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Modify or die? - RNA modification defects in metazoans

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Chemical RNA modifications are present in all kingdoms of life and many of these post-transcriptional modifications are conserved throughout evolution. However, most of the research has been performed on single cell organisms, whereas little is known about how RNA modifications contribute to the development of metazoans. In recent years, the identification of RNA modification genes in genome wide association studies (GWAS) has sparked new interest in previously neglected genes. In this review, we summarize recent findings that connect RNA modification defects and phenotypes in higher eukaryotes. Furthermore, we discuss the implications of aberrant tRNA modification in various human diseases including metabolic defects, mitochondrial dysfunctions, neurological disorders, and cancer. As the molecular mechanisms of these diseases are being elucidated, we will gain first insights into the functions of RNA modifications in higher eukaryotes and finally understand their roles during development.

Introduction

Chemical RNA modifications are a universal phenomenon of molecular biology and occur in all 3 kingdoms of life.[1] Virtually every class of RNA molecules in the cell can be post-transcriptionally modified, and certain modifications are evolutionarily conserved and may be indispensable for translation.[2] This apparent conservation has benefited our understanding of RNA modification pathways tremendously, because it allowed for the use of simple model organisms like yeast and bacteria. However, this powerful approach has left us with a puzzle. Why are so many RNA modifications evolutionarily conserved, but show no obvious phenotypes in single cell organisms when grown under laboratory conditions?

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This apparent lack of phenotypes in yeast has discouraged further studies in higher eukaryotes, preventing us from obtaining a realistic view of the function of RNA modifying pathways in developing organisms. However, it is worth taking a closer look. For example, deleting the *ELP1* gene, which is required for 5-methoxycarbonylmethyluridine (mcm^5U) and 5-carbamoylmethyluridine (ncm^5U) formation at tRNA wobble uridines (U_{34}), leads to subtle phenotypes in yeast.[3] In contrast, deleting *Ikbkap*, the *ELP1* homolog in mice, results in early embryonic lethality, revealing an essential role of these modifications in higher eukaryotes.[4] Furthermore, the prolific use of next generation sequencing and its application in genome wide association studies (GWAS) have unexpectedly linked several RNA modification genes to human diseases. While this has brought RNA modifications to center stage in certain fields, we still know very little about RNA modifying enzymes in higher eukaryotes.

In this review, we discuss recent findings that link RNA modifications to phenotypes in higher eukaryotes and consider their implication in human disease. Importantly, we will not cover capping, adenylation, deadenylation and editing, but will instead primarily focus on chemical modification of tRNA, mRNA, and to a certain degree, rRNA.

Phenotypes linked to mutations in RNA modifying enzymes have been described in many multicellular organisms (**Fig. 1**). Their analyses are complicated by several factors: First, functional orthologues of known RNA modifying enzymes have not been identified in all species.[5] Second, the modification status of many RNA molecules has been characterized only in a few metazoans,[6] and we often rely on analogies to yeast. Finally, phenotypes are complex and diverse. For an overview of phenotypes linked to RNA modification deficiencies, please refer to **Table 1** for humans, **Table 2** for mice, and **Table 3** for zebrafish and flies.

However, common themes emerge from these studies, and it is possible to separate the defects into 4 classes: i) perturbed metabolic pathways, ii) mitochondrial defects, iii) neuronal disorders, and iv) increased susceptibility to cancer. It will become apparent that this classification is somewhat artificial. Certain genes fall into more than one class and it may be a matter of preference, which one is emphasized. Furthermore, complex phenotypes can obscure classification, as it is sometimes difficult to distinguish between primary and secondary defects. Nevertheless, these categories can act as a good starting point to address the complexity.

