred into new tubes. Protein concentrations were determined by the Bradford assay.

Proteins were separated by gel electrophoresis on SDS-polyacrylamide gels as described in Table 3.8. Samples were prepared for running by adding an equal amount of 2x Laemmli Buffer (25% v/v glycerol, 5% β-mercaptoethanol, 2% w/v SDS, 0.01% w/v bromophenol blue and 65mM Tris pH 6.8) and were further heated to 95°C for 10 min. Gels were run using Mini-Protean gel electrophoresis tank (Bio-Rad Labs, USA) at 120V using Tris/glycine/SDS running buffer (1x): 25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS.

After running proteins were subsequently electrophoretically transferred from gel to polyvinylidene difluoride (PVDF) membrane (Millipore). Before use, PVDF membranes were activated by wetting the membrane in 100% methanol for a 10 sec, 2 min in ddH₂O and then equilibrating in 1x transfer blotting buffer (2.5 mM Tris, 0.192 M glycine, 20% methanol) for 5 min. The sandwich of blotting was prepared and was run for 3h 42V in a cold room. After transfer, membranes were blocked for 3h at room temperature with 5% nonfat milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.5) in order to minimize any unspecific interaction of the antibody. An incubation with primary antibodies was followed (diluted in 3% Nonfat Dry Milk-TBS buffer) for

3 h at room temperature or overnight at 4°C under rotation. The concentration of the primary antibodies ranged depending on the antibody used. After incubation, membranes were washed with TBS three times for 5 min. Further, the membranes were incubated with secondary antibodies (diluted in 2,5% Nonfat Dry Milk-TBS buffer) at room temperature for 1 h followed by washing with 1X TBS three times. Where it is necessary, the removal of primary and secondary antibodies from membranes was performed using stripping buffer (20 mL SDS 10%, 12.5 mL Tris HCl, pH 6.8, 0.5 M, 67.5 mL ddH₂O, add 0.8 mL β -mercaptoethanol). The stripping solution was first incubated at 65°C for 30 min and then was added in membrane for 30 min at RT. After incubation, the membrane was rinsed 3 times with TBS buffer. The protein bands were detected by means of the ECL kit Western Blotting detection (Bio-Rad). The secondary antibody is directly coupled to horseradish peroxidase and the band was visualized by UltraCruz® Autoradiography Film (Sant Cruz Biotechnology). To scan the X-Ray films, a flatbed scanner capable of digitizing transparencies was used. The calculation of band intensity was performed using ImageJ analysis software. The protein levels were normalized against β -actin and GAPDH levels in each separate set of experiments.

For non-radioactive measurement of translation rates, cells were treated with 1 μ M puromycin for 10 min at 37°C and after incubation total protein extracts were isolated. The levels of puromycylated nascent peptides were measured by immunoblotting according to the above-described procedure.

Table 3.8: Recipe for SDS-polyacrylamide gel

Stock Solution	Stacking Buffer	Separating Buffer
Final Concentration		
30 % (w/v) acrylamide	5%	8% -15%
1.5 M Tris-HCl pH 6.8	0.125 M	-
1 M Tris-HCl pH 8.8		0.375 M
10 % (w/v) SDS	0.1%	0.1 %
10 % (w/v) APS	0.1%	0.1 %
Temed	0.1%	0.04%
H ₂ O	up to final volume	up to final volume

3.15. Immunofluorescence microscopy and immunostaining

Cells were seeded in 12-well plates containing 13mm coverslips. If required, transfection was performed, and cells were incubated for 24h or in different time points according to the experiment procedure. After treatment, cells were washed twice and were fixed in 4% paraformaldehyde (PFA) for 10min in RT, followed by rinse twice with 1x PBS in order to remove the remaining PFA. The permeabilization with 0.3% Triton X-100 for 5min was followed. For blocking, 10% FBS and 3% BSA in PBS (blocking solution) was incubated for 60min followed by overnight incubation at 4°C with specific primary antibodies (Table 3.4). If required, a co-staining of two antibodies diluted together in blocking solution. Anti-mouse IgG secondary antibody conjugated to Alexafluor dyes (Invitrogen) was used to 1:400 dilution in blocking solution. The secondary antibodies were incubated for 60min at room temperature. The samples were washed twice in 1x PBS, and the nuclei were stained with *Hoechst* (5µg/ml) in PBS for 2min. The cover slips were washed again and were embedded on a microscope slide using Mowiol as mounting medium. Fluorescence cell imaging was performed on a Nikon Eclipse TE 2000-U. The images were analyzed using Image J.

3.16. Cell cycle

For cell cycle analysis, to synchronize cell cultures, cells were seeded on 6-well plates and were harvested for 24h with serum deprivation. After starvation, the cells were passaged and released into cell cycle by addition of serum for 24h and 48h. In the case of overexpression experiments, cells were transiently transfected with the recombinant plasmids and with empty vector as control and were harvested at 24 h post-transfection. For FACS analysis, cell samples were fixed with 70% pre-cooled ethanol for 24h. After incubation, cells were centrifuged and were washed using 1x PBS. Then, cells were incubated with 10 µg/ml Propidium Iodide (PI) staining and 100 µg/ml RNase A (both diluted in PBS) in a 37°C warm water bath for 30 min. The stained cells were analyzed using a BD Accuri C6 Plus Flow Cytometer.

3.17. Cell proliferation assay

For proliferation assay, 20,000 cells were seeded per well on 12-well plates in DMEM/10% FBS. If necessary, prior to further experimentation, cells were transiently transfected with suitable plasmids and cells were measured 24h after transfection. After incubation, cells were washed twice with 1x PBS and were stained with 1ml 0.2% crystal violet in 20% methanol for 10min. Crystal violet binds to proteins and also DNA molecules and its amount is reduced when cells undergo cell death. Then the cells were washed three times with ddH₂O and were solubilized with 500µl of 10% acetic acid for 20 minutes. The solubilized crystal violet was measured at 590nm.

3.18. Resazurin assay

This method detects the cell metabolic by converting the nonfluorescent dye, resazurin, to the highly red fluorescent dye, resorufin, in response to chemical reduction of growth medium resulting from cell growth. Cells were seeded in 96-well plates in DMEM/10% FBS in a series of dilutions (2×10^3 , 4×10^3 cells/well) and were harvested for 24h, 48h and 72h. Cells were incubated for 2h with ready resazurin solution (Biotium) at 37°C followed by fluorescence-based detection at 570nm and 600nm Excitation/Emission respectively, according to the manufacturer's instructions at FLUOSTAR. For experiments needed transfection, cells were transiently transfected with the recombinant plasmids or empty vector as control using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. 6h post-transfection, the cells were treated with trypsin (0.25%) and were seeded in 96-wells in a series of dilutions followed by the same procedure.

3.19. Wound healing assay

For the wound healing assays, cells were seeded in a 6-well plate at a density of 7×10^5 cell per well. After 24h incubation, wounds were made by scratching the monolayer using a sterile pipette tip and the detached cells were washed away with PBS. For experiments needed transfection, cells were transiently transfected with the recombinant plasmids or empty vector

as control using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions and 6h post-transfection the scratch were made. Images was captured following the wound procedure at different time points using a KERN OCM-161 inverted microscope at 10X magnification and the scratched area was quantified at each time point using ImageJ MRI tool.

3.20. Mitochondria measuring

For measurements of mitochondrial dyes using flow cytometry the following procedure was performed. Cells were seeded in 6-well plates at 0.5×10^6 density. For experiments needed transfection, cells were transiently transfected with the recombinant plasmids or empty vector as control using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions and 24h post-transfection, cell were stained using the dyes (Invitrogen) for 40 min at 37°C. Subsequently, the cells were washed three times with prewarmed complete PBS (CPBS). The stained cells were analyzed using a BD Accuri C6 Plus Flow Cytometer. The results are expressed as a histogram presenting cell fluorescence emission and were quantified using the mean fluorescence intensity (MFI). For measurement of ROS production, the MitoSOX red (Thermo Fisher) was used at 2.5 μM final concentration. MitoSOX Red reagent is permeable to live cells and is selectively targeted to mitochondria. It is oxidized by the superoxide and exhibits a red fluorescence. For measurement of mitochondrial membrane potential or mass the MitoTracker Red CMXRos and MitoTracker Green were used at 150nM and 100nM final concentration, respectively. For fluorescence detection of mitochondria, the Mitotracker Deep Red (thermo Fisher) dye was used. Cells were seeded in 12-well plates containing 13mm coverslips and 24h after incubation, the media was removed and was replaced with fresh containing 150nM of the dye. Cells were incubated for 30 minutes at 37°C. After incubation the cell were fixed according to the procedure at §3.14 and were visualized at Nikon Microscopy and the images were analyzed using Image J software.

3.21. Polysome profiling

For polysome profiling the mRNAs are separated on a sucrose gradient based on the number of ribosomes bound. Cells were seeded on 150 mm dishes and grown until they reached about

70% of confluency in order to not distort the translation process. Further, cells were incubated at 37°C with 100 µg/ml cycloheximide (CHX) for 10 min and then were washed twice with cold 1x PBS containing 100 µg/ml CHX. Cells were collected using cell scraper into eppendorfs and were centrifuged for 5 min, 1500 x g at 4°C. The supernatant was discarded, and the cells were lysed with 1 ml of lysis buffer [20mM Tris-HCl pH 7.4, 150mM KCl, 7mM MgCl₂, 1mM DTT, 1% NP-40, 1% (v/v) Protease Inhibitor Cocktail (Sigma), 40U/µL RNase Inhibitor Murine (New England Biolabs) and 100µg/ml CHX]. An incubation with rotation for 30min at 4°C was followed. After incubation, cells were centrifuged at 20.000 x g for 30 min at 4°C and 20 OD₂₆₀ units from supernatant were loaded onto a 15-50% sucrose density gradient. Ultracentrifuge in an SW41 rotor at 39,000 × rpm for 2h and 30 min at 4°C was followed. The samples were analyzed by optical scanning at 254 nm using continuous flow cytometer (Figure 3.4).

For step gradient, 5 standard sucrose solutions (55%, 45%, 35%, 25% and 15% (w/v)) were used. For 2 SW41 Beckman tubes, 25ml of Buffer G [20mM Tris-HCl pH 7.4, 100mM KCl, 3 mM MgCl₂, 100µg/ml CHX (added fresh) and 1mM DTT (added fresh)] and 20ml 55% (w/v) sucrose solution (11 g sucrose for 20 ml) were prepared, and the sucrose solutions were performed.

Sucrose solutions	50% (w/v) sucrose solution	Buffer G
γ1α 50% (w/v) sucrose	6.00ml	-
γ1α 45% (w/v) sucrose	5.40ml	0.60ml
γ1α 40% (w/v) sucrose	4.80ml	1.20ml
γ1α 35% (w/v) sucrose	4.20ml	1.80ml
γ1α 30% (w/v) sucrose	3.60ml	2.40ml
γ1α 25% (w/v) sucrose	3.00 ml	3.00ml
γ1α 20% (w/v) sucrose	2.4ml	3.6ml
γ1α 15% (w/v) sucrose	1.8ml	4.2ml
Total Volume	6ml for each solution	

For each ultracentrifuge tubes with 12ml total volume the following procedure was performed:

- 2.2 ml sucrose solution 55% (w/v) was added
- Freeze for 15 min at -80°C (cover top with parafilm to avoid contamination).

- Repeat steps 1-2 using the rest 4 sucrose solutions.
- Leave Beckman tubes at 4°C O/N to become linear.

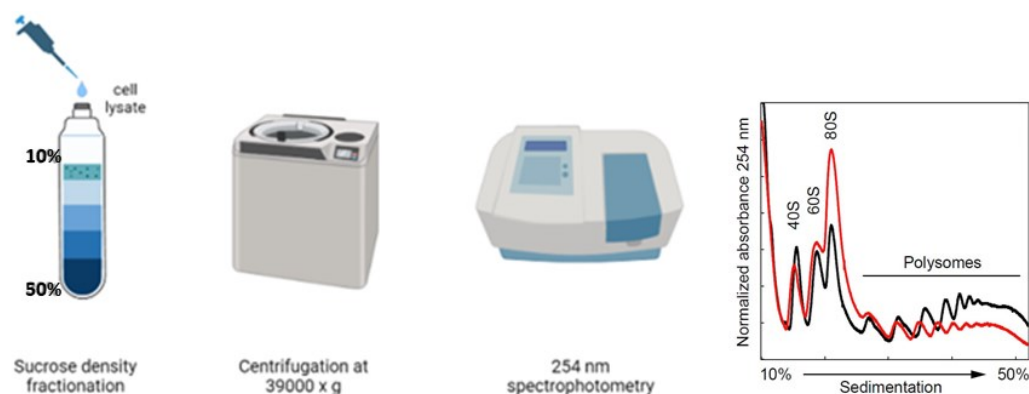


Figure 3.4: An overview of polysome prolife procedure.

3.22. Protein expression and purification

The LAM domain of human La protein (hLAM; residues 1-103) was cloned into a pET20.b(+) vector and expressed in *E. coli* BL21 (DE3) Rosetta2 pLysS cells. A single colony of BL21 cells was inoculated into 5ml LB medium and 50µg/ml Kanamycin overnight at 37°C for a starter culture. The inoculation was added to 1000 ml LB broth and were incubated at 37° C on a shaking table until OD₆₀₀ reached 0.4-0.5. Protein expression was induced by addition of 0.5mM IPTG (Isopropyl β-D-1-thiogalactopyranoside; Sigma) for 16 hours at 16° C. After induction, the culture was centrifuged at 4500 x g for 10 min at 4°C and the supernatant was discarded. The pellet was resuspended in 5 ml NP10 buffer/Wash buffer (50mM Tris-HCl pH 7.5, 100mM KCl, 5% Glycerol, 10mM imidazole) by vortexed. In order to facilitate the lysis of bacteria cell walls, 1 mg/ml of lysozyme was added followed by incubation on ice for 30 minutes and stirring at several times. The homogenization of the cells was performed using sonication with 5 bursts at a power of 25-50 W for 10 sec each, on ice. Cell debris was removed by ultracentrifugation at 4°C for 1:30 h at 10000 x g and the supernatant containing the protein was collected into Falcon tubes. 500 µl fraction of the supernatant (Input) was aliquoted. The supernatant was mixed with Ni-NTA agarose beads (Thermo Scientific) and was incubated for 30 min in a rotor at 4°C. The recombinant protein containing 6xHis tag bound to the nickel-coated beads. After incubation,

the solution (supernatant-beads) is slowly loaded onto the syringe and the column was packed. The flow-through solution which contains the unbound proteins was collected in a falcon. After the packaging, the column was washed with 50ml of NP-20 buffer/Wash Buffer (50mM Tris-HCl pH 7.5, 100mM KCl, 5% Glycerol, 20mM imidazole), thus decreasing the unspecific interactions. To elute the recombinant protein, the column was wash with a high concentration of imidazole (50mM Tris-HCl pH 7.5, 100mM KCl, 5% Glycerol, 20.mM imidazole). During elution, 500 μ l of fractions were collected. The protein concertation of each fraction was determined by the Bradford Protein Assay. The fractions with higher concertation were separated by gel electrophoresis on SDS-polyacrylamide gels (as described in §3.14). After electrophoresis, the gel was stained with Coomassie Brilliant Blue staining solution. Firstly, the solution was pre-warmed for 5 sec and then was incubated for 20 min at room temperature. The gel was destained with the de-staining solution (45% methanol, 10% acetic acid) for 1h. The fractions containing the recombinant protein were purified using size exclusion chromatography in Superdex 200 10/300 GL (GE Healthcare).

3.23. In vitro transcription and radiolabeling

For pre-tRNA^{Arg}_{CCU}, a pUC57 vector containing pre-tRNA^{Arg}_{CCU} from *Dictyostelium discoideum* downstream of a T7 RNA polymerase promoter was linearized using the DraI restriction enzyme from NEB. The reaction was incubated for 3h at 37°C. The digested plasmid was checked for accurate linearization by 1% agarose gel electrophoresis and was purified by gel extraction prior to *in vitro* transcription. For tRNA^{Gly}_{GCC}, a pcDNA3.1 vector containing pre-tRNA^{Gly}_{GCC} from *Homo sapiens* was used as template for High-fidelity DNA polymerase (§3.10). Different set of primers were designed and were used for PCR depending to desired transcript.

Reagents	Concentration
10x CutSmart Buffer	1x
Plasmid construct	10 μ g
(10,000 units/ml) DraI enzyme (NEB)	50U (0.5 μ L)
ddH ₂ O up to	50 μ L

The amplified product and the linearized plasmid were used for run-off transcription as follows. The *in vitro* transcription reaction was carried out at 37°C for 16h.

Reagents	Concentration
10X T7 RNAP buffer	1x
DTT	5mM
UTP	1mM
GTP	2mM
CTP	2mM
ATP	2mM
Linear Plasmid	10μg
RRI	10U
pyrophosphatase (PPase)	0.2U
T7 RNA Polymerase	250U
DEPC treated-H ₂ O up to 100 μL	

The reaction was treated with DNase I (New England Biolabs) followed by phenol/chloroform extraction. Transcripts were PAGE purified (10% polyacrylamide, 8M urea, 1x TBE). Briefly, the transcript was visualized by UV shadowing and the bands corresponding to tRNAs were excised and eluted by i.v.t elution buffer (1x i.v.t. elution buffer: 10 mM Tris-HCl, pH 7.5, 300 mM KCl, 1 mM EDTA) for 16h in room temperature. The eluted transcript was collected in new eppendorf, was precipitated with ethanol and was resuspended in DEPC treated-H₂O. For the radiolabeling, 10pmol of pre-tRNA was dephosphorylated using Antarctic phosphatase (5U/μL, NEB) at 37°C for 1h. After reaction, the transcript was purified by phenol/chloroform and was precipitated with ethanol. Subsequently the 5' ends of the pre-tRNA and a synthetic 10-mer oligo(U) (Eurofins) were labeled used [γ -³²P]-ATP in the presence of 10U T4 PNK (NEB) and 10U RRI (NEB).

3.24. Electrophoretic mobility shift assay (EMSA)

The binding assay was performed with the addition of increasing concentrations of LAM (5-100μM) in the presence of binding buffer (50mM Tris-HCl; pH 7.5, 10mM NH₄Cl, and 5mM MgCl₂, 1U RNase Inhibitor (NEB) and 1mM EGTA. Each labeled RNA transcript (1nM) was supplemented

with cold to a final concentration of 0.2 μ M. The pre-tRNA transcript was denatured for 5min at 65°C in presence of 10mM MgCl₂, and slowly cooled down to 37°C. The ligands were incubated with hLAM at 37°C for 15min and for 15min at RT and the mixtures after the addition of 1x loading buffer (50mM Tris-HCl; pH 7.0, 10% glycerol, 0.012% bromophenol blue, 0.012% xylene cyanol in DEPC-treated H₂O) remained on ice for 10min. The samples were further analyzed on a 10% v/v native PAGE. The gel was pre-run at 10mA for 15min and then at 8mA for 10h at 4°C. The gels were visualized using Fujifilm FLA-3000 phosphorimager scanning and the bound and free labeled RNA was analyzed by band intensity measurement using AIDA image analyzer software (Raytest version 5.0). GraphPad Prism Software (version 8.00) was used to plot and analyze binding affinities by nonlinear regression. The calculated values were used for the corresponding Scatchard plots (Bound/free versus Bound) to calculate binding constants (K_d) according to the equation: slope = $-1/K_d$.

3.25. *In vitro* pre-tRNA processing assay

The 5' end radiolabeled pre-tRNA transcript (§3.20) was used for pre-tRNA processing assay. Wild type and knockout cells were harvested and were resuspended in lysis buffer (30mM HEPES-KOH pH 7.4, 100mM KCl, 5mM MgCl₂, 10% glycerol, 1% NP-40, 0.1mM PMSF, 1mM DTT). Cells extracts were incubated for 20 min on ice and during this period the samples were suspended with vortex for 1 min every 5 min. After the incubation the samples were centrifuged at 13.000 x g for 20 min at 4°C and the supernatant were transferred in new tubes. The samples were flash frozen in liquid nitrogen and stored at -80°C. To evaluate the leader removal by RNase P, the radiolabeled pre-tRNA^{Arg}_{CCU} (up to 0.3 μ M in the presence of unlabeled transcript) was mixed with 1x reaction buffer (10mM HEPES-KOH pH 7.4, 100mM KCl, 5mM MgCl₂, 2mM DTT, 3.5mM ATP, 4.5mM Spermidine and 60U/ml RRI) and was denatured for 1 min at 95°C. After refolding for 10 min at RT, the transcript was incubated with cells extracts (final concentration 0.3mg/ml) for 30min at 30°C. pre-tRNA transcript were used as a negative control while the RNase Z^L enzyme used as positive control. All reactions were performed in a final volume of 20 μ L and after incubation was followed phenol/chloroform extraction. Samples were mixed with equal volumes of F-EDTA loading buffer (90% formamide, 10mM EDTA, 0.025% bromophenol blue, 0.025%

xylene cyanol) and were loaded on 10% denaturing polyacrylamide gel. After electrophoresis, gels were autoradiographed on a phosphorimager (Fujifilm FLA 3000 platform).

3.26. Next generation sequencing and bioinformatics analysis

RNA sequencing technology provides a powerful approach for studying the transcriptome profile of large or small RNA molecules. For our experiments, total and small RNAs were isolated using the Trizol Reagent (§3.12). The first step for next-generation sequencing (NGS analysis) is the ribosomal RNA depletion. In order to avoid deep read alignment at rRNA repeat loci, as these molecules are the most abundant, it is necessary to remove them before the sequencing. The depletion was performed using the RiboMinus kit (Ambion) which contains beads carrying oligonucleotides complementary to rRNAs. High-quality total RNA (1-5µg) was added to each reaction. The hybridization solution is pre-heated to 50°C to solubilize the salts and then was added in the reaction according to the following table:

Reagent	Concentration
2X Hybridization Buffer	1x
RiboMinus™ Eukaryote Probe Mix v2	4µl
Total RNA	5µg
ddH ₂ O	Up to 100µl

The reaction was gently mixed and incubated at 70°C for 10 min for denaturation and the 20 minutes at 37°C for hybridization. After hybridization step, the reaction was left at 37°C until to preparation of the magnetic beads.

- The magnetic beads are resuspended thoroughly by vortexing and 500µL is transferred to clear eppendorf for each sample.
- The eppendorfs are placed on a magnetic separation stand for 1 min or until the solution becomes clear and then the supernatant was discarded.
- The beads were dissolved in 500 µL of sterile ddH₂O and placed back on the magnetic separation stand for 1 minute. The wash procedure was repeated one more.

- The beads were then resuspended in 200 μ L of 1x Hybridization Buffer and were incubated at 37°C for 5 min.

After incubation, the RNA-containing samples were briefly centrifuged and transferred to the eppendorf with the magnetic beads. The sample were incubated for 15 minutes at 37°C and then were centrifuged and placed on the magnetic base for 1 minute or until the solution becomes clear. The supernatant, containing the RNA without the rRNAs was transferred to a new eppendorf. Finally, the sample is further purified and concentrated after precipitation according to the manufacturer's protocol.

Library preparation using the Ion Total RNA-Seq Kit v2

Next-generation sequencing using both ION Torrent and other similar technologies has the limitation that it cannot sequence fragments larger than 200 bases. It can therefore be understood that in order to sequence larger molecules, such as mRNA, the RNA should be fragmented into smaller fragments (up to 200nt). The fragmentation was performed with the RNase III enzyme, according to the manufacturer's instructions. Before adaptor ligation, the RNA fragments should be purified with magnetic beads according to the manufacturer's instructions.

ION TORRENT cDNA library preparation

The first step in the preparation of a cDNA library for the whole cell transcriptome is the hybridization and binding of RNA molecules with appropriate primers. More specifically, and based on the instructions provided by the manufacturer, the hybridization mixture should be prepared as follow:

Reagent	volume
Ion Adaptor Mix v2	2 μ l
Hybridization Solution	3 μ l

The hybridization mixture is added to 3 μ L of the fragmented RNA (or up to 100ng) and the mix are incubated as follow: 10 minutes at 65°C and 5 minutes at 30°C. Reagents are then added to hybridize and bind the RNA molecules with the appropriate primers.

Reagent	volume
Hybridization reaction	8 μ l
2X Ligation Buffer	10 μ l
Ligation Enzyme Mix	2 μ l

The mix is incubated for 1 hour at 30°C while preparing the reverse transcription reaction as follow:

Reagent	volume
10X RT Buffer	4 μ l
2.5mM dNTP Mix	2 μ l
Ion RT Primer v2	8 μ l
ddH ₂ O	up to 16 μ l

The reactions are combined and 4 μ L of 10X SuperScript III Enzyme Mix is added to the mix. The reaction is then incubated for 30 minutes at 42°C. The same procedure is followed for the construction of libraries for the sequencing of small RNA molecules, provided that the original sample has undergone an enrichment procedure for these molecules. In the present work this was achieved after Urea Page electrophoresis and isolation of RNA molecules of the desired size (<40 nucleotides). Before the cDNA library preparation, the cDNAs were purified according to the manufacturer's instructions.

cDNA preparation

The cDNA preparation was performed as follow:

Reagent	volume
Platinum® PCR SuperMix High Fidelity	45 μ l
Ion Xpress™ RNA 3' Barcode Primer	1 μ l

6 μ L of each cDNA was added to the above mixture and the following incubations were performed as follow: 94°C for 2 min (Hold), 94°C for 30 sec, 50°C for 30 sec, 68°C for 30 sec (2 cycles), 94°C for 30 sec, 62°C for 30 sec, 68°C for 30 sec (2cycles) and 68°C for 5 min (Hold). After incubation, the cDNA libraries were purified according to the manufacturer's instructions. At the end of the procedure, 1 μ L of each sample is analyzed in the Bioanalyzer using the Agilent® DNA 1000 Kit. The data is analyzed using 2100 Expert software to calculate the concentration of molecules in the range 50-160nt.

For small RNA library preparation prior to kit protocol, the enrichment for small RNAs was performed using 15% UREA-Page. 10 μ g of total RNA samples were runned in a 15% Urea-Polyacrylamide Gel with 1x TBE. A mix of Low Range ssRNA and miRNA ladder (NEB) was used. After the run stain the gel in 10ml 1X SYBR Gold in TE Buffer. After the SYBR Gold staining, the gel was excised from 150kDa of the RNA marker (Figure 3.5).

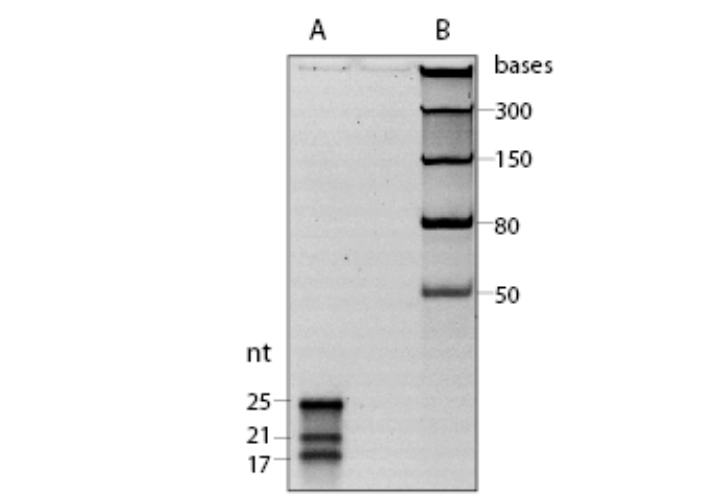


Figure 3.5: The Low Range ssRNA and miRNA ladder (NEB).

Template preparation and chip loading prior to sequencing was performed on the Ion Chef system using the Ion 540™ Chef kit and an Ion 540™ chip. The loaded chip was then sequenced on an Ion GeneStudio S5 sequencer. The facility of Ion Torrent Next Generation Sequencing platform is located in School of Medicine of the University of Patras. At the end of sequencing the results are retrieved as Fastq files from the corresponding server containing all the sequences

which are then analyzed on this website. For downstream analysis, the sequencing data were uploaded to the Galaxy web platform through the usegalaxy.eu public server (Figure 3.6). (Blankenberg et al., 2010).

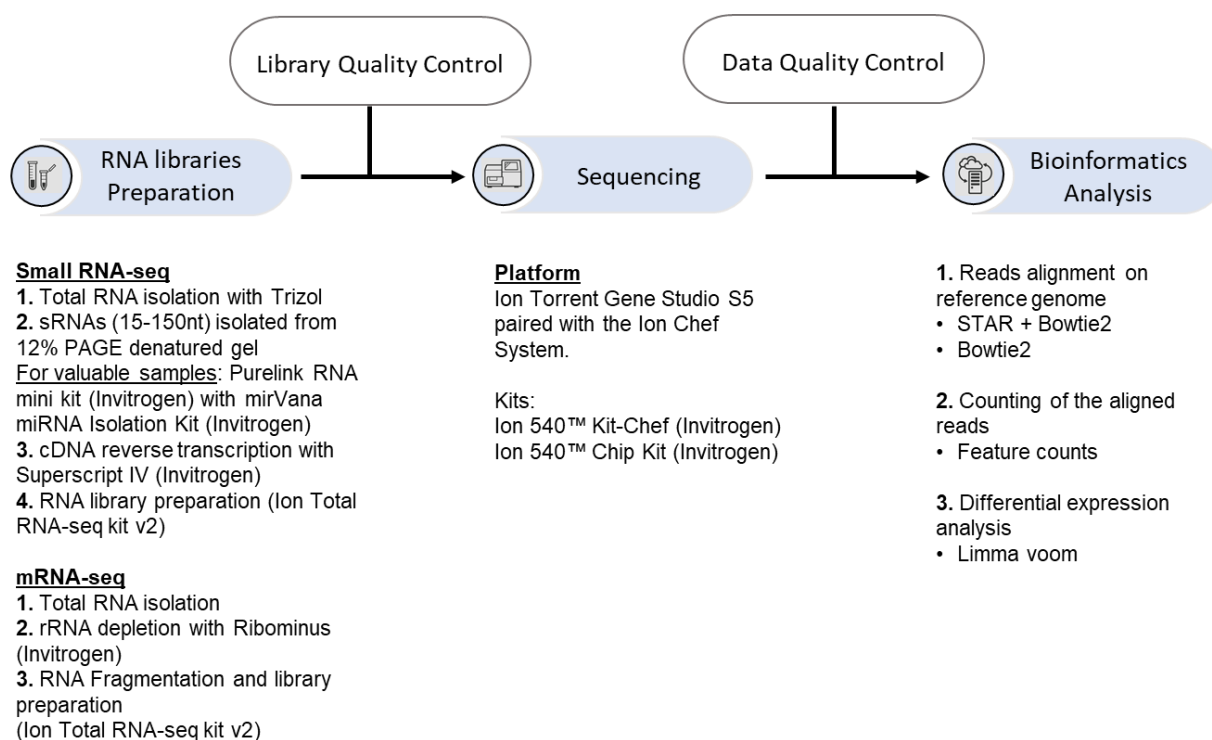


Figure 3.6: Total and small RNA sequencing pipeline.

Reads from total RNA-seq libraries were initially mapped to hg38 with the STAR aligner (v2.7.8a) with default parameters except the following (`--sjdbOverhang 75--outSAMmapqUnique 255--chimSegmentMin 18--chimScoreMin 12--outFilterType BySJout`) (Dobin et al., 2013). The unmapped reads were subsequently aligned to hg38 using bowtie2 (v2.4.5) using the `--very-sensitive-local` preset (Langmead and Salzberg, 2012a). The generated alignment files were then merged using picard MergeSamFiles (v2.18.2.1) and the unmapped reads were filtered out using samtools view (v1.15.1) (Broad Institute, 2009; Li et al., 2009). Read counts and differential gene expression analysis was performed with featureCounts (v2.0.1) and limma-voom (v3.50.1) respectively, with genes with CPM>0.5 in at least one sample only considered (Liao et al., 2014a; Liu et al., 2015a). Fastq files from small RNA libraries were quality trimmed with fastp (v0.23.2)

with default options(Chen et al., 2018). The reads were then mapped to hg38 with Bowtie2 (v2.4.5) with the `–very-sensitive-local` preset (Langmead and Salzberg, 2012b). The read counts for ncRNAs and miRNAs were generated with featureCounts (v2.0.1) using annotation files from ENSEMBL and miRbase respectively(Liao et al., 2014b). The differential gene expression analysis was performed with limma-voom (v3.50.1) (for genes with CPM>0.5 in at least one samples) (Liu et al., 2015b). In contrast, the expression levels of tRF (tRNA-derived Fragments) molecules were determined based on their exact sequences which were measured unaltered in the trimmed Fastq files (Figure 3.6).

3.27. Statistic data analysis

All experiments were performed in three independent experiments and data were presented as mean $\pm$ SD. P-values were determined by GraphPad Prism software v.6.0.1 using one-way ANOVA with multiple comparisons. * $P \leq 0.005$, *** $p \leq 0.0005$, **** $p \leq 0.0001$.

4. Results

4.1. Interactions of ncRNAs and La Protein

4.1.1. La overexpression leads to increased proliferation

Previous studies have been shown that La is overexpressed in human malignant cell lines and tissues (Al-Ejeh et al., 2007). Of note, it has been reported that La is translocated to the cytoplasm where is bound to IRES elements facilitating translation of oncogenic mRNAs. To study the role of La in lung cancer, A549 NSCLC cells were transiently transfected with the pcDNA3.1-SSB plasmid encoding La protein and with empty pCDNA3.1 which used as control. The expression levels of La protein were further verified by immunoblot analysis over the endogenous protein and an increased expression up to 12-fold was observed (Figure 4.1a).

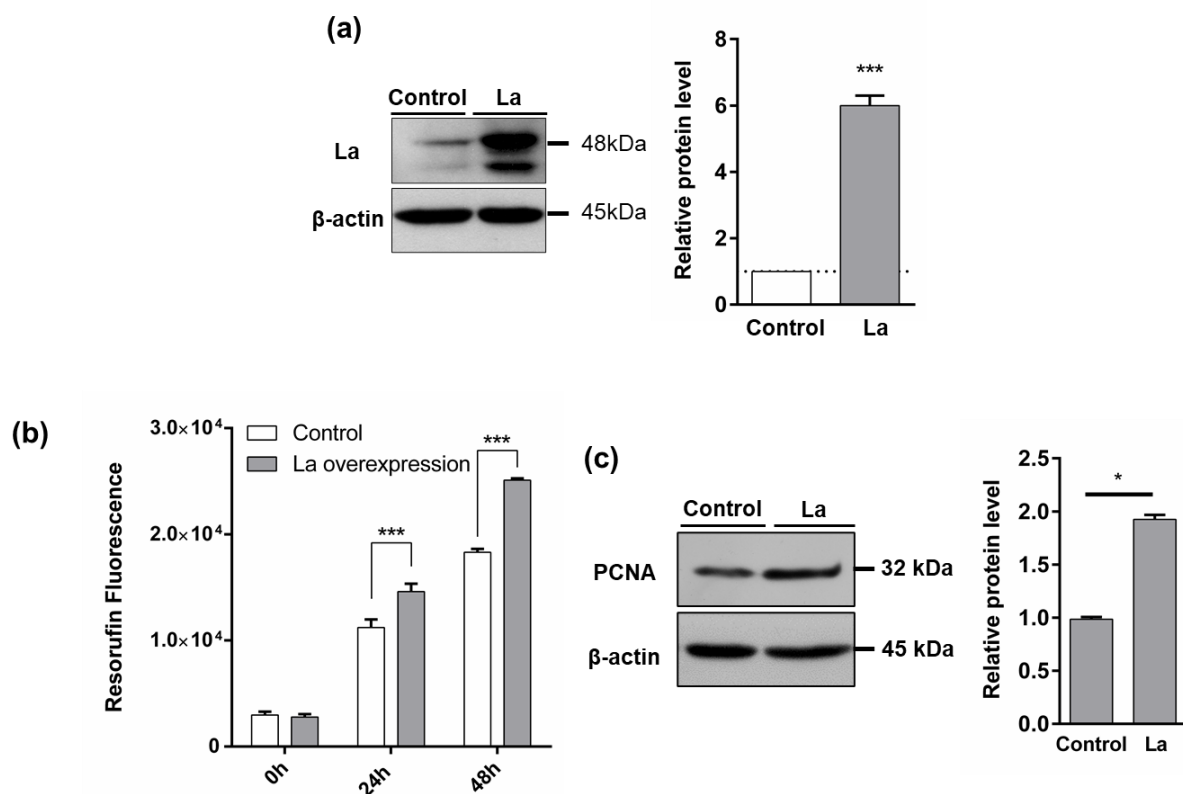


Figure 4.1: (a) Immunoblot analysis of La protein levels in transfected-A549 cells compared to control (left panel) and relative quantitation of La protein level after normalization to β -actin (right panel). (b) Resorufin fluorescence intensity measured during resazurin reduction of cells overexpressing La protein compared to control at different time points (c) Immunoblot analysis of PCNA (left panel) and quantification of its protein level (right panel) in A549 cells overexpressing La compared to control cells. The relative quantitation of PCNA level was measured after normalization to β -actin levels. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

After confirming the successful overexpression, the cell viability and consequently the proliferation rates of transfected cells using the resazurin cell viability assay were measured. After 24h and 48h of cell growth, a higher proliferation of cells, almost 20% compared to control was observed (Figure 4.1b). The proliferation was further verified by the upregulation (>2.6-fold) of PCNA (Proliferating Cell Nuclear Antigen) using immunoblotting analysis (Figure 4.1c). PCNA acts as a processivity factor for DNA polymerase δ in eukaryotic cells and is essential for replication, DNA repair and chromatin remodeling.

4.1.2. La overexpression leads to increased cell motility

To determine the functional consequences of La overexpression, the cell cycle analysis through propidium iodide (PI) staining assay was performed. After a synchronization of cells with serum starvation (G0 synchronized) for 1 day, the cells were trypsinized, re-seeded to sub-confluent density in fresh media, allowed to re-enter the cell cycle and were transiently transfected for 24 h. The results showed that the transfected cells didn't exhibit any significant changes in their cell cycle phases, however it is observed a slightly higher S-phase (Figure 4.2a). Moreover, the cells overexpressing La were used for RT-qPCR analysis of major cyclins expression levels (Cyclin A; CCNA, Cyclin B; CCNB1, Cyclin D; CCND1 and Cyclin E; CCNE1). It is observed that the cyclins were found moderately affected with no significant alterations. However, the Cyclin D was found significantly upregulated (>2-fold relative expression). Moreover, the expression of cyclin dependent kinase inhibitor 1A (P21; CDKN1A), the tumor suppressor p53 (TP53) and its inhibitor MDM2 were tested. P21 and TP53 mRNA expression levels were unaffected while a significant increase of MDM2 (>2-fold relative expression) (Figure 4.2b). The upregulation of MDM2 and the cyclin D is possibly upon the binding of La (Trotta et al., 2003, 2; Sommer et al., 2011a, 1; Nakatake et al., 2012, 2).

Since the proliferation rates of cells are affected, the cell migration of cells was examined using the wound healing assays at different time points. Remarkably, cells overexpressing La exhibited increased motility within the first 24h (Figure 4.2c). Interestingly, qPCR experiments confirmed this observation as the expression levels of MMP2 and VEGFA (>1.2-fold and >1.5-fold

respectively) were elevated. Both factors consist important migration and angiogenesis related markers in cancer (Claesson-Welsh and Welsh, 2013; Gong et al., 2014) (Figure 4.2d).

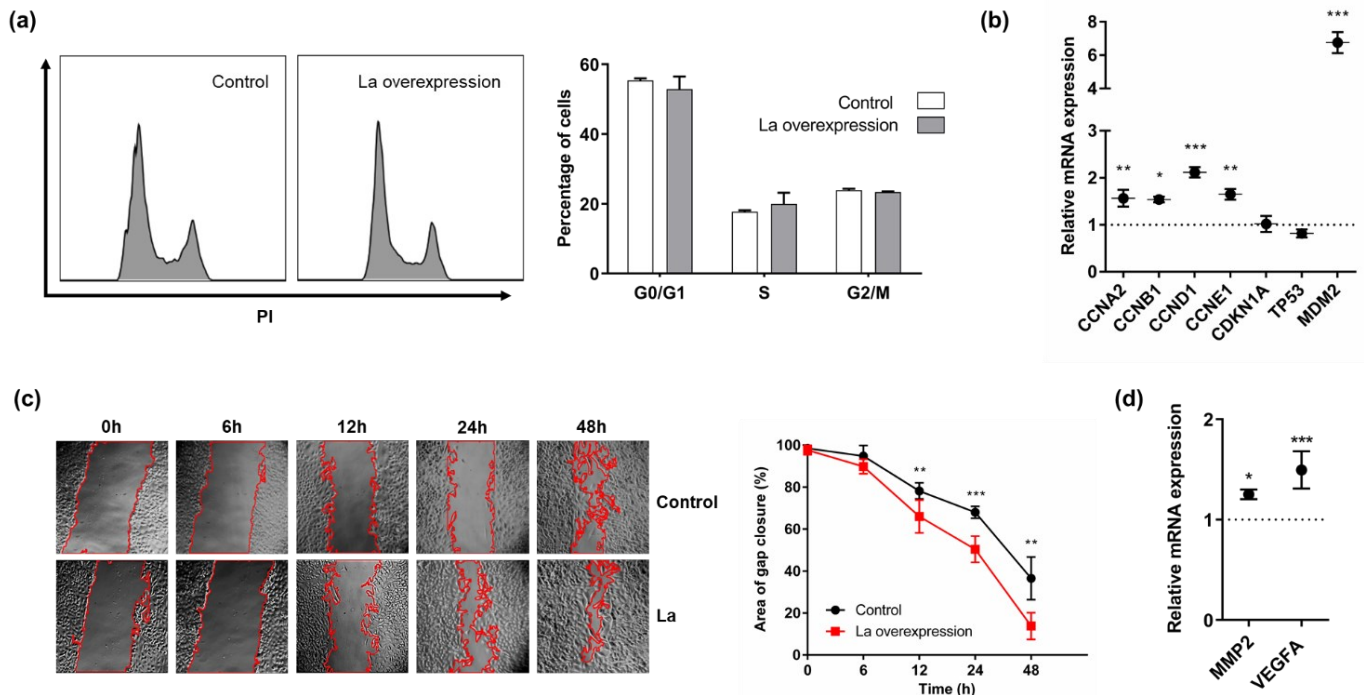


Figure 4.2: (a) A549 cells were transfected with a plasmid encoding hLa protein and DNA content was analyzed after PI-staining by flow cytometry after 24h. (b) mRNA expression levels by RT-qPCR of CCNA2, CCNB1, CCND1, CCNE1, TP53, CDKN1A, MDM2. (c) Representative images of scratch migration assay in La-transfected A549 cells compared to control. An open furrow was generated by scratching confluent cells. The distance between furrow edges in the control and La-transfected cells was measured and is presented graphically (right panel). (d) mRNA expression pattern of MMP2 and VEGFA analyzed by RT-qPCR. (*p<0.05, ** p<0.01, *** p<0.001).

