tRNA biology in the omics era: Stress signalling dynamics and cancer progression

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Recent years have seen a burst in the number of studies investigating tRNA biology. With the transition from a gene-centred to a genome-centred perspective, tRNAs and other RNA polymerase III transcripts surfaced as active regulators of normal cell physiology and disease. Novel strategies removing some of the hurdles that prevent quantitative tRNA profiling revealed that the differential exploitation of the tRNA pool critically affects the ability of the cell to balance protein homeostasis during normal and stress conditions. Furthermore, growing evidence indicates that the adaptation of tRNA synthesis to cellular dynamics can influence translation and mRNA stability to drive carcinogenesis and other pathological disorders. This review explores the contribution given by genomics, transcriptomics and epitranscriptomics to the discovery of emerging tRNA functions, and gives insights into some of the technical challenges that still limit our understanding of the RNA polymerase III transcriptional machinery.

Keywords:
epitranscriptomics; genomics; high-throughput; RNA polymerase III; transcriptomics; tRNA

Introduction

In 1958, Zamecnik and Hoagland discovered an RNA molecule capable of interacting with microsomes to effect the transfer of amino acids to a growing peptide chain [1]. This RNA intermediate, which would have later been named transfer RNA (tRNA), redefined our understanding of the process linking the genetic code to the protein sequence. The discovery stimulated much interest in this novel class of RNAs and was followed by ~50 years of experiments that contributed to their characterisation. The first tRNA sequence was determined in 1965 by Holley and coworkers [2], who also speculated about possible double strand arrangements and proposed what would have been established as the common cloverleaf structure of tRNAs. A breakthrough came in 1966, when Francis Crick published 'The Wobble Hypothesis', [3] a theory suggesting that the increased flexibility of base-pair interactions between the first nucleotide of the tRNA anticodon and the third nucleotide of the mRNA codon could explain the degeneracy of the genetic code.

With tRNAs emerging as central adapters of translation, a lot of interest developed around the mechanism of tRNA transcription. Nevertheless, it was not until 1974 that RNA polymerase III (Pol III) was recognised as the enzyme responsible for the synthesis of tRNAs and 5S rRNAs [4], based on the previous identification of three distinct forms of RNA polymerases showing different salt requirements and α-amanitin sensitivity [5, 6]. In the following years, a wealth of experiments contributed in a piecemeal manner to our understanding of Pol III transcriptional units. Biochemical approaches revealed that Pol III was driven by a small number of general transcription factors onto four different types of promoters consisting of internal or external recognition elements {reviewed in [7]} and terminated transcription at a simple run of $\geq$4 thymidine residues on the coding DNA strand [8]. In parallel, Pol III was found to provide the cell with a few additional non-coding RNAs (ncRNAs), such as the 5S rRNA, 7SK snRNA, U6 snRNA and others [9].

After many years of exciting discoveries, the main features of the Pol III machinery had been decoded, revealing a straightforward promoter organisation that contrasted with the outstanding diversity of its Pol II counterpart. tRNAs were considered to be in-excess molecules playing a marginal role as adaptors in protein synthesis, but not active regulators of

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disease or metabolism. This notion changed in recent years, when 'omics' technologies took the functional evaluation of Pol III transcripts on a completely new dimension. This review explores some of the recently emerged links between tRNA, metabolism, and disease, with a focus on high-throughput technologies that contributed to our current understanding of tRNA biology (Table 1).

The evolving Pol III landscape: From tRNA gene predictions to genomic approaches

The introduction of computational algorithms to screen the prokaryotic and eukaryotic genomes for conserved Pol III elements marked the transition from model-gene to multi-gene studies. Pol3scan successfully contributed to the definition of the first tDNA repertoire in *Saccharomyces cerevisiae* [10, 11], and was later implemented with secondary-structure prediction and covariance model algorithms into tRNAscan-SE [12], a fast-running program detecting putative Pol III-transcribed genes with higher sensitivity and specificity. tRNAscan-SE 2.0 outputs are currently deposited into GtRNAdb 2.0 (gtrnadb.ucsc.edu), an online database which includes over 367.000 tRNA gene predictions from 4.371 genomes and combines sequence information with non-canonical features such as single nucleotide polymorphisms and differential expression profiles [13].

ChIP-seq reveals Pol III binding dynamics in multiple eukaryotes

While GtRNAdb 2.0 is an excellent platform to screen for potential Pol III-transcribed tDNAs, it cannot be considered as an alternative to the systematic identification of Pol III targets by biochemical or genetic approaches. Genome-wide analyses to identify in vivo Pol III binding sites were first carried out in *S. cerevisiae* by the mean of chromatin immunoprecipitation (ChIP). With this approach, DNA is cross-linked to Pol III or its general transcription factors (GTFs), immunoprecipitated, and subjected to microarray {ChIP-chip [14–16]} or next generation sequencing {ChIP-seq [17]} analysis. These experiments revealed Pol III binding at the vast majority of known targets and an overall correlation with transcription as determined by measurement of the corresponding RNA levels. Surprisingly, only a small number of novel targets were identified in the yeast genome [15], including eight Extra TFIIIC (*ETC*) loci bound solely to TFIIIC at the exclusion of other Pol III components. Six potential Pol III genes of unknown function have recently been revealed in *S. cerevisiae* (tRNA-like transcripts, *TLT*) by combining in vivo UV crosslinking and analysis of cDNA (CRAC) [18] with immunoprecipitation of the largest yeast Pol III subunit, Rpo31 [19].

Compared to lower eukaryotes, the functional annotation of large and complex mammalian genomes makes mapping of Pol III reads a much more complicated task, which was only recently addressed in a multiplicity of ChIP-seq studies [20–31]. Thanks to the gradual improvement in sequencing technologies and downstream analysis platforms, Pol III and its general transcription factors Brf1, Brf2, Bdp1 and TFIIIC were mapped in both the mouse and human genomes. These analyses revealed an unexpectedly low number of new targets and turned out to hit genomic regions corresponding mainly to known Pol III loci {reviewed in [32]}. The leap in data volume from the Illumina GAIIX to the ~300 GB per flowcell of the HiSeq 2000/2500 platform did not result in a burst of newly identified targets, suggesting that further increasing the sequencing depth will not bring many new players into the game.

Among the non-canonical targets revealed by genome-wide experiments, tDNA-like and SINEs were by far the most abundant, the latter including mostly B2 elements in the mouse and 7SL-derived Alu elements in humans [26, 27, 33]. A comprehensive study comparing multiple human cell lines identified a total of 162 high-confidence Alus bound by Pol III factors [33], six of which were defined as transcriptionally active by an independent screening of ENCODE RNA-seq datasets [34]. Only 24 Alus were bound to Pol III in more than one cell line [33], supporting the hypothesis that Alu elements are controlled by genetic or epigenetic features limiting their expression to a certain state or tissue. As suggested by a recent work combining epigenetic studies with ChIP-bisulphite-sequencing (ChIP-BS-Seq) to map the methylation state of Pol III-bound Alus, it is the methylation of H3K9, rather than DNA, that suppresses accessibility of SINEs to the transcriptional machinery [35]. Among the novel Pol III loci, most interesting was the identification in human cells [24] and mouse liver [26] of a conserved antisense MIR (Mammalian Interspersed Repeat) located in the first intron of the *POLR3E* gene, which encodes the RPC5 subunit of Pol III. Pol III occupancy of the MIR might create a roadblock for Pol II transcription of the *POLR3E* gene and provide the cell with a negative feedback loop that helps reduce stochasticity in Pol III protein levels [26].

A large fraction of Pol III loci is silent

One of the most unexpected findings of ChIP-seq profiling in human cells was the large number of annotated loci devoid of Pol III [20, 22, 24, 30], a situation that strictly differs from the uniform binding pattern observed in yeast [14–17]. In immortalised human fibroblasts (IMR90hTert) grown under multiple conditions, Pol III was absent from ~32% of annotated loci [30], suggesting that a large fraction of Pol III genes is in a permanently repressed state. While cell-type specific differences have been observed [20], these data indicate a much narrower exploitation of the Pol III repertoire than originally thought, possibly owing to genetic or epigenetic features which differentiate Pol III-free genes from their highly occupied counterparts [26, 30]. Interestingly, Pol III-free genes present poor DNaseI hypersensitivity [36], which reflects the poor accessibility of these chromatin regions.