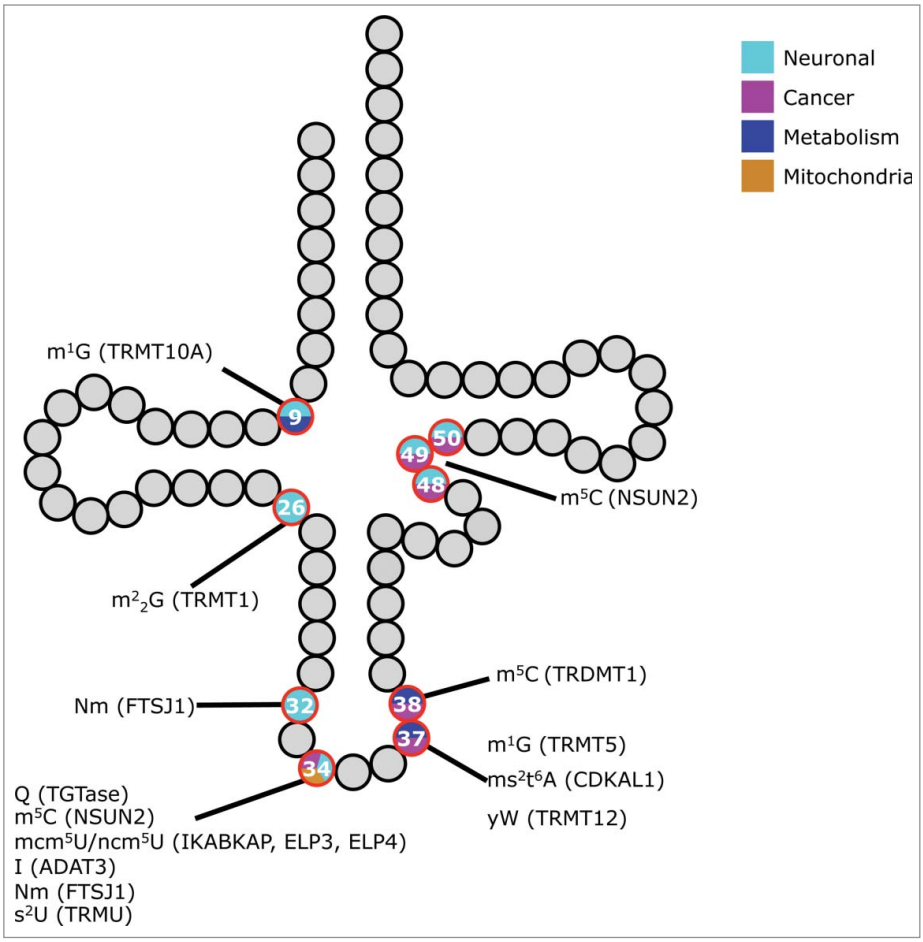

Figure 1. tRNA modification defects and phenotypes in higher eukaryotes. Schematic representation of a tRNA. Modified nucleosides that have been linked to phenotypes in higher eukaryotes are indicated as red circles. The color inside the circle denotes the type of defect observed. Chemical modifications and their causative genes (in brackets) are linked to the respective nucleoside. Gray or black residues depict nucleosides that are either unmodified or not linked to phenotypes. Abbreviations of the nucleosides follow the nomenclature of Modomics (http://modomics.genesilico.pl/).

Metabolic Defects

Metabolic defects combine changes in cellular or organismal metabolism, which lead to altered metabolite levels in the blood of patients. In the last decades, the spread of a high sugar and high fat diet has lead to an epidemic of metabolic disorders. In particular, type 2 diabetes (TIID) receives a lot of attention since it affects health and economies on a global scale.[7]

Several modification genes have been linked to metabolic defects, including the fat mass and obesity associated (FTO) gene, CDK5 regulatory subunit associated protein 1-like 1 (CDKAL1), tRNA aspartic acid methyltransferase 1 (TRDMT1), and tRNA methyltransferase 10 homolog A (TRMT10A). FTO, initially called fatso, was identified in a mouse mutant characterized by fused toes and defects in brain development and body axis control.[8] However, FTO immediately entered the limelight when independent GWAS linked single nucleotide polymorphisms (SNPs) in intron 1 of FTO to TIID and obesity.[9-12] The causative SNPs are strongly associated with early onset obesity and are predictive for average weight differences of 3 kg.[9,11,13] FTO is a 2-oxogluterate dependent oxygenase and can demethylate 6-methyladenosine (m^6A) or form N6-hydroxymethyladenosine (hm^6A) and N6-formyladenosine (f^6A) in mRNA.[14-17]

The tight regulation of m^6A methylation in mRNA is crucial during gametogenesis in Drosophila and mice and embryogenesis in Arabidopsis.[18,19] In flies and plants the gene is also essential later during development.[19,20] Thus, it is conceivable that FTO is critical in regulating mRNA by modulating their modification, which may change mRNA turnover and translation and its interaction with RNA binding proteins. For an in-depth review, please refer to. [21]

FTO is expressed in many human tissues. Expression levels in the hypothalamus are very high, which is consistent with a role in energy regulation[9,14] Mice with a FTO knockout die soon after birth as a result of a growth retardation that starts at day 2.[22,23] Both lean mass and fat mass are reduced in the neuron-specific or full knockout strains, while oxygen consumption and CO_2 production increase.[22,23] Interestingly, a dominant missense mutant, which reduces FTO activity to ~30%, develops a similar phenotype.[24] Complementary results stem from mouse models that overexpress additional cDNA copies of FTO, leading to a dose dependent increase of body weight.[25]

Analyses of the m^6A pattern in cellular transcripts identified thousands of potential mRNA targets[26-28] and different mechanisms downstream of m^6A methylation have been proposed, including dopamine receptor response and Wnt signaling.[28,29] Knockdown of FTO in zebrafish reduces brain and eye size and perturbs neural crest cell migration and cilia formation.[29] This is consistent with pathologies reported in humans. While certain SNPs were found to affect brain size of healthy individuals with no apparent damage,[30] other reports link FTO variants to mental retardation and microcephaly.[31,32]

Recently, the direct role of FTO in obesity was questioned and explained by a long-range interaction of FTO intron 1 with the promoter of the homeobox gene *IRX3*.[33] Importantly, the authors found no correlation between FTO expression levels and obesity. Even though very compelling, the described mechanism does not fully explain the phenotypes of the dominant mouse mutant and of the cDNA overexpression in mice.[24,25]

CDKAL1 was linked to TIID and obesity at the same time as FTO.[10,34-36] It is a methylthiotransferase, required for the generation of 2-methylthio-N6-threonylcarbamoyladenosine (ms^2t^6A) at

Table 1. RNA modification genes associated with disease in humans

Gene[a]	OMIM[a]	Modification	Disease	Tests[b]	Ref.
ADAT3 113179	615302	t (I)	ID	—	[123]
ELP3 55140	612722	t (mcm^5U, ncm^5U)	Amyotrophic lateral sclerosis (ALS)	—	[88]
ELP4 26610	606985	t (mcm^5U, ncm^5U)	Rolandic Epilepsy	—	[90]
FTO 79068	610966	m (m^6A=>A; hm^6A, f^6A)	TIID, ID, obesity, developmental delay	4	[9-12,31,32]
FTSJ1 24140	300499	t (Gm, Um, Cm)	Intellectual disabilities (ID)	13	[98-104]
IKBKAP 8518	603722	t (mcm^5U, ncm^5U)	Familial dysautonomia	59	[76-78]
NSUN2 54888	610916	t, m, nc, r (m^5C)	ID, developmental delay, reduced fertility	5	[106-109]
TRDMT1 1787	602478	t, m, nc, r (m^5C)	Cancer (breast), metabolism	—	[154], Om
TRMT1 55621	611669	t (m^2_2G)	ID	—	[87]
TRMT5 57570	611023	t (m^1G)	Cancer (colorectal, head and neck)	—	Om
TRMT10A 93587	–	t (m^1G)	ID, TIID	—	[126]
TRMT12 55039	611244	t (yW)	Cancer (breast, leukemia, colorectal)	1	[155,156], Om
TRMU 55687	610230	t (s^2U)mito	Mitochondriopathies	29	[124,140]
QTRT1 81890	609615	t (Q)	Cancer (lymphoma, leukemia)	4	[124,140,154], Om

t: tRNA; m: mRNA; nc: ncRNA; r: ribosome; ID: Intellectual disabilities; TIID: Type 2 diabetes; Om: Oncomine

