Attachment I

Appendix to the doctoral thesis:

ALKBH5 and FTO levels in postnatal development

Our data demonstrated that demethylases were up-regulated in hearts adapting to chronic hypoxia or fasting, both identified as cardioprotective strategies. This discovery led us to explore whether similar changes in protein levels of these demethylases occur during postnatal development, especially considering the known tolerance of newborns to H/R or I/R injury [1]. For this reason, LVs were collected from rats on postnatal days 1, 4, 7, 10, 12, 14, 18, 21, 25, 28, and 90 (P1-90). The abundance profile of both demethylases had a decreasing pattern during postnatal development (Fig. A1). However, while levels of ALKBH5 declined dramatically between P1-P4 with further indistinct changes in protein level, FTO decreased gradually throughout the investigated period.

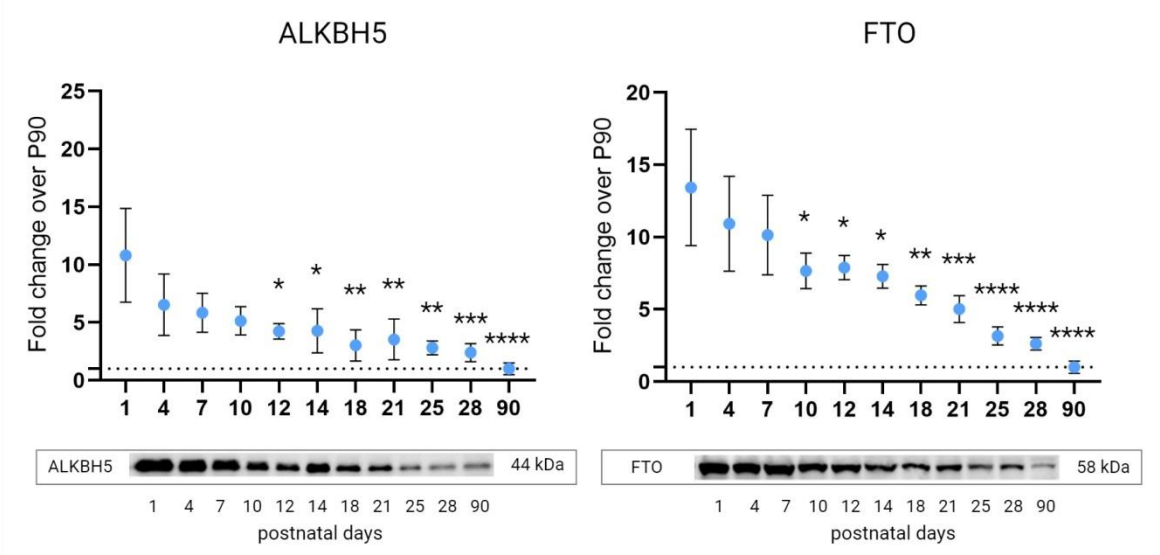

Fig. A1: Abundance profile of FTO and ALKBH5 in the left ventricles assessed by Western blot with representative Western blot membranes. Expression at P90 was established as 1. Protein loading was 15 µg. Values are means ± SD; n = 4-12; Statistical significance related to postnatal day 1: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (One-way ANOVA; Tukey's multiple comparisons test). ALKBH5 – alkB family member 5; FTO – fat mass and obesity-associated protein. Modified from Semenovykh et al. [2] (Attachment III).

Our research, as detailed in this thesis, revealed that ALKBH5 and FTO experience up-regulation in hearts undergoing adaptation to chronic hypoxia or fasting, which are linked to cardioprotective effects. This finding prompted further investigation into whether postnatal

development also witnesses alterations in the protein levels of these demethylases since an age-dependent decrease in resistance to hypoxia is well-known [3].

Indeed, our findings indicate a significant decrease in the levels of both demethylases during postnatal heart development, reinforcing the hypothesis that they play a role in cardioprotection. These results are in line with other reports. Han et al. [4] showed down-regulation of transcript and protein ALKBH5 levels throughout early development (P1, P7, and P10) in a mouse model. Yang et al. [5] reported a higher protein level of ALKBH5 at P0 compared to P7 in rats, but they did not observe any differences in FTO levels. However, a decreasing pattern of FTO protein expression from P1 to P7 and P28 was reported by another group in mouse hearts [6]. In pigs, Ferenc et al. showed that FTO protein levels were higher in neonatal samples compared to adult ones also in other tissues, including skeletal muscle, thyroid gland, or adipose tissue [7]. Interestingly, Krejci et al. [8] showed that FTO was up-regulated in the aortas, atria, and ventricles of old (24 months) male rats (interestingly not females) compared to young (3 months) males, suggesting a further regulation during aging.

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Attachment II

Molecular and Cellular Biochemistry
https://doi.org/10.1007/s11010-019-03584-x

Selection of optimal reference genes for gene expression studies in chronically hypoxic rat heart

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Received: 27 March 2019 / Accepted: 30 June 2019

Abstract
Adaptation to chronic hypoxia renders the heart more tolerant to ischemia/reperfusion injury. To evaluate changes in gene expression after adaptation to chronic hypoxia by RT-qPCR, it is essential to select suitable reference genes. In a chronically hypoxic rat model, no specific reference genes have been identified in the myocardium. This study aimed to select the best reference genes in the left (LV) and right (RV) ventricles of chronically hypoxic and normoxic rats. Sprague–Dawley rats were adapted to continuous normobaric hypoxia (CNH; 12% O_2 or 10% O_2) for 3 weeks. The expression levels of candidate genes were assessed by RT-qPCR. The stability of genes was evaluated by NormFinder, geNorm and BestKeeper algorithms. The best five reference genes in the LV were *Top1*, *Nupl2*, *Rplp1*, *Ywhaz*, *Hprt1* for the milder CNH and *Top1*, *Ywhaz*, *Sdha*, *Nupl2*, *Tomm22* for the stronger CNH. In the RV, the top five genes were *Hprt1*, *Nupl2*, *Gapdh*, *Top1*, *Rplp1* for the milder CNH and *Tomm22*, *Gapdh*, *Hprt1*, *Nupl2*, *Top1* for the stronger CNH. This study provides validation of reference genes in LV and RV of CNH rats and shows that suitable reference genes differ in the two ventricles and depend on experimental protocol.

Keywords Reference genes · RT-qPCR · Heart · Left ventricle · Chronic hypoxia · Rat

Introduction

Ischemic heart disease remains the leading cause of death worldwide [1]. The search for appropriate cardioprotective strategies is therefore crucially important. It is well known that adaptation to chronic hypoxia increases cardiac tolerance to acute ischemia/reperfusion (I/R) injury [2]. Many cellular processes are altered during adaptation to chronic hypoxia [3, 4]. Nevertheless, this phenomenon is still not fully understood. One of the possible approaches how to get a better insight into complex regulatory networks of hypoxic adaptation are the gene expression studies.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is the method of choice for quantification of the gene expression by measuring the increase of fluorescence in every RT–qPCR cycle. However, for proper evaluation of the changes in gene expression, a correct normalization is essential. One way to standardize target gene expression is to report gene expression to the total RNA mass. Nevertheless, total RNA mainly consists of ribosomal RNA (rRNA) and therefore rarely reflects messenger RNA (mRNA) amounts. For that reason, normalization to 18S rRNA molecules is also not appropriate [5]. Instead, it is recommended to select suitable internal reference genes for normalization and to analyze relative gene expression using the $2^{-\Delta\Delta Ct}$ method [6]. Gene expression of such reference genes has to be stable among studied tissues and not affected by experimental protocol [7]. The most common strategy in the past was normalization to a single reference gene. Identifying a gene with a perfectly constant expression is, however, rather problematic. Instead, normalization by geometric averaging of multiple internal reference genes is considered a better approach in the evaluation of gene expression by RT–qPCR [8, 9].

It has been shown, that reference genes may dramatically fluctuate under hypoxic conditions [10, 11]. Optimal reference genes may also vary between the left (LV) and right (RV) ventricles due to their genetic, anatomic, metabolic and physiologic differences [9]. With this knowledge, we preselected eleven candidate genes commonly used as reference genes for a stability analysis in the ventricles of the rat hypoxic heart: actin beta (*Actb*);

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Published online: 12 July 2019

beta-2-microglobulin (*B2m*); glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*); hypoxanthine phosphoribosyltransferase 1 (*Hprt1*); nucleoporin like 2 (*Nupl2*); ribosomal protein, large, P1 (*Rplp1*); succinate dehydrogenase complex flavoprotein subunit A (*Sdha*); translocase of outer mitochondrial membrane 22 (*Tomm22*); DNA topoisomerase I (*Top1*); ubiquitin C (*Ubc*); tyrosin-3-monooxygenase/tryptophan 5 monooxygenase activation protein zeta (*Ywhaz*). These genes are not specific for the heart and their RNA levels in various tissues are available in the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene). The preselection of our candidate genes was based on the study by Julian et al. [5] and further extended by several other common genes with various functions that could have stable expression in the heart and other tissues [12–14].

In the literature, there is a lack of analysis identifying reference genes suitable for hypoxic conditions in heart tissue. The aim of this study was, therefore, to select optimal reference genes in heart ventricles of rats adapted to chronic hypoxia.

Materials and methods

Animals and adaptation to chronic hypoxia

Adult (12 weeks old) male Sprague–Dawley rats were adapted to two degrees of continuous normobaric hypoxia without reoxygenation (CNH 10% O_2, $n=6$; CNH 12% O_2, $n=6$) for 3 weeks in a normobaric chamber equipped with hypoxic generators (Everest Summit, Hypoxico, NY). For each respective adaptation group, there was the control group of animals which were kept under normoxic conditions at room air for the same period of time. All the animals were kept under standard light and feeding conditions (12:12 light/dark cycle, with access to tap water and chow diet [Altromin, 1324, Velaz] ad libitum). After the adaptation, the animals were killed by cervical dislocation, hearts were excised, washed in cold (0 °C) saline and dissected into the LV, the RV and the septum. The tissue was immediately snap-frozen in liquid nitrogen and stored at − 80 °C. The experimental protocols were approved by the Animal Care and Use Committee of the Institute of Physiology of the Czech Academy of Sciences (75/2016) and were in accordance with Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

RNA isolation, cDNA synthesis and RT-qPCR analysis

Total cellular RNA was extracted from each LV and RV sample using RNAzol® RT (Sigma-Aldrich, USA) according to the manufacturer's instructions. The concentration of total RNA was measured on NanoDrop 1000 (Thermo Fisher Scientific, USA). The total RNA samples were treated by DNase I (Sigma-Aldrich, USA) to prevent DNA contamination. One µg of total RNA was used to synthesize first-strand cDNA using RevertAid H Minus First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) and random primers according to the manufacturer's protocol. RT-qPCR was performed in triplicates of 20 µL reaction volume on a LightCycler® 480 (Roche Diagnostics, Switzerland) using SYBR Green primers (Sigma-Aldrich, USA) and LightCycler® 480 SYBR Green I Master (Roche Diagnostics, Switzerland) according to the manufacturer's instructions with the following temperature profile: preincubation and enzyme activation (12 min at 95 °C) followed by 45 cycles of amplification (10 s at 95 °C, 30 s at 60 °C and 25 s at 72 °C). The specificity of primers was tested by melting curve analysis. Second Derivative Maximum method was used for identifying the Cq values. Data were analyzed in accordance with instructions from qPCR courses performed by TATAA Biocenter (http://www.tataa.com/courses/). Candidate reference genes are shown in Table 1 and their corresponding primer sequences are listed in Table 2.

Table 1 Candidate reference genes evaluated in this study

Gene symbol	Gene name	GeneID
Actb	Actin beta	81822
B2m	Beta-2 microglobulin	24223
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	24383
Hprt1	Hypoxanthine phosphoribosyltransferase 1	24465
Nupl2	Nucleoporin like 2	499974
Rplp1	Ribosomal protein, large, P1	140661
Sdha	Succinate dehydrogenase complex flavoprotein subunit A	157074
Tomm22	Translocase of outer mitochondrial membrane 22	300075
Top1	DNA topoisomerase I	64550
Ubc	Ubiquitin C	50522
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	25578

Table 2 Primer sequences for RT-qPCR

Gene symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)
Actb	AAGACCTCTATGCCAACAC	TGATCTTCATGGTGCTAGG
B2m	ACTGGTCTTTCTACATCCTG	AGATGATTCAGAGCTCCATAG
Gapdh	CACCATCTTCCAGGAGCGAG	GGCGGAGATGATGACCCTTT
Hprt1	ACTGGTAAAACAATGCAGAC	CCTGAAGTGCTCATTATAGTC
Nupl2	CAGAGCTATCTAAACTCTGTTC	CTGCATCCTTTAAGTCAGAG
Rplp1	CGGAGGATAAGATCAATGC	CTACATTGCAGATGAGGC
Sdha	ACTATTATTGCTACTGGGGG	CTGAACAAATTCTAAGTCCTGG
Tomm22	GAGCTAGATGAGACCCTATC	CCCTGGAAAATCTGTACATC
Top1	CACCAAAGACCTTTGAGAAG	TGACTCTACTACCTTCTTGG
Ubc	TGACAATGCAGATCTTTGTG	ACTCCTTCTGGATGTTGTAG
Ywhaz	TGACAAGAAAGGAATTGTGG	GGAGTTCAGGATCTCATAGTAG

Evaluation of candidate reference genes

The stability of reference genes was evaluated by NormFinder [15], geNorm [8] and BestKeeper [16] algorithms. First, the intra- and intergroup variations for each gene were analyzed by NormFinder and unsuitable genes that showed bias (variations > 0.25) were excluded from further analyses [17]. Stability values were then calculated by NormFinder and geNorm algorithms using GeNex (MultiD, Sweden), software for qPCR data processing and analysis (http://genex.gene-quantification.info/). Standard deviations (SD) of candidate reference genes Cq values were obtained by BestKeeper software tool, where the best reference genes are with the lowest SD. The final consensus was obtained by the calculation of the geometric mean of the three ranking values for each reference gene resulting in an overall stability score [8, 18].

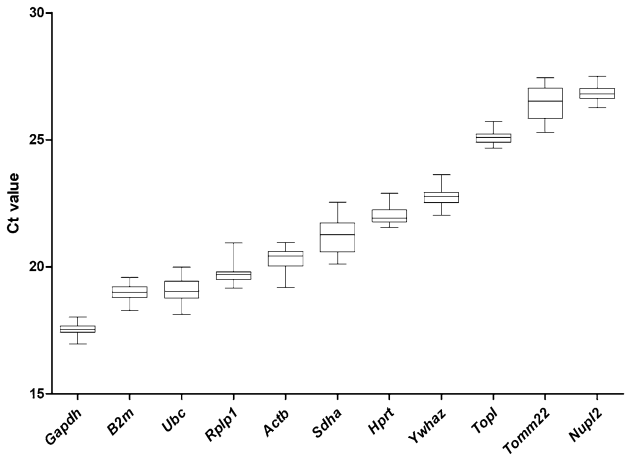

Fig. 1 Variation in candidate reference gene abundance across all samples. Box-and-whiskers plot shows the Cq values obtained from RT-qPCR of each gene analyzed in a total of 48 heart samples from 24 animals. Boxes indicate median (Q2) and quartiles first and third (Q1 and Q3) and whiskers correspond to the minimum and maximum values. Lower Cq values indicate more abundant expression in the rat myocardium

Results

Expression of 11 reference gene candidates was evaluated in heart tissue samples (LV and RV) from 24 rats (12 adapted to CNH, 12 controls) using RT–qPCR. Figure 1 shows the variation in candidate reference gene expression in all samples. The candidate reference genes are arranged by their respective abundance (lower Cq indicates higher mRNA abundance). Table 3 shows the rankings of the candidate reference genes for each particular algorithm (NormFinder, geNorm and BestKeeper) and overall ranking, which is the geometric mean of these three ranking values, for the LV. Table 4 shows the rankings for the RV. The candidate reference genes are arranged by their final ranking value. To have at least two reference genes common for both heart ventricles and both degrees of chronic hypoxia, we selected top five genes in each group.

Regarding the LV, *Sdha* in CNH (12% O_2) and *Rplp1*, *Gapdh*, *Hprt1* and *Ubc* in CNH (10% O_2) showed bias in their intra- or intergroup variations and were excluded from the analyses. The top five of the remaining genes are *Top1*, *Nupl2*, *Rplp1*, *Ywhaz* and *Hprt1* in CNH (12% O_2) and *Top1*, *Ywhaz*, *Sdha*, *Nupl2*, *Tomm22* in CNH (10% O_2). Reference genes suitable for both degrees of hypoxia are therefore *Top1*, *Ywhaz* and *Nupl2*.

Concerning the RV, *Sdha*, *Tomm22*, *B2m* and *Actb* in CNH (12% O2) and *Ywhaz*, *Rplp1*, *B2m*, *Ubc*, *Actb* and *Sdha* in CNH (10% O_2) showed bias in their intra- or intergroup variations and were excluded from the analyses. The top five of the remaining genes are *Hprt1*, *Nupl2*, *Gapdh*, *Top1* and *Rplp1* in CNH (12% O_2) and *Tomm22*, *Gapdh*, *Hprt1*, *Nupl2* and *Top1* in CNH (10% O_2). Reference genes suitable for both degrees of hypoxia are therefore *Hprt1*, *Nupl2*, *Gapdh* and *Top1*.

Table 3 Reference gene ranking for the LV

Experimental group	NormFinder		geNorm		BestKeeper		Consensus	
	Genes	Stability value	Genes	Stability value	Genes	SD	Genes	Geometric mean of ranking values
CNH 12% O_2	*Top1*	0.074	*Ywhaz*	0.107	*Rplp1*	0.103	***Top1***	1.82
($n=6$)	*Nupl2*	0.080	*Top1*	0.107	*Hprt1*	0.123	***Nupl2***	2.29
Controls	*Rplp1*	0.126	*Nupl2*	0.145	*Nupl2*	0.168	***Rplp1***	2.29
($n=6$)	*Ywhaz*	0.145	*Gapdh*	0.157	*B2m*	0.169	***Ywhaz***	3.04
	Hprt1	0.150	*Rplp1*	0.172	*Gapdh*	0.192	***Hprt1***	3.68
	Gapdh	0.157	*Hprt1*	0.181	*Top1*	0.193	*Gapdh*	4.48
	Actb	0.190	*Tomm22*	0.192	*Ywhaz*	0.208	*B2m*	6.60
	Tomm22	0.207	*Actb*	0.202	*Actb*	0.225	*Actb*	7.32
	B2m	0.230	*B2m*	0.216	*Ubc*	0.248	*Tomm22*	7.83
	Ubc	0.255	*Ubc*	0.231	*Tomm22*	0.279	*Ubc*	9.32
	Sdha	–	*Sdha*	–	*Sdha*	–	*Sdha*	–
CNH 10% O_2	*Top1*	0.066	*Ywhaz*	0.108	*Nupl2*	0.142	***Top1***	1.26
($n=6$)	*Ywhaz*	0.091	*TopI*	0.108	*Top1*	0.143	***Ywhaz***	2.00
Controls	*Sdha*	0.098	*Sdha*	0.116	*Sdha*	0.180	***Sdha***	2.62
($n=6$)	*Tomm22*	0.132	*Tomm22*	0.135	*Ywhaz*	0.182	***Nupl2***	2.88
	Actb	0.136	*Nupl2*	0.148	*Tomm22*	0.222	***Tomm22***	3.92
	Nupl2	0.137	*Actb*	0,157	*B2m*	0.233	*Actb*	5.59
	B2m	0.160	*B2m*	0.167	*Actb*	0.243	*B2m*	6.32
	Rplp1	–	*Rplp1*	–	*Rplp1*	–	*Rplp1*	–
	Gapdh	–	*Gapdh*	–	*Gapdh*	–	*Gapdh*	–
	Hprt1	–	*Hprt1*	–	*Hprt1*	–	*Hprt1*	–
	Ubc	–	*Ubc*	–	*Ubc*	–	*Ubc*	–

Genes are ranked according to their stability. Genes without values were excluded from the analysis because of the bias in their intra- or inter-group variations. The top five genes for each group are in bold

CNH continuous normobaric hypoxia; *SD* standard deviation

To identify the best reference genes among the different hypoxia regimens and heart compartments, we made a consensus of best five reference genes (according to Molina et al. [18]) for each heart ventricle and each degree of hypoxia as illustrated in Fig. 2. It shows that suitable reference genes common for both heart ventricles and both degrees of CNH are two genes - *Nupl2* and *Top1*.