4.1.3. NGS analysis of La-overexpressing cells suggests important gene expression alterations participating in translation and RNA metabolism

To better understand the biological role after La overexpression, total RNA was isolated from control and La-overexpressing cells and the gene expression profile was generated using an RNA-sequencing technique on the ION TORRENT platform. The Fastq files were further analyzed on the website <https://galaxyproject.org> using the STAR aligner (v2.7.8a). The differential gene expression was determined between control and La-overexpressing cells. Almost 3700 genes were differentially expressed (p-value <0.01). Among these 1900 were upregulated while 1800 were downregulated (Figure 4.3a). Moreover, bioinformatics analysis using Panther database and Reactome pathways category showed that La overexpression affects the regulation of

translation, RNA metabolism, ribosomes and proliferation or migration pathways (Figure 4.3b). More specifically, it was observed that the translation initiation, the formation of ribosomal subunits, the PTEN regulation, the mRNA splicing, the reorganization of the cytoskeleton, and G1/S transition of cell cycle were also affected. In addition, La is also implicated in rRNA processing, miRNA biogenesis, tRNA processing and in ATP formation (Figure 4.3b).

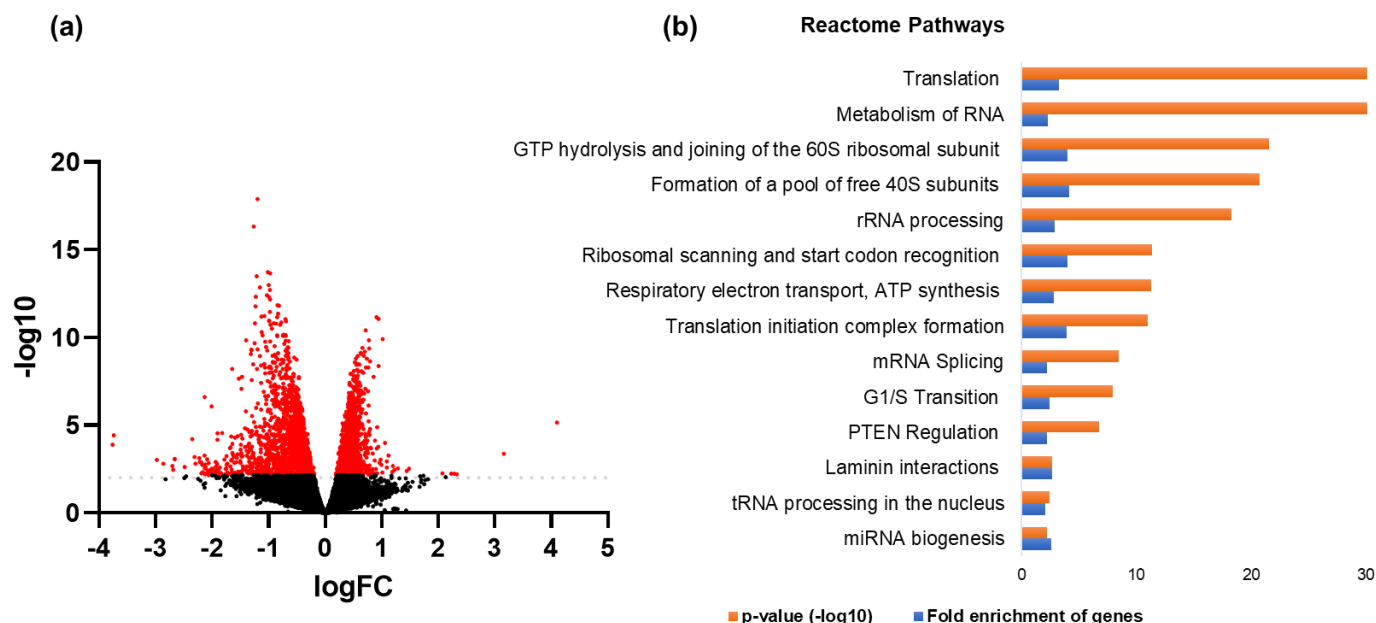


Figure 4.3. (a) Volcano plot of all genes assessed after RNA-sequencing. Red dots represent the statistically significant genes that differentially expressed. The volcano plot shows the relationship between fold change and significance using a scatter plot view. The y-axis illustrates the greater significance (higher value) while the x-axis illustrates the difference in expression levels of genes. **(b)** GO enrichment analysis for Reactome pathway category using panther database.

Furthermore, the molecular functions and biological process categories of GO analysis were analyzed after La overexpression. The results of both categories showed that the ribosome biogenesis and the translation activity consist of the significant molecular functions (Figure 4.4a). Additionally, the cell cycle, the cell communication, the cytoskeleton but also the transcription is among the significant processes that are affected in La-overexpressing cells (Figure 4.4b).

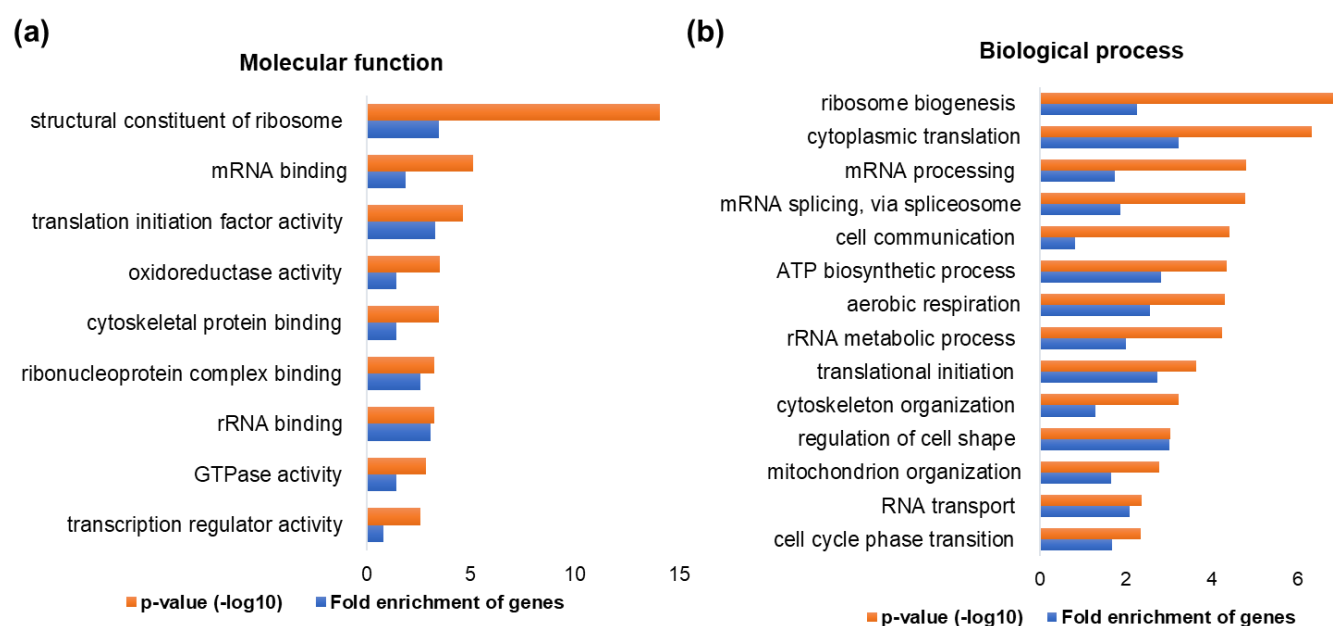


Figure 4.4. Gene ontology enrichment analysis for **(a)** molecular function and **(b)** Biological process categories after La overexpression in A549 cells.

4.1.4. La overexpression enhances the Pol III transcription

Based on GO enrichment analysis and also the involvement of La in RNA pol III transcription, the expression levels of transcription initiation factors were studied. In La-overexpressing cells the expression levels of TFIIIA, TBP and BDP1 which are required for accurate initiation transcription were upregulated almost ~2-fold, with the latest (BDP1) to exhibit the most significant increase (>2.5-fold relative expression), as it is specific to Pol III transcription. Of note, the GCN5 which consist of a histone acetyltransferase and acts as transcription activator, was also found significantly upregulated (>1.5 fold) while the TRRAP, a GCN5 adaptor protein, was increased 2.3-fold. At the same time, the mRNA expression levels of MYC were found without significant fluctuations, while MAF1, a repressor of RNA pol III transcription, was upregulated 1.3-fold (Figure 4.5a). Thus, it is observed that La stimulates the expression of important RNA pol III transcription initiation factors that could lead to increased tRNA production, as it has been shown in many cancers. It has been reported that tRNA overexpression compensates for the demand of elevated translation rates in highly proliferative cells (Goodarzi et al., 2016; Skeparnias et al.,

2020). Thus, the expression of tRF-1001, a tRNA-derived fragment from tRF-1 category, which is known for its oncogenic capacity, together with its parental tRNA^{SerUGA} were found upregulated 2.8-fold and 3.4-fold respectively (Schimmel, 2018). Moreover, the mRNA expression levels of RNase ZL, an endonuclease responsible for the cleavage of 3' trailer of pre-tRNAs that are protected by La and yield the tRF-1 type of tRNA fragment was analyzed. RNase ZL had the most significant upregulation (~3-fold) compared to angiogenin and DICER which are also involved in the formation of stress-induced tRFs (termed tRNA-halves; 2 and 1.6-fold respectively; Figure 4.5b).

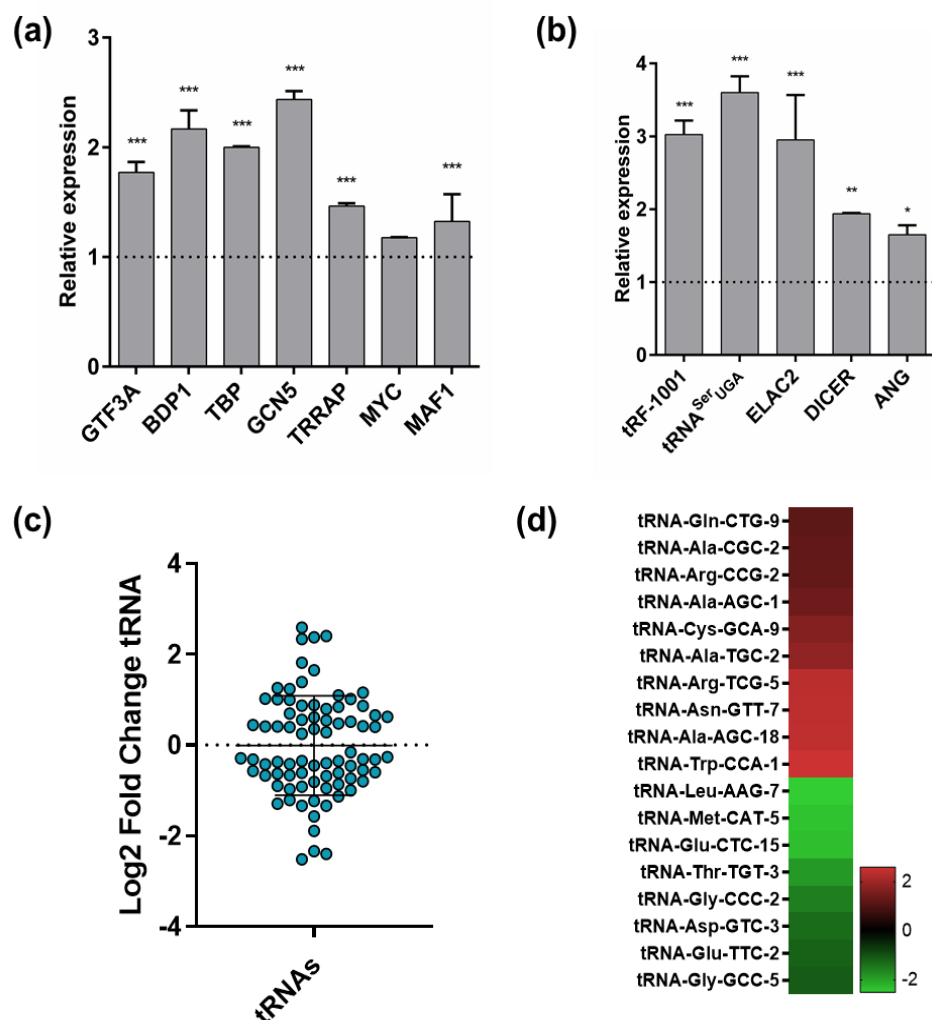


Figure 4.5: RT-qPCR analysis of **(a)** genes involved in transcription initiation factors of RNA polymerase III **(b)** ELAC2, ANG, DICER, XPOT, tRNA^{SerUGA} and tRF-1001. **(c)** Graphical presentation of significant differentially expressed tRNAs in La-overexpressing cells. **(d)** Heatmap showing the scaled expression of upregulated and downregulated tRNAs. (*p<0.05, **p<0.01, ***p<0.001)

Moreover, along with observed changes of transcription factors, the tRNA expression levels were further analyzed after RNA-seq experiments. The tRNA sequences were mapped to an artificial tRNA genome consisting of unique sequences of hg38 tRNAs using Bowtie (v.1.2.3) and further counted with featureCounts (v2.0.1). It is observed that 80 nuclear tRNAs were statistically differentially expressed with the majority to be downregulated (Figure 4.5c). However, specific tRNAs are upregulated like tRNAAla and tRNAArg (Figure 4.5d).

4.1.5. Effects on translation factors after La overexpression

In addition to transcription, it has been found that the translation pathway was affected after La overexpression thus the signaling upstream of translation initiation and also translation initiation factors were tested. It is observed that, although, MYC didn't exhibit alterations in mRNA expression levels, its protein level was upregulated ~1.3-fold, suggesting that translation of MYC is stimulated in the presence of La excess (Figure 4.6 a and b). Moreover, the transcriptional level of mTOR kinase was found unaltered while the phosphorylated protein (p-mTOR, S2448) was found significantly upregulated (1.5-fold; Figure 4.6 a,b and c). Interestingly, a 2.5-fold upregulation of RICTOR which is the mTORC2-specific subunit was observed, while the RPTOR, the mTORC1-specific subunit was found unaltered, an observation that could suggest that La overexpression affects translation initiation, through activation of the PI3K/AKT/mTORC2 axis (Figure 4.6c).

In addition, the eIF4E and 4E-BPs factors which participate in cap-dependent translation were analyzed by qPCR and WB. Although, the mRNA expression levels of eIF4E and 4E-BPs were unaffected, it is observed a lower phosphorylation level of p-4EBP1(S65) and p-4E-BP1 (T70) while a slight upregulation of 4E-BPs (Figure 4.6 a, b, c). It is known that the non-phosphorylated 4E-BPs bind on eIF4E preventing from interacting with 5'-cap of mRNAs and thus blocking cap-dependent translation. This observation may indicate that cap-dependent translation is attenuated and possibly La overexpression affects the translation initiation through another pathway. In addition, almost all translation initiation factors were upregulated, however, the eIF3C exhibited the most prominent difference (~2-fold; Figure 4.6c). It has been found the

translation initiation factor eIF3 is central to the assembly of translation preinitiation complexes and important for cell homeostasis, since binding to cell-proliferation mRNAs can either activate or repress translation in cancer (Lee et al., 2015).

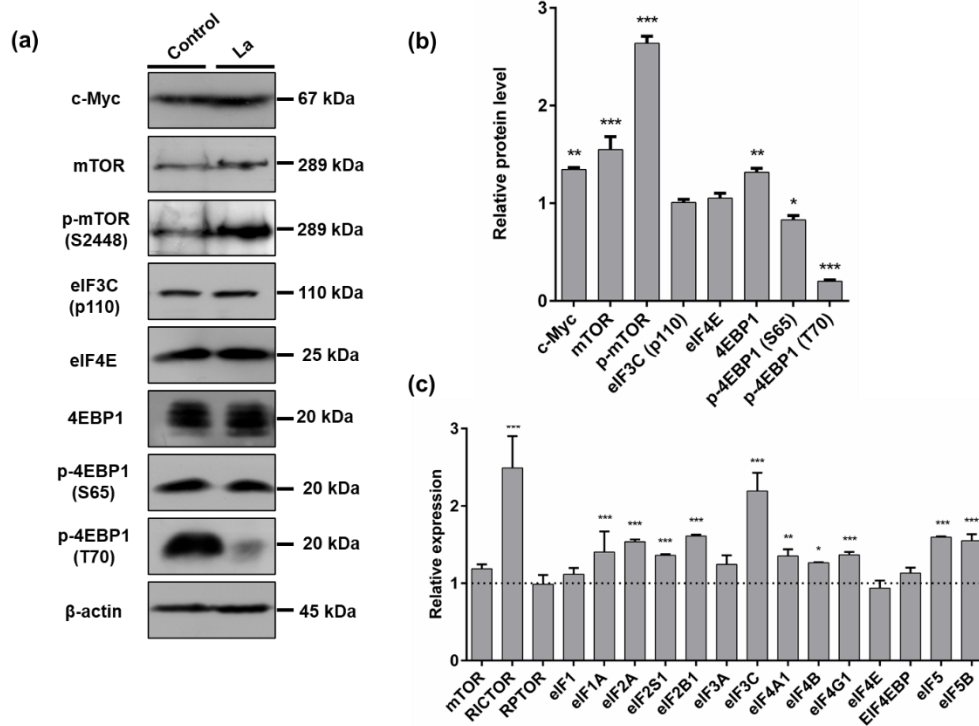


Figure 4.6: (a) Immunoblot analysis of translation initiation factors and (b) quantitation of protein levels. The quantitation was calculated after normalization to β -actin levels. (c) Relative mRNA expression levels of translation initiation genes were examined *via* RT-qPCR analysis. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

To measure the global translation rates, a puromycin incorporation assay was performed. A minor decrease (~30%) in the production of newly synthesized peptides in La-transfected was found (Figure 4.7a). As a positive control for the assay, non-transfected A549 cells treated with CHX (a known inhibitor of translation elongation) and puromycin were used (Figure 4.7b). On the other hand, to determine whether the abundance of La promotes IRES-mediated translation, a vector bearing a bicistronic reporter system including both a cap-dependent (LucR) and an IRES-dependent (LucF) cistrons was used. This vector is used to indicate the ability of specific factors to induce IRES-dependent translation (Poulin et al., 1998). A549 cells were co-transfected with the vector bearing a bicistronic system and the pcDNA3.1-La vector for La overexpression and a

significant increase of the LucF:LucR ratio was observed. In addition to A549 cells, the same experiments were further verified in HEK293 cells which exhibit better co-transfection efficiency compared to A549 cells. Similarly, an increase of the LucF:LucR ratio was found (Figure 4.7c). This observation could suggest that the excess of La enhances the IRES-mediated translation that could account for the reduced cap-dependent translation.

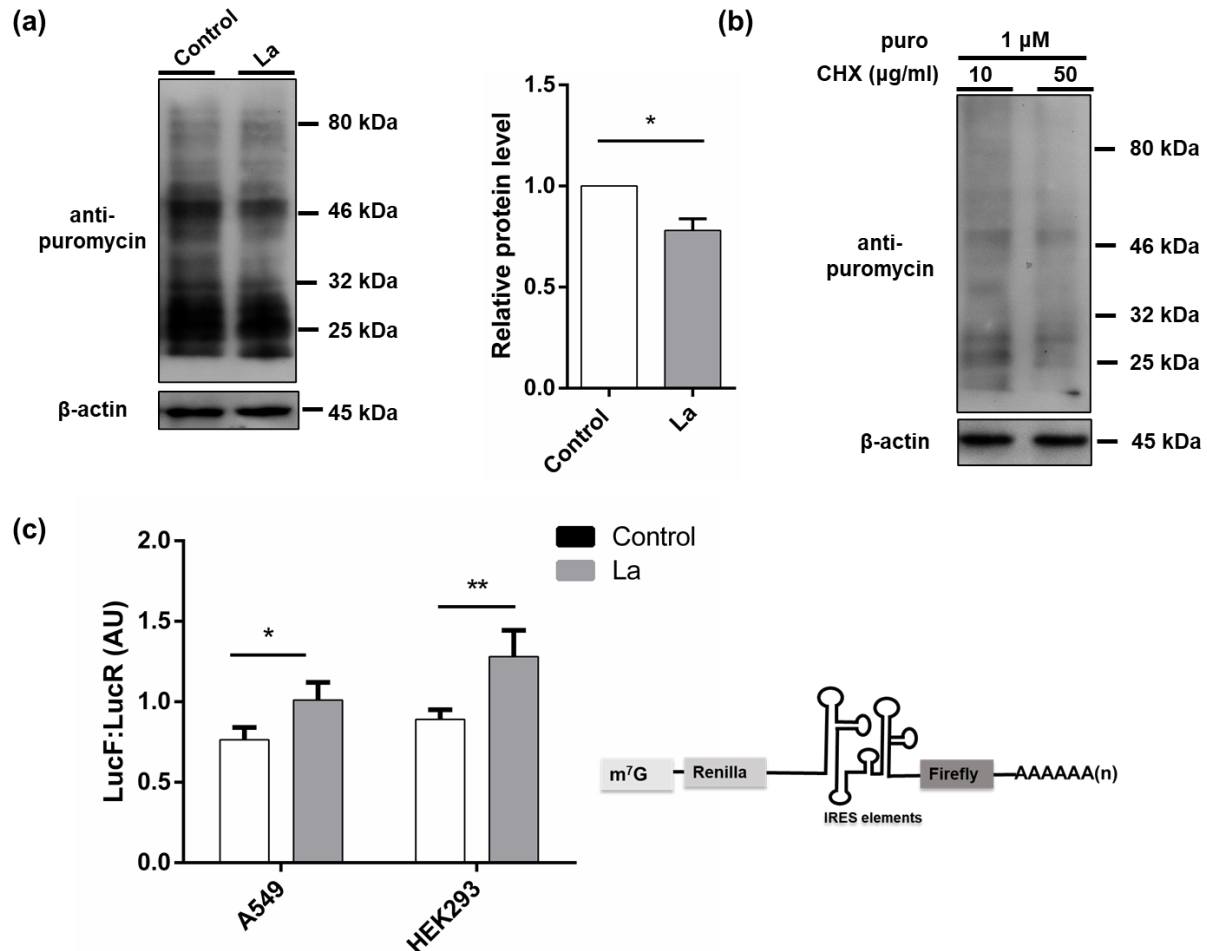


Figure 4.7: (a) An anti-puromycin immunoblot of whole cell lysate from A549 treated with 1 μ M puromycin (left) and a quantification of protein synthesis rates by measuring the signal intensity in each lane, using ImageJ, normalizing the values to that of the control lane (right). (b) A549 cells treated in presence of puromycin and different concentration of cycloheximide (CHX) and a representative image of the SUnSET-WB assay was presented. (c) Ratio of LucF to LucR activity, as inferred by the measured luminescence signals, in A549 and HEK293 cells co-transfected with a vector expressing La and vector bearing a bicistronic reporter system that allows detection of Renilla (cap-dependent) or Firefly (IRES-dependent) expression (AU, arbitrary units) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.1.6. La overexpression affects miRNA levels

To examine the role of small RNA transcriptome in La-overexpressing cells, the preparation of small RNA molecules libraries was performed. Regarding the analysis the read counts for miRNAs were generated with featureCounts (v2.0.1) using annotation files from ENSEMBL and miRbase respectively (Liao et al., 2014b). It is observed that 340 miRNAs were identified however 50 miRNAs were differentially expressed with p-value <0.05. Among them, 11 were downregulated while the 22 miRNAs were upregulated (Figure 4.8a). The miR-4467, miR-4522 and miR-138-1 had the lower expression levels while miR-3164, miR-4744, miR-940, miR-1285-1, miR-3170 and miR-632 had the higher expression levels (Figure 4.8b).

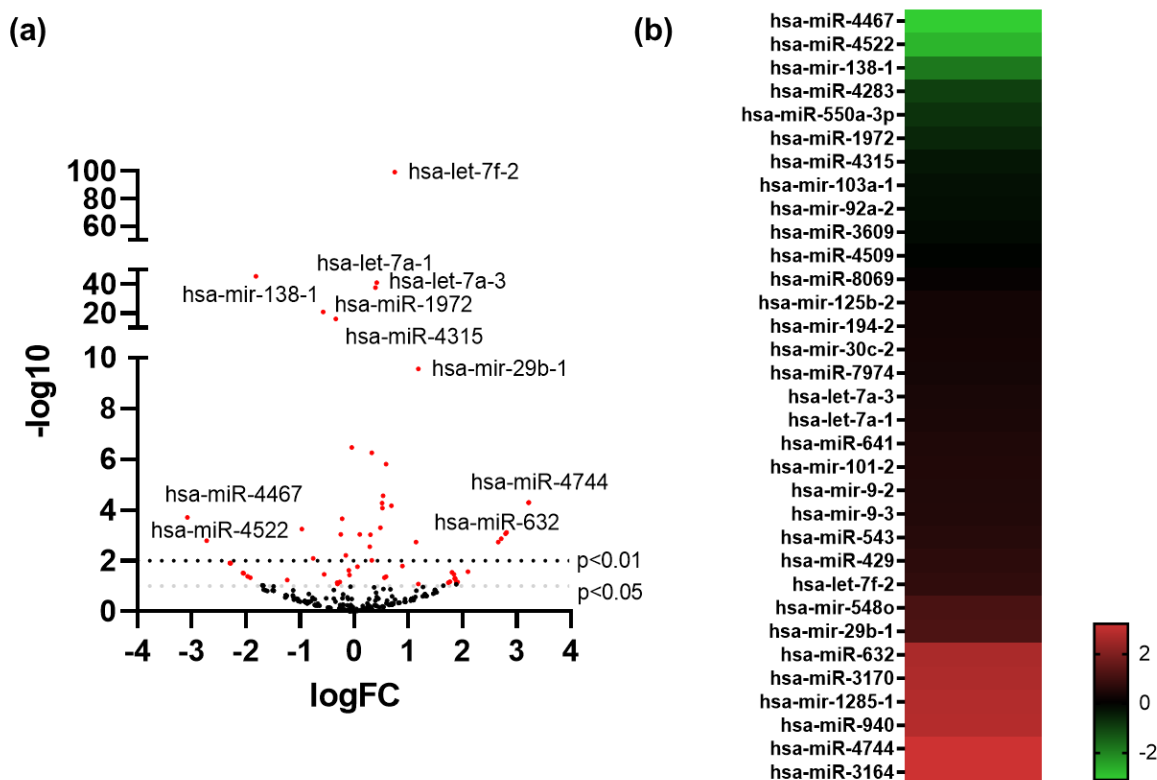


Figure 4.8 (a) Volcano plot of all identified miRNAs. The red dots show the significantly differentially expressed miRNAs. (b) Heatmap showing the scaled expression of upregulated and downregulated miRNAs with $p > 0.01$.

Subsequently, in order to find the pathways that miRNAs are involved, the predicted targets for each differentially expressed miRNA molecule were found using TarBase v8.0 database. The predicted targets were further analyzed by GO enrichment analysis using Panther database. It

was observed that the target genes from downregulated miRNAs, were mainly involved in gene silencing, in G1/S transition of cell cycle, in RNA polymerase II transcription and in translation, while the main molecular functions include chromatin, mRNA and tRNA binding as well as regulation of kinases (Figure 4.9a and b). On the other hand, GO enrichment analysis from upregulated miRNAs showed that predicted target genes of both biological and molecular categories are mainly involved in regulation of autophagy, proliferation, histone modifications, apoptotic signaling, translation regulator activity, signal transduction as well as in dsRNA binding. The latest molecular function could include the regulation of important ribonucleases like DICER which binds to dsRNA. Altogether, the results showed that miRNAs could function as modulators of protein synthesis and cell proliferation.

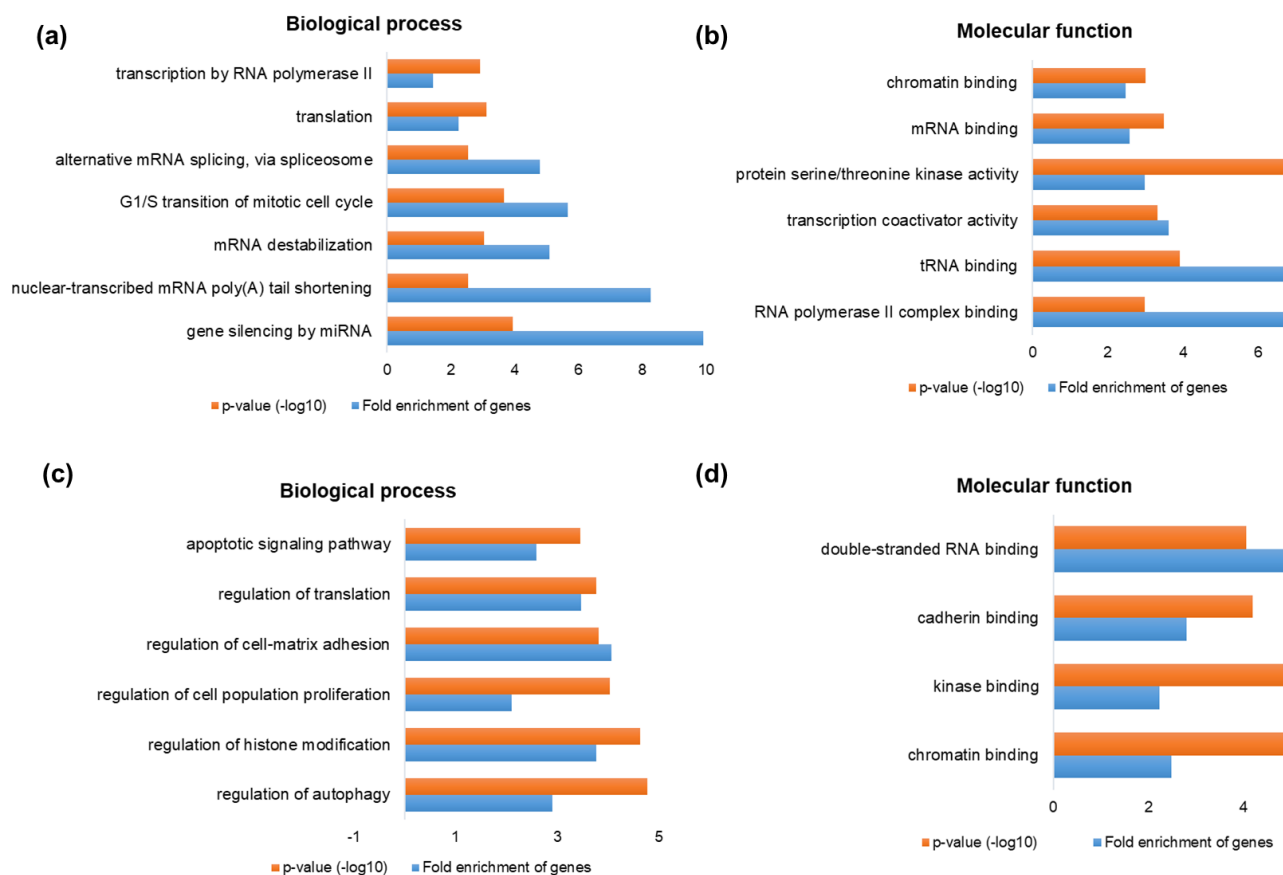


Figure 4.9. GO enrichment analysis for **(a)** molecular function and **(b)** biological process of predicted target genes from downregulated miRNAs and **(c)** molecular function and **(d)** biological process of predicted target genes from upregulated miRNAs.

4.1.7. LAM has RNA binding properties

Recent reports have been showed that that La can bind not only RNA sequence-specific but also sequence-independent binding modes, including poly(A) tails which hint on the repertoire of RNA interactors. A number of studies have been carried out to elucidate the structural and biochemical basis of the separate domains of La protein, however, the conserved LAM, although important, has been reported as incapable of interacting with model RNA ligands. The *in vitro* binding ability of the recombinant hLAM (1-103aa) was tested performing EMSA experiments in the presence of pre-tRNAArgCCU (from *D. discoideum*) and tRNAGlyGCC variants (from human), including pre-tRNAs transcripts, either bearing only 3' trailer sequence, or bearing only 5' leader sequence or mature tRNA molecules. Interestingly, a strong formation shifts of the LAM in complex with either pre-tRNAArgCCU or pre-tRNAGlyGCC bearing only 3' trailer sequence was observed, a result that coincides with the binding specificity of La protein (Figure 4.10a and b). The calculated apparent K_d values, for the pre-tRNAArgCCU and pre-tRNAGlyGCC bearing only 3' leader sequence, were found $0.96 \pm 0.173 \mu\text{M}$ and $0.12 \pm 0.06 \mu\text{M}$, respectively under the conditions tested. In addition, when a mature tRNAGlyGCC or a pre-tRNAGlyGCC bearing only 5' leader sequence was used as ligand, the binding was not observed (Figure 4.10c and d). Finally, a negative control of 70nt synthetic ssDNA which failed to produce any complex with LAM, was tested. The results indicated that LAM exhibits RNA binding capacity and specificity for binding 3' trailers of pre-tRNAs, which are among the most important ligands of La protein.

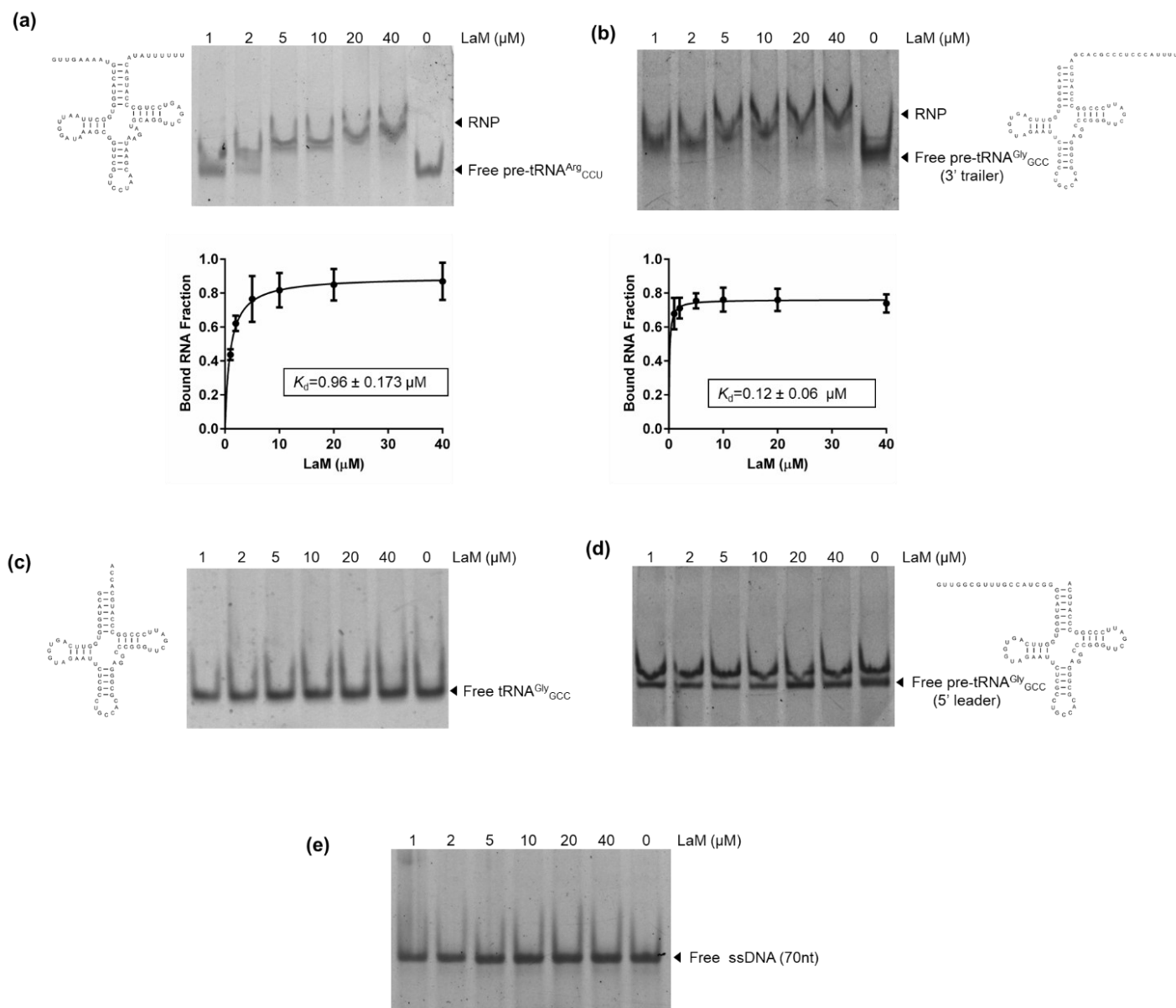


Figure 4.10: Electrophoretic Mobility Shift Assay between increasing amounts of hLAM and **(a)** 150 nM pre-tRNA^{Arg}CCU (top) and binding curve of bound pre-tRNA^{Arg}CCU fraction against LaM concentration. The estimated K_d value is listed in the box (bottom). **(b)** 150 nM pre-tRNA^{Gly}GCC bearing only 3' trailer sequence and binding curve of bound pre-tRNA^{Gly}GCC bearing 3' trailer fraction against LaM concentration. The estimated K_d value is listed in the box. **(c)** 150 nM pre-tRNA^{Gly}GCC bearing only 5' leader sequence and **(d)** 150nM mature pre-tRNA^{Gly}GCC. **(e)** Electrophoretic Mobility Shift Assay of increasing amounts of hLAM in the presence of 150nM of 70-mer synthetic ssDNA.

4.2. Interactions of ncRNAs and RNP RNase P protein subunits

4.2.1. Generation of CRISPR-Cas9 genome editing screening in RNP RNase P subunits

Several attempts have been performed to study the role of individual protein subunit beyond the holoenzyme of RNase P. The genome editing tool could provide an opportunity to produce gene mutants with deletions or insertions that could destroy the protein function. To evaluate the role of RNase P protein subunits, the CRISPR/Cas9 genome editing tool was used to knock-out each individual protein subunit of RNase P holoenzyme in HeLa cell line.

After the cloning of specific gRNAs into pX459 vector were completed, the plasmids were transiently transfected into HeLa cells. Two gRNAs for each subunit were used, and almost 10 clones for each guide were isolated. The targeted locus was amplified, sequenced and was further analyzed using CRISP-ID and Synthego tools (Figure 4.11). The sanger sequencing results showed the mutations which were introduced by CRISP/Cas9 tool and further confirmed the

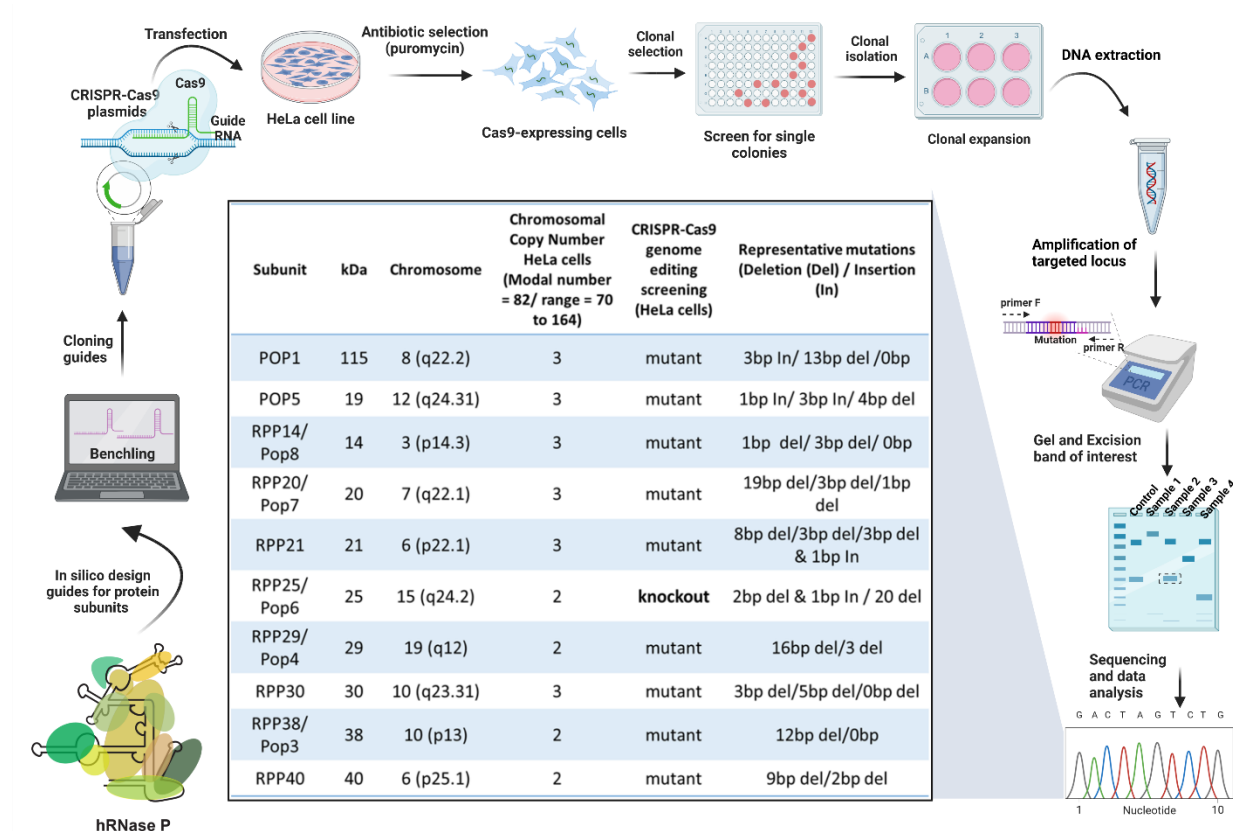


Figure 4.11. Schematic representation of genome editing experimental procedure using CRISPR/Cas9 system in HeLa cells and representative results about RNase P protein subunits.

existing genome data from HeLa cells regarding the number of alleles. The majority of isolated clones had deletion of an intervening segment while in some cases it was observed one or two nucleotides to be inserted (Figure 4.11).