[a] Source for Gene and OMIM-Identifiers: http://www.ncbi.nlm.nih.gov

[b]Source for Genetic Testing Registry: http://www.ncbi.nlm.nih.gov/gtr/

position 37 in the anticodon of cytoplasmic $tRNA^{Lys}(UUU)$.[37,38] Proinsulin processing is perturbed in CDKAL1 knockout mice, leading to ER stress and reduced insulin secretion in β-cells.[38,39] Reporter assays suggest that the insulin secretion defect is caused by misreading of Lysine-codons in proinsulin.[38] Human carriers of TIID alleles show reduced levels of $ms^2t^6A_{37}$, they fail to generate and to secrete insulin, and they may respond differently to pharmacological treatment of TIID.[40-44] Consistent with this, CDKAL1 alleles correlate strongly with an increased risk for coronary artery diseases, most likely as a downstream effect of TIID.[45] Furthermore, CDKAL1 was identified as a risk factor for Crohn's disease (CD) and for Psoriasis. However, the alleles associated with CD and Psoriasis are not linked to TIID.[46,47]

TRDMT1 causes more subtle phenotypes than FTO or CDKAL1. The protein was described as a DNA methylase but is in fact required to form 5-methylcytidine (m^5C) on tRNA and mRNA.[48-53] Knockouts in Drosophila, Arabidopsis and mice were indistinguishable from wild type.[48,49] However, TRDMT1 may influence cellular metabolism in unexpected ways. Studies in humans link a variant in TRDMT1 to increased folate levels, which may subsequently lower the risk of developing congenital heart diseases or spina bifida during pregnancy.[54,55] Furthermore, morpholino-mediated downregulation of TRDMT1 in zebrafish led to malformed eyes, as well as brain and liver defects.[50] Whether the more severe phenotype is explained by different requirements in zebrafish or by off-target effects needs to be seen. However, m^5C levels are important since the knockout of NOP2/Sun RNA methyltransferase family, member 2 (NSUN2) is characterized by a significant weight reduction.[56] Furthermore, the double knockout of TRDMT1 and NSUN2 in mice is synthetic lethal.[53] The double mutants are smaller at birth and die within the first days due to a feeding defect. They also exhibit severe skeletal and brain defects, as well as perturbed fat metabolism.[53] Interestingly, mouse embryonic fibroblasts derived from the double mutant accumulate tRNA fragments and show signs of reduced protein translation.[53] Similarly, TRDMT1 mutant flies contain stress induced tRNA fragments and constitutively upregulate the innate immune system.[57,58]

Table 2. Mouse models for RNA modification genes.

Gene	Target	Model	Phenotype	Ref.
IKAP (*ikbkap*)	t (mcm^5U; ncm^5U)	ki, ko, mut, ts	Embryonic lethal; growth defect; neuronal phenotype	[4,74,85,86]
ALKBH8	t (mcm^5U; mcm^5Um)	ko	No phenotype	[144]
NSUN2	t, m, nc, r(m^5C)	ko	Growth phenotype; stem cell defect; males sterile	[110,114,157]
Mterf4	r (m^5C)mito	ko, ts	Embryonic lethal; Cardiomyopathy	[71]
NSUN4	r (m^5C)mito	ko, ts	Embryonic lethal; Cardiomyopathy	[70]
TRDMT1	t, m, nc, r (m^5C)	ko	No phenotype	[49]
TRDMT1/NSUN2	t, m, nc, r (m^5C)		Lethal; developmental defects; brain phenotype	[53]
FTO	m (m^6A=>A; hm^6A, f^6A)	oe, ko, mut, ts	Lethal; metabolic changes; weight defects; developmental defects	[8,22-25]
ALKBH5	m (m^6A=>A)		Male sterility	[145]
WTAP	m, nc, r (m^6A)	ko	Embryonic lethal	[120,121]
CDKAL1	ms^2t^6A	ts	Pancreatic islet hypertrophy	[38]

Ko: knockout; ts: tissue-specific knockout; oe: overexpression; ki: knock in; t: tRNA; r: rRNA, m: mRNA, nc: ncRNA.

Table 3. Drosophila and zebrafish models for RNA modification genes.

Gene	Target	Model	Phenotype	Ref.
ELP3	t (mcm^5U, ncm^5U)	Dros mut	Neuronal phenotypes	88
		Zeb morph	Motor neuron defect	88
FTO	m (m^6A=>A; hm^6A, f^6A)	Zeb morph	Developmental defects; brain phenotype	29
METTL3	m, nc, r (m^6A)	Zeb morph	Tissue differentiation defects; brain phenotypes	118
TRDMT1	t, m, nc, r	Dros mut	No phenotype; Increased stress sensitivity	158
	(m^5C)	Zeb morph	Developmental defects; brain phenotype	50
U26, U44, U78	r (Nm)	Zeb morph	Multiple developmental defects; brain phenotype	122
WTAP	m, nc, r	Dros mut	Lethal for females	159
	(m^6A)	Zeb morph	Tissue differentiation defects; brain phenotypes	118

Dros mut: Drosophila mutant; Zeb morph: Zebrafish morpholino knockdown; t: tRNA; r: rRNA, m: mRNA, nc: ncRNA.