Discussion

It is a crucial step in the evaluation of RT-qPCR results to use stable reference genes for correct normalization, which is essential to control for the variance between samples that can be introduced during many stages of the sample preparation and analysis. However, there is no consensus which reference genes are suitable for individual tissues and various experimental protocols. This leads to reduced reproducibility among studies in different laboratories. Thus, the identification of optimal reference genes for the heart under different conditions would have a great impact on cardiac research. Relevant articles about the selection of reference genes in the heart ventricles are listed in Table 5.

Adaptation to chronic hypoxia is a classical way to induce cardioprotective phenotype. This experimental protocol changes expression of many genes. Hypoxia-inducible factors (HIF), nuclear factor NF-κB or nuclear factor erythroid 2-related factor 2 (Nrf2) are the key transcription factors activated under hypoxic conditions [19–21]. Signaling cascades of HIF, NF-κB or Nrf2 mediate the effects of hypoxia through the changes of gene expression profile of the cells. Genes acting on angiogenesis and oxygen supply, cellular metabolism, transcription and apoptosis are the most affected by HIF [22]. NF-κB targets mainly genes involved in inflammation, cell proliferation, apoptosis, morphogenesis and differentiation [23]. It has been shown that *Gapdh* and *Hprt* expression can be regulated by HIF-1 [24, 25]. However, expression of both of these genes was found relatively stable in our experimental protocol. Selection of reference genes is, therefore, strongly dependent on experimental conditions and also on preselection of reference genes. For illustration, studies examining reference genes in rat hearts

Table 4 Reference gene ranking for the RV

Experimental group	NormFinder		geNorm		BestKeeper		Consensus	
	Genes	Stability value	Genes	Stability value	Genes	SD	Genes	Geometric mean of ranking values
CNH 12% O_2	*Hprt1*	0.060	*Nupl2*	0.093	*Hprt1*	0.065	***Hprt1***	1.00
($n=6$)	*Nupl2*	0.090	*Hprt1*	0.093	*Nupl2*	0.101	***Nupl2***	1.59
Controls	*Top1*	0.154	*Gapdh*	0.136	*Gapdh*	0.146	***Gapdh***	2.88
($n=6$)	*Gapdh*	0.218	*Top1*	0.186	*Rplp1*	0.166	***Top1***	3.56
	Rplp1	0.224	*Rplp1*	0.213	*Top1*	0.187	***Rplp1***	4.31
	Ywhaz	0.225	*Ywhaz*	0.233	*Ywhaz*	0.252	*Ywhaz*	5.65
	Ubc	0.310	*Ubc*	0.265	*Ubc*	0.287	*Ubc*	6.65
	Sdha	–	*Sdha*	–	*Sdha*	–	*Sdha*	–
	Tomm22	–	*Tomm22*	–	*Tomm22*	–	*Tomm22*	–
	B2m	–	*B2m*	–	*B2m*	–	*B2m*	–
	Actb	–	*Actb*	–	*Actb*	–	*Actb*	–
CNH 10% O_2	*Tomm22*	0.095	*Tomm22*	0.152	*Gapdh*	0.124	***Tomm22***	1.44
($n=6$)	*Nupl2*	0.129	*Hprt1*	0.152	*Top1*	0.180	***Gapdh***	2.29
Controls	*Hprt1*	0.154	*Nupl2*	0.183	*Tomm22*	0.187	***Hprt1***	2.47
($n=6$)	*Gapdh*	0.180	*Gapdh*	0.200	*Nupl2*	0.191	***Nupl2***	2.52
	Top1	0.201	*Top1*	0.218	*Hprt1*	0.233	***Top1***	3.42
	Ywhaz	–	*Ywhaz*	–	*Ywhaz*	–	*Ywhaz*	–
	Rplp1	–	*Rplp1*	–	*Rplp1*	–	*Rplp1*	–
	B2m	–	*B2m*	–	*B2m*	–	*B2m*	–
	Ubc	–	*Ubc*	–	*Ubc*	–	*Ubc*	–
	Actb	–	*Actb*	–	*Actb*	–	*Actb*	–
	Sdha	–	*Sdha*	–	*Sdha*	–	*Sdha*	–

Genes are ranked according to their stability. Genes without values were excluded from the analysis because of the bias in their intra- or inter-group variations. The top five genes for each group are in bold

CNH continuous normobaric hypoxia; *SD* standard deviation

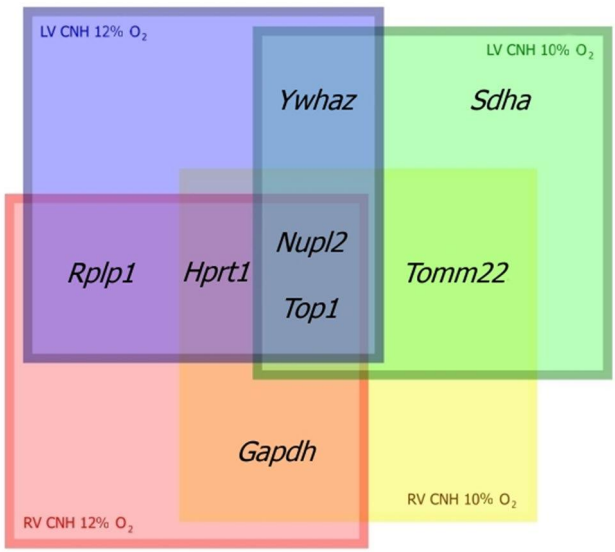

Fig. 2 Illustration of the best five reference genes in both heart ventricles under two degrees of chronic hypoxia. *CNH* continuous normobaric hypoxia; *LV* left ventricle; *RV* right ventricle

suggest different genes for normalization in heart failure, obesity, I/R and different forms of hypoxia [5, 9, 26–29].

Regarding the human heart, appropriate reference genes were determined in the LV from organ donors [30]. Among the three recommended genes only *Gapdh* was included in our study. We found that there are better reference genes suitable for the LV of rats adapted to CNH then *Gapdh*. Molina et al. [18] searched for optimal reference genes in all chambers of healthy and diseased human hearts, including heart failure, atrial dilatation, and atrial fibrillation. Li et al. [31] also studied reference genes in both ventricles from patients with heart failure. The results of both groups, similarly to our data, showed that optimal reference genes differ between different heart regions. These two studies tested various sets of candidate reference genes and only *Gapdh* was included in both. This common reference gene showed good stability between control and pathological groups and was recommended for usage [18, 31]. However, Molina et al. [18] noted that *Gapdh* is more stable in the RV than in the LV samples, which is in line with our results. Other groups even consider *Gapdh* not suitable for normalizing in

Table 5 Reference gene studies in the heart ventricles

Organism	Tissue	Condition	Candidate genes	Recommended reference genes	Study
Human	LV	Heart failure	*Arb*, ***Hprt1***, *Pgk1, Polr2a, Ppi, Rpl4, Rpl32, Tbp*	*Rpl32, Pgk1*	[26]
Mouse				*Rpl32*, ***Gapdh***, *Polr2a*	
Rat				*Polr2a, Rpl3, Tbp*	
Human	LV	LVAD	***Actb***, *Eef1a*, ***Gapdh***, ***Hprt1***, *Ppia, Rpl13a, Top2b*, ***Ywhaz***	*Eef1a, Ppia, Rpl13a*	[33]
	RV			*Rpl13a*, ***Ywhaz***, *Ppia*	
Human	LV	Heart failure	***Actb***, ***Gapdh***, *Psmb4, Rab7A, Reep5, Rpl5, Vcp*	***Gapdh***, *Reep5, Rab7a*	[31]
	RV			*Rpl5*, ***Gapdh***, *Psmb4*	
Human	LV	Heart failure	***Actb***, ***B2m***, ***Gapdh***, *Gusb, Hmbs*, ***Hprt1***, *Ipo8, Pgk1, Polr2a, Ppia, Rplp0, Tbp, Tfrc*, ***Ubc***, ***Ywhaz***, *18S*	***Ywhaz***, *Gusb, Ipo8, Pgk1, Hmbs*	[18]
	RV			***Gapdh***, *Polr2a, Gusb*, ***Hprt1***, *Ppia*	
Human	LV	Organ donors	***Actb***, ***B2m***, ***Gapdh***, ***Hprt1***, *Ppia, Rrlp, Tbp*, ***Ubc***, ***Ywhaz***, *18S*	*Ppia, Rplp*, ***Gapdh***	[30]
Human	LV	Heart failure	***Gapdh***, *Eef1a1, Rnps1, Rpl13a, Rpl22, Rpl23a, Rpl41, Rps4x, Srp1, TptI*	*Srp14, Tpt1, Eef1a1*	[32]
Minipig	LV	Heart failure	***Actb***, ***B2m***, ***Gapdh***, ***Hprt1***, *Ppia, Tbp, Top2b*, ***Ywhaz***	***Hprt1***, *Tbp*, ***Gapdh***	[35]
	RV			*Ppia*, ***Gapdh***, ***Actb***	
Mouse	LV	MI	***Actb***, ***B2m***, *Eef1a1*, ***Gapdh***, ***Hprt1***, *Polr2a, Ppia, Rpl13a, Tbp, Tpt1*	***Hprt1***, *Rpl13a, Tpt1*	[37]
Mouse	heart	Ontogenesis	*Eef1e1, H2afz*, ***Hprt1***, *Pgk1, Polr2a, Ppia, Rpl4, Rpl32, Tbp*	*Ppia, Rpl32*	[36]
	LV	MI		*Eef1e1, Rpl4*	
Rabbit	LV	LVDD	***Actb***, *Eef1e1, G6pd*, ***Gapdh***, ***Hprt1***, *Pgk1, Ppia, Rpl5*, ***Sdha***, ***Ywhaz***	***Sdha***, ***Gapdh***, ***Hprt1***, *Rpl15*	[34]
Rat	heart	Obesity	***Actb***, ***Gapdh***, ***Hprt1***, *Polr2a, Ppia, Rpl13*, ***Sdha***, *Tbp, Tfrc, Ywhag*	***Sdha,*** *Tbp*, ***Hprt1***	[27]
Rat	LV	I/R	***Actb***, ***Gapdh***, *Gusb, Hmbs*, ***Hprt1***, *Papbn1, Rpl13a, Tbp*, ***Ywhaz***	***Ywhaz***, *Pabp, Hmbs (IRR)*	[9]
				Hmbs, Tbp, ***Hprt1*** *(RR)*	
	RV			*Hmbs*, ***Hprt1***	
Rat	LV	OSA	***Actb***, ***B2m***, ***Gapdh***, ***Hprt1***, *18S*	***Actb***, ***B2m***, ***Gapdh***, ***Hprt1***	[5]
Rat	heart	CIH (7000 m)	***Actb***, *Atp5b, Cyc1*, ***Gapdh***, *Mdh*, ***Ubc***	***Gapdh***	[28]
Rat	heart	CNH (12% O_2)	***Actb***, *Arbp*, ***B2m***, ***Gapdh***, *Gusp*, ***Hprt1***, *Pgk1, Ppia, Tubb5*, ***Ywhaz***, *18S*	***Hprt1***	[29]

Reference genes tested in our study are written in bold. Recommended reference genes are arranged by their stability

CIH chronic intermittent hypoxia; *CNH* continuous normobaric hypoxia; *IRR* ischemic/reperfused region; *LV* left ventricle; *LVAD* left ventricular assist device; *LVDD* left ventricular diastolic dysfunction; *MI* myocardial infarction; *OSA* obstructive sleep apnea; *RR* myocardial infarction remote region; *RV* right ventricle

the LV of human failing myocardium at all [26, 32]. Reference genes in both ventricles of patients with left ventricular assist device support were evaluated by Caselli et al. [33]. This group suggested that *Ywhaz* is one of the usable reference genes for the RV. In contrast, we found that *Ywhaz* fits better for normalizing in the LV. The problematics of reference genes search is further complicated by the fact that reference genes vary also among humans and animal models [26]. This may explain the differences between our and other studies, which used human heart tissue.

Studies regarding reference genes in heart tissue of non-rodent animal models, which may also differ significantly from humans and rodent models, include rabbits and pigs. Nachar et al. [34] looked for optimal reference genes in the LV of rabbits with LV diastolic dysfunction. After analyzing the stability of ten candidate genes, they reported that *Sdha*, *Gapdh* and *Hprt1* had the highest stability. In our study, *Hprt1* was in our top five genes in LV at 12% O_2 and *Sdha* in LV at 10% O_2. On the contrary, *Ywhaz* is in our best five genes in both degrees of CNH, whereas it is not included in recommended genes by Nachar et al. [34]. Another study proposed optimal reference genes for failing minipig hearts. Regarding genes tested in our study, *Gapdh* was considered suitable for both heart ventricles in minipig; *Hprt1* and *Actb* for the LV and RV, respectively [35]. As mentioned earlier, we demonstrated that under our experimental conditions, *Gapdh* is more stable in the RV where it was among our best five genes in rats adapted to both degrees of CNH. *Hprt1* was stable in the RV (both degrees of CNH) and it had also the

fifth highest stability in the LV at the milder CNH degree. *Actb* is not among our recommended genes.

Concerning rodent models, reference genes were analyzed in the mouse hearts at different stages of ontogenetic development and after myocardial infarction. It has been shown that normalizing to *Gapdh* may abolish significance in the target gene expression previously observed after normalizing to reference genes which were confirmed to be stable [36]. Other authors even considered *Gapdh*, *B2m* and *Actb* selectively up- or downregulated after myocardial infarction in mice, whereas *Hprt1* is thought to be stable [37]. On the contrary, another group studied reference genes for heart failure and confirmed *Gapdh* as stable in mouse, but unstable in rats [26]. Vesentini et al. [9] studied reference genes in a rat model of I/R and suggested that gene expression should be assessed separately in each region – I/R region (IRR) of the LV, infarction remote region (RR) of the LV and also the RV. Recommended genes included *Ywhaz* (IRR) and *Hprt1* (RR and RV). *Actb* and *Gapdh* were not amongst the recommended genes. In obese rats, *Sdha* and *Hprt1* were found to be stable in heart tissue [27]. Julian et al. [5] evaluated reference genes in the LV of rats adapted to chronic intermittent hypoxia (CIH) simulating obstructive sleep apnea (repeated brief cycles of severe hypoxia and normoxia; 8 h a day; 6 weeks followed by 2 weeks recovery in normoxia). They found that *Gapdh* is the most stable reference gene, followed by *Actb*, *B2m*, *Hprt* and 18S rRNA, respectively. All of the tested genes were considered suitable for use as reference genes by this group; only 18S was inadvisable for normalization. In rats adapted to a different model of CIH (oxygen level corresponding to the altitude of 7000 m for 8 h a day; 5 days a week; 24 exposures), several reference genes, including *Gapdh*, *Actb* and *Ubc*, were tested in the heart tissue. The most stable of the tested genes was *Gapdh* followed by *Ubc*. *Actb* was the least stable gene in this case [28]. In our study, *Actb*, *B2m* and *Ubc* did not make it to the best five reference genes. *Hprt1* and *Gapdh* showed good stability in the RV, and *Hprt1* was also ranked fifth in the LV samples from animals adapted to the milder of the two CNH degrees. *Sdha* reached top five only in the LV under CNH 10% O_2. These differences could be due to the choice of different candidate genes or it could also be due to different experimental protocols. Here, we show that even minor alterations in the experimental protocol may lead to variation in reference genes: a decrease in oxygen level from 12% to 10% changed the optimal reference genes in the RV. The only study looking for reference genes in the hearts of rats adapted to CNH (12% O_2; 4 weeks) was made by Bohuslavova et al. [29]. This group highlighted the constant expression of *Hprt1*, leaving behind *Actb*, *B2m, Gapdh*, *Ywhaz*, 18S rRNA and other candidate genes. Our results confirmed that *Hprt1* is the best choice for normalization at 12% O_2 for the RV. Regarding LV, *Hprt1* was ranked fifth at 12% O_2. Interestingly, *Tomm22* and *Gapdh* were more stable in the RV then *Hprt1* at 10% O_2 and *Hprt1* in the LV was unstable according to our data.

After exclusion of genes with bias in intra- or intergroup variations, geNorm stability values (called M values) of all remaining genes were 0.265 or lower in our experiments. M values below 0.5 are typically observed for stably expressed reference genes and even genes with M values up to 1.0 can be used as reference genes in heterogeneous tissues [38]. Every gene included in our final ranking is therefore relatively stable under our experimental conditions. In spite of that, some reference genes are more suitable for the LV then for the RV and vice versa. Moreover, optimal reference genes also differ in the two degrees of CNH.

This study provides a validation of reference genes in the heart ventricles of rats adapted to two degrees of chronic hypoxia and show that reference gene studies are not generalizable not only between different tissues but also between similar experimental protocols. It is, therefore, strongly advised to make a reference gene study prior to target gene expression analysis for each experimental setting. For normalization in chronically hypoxic rat heart ventricles, we recommend usage of reference genes *Nupl2* and *Top1*.

Acknowledgements This research was funded by CHARLES UNIVERSITY GRANT AGENCY, Grant Number 200317 and GRANT AGENCY OF THE CZECH REPUBLIC, Grant Numbers 16-12420Y and 19-04790Y. We kindly thank Dr. Matus Sotak for help with manuscript editing.

Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

Ethical approval The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. All procedures have been performed in accordance with the ethical standards and with the approvement of the Ethical Committee of the Institute of Physiology CAS in Prague.

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Attachment III

SHORT COMMUNICATION

Myocardial m^{6}A Regulators in Postnatal Development: Effect of Sex

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Received September 1, 2022
Accepted November 10, 2022
Epub Ahead of Print November 25, 2022

Summary

N^6-methyladenosine (m^{6}A) is an abundant mRNA modification affecting mRNA stability and protein expression. It is a highly dynamic process, and its outcomes during postnatal heart development are poorly understood. Here we studied m^{6}A machinery in the left ventricular myocardium of Fisher344 male and female rats (postnatal days one to ninety; P1-P90) using Western Blot. A downward pattern of target protein levels (demethylases FTO and ALKBH5, methyltransferase METTL3, reader YTHDF2) was revealed in male and female rats during postnatal development. On P1, the FTO protein level was significantly higher in males compared to females.

Key words

Epitranscriptomics • N^6-methyladenosine • Postnatal development • Heart

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Introduction

Epigenetic changes have significant importance during both heart development and the manifestation of heart diseases [1]. However, the role of epitranscriptomics, RNA epigenetics, has not yet been sufficiently explored in this area.

N^6-methyladenosine (m^{6}A) is the most prevalent internal chemical mark in mRNA. It is a dynamic and reversible modification that regulates RNA splicing, export from the nucleus, stability, and degradation [2]. The deposition of m^{6}A methylation is mediated by proteins called “writers”. The most prominent one is methyltransferase-like 3 (METTL3), the catalytic subunit of a multicomponent methyltransferase complex [3]. In contrast, fat mass and obesity-associated protein (FTO) and alkB homolog 5 (ALKBH5) are “erasers” with the principal function of removing the m^{6}A modification [4,5]. Besides m^{6}A, FTO also demethylates m^{6}Am, the main target of FTO in the cytosol, and N^1-methyladenosine (m^{1}A) in tRNA [6]. The biological functions of m^{6}A are mediated by "readers" that bind to m^{6}A-containing RNAs. YTH domain family 1-3 (YTHDF1-3) proteins are eminent m^{6}A readers that all induce mRNA degradation [7]. The expression patterns of YTHDF paralogs differ across different cell types and tissues. Therefore, the dominant decay-inducing role is usually carried by the most abundant reader in particular cells. Importantly, YTHDF2 is often more highly expressed than YTHDF1 or YTHDF3 [7].