In addition, total RNA was extracted from the isolated clones and further mRNA expression levels was analyzed after qPCR compared to wild type cells (WT). In the cases of available antibodies was existed, immunoblot analysis was performed in order to test protein expression levels. Firstly, the clones of RPP21, RPP29, RPP38 subunits were studied and it was observed that both RPP21 and RPP29 CRISPR-edited clones had low mRNA expression levels (Figure 4.12a, b), while the RPP38 clones had elevated mRNA expression levels (Figure 4.12c). However, the CRISPR-edited clones 1 and 2 of RPP38 which had the lower mRNA expression levels, was further analyzed with immunoblot analysis. The RPP38 protein was detected in both clones but the protein level of clone 2 was downregulated (0.5-fold) (Figure 4.12d).

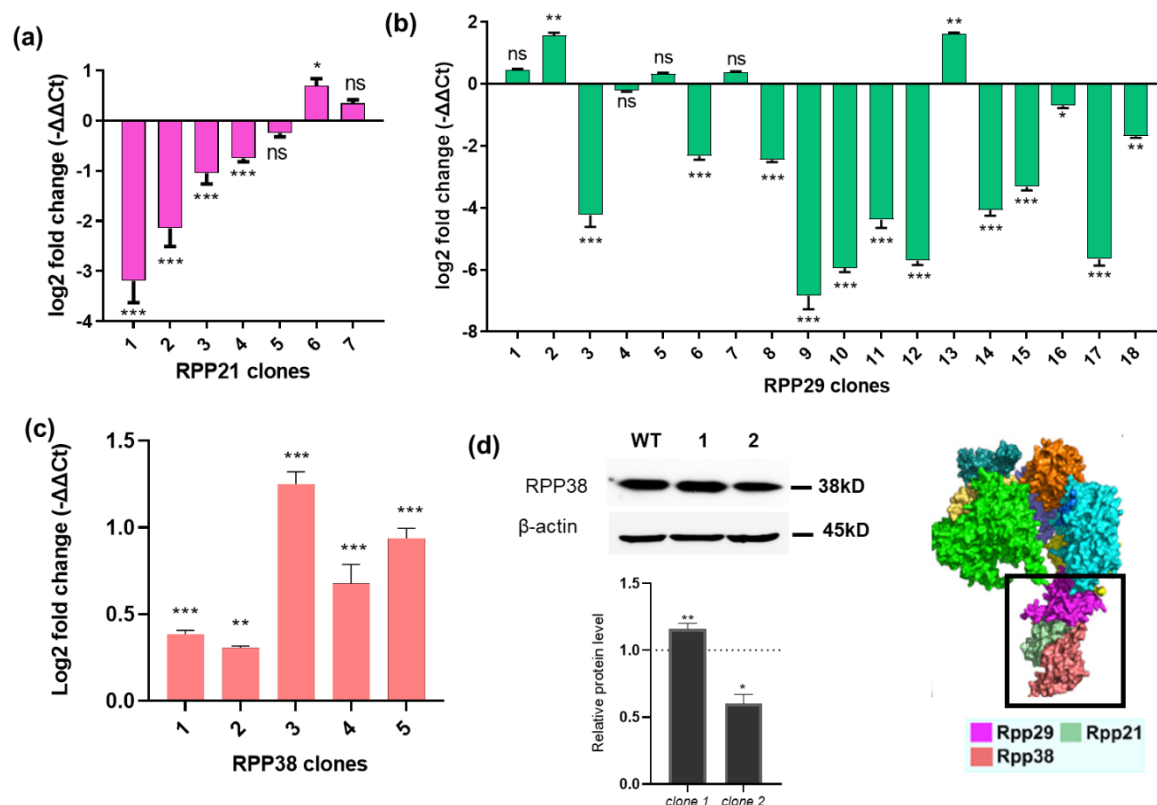


Figure 4.12. mRNA expression levels of **(a)** RPP21 **(b)** RPP29 and **(c)** RPP38 in CRISPR-edited clones compared to WT cells **(d)** immunoblot analysis of RPP38 CRISPR-edited clones (up) and their quantification of RPP38 protein level (down). (*p < 0.05, **p < 0.01, ***p < 0.001)

In addition, the clones of POP5, RPP30, RPP40, RPP14 were further analyzed. It was observed that the mRNA expression levels of CRISPR-edited clones were found with significant fluctuations (Figure 4.13a, b, c, d). Moreover, the RPP40 CRISPR-edited clones, although had lower mRNA expression levels (Figure 4.13d), the immunoblot analysis showed that the RPP40 protein was produced in all clones tested. However, the protein levels of clone 2 and 4 was reduced (0.5-fold and 0.3-fold, respectively) (Figure 4.13e).

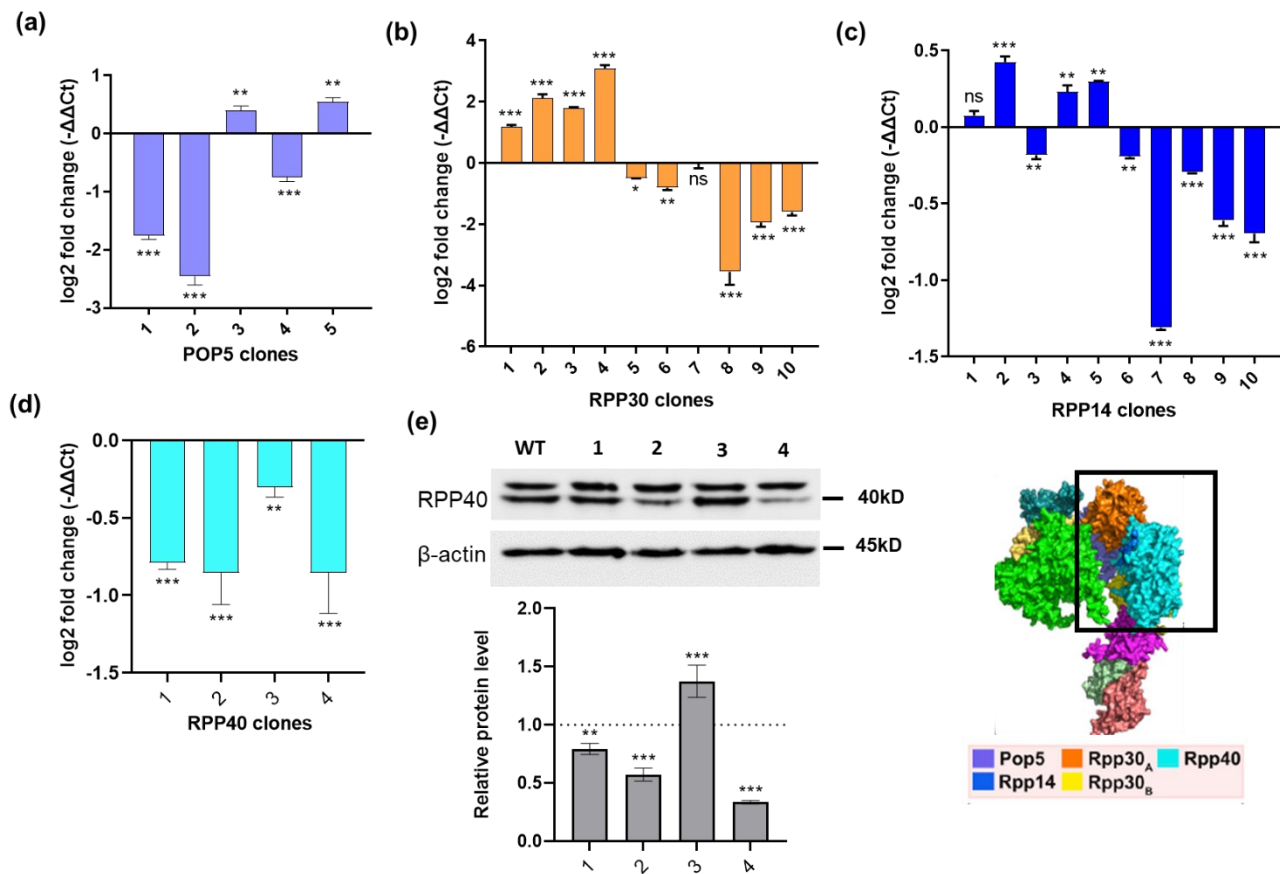


Figure 4.13. mRNA expression levels of (a) POP5 (b) RPP30 (c) RPP14 and (d) RPP40 in CRISPR-edited clones compared to WT cells (e) immunoblot analysis of RPP40 CRISPR-edited clones (up) and their quantification of RPP40 protein level (down). (*p < 0.05, **p < 0.01, ***p < 0.001)

Finally, the isolated CRISPR-edited clones of POP1, RPP20, and RPP425 were further analyzed. The number of isolated POP1 CRISPR-edited clones had mutations in two from three alleles (like +3bp/0bp/-13bp) and showed downregulation of mRNA expression level (Figure 4.14a).

However, the protein was detected in all clones tested (Figure 4.14a). In addition, both RPP20 and RPP25 CRISPR-edited clones was shown a downregulation of both mRNA and protein expression levels, however, it was observed that the protein was not detected in clone 1 of RPP25, indicating the successful ablation of RPP25 (Figure 4.14 b, c).

Of note, regarding the POP1 and RPP30 protein subunits, it was noticed that some of the clones had mutations resulted in a premature stop codon while one from the alleles hadn't mutations. Therefore, a second transfection with px459 plasmid was performed to target the one allele without mutations. The results showed that the isolated clones had not changes in the targeted allele. So, it was hypothesized that POP1 and RPP30 proteins are essential for viability. POP1 and RPP30 have been also characterized as essential subunit in yeast and *S. cerevisiae* or *Drosophila*, respectively, which in good agreement with the results (Xiao et al., 2002).

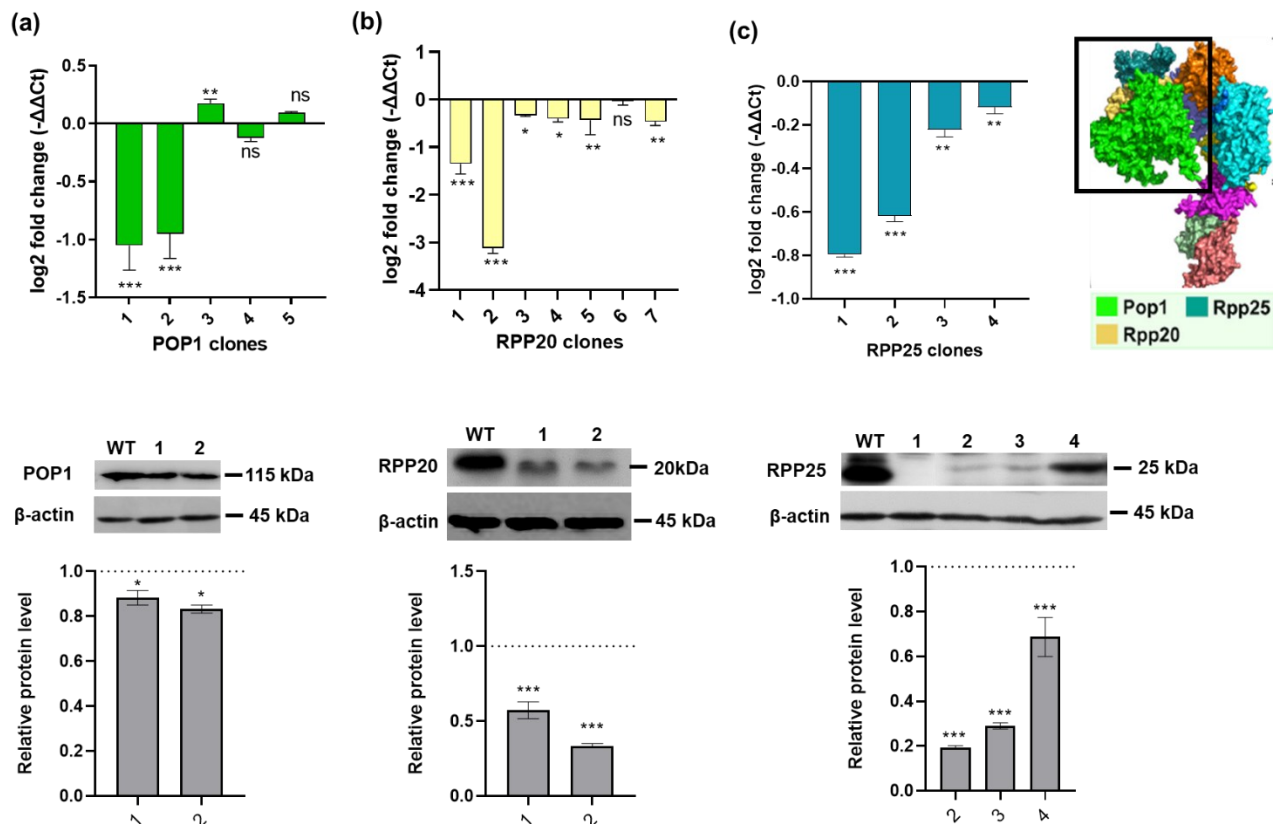


Figure 4.14. (a) mRNA expression levels of *POP1* gene (up) and immunoblot analysis of POP1 CRISPR-edited clones (down) (b) mRNA expression levels of *RPP20* gene (up) and immunoblot analysis of RPP20 CRISPR-edited clones (down) (c) mRNA expression levels of *RPP25* gene (up) and immunoblot analysis of RPP25 CRISPR-edited clones (down). (*p < 0.05, **p < 0.01, ***p < 0.001)

4.2.2. RPP25-deficient cells are viable with altered expression of RNase P protein subunits

The CRISPR/Cas9 screening analysis showed that RPP25 is dispensable for cells and thus the RPP25-deficient clone was used for further analysis. The *RPP25* knockout clone was picked, was expanded and genomic DNA was isolated. The targeted locus was amplified by PCR and the product was sequenced. The sanger sequencing results confirmed the mutations after CRISPR/Cas9 genome editing and also showed that RPP25 has two alleles as shown from the existing genomic data from HeLa cells. The analysis of mutations was performed using CRISP-ID and Synthego tools which showed that the RPP25KO has 2 bp deletion and 1 Insertion in one of the alleles while 20 bp depletion in the second allele resulted in the production of 117aa protein and in a premature stop codon, respectively (Figure 4.15a).

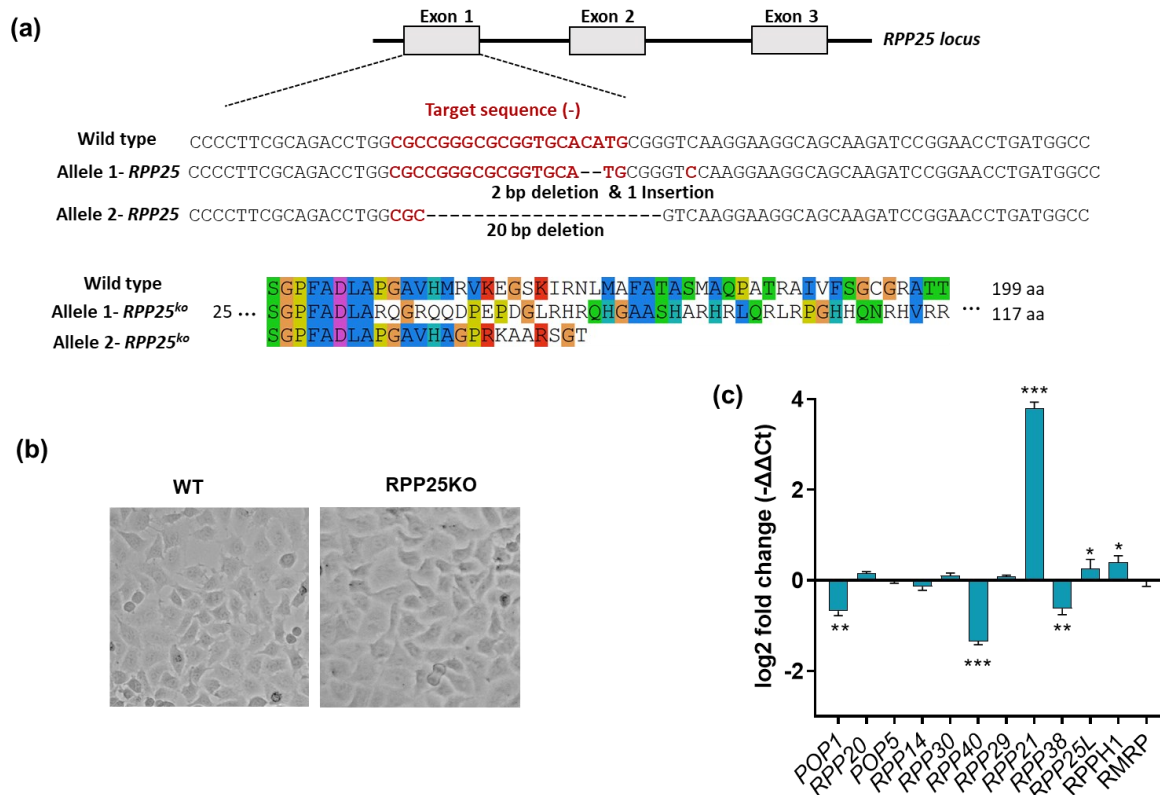


Figure 4.15. (a) Schematic representation of RPP25KO edited clone and analysis of the resulting amino acid sequence after genome editing. **(b)** Representative bright field images of WT and Rpp25KO cells **(c)** RT-qPCR analysis of all RNase P/MRP subunits in RPP25KO cells compared to WT. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

The analysis of protein sequence was performed using the ApE program and the alignment using ClustalX2 program. Furthermore, the knockout cells were observed and bright field images of HeLa WT and RPP25KO showed no obvious morphological differences (Figure 4.15b). Moreover, the mRNA expression levels of the other RNase P/MRP subunits were measured. It was observed that although the subunits did not exhibit significant changes, the POP1 and RPP40 was downregulated (0.7-fold and 1.3-fold respectively) while the RPP21 was significant upregulated (3.8-fold). Finally, the levels of RPP25L and the RPPH1 were slightly upregulated (Figure 4.15c). These alterations may affect the reconstitution of two holoenzymes but also the additional role that subunits participate like the recruitment of RPP21 to double stranded DNA breaks (Abu-Zhayia et al., 2017).

4.2.3. Ablation of RPP25 leads to increased proliferation

After the successful ablation of RPP25 protein subunit, further characterization of knockout cell was performed. As first assessment, the cell viability was measured at different time points (24h-72h) using resazurin assay which shown that knockout cells exhibited a higher growth, almost 2-fold compared to WT (Figure 4.16a). In addition, an increased proliferation rate (1.3-fold) was observed in knockout cells compared to WT (Figure 4.16b) which was further confirmed by measuring the levels of PCNA (Proliferating Cell Nuclear Antigen) using western blot (1.5-fold) (Figure 4.16c). PCNA acts as a processivity factor for DNA polymerase δ in eukaryotic cells and is essential for replication, DNA repair and chromatin remodeling (Bologna-Molina et al., 2013). Moreover, the protein expression level of the anti-apoptotic marker protein Bcl-2 were upregulated (1.6-fold) (Figure 4.16c). All the above showed that RPP25KO cells exhibited significant higher proliferation compared to WT. To further investigate the effects of RPP25 ablation, rescue experiments were performed in knockout cells. RPP25KO HeLa cells were transiently transfected with the pcDNA3.1-RPP25 plasmid encoding RPP25 protein (Rescue). As control the pcDNA3.1-empty vector (Control) was used. Total protein extracts were isolated from cells and the expression levels of RPP25 protein were further verified by Western blot (Figure 4.16d). In addition, after rescuing RPP25, the cell viability and the proliferation rates was also measured, showing the restored levels in both experiments (Figure 4.16 b and c). Moreover, the

PCNA and Bcl2 factors were also found to be downregulated almost 0.8-fold and 0.6-fold respectively (Figure 4.16d). Overall, rescue experiments showed regain of the knockout effects.

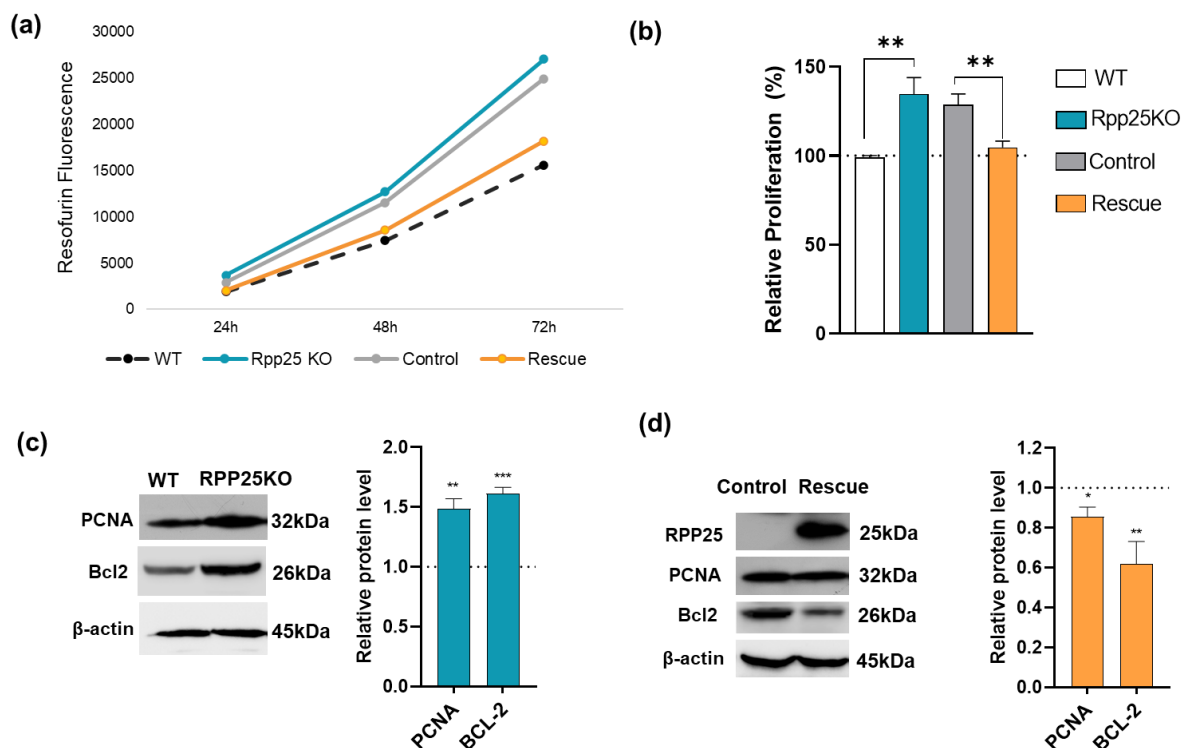


Figure 4.16. (a) Resorufin fluorescence intensity measured during resazurin reduction of cells (b) Cell proliferation assay using 0.2% crystal violet in 20% methanol and absorbance measurement. The levels are shown as a percentage (%) of relative proliferation compared to WT (c) Immunoblot analysis for proliferating (PCNA) and anti-apoptotic (Bcl-2) factors in RPP25KO cells compared WT (left panel) and quantification of protein level (right panel). (d) Western blot assay for proliferating (PCNA) and anti-apoptotic (Bcl-2) factors in rescue experiments and quantification of protein level (right panel). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.2.4. The RPP25-KO cells exhibit a prolonged S-phase

Several studies have been shown that RNase P protein subunits are recruited to chromatin in a cell cycle-dependent manner and could affect the progression of cell cycle (Reiner et al., 2016). To further determine the consequences of RPP25 ablation in the rate of cell cycle progression a propidium iodide (PI) staining assay was performed. At first, a synchronization of cells with serum starvation (G0 synchronized) for 1 day was performed and then the cells were trypsinized, re-seeded to sub-confluent density in fresh media, and allowed to re-enter the cell cycle for 24 h. The results showed a prolonged S phase in HeLa RPP25-deficient cells suggesting that the

knockout cells spend longer time in S phase of cell cycle compared to WT (Figure 4.17a). In addition, the RPP25KO cells were also used for RT-qPCR analysis of major cyclins expression levels (Cyclin A2; CCNA2, Cyclin B; CCNB1, Cyclin D; CCND1 and Cyclin E; CCNE1) as well as expression of P21, P27, TP53 and its inhibitor MDM2. All cyclins were found moderately affected with no significant alterations; however, it was observed an increased expression of p53 and p21 (1.3-fold) (Figure 4.17b). On the other hand, following rescue experiments, a G1 phase arrest was observed compared to knockout cells (Figure 4.17c).

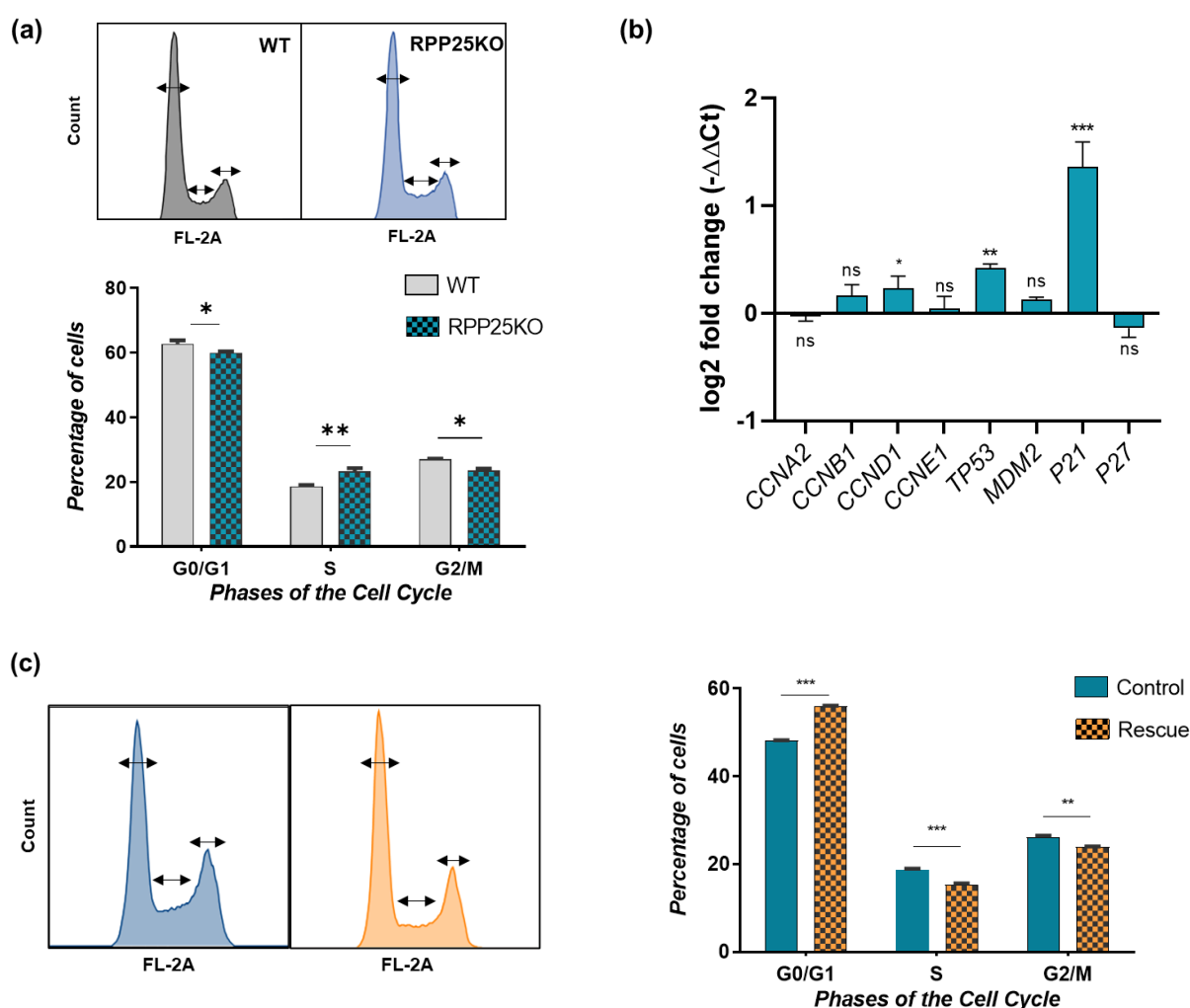


Figure 4.17. (a) The DNA content was analyzed after PI-staining by flow cytometry after 24h. Analysis of cell cycle progression (up) and graphical representation of the results (down). **(b)** RT-qPCR analysis of genes involved in cell cycle phases. **(c)** Analysis of cell cycle progression (left) and graphical representation of the results (right) after rescuing of RPP25 (*p < 0.05, **p < 0.01, ***p < 0.001)

Several studies have been identified the regulatory role of RNase P to cell cycle phases and in transcription of tRNA, 5S rRNA and rDNA transcription (Reiner, 2006; Reiner et al., 2008; Serruya et al., 2015). Regarding the RPP25 protein, it has been found that is recruited in chromatin late in G1/S phase where Pol I activity is increased. Knockdown of RPP25 showed low transcription of 5.8S rDNA (Reiner, 2006; Reiner et al., 2008). Thus, the differential expression of genes participated in RNA pol I and Pol III transcription but also the tRNA processing were tested. The results showed that the subunit of RNA pol I, *POLR1B*, was greater downregulated (-2.2-fold) in RPP25KO compared to rescue experiment (-0.5-fold) (Figure 4.18a). In addition, the region prior to 5.8s rRNA termed as pre-rRNA ITS1 is upregulated with a greater increase in knockout cells indicating a lower expression of 5.8s rRNA (Figure 4.18b). On the other hand, although the *ELAC2* and *SSB* genes were downregulated in both knockout and rescue experiment, the expression of *POLR3B* did not exhibit significant alterations, however, a slightly higher expression was observed after rescuing RPP25. Moreover, the 5S rRNA and U6 RNAs which are transcribed by pol III was slightly upregulated in knockout cells (Figure 4.18b).

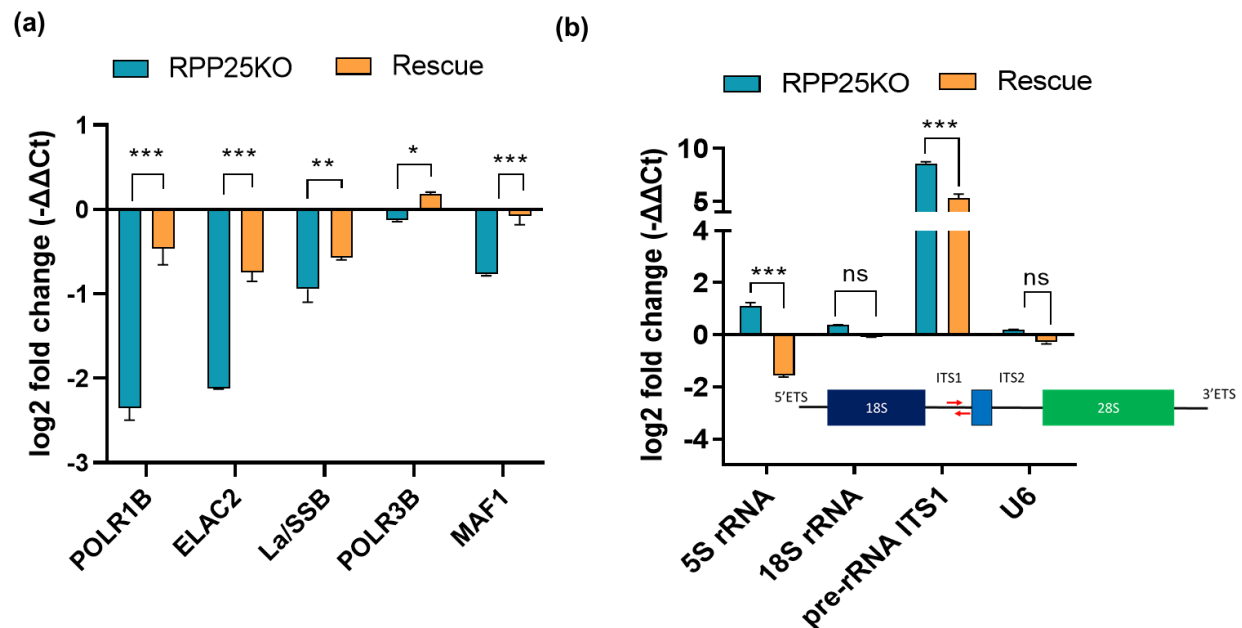


Figure 4.18. mRNA expression levels of **(a)** *POLR1B*, *ELAC2*, *SSB*, *POLR3B*, *MAF1*, **(b)** 5S rRNA, 18S rRNA, pre-rRNA ITS1 and U6. (*p < 0.05, **p < 0.01, ***p < 0.001)

Moreover, cell extracts were isolated from knockout and WT cells and were incubated with the radiolabeled pre-tRNA^{Arg}_{CCU} in different time points in order to test the RNase P activity. The samples were incubated for 30min at 30°C and as positive control a his-tagged recombinant hRNase ZL was used which were expressed in *E.coli* Rosetta cells and further purified from the extract *via* affinity chromatography. RNase ZL cleaved the 3' end of the tRNA after the discriminator base. It was observed the in the absence of RPP25 RNase P activity is present, however almost 10% of 5' leader of pre-tRNA is not cleaved (Figure 4.19).

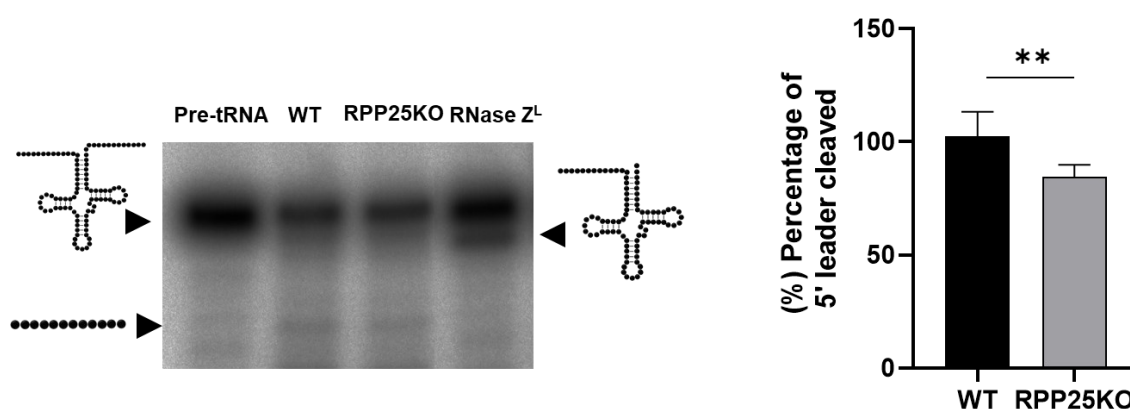


Figure 4.19. *In vitro* RNase P activity assay. Cell extracts from RPP25 knockout clone and WT cells were incubated with 5'32P-labeled substrate (pre-tRNA-tRNA^{Arg}_{CCU}) for 30 minutes (lefts). Schematic presentation of percentage of pre-tRNA processing by RNase P (right) (*p < 0.05, **p < 0.01, ***p < 0.001)

4.2.5. Ablation of RPP25 leads to increased cell motility

Since the ablation of RPP25 increase the proliferation of cells, it is possible to affect the motility of cells thus, the cell migration was examined using the wound healing assays at different time points (6h to 48h). The results showed that cells exhibited a significant increased motility within the first 12h (Figure 4.20 a and b). The migration was increased almost 35%. Interestingly, qPCR experiments confirmed the increased expression of important migration and angiogenesis related markers in cancer like MMP2 (1.5-fold), VEGFA (0.5-fold), fibronectin (1.8-fold), angiogenin (1.3-fold). However, it was observed an upregulation (1.3-fold) of hypoxia-inducible factor 1α (HIF-1α), which has a major role in cell adaptation to hypoxia (Figure 4.20c). Of note,

after rescuing RPP25, the gap was recovered similar to WT (Figure 4.20 a and b) and also all tested factors were downregulated, with exception of HIF-1 α (Figure 4.20c).

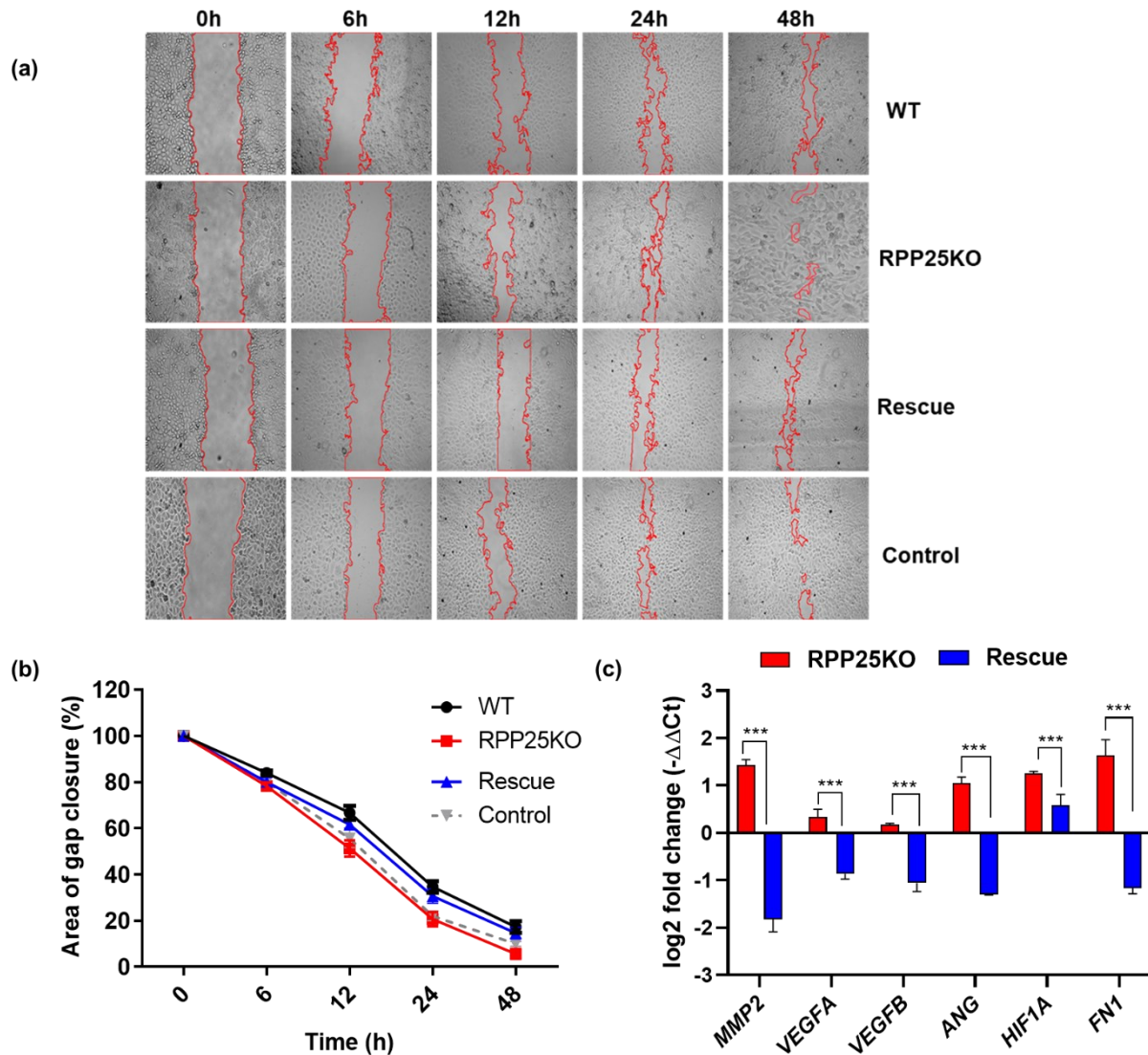


Figure 4.20. Scratch migration assay in RPP25 knockout cells and in rescue experiments. **(a)** Representative images of wound healing assay derived from three independent experiments using a KERN OCM-161 inverted microscope at 10X magnification. An open furrow was generated by scratching confluent cells. **(b)** The distance between furrow edges was measured and is presented graphically **(c)** mRNA expression pattern of MMP2, VEGFA, VEGFB, ANG, HIF1A, FN1 analyzed by RT-qPCR. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

4.2.6. Ablation of RPP25 leads to low mitochondrial potential of membrane

It is known that the increased cell proliferation of tumor cells could be also accompanied with ROS production, thus it was tested the levels of ROS production and the function of mitochondria that can impact tumor proliferation, survival and metastasis. Mitochondrial selective probes were used to measure the ROS production, the mitochondrial potential of membrane and also the mitochondrial mass using flow cytometry. The measurement of intracellular ROS production was performed by the fluorescent probe MitoSOX Red, while the mitochondrial membrane potential was measured by MitoTracker Red CMXRos which stains mitochondria in live cells and its accumulation is dependent upon membrane potential ($\Delta\Psi_m$). The mean fluorescence intensity (MFI) was used for the analysis, and the results showed an 10% increase in mitochondrial reactive oxygen species (ROS) production (Figure 4.21a) while the mitochondrial membrane potential was decreased almost 30% (Figure 4.21b).