Mitochondrial Phenotypes

Mitochondria utilize a minimal set of 22 tRNAs to decode all 60 sense codons.[59] To achieve this, only 4 post-transcriptional modifications are required at the wobble position of 10 mitochondrial tRNAs: 5-formylcytidine (f^5C), 5-taurinomethyluridine (τm^5U), 5-taurinomethyl-2-thiouridine (τm^5s^2U), and queuosine (Q).[60] In such a fine-tuned system, incomplete wobble base modification disrupts protein synthesis and causes errors that can potentiate to mitochondrial dysfunction. Cells with mitochondrial defects generally display significantly reduced oxygen consumption and mitochondrial protein synthesis, as well as reduced ATP production, decreased mitochondrial membrane potential, and increased superoxide levels.[61,62]

A single heteroplasmic A to G replacement at nucleotide position (np) 3243 in the gene encoding for mt $tRNA^{Leu}$(UAA) is associated with diverse pathological phenotypes, including ocular myopathy, diabetes with associated deafness, and the full *mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes* (MELAS) syndrome.[61,63] Roughly 80% of all MELAS patients carry this transition whereas in approx. Ten% of the cases a T to C transition occurs at np 3271. Neither of the mutations lies at the anticodon of the tRNA. However, both mutations lead to τm^5U hypomodification of mt $tRNA^{Leu}$(UAA) that manifests in identical clinical symptoms.[63,64] A to G transition at np 8344 of the mt $tRNA^{Lys}$(UUU) gene leads to loss of τm^5s^2U modification at the wobble uridine. Patients with this deficiency suffer from *mitochondrial encephalomyopathies, myoclonus epilepsy associated with ragged-red fibers* (MERRF) syndrome, which constitutes another subgroup of mitochondrial encephalomyopathia that is clinically distinct from MELAS.[60,65]

The point mutations associated with MELAS and MERRF function as negative determinants of τm^5U biosynthesis by disrupting the tertiary structure of the tRNA,[64] thereby preventing its proper recognition by a currently unknown taurine transferase. GTPBP3, the human homolog of Mto1p/Mss1p that synthesizes 5-carboxymethyluridine ($cmnm^5U$) in yeast, is a putative candidate but conclusive evidence is lacking.[66]

Loss of the τm^5U modification in MELAS patients prevents the mt $tRNA^{Leu}$(UAA) from reading UUG codons, whereas no apparent reduction in decoding capability is observed for UUA codons.[67] Decoding dysfunctions are exacerbated by the point mutations in MERRF, where τm^5s^2U hypomodification completely abrogates the ability of mt $tRNA^{Lys}$(UUU) to read the cognate codons AAA and AAG. This defect is attributed to the complete loss of s^2U modification, a critical factor for stabilizing the codon-anticodon interactions in the ribosome.[60,67] A natural suppressor tRNA in MELAS cells can largely rescue the decoding disorder. A second mutation from G to A at np 12300 changes the anticodon sequence of mt $tRNA^{Leu}$(UAG), allowing it to read the same codons as mt $tRNA^{Leu}$(UAA).[60,61,68,69] Surprisingly, this mutant tRNA can be τm^5U modified despite a mere 42% sequence homology to mt $tRNA^{Leu}$(UAA).[69] Acquisition of the wobble modification in another isoacceptor tRNA is key to suppressing MELAS and highlights the importance of translational control through nucleoside modifications.

NSUN4 performs m^5C methylation of C_{911} in mitochondrial 12S rRNA.[70] Both NSUN4 and its cofactor MTERF4 are essential and their deletion leads to early embryonic lethality in mice.[70,71] A heart specific knockout is viable but significantly shortens the lifespan. Mitochondrial translation fails despite a significant increase of transcript levels, giving rise to nonfunctional mitochondria and the ultimately lethal phenotype.[70,71]

Neurodegenerative and Neurological Defects

Several neurological defects are linked to the absence of homologues of the ELP complex, which is required for the formation of mcm^5U_{34} and ncm^5U_{34} in 11 cytoplasmic tRNAs in different organisms.[72-74] The best understood case is familial dysautonomia (FD), also termed the Riley-Day syndrome.[75] This devastating autosomal recessive disorder severely affects neurons of the autonomic nervous system, resulting in the absence of sensory neurons. The lack of autonomic control ultimately leads to premature death mainly through cardiovascular failure or respiratory defects.[76] 99.5% of the disease alleles affect a donor splice site in the *IKBKAP* gene and lead to tissue specific skipping of exon 20 and introduction of a premature stop codon, thereby drastically reducing IKAP protein levels in neurons.[77-79] However, in non-neuronal tissues the remaining wild-type transcripts suffice to ensure cell survival.[79] Importantly, reduced levels of IKAP lead to reduced levels of xm^5U in cells of FD patients.[80] Since FD is caused by a tissue specific splicing defect, attempts to

find compounds that may correct *IKBKAP* splicing and relieve some of the symptoms have been undertaken.[81-84] Several small molecules have been identified, but only Kinetin has shown positive clinical effects.[85] Thus, FD is the only RNA modification disease for which treatment options may exist through restoration of RNA modification levels.