The m^{6}A modification seems to have significant importance in the developing heart. Disruption in the

proper functionality of m^6A machinery proteins can lead to critical alterations in heart structure and function. For example, loss of enzymatic activity of FTO can lead to a ventricular septal defect, atrioventricular defect, and hypertrophic cardiomyopathy in humans [8]. Moreover, according to Su *et al.* [9], FTO levels drop in elderly murine hearts in response to acute myocardial ischemia/reperfusion injury, while those in young hearts are unaffected. The function of ALKBH5 is linked with an improvement in cardiac function and regeneration after myocardial infarction in juvenile and adult mice [10]. Recent reports also show progressive alterations in m^6A levels during heart development [10-13]. However, there is a lack of data regarding the detailed m^6A machinery protein profiles in heart tissue during postnatal development and potential sex differences.

This pilot study aimed to investigate sex-specific changes in main m^6A regulatory protein levels during postnatal development.

This study was conducted in accordance with the European Guidelines on Laboratory Animal Care. The use of animals was approved and supervised by the Animal Care and Use Committee of the Institute of Physiology of the Czech Academy of Sciences (No. 66/2021).

Animals: Fischer344 rats used for the experiments were bred and kept in the Faculty of Science of Charles University and sacrificed on postnatal days (P) 1, 4, 7, 10, 12, 14, 18, 21, 25, 28, and 90 with n = 4-12 in each group (Table 1). The higher number of individual samples in the early postnatal period was used because of their limited size. Rats were housed on a 12 h light/dark regime and were given unrestricted access to food and tap water.

Tissue processing: Hearts were dissected into the right ventricle (RV) and left ventricle (LV) with septum and frozen in liquid nitrogen. Due to the limited size of the early postnatal LVs, all samples were grouped considering their age and sex and homogenized in eight volumes of ice-cold homogenization buffer (12.5 mM Tris, 2.5 mM EGTA, 250 mM sucrose, 6 mM β-mercaptoethanol, pH 7.4) with the addition of the protease and phosphatase inhibitor cocktail (Roche Diagnostics, Switzerland) as described previously [14]. The protein concentration (Table 1) was measured using the Bradford assay (Bio-Rad, USA). Protein concentration was significantly lower at P1 compared to other days in both sexes. In males, the protein concentration increased gradually from P1 to P7, while in females there was a dramatic change between P1 and P4. The differences in protein concentration indicate significant changes in the ratio of dry mass to water in heart tissue in the early postnatal period. Our observation is in agreement with the already reported rapid postnatal decline in water content in heart tissue [15].

Table 1. The number of animals in pooled samples and concentration of total protein in samples of male and female rat hearts.

Samples	Number of animals	Concentration (μg/μl)	SD
Males			
P1	11	5.39	0.96
P4	10	8.35	1.46
P7	4	10.81	0.06
P10	4	10.09	0.40
P12	5	10.62	1.25
P14	4	10.58	1.76
P18	5	12.07	0.13
P21	5	11.27	0.30
P25	5	11.92	0.40
P28	5	11.78	0.86
P90	4	13.23	1.85
Females			
P1	9	6.43	0.87
P4	12	11.82	1.92
P7	6	11.54	2.52
P10	6	10.18	1.58
P12	4	10.75	0.60
P14	5	10.47	1.76
P18	5	10.82	1.87
P21	5	10.32	1.44
P25	5	11.73	2.15
P28	5	12.37	0.96
P90	5	11.86	1.44

P – postnatal day; SD – standard deviation

Immunoblotting: Proteins were separated by SDS-PAGE electrophoresis (10% gels) and transferred to polyvinylidene fluoride (PVDF) membranes (BioRad, USA; 1620177). The membranes were blocked using 5 % dry low-fat milk in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies against: FTO

[5-2H10] (Abcam, UK; ab92821, 1:1,000), ALKBH5 [EPR18958] (Abcam, UK; ab195377, 1:1,500), METTL3 [EPR18810] (Abcam, UK; ab195352, 1:1,000), YTHDF2 (Invitrogen, USA; PA5-70853, 1:1,000). The membranes were subsequently incubated for 1 h at room temperature with secondary anti-rabbit (Bio-Rad, USA; 170-6515, 1:10,000) or anti-mouse (Invitrogen, USA; 31432, 1:10,000) antibodies. The chemiluminescence was measured by ChemiDoc™ System (Bio-Rad, USA). Ponceau S staining (Sigma-Aldrich, USA; P7170) was used as a loading control. It was shown as an effective way of normalization of samples of different developmental phases [16]. Both male and female protein levels were expressed as fold change over the corresponding P90 male signal (equal to 1). Female protein levels were recalculated to relevant P90 male signals to enable the quantification of sex-dependent differences.

Statistics: All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc.). One-way ANOVA with Tukey's multiple comparisons test was used for the assessment of the statistical significance within sex. Two-way ANOVA with Tukey's multiple comparisons test was used for the assessment of the statistical significance of sex differences. The data were obtained from at least three experiments and are displayed as means ± standard deviation (SD). Results were recognized as statistically significant when $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Protein level profiles of m^{6}A machinery during postnatal development in male and female hearts: To investigate protein levels during postnatal development, we performed a western blot of LV tissue lysates collected from rats on postnatal days 1, 4, 7, 10, 12, 14, 18, 21, 25, 28, and 90. We examined the erasers (FTO, ALKBH5), writer (METTL3), and reader (YTHDF2) proteins of m^{6}A modification. YTHDF2 was chosen because of its highest expression among the paralogs in male LV (with the lowest Cq value indicating the highest gene abundance of *Ythdf2* (25.20 ± 0.47) compared to *Ythdf1* (25.91 ± 0.25) and *Ythdf3* (25.59 ± 0.64)). Firstly, we revealed that the abundance profile of all target proteins had a decreasing pattern during postnatal development (P1-P90) (Fig. 1). ALKBH5 and YTHDF2 declined dramatically between P1-P4 with further indistinct changes in protein levels. FTO and METTL3 protein expression dropped gradually throughout the investigated period. Concerning sex-related differences, it was found that the FTO level is significantly higher (by 40.6 ± 21.4 %) at P1 in males compared to females.

Our present study provides insights into the dynamics of m^{6}A eraser, writer, and reader protein levels through postnatal development from P1 to P90 in rat left ventricles of both sexes. We showed that all proteins revealed a downward expression pattern with either a dramatic drop during the first critical period from P1 to P4 (ALKBH5 and YTHDF2) or a gradual decline till adulthood (FTO, METTL3). The decreasing patterns of METTL3 and ALKBH5 levels correspond to previously published reports [10,12]. Han *et al.* [10] utilized a mouse model and analyzed hearts at P1, P7, and P10. They showed the downregulation of protein and gene levels of ALKBH5 throughout this early developmental period. Also, Yang *et al.* [12] found a higher protein expression of ALKBH5 and METTL3 at P0 than at P7 in the rat heart, while the FTO level remained unchanged. In contrast, Yang *et al.* [13] found that the METTL3 level in mouse hearts is higher at P7 and P28 compared to P1. FTO protein level revealed a similar decreasing pattern as was observed in our study, its level at P1 was higher than at P7 and P28 [13]. Utilizing the porcine model, Ferenc *et al.* [17] showed differences in FTO expression between neonatal samples and adult ones in other tissues: skeletal muscle along with the thyroid gland and adipose tissue displayed the higher FTO signal in the neonatal period.

Interestingly, we observed that the FTO protein level in males is higher than in females at P1. The sex-dependent differences provoked by *Fto* level disruption were found in several reports. For example, sex-specific changes in body weight were observed upon overexpression of *Fto* in mice, with females showing a slightly higher weight gain than males [18]. At the time of weaning, both male and female *Fto* knockout mice were about 65 % the weight of wild-type and heterozygous littermates. Nevertheless, *Fto* knockout male mice displayed persistent weight loss throughout their life, while female *Fto* knockout tended to make up the weight deficit by adulthood [19]. It may suggest a more significant role of FTO during the embryonic and early neonatal period in males that is in line with our data, where at P1 FTO level was higher in male samples than in female ones.

In conclusion, this study thoroughly assessed the protein levels of m^{6}A machinery in rat LVs of both sexes during postnatal development. A downward pattern of all target protein levels was revealed in both sexes. Moreover, the FTO protein level was significantly higher in males compared to females on P1.

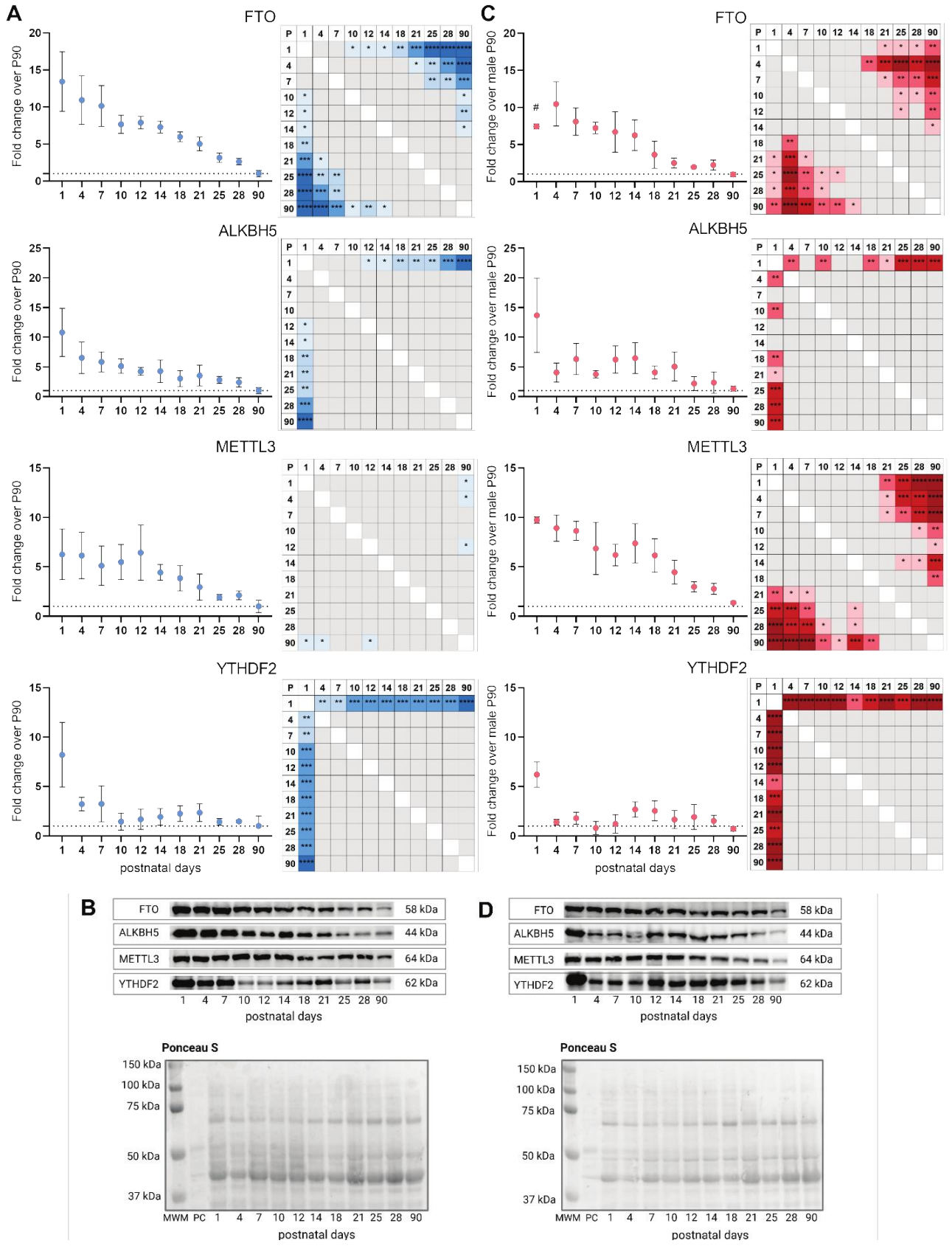

Fig. 1. The protein levels of the m^{6}A regulators in male and female rat hearts. **A**) Immunoblot analysis and multiple comparisons of the immunoblotting data of fat mass and obesity-associated protein (FTO), alkB homolog 5 (ALKBH5), methyltransferase-like 3 (METTL3), and YTHDF2 (YTH domain family 2) in LV tissue homogenates from P1-P90 male rats. **B**) Representative western blot membranes displaying FTO, ALKBH5, METTL3, and YTHDF2 protein levels in LV tissue homogenates from P1-P90 male rats and the representative total protein Ponceau S staining. **C**) Immunoblot analysis and multiple comparisons of the immunoblotting data of FTO, ALKBH5, METTL3 and YTHDF2 in LV tissue homogenates from P1-P90 female rats. **D**) Representative western blot membranes displaying FTO,

ALKBH5, METTL3, and YTHDF2 protein levels in LV tissue homogenates from P1-P90 female rats and the representative total protein Ponceau S staining. Homogenates were pooled with n = 4-12 in each group (details in Table 1). All the protein expression levels were normalized to Ponceau S staining. Both male and female protein levels were expressed as fold change over the corresponding P90 male signal (equal to 1). Experiments were performed independently three times. Protein loading was 15 µg. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (One-way ANOVA; Tukey's multiple comparisons test). #P < 0.01 compared to corresponding P1 males (Two-way ANOVA with Tukey's multiple comparisons test). MWM – molecular weight marker, P – postnatal day, PC – positive control (rat brain).

Conflict of Interest

There is no conflict of interest.

Acknowledgements

The study was supported by the Charles University Grant Agency (grant number 1076119) and the Czech Science Foundation (grant number 19-04790Y).

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Attachment IV

RNA BIOLOGY
2024, VOL. 21, NO. 1, 1–14
https://doi.org/10.1080/15476286.2024.2307732

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RESEARCH PAPER

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Epitranscriptomic regulation in fasting hearts: implications for cardiac health

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ABSTRACT

Cardiac tolerance to ischaemia can be increased by dietary interventions such as fasting, which is associated with significant changes in myocardial gene expression. Among the possible mechanisms of how gene expression may be altered are epigenetic modifications of RNA – epitranscriptomics. N^6-methyladenosine (m^6A) and N^6,2'-O-dimethyladenosine (m^6Am) are two of the most prevalent modifications in mRNA. These methylations are reversible and regulated by proteins called writers, erasers, readers, and m^6A-repelled proteins. We analysed 33 of these epitranscriptomic regulators in rat hearts after cardioprotective 3-day fasting using RT-qPCR, Western blot, and targeted proteomic analysis. We found that the most of these regulators were changed on mRNA or protein levels in fasting hearts, including up-regulation of both demethylases – FTO and ALKBH5. In accordance, decreased methylation (m^6A+m^6Am) levels were detected in cardiac total RNA after fasting. We also identified altered methylation levels in *Nox4* and *Hdac1* transcripts, both of which play a role in the cytoprotective action of ketone bodies produced during fasting. Furthermore, we investigated the impact of inhibiting demethylases ALKBH5 and FTO in adult rat primary cardiomyocytes (AVCMs). Our findings indicate that inhibiting these demethylases reduced the hypoxic tolerance of AVCMs isolated from fasting rats. This study showed that the complex epitranscriptomic machinery around m^6A and m^6Am modifications is regulated in the fasting hearts and might play an important role in cardiac adaptation to fasting, a well-known cardioprotective intervention.

ARTICLE HISTORY
Revised 5 January 2024
Accepted 16 January 2024

KEYWORDS
Fasting; heart; epitranscriptomics; m^6A; m^6Am; FTO; ALKBH5

Introduction

Ischaemic heart disease is the leading cause of death worldwide [1]. Myocardial ischaemia results in damage to cardiomyocytes which can further lead to impaired heart function. The degree of ischaemic injury, however, depends on the intensity and duration of the ischaemic stimulus and the level of cardiac tolerance to ischaemia [2]. Studies have shown that fasting can attenuate the extent of heart damage caused by myocardial infarction [3,4]. Nevertheless, the molecular mechanisms responsible for this cardioprotective phenotype are not yet fully resolved.

Epitranscriptomic modifications are dynamic changes to the chemical composition of RNA that have the potential to alter its stability or function [5]. N^6-methyladenosine (m^6A) and N^6,2'-O-dimethyladenosine (m^6Am) are among the most common modifications [6–8]. These methylations profoundly affect gene expression regulation and cellular physiology and pathophysiology. Proteins called writers (methylation deposition), erasers (methylation removal), readers (binding of modified RNA), and also m^6A-repelled proteins (binding of unmodified RNA) mediate the biological effects of these modifications [9,10] (details in Table 1).

Epitranscriptomic regulations play a wide range of roles in cardiovascular health and disease [5,11–14]. For instance, the levels of m^6A and its writer METTL3 are up-regulated in hearts after ischaemia-reperfusion (I/R) injury [15]. Changes in m^6A-RNA methylation are also associated with heart failure [16,17]. Notably, the epitranscriptomic regulators also play a role in cardioprotection [18–21]. Particularly, the well-known eraser fat mass and obesity-associated protein (FTO) is mostly associated with beneficial effects on the heart [18,19]. However, only one study has focused on fasting animal hearts so far. This study described that intermittent fasting (IF) improved high-fat diet-induced cardiomyopathy via an FTO-associated decrease in m^6A methylation [22]. These limited data suggest that epitranscriptomics might represent a crucial layer of gene regulation in fasting hearts. Thus, investigating the potential role of epitranscriptomic modifications and their regulators in the induction of cardioprotection during fasting is of great importance.

In this study, we performed a detailed analysis of 33 m^6A and m^6Am regulators in the hearts of rats subjected to 3-day fasting. We showed that most of the epitranscriptomic regulators were affected by fasting, including up-regulation of demethylases FTO and ALKBH5, and that RNA methylation levels were decreased in the fasting hearts. At the same time, some of the transcripts of genes participating in possible protective pathways were up-methylated. Moreover, we studied the inhibition of

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/15476286.2024.2307732

Table 1. The m^6A and m^6Am regulators.

Protein	Protein name	Function	Modification
METTL3	Methyltransferase-like 3	Writers	m^6A
METTL14	Methyltransferase-like 14		m^6A
WTAP	Willms' tumour 1-associating protein		m^6A
METTL5	Methyltransferase-like 5		m^6A
METTL16	Methyltransferase-like 16		m^6A
ZCCHC4	Zinc finger CCHC-type containing 4		m^6A
PCIF1	Phosphorylated CTD interacting factor 1		m^6Am
METTL4	Methyltransferase-like 4		m^6Am
FTO	Fat mass and obesity-associated protein	Erasers	m^6Am, m^6A
ALKBH5	AlkB family member 5		m^6A
YTHDF1	YTH domain-containing family protein 1	Readers	m^6A
YTHDF2	YTH domain-containing family protein 2		m^6A
YTHDF3	YTH domain-containing family protein 3		m^6A
YTHDC1	YTH domain-containing protein 1		m^6A
YTHDC2	YTH domain-containing protein 2		m^6A
eIF3a	Eukaryotic initiation factor 3a		m^6A
eIF3c	Eukaryotic initiation factor 3c		m^6A
eIF3g	Eukaryotic initiation factor 3g		m^6A
HNRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1		m^6A
HNRNPC	Heterogeneous nuclear ribonucleoprotein C		m^6A
HNRNPD	Heterogeneous nuclear ribonucleoprotein D		m^6A
RBMX (HNRNPG)	RNA-binding motif protein, X chromosome		m^6A
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1		m^6A
IGF2BP2	Insulin-like growth factor 2 mRNA binding protein 2		m^6A
IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3		m^6A
FMR1	Fragile X messenger ribonucleoprotein 1		m^6A
PRRC2A	Proline rich-coil 2A		m^6A
G3BP1	G3BP stress granule assembly factor 1	m^6A-repelled proteins	m^6A
G3BP2	G3BP stress granule assembly factor 2		m^6A
ELAVL1 (HUR)	ELAV-like protein 1		m^6A
USP10	Ubiquitin specific peptidase 10		m^6A
CAPRIN1	Cell cycle associated protein 1		m^6A
RBM42	RNA binding motif protein 42		m^6A

The key regulators are in bold.

ALKBH5 and FTO in rat adult left ventricular cardiomyocytes (AVCMs) and found that inhibition of both demethylases decreased the hypoxic tolerance of AVCMs isolated from fasting rats. Our data suggest that epitranscriptomics might play an essential role in the molecular adaptation of the heart to fasting, a promising cardioprotective intervention.