ChIP-exo: A ChIP-based tool for high-resolution mapping of Pol III components

ChIP-based approaches proved to be invaluable tools in defining target sites on the genomic scale, but their spatial

Table 1. Overview of high-throughput strategies applied to the study of Pol III transcription and tRNA modifications

Omics	High-throughput strategy	Refs.
Genomics	**ChIP-chip**, **ChIP-seq.** Chromatin immunoprecipitation-based methods to map genome wide the DNA binding site of a protein of interest or epigenetic mark.	[14–17, 20–31]
	ChIP-exo. Similar to ChIP-seq, but adding λ exonuclease digestion to achieve near single nucleotide resolution.	[40]
	DamIP-seq. High-throughput strategy to map protein-DNA transient interactions. The protein of interest is fused with *E.coli* Dam, so that a permanent methyl mark is left on the DNA at target loci.	[30]
	BioGRO. Biotin-based genomic run-on to map elongating RNA polymerases along the genome at ~50 bp resolution, starting from permeabilized cells.	[46]
	NET-seqa. It generates native elongating transcripts profiles at single nucleotide resolution to precisely reveal the strand-specific position of transcriptionally engaged RNA polymerases across the genome.	[42]
	CRAC. UV-crosslinking of nascent RNAs to RNA polymerases and analysis of cDNA to map genome-wide binding sites of the elongating enzyme at ~40–50 bp resolution.	[19]
	DNaseI-seq. It takes advantage of the hypersensitivity of cis regulatory elements to DNaseI digestion to provide a quantitative measure of chromatin accessibility.	[36]
	Bisulfite sequencing (BS-seq) and ChIP-BS-seq. A NGS strategy to identify 5-methylcitosine at single base pair resolution in DNA (BS-seq) or protein-bound DNA (ChIP-BS-seq).	[35]
Transcriptomics	**tRNA microarray.** tRNAs are hybridized to DNA probes mechanically spotted on a solid surface. Limited to 8-nt resolution, unless the oligo template is chemically modified to allow detection of single-base difference between tRNA isoacceptors (though it might not distinguish specific tRNAs within the same group of isoacceptors). A high-throughput approach based on hybridization and quantification of PCR-amplified tRNA-specific probes has been implemented.	[60, 72]
	tRNA-seq. Standard two-step ligation protocol for the conversion of tRNA to cDNA.	[87]
	ARM-seq, DM-tRNA-seq. These techniques take advantage of the dealkylating ability of *E. coli* AlkB to remove some of the roadblocks that interfere with cDNA synthesis. Improve the quantitative analysis of tRNA expression in deep sequencing studies.	[85, 86]
	tRNA-HydroSeq. tRNAs are partially hydrolyzed in alkaline conditions to facilitate deep sequencing of complex secondary structures.	[82]
	neusRNA-seq. Newly synthesized small RNAs (<400 nt) are labelled with a nucleotide analog (5-ethynyl uridine) to detect quantitative changes in the transcript population in response to various stimuli.	[30]
	cp-RNA-seq. It selectively amplifies RNAs containing a 2′,3′-cyclic phosphate at their 3′-end, such as U6 snRNA, 7SK snRNA, 5′-tiRs and SHOTRNAs.	[95]
	CLIP-seq (and variants HITS-CLIP, PAR-CLIP, iCLIP). A set of techniques based on crosslinking and immunoprecipitation to study, transcriptome-wide, in vivo binding sites of a given protein on RNA.	[93]
	CLASH. A method to identify RNA-RNA interactions through UV-crosslinking and immunoprecipitation of an interacting protein.	[92]
Epitranscriptomics	**tRNA microarray.** Post-transcriptional modifications can be identified based on the different hybridization capacity of modified versus unmodified tRNAs.	[84]
	Standard small RNA-seq. With a bioinformatics approach, it can be used to study post-transcription modifications at the precursor tRNAs level.	[80, 100, 101, 108]
	ARM-seq. The property of certain chemical modifications to induce RT roadblocks can be exploited to profile m^1A, m^3C and m^1G modified ribonucleosides in nuclear and mitochondrial tRNAs.	[85]
	ψ-seq, ICE-seq, RiboMeth-seq. High-throughput methods that use chemical reagents to convert RT-silent modifications (pseudouridine for ψ-seq, inosine for ICE-seq and 2′-O-methylation for RiboMeth-seq) in RT roadblocks.	[102, 103, 106, 110, 111]
	m6A-seq, m6A-miCLIP, m^1A-ID-seqb. Modified RNAs are captured by immunoprecipitation with an antibody that specifically recognizes m^6A or m^1A-containing ribonucleosides. m^6A-miCLIP is a single base resolution improvement over m6A-seq.	[116, 117, 120]
	m^5C profiling (RNA BS-seq, Aza-IP, miCLIP). Genome wide m^5C maps can be obtained by RNA bisulfite sequencing (RNA BS-seq), based on the same principle that m^5C in RNA are insensitive to bisulfite treatment. New methods (Aza-IP, miCLIP) enrich the RNA target of m^5C-RNA methyltransferases before directional cDNA sequencing.	[112–115]
	Quantitative mass spectrometry (MS). MS approaches can detect the full range of modifications in the global tRNA pool. Sequence mapping of modified nucleotides identified via MS is currently being implemented.	[127, 128]

[a]Pol III occupancy have not been specifically investigated in these works, but the authors detected reads mapping to Pol III genes and suggest that NET-seq is amenable to the study of the three RNA polymerases.
[b]While not yet implemented to detect m1A in Pol III transcripts, the library preparation strategy is compatible with this application.

resolution is limited by the length of sheared chromatin fragments. Nevertheless, local maxima in ChIP-seq data have been successfully used to position BDP1 and BRF1, two components of the general transcription factor (GTF) TFIIIB, at ~14–27 bp upstream of the TSS of tRNA genes [24], consistent with previous footprint analyses [37, 38]. Higher resolution maps have been obtained by ChIP-exo, an evolution of the ChIP-seq procedure, where cross-linked DNA immunoprecipitates are further degraded by a lambda (λ) exonuclease to achieve near single-base resolution [39]. This strategy was mostly applied to Pol II and its GTFs, but was also used to reveal TBP binding ~21 bp upstream of 386 tRNA genes in human K562 cells [40] and has the potential to precisely map the remaining core transcription factors of the Pol III apparatus.

Tracking Pol III transcription: Pol III occupancy as a proxy of pre-tRNAs expression

With the publication of the first ChIP-seq datasets, Pol III occupancy profiles have been established as a measure of transcriptional activity. However, given the discrepancy between RNA-seq and ChIP-seq profiles at numerous loci, stalling of Pol III was proposed [23], based on similar conclusions emerging from Pol II studies. Indeed, very generalised pausing at protein-coding genes had been suggested by genome-wide profiles revealing an uneven distribution of Pol II, with signal accumulating near the transcription start sites (TSSs) of certain gene bodies [41]. Promoter-proximal pausing of Pol II was later confirmed by NET-seq (Native Elongation Transcript sequencing) and GRO-seq (global run-on sequencing), and proposed as a mechanism to allow rapid transcriptional activation in response to environmental cues [42–44].

Pol III pausing was specifically investigated in human cells with a novel method named newly-synthesised EU-labelled small RNA-seq (neusRNA-seq); this strategy is based on the selective enrichment of pulse-labelled small RNAs generated under various conditions and provides a direct evidence of the transcriptional activity of gene-bound Pol III [30]. The good correlation between Pol III occupancy and synthesis of EU-labelled pre-tRNAs that emerged from these experiments strongly argues against the presence of unproductive, arrested Pol III. The lack of evidence for Pol III pausing suggests that, when compared to unprocessed tRNAs emerging from a given chromatin state, ChIP-seq is indeed a proxy of ongoing transcription, but not necessarily a good predictor of mature tRNA levels. Since mature tRNAs have half-lives up to 48–72 hours [45], their abundance could reflect an earlier chromatin state that does not necessarily correspond to the one captured at the moment of cross-linking; this would explain the relatively poor correlation between ChIP-seq and RNA-seq profiles that do not discriminate mature from precursor tRNAs. However, these experiments do not exclude transient Pol III pausing or slow down during the transcription cycle, for example when encountering TFIIIC or during termination. Interestingly, biotin-based genomic run-on (BioGRO) in *S. cerevisiae* revealed increased Pol III density downstream the TSS of intron-containing tDNAs, a phenomenon that was not observed at intron-less genes [46]. Pol III pausing might take place on these genes, but the increased MNase sensitivity of introns emerging from TFIIIC bootprints [47] rather suggest the formation of an intron-mediated loop that slows down elongation between the A-box and B-box. Thus, although Pol III may have to pause transiently to travel from beginning to end of its transcription units, there is presently no evidence of stable pausing. If Pol II pausing has evolved to facilitate a synchronous transcriptional response at complex promoter structures [48], the comparatively simpler organisation of Pol III promoters might have bypassed the need of an additional regulatory layer.

tRNA expression dynamics in cell physiology and disease

MAF1 drives gene-specific adaptation of Pol III transcription to environmental cues

Eukaryotic cells have developed various sensing mechanisms and signalling pathways to produce appropriate outcomes in response to changing environments, such as an accurate reorganisation of gene expression and translation dynamics [49]. A central regulator of cell growth and proliferation is the mTORC1 pathway, which integrates intracellular and extracellular signals to control energy metabolism, lipogenesis and protein synthesis. To cope with the increased protein demand that organisms face when transitioning towards optimal growth conditions, mTORC1 stimulates Pol I and Pol III activity to provide cells with sufficient amounts of rRNAs and tRNAs [50]. mTORC1-dependent regulation of Pol III transcription largely depends on MAF1, a direct mTOR substrate conserved from yeast to humans [51–53]. Whereas in yeast, Maf1 is an acute repressor affecting Pol III activity in response to stress, in mammals a recent DamIP-seq study mapping the transient interaction of MAF1 with target loci identified the protein as a chronic repressor of Pol III transcription [30]. DamIP-seq revealed that MAF1 localises at certain Pol III genes during both favorable and stress conditions, possibly to keep transcription in check when nutrients are in excess; following nutrient deprivation, stress-induced MAF1 dephosphorylation results in increased targeting of Pol III-bound genes, with a consequent decrease of the transcriptional output. Thus, in mammalian cells MAF1 functions as a stress sensor to optimise cell survival in response to environmental cues.