However, why does FD affect only sensory neurons? Are they particularly vulnerable to RNA modification defects, or are they targeted by a tissue specific splicing defect? Answers to these questions may come from animal models. The full knockout of *ikbkap* leads to lethality around embryonic day 12.5.[4] Developing embryos are small and show defects in extraembryonic tissues as well as neuronal and vascular development.[4] When a deletion allele of exon 20 is combined with a full knockout allele, the phenotypes are similar to the full knockout, suggesting that the mutant protein is non-functional.[86] A mouse model that recapitulates FD symptoms combines the exon 20 deletion with a hypomorphic allele that reduces IKAP activity to 10%.[87] These mutants are significantly smaller at birth and frequently die thereafter due to a feeding defect, reminiscent of TRDMT1/NSUN2 mutant mice.[53,87] Furthermore, the animals exhibit gastro-intestinal defects and reduction of sympathetic ganglia and other phenotypes characteristic of FD.[87] Interestingly, a tissue specific knockout in testis triggers meiotic defects and subsequent apoptosis of germ cells,[74] showing that IKAP function is essential also in non-neuronal tissues. In summary, FD is caused by reduced IKAP function in a dose dependent manner. The enzymatic nature of the ELP complex and the long half-life of tRNAs may generate sufficient levels of modified tRNA to allow for survival even at low expression levels. Thus, the reason why sensory neurons are affected may be a combination of neurons being more sensitive to perturbations together with a tissue specific splicing defect that reduces IKAP levels more in neuronal tissues than in other cells.

Not only IKAP, but almost every subunit of the ELP complex has been linked to a disease. ELP2 is a risk gene for intellectual disabilities (ID),[88] while ELP3 is associated with non-familial Amyotrophic lateral scelerosis (ALS).[89] Similarly, Drosophila mutants of ELP3 show neuronal defects and their motor neurons branch abnormally.[89] Axon length of motor neurons in zebrafish is decreased in a dose dependent manner upon knockdown of elp3,[89] and *elpc-1* and *elpc-3* mutants exhibit learning defects in *Caenorhabditis elegans*.[90] In humans, Rolandic epilepsy, a generally mild form of childhood epilepsy, has been associated with ELP4.[91,92] However, it is unclear whether ELP4 is the causal gene. Potentially, the neighboring PAX6 is controlled by regulatory elements in the introns of ELP4, as proposed for FTO and *IRX3*.[33,93] Poly, the Drosophila homolog of ELP*6*, is essential in larval development and its absence causes major metabolic changes and brain defects.[94] While the ELP complex is linked to multiple diseases, no pathologies have been associated with the *URM1* pathway, which is required for s^2U formation on 3 mcm^5-modified tRNA.[95] However, in Arabidopsis, mutants of this pathway affect root growth.[96]

FtsJ RNA methyltransferase homolog 1 (FTSJ1), a homolog of Trm7, is required for the 2'-O-ribose methylation at G34, U34, C34 and C32 of several tRNA.[97,98] It was linked to non-syndromic X-linked ID but also to general mental performance.[99-105] Most of the mutations described so far result in a loss-of-function of the protein, either by impairing the critical SAM-binding domain of the protein or by degradation of the mutant mRNA.[99,101] There are no morphological brain phenotypes in patients.[99] However, high expression levels in the fetal brain may reflect the importance of the gene during brain development.[99] Interestingly, 2 studies report that duplications of a genomic region that contains FTSJ1 lead to ID accompanied by mild dysmorphic features.[105,106] The fact that both duplication and loss of function leads to similar phenotypes may suggest that FTSJ1 levels need to be precisely controlled. To better understand this seemingly paradoxical situation, a systematic manipulation of FTSJ1 expression levels in animal models is of utmost significance. However, these models are currently not available.

Mutations in NSUN2 are linked to ID and a Dubowitz-like syndrome, characterized by mild microcephaly and congenital heart defects.[107-110] Similarly, perturbed NSUN2 function results in deficits in short-term memory and behavioral assays in mice and flies,[107] as well as reduced fertility in flies, mice and humans.[107,109,111] Expression levels in the brain are high in mice and flies, consistent with the phenotypes.[107,109,112] NSUN2 catalyzes the formation of m^5C at the wobble position 34 of $tRNA^{Leu}$(CAA), as well as positions 48-50 of several tRNA molecules. However, m^5C sites are also found in other ncRNA and mRNA, where they are enriched in the UTR and overlap with Argonaute binding sites.[52,113] Thus, most models to explain the phenotypes imply changes of RNA turnover in response to methylation of mRNA or non-coding RNAs.[113,114] A different mechanism implies tRNA as mediators of phenotypes: Interestingly, 5' tRNA-fragments accumulate in tissues and cells in NSUN2 knockout mice and both NSUN2 and the tRNA-fragments co-localize with cellular stress markers.[115] This model postulates that angiogenin, a cellular ribonuclease, generates these fragments in response to cellular stress, thereby downregulating translation rates.[115] However, it will be critical to dissect the contribution of each of the proposed mechanisms to understand the phenotypes.

SNPs in TRMT10A, the mammalian homolog of Trm10, which modifies 12 tRNA species by 1-methylguanosine (m^1G) at position 9 in yeast, were reported to cause ID, microcephaly and perturbed glucose metabolism.[115,116] Inactivation of TRMT10A by siRNA in rat β-cells sensitizes the cells against free fatty acid induced ER stress and induces apoptosis.[117]

Internal m^6A methylation of mRNA in humans is achieved by a methylase-complex, that consist of at least 3 subunits: METTL3, a methyltransferase acts in concert with it homolog METTL14 and Wilms' tumor 1-associating protein (WTAP).[118-120] WTAP targets the other subunits to mRNA but is enzymatically inactive.[119] While METTL3 and METTL14 methylate their substrates independently *in vitro*, their activity is strongly enhanced when acting cooperatively.[118] Inactivation of METTL3 or WTAP in zebrafish causes severe developmental defects characterized by smaller heads and eyes and a curved notochord accompanied by increased apoptosis.[119] Interestingly, stem cells loose their ability to maintain a pluripotent state, when

METTL3 or METTL14 are downregulated.[120] Consistently, knockout mice die during early embryonic development,[121,122] and cells of the inner cell mass fail to differentiate into mesoderm or endoderm.[122]

Malformations of the zebrafish brain are also observed in morpholino-mediated knockdowns of the 3 conserved small nucleolar RNAs (snoRNA) U26, U44 and U78, suggesting that translation defects consistently affect brain development.[123]

Finally, 2 more genes are known to cause ID: mutations in ADAT3, which is required for the formation of inosine (I_{34}) at the anticodon of several tRNAs, cause severe ID and strabismus,[124] and TRMT1, the homolog of Trm1p, is required for the formation of N2,N2-dimethylguanosine ($m^2{}_2G$).[88] However, experiments to decipher these phenotypes are still pending.