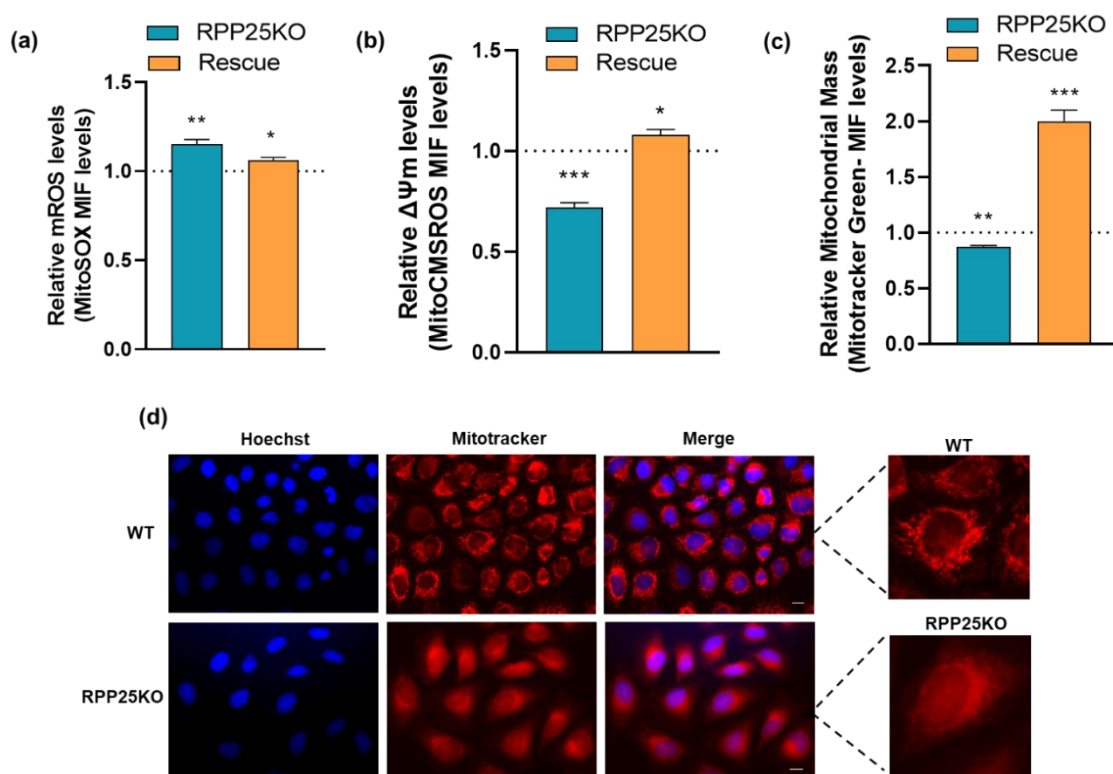


Figure 4.21. Analysis of (a) mitochondrial superoxide production (b) mitochondrial membrane potential and (c) mitochondrial mass using flow cytometry probes in both knockout and rescue experiments. All graphs represent the analysis using Mean Fluorescence intensity (MFI). (d) Representative fluorescent microscopy images to visualize the mitochondrial morphology in RPP25KO cells compared to WT (scale bar 20 μm) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Moreover, the mitochondrial mass was measured using mitotracker green fluorescence dye which showed that is reduced almost 20% in RPP25KO compared to WT (Figure 4.21c). In addition, after rescuing RPP25, although the intracellular ROS production was not reduced (Figure 4.21a), the mitochondrial potential of membrane but also the mitochondrial mass was significant increased (Figure 4.21 a, b). Finally, the changes of mitochondria between RPP25KO and WT cells, were further verified by visualization of mitochondria morphology using mitrotracker deep red dye (Figure 4.21d).

4.2.7. NGS analysis of RPP25-KO cells suggests important gene expression alterations

To elucidate the effect of RPP25 ablation on the transcriptome of HeLa cells, total RNA molecules were isolated, and the gene expression profile was generated using the indicated RNA-sequencing technique on an ION TORRENT Next Generation Sequencing platform (School of Medicine, University of Patras). Then, the Fastq files were further analyzed on the website <https://galaxyproject.org> using the STAR aligner (v2.7.8a). From the analysis, the differential gene expression was determined between WT and knockout cell lines. It was observed that 445 genes were differentially expressed (p-value <0.05) in knockout cells. Among them, 277 were significantly upregulated while 168 were downregulated (Figure 4.22a). On the other hand, the transcriptome of rescue experiments showed that among the 170 differentially expressed mRNAs (p-value <0.05), 90 were significantly upregulated while 80 were downregulated (Figure 4.22b). In addition, the genes were differentially expressed were used for further bioinformatic gene ontology (GO) analysis using the Panther database (<http://pantherdb.org/>). Firstly, the Reactome pathway category revealed that differentially expressed genes participate in pathway regarding the ECM proteoglycans, the metabolism of steroids, the extracellular matrix as well as the RNA metabolism (Figure 4.22c), while after rescuing RPP25, the analysis showed pathways regarding regulation of cell surface at vascular wall, regulation of lipid metabolism, response to Ca²⁺ and also nuclear receptor transcription pathway (Figure 4.22d). Subsequent GO analysis of RPP25KO, regarding the molecular function and biological process categories showed that genes are involved in signaling pathways, NADPH oxidases metabolic pathway, steroid or hormone

metabolism as well as regulation of cell communication and general regulation of extracellular matrix confirming the wound healing experiments (Figure 4.23a, b).

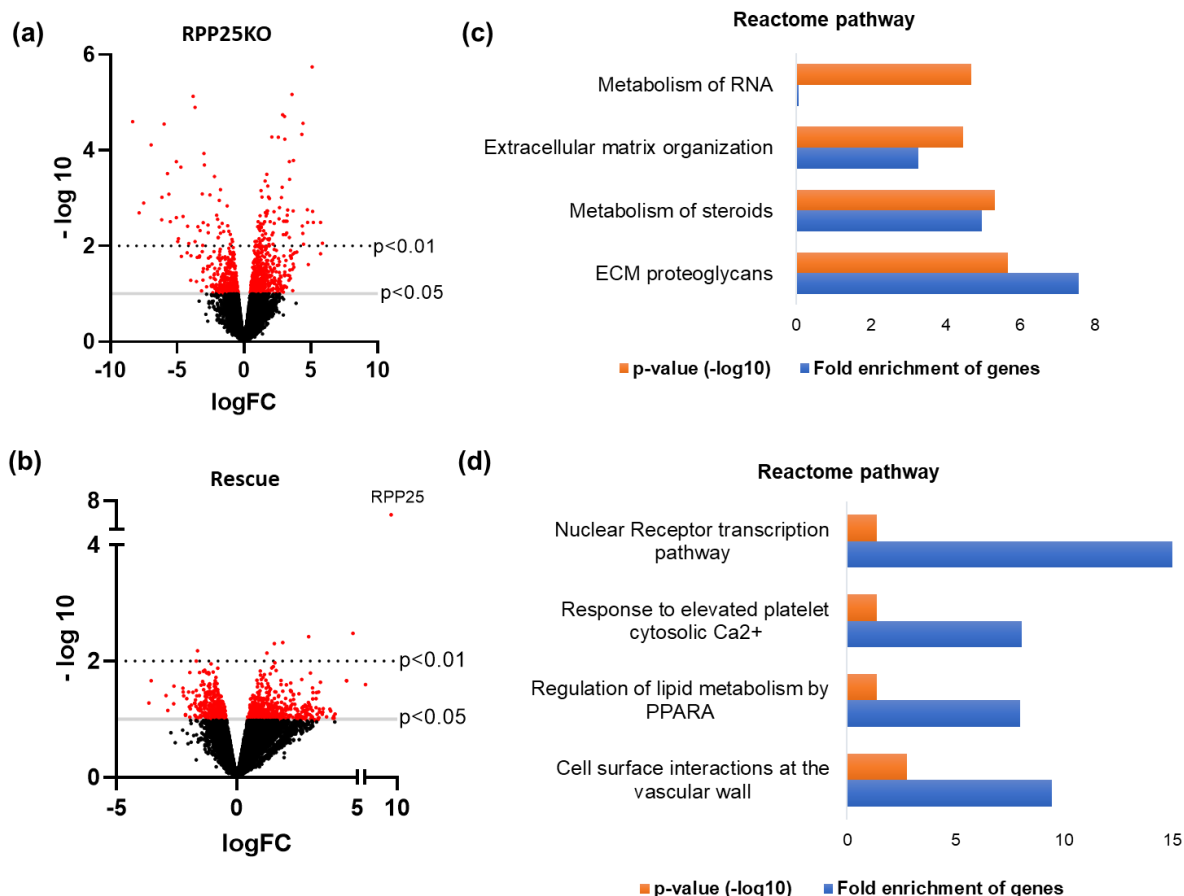


Figure 4.22. Volcano plot of differentially expressed genes in **(a)** RPP25 Knockout compared to WT HeLa cells and **(b)** after rescuing RPP25. **(c)** Reactome pathway enrichment analysis for RPP25KO cells and **(d)** after rescue experiments.

In contrast, after rescuing RPP25, the GO analysis for molecular function and biological process categories didn't show statistical significant results which may indicate that RPP25 restored the dysregulated pathways. Moreover, NGS analysis of RPP25KO cells, showed that the most upregulated genes belongs to Aldo-keto reductases (ARKs). This superfamily is an NADPH-dependent oxidoreductases that are overexpressed under oxidative stress and in different type of cancers (Figure 4.23c) (Penning et al., 2021).

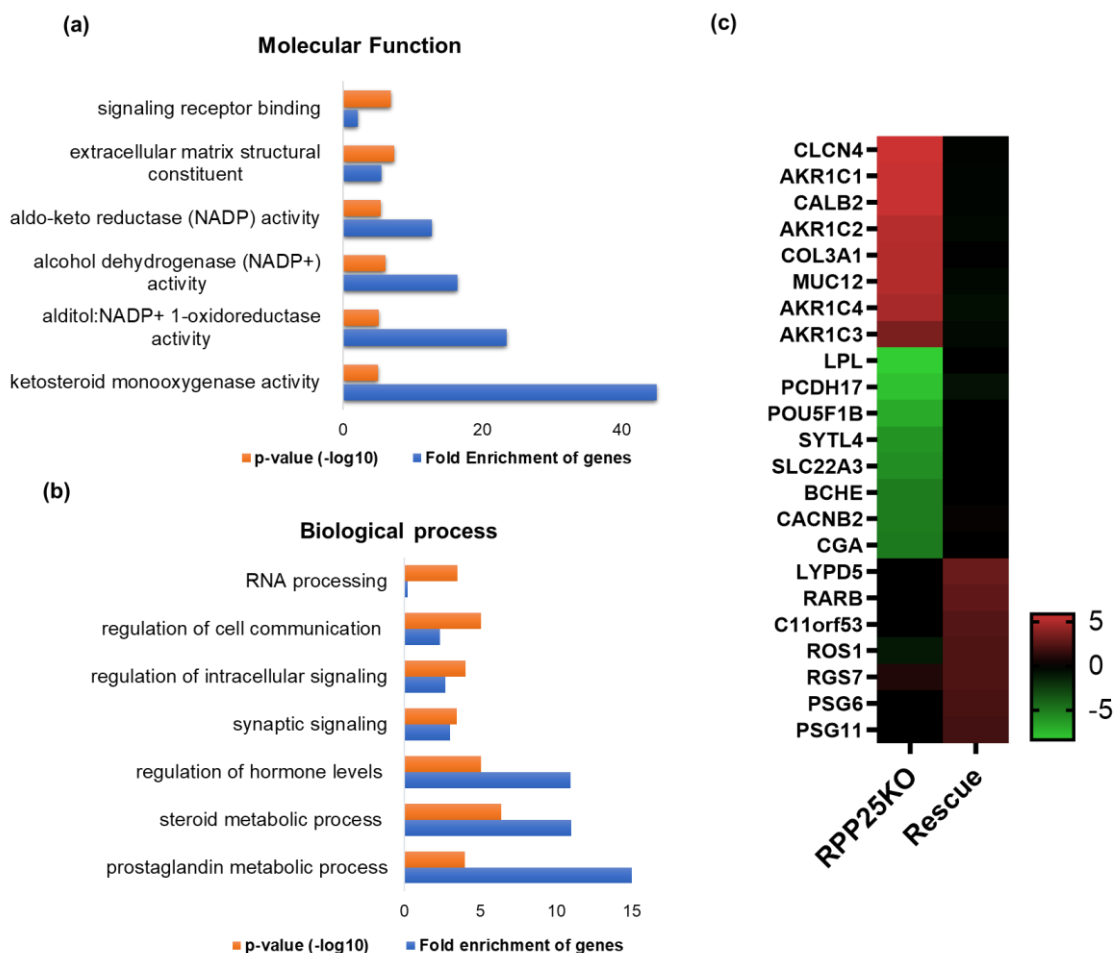


Figure 4.23. GO enrichment analysis for **(a)** molecular functions and **(b)** biological processes categories affected by dysregulation of genes following ablation of *Rpp25*.

Moreover, it was also analyzed the differentially expressed of lncRNAs as a number of lncRNA molecules has been correlated with cancer pathophysiology. The present analysis identified that the differentially expressed lncRNAs belong to Long intergenic Non-Protein Coding RNAs (lincRNAs) with unknown function. More specific, the LINC02747 and TCL6 which is known to participate in cancer progression or cell proliferation, was found to be upregulated in RPP25KO cells (Wu et al., 2019). On the other hand, the lncRNAs CCAT2, PCAT1, DSCAM-AS1a which was found to participate in miRNA pathway or in energy metabolism, were found to be downregulated. Finally, in rescue experiments, it was observed that RPP25 could regain the expression levels of lncRNA (Figure 4.24) (Shang et al., 2019, 2019; Chen et al., 2020).

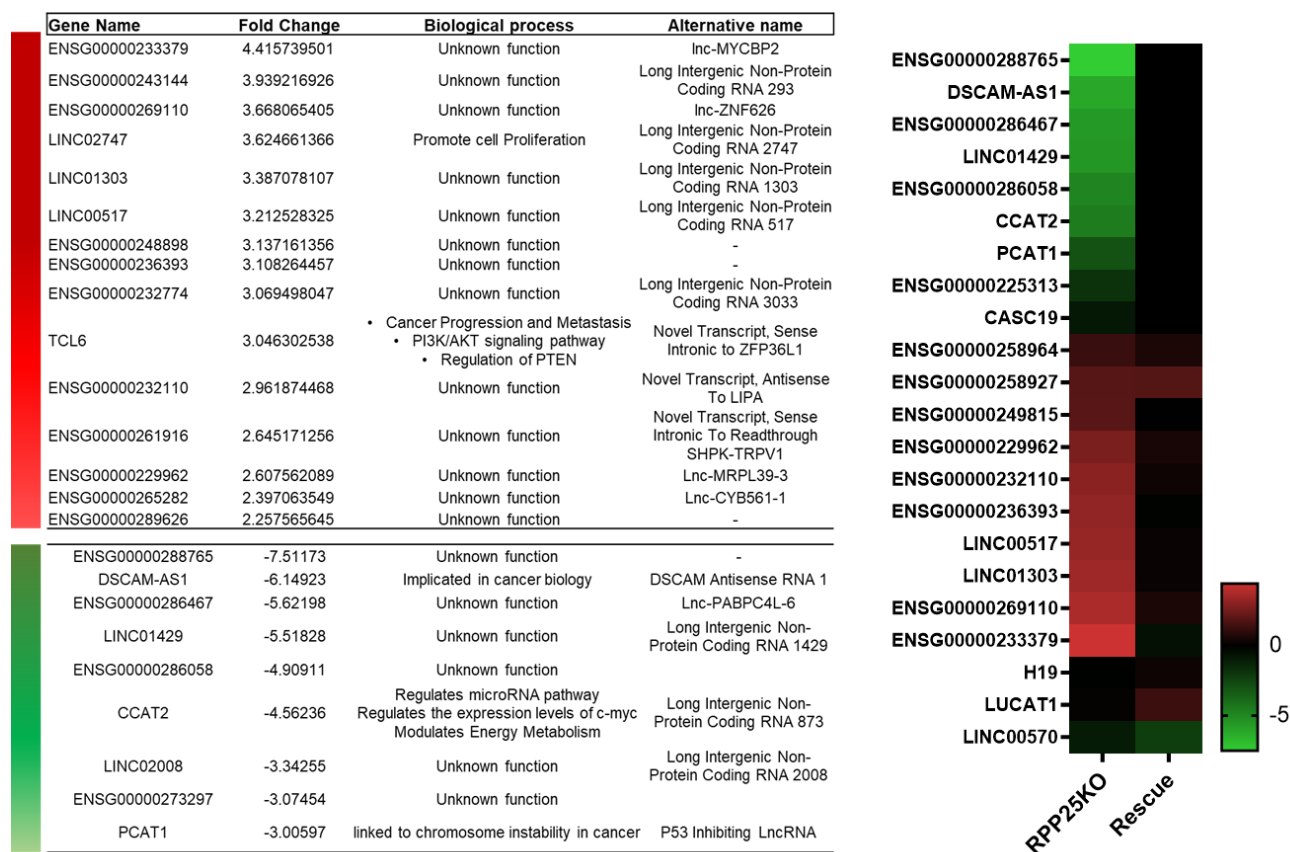


Figure 4.24. Presentation of the expression levels of long non-coding RNAs found to be upregulated (red color) or downregulated (green color) (left panel) and the expression levels of long non-coding RNAs with $p < 0.01$ compared to rescue experiments.

4.2.8. Ablation of RPP25 affects miRNAs expression

To examine the effect of RPP25 knockout on small RNA transcriptome, RNA-seq was performed using ION TORRENT platform. For the enrichment for small RNAs a 15% UREA-Page was used and the RNAs was further separated by their size. The preparation of small RNA molecules libraries was performed following the protocol (see Materials and Methods). The read counts for miRNAs were generated with featureCounts (v2.0.1) using annotation files from ENSEMBL and miRbase respectively (Liao et al., 2014b). At first, the results showed that although 350 miRNAs were identified, only 60 miRNAs were significantly differentially expressed (p -value < 0.05). Among them 24 miRNAs were significantly downregulated while 36 miRNAs were

upregulated (Figure 4.25a). In contrast, in rescue experiments, 245 miRNAs were identified but 100 miRNAs differentially expressed ($p < 0.05$) from which 24 miRNAs were significantly downregulated while almost 3 were upregulated (Figure 4.25b). The top upregulated and downregulated miRNAs from both knockout and rescue experiments (Figure 4.25 c and d) were used for further analysis.

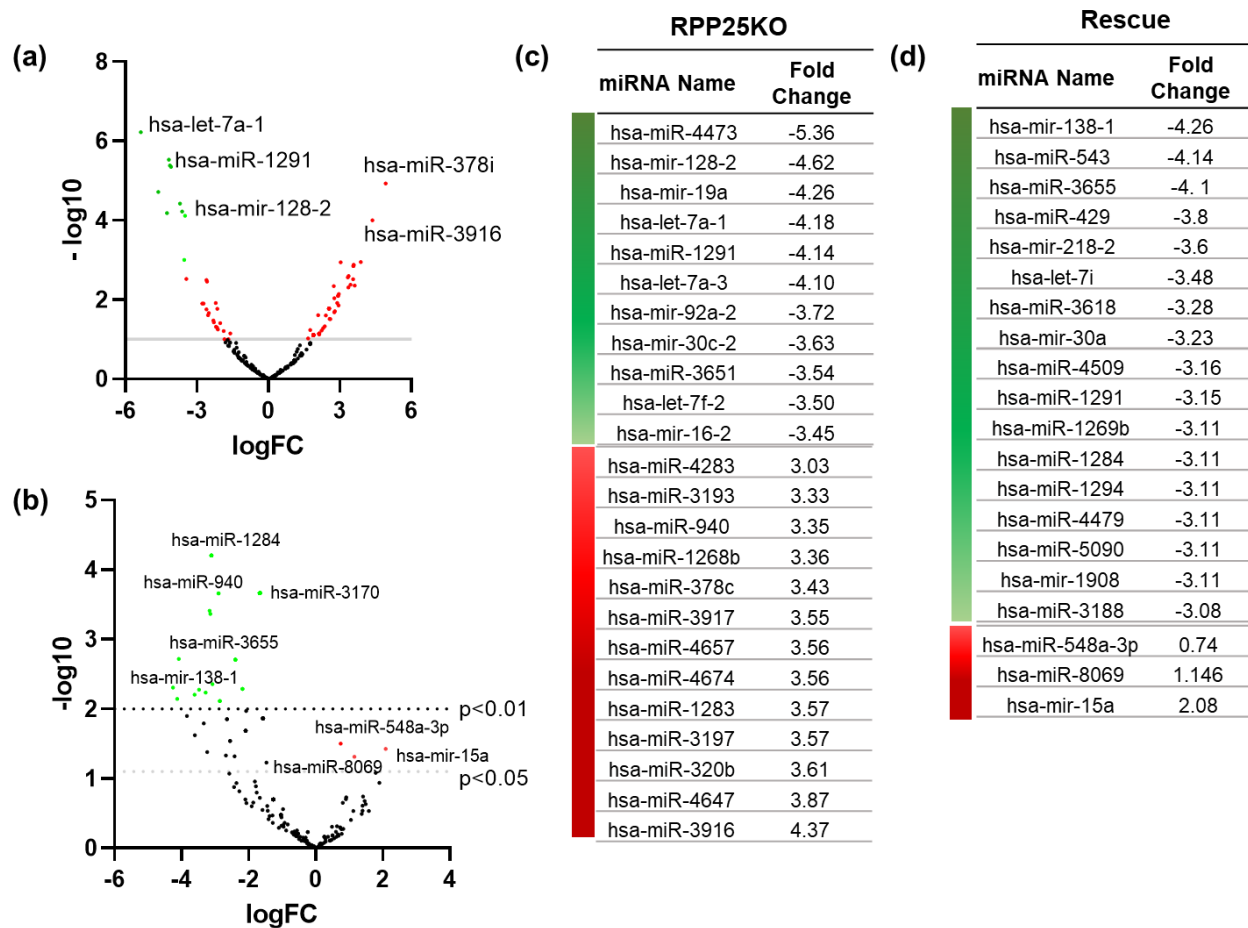


Figure 4.25. Volcano plot of all identified miRNAs from both (a) knockout and (b) rescue experiments. The upregulated miRNAs with $p < 0.05$ are visualized with red while the downregulated with green. (c) Table of representative upregulated and downregulated miRNAs in Rpp25KO and (d) after rescue experiments.

In addition, further analysis was performed to identify the role of differentially expressed miRNAs. The predicted targets of miRNAs were found from TarBase v8.0 database and they used for GO enrichment analysis using Panther database. The GO analysis for molecular function and biological process categories from the predicted target genes of downregulated miRNAs, showed

pathways related to mRNA binding, chromatin remodeling, signaling receptor activity, transcription activity and ribonucleoprotein complex binding while the main biological pathways include the G1/S transition of cell cycle, RNA polymerase II transcription, regulation of gene expression and metabolic process of RNA and other macromolecules (Figure 4.26a). On the other hand, the GO analysis for molecular function and biological process categories from the predicted target genes of upregulated miRNAs showed pathways related to chromatin regulation, in translation and transcription activity and also in RNA splicing (Figure 4.26b).

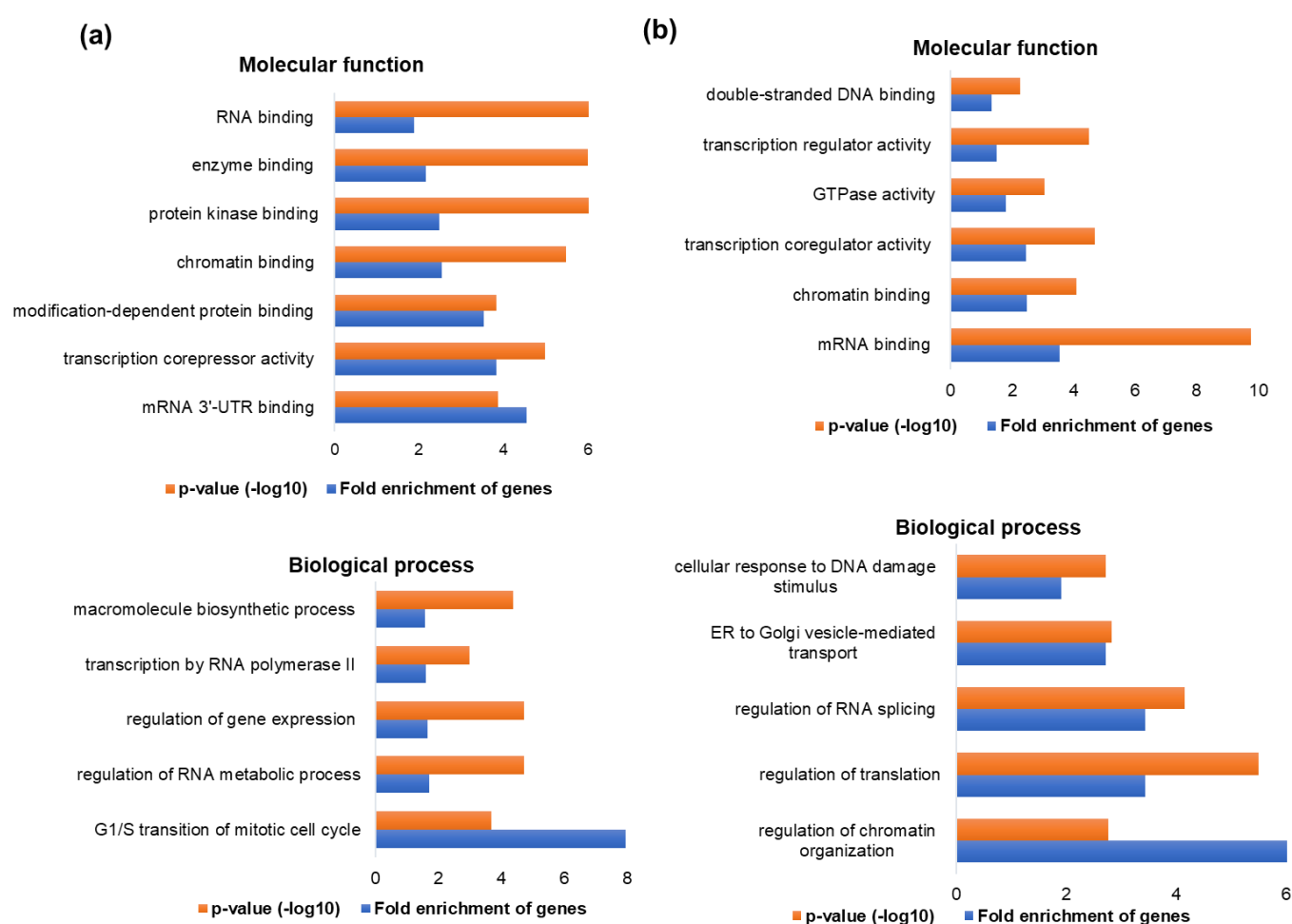


Figure 4.26. GO enrichment analysis on predicted target genes of the statistically significant altered miRNAs. **(a)** Top pathways in molecular function and biological process of predicted target genes from downregulated miRNAs **(b)** molecular function and biological process of predicted target genes from upregulated miRNAs.

Moreover, after rescuing RPP25, the predicted targets of miRNAs were also found from TarBase v8.0 database and the GO analysis was performed. The top pathways found, include the translation, chromatin organization, metabolic process, apoptosis but also signaling and RNA binding (Figure 4.27a). In addition, the 3 miRNAs that were upregulated, after rescuing RPP25, has targets which are implicated in cell cycle progression, metastasis or translation. This observation may indicate that these pathways are regulated restoring the effect of knockout (Figure 4.27b).

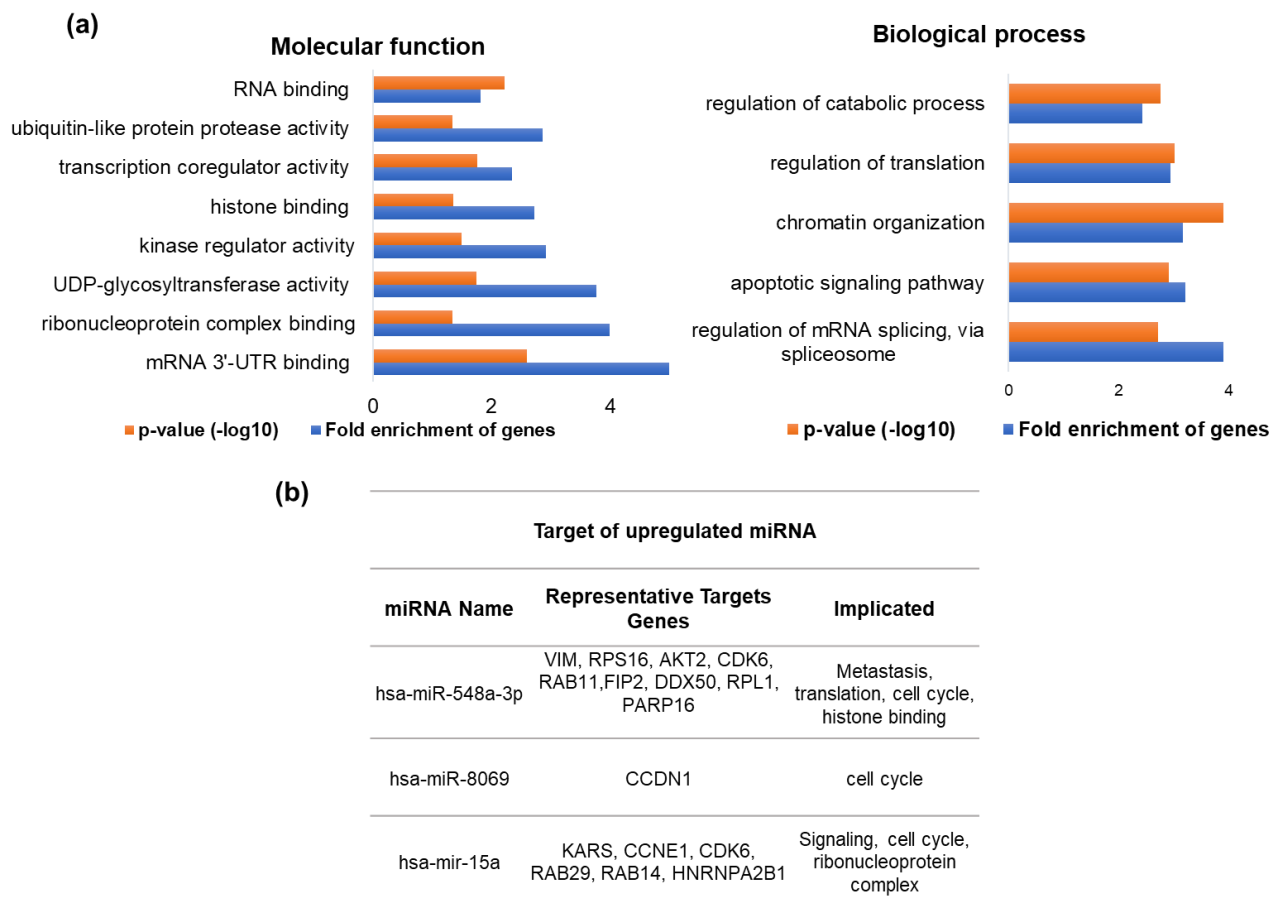


Figure 4.27. (a) GO enrichment on predicted target of the statistically significant altered miRNAs after rescue experiments **(b)** Table of three upregulated miRNA and their representative target with function that are implicated

4.2.9. Ablation of RPP25 leads to altered expression of tRNAs and tRFs

Although tRFs are an established class of regulatory molecules and they were sporadically mentioned over the last years, their function is still debatable, however, it has been reported

that could act as important modulators of various processes, including translation, apoptosis and response to nutrient starvation or oxidative stress (Schimmel, 2018). However, along with tRFs, changes in tRNA abundance have been found in many cancers. Thus, to examine the effect of RPP25 knockout on expression profile of tRNA and tRFs, NGS analysis were performed. The tRNA sequences were mapped to an artificial tRNA genome consisting of unique sequences of hg38 tRNAs using Bowtie (v.1.2.3) and further counted with featureCounts (v2.0.1). In contrast the read counts for the expression levels of tRFs molecules were determined based on their exact sequences which were measured unaltered in the Fastq files. At first, in RPP25 knockout cells, although 129 tRF sequences were detected, 18 of them were statistically significant with p-value <0.05 (11 tRF-3, 4 tRF-1, 3 tRF-5). Among the differentially expressed tRFs, the expression levels of tRF-3 and tRF-1 were found to be downregulated while only the 3 tRF-5 were upregulated (tRF-5020b, tRF-5030b and tRF-5008b) (Figure 4.28a and b).

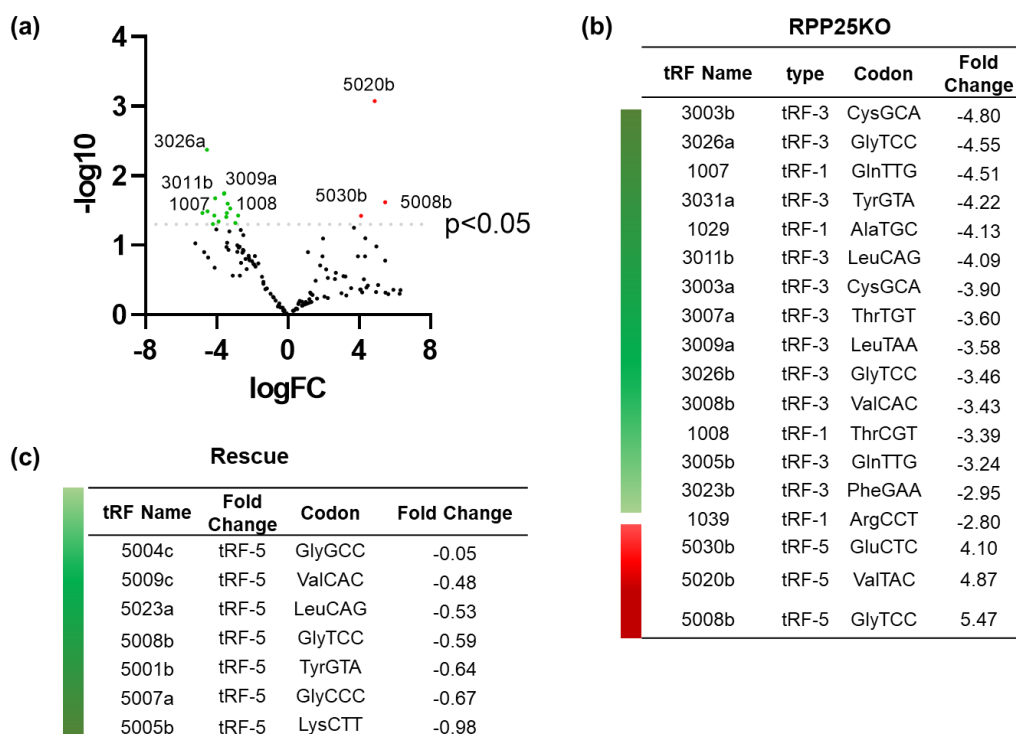


Figure 4.28. (a) Volcano plot of all identified tRFs in RPP25KO cells. The upregulated tRFs with $p < 0.05$ are visualized with red while the downregulated with green. (b) Table of representative upregulated and downregulated tRFs in Rpp25KO and (c) after rescue experiments.

In contrast, rescue experiments showed that although, 125 tRF sequences were detected, only 7 tRFs were statistically differentially expressed which are classified at tRF-5 category. All tRFs were found to be downregulated (Figure 4.28c). Moreover, in rescue experiments, among the downregulated tRFs also observed the 5008b (deriving from tRNAGlyTCC) which was also upregulated in knockout cells. Moreover, it is also observed a decrease of 5004c and 5007a derived from tRNAGlyCCC (Figure 4.28c). Of note, although different tRFs are affected in both knockout and rescue experiments, the most abundant tRFs derived from tRNAGly, thus, the tRNA expression profile was measured using next generation sequencing (Figure 4.29).

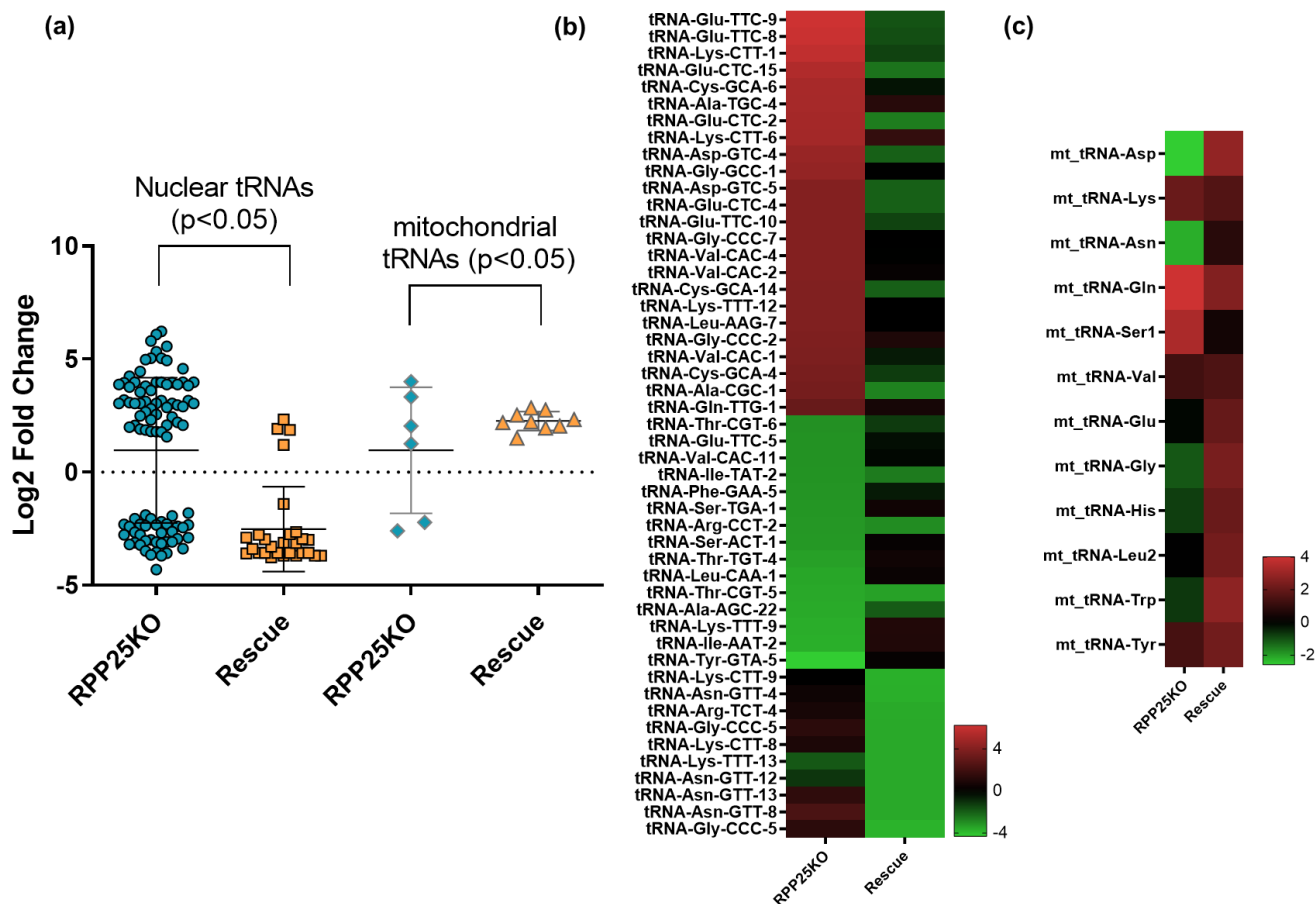


Figure 4.29. (a) Graphical presentation of significant differentially expressed of both nuclear and mitochondrial tRNAs in knockout and in rescue experiments. (b) Heatmap showing the scaled expression of upregulated and downregulated nuclear tRNAs and (c) mitochondrial tRNAs in RPP25KO cells and after rescue experiments.

It was detected 95 nuclear tRNAs and 6 mitochondrial encoded tRNAs which the statistically differentially expressed in RPP25KO cells, while 28 nuclear tRNAs and 9 mitochondrial encoded tRNAs were identified after rescue experiments (Figure 4.29a). It was observed that, in knockout cells, the nuclear tRNAs showed variations in expression profile with the abundance of them to be upregulated (almost 60%) while in rescue experiments, almost the 90% of nuclear tRNAs are downregulated. On the other hand, the mitochondrial tRNAs showed significant upregulation after rescuing RPP25, an observation which may be correlated with the restoration of mitochondria function (Figure 4.29a). In addition, the most deregulated tRNAs from both knockout and rescue experiments are those of tRNAGlu, tRNALys and tRNAGly (Figure 4.29b and c). The upregulation of tRNA Gly may be correlated with the production of specific tRFs which referred above.

Moreover, the potential targets of the tRFs were analyzed. Accumulating data allowed the development of highly specialized bioinformatic platforms, such as tRF database (tRFdb) tRFCluster, MINTmap, that can be used for the tRF identification. For GO analysis of present study the tRFdb was used as is the first database reported and it has been the basis for the meta-analysis of tRFs data from CLASH (Crosslinking, Ligation, and Sequencing of Hybrids) data obtained from PAR-CLIP (Photoactivatable Ribonucleoside-enhanced Crosslinking and Immunoprecipitation) experiments. In RPP25KO cells, all differentially expressed tRFs had specific targets with exception of tRF-1 class and tRF-5020b. The representative target genes of tRFs that were found include ribosomal proteins, helicases, RNA pol II subunits etc (Figure 4.30a). The target genes were further analyzed using Panther database to find the biological and molecular functions. Among the top pathway in biological process category include the translation, gene expression regulation, RNA processing and the protein metabolic process (Figure 4.30b) while the top pathway in molecular function category include the structural constituent of ribosome, RNA binding, translation factor activity, chromatin binding, ATP binding and the signaling receptor activity (Figure 4.30c). These observations may indicate that tRFs affect the protein synthesis, RNA processing, metabolism and also cellular signaling pathways.