Materials and methods

Animals and experimental protocol

Adult (12-week-old) male Wistar rats were divided into two groups. The experimental group was kept without food for 3 days but had free access to water [4]. The control group was fed *ad libitum*. All animals were housed in a controlled environment (23°C; 12 h light–dark cycle; light from 6:00 AM). The study followed the Guide for the Care and Use of Laboratory Animals (published by the National Academy of Science, National Academy Press, Washington, DC, USA). Experimental protocols were approved by the Animal Care and Use Committee of the Institute of Physiology, The Czech Academy of Sciences.

Blood glucose and haematocrit levels

Glucose levels in the tail blood were measured before the onset of fasting and after each day of fasting using a glucometer. Haematocrit was determined at the end of fasting by the capillary micromethod.

Echocardiography

The geometry and function of the left ventricle (LV) were assessed by echocardiography after 3 days of fasting using GE Vivid 7 Dimension (GE Vingmed Ultrasound, Horten, Norway) with a 12 MHz linear matrix probe M12L [23]. Animals were anesthetized with 2% isoflurane (Forane, Abbott Laboratories, Queenborough, United Kingdom) mixed with room air, placed on a heating pad and their rectal temperature was maintained between 35.5 and 37.5°C. Basic 2-D and M-modes were recorded in both the long and short axes. Heart rate (HR) and the following parameters of LV geometry were assessed: end-diastolic and end-systolic LV cavity diameter (LVDd, LVDs), anterior wall thickness (AWTd, AWTs), and posterior wall thickness (PWTd, PWTs). Fractional shortening (FS), relative wall thickness (RWT), and cardiac index (CI) were derived as follows: $FS = 100*[(LVDd-LVDs)/LVDd]$; $RWT = 100*[(AWTd+PWTd)/LVDd]$; $CI = [(\pi/3)*LVDd^3]-[(\pi/3)*LVDs^3]*HR/BW$.

Heart catheterization

After the echocardiographic examination, the anesthetized rats were subjected to LV catheterization through the right carotid artery using the SPR-407 microtip pressure catheter as described previously [24]. Data were acquired using MPVS 300 (Millar, Houston, Texas, USA) and PowerLab 8/30 (ADInstruments, Oxford, UK). End-diastolic pressure (Ped), end-systolic pressure (Pes), developed pressure (Pdev), and

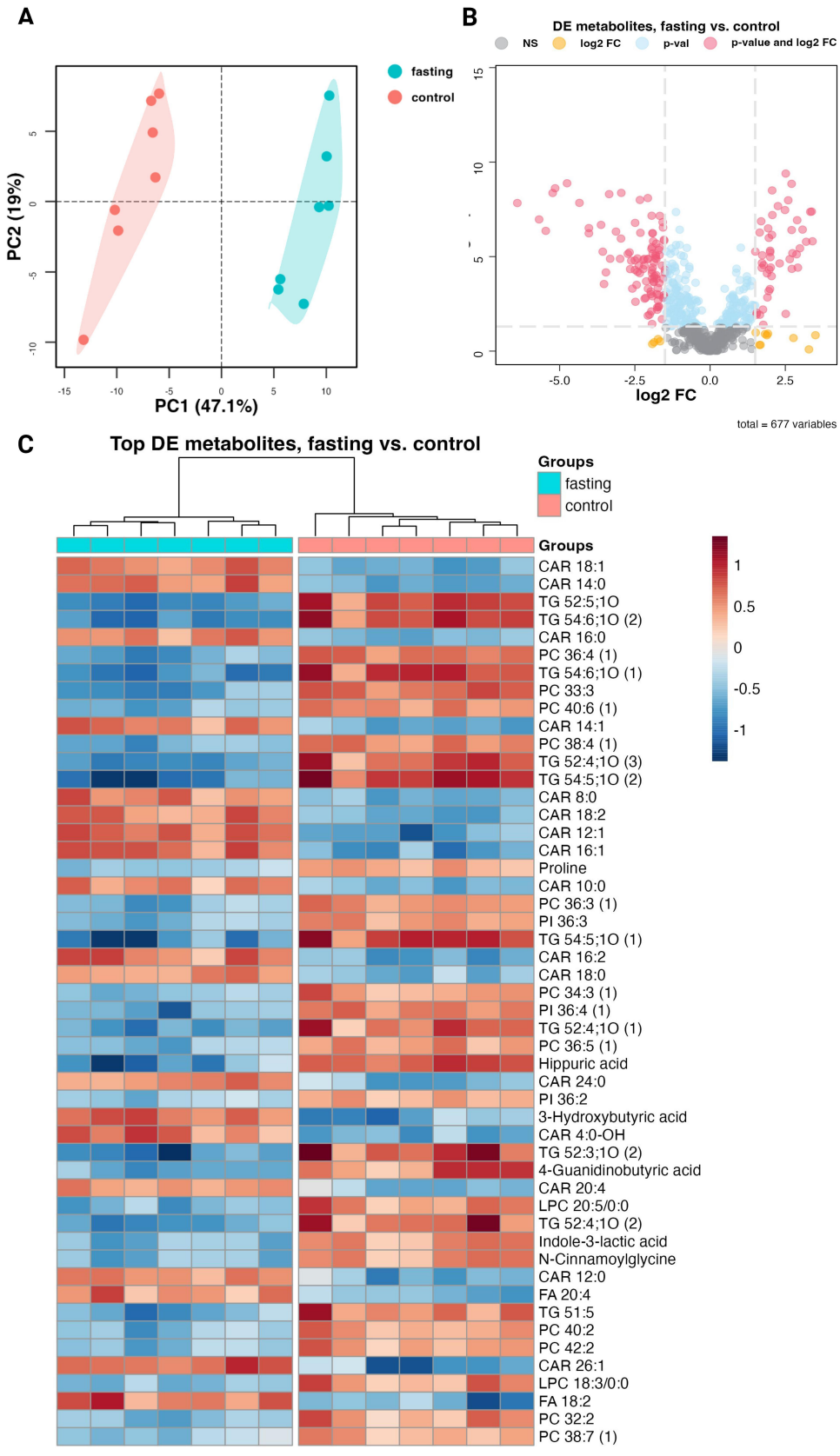

Figure 1. Effect of fasting on plasma metabolites measured by a multiplatform LC-MS-based approach (A) PCA (principal component analysis) showing a clear separation between fasting and control rat plasma samples, suggesting distinct metabolomic profiles associated with the fasting state (B) volcano plot of all (677) metabolites indicating differential levels of metabolites in plasma samples of fasting and control rats (C) heat map of the 50 most significantly affected metabolites; $n = 7$.

Table 2. Characteristics of the fasting model.

	Control rats	Fasting rats
BW change (%)	+3 ± 2.16	-17 ± 2.22*
HW/Tibia (%)	25.5 ± 1.50	22 ± 1.47***
Hematocrit (%)	40.3 ± 4.49	45.6 ± 2.97*
Glycemia (mmol/l)	6.2 ± 0.43	3.9 ± 0.77****
AWTd (mm)	1.96 ± 0.13	1.85 ± 0.15
AWTs (mm)	2.81 ± 0.09	2.66 ± 0.23
PWTd (mm)	1.83 ± 0.13	1.90 ± 0.19
PWTs (mm)	2.71 ± 0.14	2.67 ± 0.24
RWT (%)	49.88 ± 5.59	51.41 ± 3.67
LVDd (mm)	7.63 ± 0.42	7.23 ± 0.17*
LVDs (mm)	4.64 ± 0.27	4.72 ± 0.22
FS (%)	39.9 ± 1.8	35.1 ± 3.4*
HR (bpm)	350 ± 19.9	327 ± 22*
CI (ml/min/kg)	306 ± 62	276 ± 33
Pes (mmHg)	86.63 ± 5.69	89.38 ± 4.34
Ped (mmHg)	4.00 ± 1.38	4.28 ± 2.25
Pdev (mmHg)	82.63 ± 4.88	85.10 ± 4.47
$+(dP/dt)_{max}$ (mmHg/s)	7,008 ± 529	5,453 ± 417*
$-(dP/dt)_{max}$ (mmHg/s)	-7,080 ± 529	-6,592 ± 616

Values are means ± SD; $n = 8–10$; *$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$. AWTd – end-diastolic anterior wall thickness; AWTs – end-systolic anterior wall thickness; bpm – beats per minute; BW – body weight; CI – cardiac index; FS – fractional shortening; HR – heart rate; HW – heart weight; LVDd – end-diastolic LV diameter; LVDs – end-systolic LV diameter; Ped – end-diastolic pressure; Pes – end-systolic pressure; Pdev – developed pressure; PWTd – end-diastolic posterior wall thickness; PWTs – end-systolic posterior wall thickness; RWT – relative wall thickness; +(dP/dt)max – peak rate of pressure development; -(dP/dt)max – peak rate of pressure decline.

peak rate of pressure development and decline ($+(dP/dt)_{max}$, $-(dP/dt)_{max}$, respectively) were assessed from 5 consecutive pressure cycles using LabChart Pro (ADInstruments, Oxford, UK).

Collection of tissue samples

Immediately after the fasting period, the rats were killed by cervical dislocation. The hearts were rapidly excised, washed in a cold (0°C) saline, and dissected into RV, LV, and the septum [25]. All collected tissue segments were weighed, frozen, and stored in liquid nitrogen until use. The heart weight was normalized to tibia length.

RNA isolation, cDNA synthesis, and RT-qPCR analysis

Total RNA was extracted from each LV sample using RNAzol® RT according to the manufacturer's instructions. The concentration of total RNA was measured on NanoDrop 1000 (Thermo Fisher Scientific, USA). One µg of total RNA was used to synthesize first-strand cDNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) and random primers according to the manufacturer's protocol. RT-qPCR was performed in 20 µl reaction volume on a LightCycler® 480 (Roche Diagnostics, Switzerland) using TaqMan Gene Expression Assays (Tab. S1; Thermo Fisher Scientific, USA) and 5× HOT FIREPol Probe qPCR Mix Plus (NO ROX) (Solis Biodyne, Estonia) according to the manufacturer's instructions with the following temperature profile: initial enzyme activation (15 min at 95°C) followed by 45 cycles of amplification (15 s at 95°C, 1 min at 60°C) [26]. For proper normalization [27], suitable reference genes were assessed. In total, six reference genes were evaluated: hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), nucleoporin-like 2 (*Nupl2*), succinate dehydrogenase complex flavoprotein subunit A (*Sdha*), translocase of outer mitochondrial membrane 22 (*Tomm22*), DNA topoisomerase I (*Top1*), and tyrosin-3-monooxygenase/tryptophan 5 monooxygenase activation protein zeta (*Ywhaz*). *Ywhaz* and *Top1* were selected as the most stable of these genes and were used for normalization. Data were analysed by instructions from qPCR courses performed by TATAA Biocenter (http://www.tataa.com/courses/).

SDS-PAGE and Western blot analysis

Tissue homogenization, protein separation, and immunodetection were performed as described earlier [13]. Each frozen LV was pulverized in liquid nitrogen to a fine powder followed by Potter-Elvehjem homogenization in eight volumes of homogenization buffer [12.5 mM TRIS, 2.5 mM EGTA, 250 mM sucrose, 6 mM 2-mercaptoethanol, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche, Switzerland), pH 7.4]. The protein concentration in homogenates was measured by the Bradford method (Bio-Rad, USA). The LV homogenates were subjected to SDS electrophoresis on 10% polyacrylamide gels (Mini-PROTEAN TetraCell, Bio-Rad, USA) and electrotransferred onto PVDF membranes (0.2 µm pore size, Bio-Rad). Subsequently, membranes were blocked with 5% blotting-grade blocker (Bio-Rad, USA) in PBS containing Tween 20 (1%) for 1 h and incubated with appropriate primary and secondary antibodies (diluted in 1% blotting-

grade blocker and 1% Tween 20 in PBS): anti-FTO (Abcam, ab92821, 1:1,000, overnight), anti-ALKBH5 (Abcam, ab195377, 1:1,400, overnight), anti-METTL3 (Abcam, ab195352, 1:1,000, overnight), anti-PCIF1 (Invitrogen, PA5–110081, 1:1,400, overnight), anti-METTL4 (Invitrogen, PA5–97202, 1:1,400, overnight), anti-YTHDF1 (Abcam, ab157542, 1:1,400, overnight), anti-YTHDF2 (Invitrogen, PA5–70853, 1:1,400, overnight), anti-YTHDF3 (Sigma-Aldrich, SAB21022736, 1:1,400, overnight), anti-YTHDC1 (Abcam, ab220159, 1:1400, overnight), anti-YTHDC2 (Abcam, ab220160, 1:1,400, overnight), anti-mouse secondary antibody (ThermoFisher 31432, 1:10,000, 1 h) and anti-rabbit secondary antibody (Bio-Rad, 170–6515, 1:10,000, 1 h). The same amount of protein was loaded on the gels for all samples. The results were recalculated to the total protein amount gained by Ponceau S staining [28]. Each sample was analysed at least three times. The membranes were visualized by enhanced chemiluminescence (ECL) substrates (RNAzol® West Dura Extended Duration Substrate or LightCycler® West Femto Maximum Sensitivity Substrate, Thermo Scientific) using a SuperSignal™ system (Bio-Rad, Hercules, USA). Quantification of the results was performed using ImageJ software.

Targeted proteomic analysis

Samples were dissolved in 25 µl loading buffer (0.05% TFA, 2% acetonitrile) and firstly analysed using data-independent-acquisition (DIA). For targeted analysis, samples were spiked with a mix of 72 isotopically labelled peptides containing C-terminal 15N and 13C-labelled arginine and lysine residues (JPT Peptide Technologies GmbH, Berlin, Germany) to a concentration corresponding to 1 fmol/peptide on a column. Before internal standard (IS) spiking, samples were diluted to an estimated amount of 1 µg of the total peptide on a column. Due to detection limits above 1 fmol/peptide on a column for some internal standards, samples were re-spiked to 40 fmol IS peptides on a column for a second injection.

For LC-MS analysis, an Ultimate 3000 liquid chromatograph coupled to an Orbitrap Exploris 480 mass spectrometer equipped with FAIMS was used. Peptides were loaded onto a PepMap Neo 0.5 cm x 300 µm i.D., 5 µm C18, 100 A trap column (Thermo Fisher Scientific) for 2 min at 17.5 µl/min. Separation and subsequent ion spray ionization were performed on a 50 cm x 75 µm i.D. Easy-Spray column with 2 µm C18 particles and 100 A pore size. A solvent gradient from 97% mobile phase A (0.1% FA in H_2O) to 35% mobile phase B (0.1% formic acid in 80% acetonitrile) for 60 min was used for targeted acquisition and 120 min for DIA analysis. The spray voltage was set to 2,000 V for all runs. FAIMS was run in standard resolution mode for DIA runs for parallel reaction monitoring analysis (PRM) and low-resolution mode (inner electrode temp.: 100°C, outer electrode temp.: 80°C). Compensation voltage was fixed to -45 V for DIA runs but individually optimized for each of the 72 peptides for PRM analysis (CVs used: -35, -40, -45, -50, -60, -70). Analysis in data-independent mode was performed with the following settings: MS1 resolution of 60,000 FWHM with a scan range between *m/z* 350 and 1,500; injection time of 100 ms and an AGC of 300% (3×10^6). For peptide spectrum generation 2×38 staggered MS2 scans with an isolation width of *m/z* 16 including precursors from *m/z* 400 to 1,000, without overlap were defined. The corresponding instrument settings were 27% HCD collision energy 30,000 FWHM resolution, 55 ms ion injection time, and an AGC target of 1,000% (1×10^6). The targeted analysis consisted of PRM scans for light and heavy precursors with isolation widths of *m/z* 1.6, a resolution of 60,000 FWHM; 118 ms ion injection time, an AGC target of 1×10^5, and HCD collision energy set to 27%.

Acquired raw files from DIA runs were analysed in Spectronaut. PRM data were analysed in Skyline-daily. Transition areas were integrated and normalized for relative quantification to isotopically labelled heavy internal standard peptides. Normalized ratios on both peptide and protein levels were exported to Excel and relative changes between groups were computed.

Untargeted lipidomics and metabolomics

Plasma samples were extracted using a biphasic solvent system of cold methanol, methyl *tert*-butyl ether, and water [29]. Then, a multiplatform LC-MS-based approach [30] was used for metabolomic and lipidomic profiling, with details summarized in Supplementary Materials.

m^6A/m quantification in total RNA from left ventricles

The m^6A/m ($m^6A + m^6Am$) levels in the total RNA samples were detected by the EpiQuik m6A RNA Methylation Quantification Kit (Epigentek, Farmingdale, USA) according to the manufacturer's instructions. For each analysis, 300 ng of RNA was used. The absorbance was read on a microplate reader Synergy™ HT Multi-Detection Microplate Reader (BioTek, Winooski, USA) at 450 nm. The estimation of the m^6A/m percentage in RNA was done using the formula: m^6A/m (%) = [(sample OD-negative control OD)/Slope]*100%. The results of this assay were described as m^6A/m levels because this method does not differentiate between m^6A and m^6Am modifications [14].

m^6A RNA immunoprecipitation (MeRIP)

The immunoprecipitation of m^6A/m-modified RNA was done using Magna MeRIP™ m^6A Kit (Merck Millipore, Burlington, USA) following the manufacturer's instructions. Briefly, 80 µg of total RNA isolated from LVs was fragmented at 94°C for 5 min following incubation with magnetic beads at 4°C for 2 h. After that, samples were eluted with elution buffer containing N^6-Methyladenosine 5'-monophosphate sodium salt. Eluted RNA was purified using PureLink™ RNA Mini Kit (Thermo Fisher Scientific, USA). Genes potentially participating in cardioprotection induced by fasting [31] – NFE2 like BZIP transcription factor 2 (*Nfe2l2*), Sirtuin 1 (*Sirt1*), Sirtuin 3 (*Sirt3*), Protein kinase AMP-activated catalytic subunit alpha 2 (*Prkaa2*), RELA proto-oncogene, NF-KB Subunit (*Rela*), NADPH oxidase 4 (*Nox4*), Histone deacetylase 1 (*Hdac1*), Forkhead box O3 (*Foxo3*), Hypoxia-inducible factor 1 subunit alpha (*Hif1a*) – were selected for analysis of MeRIPed RNA, which was performed by RT-qPCR as described above. TaqMan Gene Expression Assays used for this analysis are listed in the supplements (Tab. S2).