Considering that MAF1 directly binds Pol III to specifically repress transcription initiation or re-initiation in vitro [54], it is reasonable to expect an uniform gene response to changing environments. While former ChIP-chip studies comparing Pol III occupancy during different growth phases in *S. cerevisiae* corroborated this hypothesis [16], more recent experiments indicate that this is the exception rather than the rule. A deep sequencing approach comparing Pol III occupancy and transcription in human fibroblasts suggests a heterogeneous

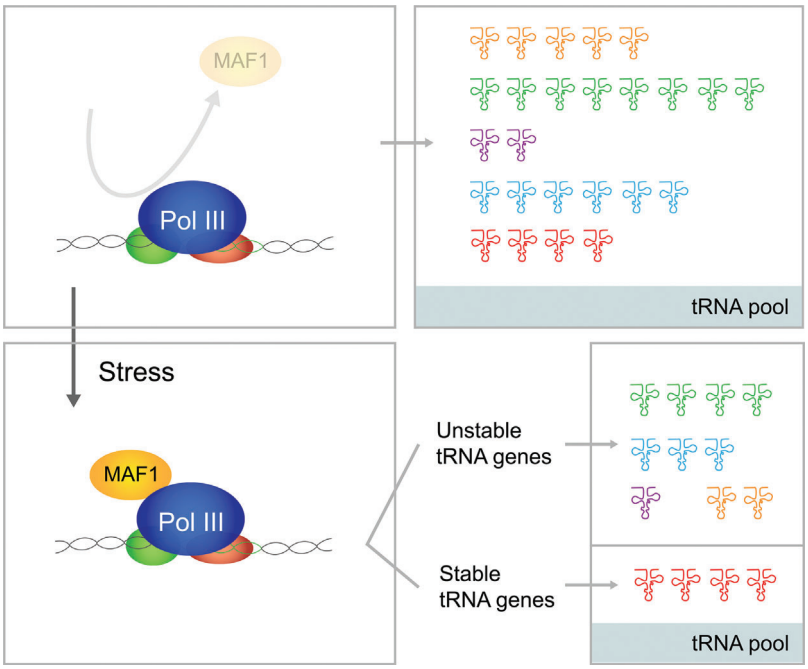

Figure 1. Pol III transcription adapts heterogeneously to stress inputs. When cells are exposed to stress such as nutrient deprivation, MAF1-targeting of transcriptionally engaged Pol III results in a different transcriptional outcome. While a certain number of genes do not respond to stress cues (stable tRNA genes) a consistent number of genes (unstable tRNA genes) adapt its expression profile. In principle, this tRNA pool could be differentially exploited to modulate expression of mRNAs enriched in cognate codons.

and dynamic adaptation of Pol III transcription to various stimuli [30]. When cells transition between optimal and suboptimal growth conditions such as absence of serum, two classes of Pol III-transcribed genes showing different levels of responsiveness are revealed. If a majority of the genes rapidly adapts to the nutrient shift, a significant fraction, including representatives of most tRNA isotypes and type III promoter loci, remains actively transcribed under both favorable and adverse conditions (Fig. 1). The mechanism driving this differential response is still unclear, but this conclusion is consistent with subsequent results obtained by high resolution CRAC in *S. cerevisiae* [19] and by ChIP-seq in mouse liver (Nicolas Bonhoure, personal communication).

The observation of dynamically responsive tRNAs suggests that phenotype modulation might be achieved through the differential exploitation of certain tRNA genes. These genes might have evolved in parallel with a subset of Pol III housekeeping genes to ensure a constant supply of essential RNAs and to avoid dramatic outcomes in environments that reduce cell viability. Yet, many questions remain; for instance, what are the factors responsible for upregulation or downregulation of specific tRNA genes? Expression of multiple isoforms or mutations in TFIIIB, TFIIIC and Pol III subunits may modulate the preference or the strength of the interaction with different tDNA isodecoders, explaining a shift in the tRNA profile under certain pathological conditions [55]. However, even within the same genetic background, Pol III transcription shows heterogeneous adaptation to external stimuli [30], suggesting that additional factors bypassing the expectedly uniform response to MAF1-mediated repression are in place. Could higher-order chromosomal structures contribute to the modulation of tRNA levels? ETC elements, identified in fission yeast [56] and higher eukaryotes, function as insulators and chromatin organiser by tethering distant sites to the nuclear periphery [56, 57]. It is tempting to speculate that, as suggested by microscopy studies [58], Pol III genes aggregate in discrete nuclear clusters for coordinated expression, mirroring co-localisation of active mRNA genes in Pol II 'transcription factories'. [59]

Adaptive translation: A new trigger of tumorigenesis

How do changes in tRNA expression influence development and disease? An increasing body of work provides evidence that changes in tRNA expression play a major role in the translational control of specific mRNAs (Fig. 2), which are often dysregulated in cancer and other pathological conditions. tRNA-specific microarrays showed elevated tRNA levels in numerous cancer types, including breast tumor samples, cancerous breast cell lines and malignant plasma cells from multiple myeloma [60–62]. Although it is not clear whether tRNA overexpression is the cause or consequence of carcinogenesis, recent work suggests that many of the mutations leading to activation of oncogenes, or to loss of oncosuppressors, require enhanced Pol III transcription to drive oncogenic transformation [63]. For example, c-Myc-driven anchorage independent growth of IHHs cells and tumor formation in mice are suppressed by tagetin inhibition of Pol III or partial knockdown of BRF1 [64, 65], providing evidence that Pol III overexpression may have functional implications in tumorigenesis. These phenotypes are mimicked by overexpression of the initiator methionine tRNA ($tRNA_i^{Met}$), which is responsible for the selection of the correct start codon during translation initiation. In human breast epithelial cells, overexpression of $tRNA_i^{Met}$ promotes increased metabolic activity and proliferation [66] and, in a recent study, a transgenic mouse carrying two extra copies of the $tRNA_i^{Met}$ gene showed enhanced tumor growth and vascularisation [67]. The latter was mainly due to $tRNA_i^{Met}$-dependent secretion of type II collagen by stromal fibroblasts, which in turn promoted angiogenesis and was generally associated with the aggressiveness of primary ovarian tumors. This is reminiscent of studies in *Drosophila* showing that elevated $tRNA_i^{Met}$ levels in the fat body promote body growth due to enhanced systemic insulin signalling [68]. While this theorise an endocrine relay for $tRNA_i^{Met}$-mediated growth triggering, overexpression of $tRNA_i^{Met}$ can also lead to changes in the global tRNA expression profile [66], suggesting that cell autonomous effects cannot be excluded.

Cellular fitness can also be affected by codon-tRNA balance [69]. Gingold et al. [70] investigated this notion and revealed that the levels of endogenous tRNAs adapt to match the codon usage of mRNAs specifically expressed in differentiated versus proliferating cells. As a result of codon adaptation to a