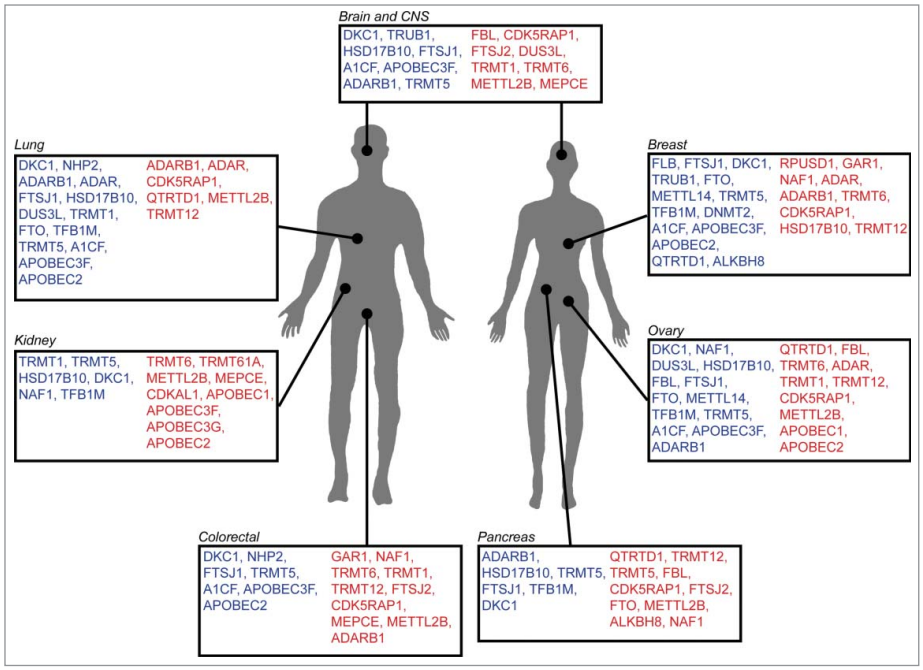

Figure 2. RNA modification genes associated with human malignancies. Schematic representation of various cancers for which increase (red) or decrease (blue) of tRNA modification gene copy number, or expression level, has been reported. (Source: Cosmic and Oncomine).

Cancer

Cancer is the collective term for a broad group of complex diseases characterized by unregulated cell growth resulting in malignant tumors. Defects in tRNA modification have been directly tied to cell proliferation and malignancy for a number of lymphomas,[125] leukemias[125,126] and carcinomas, including skin, breast, bladder, and colorectal cancers.[127-131] In the late 1960s Borek and co-workers isolated tRNA from various tumor tissues and reported elevated levels of methylated nucleosides, as well as an increased tRNA methylase activity in the cells. This suggests that tRNA hypermodification might have a specific functional role in tumor cells (reviewed in [132]). However, several subsequent studies have shown that tumor-specific tRNA species tend to be hypomodified rather than hypermodified.[133]

Insufficient queuosine Q modification in tRNA from neoplastic cells is a well-studied example of tRNA hypomodification.[125,135] Queuine, a derivative of 7-aminomethyl-7-deazaguanine, is found exclusively in the first position of the tRNA anticodon in the form of Q, mannosyl-queuosine (manQ), and galactosyl-queuosine (galQ).[135,136] Eukaryotes are unable to synthesize queuine and rely on a salvage system that acquires it as a nutrient factor. Q containing tRNAs are fully modified under normal physiological conditions, but are often hypomodified in undifferentiated rapidly growing cells, embryonic tissues, and neoplastically-transformed cells. Q deficiency is associated with lymphoma, leukemia and various kinds of tumors.[125,137,138] In fast growing tumor cells queuine is replaced by guanine at the tRNA wobble position.[134] This observation cannot be fully explained by insufficient queuine uptake, since addition of excess amounts of exogenous queuine only partially restored Q modification to tumor tRNA.[139] Furthermore, slowly growing tumors are also deficient in Q modification.[133] This suggests that Q hypomodification arises either due to functional impairment of the tRNA-guanine transglycosylase (TGTase) enzyme and subsequent lack of queuine incorporation into tRNA, or due to a queuine salvage pathway deficiency, or a combination of both.[135,136]

The physiological role of queuine remains ill defined. More recently Q modification has been implicated in protecting the cellular antioxidant defense system during malignancy.[140] It has also been suggested that replacement of queuine with guanine in the anticodon has the potential to alter mRNA codon recognition and, as a result, gene expression.[126,141] Treatment of a human promyelocytic leukemia (HL-60) cell line with 6-thioguanine, a guanine analog, resulted in growth inhibition and cell differentiation.[126] This has been attributed to altered codon recognition by tRNAs containing the guanine analogs, allowing read-through of amber (UAG) stop codons in the presence of guanine in the wobble position of $tRNA^{Tyr}$.[126,141]

Although tRNA molecules possess a considerable number of nucleoside modifications, only a handful of mammalian tRNA methyltransferases have been described to date. These include the 6 putative families of m^5C methyltransferases of which only NSUN2 and TRDMT1 have been characterized, as well as other tRNA methyltransferases including tRNA methyltransferase homolog 12 (TRMT12),[127] TRMT10A,[131] and human AlkB homolog 8 (ALKBH8).[130] Aberrant function of these methyltransferases has been linked to tumor formation. In addition, several other RNA modification enzymes have been associated with cancers in humans (please refer to **Figure 2** for a comprehensive overview).