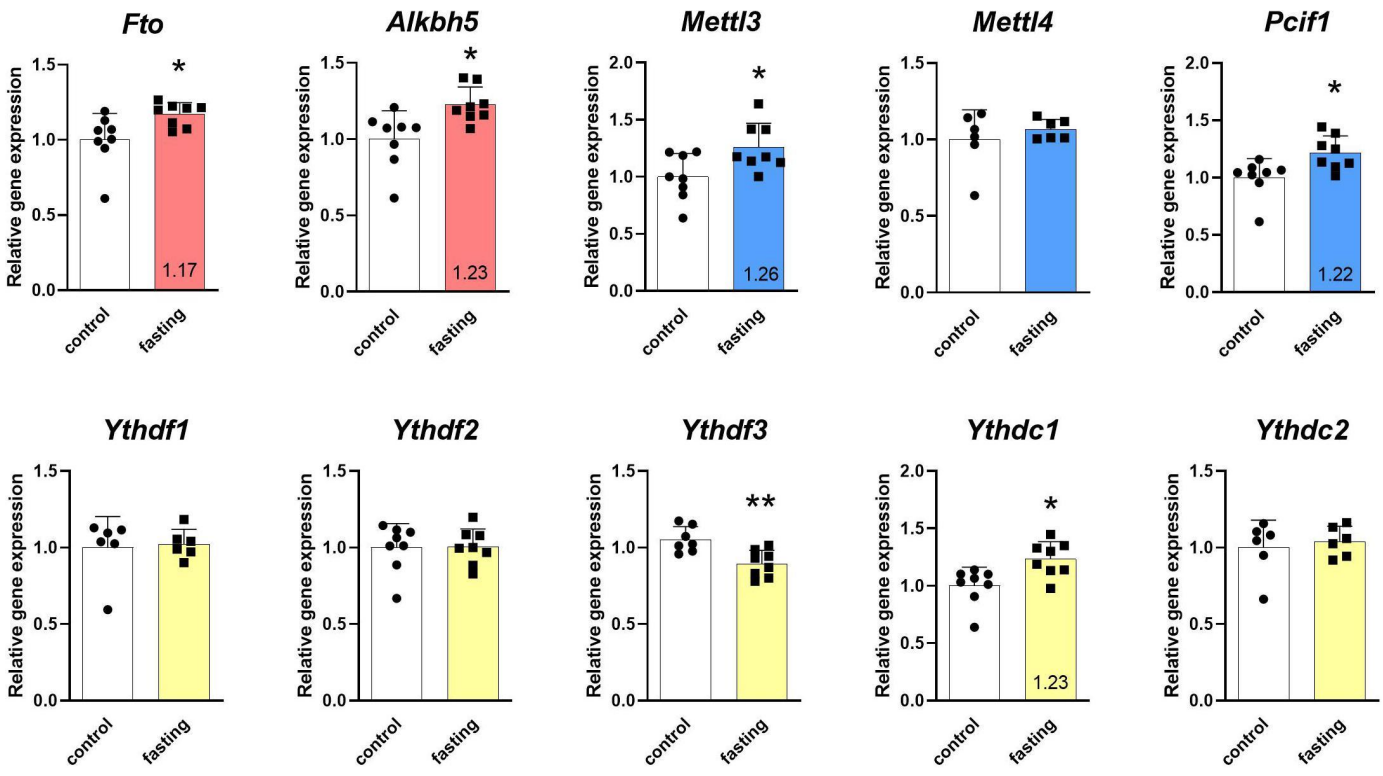

Figure 2. Effect of 3-day fasting on gene expressions of m^{6}A and m^{6}Am regulators in the left ventricle assessed by RT-qPCR. Writers are displayed in blue, erasers in red, and readers in yellow. The average of the control values is set to 1. Values are means ± SD; $n = 6–8$; *$p < 0.05$; **$p < 0.01$ (t-test). *Alkbh5* – AlkB family member 5; *Fto* – fat mass and obesity-associated; *Mettl3* – methyltransferase-like 3; *Mettl4* – methyltransferase-like 4; *Pcif1* – phosphorylated CTD interacting factor 1; *Ythdf1–3* – YTH domain-containing family protein 1–3; *Ythdc1–2* – YTH domain-containing protein 1–2.

AVCM isolation and culture

The rat AVCMs were isolated from 12-week-old male Wistar rats as described previously [32] with slight adjustments. The rats were heparinized (5,000 U/kg, i.p.), anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg), and killed by cervical dislocation. The hearts were rapidly excised and perfused for 10 min with Ca^{2+}-free buffer containing 10 mM KCl, 1.2 mM K_2HPO_4, 90 mM NaCl, 5 mM $MgSO_4$, 15 mM $NaHCO_3$, 20 mM glucose, and 30 mM taurine (pH 7.4) at 37°C. The perfusion medium was then switched to Ca^{2+}-free buffer containing collagenase Type 2 (8,000 U; Worthington, Lakewood, USA), bovine serum albumin (0.2%), and Ca^{2+} (50 µM). All solutions were gassed with 95% O_2 and 5% CO_2 for 30 min before use. After 60 min of digestion, the LV was minced, and cardiomyocytes were isolated by sedimentation in a gradually increasing Ca^{2+} concentration buffer until a final concentration of 1.2 mM. Finally, the myocytes isolated from the LV were gently resuspended in a cell culture medium-M199 (M199, containing 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin) and transferred to laminin-coated culture dishes and let in a CO_2 incubator (95% air, 5% CO_2, 37°C) for 2 h to attach.

FTO and ALKBH5 inhibitors

A pharmacological inhibitor of FTO (FTOi; MO-I-500 [33]) was dissolved in DMSO as 1 mM stocks and stored at −20°C in small aliquots. A pharmacological inhibitor of ALKBH5 (ALKBH5i; compound 3 [34]) was dissolved in DMSO as 50 mM stocks and stored at 5°C in small aliquots. DMSO (0.1% final concentration) was also used as vehicle control.

The dose-response of the viability of AVCMs to FTOi and ALKBH5i was tested in this study to select the appropriate concentration of inhibitors (Fig. S1). The viability of AVCMs was determined after 24 h incubation with FTOi (0.5, 1, 2.5, 5, 10, 50 µM) or ALKBH5i (10, 50, 75, 100, 150 µM) using SYTOX Green nucleic acid stain (S7020) (Invitrogen-Molecular Probes, Eugene, USA). Based on these results, the 1 µM (FTOi) and 50 µM (ALKBH5i) concentrations, which did not significantly affect the number of surviving cells during 24 h incubation, have been chosen for the following experiments.

Hypoxic tolerance of cardiomyocytes

AVCMs isolated either from control or fasting rats were incubated in hypoxic chamber Xvivo System X3 (BioSpherix, USA) under hypoxic conditions (1% O_2; 5% CO_2; 37°C) for 24 h in M199 medium containing 1 µM FTOi or 50 µM ALKBH5i or 0.01% DMSO. Control AVCMs were incubated under normoxic conditions (95% air, 5% CO_2, 37°C) with or without 1 µM FTOi or 50 µM ALKBH5i.

The percentage of living cells compared to the untreated normoxic cells was determined using the SYTOX Green nucleic acid stain (S7020) (Invitrogen-Molecular Probes, Eugene, USA) at the beginning of the experiments (after stabilization), after 24 h of treatment, and finally after incubation with 8% Triton X-100

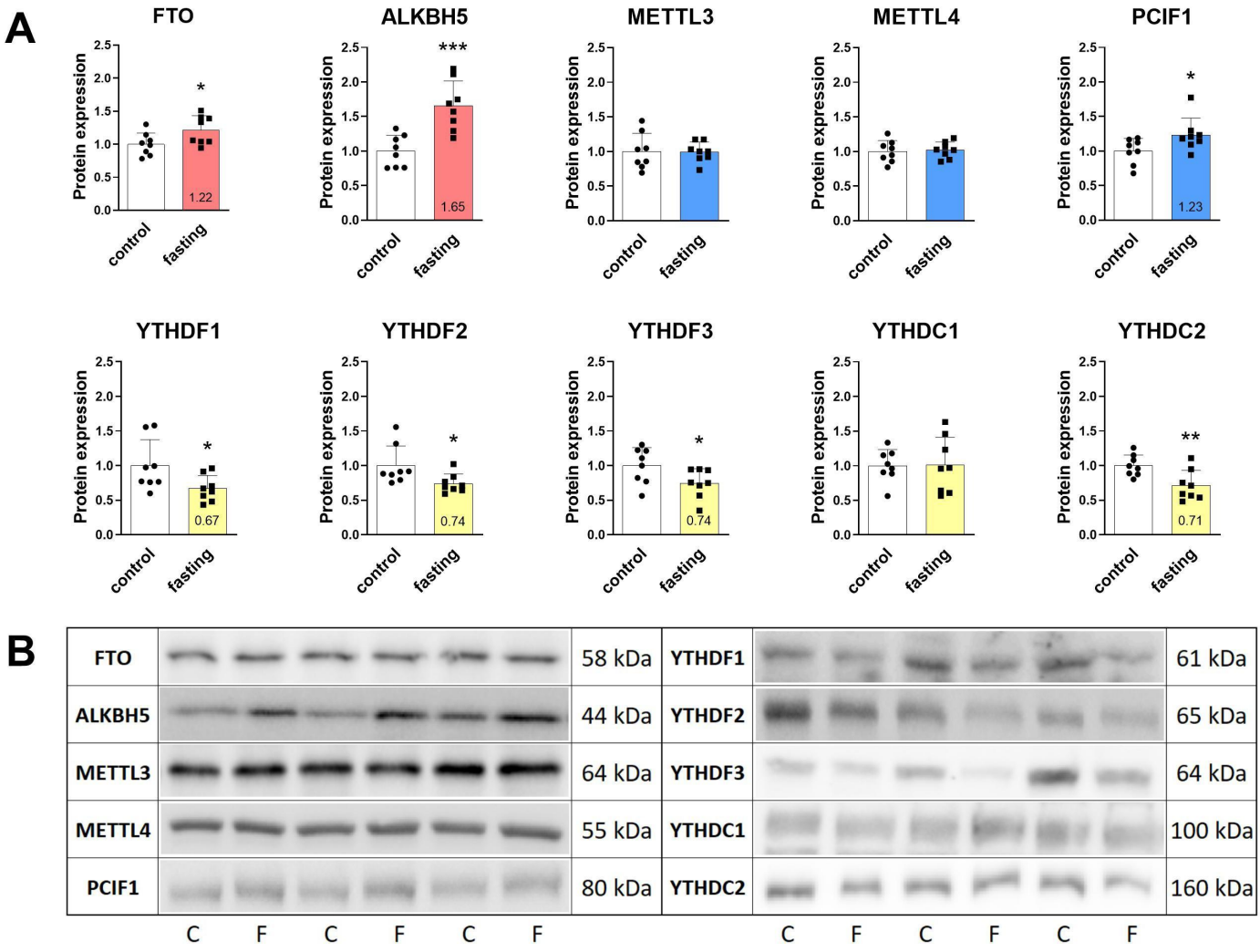

Figure 3. Effect of 3-day fasting on protein levels of m^{6}A and m^{6}Am regulators in the left ventricles assessed by Western blot (A). Writers are displayed in blue, erasers in red, and readers in yellow. The average of the control values is set to 1. Representative Western blot membranes (B). Protein loadings were 40 µg (YTHDF1, YTHDF3), 30 µg (YTHDC1), 20 µg (FTO, ALKBH5, YTHDC2), 15 µg (METTL3, YTHDF2), and 10 µg (METTL4, PCIF1). Values are means ± SD; $n = 8$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (t-test). ALKBH5 – AlkB family member 5; C – control; F – fasting; FTO – fat mass and obesity-associated protein; METTL3 – methyltransferase-like 3; METTL4 – methyltransferase-like 4; PCIF1 – phosphorylated CTD interacting factor 1; YTHDF1–3 – YTH domain-containing family protein 1–3; YTHDC1–2 – YTH domain-containing protein 1–2.

[35]. The overall fluorescence of the cells is inversely related to the intactness of the cell membranes. The fluorescence of SYTOX Green was measured at 490 nm excitation and 520 nm emission wavelengths in 96-well laminin-coated plates (at 8,000 cells per well) using the Synergy™ HT Multi-Detection Microplate Reader (BioTek, Winooski, USA).

Statistical analyses

All experiments included 6–10 biological replicates per group, except for MeRIP analysis ($n = 3$). Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, USA). Data were expressed as means ± SD. Unpaired two-sided Student's t-test or one-way ANOVA followed by Tukey's multiple comparisons test were used to assess statistical significance when comparing two or more groups, respectively. Differences with a p-value ≤0.05 were considered statistically significant.

Results

Characteristics of the fasting model

The average body weight (BW) of control (421 g) and fasting (426 g) rats did not differ before the onset of the experimental protocol. On average, after 3 days of fasting, the rats lost 71 g (17%) of BW, while control rats gained 4 g (+3%) of BW by the same period. The hearts of fasting rats were smaller by 16% compared to control rats after normalization to tibia length (Table 2).

The haematocrit in fasting rats (45.6%) was significantly higher than in controls (40.3%) The glycaemia dropped from 6.2 mmol/l to 3.5, 3.7, and 3.9 mmol/l after the first, second, and third day, respectively (Table 2). In total, 677 metabolites were detected in plasma samples. Fasting rats exhibited 171 down-regulated and 79 up-regulated metabolites (Figure 1). The most down-regulated included triacylglycerols, proline, hippuric acid, phosphatidylinositols, phosphatidylcholines, and lysophosphatidylcholines. The most up-regulated included free fatty acids, acylcarnitines, and 3-

hydroxybutyric acid. These results demonstrated a shift in metabolism towards lipids and the generation of ketone bodies typical for fasting [4,36].

Echocardiographic assessment of LV geometry and function (Table 2) did not show differences in wall thicknesses in control and fasting rats. LVDd was decreased in fasting rats and LVDs did not differ between the two groups. FS and HR were lower in fasting rats. CI, a parameter describing the cardiac output corrected to the BW, did not differ significantly between the two groups.

LV catheterization (Table 2) showed differences between experimental groups in neither Pes nor Ped. Developed pressure was not altered by fasting. While $+(dP/dt)_{max}$ decreased in fasting rats, $-(dP/dt)_{max}$ was not significantly affected by fasting.

While the observed metabolic and physiological changes, including weight loss and altered plasma metabolites, are consistent with a prolonged fasting response, it is important to note that many cardiac function parameters remained unaffected. This highlights a selective impact of fasting on different body systems, indicating a complex adaptive response to nutritional stress.

Effect of fasting on levels of m^{6}A and m^{6}Am regulatory proteins and transcripts in the left ventricles

Firstly, the effect of fasting on the main m^{6}A and m^{6}Am machinery gene expression was evaluated in LV samples of fasting and control rats by RT-qPCR (Figure 2). Both erasers were up-regulated, *Fto* by 17%, and *Alkbh5* by 23%. Two out of three writers were also up-regulated, *Mettl3* by 26%, and *Pcif1* by 22%. Regarding readers, only two were affected by fasting. *Ythdf3* levels were decreased by 15% and *Ythdc1* levels were increased by 23%. Other transcript levels (*Mettl4*, *Ythdf1*, *Ythdf2*, *Ythdc2*) were stable in the LVs of starving rats.

Secondly, the protein levels of the main m^{6}A and m^{6}Am regulators were measured in LV samples using Western blot (Figure 3). Both erasers were up-regulated, FTO by 22%, and ALKBH5 by 65%, which corresponded to the transcript changes. Regarding writers, only m^{6}Am methyltransferase PCIF1 was increased by 23%. Readers YTHDF1, YTHDF2, YTHDF3, and YTHDC2 were down-regulated by 33%, 26%, 26%, and 29%, respectively. Levels of other regulators (METTL3, METTL4, YTHDC1) were not affected on protein level.

Lastly, the peptide levels of m^{6}A and m^{6}Am regulators (2 peptides analysed for each protein) were also assessed using targeted proteomic analysis (Table 3). Out of the two peptides measured for each protein, the peptide with more profound changes was mentioned in the text. Regarding the main regulators, peptide levels of all YTHDF readers were decreased: YTHDF1 by 25%, YTHDF2 by 34%, and YTHDF3 by 27%. Demethylase ALKBH5, methyltransferase PCIF1, and reader YTHDC2 did not change significantly, but an increasing (ALKBH5, PCIF1) and decreasing (YTHDC2) trend was evident. FTO, METTL3, and YTHDC1 were unchanged at peptide levels. In addition to the main regulators, proteomic analysis revealed significant down-regulation also in peptide levels of other important proteins belonging to m^{6}A machinery: methyltransferase METTL5 (by 50%); readers eIF3a (by 37%), eIF3g (by 26%), eIF3c (by 23%),

Table 3. Targeted proteomic analysis – changes in peptide levels.

Changes in peptide levels of epitranscriptomic regulators				
Protein	Protein Accession	Peptide	Change	P-value
Writers				
METTL5	B0BNB3	LFDTVIMNPPFGTK	−50%	0.004
		YDLPALYNFHK	−41%	0.038
WTAP	D3ZPY0	TTSSEPVDQAEATSK	−13%	0.076 ×
Erasers				
ALKBH5	D3ZKD3	YFFGEGYTYGAQLQK	67%	0.060 ×
Readers				
eIF3a	Q1JU68	ALEVIKPAHILQEK	−37%	0.049
YTHDF2	E9PU11	LGSTEVASSVPK	−34%	0.003
		APGMNTIDQGMAALK	−30%	0.021
eIF3a	Q1JU68	LLDMDGIIVEK	−28%	0.003
YTHDF3	D3ZIY3	AITDGQAGFGNDTLSK	−27%	0.002
eIF3g	Q5RK09	GFAFISFHR	−26%	0.009
YTHDF3	D3ZIY3	HTTSIFDDFAHYEK	−26%	0.012
YTHDF1	Q4V8J6	HTTSIFDDFSHYEK	−25%	0.013
eIF3c	B5DFC8	LNEILQVR	−23%	0.011
RBMX	Q4V898	GGHMDDGGYSMNFTLSSSR	−16%	0.025
eIF3g	Q5RK09	LPGELEPVQAAQNK	−17%	0.086 ×
m^{6}A-repelled proteins				
USP10	Q3KR59	QADFVQTPITGIFGGHIR	−29%	0.021
CAPRIN1	Q5M9G3	TVLELQYVLDK	−28%	0.005
G3BP2	Q6AY21	VDAKPEVQSQPPR	−26%	0.007
CAPRIN1	Q5M9G3	YQEVTNNLEFAK	−24%	0.039
G3BP1	D3ZYS7	DFFQSYGNVVELR	−23%	0.023
ELAVL1	B5DF91	VAGHSLGYGFVNYVTAK	−21%	0.033

Changes at the edge of significance are marked by '×'. ALKBH5 – AlkB family member 5; CAPRIN1 – Cell cycle associated protein 1; eIF3a/c/g – Eukaryotic initiation factor 3a/c/g; ELAVL1 – ELAV-like protein 1; G3BP1 – G3BP stress granule assembly factor 1; G3BP2 – G3BP stress granule assembly factor 2; RBMX – RNA-binding motif protein, X chromosome; USP10 – Ubiquitin specific peptidase 10; WTAP – Willms' tumour 1-associating protein; YTHDC1 – YTH domain-containing protein 1; YTHDF1–3 – YTH domain-containing family protein 1–3.

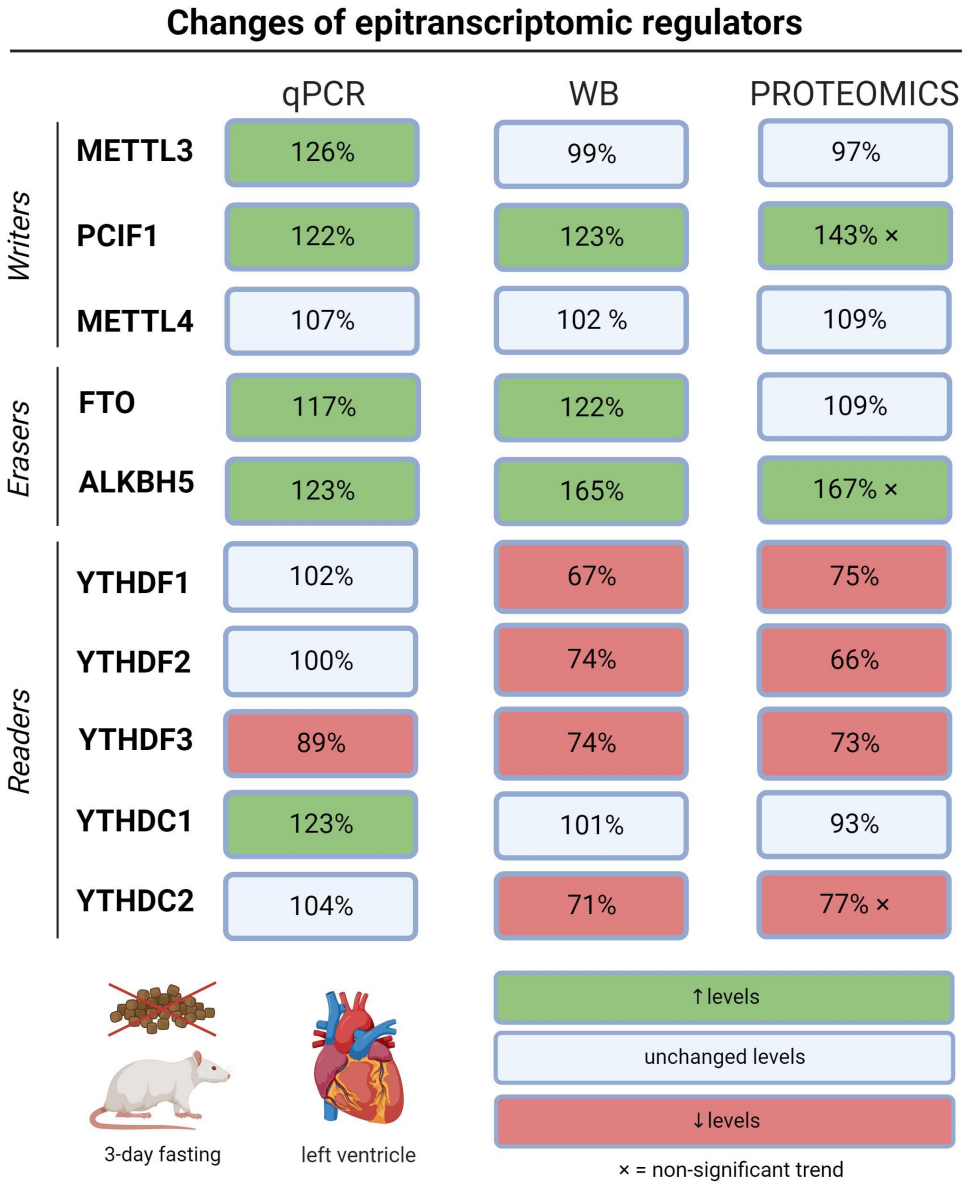

Figure 4. Levels of epitranscriptomic regulators in left ventricles of fasting rats assessed by RT-qPCR, Western blot, and proteomic analysis. Out of the two peptides measured for each protein in proteomic analyses, the peptide with more profound changes was depicted. ALKBH5 – AlkB family member 5; FTO – fat mass and obesity-associated protein; METTL3 – methyltransferase-like 3; METTL4 – methyltransferase-like 4; PCIF1 – phosphorylated CTD interacting factor 1; YTHDF1–3 – YTH domain-containing family protein 1–3; YTHDC1–2 – YTH domain-containing protein 1–2.

and RBMX (by 16%); and repelled proteins USP10 (by 29%), CAPRIN1 (by 28%), G3BP2 (by 26%), G3BP1 (by 23%), and ELAVL1 (by 21%).