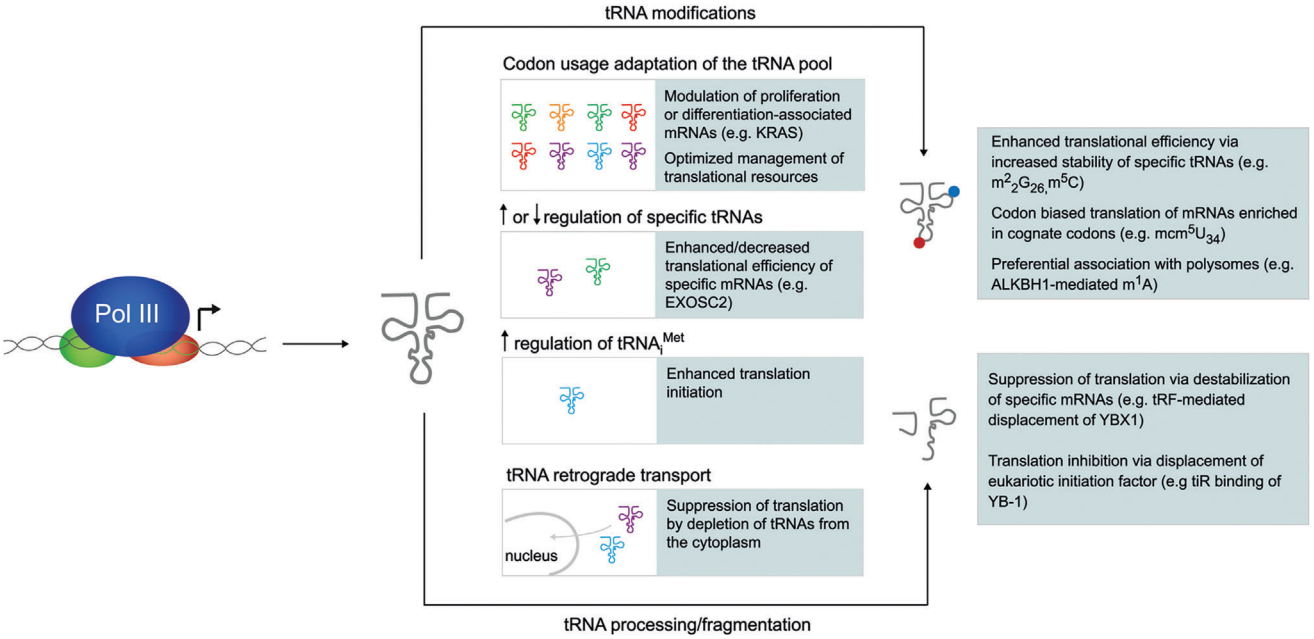

Figure 2. Mechanisms for tRNA-mediated translational control. Translation efficiency of mRNAs can be modulated by global or specific changes in the tRNA pool. Changes in tRNA expression are often linked to cancer development, either via a global increase in protein synthesis driven by $tRNA_i^{Met}$ upregulation, tRNA localization, or adaptation of the tRNA pool to the codon usage of tumor-specific mRNAs. The factors leading to differential tRNA expression are not fully understood. tRNA processing and modification can further regulate the translational output and play a role in stress adaptation and pathological disorders.

specific tRNA pool, pro-tumorigenic mRNAs might be translated more efficiently, though recent studies suggest that preferential codon usage might simply be a strategy to optimise available translational resources under certain cellular programs [71]. Specific phenotypic consequences can also be caused by the alteration of single tRNA levels. For example, upregulation of $tRNA^{Arg}$ CCG and $tRNA^{Glu}$ UUC in breast cancer cells enhances the translation efficiency of core target transcripts associated with higher metastatic capacity and invasiveness [72]. In mice, rare codon-bias influences the ability of KRAS to drive de novo tumorigenesis [73, 74]. Moreover, loss of function of one of the $tRNA^{Arg}$ UCU isodecoders specifically expressed in the mouse central nervous system is associated with increased ribosome stalling and leads to neurodegeneration in GTPBP2-deficient mice [75]. These studies support the idea that the large number of isodecoder tRNA genes in complex organisms is not fully redundant, but it is rather precisely exploited to mirror the codon usage of selected transcripts expressed in certain programmes or developmental states.

Overcoming the hurdles: Towards quantitative profiling of tRNAs and tRNA-derived fragments

Custom-made microarrays have been for several years the gold standard for the quantitative assessment of tRNAs in biological samples. This approach has proven useful in pioneering studies [76, 77], but it has been limited by its poor dynamic range, resolution {though the more recent use of chemically modified oligos allows detection of single-base differences among tRNA isoacceptors [60]} and scalability. The emergence of high-throughput technologies has opened unforeseen possibilities for biologist studying tRNA expression, offering increased sensitivity and the possibility to analyse whole transcriptomes at a fraction of the cost.

Despite numerous advantages, high-throughput technologies have been intrinsically hampered by the extensive secondary structure and abundant post-transcriptional modifications of tRNAs that interfere with cDNA synthesis, a step required in most RNA sequencing library preparation protocols. Depending on the dNTP concentrations and on the enzyme used, some of these modifications (e.g. m^1A, m^1G, m^3C, $m^2{}_2G$, 2′-O-methylation) slow down reverse transcription or result in nucleotide misincorporation during sequencing library preparation [78–80]. Other modifications, such as the 2′-3′-cyclic phosphate at the 3′-end of the human U6 snRNA, can interfere with standard sequencing methods based on ligation of RNA adapters [81]. tRNA-HydroSeq overcomes some of these hurdles via partial alkaline hydrolysis of purified tRNAs, which generates smaller fragments less likely of harboring complex secondary structures and modifications that interfere with deep RNA sequencing [82]. The method represents a considerable improvement over standard tRNA-seq protocols, but fragments carrying modifications that hamper regular reverse transcriptase (RT) activity might still be overlooked in the final data set. An alternative approach relies on the selection of tRNAs by hybridisation to specific probes, which are then used to quantify the levels of cognate tRNAs by high-throughput sequencing [72]. Although RNA hybridisation to DNA oligonucleotides would still be hindered by modifications that interfere with Watson–Crick base pairing {e.g. m^1G, m^1A, $m^2{}_2G$, i^6A and t^6A [82–84]}, this method has the significant advantage of bypassing the cDNA synthesis step. However, since it is based on the splinted ligation of probe pairs specific for each family of

mature tRNA isoacceptors, it cannot discriminate individual tRNAs within the same group of isoacceptors, nor identify precursors or truncated transcripts.

To facilitate cDNA extension, two groups took an enzymatic approach that significantly increased the number and the length of reads mapping on tRNA genes. In ARM-seq, the RNA is treated with *Escherichia coli* AlkB, a dealkylating enzyme which efficiently demethylates m^1A and m^3C before adapter binding and reverse transcription [85]. The demethylating ability of this enzyme is further exploited in DM-tRNA-seq, which combines wild-type AlkB with a D135S mutant that efficiently targets m^1G in addition to the regular substrates [86]. The DM-tRNA-seq downstream library preparation protocol is reminiscent of tRNA-seq [87], in that it also has the capacity to capture truncated tRNA fragments generated by premature RT termination at modified ribonucleotides. However, compared to the two-step ligation approach of tRNA-seq, DM-tRNA-seq bypasses the adapter ligation step by using a thermostable group II intron reverse transcriptase (TGIRT), which synthesises cDNA via a template-switching mechanism and deals more easily with highly structured tRNAs. On the other hand, owing to the binding of adapters at both ends of the template, which guarantees end-to-end sequencing, ARM-seq is the only strategy that can discriminate tRNA fragments from full-length mature tRNAs and pre-tRNAs. Thanks to this peculiarity, ARM-seq identified a large number of m^1A_{58}-modified pre-tRNAs, consistent with previous literature showing extensive pre-tRNA modification [88].

Overall, tRNA-HydroSeq, ARM-seq and DM-tRNA-seq represent powerful tools for researchers looking for more efficient and sensitive ways to quantitatively monitor changes in tRNA abundance and modifications. However, these strategies might still overlook tRNAs harboring modifications that interfere with seamless RT activity and each of them has advantages or disadvantages in terms of discriminating full-length from truncated tRNAs. This last consideration is particularly relevant at the light of recent studies revealing that tRNAs are not always the end product of Pol III transcription. High-throughput sequencing and analysis of small RNA libraries identified a novel class of ncRNAs that can be broadly divided into three different subtypes based on their primary cleavage location {reviewed in [89]}: tRNA-derived fragments (tRFs), stress-induced tRNA halves (tiRs) and sex hormone-dependent tRNA-derived RNAs (SHOTRNAs). Over the past few years, a number of studies have implicated these ncRNAs in distinct biological functions, such as tumor suppression, oncogenic activity, regulation of gene expression and protein synthesis [90–92]. For instance, CLIP-seq revealed that specific tRFs can act as tumor suppressors by displacing YBX1 from the 3′UTR of oncogenic transcripts [93]. Also, 5′-tiR^{Ala} cooperates with the translational silencer YB-1 to inhibit translation initiation by interfering with the cap-binding complex eIF4F [94]. Given the emerging roles of these tRNA fragments in normal cell physiology and human disease, several computational and technical tools have been developed to differentiate them from small RNAs that arise from degradation of longer transcripts. For example, cp-RNA-seq was developed to selectively amplify 5′-tiRs and 5′-SHOTRNAs, which contain a characteristic 2′,3′-cyclic phosphate generated by angiogenin-specific cleavage in the anticodon loop of mature tRNAs [95].

Epitranscriptomics: The new frontier of tRNA research?

tRNA modifications provide a new layer of translational control

In eukaryotes, nuclear and mitochondrial-encoded tRNAs carry a myriad of post-transcriptional chemical modifications (http://modomics.genesilico.pl/; http://www.genesilico.pl/rnapathwaysdb/). Some of these modifications are crucial for correct tRNA folding and stability, while others can have a dramatic effect on the decoding capacity of certain tRNAs (Fig. 2). For instance, the tRNA modification N2,N2-dimethylguanosine-26 ($m^2_2G_{26}$) is differentially incorporated into tRNAs due to a limiting amount of Trm1, so that in case of tRNA overproduction a subset of tRNAs becomes $m^2_2G_{26}$ hypomodified in response to saturation of the modification apparatus. Under conditions that decreases RNA polymerase III activity in a *maf1*-dependent manner, such as serum starvation or rapamycin treatment, a subset of tRNAs shows increased $m^2_2G_{26}$ modification [82]. This phenomenon, which is conserved from yeast to humans, has the effect to stabilise correctly folded tRNAs, affecting the translational efficiency of certain mRNA codons and potentially proteome composition.