NSUN2 is a direct target gene of c-Myc, a well-known proto-oncogene that triggers proliferation and decrease cell adhesion. Significant upregulation of NSUN2 expression was detected both in primary tumors and metastases of breast carcinomas and in malignant squamous cell carcinomas, whereas benign papillomas showed slightly lower expression levels. Knockdown of NSUN2 expression by RNAi inhibits tumor growth in squamous cell carcinoma xenografts in a dose-dependent manner,[142] making it a potential target for cancer therapy. Interestingly, the cancer drug Azacytidine, which inhibits DNA methylation, reduces m^5C levels in RNA in tissue culture cells, suggesting that RNA demethylation may contribute to the efficacy of Azacytidine.[143]

Correct function of the human tRNA-(N1G37) methyltransferase (TRMT5), which catalyzes methyl transfer from S-adenosyl methionine (AdoMet) to guanine to synthesize m^1G at position 37 in many tRNAs, is a critical determinant in preventing ribosomal frameshifts.[144] TRMT5 expression is significantly downregulated in several colorectal cancers whereas overexpression occurs in head and neck cancers (Oncomine). Another modification occurring at position 37 of $tRNA^{Phe}$ is the formation of wybutosine by TRMT12, whose expression is significantly upregulated in several breast cancer cell lines[127] and is also prevalent in leukemia, but tends to be downregulated in colorectal cancers (Oncomine).

Weak Phenotypes

Mice deleted for ALKBH8, which is required for the last step of mcm^5U synthesis in cytoplasmic tRNA, appear normal up to 20 months of age.[145] However, tRNASec, which is required for the recoding of UGA stop-codons to selenocysteine, lacks mcm^5U and 5-methoxycarbonylmethyl-2'-O-methyluridine (mcm^5Um),[145] leading to reduced levels of the selenoprotein glutathione peroxidase 1 (Gpx1), as well as glutathione peroxidase activity.[145] Nevertheless, this deficiency does not cause apparent morphological phenotypes.

Like for ALKBH8, knockout mice of ALKBH5 - a sister enzyme of FTO - overall seem anatomically normal.[146] However, males have small testes with perturbed morphology and a reduced number of dysfunctional spermatozoa. It is unclear whether the localization of ALKBH5 to nuclear speckles, and its effects for the recruitment of certain RNA processing factors and mRNA, contribute to this phenotype.[146]

Discussion

We observe diverse phenotypes when RNA modification genes are perturbed in higher eukaryotes, but we are only starting to grasp the complexity of these pathways. It appears relatively straightforward to rationalize that mitochondrial dysfunction severely affects different tissues. However, to connect other deficiencies to the observed pathologies is much more challenging. In yeast, certain modification defects can be rescued by overexpressing their respective modification target.[3,98,147] However, similar experiments have not been performed in metazoans. Hence, it needs to be shown that these phenotypes are actually caused by RNA modification defects.

Why do phenotypes differ between single cell and multicellular organisms? Yeasts and bacteria are generally grown under favorable conditions in the lab and have been selected for rapid growth under this regime for thousands of generations. Thus, they can by no means be compared to the complexity of a developing organism that coordinates multiple differentiation events in parallel. Despite a high degree of buffering and compensatory mechanisms, seemingly subtle but systematic perturbations may trigger processes that will ultimately affect cellular and organismal function.

It will be interesting albeit challenging to understand phenotypes that were identified in GWAS. Even in the well-defined case of IKAP, several attempts were necessary to generate a mouse model that recapitulates FD, suggesting that the relevant phenotype is not a full knockout. Thus, the generation of hypomorphic mutants or introduction of relevant SNPs into model organisms may be required to generate realistic disease models. Furthermore, the ratio of cognate versus near-cognate tRNA genes can potentially affect the interference by unmodified tRNAs.[148] Finally, tissue specific RNA expression or different genetic mouse backgrounds can affect the outcome of a phenotype significantly (please refer also to the checklist in **Table 4**).[149,150] All these factors need to be kept in mind, when modeling RNA modification pathologies, and it is possible that certain defects in humans can be best mimicked by studying inducible pluripotent stem cells (iPSC) and differentiated cells derived from them.

What are the potential mechanisms by which RNA modifications may affect multicellular species? First, perturbation of RNA modification pathways may reroute certain metabolites, thereby affecting other pathways and causing toxicity (**Fig. 3A**). Second, translation is likely to be affected in many modification defects of tRNA, rRNA or mRNA. However, direct evidence *in vivo* is scarce. Codon specific translation defects may lead to decreased production of individual proteins that are required either in certain cell types or under specific stress conditions (**Fig. 3B**). Third, codon specific translation defects may give rise to protein aggregates and subsequent failure of protein homeostasis (S.L unpublished

Table 4. Factors to consider:

1) Are aspects of the pathology tissue specific?
- expression of RNA targets
- expression of modification enzymes
- splicing
- turnover (RNA or protein)
- metabolite levels
2) Genome characteristics:
- tRNA abundance (gene copy number)
- Codon usage
- GC content