Despite the slight differences between the data from the three distinct methods (Figure 4), our results revealed that epitranscriptomic machinery was regulated in LV of rats subjected to 3-day fasting. A different gene expression regulation on transcriptional and translational levels can explain the minor discrepancies between transcript and protein levels.

Effect of fasting on m^{6}A/m levels in the left ventricles

The effect of fasting on m^{6}A/m methylation was evaluated in LV samples of fasting and control rats (Figure 5). The methylation levels were significantly decreased from 0.008% of total RNA to 0.006% of total RNA, which corresponded with increased protein levels of demethylases in fasting animals. These results demonstrated the cardiac epitranscriptomic regulation in the fasting heart.

Methylation status of transcripts associated with cytoprotective functions of ketone bodies

Ketolysis is the hallmark of fasting. We assessed the methylation levels in cardiac transcripts potentially associated with cell-protective functions of ketone bodies (Figure 6). Out of 9 selected transcripts, we found a 5-fold up-methylation of *Nox4* and 4-fold up-methylation of *Hdac1*. Other transcripts (*Nfe2l2*, *Sirt1*,

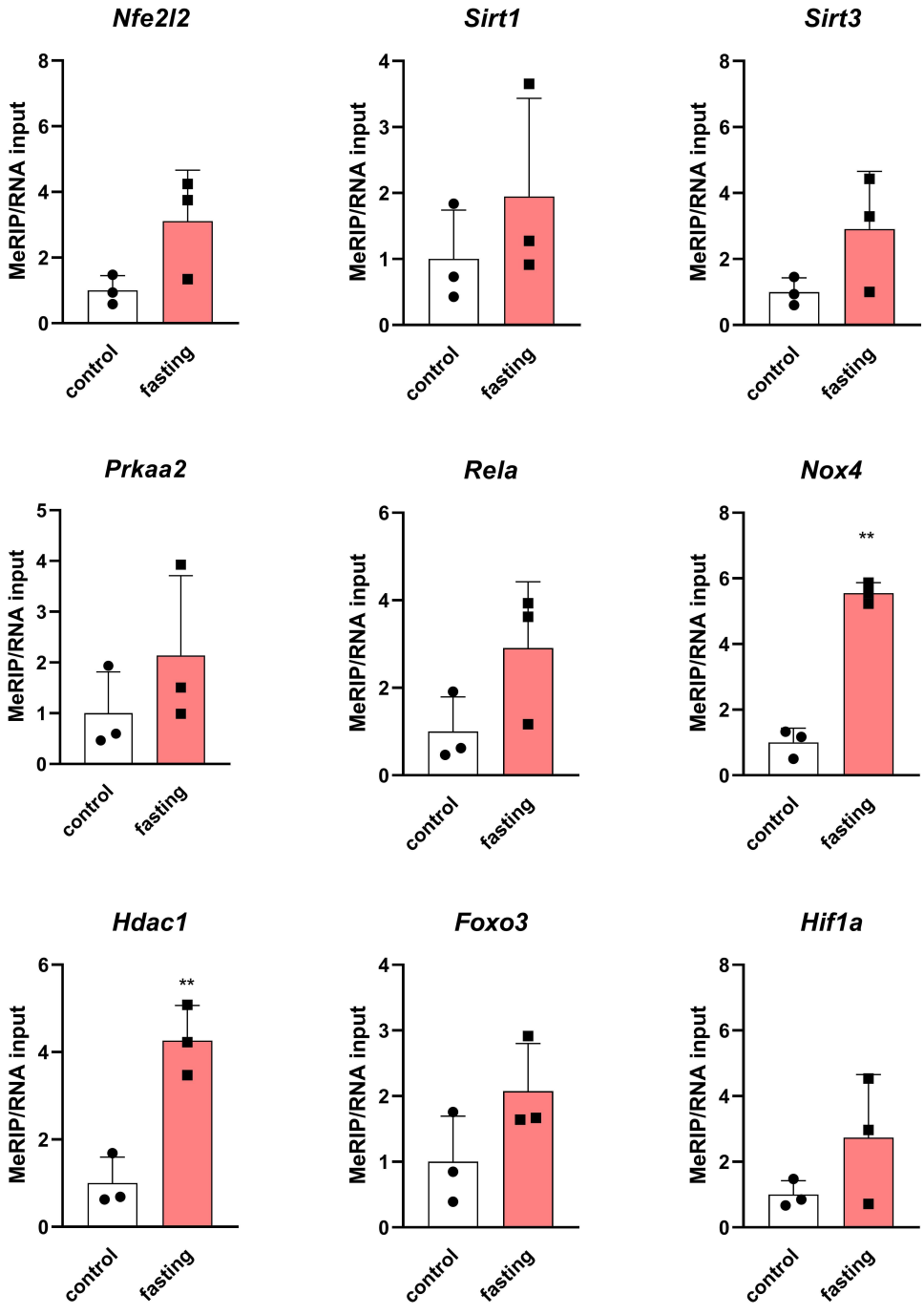

Figure 6. The m^{6}A/m enrichment in specific mRnas isolated from left ventricles of fasting rats. The average of the control values is set to 1. Values are means ± SD; $n = 3$; ** $p < 0.01$ (t-test). *Nfe2l2* – NFE2 like BZIP transcription factor 2; *Sirt1* – sirtuin 1; *Sirt3* – sirtuin 3; *Prkaa2* – protein kinase AMP-activated catalytic subunit alpha 2; *Rela* – RELA proto-oncogene, NF-KB Subunit; *Nox4* – NADPH oxidase 4; *Hdac1* – histone deacetylase 1; *Foxo3* – forkhead box O3; *Hif1a* – hypoxia inducible factor 1 subunit alpha.

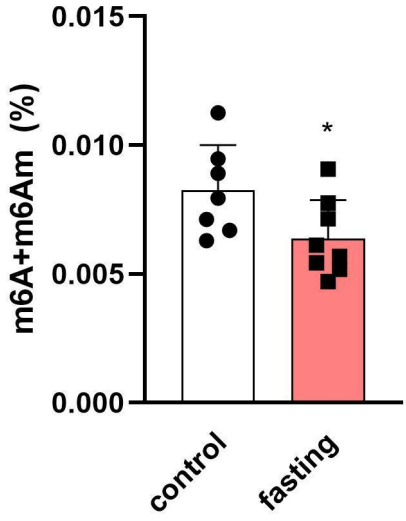

Figure 5. The difference between m^{6}A/m methylation levels in total RNA from left ventricles of fasting rats. Values are means ± SD; $n = 7–8$; * $p < 0.05$ (t-test).

Sirt3, *Prkaa2*, *Rela*, *Foxo3*, *Hif1a*) did not differ significantly between the control and fasting groups. However, upward trends were also obvious. These results showed altered epitranscriptomic regulations in possible cytoprotective pathways induced by fasting.

Effect of FTO and ALKBH5 inhibition on AVCMs exposed to hypoxia

To study the role of m^{6}A and m^{6}Am demethylases in the hypoxic tolerance of AVCMs, we examined the effect of FTO and ALKBH5 inhibitors on the viability of AVCMs from fasting and control rats using the SYTOX staining (Figure 7).

Administration of inhibitors did not affect the viability of AVCMs under normoxic conditions. Hypoxia significantly decreased the viability in untreated cells isolated from control and fasting rats to 87% and 89%, respectively. Under hypoxic conditions, inhibition of each demethylase further significantly decreased the viability of the cells from fasting rats to 81% (ALKBH5i) and 78% (FTOi), whereas the decrease in viability of cells from control rats did not reach statistical significance.

Discussion

Our data indicate that the cardioprotective regime of 3-day fasting is associated with cardiac regulation of m^{6}A and m^{6}Am machinery. Out of the 33 epitranscriptomic regulators studied, 22 were affected by fasting on either mRNA or protein levels, including up-regulation of demethylases FTO and ALKBH5. Together with that, we also observed decreased m^{6}A/m methylation in the hearts of fasting animals. Fasting was also associated with the up-methylation of *Nox4* and *Hdac1* transcripts, potentially participating in cell-protective functions of ketone bodies produced during fasting [31]. Inhibition of either demethylase resulted in decreased hypoxic tolerance in AVCMs isolated from fasting animals. Hence, we suggest that epitranscriptomics might act as an important layer of gene expression regulation in the fasting heart and contribute to cardioprotection.

Fasting affects cardiac epitranscriptomic regulations

Epitranscriptomic modification m^{6}A and its main regulators are affected in the heart under various physiological and pathophysiological conditions [37]. However, the role of m^{6}A and m^{6}Am in cardioprotection and cardioprotective models remains poorly described. In the past, the demethylases FTO and ALKBH5 were mostly associated with cardioprotective effects [18–20]. We also previously showed that the cardioprotective 4-week adaptation of rats to chronic hypoxia increased the protein expression of these demethylases [38]. However, the participation of epitranscriptomic regulations in fasting, another cardioprotective model, was still hypothetical. Recently, Xu et al. [22] discovered that intermittent fasting (IF) protects the mouse heart via a mechanism associated with decreased m^{6}A-RNA methylation levels. In line with this, they found that METTL3 levels were down-regulated and FTO levels were up-regulated after IF intervention. In our experiments, we found decreased methylation levels after 3 days of fasting as well. Regarding FTO, we observed an increase in its expression by RT-qPCR and Western blot techniques; however, the proteomic analysis did not confirm significant up-regulation. The discrepancy appeared in the case of METTL3, where we found increased gene expression by RT-qPCR and no changes in protein levels. This might be explained by the different fasting models (fasting every other day for 8 weeks vs. 'short-term' fasting for 3 days) and also different animal models used (mice vs. rats). Regarding the other main regulators not analysed by Xu et al., we observed up-regulation of the second demethylase ALKBH5 and methyltransferase PCIF1 at both gene and protein levels. Gene expression of reader *Ythdc1* was also increased. Other readers (YTHDF1–3 and YTHDC2) decreased their protein levels, and only *Ythdf3* was down-regulated on the gene level.

In addition to the main m^{6}A and m^{6}Am machinery, our study analysed less-known regulators in fasting hearts by targeted proteomic analysis. Their role in the fasting heart is unclear, but important functions in cardiac biology were suggested for these proteins. The most down-regulated protein was methyltransferase METTL5. This m^{6}A writer has been shown to regulate mRNA translation via 18S rRNA methylation [39]. Cardiac-specific depletion of METTL5 promoted pressure overload-induced cardiomyocyte hypertrophy and adverse remodelling [40]. Other affected proteins, which were down-regulated, included eIF3 reader subunits (eIF3a/c/g) and reader RBMX. eIF3 is a key factor in translation regulation. The largest and most well-known member of the eIF family is eIF3a. A mutation in the *eIF3a* gene was uncovered in patients with left ventricular non-compaction cardiomyopathy, a hereditary disease manifested by thromboembolic complications, arrhythmias, and heart failure. Further analyses on

H9c2 cells showed that this mutation was associated with decreased proliferation and induction of apoptosis [41]. The role of *eIF3a* was also described in cardiac fibrosis [42]. Another eIF3 subunit, eIF3c, was identified as a direct target of the reader YTHDF1, which augmented the translation of eIF3c in an m^{6}A-dependent manner [43]. In fasting hearts, we observed down-regulation of the YTHDF1-eIF3c axis as levels of both regulators were decreased. In addition to m^{6}A readers, the majority of m^{6}A-repelled proteins were less expressed in fasting hearts (G3BP1/2, ELAVL1, USP10, CAPRIN1). G3BP1 was found to be an important regulator of cardiac hypertrophy, atrial fibrillation, and coronary heart disease [44]. G3BP2 was also involved in the induction of cardiac hypertrophy and contributed to the development of atherosclerosis [45–47]. Moreover, overexpression of G3BP2 partially reversed the hypoxia/reoxygenation (H/R)-induced apoptosis in H9c2 cells [48]. However, in the cardioprotective fasting model, we found decreased G3BP2 levels. ELAVL1 is another RNA-binding protein with diverse cellular roles. Among other functions, it associates with mRNAs encoding hypoxia-response proteins such as hypoxia-inducible factor 1α (HIF-1α) or vascular endothelial growth factor (VEGF) and enhances their expression after hypoxia [49]. Myocardial I/R injury was linked with the up-regulation of ELAVL1 level [50]. Knockdown of ELAVL1 reduced MI-induced cardiomyocyte apoptosis, infarct size, and fibrosis area [50,51]. Therefore, decreased ELAVL1 levels observed in fasting hearts could play a role in the induction of cardioprotective phenotype. Protein USP10 was associated with cardiac hypertrophy [52,53]. Also, levels of this m^{6}A-repelled protein were decreased in H9c2 cells after the H/R insult, and overexpression of USP10 increased the viability and suppressed the apoptosis of H/R-induced cells [54]. However, this regulator was down-regulated in fasting hearts.

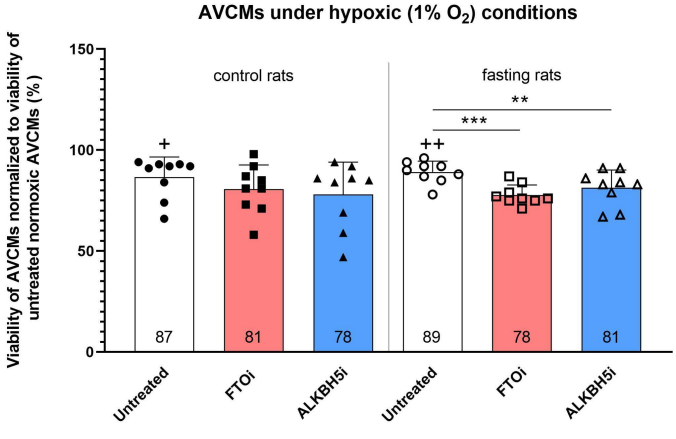

Figure 7. Effect of ALKBH5 and FTO inhibition on hypoxic tolerance of AVCMs isolated from control and fasting rats. Values are means ± SD; $n = 9$; ** $p < 0.01$; *** $p < 0.001$ (one-way ANOVA); + $p < 0.05$ compared to normoxic untreated AVCMs; ++ $p < 0.01$ compared to normoxic untreated AVCMs. ALKBH5i – ALKBH5 inhibitor; AVCMs – adult rat left ventricular cardiomyocytes; FTOi – FTO inhibitor.

Fasting is associated with up-methylation of *Nox4* and *Hdac1* transcripts in the heart

Given the association between fasting and ketolysis, our study focused on transcripts potentially related to the cytoprotective functions of ketone bodies, examining their m^{6}A/m methylation levels. We found that *Nox4* was significantly up-methylated in fasting hearts. NOX4 generates reactive oxygen species, which are involved in various signalling pathways including cardiac adaptation to different types of physiological and pathophysiological stresses. The protective role of NOX4 in the heart has been described [55]. However, ROS production by the NOX4 enzyme may also be harmful to the heart since ROS are a double-edged sword. It has been reported that NOX4 underwent extensive alternative splicing in human hearts and that the full-length NOX4 was significantly up-regulated in ischaemic cardiomyopathy [56]. As splicing regulation is one of the primary functions of m^{6}A and m^{6}Am modifications [8,14], alterations in methylation levels in the *Nox4* transcript may be crucial for determining the heart's fate. Besides *Nox4*, we also detected *Hdac1* up-methylation after fasting. HDAC1 functions as an epigenetic regulator by removing acetyl groups from histones and is inhibited by 3-hydroxybutyrate [31], the main ketone body increased in our fasting model. Inhibition of this protein was also associated with protecting cardiomyocytes against hypoxia [57]. Thus, these results revealed altered epitranscriptomic regulation in cell-protective pathways induced by ketone bodies in fasting hearts.

Inhibition of demethylases decreases the hypoxic tolerance of AVCMs isolated from fasting rats

The changes observed in fasting hearts included up-regulation of both demethylases on both transcript and protein levels. We did not observe a significant effect of FTO inhibition or ALKBH5 inhibition on hypoxic tolerance (1% O_2, 24 h) in cells from control animals, even though the decreasing trend was evident. However, a significant reduction of AVCM viability appeared in the fasting group after FTOi and ALKBH5i treatment.

It was already reported that FTO affects the survival of cardiomyocytes subjected to H/R. FTO was poorly expressed in human cardiomyocyte cell line AC16 exposed to H/R while FTO up-regulation improved the viability after H/R insult [18]. Similarly, in mouse cardiomyocytes, FTO overexpression inhibited apoptosis induced by acute H/R, while FTO knockdown had the opposite effect [19]. Moreover, the downexpression of FTO was observed in mouse hearts and isolated mouse cardiomyocytes subjected to I/R and acute H/R insults, respectively. FTO overexpression then attenuated the H/R-induced apoptosis in these cells [20]. Similarly, ALKBH5 overexpression also inhibited apoptosis of H/R-treated cardiomyocytes [15]. In line with these observations, our data confirm that the activity of RNA demethylases FTO and ALKBH5 is important for cardiomyocyte tolerance to hypoxic insult.

Conclusion

This study revealed that the cardioprotective regime of fasting altered m^6A/m modifications and its regulators in the heart, including demethylases FTO and ALKBH5, which were up-regulated after fasting. Specific transcripts potentially associated with the cell-protective functions of ketone bodies induced by fasting showed differential methylation in fasting hearts. Moreover, the inhibition of demethylases FTO and ALKBH5 decreased the hypoxic tolerance of cardiomyocytes isolated from fasting rats. In summary, these results suggest that epitranscriptomic regulations participate in the induction of cardioprotective phenotype induced by fasting.

Acknowledgments

The authors would like to acknowledge the Proteomics Core Facility (proteomics.fgu.cas.cz) at the Institute of Physiology of the Czech Academy of Sciences and Marek Vrbacky Ph.D. for proteomic analysis; and the Metabolomics Core Facility (metabolomics.fgu.cas.cz) at the Institute of Physiology of the Czech Academy of Sciences and Tomas Cajka Ph.D. for metabolomics and lipidomics profiling. Figures were created with BioRender.com.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Czech Science Foundation under Grant [1904790Y] to M.H.; the Charles University Grant Agency under Grant (GA UK 668220) to D.B.; and the project National Institute for Research of Metabolic and Cardiovascular Diseases [Programme EXCELES, ID Project No. LX22NPO5104] - Funded by the European Union – Next Generation EU.