The wobble nucleoside is well known for its ability to decode multiple synonymous codons and modifications at this position play a prominent role in the stress response pathway and human diseases [45, 96, 97]. Trm9-catalysed modifications at wobble U_{34} (mcm^5U_{34} and $mcm^5s^2U_{34}$) of specific tRNAs regulate protein expression via codon-biased translation of mRNAs enriched in cognate codons [98]. Loss of U_{34} modifications slows down translation elongation and elicits proteins aggregation, impairing the ability of cells to maintain proteome integrity during stress [99].

High-throughput methods for detection of tRNA modifications: Bioinformatics, chemical and antibody-based strategies

Early methods for detection and quantitation of RNA modifications were based on the differential hybridisation capacity of modified versus unmodified RNAs. The principle has been implemented in a microarray system and successfully employed to identify a number of tRNA modifications in *S. cerevisiae* [84]. An alternative approach relies on the ability of certain RNA modifications to induce misincorporation of specific nucleotides when read by reverse transcriptase. Since standard small RNA-seq library preparation protocols require cDNA synthesis, with the right bioinformatics pipeline it is possible to identify tRNA modifications that affect RT activity. Based on the observation that specific nucleotides misincorporation occur at higher frequency than the standard error rate, several authors identified known and novel tRNA post-transcriptional modifications by comparing small RNA-seq data sets with the underlying genomic sequence [80, 100, 101].

A similar principle is applied to the identification of known tRNA modifications via tRNA-HydroSeq, which are detected as misincorporations in the final data set and further validated with genetic studies [82].

An advantage of the bioinformatics strategy is the possibility to retrospectively validate, against existing RNA-seq data sets, tRNA modification profiles obtained with other tools or specific antibodies. Nevertheless, this approach is limited to modifications that modulate RT activity. For RT-silent modifications, such as pseudouridine (Ψ), 2′-O-methylated nucleosides and others [78], specific chemical treatments that add a bulky group to existing modifications must be further applied. For example, the CMC reagent creates with pseudouridine a stable adduct which is not hydrolysed at basic pH. This property was implemented in Ψ-seq for transcriptome-wide mapping of pseudouridinylation, which revealed Ψ-residues in numerous transcripts including tRNAs and the 7SK snRNA [102, 103].

2′-O-methylation is another widespread modification present in a plethora of RNAs. Defective 2′-O-methylation in the anticodon loop of the $tRNA^{Phe}$ isotype contributes to X-linked intellectual disability [104] and 2′-O-methylation of G_{18} in certain bacterial tRNAs can suppress activation of the innate immune system [105]. RiboMeth-seq [106] takes advantage of the differential alkaline-cleavage properties of nucleotides adjacent to a 2′-O-methylated site and provides a promising avenue for detection of modified tRNAs from limiting biological and clinical samples.

Inosine is a modified nucleoside produced by adenosine deamination and detected as an A-to-G substitution in cDNA. Based on the widespread differences between the RNA and DNA sequences, standard RNA-seq experiments have been used to identify numerous instances of A-to-I editing [107], and revealed that inosine is incorporated both at the pre-tRNA and mature tRNA level [108]. While this bioinformatics approach is extremely powerful, great care must be taken in the downstream analysis to discriminate true modifications from potential false positives [109]. Additional biochemical methods that more directly map inosine modifications in the RNA strands should also be employed for further validation, such as the recently proposed Inosine Chemical Erasing followed by sequencing (ICE-seq). Studies using ICE-seq found many instances of A-to-I editing in intronic Alu elements, leading to the interesting conclusion that intronic editing prevents aberrant exonisation of Alu sequences in the mature mRNA [110, 111].

Among the most studied tRNA post-transcriptional modification is 5-methylcytosine (m^5C), which promotes protein synthesis by protecting tRNAs from angiogenin-mediated cleavage [112]. RNA bisulfite sequencing (RNA BS-seq), a protocol based on the selective chemical deamination of cytosine to uracil by bisulfite treatment, was the first NGS method to provide a single nucleotide-resolution map of m^5C in tRNAs and other Pol III transcripts [112, 113]. To overcome some of the intrinsic RNA BS-seq caveats leading to false positives detection, such as the presence of conversion-resistant cytosines embedded in double stranded RNA stretches and the inability to discriminate m^5C from 5-hydroxymethylcytosine (5hmC), additional methods have been developed. 5-azacytidine-mediated RNA immunoprecipitation {Aza-IP [114]} and methylation iCLIP {miCLIP [112, 115]} selectively capture m^5C-modified RNAs thanks to the covalent bond formed with the m^5C RNA methyltransferases NSUN2 or DNMT2. Base-pair resolution maps obtained with these two strategies, combined with RNA BS-seq output, offer a high-fidelity profile of the m^5C epitranscriptome.

One of the main achievements in the field of RNA modifications was the development of antibodies that bind directly to modified nucleosides. For instance, low resolution transcriptome-wide maps of N6-methyladenosine (m^6A) were obtained thanks to anti-m^6A immunoprecipitation and deep-sequencing (m^6A-seq) of captured RNA fragments {reviewed in [116]}. Recently, a single nucleotide resolution map of m^6A (m^6A-miCLIP) was achieved, thanks to the ability of certain antibodies to leave a unique signature mutation when UV-crosslinked to the modified nucleoside [117]. While m^6A is absent from eukaryotic tRNAs, the presence of m^6A within an mRNA codon can slow down cognate tRNA decoding and translation elongation [118], potentially perturbing accurate protein folding [119].

A similar high-throughput methodology named m^1A-ID-seq recently enabled the detection of N^1-methyladenosine (m^1A) across human mRNAs, revealing a dynamic adaptation of the m^1A methylome to serum starvation and oxidative stress [120]. m^1A occurs in both mRNAs and tRNAs and has important implications in cell growth and protein synthesis. m^1A_{58} protects pre-$tRNAi^{Met}$ from degradation by the nuclear surveillance pathway [88, 121–123] and m^1A-modified tRNAs preferentially associate with polysomes to support translation via an ALKBH1-dependent mechanism [124]. Since m^1A-ID-seq was developed on a tRNA-depleted RNA fraction [120], these data are currently of limited use to tRNA biologist. However, the procedure could be easily adapted to include tRNAs, providing a welcomed addition to techniques (ARM-seq, DM-tRNA-seq, tRNA-HydroSeq and microarrays) that are currently used to identify high-confidence m^1A sites in tRNAs.

Looking ahead: Mass spectrometry and direct RNA sequencing for global profiling of the Pol III epitranscriptome

Despite technological advancements, a truly comprehensive strategy for precise quantification and characterisation of Pol III-derived transcripts is still much needed. The golden standard would be a method coupling direct RNA sequencing with detection of post-transcriptional modifications, similar to the SMRT (Single Molecule, Real-Time) sequencing technology [125] which is routinely applied to DNA templates. Recent advances have already proven the feasibility of direct RNA-seq {[126], https://cws.nanoporetech.com/publications/}, which hopefully will be made available on a commercial scale to the broader scientific community. Besides NGS-based mapping, mass spectrometry (MS) represents an alternative, reliable technique to detect post-transcriptional modifications in any given RNA. An MS-based approach was used to quantify the majority of tRNA modifications in yeast [127], but the technique has been traditionally limited by the inability to

place a certain modification within its sequence context. Novel approaches combining RNase digestion of tRNAs with liquid chromatography tandem mass spectrometry (LC-MS/MS) for sequence attribution [128] promise exciting developments in the near future.

Conclusions and prospects

In recent years, a growing number of studies have focused on the implications of tRNAs and other Pol III transcripts in disease and homeostasis. The discovery of differential tRNA expression, the link between codon usage and the abundance of the corresponding tRNA, and the impact of tRNA modifications on translational control, were made possible by the complementation of traditional biochemical studies with omics technologies. To understand the mechanisms linking Pol III with phenotype modulation, I suggest that at least four questions will need to be addressed in the near future; (i) What specifies MAF1 recruitment on tRNA genes and how does this contribute to the selective activation or repression of target genes? (ii) Is translational control by specific tRNAs a widespread mechanism that applies to more cancer types and diseases? (iii) Is there a crosstalk between tRNAs, tiRs and tRFs, and how does this impact on pathological outcomes? (iv) What is the impact of reversible tRNA modifications on tRNA expression, tRNA processing and translational efficiency? The development of refined omics technologies will greatly help to answer these outstanding questions.