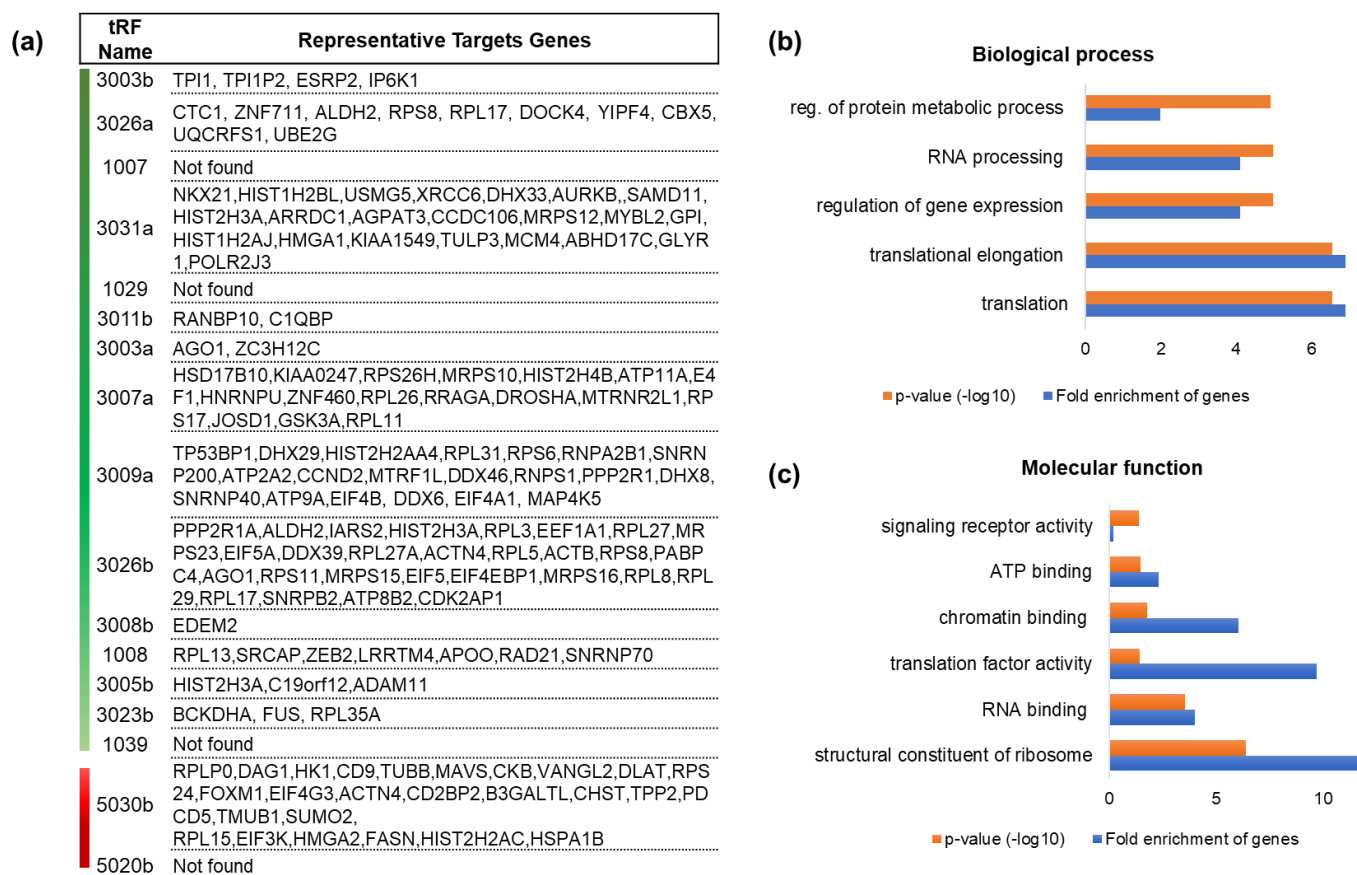


Figure 4.30. (a) Table of representative predicted target genes of statistically significant altered tRFs in RPP25KO cells. (b) GO enrichment analysis on predicted targets of the statistically significant altered tRFs in RPP25KO cells. Top pathways of molecular functions and (c) biological processes categories were presented.

Moreover, the GO analysis was also performed for the predicted targets of tRFs after rescuing RPP25 (Figure 4.31a). The results showed that the top pathways of molecular function category include translation, heat shock proteins, rRNA binding and the ribosome structure (Figure 4.31b) whereas the pathways of biological process category include the RNA processing, RNPs complex, ribosome biogenesis or rRNA metabolic process, translation and protein folding (Figure 4.31c).

Altogether, the results of sequencing of small non-coding RNA molecules (miRNAs and tRFs) indicated that RPP25 is directly or indirectly involved in the regulation of the expression of RNA molecules which in turn influence the gene expression of the cell.

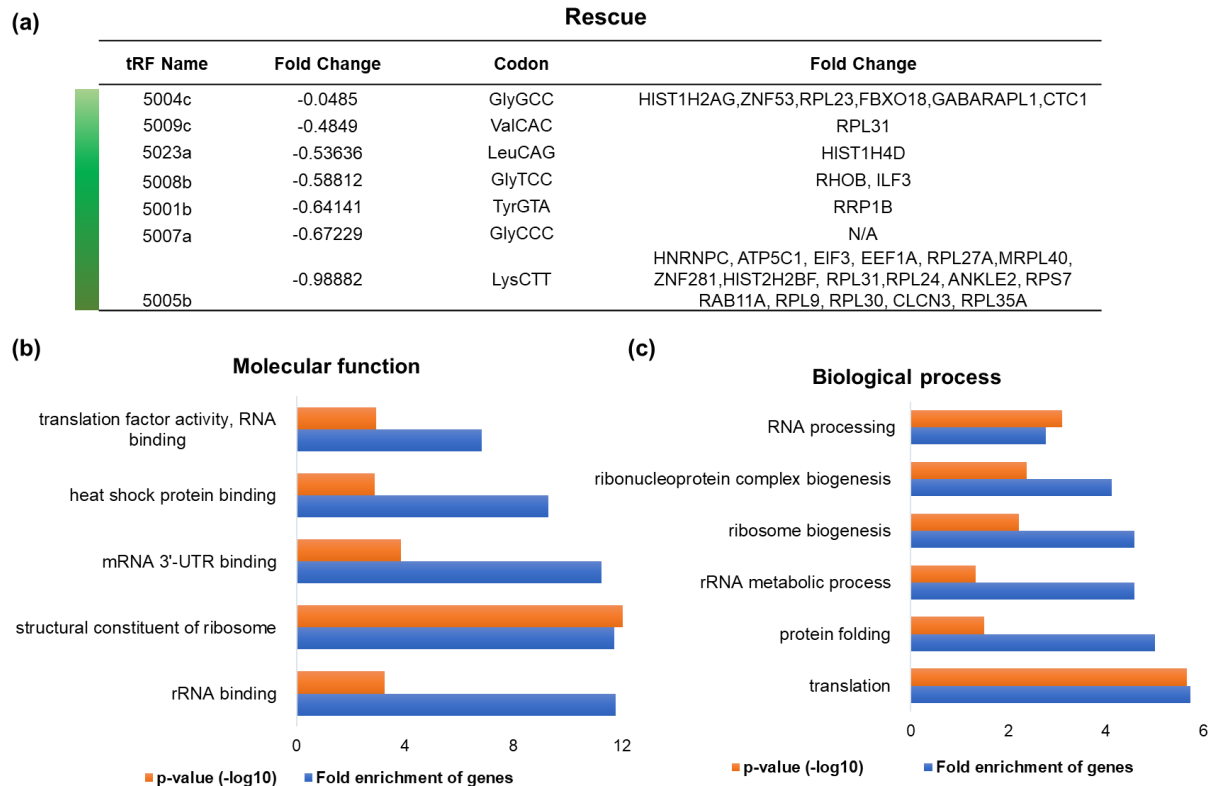


Figure 4.31. GO enrichment analysis on predicted targets of the statistically significant altered tRFs after rescuing RPP25 **(a)** Table of representative predicted target genes **(b)** Top pathway of molecular functions and **(c)** biological processes categories.

4.2.10. Ablation of RPP25 affects translational levels

Based on the transcriptomic profile of tRFs and their GO enrichment analysis, it was observed that among the cellular functions that displayed significant changes are the translation regulation. Thus, it was performed a first assessment of translation activity was obtained by the puromycin incorporation assay in both knockout and WT cells. This method is based on translation inhibition by puromycin. Puromycin enters into A-site of ribosome and is incorporated into newly synthesized peptides (Enam et al., 2020). After treating cells with puromycin, its detection was performed with an anti-puromycin antibody visualizing the nascent peptides. The results showed that knockout cells have an increase (~60%) in the production of newly synthesized peptides compared to WT (Figure 4.32a). In addition, to verify this observation, polysome profile analysis was performed using sucrose density gradient. The samples were

treated with cycloheximide (CHX) which is known to block translation elongation. The ribosomal particles were evaluated by optical scanning at 254 nm using continuous flow cytometer. The analysis showed an increased quantity of polysome formation in knockout cells compared to WT, verifying the stimulation of translation rates. However, the difference between the 40S, 60S as well as the 80S ribosomal peaks is possible due to the formation of polysomes. The percentage of both polysome and free ribosomal subunit was calculated from the area of each region and the results showed a 20% increase (Figure 4.32b). To verify this hypothesis, we performed a second sucrose density gradient analysis using only EDTA treatment in samples. EDTA treatment disrupts polysome association and increased peaks for monosomes and ribosomal subunits. The EDTA-treated sample showed no significant alterations in ribosomal subunits in both cell lines

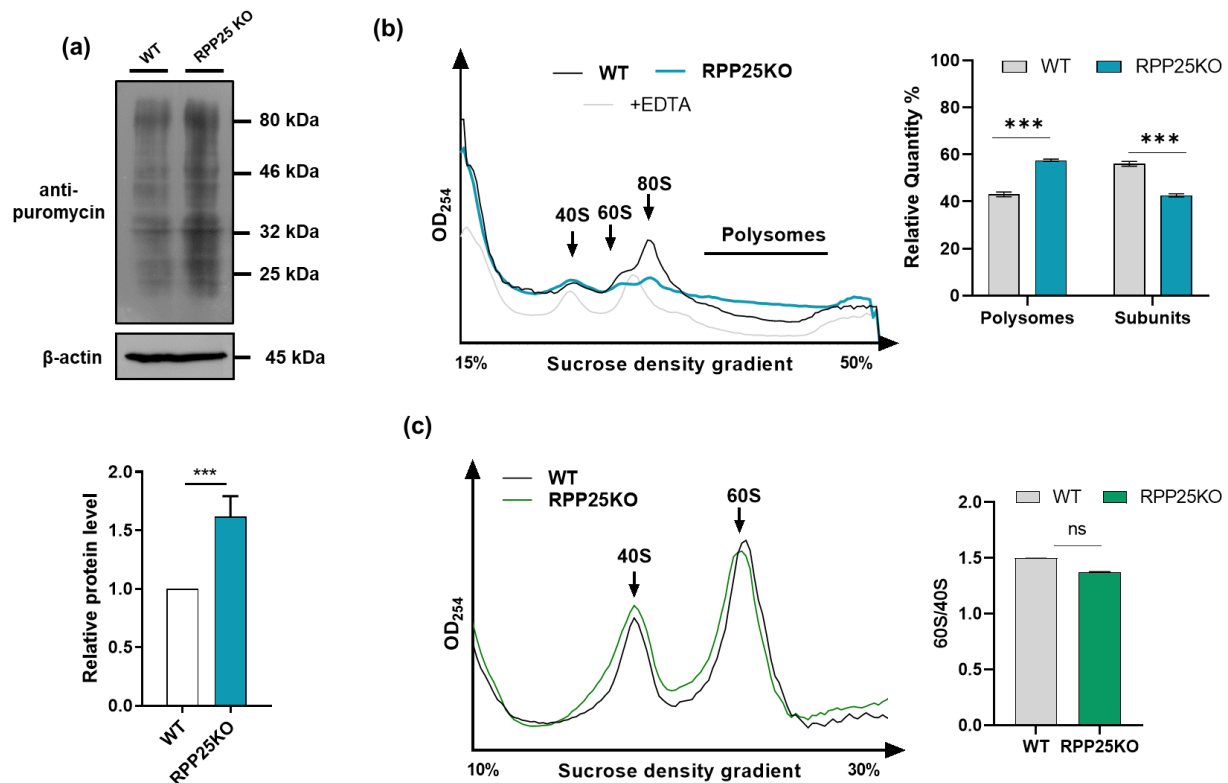


Figure 4.32. Global analysis of translation in absence of RPP25. **(a)** SUNSET-WB assay. An anti-puromycin immunoblot of whole cell lysates treated with 1 μM puromycin (left) and a quantification of protein synthesis rates by measuring the signal intensity in each lane, using ImageJ, normalizing the values to that of the WT lane (right). **(b)** Polysome profiling in both WT (black line) and RPP25KO (blue line) in the presence of CHX (left) and the relative quantity (%) of each peak corresponding to polysomes or free ribosomal subunits. **(c)** Ribosomal subunits analysis in both WT (black line) and RPP25KO (green line) in the presence of EDTA (left) and measurements of 60S/40S ratio of each peak (right) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

(Figure 4.32c). The polysome profiling is consistent with the above results showing an increased cell proliferation and motility. Subsequently, it was studied the expression patterns of key components participating in translation. The mTOR levels which participates in both mTORC1 and mTORC2 complexes didn't exhibit significant alterations in both transcriptional and translational level (Figure 4.33 a and b), while a significant decrease of both RICTOR and RPTOR (1-fold and 2.2-fold, respectively) which participated in the mTORC2 and the mTORC1 complex was observed (Figure 4.33a). This observation could suggest that translation is activated through another pathway like MAPK pathway. MAPK/ERK signaling pathway, is related to cell proliferation and differentiation (Zhang and Liu, 2002), thus, the phosphorylation of ERK, downstream of MAPK kinase was tested and further observed an upregulation of the p-ERK in RPP25 knockout cells compared to control. In addition, it was also tested the eIF2 α , an important initiation factor which recruits the initiator Met-tRNA^{Met} and binds the 40S ribosomal subunit to form the 43S pre-initiation complex (Aitken et al., 2016). It was found that eIF2 α was upregulated while its phosphorylated (Ser51) levels tended to be lower (Figure 4.33b and c). This observation confirmed the increased rates of protein synthesis as it has been found that eIF2 α is overexpressed in cancers stimulating the translation (Holcik, 2015). Furthermore, the eIF4E and 4E-BPs factors which participate in cap-dependent translation were tested and it was observed an increased phosphorylation levels of p-4EBP1(S65) and p-4E-BP1 (T37/46), while the levels of 4E-BP1 were unaffected. Moreover, although the protein levels of eIF4E were found downregulated, its phosphorylation levels (p-eIF4E) were significant increased indicating that 4E-BPs is phosphorylated and eIF4E is released leading to enhanced cap-dependent translation. (Figure 4.34b, c). Finally, the expression levels of eEF1A and eEF2 were studied in order to find possible alterations that are related to translation elongation. The results showed an upregulation of eEF2 (2-fold; Figure 4.33a).

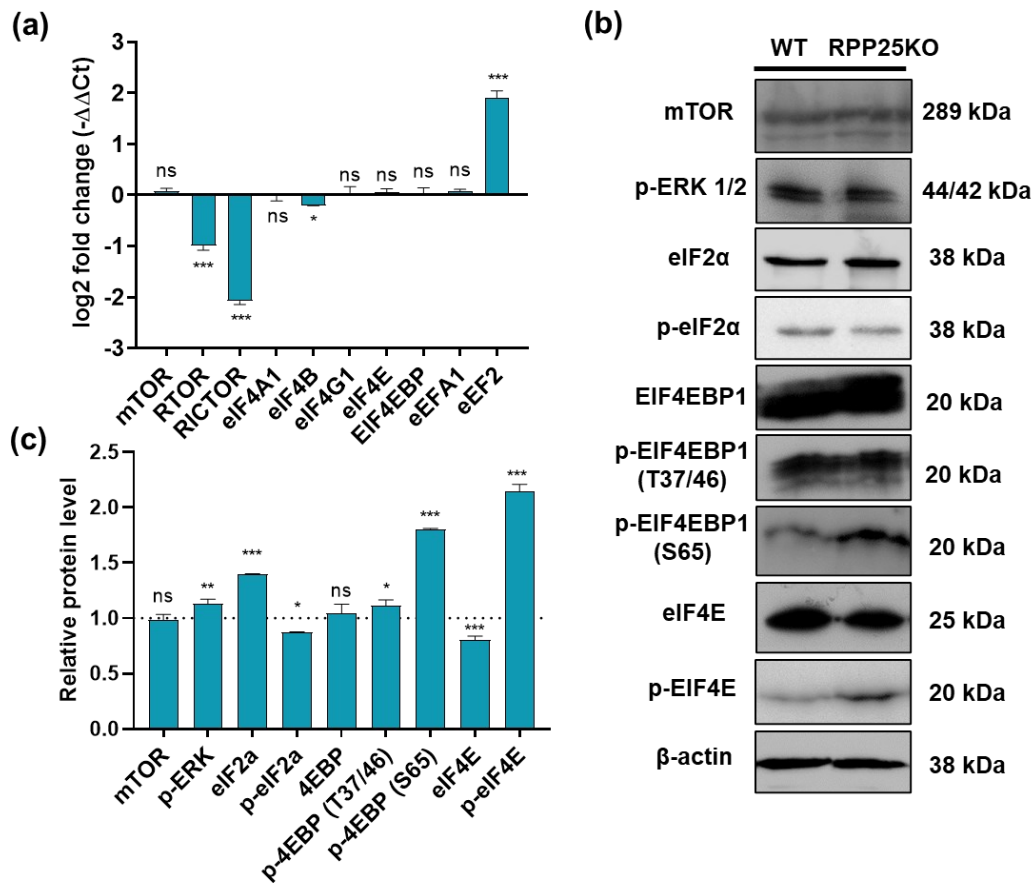


Figure 4.33: (a) mRNA expression levels of translation initiation genes were examined *via* RT-qPCR analysis. (b) Immunoblot analysis of translation initiation factors and binding proteins and (c) the quantitation of protein levels. The quantitation of protein levels was calculated after normalization to β -actin levels. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

4.2.11. RPP25 is co-localized with coilin and possibly interacts with scaRNA molecules

A recent study has reported that human RPP20-RPP25 complex are likely bound to RNA molecules with unknown cellular functions (Yin et al., 2021). To study this hypothesis and using the NGS data and the matched whole-genome sequencing analysis, alterations in expression levels of specific scaRNAs (Small Cajal body-specific RNAs) were detected. ScaRNAs are mainly localizes in the Cajal bodies and guide post-transcriptional modifications on snRNAs (Meier, 2017). They have similar structure and function with snoRNAs that are involved in modification of rRNAs. It was observed a downregulation of scaRNA23, scaRNA20, scaRNA1, scaRNA14 (Figure 4.34a). To monitor any possible participation of RPP25 in Cajal bodies (CB), immunofluorescent

experiments were performed. Both WT and RPP25KO cells were transiently transfected with pcDNA3.1 construct for expression of RPP25 tagged with EGFP in C-terminus (pcDNA3.1-RPP25-EGFP; green). Hoechst fluorescence dye was used for staining of nucleus while for staining of CB or nucleolus the coilin and fibrillarin antibodies were used, respectively. It was observed that RPP25 was detected in the nucleus and nucleoli of both cell lines and also it co-localized with fibrillarin in nucleolus (Figure 4.35b). Moreover, RPP25 is co-localized with coilin, suggesting that RPP25 could interact with coilin for RNA processing in CB (Figure 4.34b).

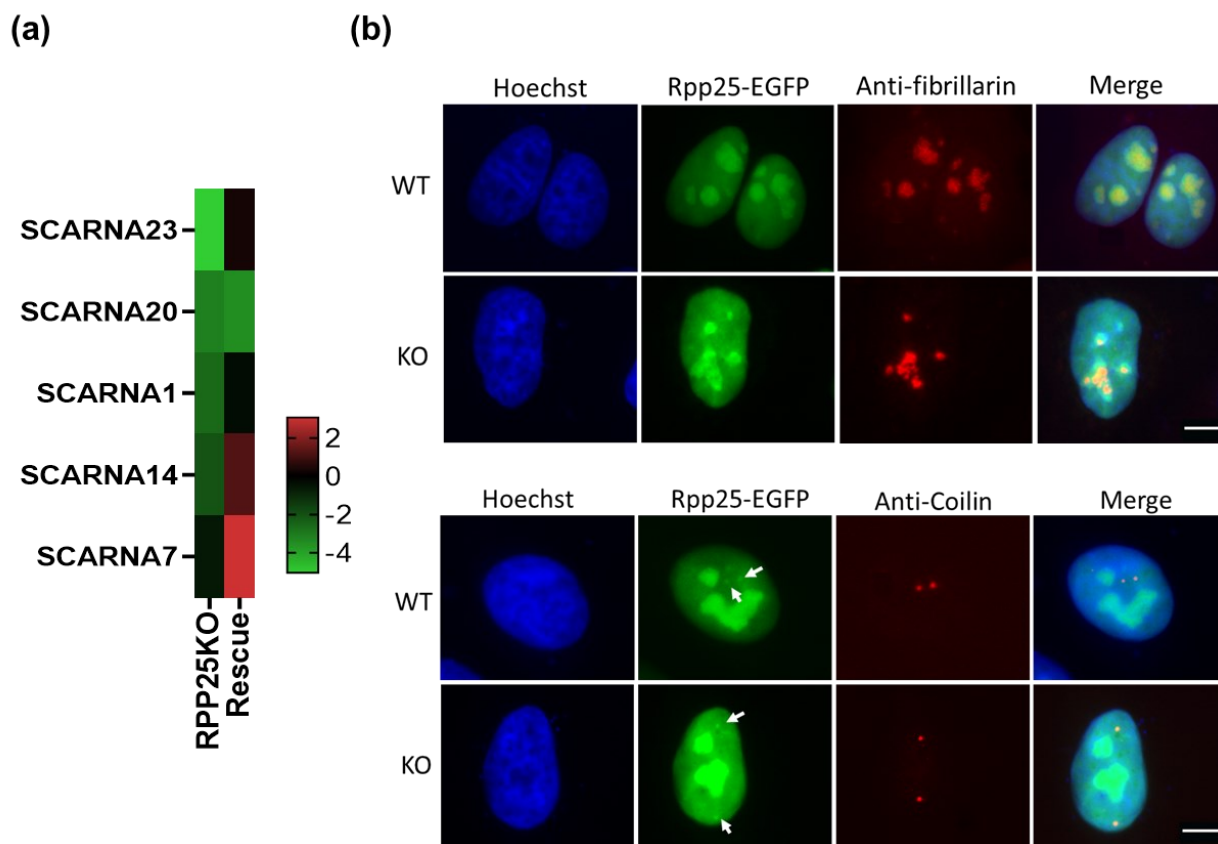


Figure 4.34: (a) Heatmap analysis of specific altered scaRNAs in both RPP25KO and rescue experiments. **(b)** Fluorescence microscopy images showing the subcellular localization of RPP25 tagged with EGFP. The nuclei were stained with Hoechst (blue color) and anti-Fibrillarin (red color; up) and with (red; up) and anti-coilin (red; down). The merge channels contain a 20 µm scale bar.

The specificity of RPP25 localization signal was compared with cells bearing an empty vector under the same conditions (Figure 4.35a). In addition, further immunoblot analysis showed that in knockout cells the non-phosphorylated coilin is upregulated (1.8-fold) while in rescue experiments the non-phosphorylated coilin are downregulated (0.8-fold) (Figure 4.35b). Based

on this observation it could be hypothesized that RPP25 possibly recognized snoRNA-like or snRNA-like structure and may synergistically act with coilin for binding of scaRNAs.

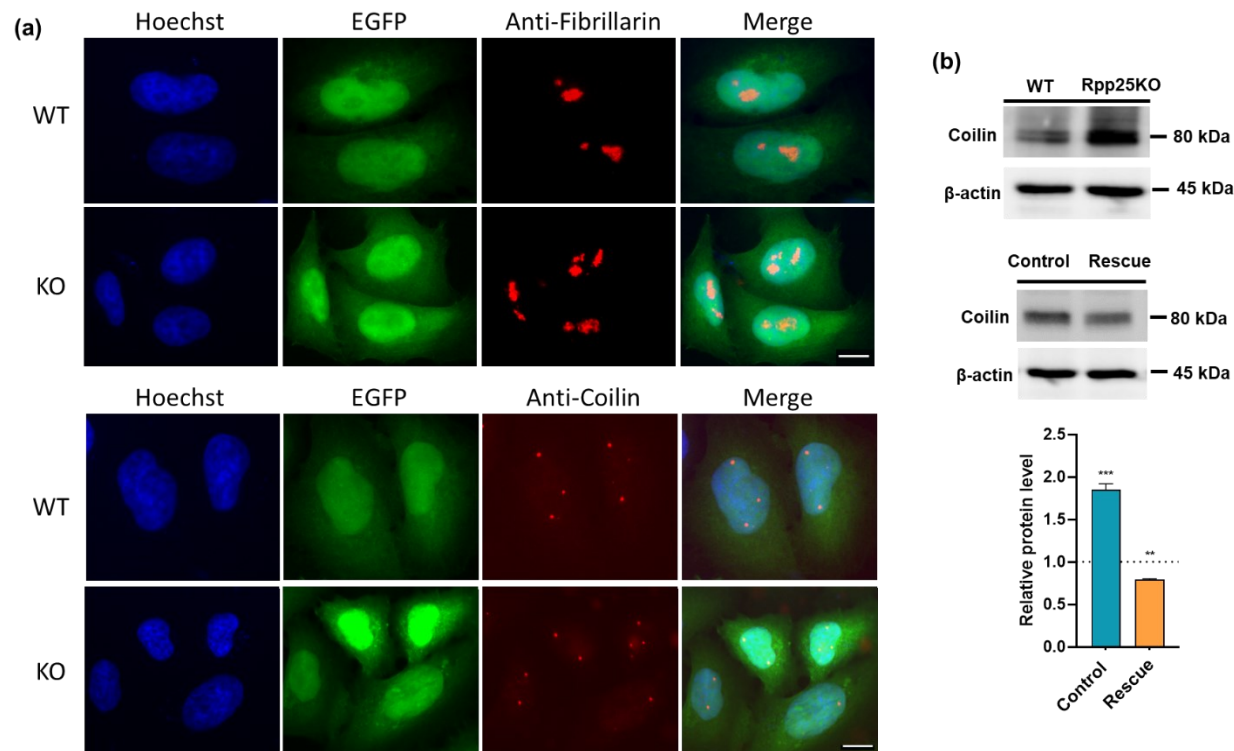


Figure 4.36: (a) Fluorescence microscopy images showing the subcellular distribution of empty pcDNA3.1 vector tagged with EGFP. The nuclei were stained with Hoechst (blue color) and anti-Fibrillarin (red color; up) and with (red; up) and anti-coilin (red; down). The merge channels contain a 20 μm scale bar. **(b)** Immunoblot analysis of coilin in RPP25KO cells and after rescuing RPP25 (up) and quantitation of protein levels (down). The quantitation of protein levels was calculated after normalization to β-actin levels (*p < 0.05, **p < 0.01, ***p < 0.001).

4.2.12. RPP25-Like is localized in nucleus and nucleolus

RPP25 protein is known as a protein component of RNase P/MRP holoenzyme. However, it has been found that a paralogue gene exists that encodes for RPP25L protein. RPP25L is an uncharacterized protein with unknown function. Although, the predicted RPP25L showed 43% identity (in amino acid sequence) to hRPP25 protein, a superposition of both proteins revealed structural similarities that may imply that RPP25L serves as an alternative protein subunit of RNase P. To characterize this protein, immunofluorescence experiments were performed. Both

WT and RPP25KO cells were transiently transfected with pcDNA3.1 construct for expression of RPP25L tagged with EGFP in C-terminus (pcDNA3.1-RPP25L-GFP; green). Hoechst fluorescence dye was used for staining of nucleus while for staining of CB or nucleolus the coilin and fibrillarin antibodies were used, respectively. It was observed that RPP25L was mainly detected in the nucleus and nucleoli where it is co-localized with fibrillarin and coilin (Figure 4.36).

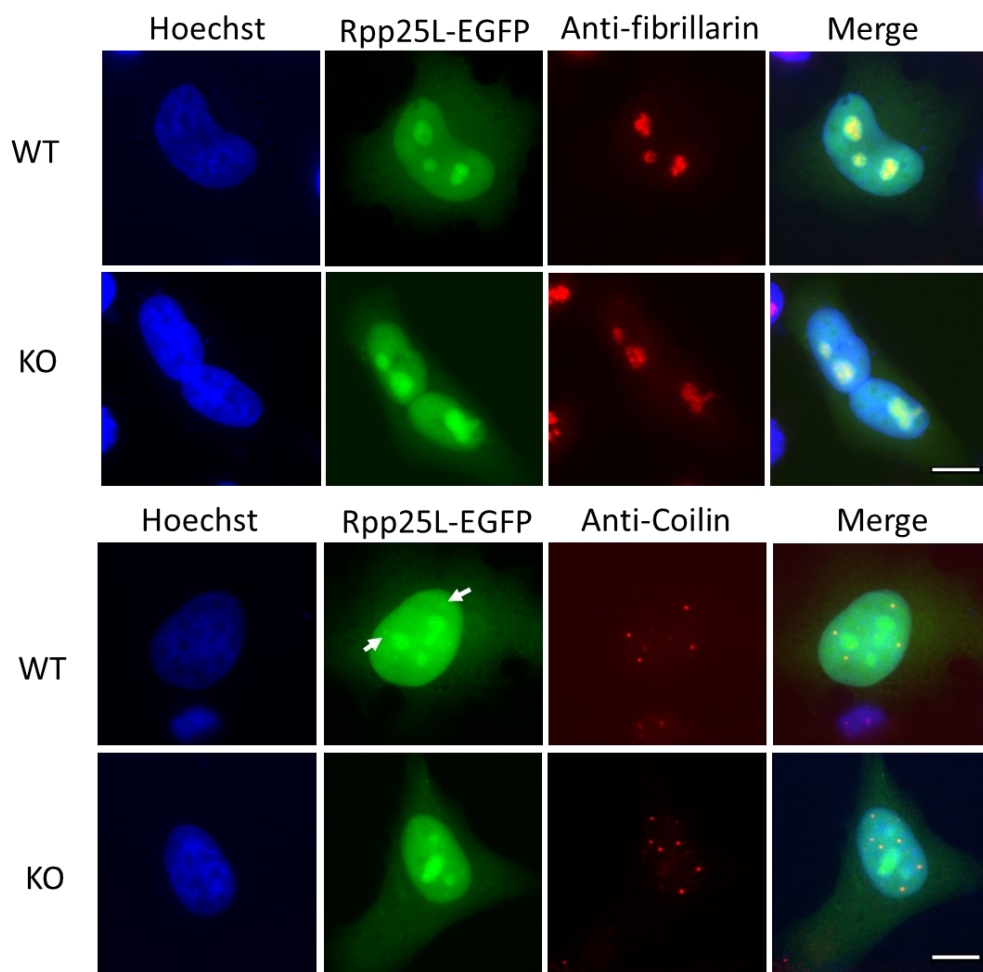


Figure 4.36: Fluorescence microscopy images showing the subcellular localization of RPP25L tagged with EGFP. The nuclei were stained with Hoechst (blue color) and anti-Fibrillarin (red color; up) and with (red; up) and anti-coilin (red; down). The merge channels contain a 20 μm scale bar.

5. Discussion

The milestone of the human genome draft complemented existing genomes from a variety of model organisms and conformed that a vast amount of genomic content could be transcribed in a myriad of small and long noncoding RNA molecules (ncRNAs). The limited number of annotated protein-coding genes, which neither correlates with the complexity of organisms nor accounts for the selection pressure during evolutionary processes occupy less than 2% of the total genome and more surprisingly, more than 80% of remaining genomic DNA corresponds to transcripts of various sizes and sequence conservation. Today, the major classes of ncRNAs include also RNA groups that were previously considered housekeeping like rRNAs, tRNAs, snRNAs, snoRNAs and have enriched the known groups of more extensively characterized ncRNAs like miRNAs and lncRNAs (Figure 5.1) (Amin et al., 2019). At the same time, the role of a variety of ncRNAs like piRNAs, scaRNAs, vaultRNAs, YRNAs and many others remains essentially uncharacterized.

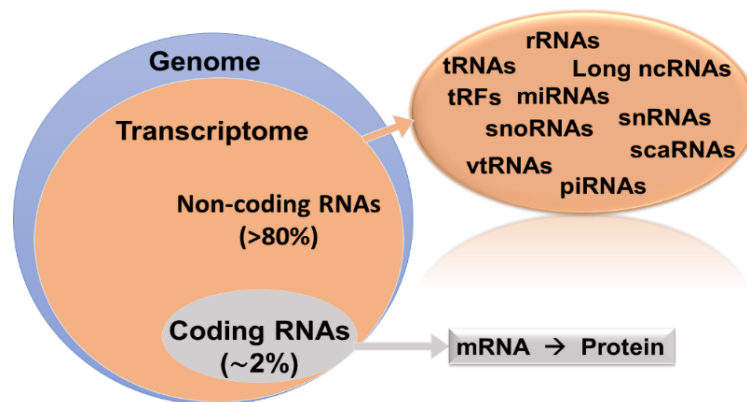


Figure 5.1. Coding and non-coding RNAs of human transcriptome.

The accumulated observations of thousands of noncoding RNAs which exist to regulate and decode the information which is encrypted in a rather small number of mRNAs, is characteristic of the contemporary RNA world. Moreover, the sensitivity of all RNA molecules to random hydrolysis or degradation by hundreds of endo- and exo-ribonucleases, imposes the necessity for all RNA molecules to be protected either by post-transcriptional modifications, or *via* interactions with specific proteins. Therefore, it is critical not only to identify the expanding universe of ncRNAs, but also to recognize the RNA-RNA and RNA-protein interactions that mediate events, like RNA folding, RNA maturation, RNA post-transcriptional modifications, RNA intracellular

transport and diffusion and RNA-protein-mediated formation of speckles or condensates that are important for RNA processing or turnover (Figure 5.2) (Liao and Regev, 2021; Wiedner and Giudice, 2021).

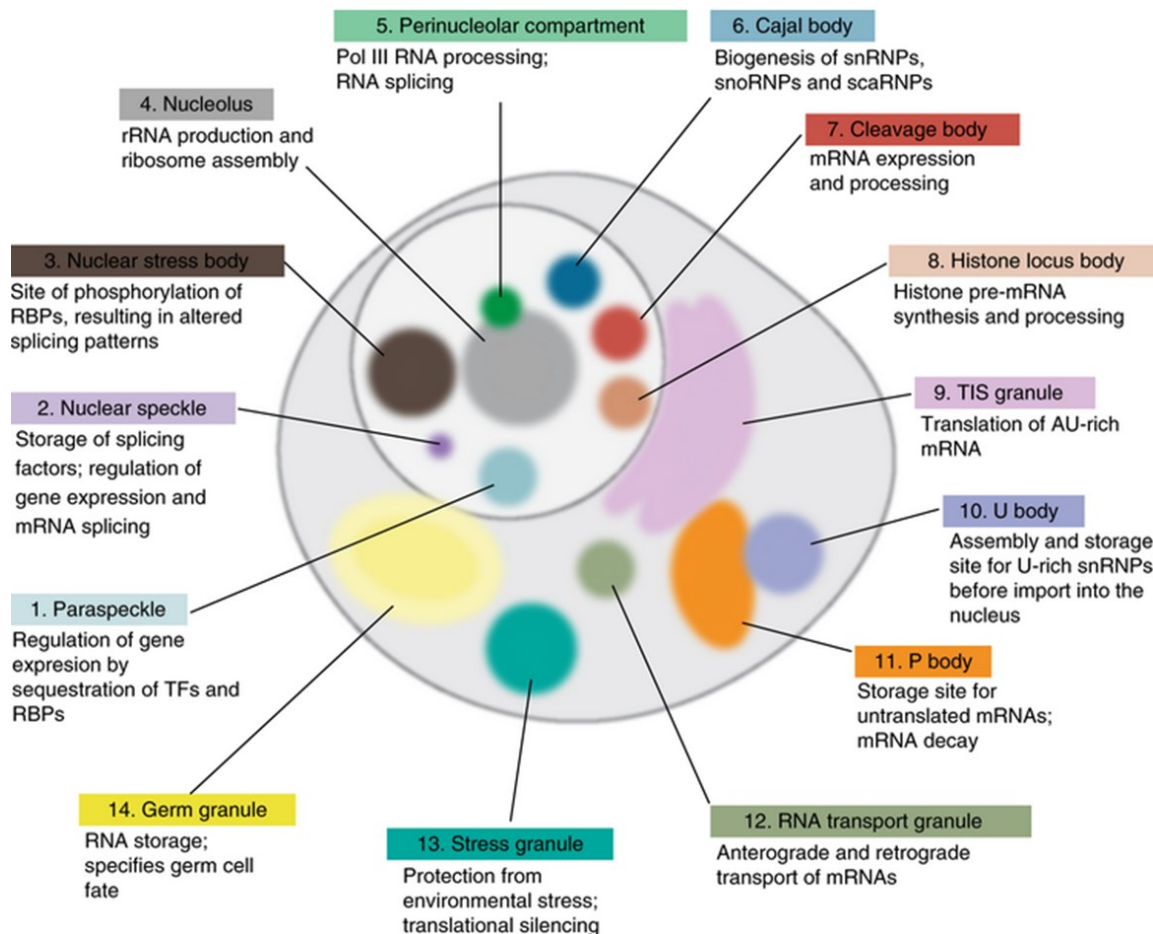


Figure 5.2. Membraneless distinct speckles in cells and their proposed functions.

In addition, the fact that several paralogue genes exist that encode for putative ribonucleases or protein components which participate in important RNPs (like the ribosome, RNase P, RNase MRP etc), suggests alternative roles for the orthologue gene products. Considered, that ncRNAs control more than 75% of the human transcriptional output and accomplish a remarkable variety of biological functions including chromatin remodeling, cell cycle arrest, gene expression regulation at both the transcriptional and translational level, highlights the importance of the above-mentioned interactions ((Schimmel, 2018; Slack and Chinnaiyan, 2019; Kaliatsi et al., 2020;

Pirola et al., 2021). Deregulation of expression, unstructured misfolding or aberrant processing and metabolism of all ncRNAs and their related proteins has been correlated to many diseases with mild to severe clinical phenotypes including cancer (Lekka and Hall, 2018; Li et al., 2020; Bhatti et al., 2021; Pirola et al., 2021). Of note, a flawless and accurate processing of all ncRNA transcripts requires multiple RNA-RNA and RNA-protein interactions that are crucial to produce structured and functional RNAs. These interactions mediated by scaffold proteins like La protein or by ribonucleoprotein complexes, many of which contain both ncRNAs and RNA-binding proteins that act synergistically, like RNase P or RNase MRP (Kessler and Maraia, 2021). The RNA-binding proteins which shape mode action of ncRNAs are found often deregulated in many diseases like cancer, thus the characterization is critical for in-depth understanding of RNA regulatory processes.

5.1. The possible role of La protein as gatekeeper of Pol III ncRNAs

One of the most well-studied RNA-binding proteins is La (Lupus antigen). La is present across eukaryotes and its function in RNA metabolism is highly conserved. Its actual cellular role is to facilitate RNA folding and maturation of 3' end of pre-tRNAs (Maraia, 2001; Wolin and Cedervall, 2002). Interestingly, in mammals, La is essential for viability and previous studies showed that its depletion results in defective pre-tRNA maturation (Bayfield and Maraia, 2009; Blewett et al., 2017). However, La is an important RNA chaperone not only for tRNAs but also for a number of ncRNAs, like miRNAs, 5S rRNA, YRNAs, vtRNAs, 7SK and 7SL RNAs (Figure 5.3) (Wolin and Cedervall, 2002). Since La has also dual localization (nucleus/nucleolus or cytoplasm), current knowledge expands its RNA binding repertoire, beyond pre-tRNAs, displayed its involvement in IRES and poly(A) binding under specific cases (Sommer and Heise, 2021). Although La has an important “gatekeeping” role, the interplay between the different ncRNA pools, is nodal and could lead to aberrant translation rates.