Author contributions

Conceptualization: D.B., and M.H.; Formal Analysis: D.B., and M.H.; Funding acquisition: D.B., F.K., and M.H.; Investigation: D.B., K.H., J.H., F.K. and M.H.; Methodology: D.B., K.H., J.H., F.K., and M.H.; Project administration: D.B., and M.H.; Resources: M.O., M.K.; Supervision: F.K., and M.H.; Visualization: D.B., and M.H.; Writing – original draft: D.B.; Writing – review & editing: K.H., J.H., F.K., M.O., M.K., and M.H. All authors read and approved the final submitted manuscript.

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Attachment V

EPIGENETICS
2023, VOL. 18, NO. 1, 2218771
https://doi.org/10.1080/15592294.2023.2218771

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REVIEW

RNA modification m^{6}Am: the role in cardiac biology

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ABSTRACT

Epitranscriptomic modifications have recently emerged into the spotlight of researchers due to their vast regulatory effects on gene expression and thereby cellular physiology and pathophysiology. N^6,2′-O-dimethyladenosine (m^{6}Am) is one of the most prevalent chemical marks on RNA and is dynamically regulated by writers (PCIF1, METTL4) and erasers (FTO). The presence or absence of m^{6}Am in RNA affects mRNA stability, regulates transcription, and modulates pre-mRNA splicing. Nevertheless, its functions in the heart are poorly known. This review summarizes the current knowledge and gaps about m^{6}Am modification and its regulators in cardiac biology. It also points out technical challenges and lists the currently available techniques to measure m^{6}Am. A better understanding of epitranscriptomic modifications is needed to improve our knowledge of the molecular regulations in the heart which may lead to novel cardioprotective strategies.

ARTICLE HISTORY
Received 7 December 2022
Revised 19 May 2023
Accepted 23 May 2023

KEYWORDS
heart; epitranscriptomics; N^6,2′-O-dimethyladenosine; m^{6}Am; N^6-methyladenosine; m^{6}A

Introduction

The rapidly developing research field of epitranscriptomics has recently introduced a novel layer of gene expression regulation into cardiac biology. Over 170 chemical modifications have been found in RNA so far [1]. One of the most prevalent and characterized modifications is N^6-methyladenosine (m^{6}A) [2,3]. Similar N^6,2'-O-dimethyladenosine (m^{6}Am) is also a common form of modified adenosine (Table 1), but it is much less studied than m^{6}A. This modification is formed by the methylation of a 2'-O-methyladenosine (Am). It has been described in two RNA classes: messenger RNA (mRNA) and small nuclear RNA (snRNA). In mRNA, m^{6}Am is a common part of the mRNA cap and it is located at the transcription start site just next to the well-known 5-terminal modification – 7-methylguanosine (m^{7}G) [4,5]. It has been found in at least 30–40% of all transcripts in vertebrate mRNA [4]. However, in specific cell lines, m^{6}Am is even more dominant. For example, HEK293T cells have 92% of 5'capped mRNAs with m^{6}Am and only 8% with single methylated Am [6]. The presence of m^{6}Am in mRNA markedly enhances its stability due to the increased resistance of m^{6}Am-modified mRNA to the mRNA-decapping enzyme DCP2 [7]. Two different isoforms of snRNAs reflecting the methylation state of A adjacent to the 5' cap exist – m_1 (Am) and m_2 (m^{6}Am). Cells that exhibit high m_2 snRNA levels show modified patterns of alternative mRNA splicing [8]. m^{6}Am is also present at the internal sites of snRNAs [9]. The m^{6}Am/A percentage is approximately 0.01% of total RNA from human hearts [10]. This modification is dynamic, and it is regulated by proteins called writers (methylation deposition) and erasers (methylation removal) (Figure 1). No readers mediating the biological functions of m^{6}Am were described so far. However, many readers binding to the more explored m^{6}A are known. The question arises whether these RNA-binding proteins also recognize the similar m^{6}Am modification. At present, it is known that YTH domain-containing family protein 3 (YTHDF3), one of the key m^{6}A readers, does not bind m^{6}Am-containing transcripts [10]. For a better understanding of m^{6}Am biology, the search for m^{6}Am readers is needed.

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Table 1. Basic overview of adenosine methylations in RNA.

A	adenosine	One of the four nucleosides in RNA.
Am	2′-O-methyladenosine	Modified adenosine positioned typically next to the first nucleotide (m^7G) in the 5′ cap. N^6-methylation of Am forms m^6Am.
m^6A	N^6-methyladenosine	A prevalent modification in mRNA, but also other types of RNA (lncRNA, rRNA, tRNA, or snRNA). The primary target of FTO in the nucleus.
m^6Am	N^6,2′-O-dimethyladenosine	A modification formed by N^6-methylation of Am in the 5′ cap (mRNA, snRNA) and also internal RNA sites (snRNA). The main target of FTO in the cytosol.
m^1A	N^1-methyladenosine	A modification found mainly in tRNA and rRNA. An ancillary target of FTO.

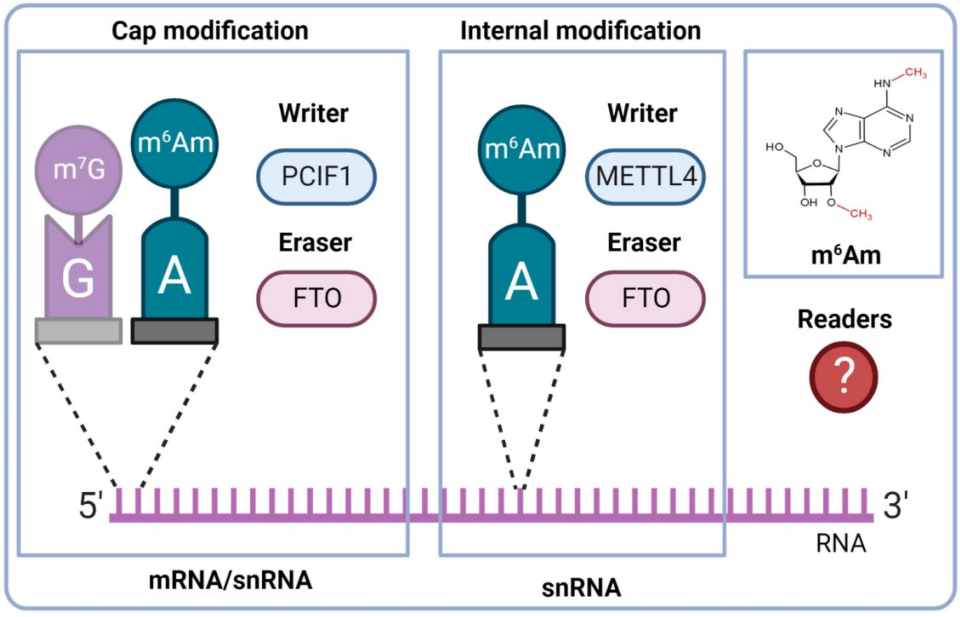

Figure 1. Basic overview of m^6Am modification. A – adenosine; G – guanosine; FTO – fat mass and obesity-associated; m^6Am – N^6,2′-O-dimethyladenosine; m^7G –7-methylguanosine; METTL4 – methyltransferase-like 4; PCIF1 – phosphorylated CTD interacting factor 1.

The pathological significance of m^6Am and its regulation remains largely unknown. Recent data suggest that this modification plays a role in obesity [11], cancer [12–15], or virus–host interaction [16–18], but its function in cardiac diseases has not yet been properly addressed. However, several studies have suggested the importance of fat mass and obesity-associated protein (FTO) for cardiac function. Since this demethylase has an affinity for both m^6A and m^6Am, the recognition of the m^6Am- function of FTO is important to unravel the role of m^6Am modification in cardiac physiology and pathophysiology.

m^6Am writers

N^6-methylation of Am to m^6Am is catalysed by two known writers: phosphorylated CTD interacting factor 1 (PCIF1) and methyltransferase-like 4 (METTL4). In 2019, PCIF1 has been described as a cap-specific adenosine-N^6-methyltransferase (also called CAPAM) which does not methylate adenosine residues in the RNA body [6,19]. However, recently it was reported that PCIF1 also has ancillary methylation activities on internal adenosines (both A and Am), although with lower affinities [20]. This writer has direct and indirect impacts on RNA stability and transcription [7,21–23]. The second m^6Am writer – METTL4 – has been described one year later in 2020. It is responsible for internal m^6Am formation within U2 snRNA and affects pre-mRNA splicing [24,25]. METTL4 also catalyses methylation of N^6-methyldeoxyadenosine (6mA), a modification in mitochondrial DNA (mtDNA), particularly under stress conditions [26].

m^6Am erasers

So far, the only described m^6Am eraser is FTO. Originally, FTO was described as an m^6A demethylase [27]. However, in 2017, FTO was reported to preferentially demethylate m^6Am rather than m^6A [7,28]. Recently, it was suggested that the substrate preference of FTO might depend on its cellular localization, which varies between cell types. In the nucleus, FTO preferably targets m^6A whereas cytosolic FTO demethylates especially m^6Am [9]. The cytosolic demethylation of m^6Am was later confirmed by others [12]. In cardiomyocytes, FTO is present in both the cytosol and the nucleus [29]. FTO demethylates m^6Am in both mRNA and snRNA [9]. Additionally to m^6A and m^6Am, FTO can also target N^1-methyladenosine (m^1A) in transfer RNA (tRNA) [7,9].

m^6Am regulation in cardiac physiology and pathophysiology

Epitranscriptomics is an emerging research field in cardiovascular physiology and pathophysiology, however, attention is mainly focused on m^6A [30–46]. The role of m^6Am in the heart is poorly understood (Figure 2). In rRNA-depleted RNA from rat adult cardiomyocytes, the m^6Am levels were 9-fold higher than m^6A levels, indicating the importance of m^6Am modification in cellular physiology (unpublished data).

m^6Am writers in cardiac physiology and pathophysiology

The role of m^6Am methyltransferases in the heart is unknown. Recently, we found that METTL4 is down-regulated in the hearts of rats adapted to chronic hypoxia, a well-known cardioprotective phenomenon, while PCIF1 is not affected under these conditions (unpublished data). A regulation in protein levels of METTL4 might indicate altered mRNA splicing in hearts from chronically hypoxic animals. PCIF1, on the other hand, increased in the hearts of rats subjected to fasting, another cardioprotective intervention [47]. Publicly available RNA-seq datasets generated from human left ventricles of failing and non-failing hearts report some degree of regulation of *METTL4* and *PCIF1*. These genes were especially regulated in a large RNA-seq dataset including 356 left ventricular samples from subjects with heart failure (HF) undergoing transplantation and non-failing donors from the MAGNet consortium (Table 2). However, further research is needed to decipher the functional role of m^6Am writers in HF, as there are no experimental data in animal models to date.

m^6Am erasers in cardiac physiology and pathophysiology

Undoubtedly, the best-studied m^6Am regulator is FTO. This eraser is essential for the normal development of the cardiovascular system in humans. Loss-of-function mutation in the human *FTO* gene caused several heart defects (ventricular septal defect, atrioventricular defect, patent ductus arteriosus) and hypertrophic cardiomyopathy [48]. Regulated expression of FTO was observed in myocardial infarction (MI) and HF patients and respective animal models [49–56]. Interestingly, RNA-seq datasets that revealed regulation of m^6Am writers in HF patients did not show significant changes in *FTO* expression (Table 2). Since HF is a heterogeneous clinical syndrome with complex pathophysiology, more studies with comparable methodology are necessary to reliably assess the role of FTO in different HF aetiologies. Gene variants of *FTO* were linked with various cardiovascular diseases, including MI, acute coronary syndrome, and an increased risk of rejection in heart transplant patients [57–61].

Global knockout (KO) of the mouse *Fto* gene led to a proarrhythmic remodelling of the heart. KO mice displayed higher heart rate and heart rate variability compared to their wild-type counterparts. Moreover, they were more vulnerable to stress-induced tachyarrhythmias, their ventricular repolarization was altered, and they also developed myocardial hypertrophy [62]. Studies on cultured neonatal rat cardiomyocytes showed that myocyte hypertrophy can be caused by a leptin-induced increase in FTO expression and that FTO knockdown with siRNA abrogated this event [63]. Mice lacking FTO specifically in cardiomyocytes showed worsened cardiac phenotype characterized by

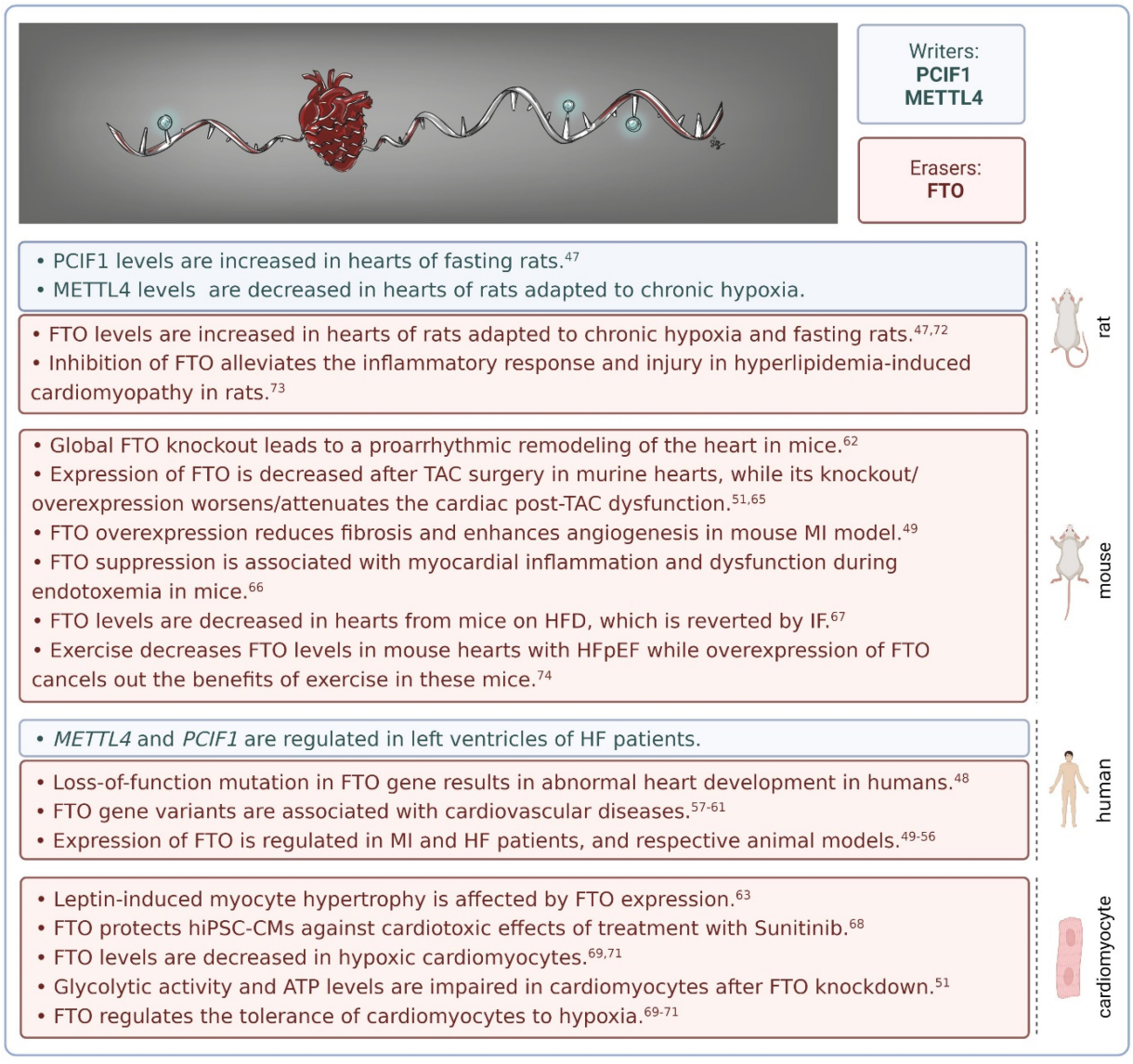

Figure 2. m^6Am modification in cardiac biology. FTO – fat mass and obesity-associated; HF – heart failure; HFD – high-fat diet; hiPSC-CMs – human-induced pluripotent stem cell-derived cardiomyocytes; IF – intermittent fasting; m^6Am – N^6,2′-O-dimethyladenosine; METTL4 – methyltransferase-like 4; MI – myocardial infarction; PCIF1 – phosphorylated CTD interacting factor 1; TAC – transverse aortic constriction.

reduced ejection fraction and increased dilatation upon transverse aortic constriction (TAC) surgery, an experimental model for pressure overload-induced cardiac hypertrophy and HF [64]. TAC surgery itself led to a decreased expression of FTO while FTO overexpression attenuated the cardiac post-TAC dysfunction [51,65]. FTO suppression was also associated with myocardial inflammation and dysfunction during endotoxemia in mice [66]. In murine isolated primary cardiomyocytes, FTO knockdown impaired the glycolytic activity and decreased ATP levels [51].

Recently, it has been shown that FTO plays a role in cardioprotection. Heart tissues from mice on a high-fat diet (HFD) contained decreased levels (both mRNA and protein) of FTO, which were reversed by intermittent fasting, a nutritional approach improving HFD-induced obesity cardiomyopathy [67]. These changes in FTO expression were also associated with corresponding changes in m^6A/m levels (detection of m^6A which does not differentiate between m^6A and m^6Am). Ma et al. [68] showed that FTO protected human-induced pluripotent stem cell-derived cardiomyocytes

Table 2. Changes of m^6Am regulators in left ventricles from heart failure patients (RNA-seq data).

Gene	Expression	Log2 Fold Change (disease/control)	P-value	FDR	Sample size	Dataset ID
METTL4	187	0.102	0.546	0.822	11	GSE108157
PCIF1	917	0.046	0.662	0.879	11	GSE108157
FTO	2132	−0.135	0.193	0.578	11	GSE108157
METTL4	256	−0.123	0.142	0.223	64	GSE116250
PCIF1	**988**	**0.336**	**7.48E−08**	**8.65E−07**	**64**	**GSE116250**
FTO	1703	0.077	0.151	0.235	64	GSE116250
METTL4	254	−0.151	0.105	0.213	30	GSE135055
PCIF1	845	0.025	0.708	0.807	30	GSE135055
FTO	1327	−0.039	0.640	0.755	30	GSE135055
METTL4	**319**	**−0.138**	**3.95E−03**	**0.007**	**356**	**GSE141910**
PCIF1	**725**	**0.206**	**6.27E−07**	**1.88E−06**	**356**	**GSE141910**
FTO	1156	−0.054	0.238	0.293	356	GSE141910
METTL4	81	0.051	0.709	0.847	23	GSE150736
PCIF1	268	0.193	0.0512	0.190	23	GSE150736
FTO	690	0.133	0.169	0.387	23	GSE150736
METTL4	174	0.061	0.552	0.750	15	GSE203160
PCIF1	801	−0.152	0.037	0.145	15	GSE203160
FTO	2119	0.004	0.942	0.974	15	GSE203160

Comparison of the expression of m^6Am regulators in the left ventricles of heart failure patients and healthy controls based on analysis of 6 RNA-seq experiments obtained from the GEO database. Changes significant according to FDR (false discovery rate) value are in bold. FTO – fat mass and obesity-associated; METTL4 - methyltransferase-like 4; PCIF1 - phosphorylated CTD interacting factor 1.

(hiPSC-CMs) against treatment with Sunitinib, a tyrosine kinase inhibitor with cardiotoxic effects. Importantly, FTO also affected the tolerance of cardiomyocytes to hypoxia, a key parameter in the possible prevention of ischaemic heart disease. FTO was poorly expressed in human cardiomyocyte cell line AC16 exposed to acute hypoxia/reoxygenation (H/R) and its up-regulation improved cell viability after H/R insult [69]. Likewise, in mouse cardiomyocytes, FTO overexpression inhibited apoptosis induced by acute H/R while FTO knockdown had the opposite effect [70]. Moreover, Ke et al. [71] observed the down expression of FTO in mouse hearts and isolated cardiomyocytes subjected to ischaemia-reperfusion and acute H/R insults, respectively. FTO overexpression then led to attenuation of the H/R-induced apoptosis and inflammation in these cells. In our lab, we found that administration of FTO inhibitor MO-I-500 increased the lactate dehydrogenase release (which indicates cell damage) and decreased cell viability in rat cardiomyocytes under acute hypoxic conditions (unpublished data). Adaptation of rats to 3 weeks of chronic hypoxia and 3-day fasting, both cardioprotective regimes, was associated with increased myocardial levels of FTO, which might thus participate in the induction of the protection [47,72]. FTO overexpression also reduced fibrosis and enhanced angiogenesis in mouse models of myocardial infarction [49].