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References

1. **Hoagland MB, Stephenson ML, Scott JF, Hecht LI**, et al. 1958. A soluble ribonucleic acid intermediate in protein synthesis. *J Biol Chem* **231**: 241–57.
2. **Holley RW**. 1965. Structure of an alanine transfer ribonucleic acid. *JAMA* **194**: 868.
3. **Crick FH**. 1966. Codon-anticodon pairing: the wobble hypothesis. *J Mol Biol* **19**: 548–55.
4. **Weinmann R, Roeder RG**. 1974. Role of DNA-dependent RNA polymerase 3 in the transcription of the tRNA and 5S RNA genes. *Proc Natl Acad Sci USA* **71**: 1790–4.
5. **Roeder RG, Rutter WJ**. 1969. Multiple Forms of DNA-dependent RNA polymerase in eukaryotic organisms. *Nature* **224**: 234–7.
6. **Kedinger C, Gniazdowski M, Mandel JL, Gissinger F**, et al. 1970. α-Amanitin: a specific inhibitor of one of two DNA-dependent RNA polymerase activities from calf thymus. *Biochem Biophys Res Commun* **38**: 165–71.
7. **Orioli A, Pascali C, Pagano A, Teichmann M**, et al. 2012. RNA polymerase III transcription control elements: themes and variations. *Gene* **493**: 185–94.
8. **Bogenhagen DF, Brown DD**. 1981. Nucleotide sequences in Xenopus 5S DNA required for transcription termination. *Cell* **24**: 261–70.
9. **White RJ**. 2011. Transcription by RNA polymerase III: more complex than we thought. *Nat Rev Genet* **12**: 459–63.
10. **Pavesi A, Conterio F, Bolchi A, Dieci G**, et al. 1994. Identification of new eukaryotic tRNA genes in genomic DNA databases by a multistep weight matrix analysis of transcriptional control regions. *Nucleic Acids Res* **22**: 1247–56.
11. **Percudani R, Pavesi A, Ottonello S**. 1997. Transfer RNA gene redundancy and translational selection in *Saccharomyces cerevisiae*. *J Mol Biol* **268**: 322–30.
12. **Lowe TM, Eddy SR**. 1997. TRNAscan-SE: a program for inproved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res* **25**: 955–64.
13. **Chan PP, Lowe TM**. 2016. GtRNAdb 2.0: an expanded database of transfer RNA genes identified in complete and draft genomes. *Nucleic Acids Res* **44**: D184–9.
14. **Roberts DN, Stewart AJ, Huff JT, Cairns BR**. 2003. The RNA polymerase III transcriptome revealed by genome-wide localization and activity-occupancy relationships. *Proc Natl Acad Sci USA* **100**: 14695–700.
15. **Moqtaderi Z, Struhl K**. 2004. Genome-wide occupancy profile of the RNA polymerase III machinery in *Saccharomyces cerevisiae* reveals loci with incomplete transcription complexes. *Mol Cell Biol* **24**: 4118–27.
16. **Harismendy O, Gendrel C-G, Soularue P, Gidrol X**, et al. 2003. Genome-wide location of yeast RNA polymerase III transcription machinery. *EMBO J* **22**: 4738–47.
17. **Kumar Y, Bhargava P**. 2013. A unique nucleosome arrangement, maintained actively by chromatin remodelers facilitates transcription of yeast tRNA genes. *BMC Genomics* **14**: 402.
18. **Granneman S, Kudla G, Petfalski E, Tollervey D**. 2009. Identification of protein binding sites on U3 snoRNA and pre-rRNA by UV cross-linking and high-throughput analysis of cDNAs. TL – 106 *Proc Natl Acad Sci USA* **106 VN-**: 9613–8.
19. **Turowski TW, Leśniewska E, Delan-Forino C, Sayou C**, et al. 2016. Global analysis of transcriptionally engaged yeast RNA polymerase III reveals extended tRNA transcripts. *Genome Res* **26**: 933–44.
20. **Oler AJ, Alla RK, Roberts DN, Wong A**, et al. 2010. Human RNA polymerase III transcriptomes and relationships to Pol II promoter chromatin and enhancer-binding factors. *Nat Struct Mol Biol* **17**: 620–8.
21. **Barski A, Chepelev I, Liko D, Cuddapah S**, et al. 2010. Pol II and its associated epigenetic marks are present at Pol III-transcribed noncoding RNA genes. *Nat Struct Mol Biol* **17**: 629–34.
22. **Moqtaderi Z, Wang J, Raha D, White RJ**, et al. 2010. Genomic binding profiles of functionally distinct RNA polymerase III transcription complexes in human cells. *Nat Struct Mol Biol* **17**: 635–40.
23. **Raha D, Wang Z, Moqtaderi Z, Wu L**, et al. 2010. Close association of RNA polymerase II and many transcription factors with Pol III genes. *Proc Natl Acad Sci USA* **107**: 3639–44.
24. **Canella D, Praz V, Reina JH, Cousin P**, et al. 2010. Defining the RNA polymerase III transcriptome: genome-wide localization of the RNA polymerase III transcription machinery in human cells. *Genome Res* **20**: 710–21.
25. **Kutter C, Brown GD, Gonçalves A, Wilson MD**, et al. 2011. Pol III binding in six mammals shows conservation among amino acid isotypes despite divergence among tRNA genes. *Nat Genet* **43**: 948–55.
26. **Canella D, Bernasconi D, Gilardi F, LeMartelot G**, et al. 2012. A multiplicity of factors contributes to selective RNA polymerase III occupancy of a subset of RNA polymerase III genes in mouse liver. *Genome Res* **22**: 666–80.
27. **Renaud M, Praz V, Vieu E, Florens L**, et al. 2014. Gene duplication and neofunctionalization: POLR3G and POLR3GL. *Genome Res* **24**: 37–51.
28. **Schmitt BM, Rudolph KLM, Karagianni P, Fonseca NA**, et al. 2014. High-resolution mapping of transcriptional dynamics across tissue development reveals a stable mRNA-tRNA interface. *Genome Res* **24**: 1797–807.
29. **Carrire L, Graziani S, Alibert O, Ghavi-Helm Y**, et al. 2012. Genomic binding of Pol III transcription machinery and relationship with TFIIS transcription factor distribution in mouse embryonic stem cells. *Nucleic Acids Res* **40**: 270–83.
30. **Orioli A, Praz V, Lhôte P, Hernandez N**. 2016. Human MAF1 targets and represses active RNA polymerase III genes by preventing recruitment rather than inducing long-term transcriptional arrest. *Genome Res* **26**: 624–35.
31. **Bonhoure N, Bounova G, Bernasconi D, Praz V**, et al. 2014. Quantifying ChIP-seq data: a spiking method providing an internal reference for sample-to-sample normalization. *Genome Res* **24**: 1157–68.