Recently, several studies have highlighted the participation of La in malignancies and its role in protein synthesis (Stavraka and Blagden, 2015; Sommer and Heise, 2021). Based on the observation that La is significantly expressed in numerous cancers, including NSCLC, we examined the effects of human La protein overexpression in A549 lung adenocarcinoma cell line. In this

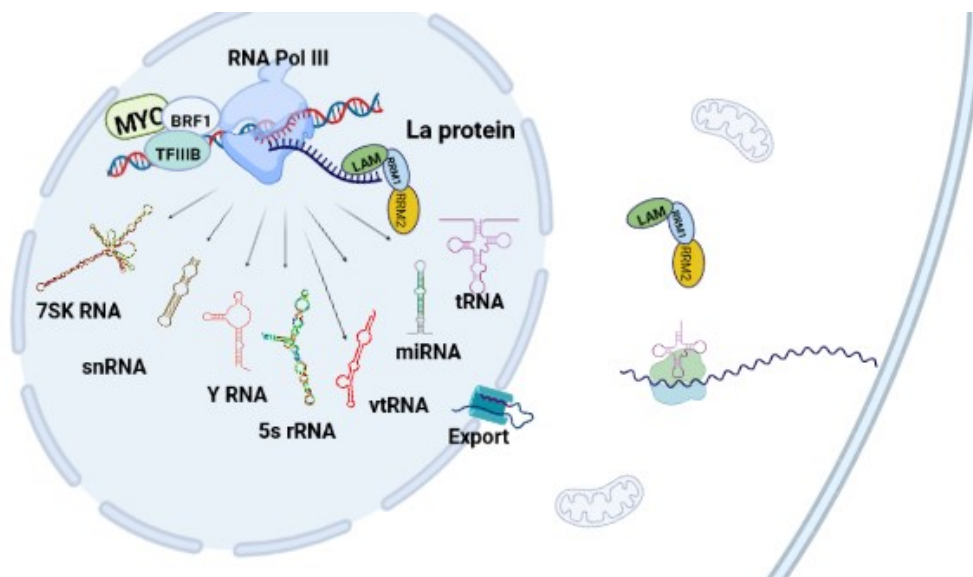


Figure 5.3: The RNA polymerase III transcriptome that interact with La protein.

context, our results suggest that up-regulation of La protein in A549 cells is strongly correlated with cancer progression. As we observed, ectopic expression of La lead to enhance the growth rates which was confirmed by monitoring the cell viability using the resazurin assay and measuring the expression levels of PCNA which is expressed in all phases of the cell cycle and is used as a marker of mitotic activity (Bologna-Molina et al., 2013). Given the upregulation of La, we also tested the cell cycle, but we didn't notice significant alterations between the phases, however, results from RT-qPCR analysis showed upregulation of all cyclins expression levels with important increase of cyclin D1 (CCDN1). CCND1 has been reported to moonlight as a regulator of migration and invasion of non-small cell lung cancer but also it has been correlated with La (Gautschi et al., 2007; Sommer et al., 2011b). More specific, it has been found that AKT kinase which is often overactivated in many cancers (including lung cancer) phosphorylates La at T389. This modification directly affects La binding to an IRES responsible for translation of cyclin D1 (Kuehnert et al., 2015). In addition, we specifically analyzed the expression of MDM2, which were also upregulated. MDM2 promotes cell motility in a p53-independent manner. More importantly, La overexpression stabilizes the expression of p53-negative regulator MDM2, in BCR/ABL-expressing myeloid precursor cell line, an observation which suggests that La-dependent activation of MDM2 translation might represent a mechanism involved in carcinogenesis (Huang et al., 2009; Chen et al., 2017). Interestingly, La also increased motility of cells as shown in wound

healing assays (Figure 4.2), an important finding which are in agreement with previous reports describing the role of La in cancer (Sommer and Heise, 2021). Increased cell motility, which is the hallmark of aggressive malignant cells, requires a general increase the expression of tumor marker like MMP2 and VEGF as we shown in present study, that both largely contribute to growth, invasion and metastasis (Claesson-Welsh and Welsh, 2013; Gong et al., 2014).

Growing evidence support the participation of La in different cellular process, like miRNA biogenesis, turnover by RISC, sorting of tRFs, in the promotion of IRES-dependent mRNA translation but even more interesting is the observation that La can enhance mRNA stability or consolidate interactions with translation factors and ribosomes (Liang et al., 2013; Hasler et al., 2016; Vinayak et al., 2018). All the above cellular process was further confirmed after the analysis of the transcriptomic profile in La overexpressing cells. Moreover, the genes found to be dysregulated were analyzed and were found that La affects proliferation or migration pathways confirming the previous observations (Figure 4.3). However, the translation process was among of the function with the largest and statistically significant alterations, confirming the subsequently observation regarding the translation initiation factors. Based on NGS analysis, it was further analyzed the signaling upstream of translation initiation. La modulates important components of the PI3K/AKT signaling and translation initiation axis. Interestingly, the upregulation of both phosphorylated mTOR and RICTOR could suggest that signals are transduced mainly through the mTORC2 complex (Figure 4.6). This observation is in the same line with migration profile as the mTORC2 complex subunits could regulate cytoskeleton, probably by activating one or more Rho-type guanine nucleotide exchange factors (Jacinto et al., 2004). In addition, the results showed the eIF4E expression was unaffected and the level of 4E-BPs phosphorylation was very low, thus promoting an eIF4E/4E-BPs stable complex formation that prevents eIF4E to initiate cap-dependent translation an observation which confirmed by the decreased of global translation rates (Figure 4.7).

However, an upregulation expression level of all other initiation factors and especially of eIF3C was observed. The eIF-3 complex could specifically targets and initiates translation of a subset of mRNAs involved in cell proliferation through IRES-dependent translation (Lee et al., 2015). The IRESs are recognized by components of the translation machinery, like initiation

translation factors (eIFs) and 40S ribosomal subunits and additional proteins, known as transacting factors (ITAFs), which all are required for efficient IRES-translation initiation (Bera and Lewis, 2020). So far, the literature highlights the role of La protein as an ITAF enhancing the translation not only of viral RNAs but also of cellular mRNAs containing IRES-elements like XIAP. Interestingly, XIAP IRES not only interacts with La, but also requires direct interaction with eIF3 to recruit the ribosome (Holcik and Korneluk, 2000; Petz et al., 2012a). Moreover, overexpression of La alters the protein levels of MYC which it has been reported that, in lymphoma, it can be stimulated by IL-6 through enhancement of IRES-mediated translation and can upregulate VEGF expression, as it was also observed also in the present study (Mezquita et al., 2005). This is good agreement with the notion that La could possibly play a modulatory role in IRES dependent translation of important oncogenes (like MYC), in a way similar to its ability to promote viral IRES-mediated translation stimulation. Thus, our findings indicate that La overexpression possibly results in an increased translation initiation in an IRES-dependent manner in these cells.

Furthermore, in concomitant with the translation, La could affect RNA processing suggesting an indirect role of La in tRNA gene expression, rRNA processing, miRNA biogenesis, but also in upregulation of important ribonucleases which participate in RNA processing. In fact, small RNA-sequencing analysis showed that the La affected specific miRNA molecules which interact with Argonaut proteins (AGO) to form RNA-induced silencing complexes (RISCs) repressing the translation mRNA molecule (Wilczynska and Bushell, 2015; Hasler et al., 2016). The miRNA-mediated regulation in the La-overexpressing cells affects, among others, gene silencing, signaling, transcription and apoptosis suggesting that miRNAs upregulated or downregulated in order to modulate the molecular function inside cells, promoting the cancer progression. In the current study, an upregulation of the reports as oncogenic tRF-1001 and its parental tRNA (tRNA^{Ser}UGA) was observed (Figure 4.5). Since the production of type-1 tRNA-derived fragments depends on the activity of RNase Z (ELAC2), an increased expression level of RNase ZL (ELAC2) was observed. Recently, it was reported that tRF-1 fragments sequester La and compete with viral IRES for La binding, thus inhibiting viral RNA translation (Cho et al., 2019).

It is established that La has the ability to interact with a large proportion of RNA molecules due to its characteristic structural motifs. However, recent studies have shown that LAM and RRM1 can bind mRNAs' poly(A) tails in a non-specific binding mode, a novel and intriguing La feature which seems to be more conserved among LARPs than it was previously thought (Vinayak et al., 2018). This observation raised questions on the ability of La to recognize and bind additional RNA ligands, but also regarding its modulatory role beyond the folding of Pol III transcripts. One of the most interesting and novel findings of this study, is the observation that LAM (1-103 aa) alone has RNA binding properties. So far, the literature highlighted the importance of the entirety of LAM-RRM1 (La module) in RNA recognition which has been proposed to act as a functional unit (Stefano, 1984; Alfano et al., 2004; Dong et al., 2004; Teplova et al., 2006; Kotik-Kogan et al., 2008). However, in the present study, it was identified that hLAM alone has oligo(U) and pre-tRNA binding properties, independently of the RRM1 presence. This result pinpoints the intrinsic property of LAM as an RNA binding module which could also facilitate interactions with RNA domains that exhibit local tRNA mimicry, as in the case of maturation of MALAT1 lncRNA for the production of mascRNA (Figure 5.4). It is known that both

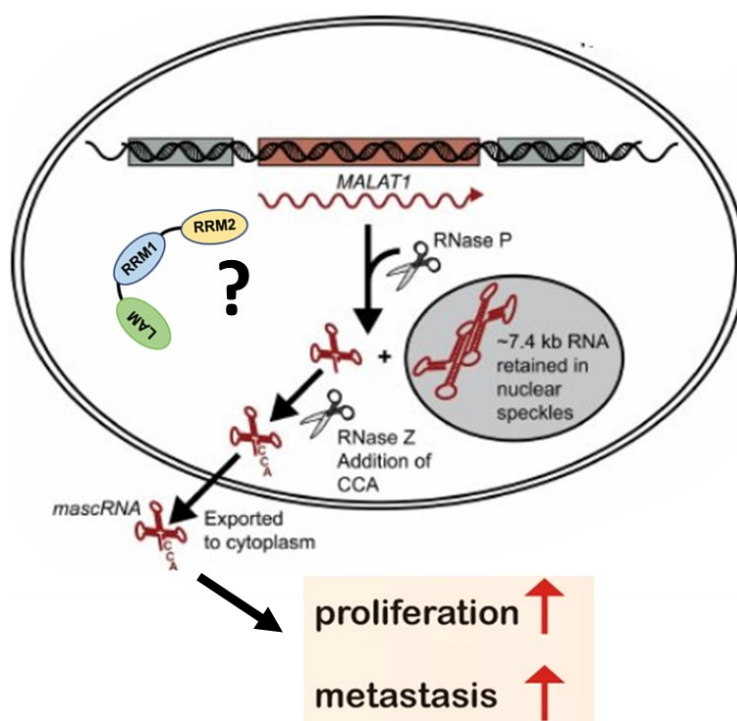


Figure 5.4: Schematic representation of processing of MALAT1 transcripts and a possible involvement La protein in tRNA mimicry.

ncRNAs are involved in carcinogenesis and therefore, could explain that modulatory role of La in malignancies, beyond translation (Li, 2011; Xu et al., 2022).

Collectively, our results highlight the important modulatory role of La in translation and they provide new functional and structural information that show clearly that LAM has RNA binding properties. Given the dynamic nature of La domains, the RNA binding ability of LAM provides a new perspective regarding the ability of La to interact with diverse RNA molecules, an ability that is reflected to La involvement in important cellular events linked to carcinogenesis (Figure 5.5).

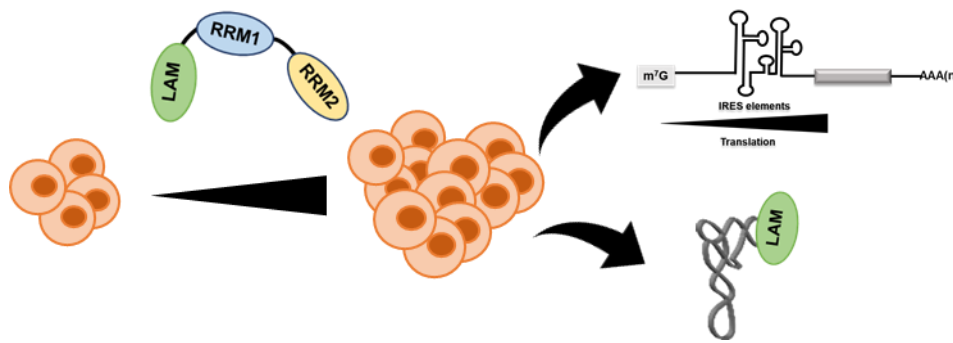


Figure 5.5: Schematic representation of possible role of La protein in cancer

5.2. The dynamic roles of RNase P RNP protein subunits

RNase P represents a ubiquitous but unique ribonucleoprotein complex that resembles ribosome and represents the evolutionary transition from the RNA to the protein world. It endonucleolytically cleaves the pre-tRNAs to produce their mature 5' ends, but also, it recognizes tRNA-lookalike structures, and it has been found to cleave also lncRNAs like MALAT1 and MEN β /NEAT1 (Sunwoo et al., 2008). RNase P occurs in representatives of all domains of life (bacteria, archaea, eukarya), as well as in organelles like mitochondria and chloroplasts (Randau et al., 2005). In almost every organism, RNase P is a ribonucleoprotein complex, with one RNA subunit and a varying number of protein subunits. However, the human mitochondria and the plastids from *A. thaliana*, are two exceptional cases, whose RNase P lacks the RNA subunit (Figure 5.6) (Holzmann et al., 2008; Gutmann et al., 2012). In human, the nuclear RNase P is consisted of the RNA subunit termed as RPPH1 and eleven protein subunits: RPP14, RPP20, RPP21, RPP25,

RPP29, two copies of RPP30, RPP38, RPP40, POP5 and POP1. The RNA subunit is responsible for the main catalytic function of the RNase P holoenzyme while the protein subunits act as scaffold to stabilize the RNA core to an optimal conformation for tRNA binding (Jarrous, 2002). Moreover, eukaryotes possess an enzyme that is related to RNase P, called RNase MRP (mitochondrial RNA processing). RNase MRP shares nine out of ten protein subunits (except for RPP21) with RNase P and get involved in pre-rRNA and mRNA processing (Welting et al., 2006; Esakova and Krasilnikov, 2010). While RNase P shares three subunits with yeast telomerase (yeast homologs of RPP20, RPP25 and POP1), playing a role in stability and the transportation of the telomerase RNA component (TERC) from cytoplasm to nucleus (Garcia and Zakian, 2020; Garcia et al., 2020). This observation strongly suggests a synchronized expression of the common protein subunits for all three ribonucleoproteins but also the participation in other nodal biological processes.

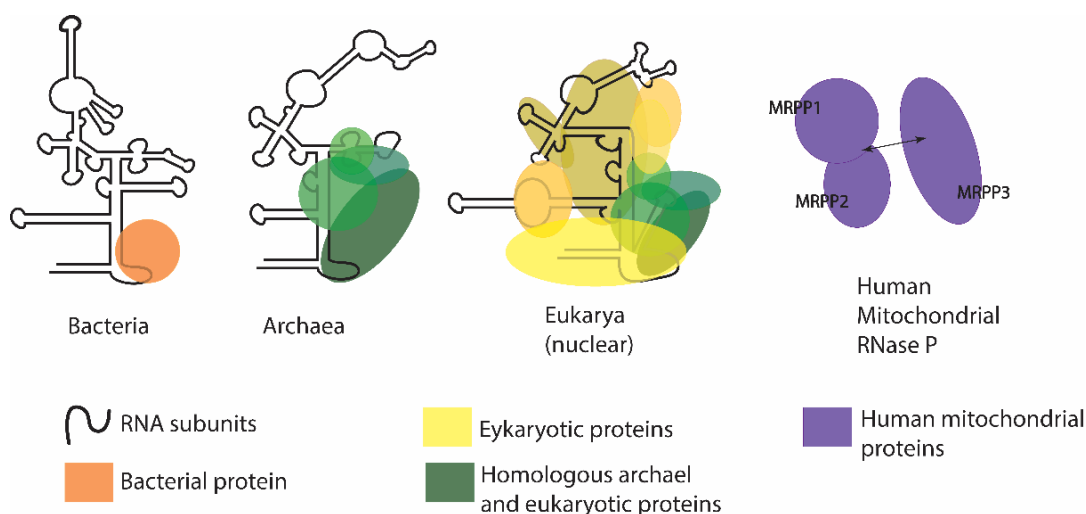


Figure 5.6. The evolution of RNase P holoenzymes.

In fact, several studies have shown that beyond the synthesis of the RNPs, some of the subunits can dissociate from the holoenzyme to carry out alternative tasks in cellular process such as transcription, DNA damage repair (DDR) and chromatin assembly (Reiner, 2006; Serruya et al., 2015; Abu-Zhayia et al., 2017). For example, RNase P regulates the transcription of tRNA, 5S rRNA, 7SL RNA, U6 snRNA and as well as it is also required for efficient rDNA transcription by RNA Polymerase (Pol) I (Jarrous, 2017). The possible proposed mechanism for transcription is that either RNase P acts as auxiliary associated with the transcription factors of RNA polymerase

III (TFIIIA, TFIIIB, TFIIIC), or possibly physical interacts with the polymerase (Jarrous and Reiner, 2007). In case of rDNA transcription, coimmunoprecipitation experiments showed that an active RNase P copurified with the components of RNA Pol I (such as RPA194 and RPA43) and its associated transcription factors (UBF and TBP-associated factor, TAF1110) (Reiner, 2006). In addition, extensive work on RPP21 and RPP29 showed that they participate in DNA damage response (DDR) or in nucleosome remodeling, while RPP30 the most well-studied protein, has been found to affects piRNAs transcription or tRF biogenesis (Jarrous, 2017). Importantly, some protein subunits, like POP1, RPP25, RPP38 and RPP30 have been identified as main component of Th/To autoantigen present in the sera of patients suffer from systemic sclerosis (scleroderma) (Van Eenennaam et al., 2002; Koenig et al., 2019).

In the present study, a combination of cutting-edge and conventional methodologies (genome editing screening, NGS experiments, fluorescence microscopy etc.), was used to study the contribution of each individual subunit in alternatives functions and to assess their importance and their dispensability performing CRISPR/Cas9 genome editing tool in HeLa cell line. The screening showed that POP1 as well as RPP30 protein subunits is essential for cell viability, as after CRISPR/cas9 genome editing tool, cells were not viable. On the other hand, lower editing efficiencies were observed for the other subunits in HeLa cell line possible due to the polyploidy of HeLa cell lines or due to tight conformation of chromatin around of the locus and hence the limited access of Cas9 to each exon. The knockout efficiency could also assess in Hap1 cell line which is a near-haploid cells containing a single copy of each gene and the characterization of knockout of our gene-of-interest will be more easily. It is noteworthy to mention that, in yeast, depletion of each protein subunit led to dispensability of cells with exception of RPP38 (Xiao et al., 2002). From structural analysis point of view, it is evident that both in yeast and human RNase P, the protein subunits interlock and wrap around the RNA subunit and loss of even one could weaken the inter-subunit interactions, suggesting the possibility that all proteins could be essential for the cells.

However, the present study indicated that RPP25 is dispensable for HeLa cells as a knockout clone was successful generated through CRISPR/Cas9 genome editing tool (Figure 4.14 and 4.15) Although, RPP25 is not well characterized in cancer, its expression levels are elevated in most

type of tumors as has been referred from the Cancer Genome Atlas (Figure 5.7). A recent study in glioblastoma showed that the RPP25 expression could be altered depending on the tumour stages by acting either as an oncogene or as tumor suppressor (Huang et al., 2021; Xiao et al., 2022). Our results indicated that ablation of RPP25 lead to enhanced growth rates as monitored by cell viability assays using resazurin and proliferation assays. Also, the expression levels of the proliferation marker PCNA and the anti-apoptotic marker Bcl2, which were both found upregulated, confirm that RPP25 deficient cells exhibit higher proliferation rates (Figure 4.17).

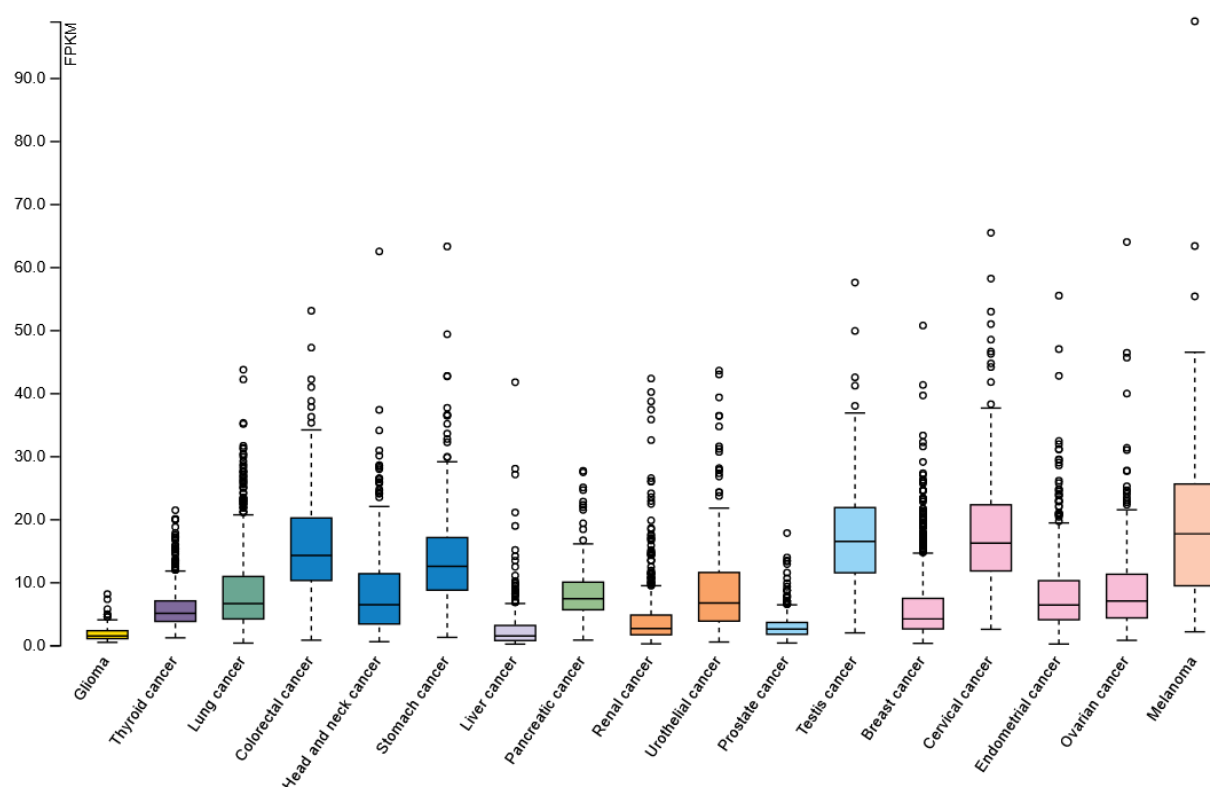


Figure 5.7. Expression levels of RPP25 in different cancer from Cancer Genome Atlas (TCGA).

Notably, the rescue experiments showed that the effect of knockout was restored, an observation which suggests that indeed, RPP25 could play role in cell proliferation independently of its association as a subunit of the RNase P RNP. In addition, RPP25 deficient cells exhibited a prolonged S phase together with a slight decrease in the G2/M phase. Although no significant alterations were observed in expression levels of cyclins, the Cyclin D1 was found slightly upregulated, an observation which indicates possible induction of phosphorylation of the

retinoblastoma protein (Rb) to promote the cell cycle transition from G1 to S phase (Hume et al., 2020). In addition, it was observed that mediators of cell cycle like p53 and p21 were also upregulated. Although both proteins have been correlated with apoptosis, senescence, or autophagy, its expression may be correlated to stress response mechanisms. For example, p21 could regulate both G1/S transition and G2/M phases of cell cycle (P21 is also known to inhibit the kinase activity of cyclin A/CDK1, 2, resulting in cell cycle inhibition through and into S phase (Karimian et al., 2016; Manu et al., 2019) while p53 could be localized either inside the mitochondrial inner membrane-matrix compartment playing a protective role on mitochondrial function, or it could act as safeguard in response to nucleolar stress (Marcel et al., 2014). Furthermore, rescue experiment was shown that RPP25 expression causes arrest in G0/G1 phase of cells cycle, which is good agreement with the finding of recruitment of RPP25 in chromatin late in G1/S phase. Therefore, it is highly likely that using either way, RPP25 is involved in the deregulation of cell cycle and its association with tumorigenesis (Reiner, 2006; Reiner et al., 2008).

It has been reported that knockdown of RPP25 resulted in low transcription of 5.8S rDNA (Reiner, 2006; Jarrous, 2017) as it was also observed in our study. However, in the RPP25 deficient cells, the processing of rRNA could occur through alternative pathways. In the absence of RPP25, the RNase P activity is present, albeit lower and the NGS analysis showed that the levels of total mature tRNAs measured do not significantly fluctuate. However, specific tRNA isoacceptors like tRNA^{Gly}, tRNA^{Arg} are overexpressed and may affect the translation rates of specific genes, as it has been previously suggested (Skeparnias et al., 2020).

Interestingly, in RPP25KO cells also increased the cell motility as shown in wound healing assays with further upregulation of important tumor marker like MMP2 and VEGF, that both largely contribute to growth, invasion and metastasis (Claesson-Welsh and Welsh, 2013; Gong et al., 2014). As shown in previous studies, the EMT process could enhanced after downregulation of RPP25, which are in agreement with our results (Yang et al., 2020). Of note, an upregulation of hypoxia-induced transcription factor 1 α (HIF-1 α) was observed. It is well established that HIF-1 α , under hypoxia or genetic alterations, enters to the nucleus and then induces the transcription of genes involved in angiogenesis, metastasis or metabolism reprogramming like VEGF or GLUT1

(Nagao et al., 2019; Rashid et al., 2021). Therefore, it could be suggested that RPP25 affects indirectly through HIF-1 α , the metabolic reprogramming that occurs in many cases where translation is deregulated.

Under hypoxia, cancer cells are characterized by elevated ROS levels, as it is reported herein. However, a reduction of both mitochondrial membrane potential ($\Delta\psi_m$) and mitochondrial mass was also observed, which may lead to overall mitochondrial dysfunction. It has been reported that the reduction of aerobic oxidative respiration causes decrease in electron-transport rate in the mitochondria leading to reduction of mitochondrial membrane potential ($\Delta\psi_m$) as a consequent in mitochondrial dysfunction (Bell et al., 2007; Aggarwal et al., 2019). However, cancer cells, in order to fulfill energy requirements and maintain high proliferation rates, alter their metabolic pathways from OXPHOS to glycolysis to optimize cellular nutrient utilization (Brillo et al., 2021; Luengo et al., 2021; Tang et al., 2022). The glycolysis pathway was further accompanied by activation of downstream pathways like pentose phosphate pathway for NADPH generation, nucleic acid synthesis and amino acid or fatty acid biosynthesis (Tran et al., 2021). In the present study, Gene Ontology enrichment analysis revealed that knockout of RPP25 affects the expression of gene networks that are involved in tumor metabolism. Important mediators in this process seem to be the aldo-keto reductases (AKRs) that have been found to be increased in the present NGS analysis. All AKR1C isoforms (AKR1C1-AKR1C4) catalyze the NADPH-dependent reduction used for further metabolism of steroids. Finally, these enzymes have been found implicated in prostate or breast cancer progression (Zeng et al., 2017; Penning et al., 2021). Overexpression of AKR1C3 has been found to regulate the tumor suppressor PTEN and activate the AKT signaling pathway in chemotherapy drug resistance in MCF7 cell line (Zhong et al., 2015) while overexpression of both AKR1C1 and AKR1C3 could increase the ratio of 17 β -estradiol:progesterone in estrogen-dependent malignancies (Hofman et al., 2014). Both NGS results and the observed mitochondrial dysfunction may indicate that RPP25 is associated with metabolic reprogramming through glycolysis or pyrimidine metabolism, an implication also referred in glioblastoma (Xiao et al., 2022).

The function of RPP25 in cancer has been also linked with lncRNAs and miRNAs. In cervical cancer LINC00319 lncRNAs could promote the EMT progression through regulating miR-3127-

5p/RPP25 axis (Yang et al., 2020). More specifically, knockdown of RPP25 leads to higher expression levels of miR-3127-5p, which binds to LINC00319 and stimulates the cancer development. In the present study, changes of both large and small non-coding RNA expression were observed like TCL6 which promote metastasis through PTEN/AKT signaling (Wu et al., 2019). In addition, long intergenic non-protein coding RNAs like LINC02747 or LINC01303 that were found to be upregulated may induce the metastatic phenotype through miRNA axis after RPP25 knockout. It is worth-mentioned that lincRNAs could be produced by alternative splicing and based on the role of RPP25 in *Drosophila*, as protector by alternative splicing, one could hypothesize that RPP25 retains this role in mammals, and its ablation induced alternative splicing producing specific lincRNAs that function as sponge of miRNA molecular which has as target oncogenic mRNAs (Figure 5.8). Regarding the GO enrichment analysis of miRNAs and the biological processes which include transcriptional activity, GTPase activity, translation regulation and chromatin organization, it could be hypothesized that miRNA-mediated mechanism participate in cancer progression or metabolism.

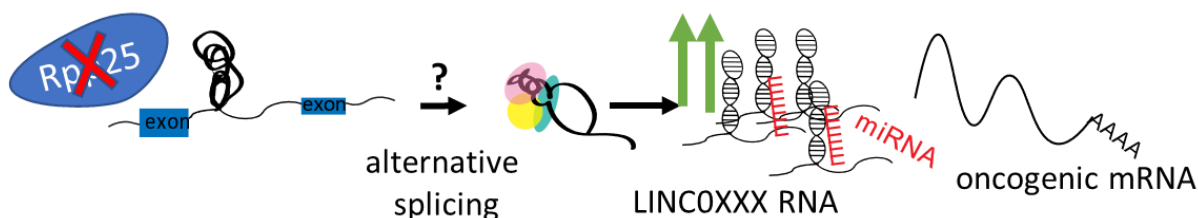


Figure 5.8. Hypothesized model of the additional role of RPP25 in cancer.

Even so, the deregulation of small RNAs was also observed inside the new class of RNA molecules known as tRNA-derived fragments (tRFs). tRFs are an established class of small non-coding RNAs that are produced by precise endonucleolytic cleavage of precursor or mature tRNAs and are categorized in tRF-1s, tRF-5s, tRF-3s, 5'-tRNA halves, 3' tRNA halves and itRFs (internal tRFs), depending on their origin site from the tRNA molecule. tRFs could act as the miRNA molecules in gene expression regulation and thus are characterized as either tumor promoters or tumor suppressors (Li et al., 2021). In our study, it has been observed a significant increase of specific tRF-5: 5030b, 5020b and 5008b which derived from tRNA-Glu-CTC, tRNA- Val-TAC and

tRNA-Gly-TCC, respectively, while the tRF-3 and tRF-1 were decreased. This observation may indicate the role of RPP25 in tRF-5 fragmentation as in rescue experiments the levels of tRF-5 is downregulated. In fact, several tRF-5 fragments like tRF-5023a and tRF-5008b have been found to be upregulated in lung cancer tissues promoting cell proliferation and cell cycle progression (Shao et al., 2017; Skeparnias et al., 2020). In addition to tRFs, modulated expression of specific tRNAs, including those of tRNA-Glu, tRNA-Gly and tRNA-Cys was observed in knockout cell, while in rescue experiments, the levels of nuclear tRNAs were reduced. It is not surprising that deregulation of specific tRNAs could lead to elevated protein synthesis rates of specific transcripts that could contribute to carcinogenesis. Breast cancer was the first example showed that the global upregulation of tRNAs but also upregulation of specific tRNAs like tRNA-Glu or tRNA-Arg lead to enhance the cancer cell invasion and metastasis through a “codon-biased” translation of specific transcripts (Kirchner and Ignatova, 2015; Goodarzi et al., 2016). Moreover, the elevated levels of tRNAs along with the increase of translation rates as well as elevated polysomes confirm our observations. However, the findings of reduced expression levels both RICTOR and RAPTOR which participated in the mTORC2 and the mTORC1 complex and upregulation of the p-ERK downstream of MAPK kinase may indicate that the elevated translation, in RPP25KO cells, is mediated by MAPK pathway though inhibition of mTOR. In fact, MAPK/ERK signaling pathway, is related to cell proliferation and differentiation (Zhang and Liu, 2002). Further analysis showed, lower phosphorylation of eIF2 α (Ser51) and upregulation of p-eIF4E and p-4E-BPs confirming the availability of eIF4E to interact with the 5' cap and the enhanced translation rates. In addition, the elongation factor eEF2 exhibited significant increase that could explain the ribosomal profile analysis as previous shown in ovarian cancer (White-Gilbertson et al., 2009). Moreover, phosphorylation of eEF2 is catalyzed by the eEF2-kinase leading to inactivation of this factor, however, eEF2-kinase could be inhibited by p70S6 kinase, or ERK and p38 MAPK-activated protein kinase, allowing the translation elongation to proceed (Kenney et al., 2014).

From all the above, it is evident that ablation of RPP25 directly or indirectly affects the gene expression of important RNA molecules that in turn, participate in the regulation of cell proliferation and translation. Similar to RPP21, overexpressed RPP25 is localized in Cajal bodies

and may be implicated in RNA processing. Cajal bodies (CB) are distinct sub-nuclear structures that are enriched in RNP complexes, including small nuclear RNPs (snRNPs), small Cajal body specific RNPs (scaRNPs) as well as telomerase RNP complex which involved in splicing, modifications and telomere maintenance, respectively (Meier, 2017). Interestingly, the NGS analysis showed that some specific scaRNAs (Small Cajal body specific RNAs) are downregulated after RPP25 ablation. ScaRNAs have similar structure and function with snoRNAs whereas the main difference is the subcellular localization. ScaRNAs are localized in CB and guide post-transcriptional modifications on snRNAs, while the snoRNAs are mainly localized in the nucleolus and are involved in modification of rRNAs (Darzacq, 2002; Deryusheva and Gall, 2019). Although, scaRNAs have not yet fully characterized, has been found that could be implicated in cancer progression, however, their role remains elusive (Cao et al., 2018; Beneventi et al., 2021). Moreover, the specific binding of RPP25 along with RPP20 in the P3 stem of both RPPH1 and RMRP but also its binding of a P3-Like domain in the telomerase indicates that RPP25 could bind additional RNAs with similar structure (Figure 5.9).

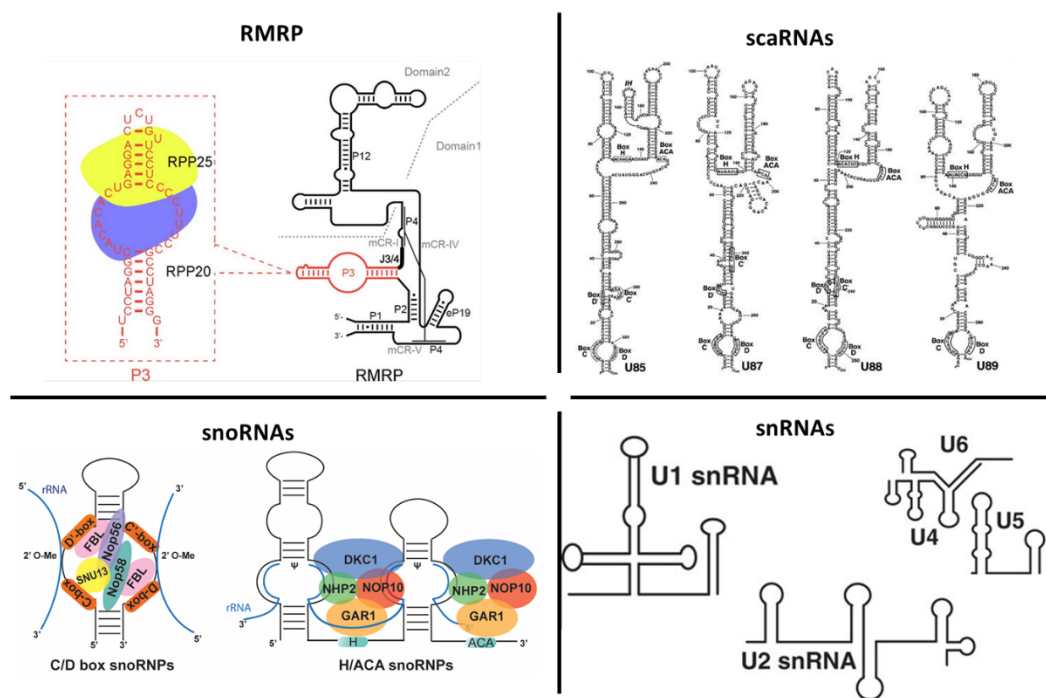


Figure 5.9. Secondary structure of RMRP, snoRNAs, scaRNAs and snRNAs.

Interestingly, snoRNAs, snRNAs but also scaRNAs share similar structure with P3 domain including stems and internal loops that could be recognized by RPP25. This observation could also explain the localization of RPP25 in three compartments (nucleus/nucleolus/CB) (Perederina and Krasilnikov, 2010; Dupuis-Sandoval et al., 2015; Deryusheva and Gall, 2019).

Of note, it is important to mention that in human cell lines also exist a paralogue gene that encodes a protein similar to RPP25, termed as RPP25-like protein. The exact function of this protein is not known. However, a comparison of the structure of RPP25 with the structure expected for the RPP25-like protein has shown that there is a high structural similarity (Figure 5.10). It is speculated that the RPP25-like protein functions as a substitute for RPP25 in the RNase P holoenzyme under specific conditions (Köferle et al., 2022). Fluorescence microscopy analysis showed that RPP25L has similar localization with RPP25 confirming the hypothesis that it could be a potential RNase P/MRP subunits, however, a small amount of RPP25L is also localized in the cytoplasm, indicating an additional role beyond of assembly in the holoenzymes. However, the role of RPP25L should be further studied.

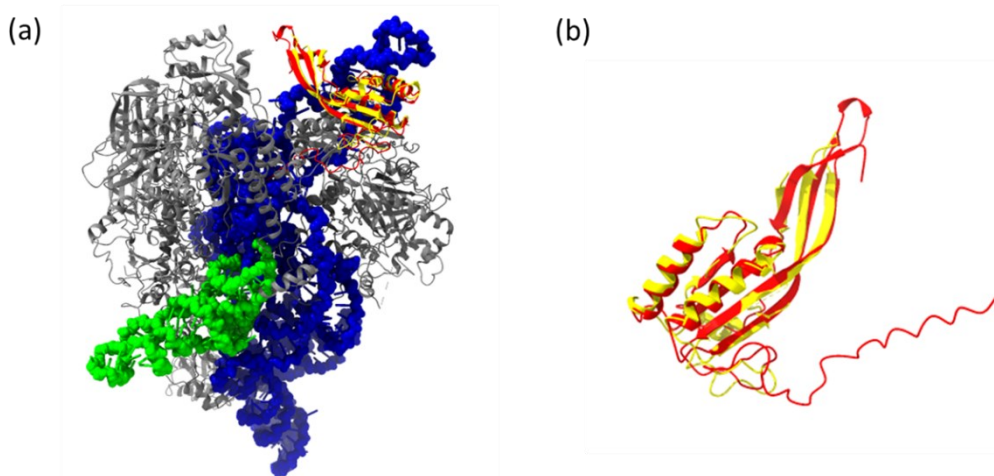


Figure 5.10: Superposition of Rpp25-like structural prediction model obtained from the AlphaFold database (red color; Uniprot ID: Q8N5L8) with (a) RNase P holoenzyme (PDB ID: 6AHU). The RppH1 and the tRNA are colored blue and green respectively, while all protein subunits are colored with grey, except for Rpp25 (yellow) (b) RPP25 Cryo-EM structure (yellow, PDB ID: 6AHU). The comparison was visualized using the Matchmaker tool in ChimeraX v1.2. Secondary structure of eukaryotic RNase P RNA subunit.

Overall, the existence of this paralogue gene may indicate its involvement as putative mediators in biogenesis, folding and action of ncRNAs, through elusive so far RNA-protein interactions that are crucial for cellular process. The elucidation of the role of this paralogue gene is a future goal.

6. References

- Abdulahdi-Atwan, M., Klopstock, T., Sharaf, M., Weinberg-Shukron, A., Renbaum, P., Levy-Lahad, E., et al. (2020). The novel R211Q *POP1* homozygous mutation causes different pathogenesis and skeletal changes from those of previously reported *POP1* -associated anauxetic dysplasia. *Am J Med Genet* 182, 1268–1272. doi: 10.1002/ajmg.a.61538.
- Abu-Zhayia, E. R., Khoury-Haddad, H., Guttmann-Raviv, N., Serruya, R., Jarrous, N., and Ayoub, N. (2017). A role of human RNase P subunits, Rpp29 and Rpp21, in homology directed-repair of double-strand breaks. *Sci Rep* 7, 1002. doi: 10.1038/s41598-017-01185-6.
- Aggarwal, V., Tuli, H., Varol, A., Thakral, F., Yerer, M., Sak, K., et al. (2019). Role of Reactive Oxygen Species in Cancer Progression: Molecular Mechanisms and Recent Advancements. *Biomolecules* 9, 735. doi: 10.3390/biom9110735.
- Aitken, C. E., Beznosková, P., Vlčková, V., Chiu, W.-L., Zhou, F., Valášek, L. S., et al. (2016). Eukaryotic translation initiation factor 3 plays distinct roles at the mRNA entry and exit channels of the ribosomal preinitiation complex. *eLife* 5, e20934. doi: 10.7554/eLife.20934.
- Akhtar, W., and Veenstra, G. J. C. (2011). TBP-related factors: a paradigm of diversity in transcription initiation. *Cell Biosci* 1, 23. doi: 10.1186/2045-3701-1-23.
- Alderson, T. R., Roche, J., Gastall, H. Y., Dias, D. M., Pritišanac, I., Ying, J., et al. (2019). Local unfolding of the HSP27 monomer regulates chaperone activity. *Nat Commun* 10, 1068. doi: 10.1038/s41467-019-08557-8.
- Al-Ejeh, F., Darby, J. M., and Brown, M. P. (2007). The La Autoantigen Is a Malignancy-Associated Cell Death Target That Is Induced by DNA-Damaging Drugs. *Clinical Cancer Research* 13, 5509s–5518s. doi: 10.1158/1078-0432.CCR-07-0922.
- Alfano, C., Sanfelice, D., Babon, J., Kelly, G., Jacks, A., Curry, S., et al. (2004). Structural analysis of cooperative RNA binding by the La motif and central RRM domain of human La protein. *Nat Struct Mol Biol* 11, 323–329. doi: 10.1038/nsmb747.
- Ali, N., Puijn, G. J. M., Kenan, D. J., Keene, J. D., and Siddiqui, A. (2000). Human La Antigen Is Required for the Hepatitis C Virus Internal Ribosome Entry Site-mediated Translation. *Journal of Biological Chemistry* 275, 27531–27540. doi: 10.1074/jbc.M001487200.
- Alspaugh, M. A., and Tan, E. M. (1975). Antibodies to cellular antigens in Sjögren's syndrome. *J. Clin. Invest.* 55, 1067–1073. doi: 10.1172/JCI108007.
- Amin, N., McGrath, A., and Chen, Y.-P. P. (2019). Evaluation of deep learning in non-coding RNA classification. *Nat Mach Intell* 1, 246–256. doi: 10.1038/s42256-019-0051-2.
- Anderson, J. R., Mukherjee, D., Muthukumaraswamy, K., Moraes, K. C. M., Wilusz, C. J., and Wilusz, J. (2006). Sequence-specific RNA binding mediated by the RNase PH domain of components of the exosome. *RNA* 12, 1810–1816. doi: 10.1261/rna.144606.
- Apostolidi, M., Vourtsis, D. J., Chasapis, C. T., Stathopoulos, C., Bentrop, D., and Spyroulias, G. A. (2014). 1H, 15N, 13C assignment and secondary structure determination of two domains of La protein from *D. discoideum*. *Biomol NMR Assign* 8, 47–51. doi: 10.1007/s12104-012-9450-6.