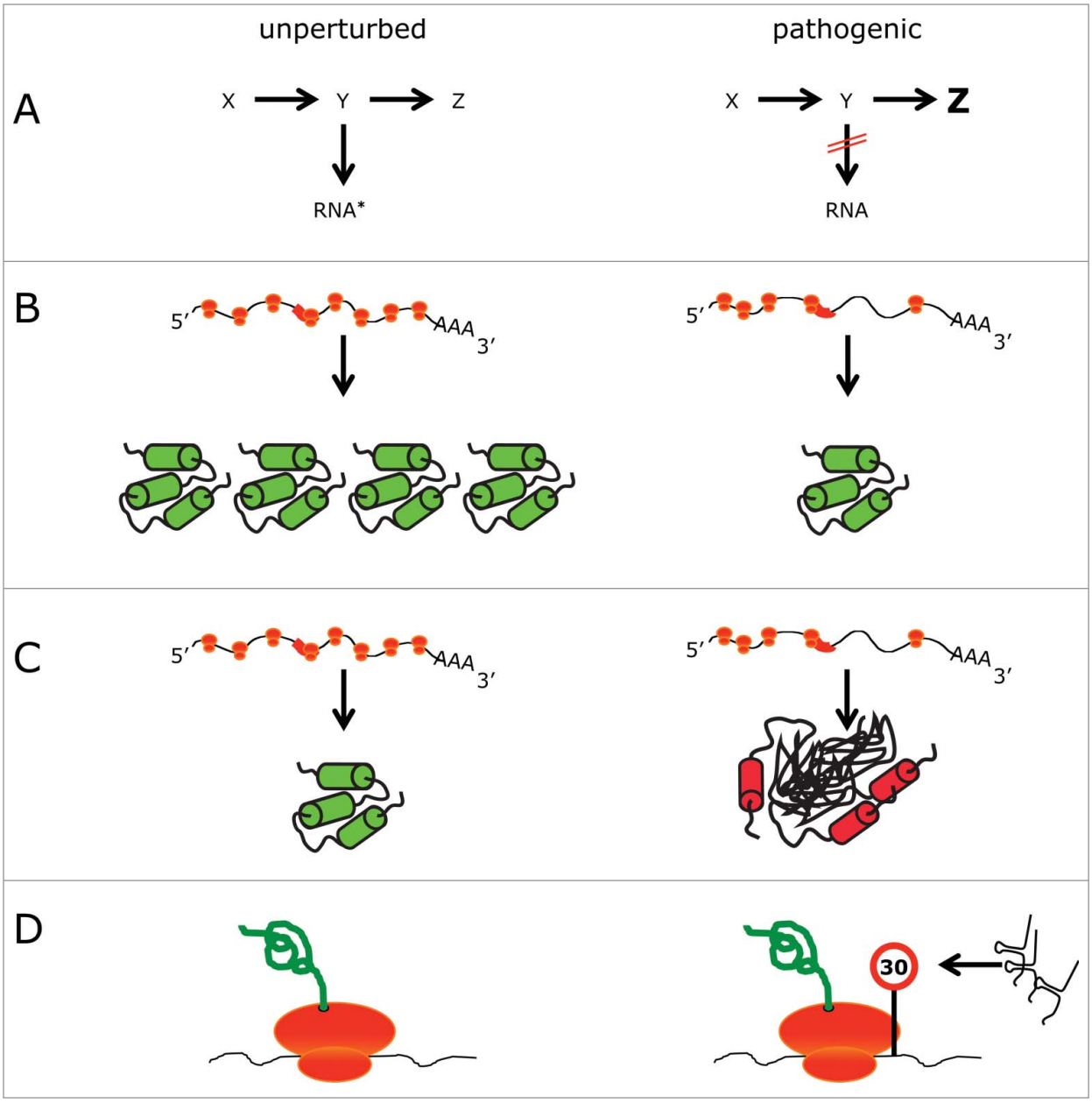

Figure 3. Models of how RNA modification defects cause phenotypes. Comparison of different scenarios in an unperturbed (left) and pathogenic (right) situation. (**A**) A metabolic pathway is blocked, leading to the absence of modified RNA (indicated by an asterisk) and the build up of a different metabolite (in this case Z). (**B**) Ribosomes (orange) translating an mRNA, which contains a region that is difficult to translate (red box). In the pathogenic situation translation is perturbed, leading to a lower amount of protein. (**C**) As in (**B**). Perturbed translation prevents the folding of some proteins into their native state resulting in perturbed protein homeostasis. (**D**) tRNA fragments cause a slowdown of translation.

results; **Fig. 3C**). Finally, the occurrence of tRNA fragments in a number of defects is an interesting observation.[53,115] In this scenario, 5' halves of tRNA reduce cellular translation rates, which perturbs the cell (**Fig. 3D**). Similarly, accumulation of tRNA fragments was also reported in patients suffering from neurodegeneration and microcephaly upon loss of tRNA kinase *CLP1* activity.[151,152] However, this effect may not be independent of protein aggregation and further analysis of the causal relationship between these phenomena will be imperative. At this point it is conceivable that tRNA fragments are both a causal factor as well as a downstream marker of cellular defects. Nevertheless, translational modulation is a likely mechanism underlying many pathologies, since a large number of mutations affect the tRNA anticodon and, in particular, the wobble position (**Fig. 1**).

Finally, why do certain alleles occur at such high frequency in the population? For example, certain FTO variants account for adverse phenotypes, such as predisposition to obesity[9-12] and

reduced brain volume,[30] while the same allele protects against depression[153] and the risk for alcohol dependence.[154] It is therefore difficult to decide whether a specific allele is problematic or not. This Janus faced character of certain alleles may explain their high prevalence in the population.

We live and perform research in exciting times. Unbiased approaches identify new links between hitherto "boring" genes and new phenotypes. At the same time the role of classical genes like FTO is questioned. It will require an enormous effort to combine new animal models and iPSC with state of the art biochemistry and molecular biology. Only when we look systematically, we will be able to understand why certain mutants "modify or die" and others don't!

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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