32. **Dieci G, Conti A, Pagano A, Carnevali D**. 2013. Identification of RNA polymerase III-transcribed genes in eukaryotic genomes. *Biochim Biophys Acta* **1829**: 296–305.
33. **Oler AJ, Traina-Dorge S, Derbes RS, Canella D**, et al. 2012. Alu expression in human cell lines and their retrotranspositional potential. *Mob DNA* **3**: 11.
34. **Conti A, Carnevali D, Bollati V, Fustinoni S**, et al. 2015. Identification of RNA polymerase III-transcribed Alu loci by computational screening of RNA-Seq data. *Nucleic Acids Res* **43**: 817–35.
35. **Varshney D, Vavrova-Anderson J, Oler AJ, Cowling VH**, et al. 2015. SINE transcription by RNA polymerase III is suppressed by histone methylation but not by DNA methylation. *Nat Commun* **6**: 6569.
36. **Thurman RE, Rynes E, Humbert R, Vierstra J**, et al. 2012. The accessible chromatin landscape of the human genome. *Nature* **489**: 75–82.
37. **Bartholomew B, Durkovich D, Kassavetis GA, Geiduschek EP**. 1993. Orientation and topography of RNA polymerase III in transcription complexes. *Mol. Cell. Biol* **13**: 942–52.
38. **Kassavetis GA, Bardeleben C, Kumar A, Ramirez E**, et al. 1997. Domains of the Brf component of RNA polymerase III transcription factor IIIB (TFIIIB): functions in assembly of TFIIIB-DNA complexes and recruitment of RNA polymerase to the promoter. *Mol Cell Biol* **17**: 5299–306.
39. **Rhee HS, Pugh BF**. 2011. Comprehensive genome-wide protein-DNA interactions detected at single-nucleotide resolution. *Cell* **147**: 1408–19.
40. **Pugh BF, Venters BJ**. 2016. Genomic organization of human transcription initiation complexes. *PLoS ONE* **11**: e0149339.
41. **Guenther MG, Levine SS, Boyer LA, Jaenisch R**, et al. 2007. A chromatin landmark and transcription initiation at most promoters in human cells. *Cell* **130**: 77–88.
42. **Mayer A, Di Iulio J, Maleri S, Eser U**, et al. 2015. Native elongating transcript sequencing reveals human transcriptional activity at nucleotide resolution. *Cell* **161**: 541–4.
43. **Danko CG, Hah N, Luo X, Martins AL**, et al. 2013. Signaling pathways differentially affect RNA polymerase II initiation, pausing, and elongation rate in cells. *Mol Cell* **50**: 212–22.
44. **Core LJ, Martins AL, Danko CG, Waters CT**, et al. 2014. Analysis of nascent RNA identifies a unified architecture of initiation regions at mammalian promoters and enhancers. *Nat Genet* **46**: 1311–20.
45. **Huang H-Y, Hopper A**. 2016. Multiple layers of stress-induced regulation in tRNA biology. *Life* **6**: 16.
46. **Jordan-Pla A, Gupta I, de Miguel-Jimenez L, Steinmetz LM**, et al. 2015. Chromatin-dependent regulation of RNA polymerases II and III activity throughout the transcription cycle. *Nucleic Acids Res* **43**: 787–802.
47. **Nagarajavel V, Iben JR, Howard BH, Maraia RJ**, et al. 2013. Global "bootprinting" reveals the elastic architecture of the yeast TFIIIB-TFIIIC transcription complex in vivo. *Nucleic Acids Res* **41**: 8135–43.
48. **Adelman K, Lis JT**. 2012. Promoter-proximal pausing of RNA polymerase II: emerging roles in metazoans. *Nat Rev Genet* **13**: 720–31.
49. **de Nadal E, Ammerer G, Posas F**. 2011. Controlling gene expression in response to stress. *Nat Rev Genet* **12**: 833–45.
50. **Laplante M, Sabatini DM**. 2013. Regulation of mTORC1 and its impact on gene expression at a glance. *J Cell Sci* **126**: 1713–9.
51. **Shor B, Wu J, Shakey Q, Toral-Barza L**, et al. 2010. Requirement of the mTOR kinase for the regulation of Maf1 phosphorylation and control of RNA polymerase III-dependent transcription in cancer cells. *J Biol Chem* **285**: 15380–92.
52. **Michels AA, Robitaille AM, Buczynski-Ruchonnet D, Hodroj W**, et al. 2010. MTORC1 directly phosphorylates and regulates human MAF1. *Mol Cell Biol* **30**: 3749–57.
53. **Boguta M, Graczyk D**. 2011. RNA polymerase III under control: repression and de-repression. *Trends Biochem Sci* **36**: 451–6.
54. **Vannini A, Ringel R, Kusser AG, Berninghausen O**, et al. 2010. Molecular basis of RNA polymerase III transcription repression by Maf1. *Cell* **143**: 59–70.
55. **Arimbasseri AG, Maraia RJ**. 2016. RNA polymerase III advances: structural and tRNA functional views. *Trends Biochem Sci* **41**: 546–59.
56. **Noma K, Cam HP, Maraia RJ, Grewal SI**. 2006. A role for TFIIIC transcription factor complex in genome organization. *Cell* **125**: 859–72.
57. **Simms TA, Dugas SL, Gremillion JC, Ibos ME**, et al. 2008. TFIIIC binding sites function as both heterochromatin barriers and chromatin insulators in Saccharomyces cerevisiae. *Eukaryot Cell* **7**: 2078–86.
58. **Pombo A, Jackson DA, Hollinshead M, Wang Z**, et al. 1999. Regional specialization in human nuclei: visualization of discrete sites of transcription by RNA polymerase III. *EMBO J* **18**: 2241–53.
59. **Sutherland H, Bickmore WA**. 2009. Transcription factories: gene expression in unions? *Nat Rev Genet* **10**: 457–66.
60. **Pavon-Eternod M, Gomes S, Geslain R, Dai Q**, et al. 2009. TRNA over-expression in breast cancer and functional consequences. *Nucleic Acids Res* **37**: 7268–80.
61. **Mahlab S, Tuller T, Linial M**. 2012. Conservation of the relative tRNA composition in healthy and cancerous tissues. *RNA* **18**: 640–52.
62. **Zhou Y, Goodenbour JM, Godley LA, Wickrema A**, et al. 2009. High levels of tRNA abundance and alteration of tRNA charging by bortezomib in multiple myeloma. *Biochem Biophys Res Commun* **385**: 160–4.
63. **Grewal SS**. 2015. Why should cancer biologists care about tRNAs? tRNA synthesis, mRNA translation and the control of growth. *Biochim Biophys Acta* **1849**: 898–907.
64. **Shukla SK, Kumar V.** 2012. Hepatitis B virus X protein and c-Myc cooperate in the upregulation of ribosome biogenesis and in cellular transformation. *FEBS J* **279**: 3859–71.
65. **Johnson SA, Dubeau L, Johnson DL**. 2008. Enhanced RNA polymerase III-dependent transcription is required for oncogenic transformation. *J Biol Chem* **283**: 19184–91.
66. **Pavon-Eternod M, Gomes S, Rosner MR, Pan T**. 2013. Over-expression of initiator methionine tRNA leads to global reprogramming of tRNA expression and increased proliferation in human epithelial cells. *RNA* **19**: 461–6.
67. **Clarke CJ, Berg TJ, Birch J, Zanivan S**, et al. 2016. The initiator methionine tRNA drives secretion of type II collagen from stromal fibroblasts to promote article the initiator methionine tRNA drives secretion of type II collagen from stromal fibroblasts to promote tumor growth and angiogenesis. *Curr Biol* **26**: 755–65.
68. **Rideout EJ, Marshall L, Grewal SS**. 2012. Drosophila RNA polymerase III repressor Maf1 controls body size and developmental timing by modulating tRNAiMet synthesis and systemic insulin signaling. *Proc Natl Acad Sci USA* **109**: 1139–44.
69. **Qian W, Yang J, Pearson NM, Maclean C**, et al. 2012. Balanced codon usage optimizes eukaryotic translational efficiency. *PLoS Genet* **8**: e1002603.
70. **Gingold H, Tehler D, Christoffersen NR, Nielsen MM**, et al. 2014. A dual program for translation regulation in cellular proliferation and differentiation. *Cell* **158**: 1281–92.
71. **Rudolph KLM, Schmitt BM, Villar D, White RJ**, et al. 2016. Codon-driven translational efficiency is stable across diverse mammalian cell states. *PLoS Genet* **12**: e1006024.
72. **Goodarzi H, Nguyen HCB, Zhang S, Dill BD**, et al. 2016. Modulated expression of specific tRNAs drives gene expression and cancer progression. *Cell* **165**: 1416–27.
73. **Lampson BL, Pershing NLK, Prinz JA, Lacsina JR**, et al. 2013. Rare codons regulate KRas oncogenesis. *Curr Biol* **23**: 70–5.
74. **Pershing NLK, Lampson BL, Belsky JA, Kaltenbrun E**, et al. 2015. Rare codons capacitate Kras-driven de novo tumorigenesis. *J Clin Invest* **125**: 222–33.
75. **Ishimura R, Nagy G, Dotu I, Zhou H**, et al. 2014. RNA function. Ribosome stalling induced by mutation of a CNS-specific tRNA causes neurodegeneration. *Science* **345**: 455–9.
76. **Dittmar KA, Mobley EM, Radek AJ, Pan T**. 2004. Exploring the regulation of tRNA distribution on the genomic scale. *J Mol Biol* **337**: 31–47.
77. **Dittmar KA, Goodenbour JM, Pan T**. 2006. Tissue-specific differences in human transfer RNA expression. *PLoS Genet* **2**: 2107–15.
78. **Basturea GN**. 2013. Research methods for detection and quantitation of RNA modifications. *Mater Methods* **3**: 1–0.
79. **Behm-Ansmant I, Helm M, Motorin Y**. 2011. Use of specific chemical reagents for detection of modified nucleotides in RNA. *J Nucleic Acids* **2011**: 408053.
80. **Ryvkin P, Leung YY, Silverman IM, Childress M**, et al. 2013. HAMR: high-throughput annotation of modified ribonucleotides. *RNA* **19**: 1684–92.
81. **Lund E, Dahlberg J**. 1992. Cyclic 2′,3′-phosphates and nontemplated nucleotides at the 3′ end of spliceosomal U6 small nuclear RNA's. *Science* (80-.) **255**: 327–30.
82. **Arimbasseri AG, Blewett NH, Iben JR, Lamichhane TN**, et al. 2015. RNA polymerase III output is functionally linked to tRNA dimethyl-G26 modification. *PLoS Genet* **11**: 1–22.
83. **Hou YM, Li Z, Gamper H**. 2006. Isolation of a site-specifically modified RNA from an unmodified transcript. *Nucleic Acids Res* **34**: 1–6.
84. **Hiley SL, Jackman J, Babak T, Trochesset M**, et al. 2005. Detection and discovery of RNA modifications using microarrays. *Nucleic Acids Res* **33**: e2.