- Argyriou, A. I., Chasapis, C. T., Apostolidi, M., Konstantinidou, P., Stathopoulos, C., Bentrop, D., et al. (2015). Backbone and side chain NMR assignment, along with the secondary structure prediction of RRM2 domain of La protein from a lower eukaryote exhibiting identical structural organization with its human homolog. *Biomol NMR Assign* 9, 219–222. doi: 10.1007/s12104-014-9578-7.
- Arimbasseri, A. G., Rijal, K., and Maraia, R. J. (2013). Transcription termination by the eukaryotic RNA polymerase III. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1829, 318–330. doi: 10.1016/j.bbagr.2012.10.006.
- Ayukawa, K., Taniguchi, S., Masumoto, J., Hashimoto, S., Sarvotham, H., Hara, A., et al. (2000). La Autoantigen Is Cleaved in the COOH Terminus and Loses the Nuclear Localization Signal during Apoptosis. *Journal of Biological Chemistry* 275, 34465–34470. doi: 10.1074/jbc.M003673200.
- Bachmann, M., Chang, S., Slor, H., Kukulies, J., and Müller, W. E. G. (1990). Shuttling of the autoantigen La between nucleus and cell surface after uv irradiation of human keratinocytes. *Experimental Cell Research* 191, 171–180. doi: 10.1016/0014-4827(90)90002-R.
- Bai, S., Herrera-Abreu, M., Rohn, J. L., Racine, V., Tajadura, V., Suryavanshi, N., et al. (2011). Identification and characterization of a set of conserved and new regulators of cytoskeletal organization, cell morphology and migration. *BMC Biol* 9, 54. doi: 10.1186/1741-7007-9-54.
- Barraza-García, J., Rivera-Pedroza, C. I., Hisado-Oliva, A., Belinchón-Martínez, A., Sentchordi-Montané, L., Duncan, E. L., et al. (2017). Broadening the phenotypic spectrum of POP1-skeletal dysplasias: identification of POP1 mutations in a mild and severe skeletal dysplasia: Broadening the phenotypic spectrum of POP1-skeletal dysplasias. *Clin Genet* 92, 91–98. doi: 10.1111/cge.12964.
- Bayfield, M. A., Kaiser, T. E., Intine, R. V., and Maraia, R. J. (2007). Conservation of a Masked Nuclear Export Activity of La Proteins and Its Effects on tRNA Maturation. *Mol Cell Biol* 27, 3303–3312. doi: 10.1128/MCB.00026-07.
- Bayfield, M. A., and Maraia, R. J. (2009). Precursor-product discrimination by La protein during tRNA metabolism. *Nat Struct Mol Biol* 16, 430–437. doi: 10.1038/nsmb.1573.
- Bayfield, M. A., Vinayak, J., Kerkhofs, K., and Mansouri-Noori, F. (2021). La proteins couple use of sequence-specific and non-specific binding modes to engage RNA substrates. *RNA Biology* 18, 168–177. doi: 10.1080/15476286.2019.1582955.
- Bayfield, M. A., Yang, R., and Maraia, R. J. (2010). Conserved and divergent features of the structure and function of La and La-related proteins (LARPs). *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1799, 365–378. doi: 10.1016/j.bbagr.2010.01.011.
- Bell, E. L., Klimova, T. A., Eisenbart, J., Schumacker, P. T., and Chandel, N. S. (2007). Mitochondrial Reactive Oxygen Species Trigger Hypoxia-Inducible Factor-Dependent Extension of the Replicative Life Span during Hypoxia. *Mol Cell Biol* 27, 5737–5745. doi: 10.1128/MCB.02265-06.
- Beneventi, G., Munita, R., Cao Thi Ngoc, P., Madej, M., Cieřla, M., Muthukumar, S., et al. (2021). The small Cajal body-specific RNA 15 (SCARNA15) directs p53 and redox homeostasis via selective splicing in cancer cells. *NAR Cancer* 3, zcab026. doi: 10.1093/narcan/zcab026.

- Bera, A., and Lewis, S. M. (2020). Regulation of Epithelial-to-Mesenchymal Transition by Alternative Translation Initiation Mechanisms and Its Implications for Cancer Metastasis. *IJMS* 21, 4075. doi: 10.3390/ijms21114075.
- Berg, M. D., and Brandl, C. J. (2021). Transfer RNAs: diversity in form and function. *RNA Biology* 18, 316–339. doi: 10.1080/15476286.2020.1809197.
- Betat, H., and Mörl, M. (2015). The CCA-adding enzyme: A central scrutinizer in tRNA quality control. *BioEssays* 37, 975–982. doi: 10.1002/bies.201500043.
- Bhatta, A., Dienemann, C., Cramer, P., Hillen, H.S. (2021). Structural basis of RNA processing by human mitochondrial RNase P. *Nat Struct Mol Biol* In press. doi: 10.1038/s41594-021-00637-y.
- Bhatti, G. K., Khullar, N., Sidhu, I. S., Navik, U. S., Reddy, A. P., Reddy, P. H., et al. (2021). Emerging role of non-coding RNA in health and disease. *Metab Brain Dis* 36, 1119–1134. doi: 10.1007/s11011-021-00739-y.
- Birch, J., Clarke, C. J., Campbell, A. D., Campbell, K., Mitchell, L., Liko, D., et al. (2016). The initiator methionine tRNA drives cell migration and invasion leading to increased metastatic potential in melanoma. *Biology Open* 5, 1371–1379. doi: 10.1242/bio.019075.
- Blankenberg, D., Gordon, A., Von Kuster, G., Coraor, N., Taylor, J., Nekrutenko, A., et al. (2010). Manipulation of FASTQ data with Galaxy. *Bioinformatics (Oxford, England)* 26, 1783–5. doi: 10.1093/bioinformatics/btq281.
- Blewett, N. H., Iben, J. R., Gaidamakov, S., and Maraia, R. J. (2017). La Deletion from Mouse Brain Alters Pre-tRNA Metabolism and Accumulation of Pre-5.8S rRNA, with Neuron Death and Reactive Astrocytosis. *Mol Cell Biol* 37, e00588-16. doi: 10.1128/MCB.00588-16.
- Blewett, N. H., and Maraia, R. J. (2018). La involvement in tRNA and other RNA processing events including differences among yeast and other eukaryotes. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1861, 361–372. doi: 10.1016/j.bbagr.2018.01.013.
- Bologna-Molina, R., Mosqueda-Taylor, A., Molina-Frechero, N., Mori-Estevez, Ad., and Sanchez-Acuna, G. (2013). Comparison of the value of PCNA and Ki-67 as markers of cell proliferation in ameloblastic tumors. *Med Oral*, e174–e179. doi: 10.4317/medoral.18573.
- Bousquet-Antonelli, C., and Deragon, J.-M. (2009). A comprehensive analysis of the La-motif protein superfamily. *RNA* 15, 750–764. doi: 10.1261/rna.1478709.
- Bravo, R., Parra, V., Gatica, D., Rodriguez, A. E., Torrealba, N., Paredes, F., et al. (2013). “Endoplasmic Reticulum and the Unfolded Protein Response,” in *International Review of Cell and Molecular Biology* (Elsevier), 215–290. doi: 10.1016/B978-0-12-407704-1.00005-1.
- Brenet, F., Socci, N. D., Sonenberg, N., and Holland, E. C. (2009). Akt phosphorylation of La regulates specific mRNA translation in glial progenitors. *Oncogene* 28, 128–139. doi: 10.1038/onc.2008.376.
- Brillo, V., Chieragato, L., Leanza, L., Muccioli, S., and Costa, R. (2021). Mitochondrial Dynamics, ROS, and Cell Signaling: A Blended Overview. *Life* 11, 332. doi: 10.3390/life11040332.
- Broad Institute (2009). Picard Toolkit. *GitHub Repository*.
- Brown, K. A., Sharifi, S., Hussain, R., Donaldson, L., Bayfield, M. A., and Wilson, D. J. (2016). Distinct Dynamic Modes Enable the Engagement of Dissimilar Ligands in a Promiscuous Atypical RNA Recognition Motif. *Biochemistry* 55, 7141–7150. doi: 10.1021/acs.biochem.6b00995.

- Buchkovich, N. J., Yu, Y., Pierciey, F. J., and Alwine, J. C. (2010). Human cytomegalovirus induces the endoplasmic reticulum chaperone BiP through increased transcription and activation of translation by using the BiP internal ribosome entry site. *J Virol* 84, 11479–11486. doi: 10.1128/JVI.01330-10.
- Canella, D., Praz, V., Reina, J. H., Cousin, P., and Hernandez, N. (2010). Defining the RNA polymerase III transcriptome: Genome-wide localization of the RNA polymerase III transcription machinery in human cells. *Genome Res.* 20, 710–721. doi: 10.1101/gr.101337.109.
- Cao, T., Rajasingh, S., Samanta, S., Dawn, B., Bittel, D. C., and Rajasingh, J. (2018). Biology and clinical relevance of noncoding sno/scaRNAs. *Trends in Cardiovascular Medicine* 28, 81–90. doi: 10.1016/j.tcm.2017.08.002.
- Chaliotis, A., Vlastaridis, P., Mossialos, D., Ibba, M., Becker, H. D., Stathopoulos, C., et al. (2017). The complex evolutionary history of aminoacyl-tRNA synthetases. *Nucleic Acids Research* 45, 1059–1068. doi: 10.1093/nar/gkw1182.
- Chambers, J. C., Kurilla, M. G., and Keene, J. D. (1983). Association between the 7 S RNA and the lupus La protein varies among cell types. *J Biol Chem* 258, 11438–11441.
- Chan, S.-W. (2016). Hydrogen peroxide induces La cytoplasmic shuttling and increases hepatitis C virus internal ribosome entry site-dependent translation. *Journal of General Virology* 97, 2301–2315. doi: 10.1099/jgv.0.000556.
- Chang, Y. N., Kenan, D. J., Keene, J. D., Gatignol, A., and Jeang, K. T. (1994). Direct interactions between autoantigen La and human immunodeficiency virus leader RNA. *J Virol* 68, 7008–7020. doi: 10.1128/jvi.68.11.7008-7020.1994.
- Chen, B., Dragomir, M. P., Fabris, L., Bayraktar, R., Knutsen, E., Liu, X., et al. (2020). The Long Noncoding RNA CCAT2 Induces Chromosomal Instability Through BOP1-AURKB Signaling. *Gastroenterology* 159, 2146–2162.e33. doi: 10.1053/j.gastro.2020.08.018.
- Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560.
- Chen, Y., Wang, D.-D., Wu, Y.-P., Su, D., Zhou, T.-Y., Gai, R.-H., et al. (2017). MDM2 promotes epithelial–mesenchymal transition and metastasis of ovarian cancer SKOV3 cells. *Br J Cancer* 117, 1192–1201. doi: 10.1038/bjc.2017.265.
- Cho, H., Lee, W., Kim, G.-W., Lee, S.-H., Moon, J.-S., Kim, M., et al. (2019). Regulation of La/SSB-dependent viral gene expression by pre-tRNA 3' trailer-derived tRNA fragments. *Nucleic Acids Research* 47, 9888–9901. doi: 10.1093/nar/gkz732.
- Cho, I.-M., Lai, L. B., Susanti, D., Mukhopadhyay, B., and Gopalan, V. (2010). Ribosomal protein L7Ae is a subunit of archaeal RNase P. *Proc Natl Acad Sci U S A* 107, 14573–14578. doi: 10.1073/pnas.1005556107.
- Claesson-Welsh, L., and Welsh, M. (2013). VEGFA and tumour angiogenesis. *J Intern Med* 273, 114–127. doi: 10.1111/joim.12019.
- Clarke, C. J., Berg, T. J., Birch, J., Ennis, D., Mitchell, L., Cloix, C., et al. (2016). The Initiator Methionine tRNA Drives Secretion of Type II Collagen from Stromal Fibroblasts to Promote Tumor Growth and Angiogenesis. *Current Biology* 26, 755–765. doi: 10.1016/j.cub.2016.01.045.

- Collins, K., and Mitchell, J. R. (2002). Telomerase in the human organism. *Oncogene* 21, 564–579. doi: 10.1038/sj.onc.1205083.
- Copela, L. A., Fernandez, C. F., Sherrer, R. L., and Wolin, S. L. (2008). Competition between the Rex1 exonuclease and the La protein affects both Trf4p-mediated RNA quality control and pre-tRNA maturation. *RNA* 14, 1214–1227. doi: 10.1261/rna.1050408.
- Craig, A. W., Svitkin, Y. V., Lee, H. S., Belsham, G. J., and Sonenberg, N. (1997). The La autoantigen contains a dimerization domain that is essential for enhancing translation. *Mol Cell Biol* 17, 163–169. doi: 10.1128/MCB.17.1.163.
- Daly, N. L., Arvanitis, D. A., Fairley, J. A., Gomez-Roman, N., Morton, J. P., Graham, S. V., et al. (2005). Deregulation of RNA polymerase III transcription in cervical epithelium in response to high-risk human papillomavirus. *Oncogene* 24, 880–888. doi: 10.1038/sj.onc.1208031.
- Darzacq, X. (2002). Cajal body-specific small nuclear RNAs: a novel class of 2'-O-methylation and pseudouridylation guide RNAs. *The EMBO Journal* 21, 2746–2756. doi: 10.1093/emboj/21.11.2746.
- Dehairs, J., Talebi, A., Cherifi, Y., and Swinnen, J. V. (2016). CRISP-ID: decoding CRISPR mediated indels by Sanger sequencing. *Sci Rep* 6, 28973. doi: 10.1038/srep28973.
- Deryusheva, S., and Gall, J. G. (2019). scaRNAs and snoRNAs: Are they limited to specific classes of substrate RNAs? *RNA* 25, 17–22. doi: 10.1261/rna.068593.118.
- Deutschmann, A. J., Amberger, A., Zavadil, C., Steinbeisser, H., Mayr, J. A., Feichtinger, R. G., et al. (2014). Mutation or knock-down of 17 β -hydroxysteroid dehydrogenase type 10 cause loss of MRPP1 and impaired processing of mitochondrial heavy strand transcripts. *Human Molecular Genetics* 23, 3618–3628. doi: 10.1093/hmg/ddu072.
- Dieci, G., Fiorino, G., Castelnuovo, M., Teichmann, M., and Pagano, A. (2007). The expanding RNA polymerase III transcriptome. *Trends in Genetics* 23, 614–622. doi: 10.1016/j.tig.2007.09.001.
- Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. doi: 10.1093/bioinformatics/bts635.
- Doench, J. G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E. W., Donovan, K. F., et al. (2016). Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol* 34, 184–191. doi: 10.1038/nbt.3437.
- Doench, J. G., Hartenian, E., Graham, D. B., Tothova, Z., Hegde, M., Smith, I., et al. (2014). Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat Biotechnol* 32, 1262–1267. doi: 10.1038/nbt.3026.
- Dong, G., Chakshusmathi, G., Wolin, S. L., and Reinisch, K. M. (2004). Structure of the La motif: a winged helix domain mediates RNA binding via a conserved aromatic patch. *EMBO J* 23, 1000–1007. doi: 10.1038/sj.emboj.7600115.
- Dupuis-Sandoval, F., Poirier, M., and Scott, M. S. (2015). The emerging landscape of small nucleolar RNAs in cell biology: Emerging landscape of small nucleolar RNAs. *WIREs RNA* 6, 381–397. doi: 10.1002/wrna.1284.
- Egiz, M., Usui, T., Ishibashi, M., Zhang, X., Shigeta, S., Toyoshima, M., et al. (2019). La-Related Protein 4 as a Suppressor for Motility of Ovarian Cancer Cells. *Tohoku J. Exp. Med.* 247, 59–67. doi: 10.1620/tjem.247.59.

- Egloff, S., Studniarek, C., and Kiss, T. (2018). 7SK small nuclear RNA, a multifunctional transcriptional regulatory RNA with gene-specific features. *Transcription* 9, 95–101. doi: 10.1080/21541264.2017.1344346.
- Elalaoui, S. C., Laarabi, F. Z., Mansouri, M., Mrani, N. A., Nishimura, G., and Sefiani, A. (2016). Further evidence of *POP1* mutations as the cause of anauxetic dysplasia. *Am. J. Med. Genet.* 170, 2462–2465. doi: 10.1002/ajmg.a.37839.
- Enam, S. U., Zinshteyn, B., Goldman, D. H., Cassani, M., Livingston, N. M., Seydoux, G., et al. (2020). Puromycin reactivity does not accurately localize translation at the subcellular level. *eLife* 9, e60303. doi: 10.7554/eLife.60303.
- Esakova, O., and Krasilnikov, A. S. (2010). Of proteins and RNA: The RNase P/MRP family. *RNA* 16, 1725–1747. doi: 10.1261/rna.2214510.
- Esakova, O., Perederina, A., Quan, C., Schmitt, M. E., and Krasilnikov, A. S. (2008). Footprinting analysis demonstrates extensive similarity between eukaryotic RNase P and RNase MRP holoenzymes. *RNA* 14, 1558–1567. doi: 10.1261/rna.1106408.
- Ezkurdia, I., Juan, D., Rodriguez, J. M., Frankish, A., Diekhans, M., Harrow, J., et al. (2014). Multiple evidence strands suggest that there may be as few as 19 000 human protein-coding genes. *Human Molecular Genetics* 23, 5866–5878. doi: 10.1093/hmg/ddu309.
- Fan, H., Sakulich, A. L., Goodier, J. L., Zhang, X., Qin, J., and Maraia, R. J. (1997). Phosphorylation of the Human La Antigen on Serine 366 Can Regulate Recycling of RNA Polymerase III Transcription Complexes. *Cell* 88, 707–715. doi: 10.1016/S0092-8674(00)81913-3.
- Francoeur, A. M., and Mathews, M. B. (1982). Interaction between VA RNA and the lupus antigen La: formation of a ribonucleoprotein particle in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 79, 6772–6776. doi: 10.1073/pnas.79.22.6772.
- Gallo, S., Kong, E., Ferro, I., and Polacek, N. (2022). Small but Powerful: The Human Vault RNAs as Multifaceted Modulators of Pro-Survival Characteristics and Tumorigenesis. *Cancers* 14, 2787. doi: 10.3390/cancers14112787.
- Gao, W., Li, Q., Zhu, R., and Jin, J. (2016). La Autoantigen Induces Ribosome Binding Protein 1 (RRBP1) Expression through Internal Ribosome Entry Site (IRES)-Mediated Translation during Cellular Stress Condition. *IJMS* 17, 1174. doi: 10.3390/ijms17071174.
- Garcia, P. D., Leach, R. W., Wadsworth, G. M., Choudhary, K., Li, H., Aviran, S., et al. (2020). Stability and nuclear localization of yeast telomerase depend on protein components of RNase P/MRP. *Nat Commun* 11, 2173. doi: 10.1038/s41467-020-15875-9.
- Garcia, P. D., and Zakian, V. A. (2020). A new role for proteins subunits of RNase P: stabilization of the telomerase holoenzyme. *Microb Cell* 7, 250–254. doi: 10.15698/mic2020.09.730.
- Gautschi, O., Ratschiller, D., Gugger, M., Betticher, D. C., and Heighway, J. (2007). Cyclin D1 in non-small cell lung cancer: A key driver of malignant transformation. *Lung Cancer* 55, 1–14. doi: 10.1016/j.lungcan.2006.09.024.
- Genzor, P., Konstantinidou, P., Stoyko, D., Manzourolajdad, A., Marlin Andrews, C., Elchert, A. R., et al. (2021). Cellular abundance shapes function in piRNA-guided genome defense. *Genome Res.* 31, 2058–2068. doi: 10.1101/gr.275478.121.
- Giege, R., Sissler, M., and Florentz, C. (1998). Universal rules and idiosyncratic features in tRNA identity. *Nucleic Acids Research* 26, 5017–5035. doi: 10.1093/nar/26.22.5017.

- Gill, T., Cai, T., Aulds, J., Wierzbicki, S., and Schmitt, M. E. (2004). RNase MRP Cleaves the *CLB2* mRNA To Promote Cell Cycle Progression: Novel Method of mRNA Degradation. *Mol Cell Biol* 24, 945–953. doi: 10.1128/MCB.24.3.945-953.2004.
- Girbig, M., Misiaszek, A. D., and Müller, C. W. (2022). Structural insights into nuclear transcription by eukaryotic DNA-dependent RNA polymerases. *Nat Rev Mol Cell Biol* 23, 603–622. doi: 10.1038/s41580-022-00476-9.
- Gobert, A., Pinker, F., Fuchsbauer, O., Gutmann, B., Boutin, R., Roblin, P., et al. (2013). Structural insights into protein-only RNase P complexed with tRNA. *Nat Commun* 4, 1353. doi: 10.1038/ncomms2358.
- Gong, J., Zhu, S., Zhang, Y., and Wang, J. (2014). Interplay of VEGFa and MMP2 regulates invasion of glioblastoma. *Tumor Biol.* 35, 11879–11885. doi: 10.1007/s13277-014-2438-3.
- Goodarzi, H., Nguyen, H. C. B., Zhang, S., Dill, B. D., Molina, H., and Tavazoie, S. F. (2016). Modulated Expression of Specific tRNAs Drives Gene Expression and Cancer Progression. *Cell* 165, 1416–1427. doi: 10.1016/j.cell.2016.05.046.
- Govaere, O., Petz, M., Wouters, J., Vandewynckel, Y.-P., Scott, E. J., Topal, B., et al. (2017). The PDGFR α -laminin B1-keratin 19 cascade drives tumor progression at the invasive front of human hepatocellular carcinoma. *Oncogene* 36, 6605–6616. doi: 10.1038/onc.2017.260.
- Goyal, M., Banerjee, C., Nag, S., and Bandyopadhyay, U. (2016). The Alba protein family: Structure and function. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 1864, 570–583. doi: 10.1016/j.bbapap.2016.02.015.
- Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., and Altman, S. (1983). The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell* 35, 849–857. doi: 10.1016/0092-8674(83)90117-4.
- Guerrier-Takada, C., McClain, W. H., and Altman, S. (1984). Cleavage of tRNA precursors by the RNA subunit of E. coli ribonuclease P (M1 RNA) is influenced by 3'-proximal CCA in the substrates. *Cell* 38, 219–224. doi: 10.1016/0092-8674(84)90543-9.
- Guglas, K., Kołodziejczak, I., Kolenda, T., Kopczyńska, M., Teresiak, A., Sobocińska, J., et al. (2020). YRNAs and YRNA-Derived Fragments as New Players in Cancer Research and Their Potential Role in Diagnostics. *IJMS* 21, 5682. doi: 10.3390/ijms21165682.
- Gutmann, B., Gobert, A., and Giegé, P. (2012). PRORP proteins support RNase P activity in both organelles and the nucleus in *Arabidopsis*. *Genes Dev.* 26, 1022–1027. doi: 10.1101/gad.189514.112.
- Hall, T. A., and Brown, J. W. (2002). Archaeal RNase P has multiple protein subunits homologous to eukaryotic nuclear RNase P proteins. *RNA* 8, 296–306. doi: 10.1017/S1355838202028492.
- Hashimoto, C., and Steitz, J. A. (1983). Sequential association of nucleolar 7-2 RNA with two different autoantigens. *J Biol Chem* 258, 1379–1382.
- Hasler, D., Lehmann, G., Murakawa, Y., Klironomos, F., Jakob, L., Grässer, F. A., et al. (2016). The Lupus Autoantigen La Prevents Mis-channeling of tRNA Fragments into the Human MicroRNA Pathway. *Molecular Cell* 63, 110–124. doi: 10.1016/j.molcel.2016.05.026.
- Hasler, D., and Meister, G. (2016). From tRNA to miRNA: RNA-folding contributes to correct entry into noncoding RNA pathways. *FEBS Lett* 590, 2354–2363. doi: 10.1002/1873-3468.12294.

- Hasler, D., Meister, G., and Fischer, U. (2021). Stabilize and connect: the role of LARP7 in nuclear non-coding RNA metabolism. *RNA Biology* 18, 290–303. doi: 10.1080/15476286.2020.1767952.
- Hayne, C. K., Schmidt, C. A., Haque, M. I., Matera, A. G., and Stanley, R. E. (2020). Reconstitution of the human tRNA splicing endonuclease complex: insight into the regulation of pre-tRNA cleavage. *Nucleic Acids Research* 48, 7609–7622. doi: 10.1093/nar/gkaa438.
- Heise, T., Kota, V., Brock, A., Morris, A. B., Rodriguez, R. M., Zierk, A. W., et al. (2016). The La protein counteracts cisplatin-induced cell death by stimulating protein synthesis of anti-apoptotic factor Bcl2. *Oncotarget* 7. doi: 10.18632/oncotarget.8819.
- Hermanns, P., Tran, A., Munivez, E., Carter, S., Zabel, B., Lee, B., et al. (2006). *RMRP* mutations in cartilage-hair hypoplasia. *Am. J. Med. Genet.* 140A, 2121–2130. doi: 10.1002/ajmg.a.31331.
- Hofman, J., Malcekova, B., Skarka, A., Novotna, E., and Wsol, V. (2014). Anthracycline resistance mediated by reductive metabolism in cancer cells: The role of aldo-keto reductase 1C3. *Toxicology and Applied Pharmacology* 278, 238–248. doi: 10.1016/j.taap.2014.04.027.
- Holcik, M. (2015). Could the eIF2 α -Independent Translation Be the Achilles Heel of Cancer? *Front. Oncol.* 5. doi: 10.3389/fonc.2015.00264.
- Holcik, M., and Korneluk, R. G. (2000). Functional Characterization of the X-Linked Inhibitor of Apoptosis (XIAP) Internal Ribosome Entry Site Element: Role of La Autoantigen in XIAP Translation. *Mol Cell Biol* 20, 4648–4657. doi: 10.1128/MCB.20.13.4648-4657.2000.
- Holzmann, J., Frank, P., Löffler, E., Bennett, K. L., Gerner, C., and Rossmanith, W. (2008). RNase P without RNA: Identification and Functional Reconstitution of the Human Mitochondrial tRNA Processing Enzyme. *Cell* 135, 462–474. doi: 10.1016/j.cell.2008.09.013.
- Hopper, A. K. (2003). tRNA transfers to the limelight. *Genes & Development* 17, 162–180. doi: 10.1101/gad.1049103.
- Hopper, A. K., and Nostramo, R. T. (2019). tRNA Processing and Subcellular Trafficking Proteins Multitask in Pathways for Other RNAs. *Front. Genet.* 10, 96. doi: 10.3389/fgene.2019.00096.
- Horke, S., Reumann, K., Schweizer, M., Will, H., and Heise, T. (2004). Nuclear Trafficking of La Protein Depends on a Newly Identified Nucleolar Localization Signal and the Ability to Bind RNA. *Journal of Biological Chemistry* 279, 26563–26570. doi: 10.1074/jbc.M401017200.
- Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., et al. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* 31, 827–832. doi: 10.1038/nbt.2647.
- Hua, Y., and Zhou, J. (2004). Rpp20 interacts with SMN and is re-distributed into SMN granules in response to stress. *Biochemical and Biophysical Research Communications* 314, 268–276. doi: 10.1016/j.bbrc.2003.12.084.
- Huang, H., Hu, Y., Li, N., and Zhu, Y. (2009). Inhibition of Tumor Growth and Metastasis by Non-Small Cell Lung Cancer Cells Transfected With Cyclin D1-Targeted siRNA. *Oligonucleotides* 19, 151–161. doi: 10.1089/oli.2008.0174.
- Huang, Z., Li, F., and Li, Q. (2021). Expression profile of RNA binding protein in cervical cancer using bioinformatics approach. *Cancer Cell Int* 21, 647. doi: 10.1186/s12935-021-02319-7.
- Hume, S., Dianov, G. L., and Ramadan, K. (2020). A unified model for the G1/S cell cycle transition. *Nucleic Acids Research* 48, 12483–12501. doi: 10.1093/nar/gkaa1002.

- Intine, R. V., Dundr, M., Misteli, T., and Maraia, R. J. (2002). Aberrant Nuclear Trafficking of La Protein Leads to Disordered Processing of Associated Precursor tRNAs. *Molecular Cell* 9, 1113–1123. doi: 10.1016/S1097-2765(02)00533-6.
- Intine, R. V., Dundr, M., Vassilev, A., Schwartz, E., Zhao, Y., Zhao, Y., et al. (2004). Nonphosphorylated Human La Antigen Interacts with Nucleolin at Nucleolar Sites Involved in rRNA Biogenesis. *Mol Cell Biol* 24, 10894–10904. doi: 10.1128/MCB.24.24.10894-10904.2004.
- Intine, R. V., Tenenbaum, S. A., Sakulich, A. L., Keene, J. D., and Maraia, R. J. (2003). Differential Phosphorylation and Subcellular Localization of La RNPs Associated with Precursor tRNAs and Translation-Related mRNAs. *Molecular Cell* 12, 1301–1307. doi: 10.1016/S1097-2765(03)00429-5.
- Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M. A., Hall, A., et al. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol* 6, 1122–1128. doi: 10.1038/ncb1183.
- Jacks, A., Babon, J., Kelly, G., Manolaridis, I., Cary, P. D., Curry, S., et al. (2003). Structure of the C-Terminal Domain of Human La Protein Reveals a Novel RNA Recognition Motif Coupled to a Helical Nuclear Retention Element. *Structure* 11, 833–843. doi: 10.1016/S0969-2126(03)00121-7.
- Jagut, M., Mihaila-Bodart, L., Molla-Herman, A., Alin, M.-F., Lepesant, J.-A., and Huynh, J.-R. (2013). A Mosaic Genetic Screen for Genes Involved in the Early Steps of Drosophila Oogenesis. *G3 Genes/Genomes/Genetics* 3, 409–425. doi: 10.1534/g3.112.004747.
- Jarrous, N. (2002). Human ribonuclease P: Subunits, function, and intranuclear localization. *RNA* 8, 1–7. doi: 10.1017/S1355838202011184.
- Jarrous, N. (2017). Roles of RNase P and Its Subunits. *Trends in Genetics* 33, 594–603. doi: 10.1016/j.tig.2017.06.006.
- Jarrous, N., Eder, P. S., Wesolowski, D., and Altman, S. (1999a). Rpp14 and Rpp29, two protein subunits of human ribonuclease P. *RNA* 5, 153–157. doi: 10.1017/S135583829800185X.
- Jarrous, N., and Gopalan, V. (2010). Archaeal/Eukaryal RNase P: subunits, functions and RNA diversification. *Nucleic Acids Research* 38, 7885–7894. doi: 10.1093/nar/gkq701.
- Jarrous, N., and Reiner, R. (2007). Human RNase P: a tRNA-processing enzyme and transcription factor. *Nucleic Acids Research* 35, 3519–3524. doi: 10.1093/nar/gkm071.
- Jarrous, N., Reiner, R., Wesolowski, D., Mann, H., Guerrier-Takada, C., and Altman, S. (2001). Function and subnuclear distribution of Rpp21, a protein subunit of the human ribonucleoprotein ribonuclease P. *RNA* 7, 1153–1164. doi: 10.1017/S1355838201010469.
- Jarrous, N., Wolenski, J. S., Wesolowski, D., Lee, C., and Altman, S. (1999b). Localization in the Nucleolus and Coiled Bodies of Protein Subunits of the Ribonucleoprotein Ribonuclease P. *Journal of Cell Biology* 146, 559–572. doi: 10.1083/jcb.146.3.559.
- Jiang, T., and Altman, S. (2001). Protein-protein interactions with subunits of human nuclear RNase P. *Proceedings of the National Academy of Sciences* 98, 920–925. doi: 10.1073/pnas.98.3.920.
- Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., et al. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature* 596, 583–589. doi: 10.1038/s41586-021-03819-2.

- Kaliatsi, E. G., Giarimoglou, N., Stathopoulos, C., and Stamatopoulou, V. (2020). Non-Coding RNA-Driven Regulation of rRNA Biogenesis. *IJMS* 21, 9738. doi: 10.3390/ijms21249738.
- Karasik, A., Wilhelm, C. A., Fierke, C. A., and Koutmos, M. (2021). Disease-associated mutations in mitochondrial precursor tRNAs affect binding, m1R9 methylation, and tRNA processing by mtRNase P. *RNA* 27, 420–432. doi: 10.1261/rna.077198.120.
- Karimian, A., Ahmadi, Y., and Yousefi, B. (2016). Multiple functions of p21 in cell cycle, apoptosis and transcriptional regulation after DNA damage. *DNA Repair* 42, 63–71. doi: 10.1016/j.dnarep.2016.04.008.
- Kassavetis, G. A., and Geiduschek, E. P. (2006). Transcription factor TFIIIB and transcription by RNA polymerase III. *Biochemical Society Transactions* 34, 1082–1087. doi: 10.1042/BST0341082.
- Katsiogiannis, S., Tenta, R., and Skopouli, F. N. (2015). Endoplasmic reticulum stress causes autophagy and apoptosis leading to cellular redistribution of the autoantigens Ro/Sjögren's syndrome-related antigen A (SSA) and La/SSB in salivary gland epithelial cells. *Clinical and Experimental Immunology* 181, 244–252. doi: 10.1111/cei.12638.
- Kenneth, N. S., Ramsbottom, B. A., Gomez-Roman, N., Marshall, L., Cole, P. A., and White, R. J. (2007). TRRAP and GCN5 are used by c-Myc to activate RNA polymerase III transcription. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14917–14922. doi: 10.1073/pnas.0702909104.
- Kenney, J. W., Moore, C. E., Wang, X., and Proud, C. G. (2014). Eukaryotic elongation factor 2 kinase, an unusual enzyme with multiple roles. *Advances in Biological Regulation* 55, 15–27. doi: 10.1016/j.jbior.2014.04.003.
- Kessler, A. C., and Maraia, R. J. (2021). The nuclear and cytoplasmic activities of RNA polymerase III, and an evolving transcriptome for surveillance. *Nucleic Acids Research* 49, 12017–12034. doi: 10.1093/nar/gkab1145.
- Khattar, E., Kumar, P., Liu, C. Y., Akıncılar, S. C., Raju, A., Lakshmanan, M., et al. (2016). Telomerase reverse transcriptase promotes cancer cell proliferation by augmenting tRNA expression. *Journal of Clinical Investigation* 126, 4045–4060. doi: 10.1172/JCI86042.
- Kim, Y. K. (2001). La autoantigen enhances translation of BiP mRNA. *Nucleic Acids Research* 29, 5009–5016. doi: 10.1093/nar/29.24.5009.
- Kirchner, S., and Ignatova, Z. (2015). Emerging roles of tRNA in adaptive translation, signalling dynamics and disease. *Nat Rev Genet* 16, 98–112. doi: 10.1038/nrg3861.
- Koenig, M., Bentow, C., Satoh, M., Fritzler, M. J., Senécal, J.-L., and Mahler, M. (2019). Autoantibodies to a novel Rpp38 (Th/To) derived B-cell epitope are specific for systemic sclerosis and associate with a distinct clinical phenotype. *Rheumatology*, kez123. doi: 10.1093/rheumatology/kez123.
- Köferle, A., Schlattl, A., Hörmann, A., Thatikonda, V., Popa, A., Spreitzer, F., et al. (2022). Interrogation of cancer gene dependencies reveals paralog interactions of autosome and sex chromosome-encoded genes. *Cell Reports* 39, 110636. doi: 10.1016/j.celrep.2022.110636.
- Kotik-Kogan, O., Valentine, E. R., Sanfelice, D., Conte, M. R., and Curry, S. (2008). Structural Analysis Reveals Conformational Plasticity in the Recognition of RNA 3' Ends by the Human La Protein. *Structure* 16, 852–862. doi: 10.1016/j.str.2008.02.021.

- Kouvela, A., Zaravinos, A., and Stamatopoulou, V. (2021). Adaptor Molecules Epitranscriptome Reprograms Bacterial Pathogenicity. *IJMS* 22, 8409. doi: 10.3390/ijms22168409.
- Kovrigina, E., Wesolowski, D., and Altman, S. (2003). Coordinate inhibition of expression of several genes for protein subunits of human nuclear RNase P. *Proceedings of the National Academy of Sciences* 100, 1598–1602. doi: 10.1073/pnas.0337661100.
- Krasilnikov, A. S., Yang, X., Pan, T., and Mondragón, A. (2003). Crystal structure of the specificity domain of ribonuclease P. *Nature* 421, 760–764. doi: 10.1038/nature01386.
- Kuehnert, J., Sommer, G., Zierk, A. W., Fedarovich, A., Brock, A., Fedarovich, D., et al. (2015). Novel RNA chaperone domain of RNA-binding protein La is regulated by AKT phosphorylation. *Nucleic Acids Research* 43, 581–594. doi: 10.1093/nar/gku1309.
- Kufel, J., Allmang, C., Chanfreau, G., Petfalski, E., Lafontaine, D. L. J., and Tollervey, D. (2000). Precursors to the U3 Small Nucleolar RNA Lack Small Nucleolar RNP Proteins but Are Stabilized by La Binding. *Mol Cell Biol* 20, 5415–5424. doi: 10.1128/MCB.20.15.5415-5424.2000.
- Kurilla, M. G., Cabradilla, C. D., Holloway, B. P., and Keene, J. D. (1984). Nucleotide sequence and host La protein interactions of rabies virus leader RNA. *J Virol* 50, 773–778. doi: 10.1128/jvi.50.3.773-778.1984.
- Kurilla, M. G., and Keene, J. D. (1983). The leader RNA of vesicular stomatitis virus is bound by a cellular protein reactive with anti-La lupus antibodies. *Cell* 34, 837–845. doi: 10.1016/0092-8674(83)90541-X.
- Langmead, B., and Salzberg, S. L. (2012a). Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9, 357–359. doi: 10.1038/nmeth.1923.
- Langmead, B., and Salzberg, S. L. (2012b). Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9, 357–359. doi: 10.1038/nmeth.1923.
- Lee, A. S. Y., Kranzusch, P. J., and Cate, J. H. D. (2015). eIF3 targets cell-proliferation messenger RNAs for translational activation or repression. *Nature* 522, 111–114. doi: 10.1038/nature14267.
- Lekka, E., and Hall, J. (2018). Noncoding RNA s in disease. *FEBS Lett* 592, 2884–2900. doi: 10.1002/1873-3468.13182.
- Lemieux, B., Laterreur, N., Perederina, A., Noël, J.-F., Dubois, M.-L., Krasilnikov, A. S., et al. (2016). Active Yeast Telomerase Shares Subunits with Ribonucleoproteins RNase P and RNase MRP. *Cell* 165, 1171–1181. doi: 10.1016/j.cell.2016.04.018.
- Lerner, M. R., Andrews, N. C., Miller, G., and Steitz, J. A. (1981). Two small RNAs encoded by Epstein-Barr virus and complexed with protein are precipitated by antibodies from patients with systemic lupus erythematosus. *Proc. Natl. Acad. Sci. U.S.A.* 78, 805–809. doi: 10.1073/pnas.78.2.805.
- Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–2079. doi: 10.1093/bioinformatics/btp352.
- Li, K., and Williams, R. S. (1995). Cloning and Characterization of Three New Murine Genes Encoding Short Homologues of RNase P RNA. *Journal of Biological Chemistry* 270, 25281–25285. doi: 10.1074/jbc.270.42.25281.

- Li, X. (2004). Identification of a functional core in the RNA component of RNase MRP of budding yeasts. *Nucleic Acids Research* 32, 3703–3711. doi: 10.1093/nar/gkh689.
- Li, X., Liu, X., Zhao, D., Cui, W., Wu, Y., Zhang, C., et al. (2021). tRNA-derived small RNAs: novel regulators of cancer hallmarks and targets of clinical application. *Cell Death Discov.* 7, 249. doi: 10.1038/s41420-021-00647-1.
- Li, Y., and Altman, S. (2001). A subunit of human nuclear RNase P has ATPase activity. *Proceedings of the National Academy of Sciences* 98, 441–444. doi: 10.1073/pnas.98.2.441.
- Li, Y., Shan, G., Teng, Z.-Q., and Wingo, T. S. (2020). Editorial: Non-Coding RNAs and Human Diseases. *Front. Genet.* 11, 523. doi: 10.3389/fgene.2020.00523.
- Li, Z. (2011). MALAT-1: A long non-coding RNA and its important 3' end functional motif in colorectal cancer metastasis. *Int J Oncol.* doi: 10.3892/ijo.2011.1007.
- Liang, C., Xiong, K., Szulwach, K. E., Zhang, Y., Wang, Z., Peng, J., et al. (2013). Sjögren Syndrome Antigen B (SSB)/La Promotes Global MicroRNA Expression by Binding MicroRNA Precursors through Stem-Loop Recognition. *Journal of Biological Chemistry* 288, 723–736. doi: 10.1074/jbc.M112.401323.
- Liao, S. E., and Regev, O. (2021). Splicing at the phase-separated nuclear speckle interface: a model. *Nucleic Acids Research* 49, 636–645. doi: 10.1093/nar/gkaa1209.
- Liao, Y., Smyth, G. K., and Shi, W. (2014a). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923–930. doi: 10.1093/bioinformatics/btt656.
- Liao, Y., Smyth, G. K., and Shi, W. (2014b). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923–930. doi: 10.1093/bioinformatics/btt656.
- Lindahl, L., Fretz, S., Epps, N., and Zengel, J. M. (2000). Functional equivalence of hairpins in the RNA subunits of RNase MRP and RNase P in *Saccharomyces cerevisiae*. *RNA* 6, 653–658. doi: 10.1017/S1355838200992574.
- Lin-Marq, N., and Clarkson, S. G. (1995). A Yeast RNA Binding Protein that Resembles the Human Autoantigen La. *Journal of Molecular Biology* 245, 81–85. doi: 10.1006/jmbi.1994.0008.
- Liu, R., Holik, A. Z., Su, S., Jansz, N., Chen, K., Leong, H. S., et al. (2015a). Why weight? Modelling sample and observational level variability improves power in RNA-seq analyses. *Nucleic Acids Research* 43, e97–e97. doi: 10.1093/nar/gkv412.
- Liu, R., Holik, A. Z., Su, S., Jansz, N., Chen, K., Leong, H. S., et al. (2015b). Why weight? Modelling sample and observational level variability improves power in RNA-seq analyses. *Nucleic Acids Research* 43, e97–e97. doi: 10.1093/nar/gkv412.
- López, M. D., Rosenblad, M. A., and Samuelsson, T. (2009). Conserved and variable domains of RNase MRP RNA. *RNA Biology* 6, 208–221. doi: 10.4161/rna.6.3.8584.
- Lopez Sanchez, M. I. G., Mercer, T. R., Davies, S. M. K., Shearwood, A.-M. J., Nygård, K. K. A., Richman, T. R., et al. (2011). RNA processing in human mitochondria. *Cell Cycle* 10, 2904–2916. doi: 10.4161/cc.10.17.17060.
- Lorenzi, L., Chiu, H.-S., Avila Cobos, F., Gross, S., Volders, P.-J., Cannoodt, R., et al. (2021). The RNA Atlas expands the catalog of human non-coding RNAs. *Nat Biotechnol* 39, 1453–1465. doi: 10.1038/s41587-021-00936-1.