85. **Cozen AE, Quartley E, Holmes AD, Hrabeta-Robinson E**, et al. 2015. ARM-seq: AlkB-facilitated RNA methylation sequencing reveals a complex landscape of modified tRNA fragments. *Nat Methods* **12**: 879–84.
86. **Zheng G, Qin Y, Clark WC, Dai Q**, et al. 2015. Efficient and quantitative high-throughput tRNA sequencing. *Nat Methods* **12**: 835–7.
87. **Pang YLJ, Abo R, Levine SS, Dedon PC**. 2014. Diverse cell stresses induce unique patterns of tRNA up- and down-regulation: tRNA-seq for quantifying changes in tRNA copy number. *Nucleic Acids Res* **42**.
88. **Phizicky EM, Hopper AK**. 2010. TRNA biology charges to the front. *Genes Dev* **24**: 1832–60.
89. **Kumar P, Kuscu C, Dutta A**. 2016. Biogenesis and function of transfer RNA-related fragments (tRFs). *Trends Biochem Sci* **41**: 679–89.
90. **Saikia M, Hatzoglou M**. 2015. The many virtues of tRNA-derived stress-induced RNAs (tiRNAs): discovering novel mechanisms of stress response and effect on human health. *J Biol Chem* **290**: 29761–8.
91. **Anderson P, Ivanov P**. 2014. TRNA fragments in human health and disease. *FEBS Lett* **588**: 4297–304.
92. **Kumar P, Anaya J, Mudunuri SB, Dutta A**. 2014. Meta-analysis of tRNA derived RNA fragments reveals that they are evolutionarily conserved and associate with AGO proteins to recognize specific RNA targets. *BMC Biol* **12**: 78.
93. **Goodarzi H, Liu X, Nguyen HCB, Zhang S**, et al. 2015. Endogenous tRNA-derived fragments suppress breast cancer progression via YBX1 displacement. *Cell* **161**: 790–802.
94. **Ivanov P, Emara MM, Villen J, Gygi SP**, et al. 2011. Angiogenin-induced tRNA fragments inhibit translation initiation. *Mol Cell* **43**: 613–23.
95. **Honda S, Loher P, Shigematsu M, Palazzo JP**, et al. 2015. Sex hormone-dependent tRNA halves enhance cell proliferation in breast and prostate cancers. *Proc Natl Acad Sci USA* **112**: E3816–25.
96. **Duechler M, Leszczyńska G, Sochacka E, Nawrot B**. 2016. Nucleoside modifications in the regulation of gene expression: focus on tRNA. *Cell Mol Life Sci* **73**: 3075–95.
97. **Torres AG, Batlle E, Ribas de Pouplana L**. 2014. Role of tRNA modifications in human diseases. *Trends Mol Med* **20**: 306–14.
98. **Deng W, Babu IR, Su D, Yin S**, et al. 2015. Trm9-catalyzed tRNA modifications regulate global protein expression by Codon-biased translation. *PLoS Genet* **11**: 1–23.
99. **Nedialkova DD, Leidel SA**. 2015. Optimization of codon translation rates via tRNA modifications maintains proteome integrity. *Cell* **161**: 1606–18.
100. **Iida K, Jin H, Zhu J-K**. 2009. Bioinformatics analysis suggests base modifications of tRNAs and miRNAs in Arabidopsis thaliana. *BMC Genomics* **10**: 155.
101. **Ebhardt HA, Tsang HH, Dai DC, Liu Y**, et al. 2009. Meta-analysis of small RNA-sequencing errors reveals ubiquitous post-transcriptional RNA modifications. *Nucleic Acids Res* **37**: 2461–70.
102. **Carlile TM, Rojas-Duran MF, Zinshteyn B, Shin H**, et al. 2014. Pseudouridine profiling reveals regulated mRNA pseudouridylation in yeast and human cells. *Nature* **515**: 143–6.
103. **Schwartz S, Bernstein DA, Mumbach MR, Jovanovic M**, et al. 2014. Transcriptome-wide mapping reveals widespread dynamic-regulated pseudouridylation of ncRNA and mRNA. *Cell* **159**: 148–62.
104. **Guy MP, Shaw M, Weiner CL, Hobson L**, et al. 2015. Defects in tRNA anticodon loop 2′-O-methylation are implicated in nonsyndromic X-linked intellectual disability due to mutations in FTSJ1. *Hum. Mutat* **36**: 1176–87.
105. **David R**. 2012. Immune evasion: Gm18, a bacterial "invisibility cloak. *Nat Rev Micro* **10**: 238–9.
106. **Marchand V, Blanloeil-Oillo F, Helm M, Motorin Y**. 2016. Illumina-based RiboMethSeq approach for mapping of 2′-O-Me residues in RNA. *Nucleic Acids Res* **44**: e135.
107. **Li M, Wang IX, Li Y, Bruzel A**, et al. 2011. Widespread RNA and DNA sequence differences in the human transcriptome. *Science* (80-.) **333**: 53–8.
108. **Torres AG, Pineyro D, Rodriguez-Escriba M, Camacho N**, et al. 2015. Inosine modifications in human tRNAs are incorporated at the precursor tRNA level. *Nucleic Acids Res* **43**: 5145–57.
109. **Lin W, Piskol R, Tan MH, Li JB**. 2012. Comment on "Widespread RNA and DNA sequence differences in the human transcriptome." *Science* (80-.) **335**: 1302.
110. **Sakurai M, Yano T, Kawabata H, Ueda H**, et al. 2010. Inosine cyanoethylation identifies A-to-I RNA editing sites in the human transcriptome. *Nat Chem Biol* **6**: 733–40.
111. **Sakurai M, Ueda H, Yano T, Okada S**, et al. 2014. A biochemical landscape of A-to-I RNA editing in the human brain transcriptome. *Genome Res* **24**: 522–34.
112. **Blanco S, Dietmann S, Flores JV, Hussain S**, et al. 2014. Aberrant methylation of tRNAs links cellular stress to neuro-developmental disorders. *EMBO J* **33**: 1–20.
113. **Schaefer M, Pollex T, Hanna K, Lyko F**. 2009. RNA cytosine methylation analysis by bisulfite sequencing. *Nucleic Acids Res* **37**.
114. **Khoddami V, Cairns BR**. 2013. Identification of direct targets and modified bases of RNA cytosine methyltransferases. *Nat Biotechnol* **31**: 458–64.
115. **Hussain S, Sajini AA, Blanco S, Dietmann S**, et al. 2013. NSun2-mediated cytosine-5 methylation of vault noncoding RNA determines its processing into regulatory small RNAs. *Cell Rep* **4**: 255–61.
116. **Liu N, Pan T**. 2016. N6-methyladenosine-encoded epitranscriptomics. *Nat Struct Mol Biol* **23**: 98–102.
117. **Linder B, Grozhik AV, Olarerin-George AO, Meydan C**, et al. 2015. Single-nucleotide-resolution mapping of m6A and m6Am throughout the transcriptome. *Nat Methods* **12**: 767–72.
118. **Choi J, Ieong K-W, Demirci H, Chen J**, et al. 2016. N(6)-methyladenosine in mRNA disrupts tRNA selection and translation-elongation dynamics. *Nat Struct Mol Biol* **23**: 110–5.
119. **Kim SJ, Yoon JS, Shishido H, Yang Z**, et al. 2015. Translational tuning optimizes nascent protein folding in cells. *Science* (80-.) **348**: 444–8.
120. **Li X, Xiong X, Wang K, Wang L**, et al. 2016. Transcriptome-wide mapping reveals reversible and dynamic N1-methyladenosine methylome. *Nat Chem Biol* **12**: 311–6.
121. **Saikia M, Fu Y, Pavon-Eternod M, He C**, et al. 2010. Genome-wide analysis of N1-methyl-adenosine modification in human tRNAs. *RNA* **16**: 1317–27.
122. **Anderson J, Phan L, Cuesta R, Carlson BA**, et al. 1998. The essential Gcd10p – Gcd14p nuclear complex is required for 1-methyladenosine modification and maturation of initiator methionyl-tRNA. *Genes Dev* **3**: 3650–62.
123. **Kadaba S, Krueger A, Trice T, Krecic AM**, et al. 2004. Nuclear surveillance and degradation of hypomodified initiator tRNA Met in S. cerevisiae. *Genes Dev* **18**: 1227–40.
124. **Liu F, Clark W, Luo G, Klungland A**, et al. 2016. ALKBH1-mediated tRNA demethylation regulates translation. *Cell* **167**: 1–3.
125. **Flusberg BA, Webster D, Lee J, Travers K**, et al. 2010. Direct detection of DNA methylation during single-molecule, real-time sequencing. *Nat Methods* **7**: 461–5.
126. **Vilfan ID, Tsai Y-C, Clark TA, Wegener J**, et al. 2013. Analysis of RNA base modification and structural rearrangement by single-molecule real-time detection of reverse transcription. *J Nanobiotechnology* **11**: 1.
127. **Chan CTY, Dyavaiah M, DeMott MS, Taghizadeh K**, et al. 2010. A quantitative systems approach reveals dynamic control of tRNA modifications during cellular stress. *PLoS Genet* **6**: 1–9.
128. **Ross R, Cao X, Yu N, Limbach PA**. 2016. Sequence mapping of transfer RNA chemical modifications by liquid chromatography tandem mass spectrometry. *Methods* **107**: 73–8.