- Luengo, A., Li, Z., Gui, D. Y., Sullivan, L. B., Zagorulya, M., Do, B. T., et al. (2021). Increased demand for NAD⁺ relative to ATP drives aerobic glycolysis. *Mol Cell* 81, 691–707.e6. doi: 10.1016/j.molcel.2020.12.012.
- Lygerou, Z., Mitchell, P., Petfalski, E., Seraphin, B., and Tollervey, D. (1994). The POP1 gene encodes a protein component common to the RNase MRP and RNase P ribonucleoproteins. *Genes & Development* 8, 1423–1433. doi: 10.1101/gad.8.12.1423.
- Lygerou, Z., Pluk, H., van Venrooij, W. J., and Séraphin, B. (1996). hPop1: an autoantigenic protein subunit shared by the human RNase P and RNase MRP ribonucleoproteins. *EMBO J* 15, 5936–5948.
- Madore, S. J., Wieben, E. D., and Pederson, T. (1984). Eukaryotic small ribonucleoproteins. Anti-La human autoantibodies react with U1 RNA-protein complexes. *J Biol Chem* 259, 1929–1933.
- Mann, H., Ben-Asouli, Y., Schein, A., Moussa, S., and Jarrous, N. (2003). Eukaryotic RNase P: role of RNA and protein subunits of a primordial catalytic ribonucleoprotein in RNA-based catalysis. *Molecular Cell* 12, 925–935. doi: 10.1016/S1097-2765(03)00357-5.
- Manu, K., Cao, P., Chai, T., Casey, P., and Wang, M. (2019). p21cip1/waf1 Coordinates Autophagy, Proliferation and Apoptosis in Response to Metabolic Stress. *Cancers* 11, 1112. doi: 10.3390/cancers11081112.
- Maraia, R. J. (2001). La Protein and the Trafficking of Nascent RNA Polymerase III Transcripts. *Journal of Cell Biology* 153, F13–F18. doi: 10.1083/jcb.153.4.F13.
- Maraia, R. J., and Lamichhane, T. N. (2011). 3' processing of eukaryotic precursor tRNAs: 3' processing of eukaryotic precursor tRNAs. *WIREs RNA* 2, 362–375. doi: 10.1002/wrna.64.
- Maraia, R. J., Mattijssen, S., Cruz-Gallardo, I., and Conte, M. R. (2017). The La and related RNA-binding proteins (LARPs): structures, functions, and evolving perspectives. *WIREs RNA* 8. doi: 10.1002/wrna.1430.
- Marcel, V., Fernandes, K., Terrier, O., Lane, D. P., and Bourdon, J.-C. (2014). Modulation of p53 β and p53 γ expression by regulating the alternative splicing of TP53 gene modifies cellular response. *Cell Death Differ* 21, 1377–1387. doi: 10.1038/cdd.2014.73.
- Maris, C., Dominguez, C., and Allain, F. H.-T. (2005). The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression: The RRM domain, a plastic RNA-binding platform. *FEBS Journal* 272, 2118–2131. doi: 10.1111/j.1742-4658.2005.04653.x.
- Martino, L., Pennell, S., Kelly, G., Bui, T. T. T., Kotik-Kogan, O., Smerdon, S. J., et al. (2012). Analysis of the interaction with the hepatitis C virus mRNA reveals an alternative mode of RNA recognition by the human La protein. *Nucleic Acids Research* 40, 1381–1394. doi: 10.1093/nar/gkr890.
- Mattijssen, S., Hinson, E. R., Onnekink, C., Hermanns, P., Zabel, B., Cresswell, P., et al. (2011). Viperin mRNA is a novel target for the human RNase MRP/RNase P endoribonuclease. *Cell. Mol. Life Sci.* 68, 2469–2480. doi: 10.1007/s00018-010-0568-3.
- Mattioli, M., and Reichlin, M. (1974). Heterogeneity of RNA protein antigens reactive with sera of patients with systemic lupus erythematosus description of a cytoplasmic nonribosomal antigen. *Arthritis & Rheumatism* 17, 421–429. doi: 10.1002/art.1780170413.

- Meier, U. T. (2017). RNA modification in Cajal bodies. *RNA Biology* 14, 693–700. doi: 10.1080/15476286.2016.1249091.
- Mezquita, P., Parghi, S. S., Brandvold, K. A., and Ruddell, A. (2005). Myc regulates VEGF production in B cells by stimulating initiation of VEGF mRNA translation. *Oncogene* 24, 889–901. doi: 10.1038/sj.onc.1208251.
- Molla-Herman, A., Angelova, M. T., Ginestet, M., Carré, C., Antoniewski, C., and Huynh, J.-R. (2020). tRNA Fragments Populations Analysis in Mutants Affecting tRNAs Processing and tRNA Methylation. *Front. Genet.* 11, 518949. doi: 10.3389/fgene.2020.518949.
- Molla-Herman, A., Vallés, A. M., Ganem-Elbaz, C., Antoniewski, C., and Huynh, J. (2015). tRNA processing defects induce replication stress and Chk2-dependent disruption of piRNA transcription. *EMBO J* 34, 3009–3027. doi: 10.15252/embj.201591006.
- Morris, K. V., and Mattick, J. S. (2014). The rise of regulatory RNA. *Nat Rev Genet* 15, 423–437. doi: 10.1038/nrg3722.
- Naeeni, A. R., Conte, M. R., and Bayfield, M. A. (2012). RNA Chaperone Activity of Human La Protein Is Mediated by Variant RNA Recognition Motif. *Journal of Biological Chemistry* 287, 5472–5482. doi: 10.1074/jbc.M111.276071.
- Nagao, A., Kobayashi, M., Koyasu, S., Chow, C. C. T., and Harada, H. (2019). HIF-1-Dependent Reprogramming of Glucose Metabolic Pathway of Cancer Cells and Its Therapeutic Significance. *IJMS* 20, 238. doi: 10.3390/ijms20020238.
- Nakatake, M., Monte-Mor, B., Debili, N., Casadevall, N., Ribrag, V., Solary, E., et al. (2012). JAK2V617F negatively regulates p53 stabilization by enhancing MDM2 via La expression in myeloproliferative neoplasms. *Oncogene* 31, 1323–1333. doi: 10.1038/onc.2011.313.
- Newhart, A., Powers, S. L., Shastrula, P. K., Sierra, I., Joo, L. M., Hayden, J. E., et al. (2016). RNase P protein subunit Rpp29 represses histone H3.3 nucleosome deposition. *MBoC* 27, 1154–1169. doi: 10.1091/mbc.E15-02-0099.
- Nickel, A. I., Wäber, N. B., Gößringer, M., Lechner, M., Linne, U., Toth, U., et al. (2017). Minimal and RNA-free RNase P in *Aquifex aeolicus*. *Proc. Natl. Acad. Sci. U.S.A.* 114, 11121–11126. doi: 10.1073/pnas.1707862114.
- Oshima, K., Gao, X., Hayashi, S., Ueda, T., Nakashima, T., and Kimura, M. (2018). Crystal structures of the archaeal RNase P protein Rpp38 in complex with RNA fragments containing a K-turn motif. *Acta Crystallogr F Struct Biol Commun* 74, 57–64. doi: 10.1107/S2053230X17018039.
- Palazzo, A. F., and Lee, E. S. (2015). Non-coding RNA: what is functional and what is junk? *Front. Genet.* 6. doi: 10.3389/fgene.2015.00002.
- Palsule, G., Gopalan, V., and Simcox, A. (2019). Biogenesis of RNase P RNA from an intron requires co-assembly with cognate protein subunits. *Nucleic Acids Research* 47, 8746–8754. doi: 10.1093/nar/gkz572.
- Park, O. H., Ha, H., Lee, Y., Boo, S. H., Kwon, D. H., Song, H. K., et al. (2019). Endoribonucleolytic Cleavage of m6A-Containing RNAs by RNase P/MRP Complex. *Molecular Cell* 74, 494–507.e8. doi: 10.1016/j.molcel.2019.02.034.
- Paushkin, S. V., Patel, M., Furia, B. S., Peltz, S. W., and Trotta, C. R. (2004). Identification of a Human Endonuclease Complex Reveals a Link between tRNA Splicing and Pre-mRNA 3' End Formation. *Cell* 117, 311–321. doi: 10.1016/S0092-8674(04)00342-3.

- Peck-Miller, K. A., and Altman, S. (1991). Kinetics of the processing of the precursor to 4-5 S RNA, a naturally occurring substrate for RNase P from *Escherichia coli*. *Journal of Molecular Biology* 221, 1–5. doi: 10.1016/0022-2836(91)80194-Y.
- Penning, T. M., Jonnalagadda, S., Trippier, P. C., and Rižner, T. L. (2021). Aldo-Keto Reductases and Cancer Drug Resistance. *Pharmacol Rev* 73, 1150–1171. doi: 10.1124/pharmrev.120.000122.
- Perederina, A., Esakova, O., Quan, C., Khanova, E., and Krasilnikov, A. S. (2010). Eukaryotic ribonucleases P/MRP: the crystal structure of the P3 domain. *EMBO J* 29, 761–769. doi: 10.1038/emboj.2009.396.
- Perederina, A., and Krasilnikov, A. S. (2010). The P3 domain of eukaryotic RNases P/MRP: Making a protein-rich RNA-based enzyme. *RNA Biology* 7, 534–539. doi: 10.4161/rna.7.5.12302.
- Petz, M., Them, N. C. C., Huber, H., and Mikulits, W. (2012a). PDGF enhances IRES-mediated translation of Laminin B1 by cytoplasmic accumulation of La during epithelial to mesenchymal transition. *Nucleic Acids Research* 40, 9738–9749. doi: 10.1093/nar/gks760.
- Petz, M., Them, N., Huber, H., Beug, H., and Mikulits, W. (2012b). La enhances IRES-mediated translation of laminin B1 during malignant epithelial to mesenchymal transition. *Nucleic Acids Research* 40, 290–302. doi: 10.1093/nar/gkr717.
- Phan, H.-D., Lai, L. B., Zahurancik, W. J., and Gopalan, V. (2021). The many faces of RNA-based RNase P, an RNA-world relic. *Trends in Biochemical Sciences* 46, 976–991. doi: 10.1016/j.tibs.2021.07.005.
- Pinker, F., Bonnard, G., Gobert, A., Gutmann, B., Hammani, K., Sauter, C., et al. (2013). PPR proteins shed a new light on RNase P biology. *RNA Biology* 10, 1457–1468. doi: 10.4161/rna.25273.
- Pirola, L., Ciesielski, O., Biesiekierska, M., and Balcerczyk, A. (2021). “Modulation of noncoding RNAs (ncRNAs) and their potential role as therapeutics,” in *Medical Epigenetics* (Elsevier), 721–740. doi: 10.1016/B978-0-12-823928-5.00041-4.
- Popow, J., Englert, M., Weitzer, S., Schleiffer, A., Mierzwa, B., Mechtler, K., et al. (2011). HSPC117 Is the Essential Subunit of a Human tRNA Splicing Ligase Complex. *Science* 331, 760–764. doi: 10.1126/science.1197847.
- Poulin, F., Gingras, A.-C., Olsen, H., Chevalier, S., and Sonenberg, N. (1998). 4E-BP3, a New Member of the Eukaryotic Initiation Factor 4E-binding Protein Family. *Journal of Biological Chemistry* 273, 14002–14007. doi: 10.1074/jbc.273.22.14002.
- Raina, M., and Ibba, M. (2014). tRNAs as regulators of biological processes. *Front. Genet.* 5. doi: 10.3389/fgene.2014.00171.
- Ramsay, E. P., Abascal-Palacios, G., Daiß, J. L., King, H., Gouge, J., Pilsl, M., et al. (2020). Structure of human RNA polymerase III. *Nat Commun* 11, 6409. doi: 10.1038/s41467-020-20262-5.
- Ramsay, E. P., and Vannini, A. (2018). Structural rearrangements of the RNA polymerase III machinery during tRNA transcription initiation. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1861, 285–294. doi: 10.1016/j.bbagr.2017.11.005.
- Randau, L., Münch, R., Hohn, M. J., Jahn, D., and Söll, D. (2005). Nanoarchaeum equitans creates functional tRNAs from separate genes for their 5'- and 3'-halves. *Nature* 433, 537–541. doi: 10.1038/nature03233.

- Rashid, M., Zadeh, L. R., Baradaran, B., Molavi, O., Ghesmati, Z., Sabzichi, M., et al. (2021). Up-down regulation of HIF-1 α in cancer progression. *Gene* 798, 145796. doi: 10.1016/j.gene.2021.145796.
- Reiner, R. (2006). A role for the catalytic ribonucleoprotein RNase P in RNA polymerase III transcription. *Genes & Development* 20, 1621–1635. doi: 10.1101/gad.386706.
- Reiner, R., Krasnov-Yoeli, N., Dehtiar, Y., and Jarrous, N. (2008). Function and Assembly of a Chromatin-Associated RNase P that Is Required for Efficient Transcription by RNA Polymerase I. *PLoS ONE* 3, e4072. doi: 10.1371/journal.pone.0004072.
- Reinhard, L., Sridhara, S., and Hällberg, B. M. (2017). The MRPP1/MRPP2 complex is a tRNA-maturation platform in human mitochondria. *Nucleic Acids Research* 45, 12469–12480. doi: 10.1093/nar/gkx902.
- Reiter, N. J., Osterman, A., Torres-Larios, A., Swinger, K. K., Pan, T., and Mondragón, A. (2010). Structure of a bacterial ribonuclease P holoenzyme in complex with tRNA. *Nature* 468, 784–789. doi: 10.1038/nature09516.
- Rinke, J., and Steitz, J. A. (1982). Precursor molecules of both human 5S ribosomal RNA and transfer RNAs are bound by a cellular protein reactive with anti-La Lupus antibodies. *Cell* 29, 149–159. doi: 10.1016/0092-8674(82)90099-X.
- Rosenblad, M. A., López, M. D., Piccinelli, P., and Samuelsson, T. (2006). Inventory and analysis of the protein subunits of the ribonucleases P and MRP provides further evidence of homology between the yeast and human enzymes. *Nucleic Acids Res* 34, 5145–5156. doi: 10.1093/nar/gkl626.
- Saikia, M., Jobava, R., Parisien, M., Putnam, A., Krokowski, D., Gao, X.-H., et al. (2014). Angiogenin-Cleaved tRNA Halves Interact with Cytochrome c, Protecting Cells from Apoptosis during Osmotic Stress. *Molecular and Cellular Biology* 34, 2450–2463. doi: 10.1128/MCB.00136-14.
- Salzberg, S. L. (2018). Open questions: How many genes do we have? *BMC Biol* 16, 94. doi: 10.1186/s12915-018-0564-x.
- Saoji, M., Petersen, C. E., Sen, A., Tripoli, B. A., Smyth, J. T., and Cox, R. T. (2022). Reduction of Drosophila Mitochondrial RNase P in Skeletal and Heart Muscle Causes Muscle Degeneration, Cardiomyopathy, and Heart Arrhythmia. *Front. Cell Dev. Biol.* 10, 788516. doi: 10.3389/fcell.2022.788516.
- Saoji, M., Sen, A., and Cox, R. T. (2021). Loss of Individual Mitochondrial Ribonuclease P Complex Proteins Differentially Affects Mitochondrial tRNA Processing In Vivo. *IJMS* 22, 6066. doi: 10.3390/ijms22116066.
- Scherly, D., Stutz, F., Lin-Marq, N., and Clarkson, S. G. (1993). La Proteins from *Xenopus laevis* cDNA Cloning and Development Expression. *Journal of Molecular Biology* 231, 196–204. doi: 10.1006/jmbi.1993.1275.
- Schimmel, P. (2018). The emerging complexity of the tRNA world: mammalian tRNAs beyond protein synthesis. *Nat Rev Mol Cell Biol* 19, 45–58. doi: 10.1038/nrm.2017.77.
- Schmidt, C. A., and Matera, A. G. (2020). tRNA introns: Presence, processing, and purpose. *WIREs RNA* 11. doi: 10.1002/wrna.1583.

- Schmitt, M. E., and Clayton, D. A. (1993). Nuclear RNase MRP is required for correct processing of pre-5.8S rRNA in *Saccharomyces cerevisiae*. *Mol Cell Biol* 13, 7935–7941. doi: 10.1128/mcb.13.12.7935-7941.1993.
- Schwartz, E. I., Intine, R. V., and Maraia, R. J. (2004). CK2 Is Responsible for Phosphorylation of Human La Protein Serine-366 and Can Modulate rpL37 5J-Terminal Oligopyrimidine mRNA Metabolism. *MOL. CELL. BIOL.* 24, 12.
- Sen, A., Karasik, A., Shanmuganathan, A., Mirkovic, E., Koutmos, M., and Cox, R. T. (2016). Loss of the mitochondrial protein-only ribonuclease P complex causes aberrant tRNA processing and lethality in *Drosophila*. *Nucleic Acids Res* 44, 6409–6422. doi: 10.1093/nar/gkw338.
- Serruya, R., Orlovetskie, N., Reiner, R., Dehtiar-Zilber, Y., Wesolowski, D., Altman, S., et al. (2015). Human RNase P ribonucleoprotein is required for formation of initiation complexes of RNA polymerase III. *Nucleic Acids Research* 43, 5442–5450. doi: 10.1093/nar/gkv447.
- Shang, Z., Yu, J., Sun, L., Tian, J., Zhu, S., Zhang, B., et al. (2019). LncRNA PCAT1 activates AKT and NF- κ B signaling in castration-resistant prostate cancer by regulating the PHLPP/FKBP51/IKK α complex. *Nucleic Acids Research* 47, 4211–4225. doi: 10.1093/nar/gkz108.
- Shao, Y., Sun, Q., Liu, X., Wang, P., Wu, R., and Ma, Z. (2017). tRF-Leu-CAG promotes cell proliferation and cell cycle in non-small cell lung cancer. *Chem Biol Drug Des* 90, 730–738. doi: 10.1111/cbdd.12994.
- Shastrula, P. K., Lund, P. J., Garcia, B. A., and Janicki, S. M. (2018). Rpp29 regulates histone H3.3 chromatin assembly through transcriptional mechanisms. *Journal of Biological Chemistry* 293, 12360–12377. doi: 10.1074/jbc.RA118.001845.
- Shaukat, A.-N., Kaliatsi, E. G., Skeparnias, I., and Stathopoulos, C. (2021). The Dynamic Network of RNP RNase P Subunits. *IJMS* 22, 10307. doi: 10.3390/ijms221910307.
- Skeparnias, I., Anastasakis, D., Grafanaki, K., Kyriakopoulos, G., Alexopoulos, P., Dougenis, D., et al. (2020). Contribution of miRNAs, tRNAs and tRFs to Aberrant Signaling and Translation Deregulation in Lung Cancer. *Cancers* 12, 3056. doi: 10.3390/cancers12103056.
- Slack, F. J., and Chinnaiyan, A. M. (2019). The Role of Non-coding RNAs in Oncology. *Cell* 179, 1033–1055. doi: 10.1016/j.cell.2019.10.017.
- Sloan, K. E., Mattijssen, S., Lebaron, S., Tollervey, D., Pruijn, G. J. M., and Watkins, N. J. (2013). Both endonucleolytic and exonucleolytic cleavage mediate ITS1 removal during human ribosomal RNA processing. *Journal of Cell Biology* 200, 577–588. doi: 10.1083/jcb.201207131.
- Sommer, G., Dittmann, J., Kuehnert, J., Reumann, K., Schwartz, P. E., Will, H., et al. (2011a). The RNA-binding protein La contributes to cell proliferation and CCND1 expression. *Oncogene* 30, 434–444. doi: 10.1038/onc.2010.425.
- Sommer, G., and Heise, T. (2021). Role of the RNA-binding protein La in cancer pathobiology. *RNA Biology* 18, 218–236. doi: 10.1080/15476286.2020.1792677.
- Sommer, G., Rossa, C., Chi, A. C., Neville, B. W., and Heise, T. (2011b). Implication of RNA-Binding Protein La in Proliferation, Migration and Invasion of Lymph Node-Metastasized Hypopharyngeal SCC Cells. *PLoS ONE* 6, e25402. doi: 10.1371/journal.pone.0025402.

- Statello, L., Guo, C.-J., Chen, L.-L., and Huarte, M. (2021). Gene regulation by long non-coding RNAs and its biological functions. *Nat Rev Mol Cell Biol* 22, 96–118. doi: 10.1038/s41580-020-00315-9.
- Stathopoulos, C., Ahel, I., Ali, K., Ambrogelly, A., Becker, H., Bunjun, S., et al. (2001). Aminoacyl-tRNA Synthesis: A Postgenomic Perspective. *Cold Spring Harbor Symposia on Quantitative Biology* 66, 175–184. doi: 10.1101/sqb.2001.66.175.
- Stavraka, C., and Blagden, S. (2015). The La-Related Proteins, a Family with Connections to Cancer. *Biomolecules* 5, 2701–2722. doi: 10.3390/biom5042701.
- Stefano, J. (1984). Purified lupus antigen la recognizes an oligouridylate stretch common to the 3' termini of RNA polymerase III transcripts. *Cell* 36, 145–154. doi: 10.1016/0092-8674(84)90083-7.
- Steinbusch, M. M. F., Caron, M. M. J., Surtel, D. A. M., Friedrich, F., Lausch, E., Pruijn, G. J. M., et al. (2017). Expression of RMRP RNA is regulated in chondrocyte hypertrophy and determines chondrogenic differentiation. *Sci Rep* 7, 6440. doi: 10.1038/s41598-017-06809-5.
- Stolc, V., and Altman, S. (1997). Rpp1, an essential protein subunit of nuclear RNase P required for processing of precursor tRNA and 35S precursor rRNA in *Saccharomyces cerevisiae*. *Genes & Development* 11, 2926–2937. doi: 10.1101/gad.11.21.2926.
- Stolc, V., Katz, A., and Altman, S. (1998). Rpp2, an essential protein subunit of nuclear RNase P, is required for processing of precursor tRNAs and 35S precursor rRNA in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences* 95, 6716–6721. doi: 10.1073/pnas.95.12.6716.
- Stribinskis, V., Gao, G. J., Sulo, P., Dang, Y. L., and Martin, N. C. (1996). Yeast mitochondrial RNase P RNA synthesis is altered in an RNase P protein subunit mutant: insights into the biogenesis of a mitochondrial RNA-processing enzyme. *Mol Cell Biol* 16, 3429–3436. doi: 10.1128/MCB.16.7.3429.
- Su, Z., Wilson, B., Kumar, P., and Dutta, A. (2020). Noncanonical Roles of tRNAs: tRNA Fragments and Beyond. *Annual Review of Genetics* 54, 47–69. doi: 10.1146/annurev-genet-022620-101840.
- Sunwoo, H., Dinger, M. E., Wilusz, J. E., Amaral, P. P., Mattick, J. S., and Spector, D. L. (2008). MEN / nuclear-retained non-coding RNAs are up-regulated upon muscle differentiation and are essential components of paraspeckles. *Genome Research* 19, 347–359. doi: 10.1101/gr.087775.108.
- Tang, Y., Zhang, Z., Chen, Y., Qin, S., Zhou, L., Gao, W., et al. (2022). Metabolic Adaptation-Mediated Cancer Survival and Progression in Oxidative Stress. *Antioxidants* 11, 1324. doi: 10.3390/antiox11071324.
- Teplova, M., Yuan, Y.-R., Phan, A. T., Malinina, L., Ilin, S., Teplov, A., et al. (2006). Structural Basis for Recognition and Sequestration of UUUOH 3' Termini of Nascent RNA Polymerase III Transcripts by La, a Rheumatic Disease Autoantigen. *Molecular Cell* 21, 75–85. doi: 10.1016/j.molcel.2005.10.027.
- Tian, S., Curnutte, H. A., and Trcek, T. (2020). RNA Granules: A View from the RNA Perspective. *Molecules* 25, 3130. doi: 10.3390/molecules25143130.

- Topfer, F., Gordon, T., and McCluskey, J. (1993). Characterization of the mouse autoantigen La (SS-B). Identification of conserved RNA-binding motifs, a putative ATP binding site and reactivity of recombinant protein with poly(U) and human autoantibodies. *J Immunol* 150, 3091–3100.
- Torres-Larios, A., Swinger, K. K., Krasilnikov, A. S., Pan, T., and Mondragón, A. (2005). Crystal structure of the RNA component of bacterial ribonuclease P. *Nature* 437, 584–587. doi: 10.1038/nature04074.
- Toumpeki C., Vourekas A., Kalavrizioti D., Stamatopoulou V., Drainas D. (2008). Activation of bacterial ribonuclease P by macrolides. *Biochemistry* 47, 4112–8. doi: 10.1021/bi701488q.
- Tran, D. H., Kesavan, R., Rion, H., Soflaee, M. H., Solmonson, A., Bezwada, D., et al. (2021). Mitochondrial NADP⁺ is essential for proline biosynthesis during cell growth. *Nat Metab* 3, 571–585. doi: 10.1038/s42255-021-00374-y.
- Trotta, C. R., Miao, F., Arn, E. A., Stevens, S. W., Ho, C. K., Rauhut, R., et al. (1997). The Yeast tRNA Splicing Endonuclease: A Tetrameric Enzyme with Two Active Site Subunits Homologous to the Archaeal tRNA Endonucleases. *Cell* 89, 849–858. doi: 10.1016/S0092-8674(00)80270-6.
- Trotta, R., Vignudelli, T., Candini, O., Intine, R. V., Pecorari, L., Guerzoni, C., et al. (2003). BCR/ABL activates mdm2 mRNA translation via the La antigen. *Cancer Cell* 3, 145–160. doi: 10.1016/S1535-6108(03)00020-5.
- Turowski, T. W., and Tollervey, D. (2016). Transcription by RNA polymerase III: insights into mechanism and regulation. *Biochemical Society Transactions* 44, 1367–1375. doi: 10.1042/BST20160062.
- Vakkilainen, S., Skoog, T., Einarsdottir, E., Middleton, A., Pekkinen, M., Öhman, T., et al. (2019). The human long non-coding RNA gene RMRP has pleiotropic effects and regulates cell-cycle progression at G2. *Sci Rep* 9, 13758. doi: 10.1038/s41598-019-50334-6.
- van Eenennaam, H., Lugtenberg, D., Vogelzangs, J. H. P., van Venrooij, W. J., and Pruijn, G. J. M. (2001). hPop5, a Protein Subunit of the Human RNase MRP and RNase P Endoribonucleases. *Journal of Biological Chemistry* 276, 31635–31641. doi: 10.1074/jbc.M103399200.
- Van Eenennaam, H., Vogelzangs, J. H. P., Lugtenberg, D., Van Den Hoogen, F. H. J., Van Venrooij, W. J., and Pruijn, G. J. M. (2002). Identity of the RNase MRP- and RNase P-associated Th/To autoantigen. *Arthritis & Rheumatism* 46, 3266–3272. doi: 10.1002/art.10673.
- Van Horn, D. J., Yoo, C. J., Xue, D., Shi, H., and Wolin, S. L. (1997). The La protein in *Schizosaccharomyces pombe*: a conserved yet dispensable phosphoprotein that functions in tRNA maturation. *RNA* 3, 1434–1443.
- van Niekerk, E. A., Willis, D. E., Chang, J. H., Reumann, K., Heise, T., and Twiss, J. L. (2007). Sumoylation in axons triggers retrograde transport of the RNA-binding protein La. *Proc. Natl. Acad. Sci. U.S.A.* 104, 12913–12918. doi: 10.1073/pnas.0611562104.
- Vilardo, E., Nachbagauer, C., Buzet, A., Taschner, A., Holzmann, J., and Rossmanith, W. (2012). A subcomplex of human mitochondrial RNase P is a bifunctional methyltransferase—extensive moonlighting in mitochondrial tRNA biogenesis. *Nucleic Acids Research* 40, 11583–11593. doi: 10.1093/nar/gks910.
- Vilardo, E., and Rossmanith, W. (2015). Molecular insights into HSD10 disease: impact of SDR5C1 mutations on the human mitochondrial RNase P complex. *Nucleic Acids Research* 43, 5112–5119. doi: 10.1093/nar/gkv408.

- Vinayak, J., Marrella, S. A., Hussain, R. H., Rozenfeld, L., Solomon, K., and Bayfield, M. A. (2018). Human La binds mRNAs through contacts to the poly(A) tail. *Nucleic Acids Research* 46, 4228–4240. doi: 10.1093/nar/gky090.
- Walker, S. C., and Engelke, D. R. (2008). A Protein-Only RNase P in Human Mitochondria. *Cell* 135, 412–414. doi: 10.1016/j.cell.2008.10.010.
- Walter, P., and Blobel, G. (1982). Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. *Nature* 299, 691–698. doi: 10.1038/299691a0.
- Wan, F., Wang, Q., Tan, J., Tan, M., Chen, J., Shi, S., et al. (2019). Cryo-electron microscopy structure of an archaeal ribonuclease P holoenzyme. *Nat Commun* 10, 2617. doi: 10.1038/s41467-019-10496-3.
- Wang, S.-Q., Shi, D.-Q., Long, Y.-P., Liu, J., and Yang, W.-C. (2012). GAMETOPHYTE DEFECTIVE 1, a Putative Subunit of RNases P/MRP, Is Essential for Female Gametogenesis and Male Competence in Arabidopsis. *PLoS ONE* 7, e33595. doi: 10.1371/journal.pone.0033595.
- Wang, X., Chow, C. R., Ebine, K., Lee, J., Rosner, M. R., Pan, T., et al. (2016). Interaction of tRNA with MEK2 in pancreatic cancer cells. *Sci Rep* 6, 28260. doi: 10.1038/srep28260.
- Weinberg, C. E., Weinberg, Z., and Hammann, C. (2019). Novel ribozymes: discovery, catalytic mechanisms, and the quest to understand biological function. *Nucleic Acids Research* 47, 9480–9494. doi: 10.1093/nar/gkz737.
- Welting, T. J. M., Peters, F. M. A., Hensen, S. M. M., van Doorn, N. L., Kikkert, B. J., Raats, J. M. H., et al. (2006). Heterodimerization regulates RNase MRP/RNase P association, localization, and expression of Rpp20 and Rpp25. *RNA* 13, 65–75. doi: 10.1261/rna.237807.
- Westermann, S., and Weber, K. (2000). Cloning and recombinant expression of the La RNA-binding protein from *Trypanosoma brucei*. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* 1492, 483–487. doi: 10.1016/S0167-4781(00)00113-5.
- White-Gilbertson, S., Kurtz, D. T., and Voelkel-Johnson, C. (2009). The role of protein synthesis in cell cycling and cancer. *Molecular Oncology* 3, 402–408. doi: 10.1016/j.molonc.2009.05.003.
- Wiedner, H. J., and Giudice, J. (2021). It's not just a phase: function and characteristics of RNA-binding proteins in phase separation. *Nat Struct Mol Biol* 28, 465–473. doi: 10.1038/s41594-021-00601-w.
- Wilczynska, A., and Bushell, M. (2015). The complexity of miRNA-mediated repression. *Cell Death Differ* 22, 22–33. doi: 10.1038/cdd.2014.112.
- Wilusz, J. E., Freier, S. M., and Spector, D. L. (2008). 3' End Processing of a Long Nuclear-Retained Noncoding RNA Yields a tRNA-like Cytoplasmic RNA. *Cell* 135, 919–932. doi: 10.1016/j.cell.2008.10.012.
- Winkle, M., El-Daly, S. M., Fabbri, M., and Calin, G. A. (2021). Noncoding RNA therapeutics — challenges and potential solutions. *Nat Rev Drug Discov* 20, 629–651. doi: 10.1038/s41573-021-00219-z.
- Wolin, S. L., and Cedervall, T. (2002). The La Protein. *Annu. Rev. Biochem.* 71, 375–403. doi: 10.1146/annurev.biochem.71.090501.150003.
- Wu, J., Niu, S., Tan, M., Huang, C., Li, M., Song, Y., et al. (2018). Cryo-EM Structure of the Human Ribonuclease P Holoenzyme. *Cell* 175, 1393–1404.e11. doi: 10.1016/j.cell.2018.10.003.

- Wu, J.-L., Wang, Y.-G., Gao, G.-M., Feng, L., Guo, N., and Zhang, C.-X. (2019). Overexpression of lncRNA TCL6 promotes preeclampsia progression by regulating PTEN. *European Review for Medical and Pharmacological Sciences* 23, 4066–4072. doi: 10.26355/eurrev_201905_17907.
- Xiao, D., Wu, J., Zhao, H., Jiang, X., and Nie, C. (2022). RPP25 as a Prognostic-Related Biomarker That Correlates With Tumor Metabolism in Glioblastoma. *Front. Oncol.* 11, 714904. doi: 10.3389/fonc.2021.714904.
- Xiao, S., Scott, F., Fierke, C. A., and Engelke, D. R. (2002). Eukaryotic Ribonuclease P: A Plurality of Ribonucleoprotein Enzymes. *Annu. Rev. Biochem.* 71, 165–189. doi: 10.1146/annurev.biochem.71.110601.135352.
- Xiong, Y., and Steitz, T. A. (2004). Mechanism of transfer RNA maturation by CCA-adding enzyme without using an oligonucleotide template. *Nature* 430, 640–645. doi: 10.1038/nature02711.
- Xu, W.-W., Jin, J., Wu, X., Ren, Q.-L., and Farzaneh, M. (2022). MALAT1-related signaling pathways in colorectal cancer. *Cancer Cell Int* 22, 126. doi: 10.1186/s12935-022-02540-y.
- Xue, D. (2000). U snRNP assembly in yeast involves the La protein. *The EMBO Journal* 19, 1650–1660. doi: 10.1093/emboj/19.7.1650.
- Xue, S., Calvin, K., and Li, H. (2006). RNA Recognition and Cleavage by a Splicing Endonuclease. *Science* 312, 906–910. doi: 10.1126/science.1126629.
- Yada, R. C., Ostrominski, J. W., Tunc, I., Hong, S. G., Zou, J., and Dunbar, C. E. (2017). CRISPR/Cas9-Based Safe-Harbor Gene Editing in Rhesus iPSCs. *Current Protocols in Stem Cell Biology* 43. doi: 10.1002/cpsc.37.
- Yang, J., Hou, S., and Liang, B. (2020). LINC00319 promotes migration, invasion and epithelial-mesenchymal transition process in cervical cancer by regulating miR-3127-5p/RPP25 axis. *In Vitro Cell.Dev.Biol.-Animal* 56, 145–153. doi: 10.1007/s11626-019-00425-5.
- Yeganeh, M., and Hernandez, N. (2020). RNA polymerase III transcription as a disease factor. *Genes Dev.* 34, 865–882. doi: 10.1101/gad.333989.119.
- Yin, C., Bai, G., Zhang, Y., and Huang, J. (2021). Crystal structure of human RPP20-RPP25 proteins in complex with the P3 domain of lncRNA RMRP. *Journal of Structural Biology* 213, 107704. doi: 10.1016/j.jsb.2021.107704.
- Yip, M. C. J., Savickas, S., Gygi, S. P., and Shao, S. (2020). ELAC1 Repairs tRNAs Cleaved during Ribosome-Associated Quality Control. *Cell Reports* 30, 2106–2114.e5. doi: 10.1016/j.celrep.2020.01.082.
- Yoo, C. J., and Wolin, S. L. (1994). La proteins from *Drosophila melanogaster* and *Saccharomyces cerevisiae*: a yeast homolog of the La autoantigen is dispensable for growth. *Mol Cell Biol* 14, 5412–5424. doi: 10.1128/mcb.14.8.5412-5424.1994.
- Yoshihisa, T. (2014). Handling tRNA introns, archaeal way and eukaryotic way. *Front. Genet.* 5. doi: 10.3389/fgene.2014.00213.
- Zeng, C.-M., Chang, L.-L., Ying, M.-D., Cao, J., He, Q.-J., Zhu, H., et al. (2017). Aldo-Keto Reductase AKR1C1–AKR1C4: Functions, Regulation, and Intervention for Anti-cancer Therapy. *Front. Pharmacol.* 8. doi: 10.3389/fphar.2017.00119.

- Zhang, J., Dinh, T. N., Kappeler, K., Tsaprailis, G., and Chen, Q. M. (2012). La Autoantigen Mediates Oxidant Induced De Novo Nrf2 Protein Translation. *Molecular & Cellular Proteomics* 11, M111.015032. doi: 10.1074/mcp.M111.015032.
- Zhang, J., and Ferré-D'Amaré, A. (2016). The tRNA Elbow in Structure, Recognition and Evolution. *Life* 6, 3. doi: 10.3390/life6010003.
- Zhang, J., and Ferré-D'Amaré, A. (2016). Trying on tRNA for Size: RNase P and the T-box Riboswitch as Molecular Rulers. *Biomolecules* 6, 18. doi: 10.3390/biom6020018.
- Zhang, P., Wu, W., Chen, Q., and Chen, M. (2019). Non-Coding RNAs and their Integrated Networks. *Journal of Integrative Bioinformatics* 16. doi: 10.1515/jib-2019-0027.
- Zhang, W., Foo, M., Eren, A. M., and Pan, T. (2022). tRNA modification dynamics from individual organisms to metaepitranscriptomics of microbiomes. *Molecular Cell* 82, 891–906. doi: 10.1016/j.molcel.2021.12.007.
- Zhang, W., and Liu, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res* 12, 9–18. doi: 10.1038/sj.cr.7290105.
- Zhang, Y., and Stefanovic, B. (2016). LARP6 Meets Collagen mRNA: Specific Regulation of Type I Collagen Expression. *IJMS* 17, 419. doi: 10.3390/ijms17030419.
- Zheng, Q., Yang, H.-J., and Yuan, Y. A. (2017). Autoantigen La Regulates MicroRNA Processing from Stem–Loop Precursors by Association with DGCR8. *Biochemistry* 56, 6098–6110. doi: 10.1021/acs.biochem.7b00693.
- Zhong, T., Xu, F., Xu, J., Liu, L., and Chen, Y. (2015). Aldo-keto reductase 1C3 (AKR1C3) is associated with the doxorubicin resistance in human breast cancer via PTEN Loss. *Biomedicine & Pharmacotherapy* 69, 317–325. doi: 10.1016/j.biopha.2014.12.022.
- Zhou, Y., Goodenbour, J. M., Godley, L. A., Wickrema, A., and Pan, T. (2009). High levels of tRNA abundance and alteration of tRNA charging by bortezomib in multiple myeloma. *Biochemical and Biophysical Research Communications* 385, 160–164. doi: 10.1016/j.bbrc.2009.05.031.
- Ziehler, W. A., Morris, J., Scott, F. H., Millikin, C., and Engelke, D. R. (2001). An essential protein-binding domain of nuclear RNase P RNA. *RNA* 7, 565–575. doi: 10.1017/S1355838201001996.

7. Publications

1. Argyriou A.I., Machaliotis G.A., Makrynitsa G.I., **Kaliatsi E.G.**, Stathopoulos C., Spyroulias G.A. (2023). NMR Analysis Suggests Synergy between the RRM2 and the Carboxy-Terminal Segment of Human La Protein in the Recognition and Interaction with HCV IRES. *Int J Mol Sci.*, doi: 24:25720.3390/ijms24032572
2. Shaukat A.N.*, **Kaliatsi E.G.***, Skeparnias I., Stathopoulos C. (2021). The Dynamic Network of RNP RNase P Subunits. *Int. J. Mol. Sci.*, 22:10307. doi: 10.3390/ijms221910307. (*equal contribution)
3. Shaukat A.N.*, **Kaliatsi E.G.***, Stamatopoulou V., Stathopoulos C. (2021). Mitochondrial tRNA-Derived Fragments and Their Contribution to Gene Expression Regulation. *Frontiers in physiology*, 12, 729452. doi:10.3389/fphys.2021.729452. (*equal contribution)
4. Kalantzi S., Leonardi S., Vachlioti E., **Kaliatsi E.G.**, Papachristopoulou K., Stathopoulos C., Vainos N., Papaioannou D. (2021). Studies towards the Synthesis of Novel 3-Aminopropoxy-Substituted Dioxins Suitable for the Development of Aptamers for Photonic Biosensor Applications. *Materials (Basel)*. 14:4727. doi: 10.3390/ma14164727.
5. Kyriakopoulos G., Katopodi V., Skeparnias I., **Kaliatsi E.G.**, Grafanaki K., Stathopoulos C. (2021). KRAS G12C Can Either Promote or Impair Cap-Dependent Translation in Two Different Lung Adenocarcinoma Cell Lines. *Int J Mol Sci.*, 22:2222. doi: 10.3390/ijms22042222.
6. **Kaliatsi E.G.**, Giarimoglou N, Stathopoulos C, Stamatopoulou V. (2020). Non-Coding RNA-Driven Regulation of rRNA Biogenesis. *Int J Mol Sci.*, 21: 9738. doi: 10.3390/ijms21249738.
7. **Kaliatsi E.G.**, Argyriou A.I., Bouras G., Apostolidi M., Konstantinidou P., Shaukat A.N, Spyroulias G.A, Stathopoulos C. (2020). Functional and Structural Aspects of La Protein Overexpression in Lung Cancer. *J Mol Biol.*, 432: 166712. doi: 10.1016/j.jmb.2020.11.011