Contrary to the data showing the beneficial effects of FTO on the heart, Yu et al. [73] reported that inhibition of FTO using LuHui Derivative (LHD) compound alleviated the inflammatory response and injury in hyperlipidaemia-induced cardiomyopathy in rats. Similarly, mice with heart failure (HFpEF) exhibited decreased FTO levels in the heart after physical training while overexpression of FTO abolished the health benefits of exercise in these mice by promoting myocyte apoptosis, myocardial fibrosis, and myocyte hypertrophy [74].

It is worth mentioning that FTO regulations in the heart might be age-dependent. According to Su et al. [75], FTO levels dropped in elderly murine hearts in response to acute myocardial ischaemia/reperfusion injury while in young hearts it remained unaffected. FTO levels also changed during postnatal development [76]. Moreover, the hearts of newborn male rats exhibited higher FTO protein levels than females, suggesting possible sex-dependent differences in m^6Am regulations [76].

These data show that FTO can have both beneficial and detrimental effects on the heart. However, current FTO studies have focused on m^6A and have not included m^6Am. Since many m^6A detection methods do not distinguish between these two modifications, the potential involvement of m^6Am could be masked. Separating the m^6A-specific and m^6Am-specific mechanisms of action in the future is essential for understanding the role of m^6Am in cardiac

biology. In rRNA-depleted RNA isolated from rat cardiomyocytes, the m^{6}Am levels are 9-fold higher compared to m^{6}A. Nevertheless, according to RM2Target (database for targets of writers, erasers, and readers), data on the m^{6}Am-targets of FTO in the heart are virtually missing [77].

m^{6}Am regulators as pharmacological targets

At present, several inhibitors of demethylase FTO are available. In 2012, the natural product rhein was identified as the first cell-active inhibitor of FTO that was capable to increase the modification level of m^{6}A in mRNA [78]. Two years later, compound MO-I-500 was introduced as another specific inhibitor of this demethylase [79]. A known anti-inflammatory drug meclofenamic acid (MA) was also identified as a highly selective inhibitor of FTO [80]. Treatment of HeLa cells with either MO-I-500 or MA resulted in the elevation of m^{6}A levels in mRNA [79,80]. Fluorescein and its derivatives were introduced as bifunctional molecules that can be used for FTO inhibition or labelling [81]. Selective inhibition of FTO was also achieved by other small molecule inhibitors, such as FB23, FB232, CS1, CS2, Dac51, LHD, FTO-02, or FTO-04 [73,82–89]. Since FTO is a non-specific demethylase, it is expectable that its inhibition may affect levels of all its substrates. For instance, treatment of glioblastoma stem cells with FTO-04 resulted in increases in both m^{6}A and m^{6}Am levels [87]. Unfortunately, current FTO inhibitors are not suitable for clinical use due to either low target selectivity or pharmacokinetic properties [87]. Compounds affecting other m^{6}Am regulators were not described so far. Future identification of effective pharmacological agents targeting m^{6}Am regulation is important for the development of novel therapies for cardiac diseases.

m^{6}Am research methods

To determine the effects of m^{6}Am, it is necessary to properly distinguish m^{6}Am from similar m^{6}A. Antibodies used in the m^{6}A detection bind both m^{6}A and m^{6}Am and therefore incorrectly annotate also m^{6}Am as m^{6}A [7]. Another problem is that m^{6}Am regulators are not m^{6}Am-specific. METTL4 can also catalyse methylation of 6mA and FTO can demethylate m^{6}A and m^{1}A, so both of these regulators might affect the cardiac biology also in an m^{6}Am-independent manner. Thus, future studies should focus on the differentiation of m^{6}A and m^{6}Am regulations. Various novel techniques can deal with the similarity of these two modifications and distinguish m^{6}Am from m^{6}A (Figure 3).

Similarly to other modifications, relative quantification of m^{6}Am levels can be achieved by a thin layer chromatography (TLC) method or mass spectrometry-based detections such as liquid chromatography with tandem mass spectrometry (LC-MS/MS) or CapQuant technique [6,19,90,91]. Moreover, several high-throughput sequencing methods are used for m^{6}Am profiling. The m^{6}A antibodies used in these techniques do not differentiate between m^{6}A - and m^{6}Am. Various approaches have been used to overcome this issue. For example, MeRIPseq (also called m^{6}A-seq), miCLIP, or m6ACE-seq require either a dedicated bioinformatics analysis (as m^{6}Am is present at the 5'end of mRNAs while m^{6}A is enriched in its internal regions) or signal depletion of m^{6}Am in the PCIF1-KO cell lines to reveal m^{6}Am profiles [92–95]. m^{6}Am-exo-seq relies on the elimination of uncapped RNA fragments (with m^{6}A modification), resulting in the enrichment of 5'-end RNA fragments containing m^{6}Am. Subsequent decapping of 5'-end RNA fragments facilitates m^{6}Am recognition by m^{6}A antibody [22]. Likewise, m^{6}Am-seq utilizes cap-m^{7}G immunoprecipitation to purify 5'-end RNA fragments and then uses recombinant FTO treatment to erase m^{6}Am before sequencing. Since FTO has a higher affinity to m^{6}Am compared to m^{6}A *in vitro*, the comparison of FTO-treated and untreated samples allows the specific identification of m^{6}Am sites [96,97]. Recently, CAPturAM, an antibody-independent method to selectively enrich and detect physiological targets of PCIF1 has been introduced. This technique is based on RNA labelling with recombinantly produced PCIF1 and assessment by RT-qPCR. CAPturAm allows the identification of the transcription start nucleotide N^6-methylation status by comparing enrichment between WT and PCIF1-KO cells [98]. In addition to these experimental methods, several computational approaches were introduced to accurately identify m^{6}Am sites based on sequence-derived data, such as m6AmPred, MultiRM, or DLm6Am [99–101].

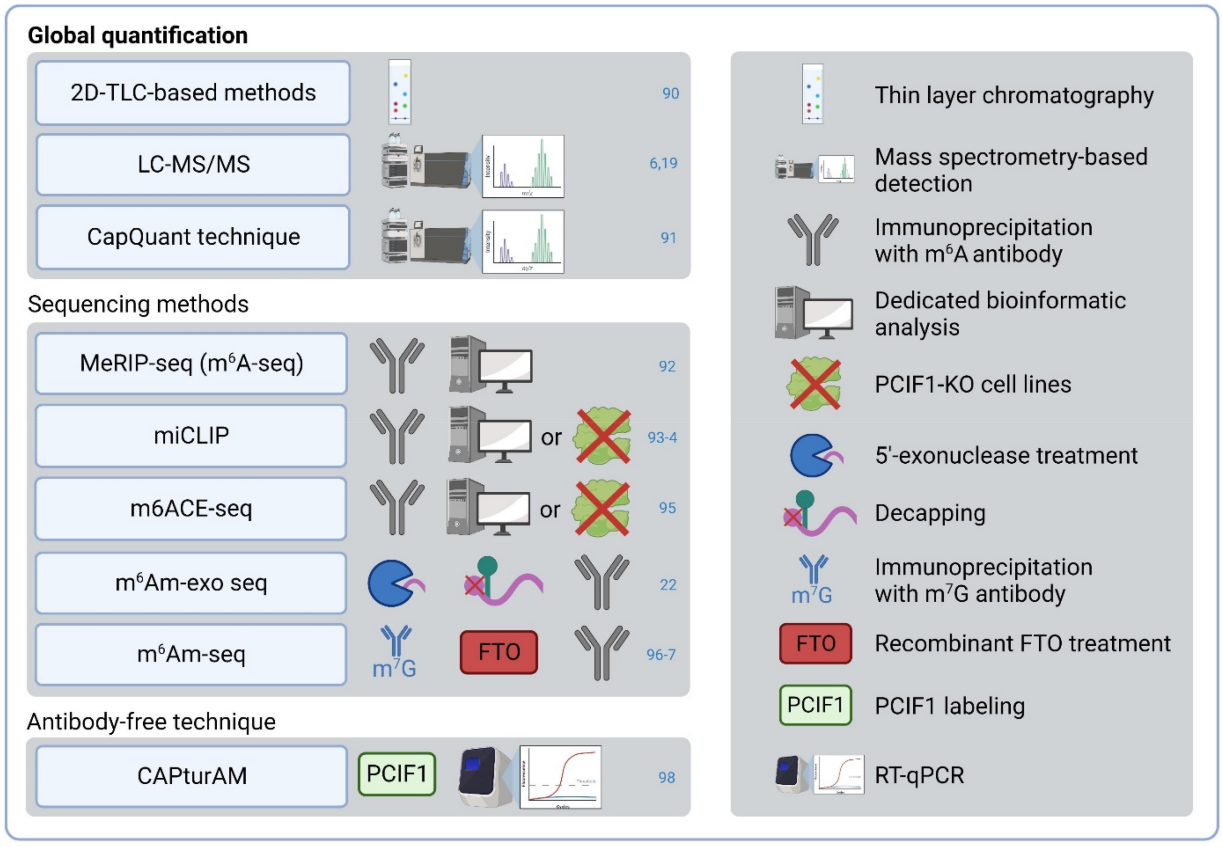

Figure 3. m^{6}Am detection methods. 2D-TLC – two-dimensional thin-layer chromatography; FTO – fat mass and obesity-associated protein; LC-MS/MS – liquid chromatography-tandem mass spectrometry; m6ACE-seq – m^{6}A-crosslinking-exonuclease-sequencing; m^{6}Am-exo-seq – m^{6}Am-exonuclease-assisted-sequencing; m^{6}Am-seq – m^{6}Am-sequencing; MeRIP-seq – methyl RNA immunoprecipitation and sequencing; miCLIP – m^{6}A individual-nucleotideresolution crosslink and immunoprecipitation; PCIF1 – phosphorylated CTD interacting factor 1; RT-Qpcr – reverse transcription quantitative real-time polymerase chain reaction.

Conclusion

m^{6}Am is one of the most prevalent modifications of RNA. Its regulation might have a profound effect on cardiac physiology, yet the knowledge of its functional role in cardiac disease development as well as its potential value as a therapeutic target and biomarker deserves further investigation. The epitranscriptomics field remains uncharted territory that might reveal clinically relevant discoveries in the future.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The work was supported by the Charles University Grant Agency [200317]; the Czech Science Foundation [19-04790Y]; the project National Institute for Research of Metabolic and Cardiovascular Diseases (Programme EXCELES) - Funded by the European Union – Next Generation EU [LX22NPO5104]; the EU Horizon 2020 project COVIRNA [101016072]; the National Research Fund [C14/BM/8225223, C17/BM/11613033, and COVID-19/2020-1/14719577/miRCOVID]; the Ministry of Higher Education and Research; the Heart Foundation-Daniel Wagner of Luxembourg ; EU-CardioRNA COST Action [CA17129].

Data availability statement

The RNA-seq data analysed in this study (Table 2) are available at the Gene Expression Omnibus (GEO) data repository, which is accessible at https://www.ncbi.nlm.nih.gov/geo/.

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Attachment VI

frontiers | Frontiers in Endocrinology

TYPE Review
PUBLISHED 07 July 2023
DOI 10.3389/fendo.2023.1223583

OPEN ACCESS

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RECEIVED 16 May 2023
ACCEPTED 26 June 2023
PUBLISHED 07 July 2023

CITATION
Benak D, Benakova S, Plecita-Hlavata L and Hlavackova M (2023) The role of m^{6}A and m^{6}Am RNA modifications in the pathogenesis of diabetes mellitus. *Front. Endocrinol.* 14:1223583. doi: 10.3389/fendo.2023.1223583

The role of m^{6}A and m^{6}Am RNA modifications in the pathogenesis of diabetes mellitus

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The rapidly developing research field of epitranscriptomics has recently emerged into the spotlight of researchers due to its vast regulatory effects on gene expression and thereby cellular physiology and pathophysiology. N^6-methyladenosine (m^{6}A) and N^6,2'-O-dimethyladenosine (m^{6}Am) are among the most prevalent and well-characterized modified nucleosides in eukaryotic RNA. Both of these modifications are dynamically regulated by a complex set of epitranscriptomic regulators called writers, readers, and erasers. Altered levels of m^{6}A and also several regulatory proteins were already associated with diabetic tissues. This review summarizes the current knowledge and gaps about m^{6}A and m^{6}Am modifications and their respective regulators in the pathophysiology of diabetes mellitus. It focuses mainly on the more prevalent type 2 diabetes mellitus (T2DM) and its treatment by metformin, the first-line antidiabetic agent. A better understanding of epitranscriptomic modifications in this highly prevalent disease deserves further investigation and might reveal clinically relevant discoveries in the future.

KEYWORDS

type 2 diabetes mellitus, T2DM, diabetes, RNA, epigenetics, epitranscriptomics, m6A, m6Am

1 Introduction

Diabetes mellitus is one of the most common chronic diseases with an increasing prevalence (1). Type 2 diabetes mellitus (T2DM) is more frequent than type 1 diabetes mellitus (T1DM) and accounts for approximately 90% of all cases of diabetes (2). This heterogeneous systemic disorder is mainly characterized by two factors: deficient insulin secretion by pancreatic β-cells and insulin resistance of insulin-sensitive tissues (3). The subsequent chronic hyperglycemia, a hallmark of T2DM, damages glucose-sensitive organs and results in downstream deficits in vital functions (4). Despite a considerable amount of

data collected regarding T2DM, the molecular mechanism of its development is still unclear. However, it is known that T2DM is linked with the dysregulation of gene expression profiles in cells (5–7). Epitranscriptomic modifications of RNA are one of the possible mechanisms by which gene expression could be affected during the pathogenesis of T2DM.

To date, over 170 chemical modifications have been described in RNA (8). N^6-methyladenosine (m^6A) and N^6,2'-O-dimethyladenosine (m^6Am) are among the most prevalent and well-characterized RNA-modified nucleosides (9–12). The biological effects of these modifications are regulated by proteins called writers (methylation deposition), readers (binding of modified RNA), and erasers (methylation removal). The presence or absence of m^6A and m^6Am in mRNA affects key stages of its life cycle, including splicing, export, decay, and translation (Figure 1) (13, 14). These dynamic modifications with profound impact on gene expression regulation might thereby play an important role in the pathogenesis of T2DM and become the future targets in the search for the next generation of anti-diabetic drugs.

2 N^6-methyladenosine

The most prevalent modification in eukaryotic mRNA is m^6A (9, 10). Besides mRNA, m^6A also occurs in other types of RNA, including ribosomal RNA (rRNA), long non-coding RNA (lncRNA), small nuclear RNA (snRNA), or microRNA (miRNA) (15). The deposition of the methyl group to adenosine (A) is performed by a multicomponent methyltransferase complex (MTC) with a stable core component formed between methyltransferase-like 3 (METTL3) and methyltransferase-like 14 (METTL14). METTL3 functions as a catalytic subunit and METTL14 facilitates RNA binding (16, 17). The third major component of the MTC is the Willms' tumor 1-associating protein (WTAP) which interacts with the METTL3/METTL14 heterodimer and promotes the localization of the MTC to nuclear speckles (18). The reverse process, demethylation of m^6A back to A, is mediated by enzymes called demethylases. In 2011, Fat mass and obesity-associated protein (FTO) was the first described demethylase of m^6A (19). This discovery provided evidence of reversible posttranscriptional modifications in mRNAs and renewed the interest of researchers in mRNA modifications (20). After 2 years, alkB homolog 5 (ALKBH5) was reported as another m^6A eraser (21). The biological functions of m^6A can be mediated by m^6A readers which recognize and selectively bind to m^6A-decorated RNAs. The most prominent readers are YTH domain-containing family proteins 1-3 (YTHDF1-3) which mediate the degradation of methylated mRNAs, and YTH domain-containing proteins 1-2 (YTHDC1-2) which regulate mRNA splicing and facilitate translation initiation (22–28). In addition to YTH proteins, other readers described include insulin-like growth factor 2 mRNA-binding proteins 1-3 (IGF2BP1-3) which promote the stability of their target mRNAs in an m^6A-dependent manner under normal and stress conditions and therefore also affect gene expression output (29).

3 N^6,2'-O-dimethyladenosine

m^6Am is another prevalent form of modified adenosine, but it is much less studied than m^6A. This modification is formed by the methylation of a 2'-O-methyladenosine (Am). It has been described only in mRNA and snRNA. In mRNA, m^6Am is found directly downstream to the 7-methylguanosine (m^7G), forming the extended cap structure (11, 12). It has been found in at least 30-40% of all transcripts in vertebrate mRNA (11). However, in specific cell lines, m^6Am is even more dominant. For instance, HEK293T cells have 92% of 5' capped mRNAs with m^6Am and only 8% with

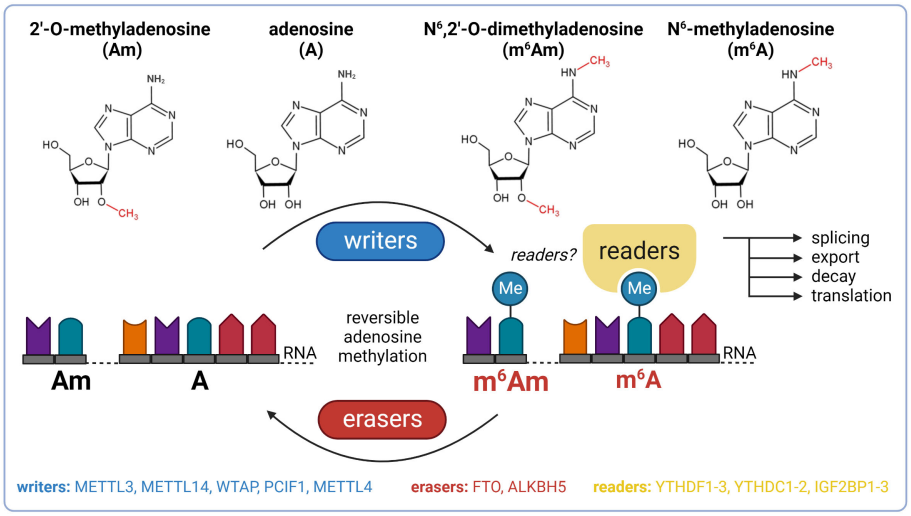

FIGURE 1
Basic overview of m^6A and m^6Am epitranscriptomics. ALKBH5, AlkB family member 5; FTO, fat mass and obesity-associated; IGF2BP1-3, insulin-like growth factor 2 mRNA binding proteins 1-3; METTL3, methyltransferase-like 3; METTL14, methyltransferase-like 14; WTAP, Willms' tumor 1-associating protein; YTHDC1-2, YTH domain-containing protein 1-2; YTHDF1-3, YTH domain-containing family proteins 1-3.

single methylated Am (30). The presence of m^6Am in mRNA markedly enhances its stability (31). In snRNA, m^6Am is also present at its internal sites and influences pre-mRNA splicing (11, 32). N^6-methylation of Am to m^6Am is catalyzed by two known writers: phosphorylated CTD interacting factor 1 (PCIF1) and methyltransferase-like 4 (METTL4). PCIF1 has been described as a cap-specific adenosine-N^6-methyltransferase (also called CAPAM) which does not methylate adenosine residues in the RNA body (30, 33). However, recently it was reported that PCIF1 also has ancillary methylation activities on internal adenosines (both A and Am), although with lower affinities (34). Importantly, before the recognition of methyltransferase activity of PCIF1, this protein was known to inhibit pancreatic and duodenal homeobox protein 1 (PDX1), a transcription factor crucial for normal pancreas development and function (35, 36). METTL4, the second methyltransferase, is responsible for internal m^6Am formation within U2 snRNA (37, 38). The only described m^6Am eraser so far is FTO, the well-known m^6A demethylase. In 2017, it was reported that FTO preferentially demethylates m^6Am rather than m^6A (31, 39), but recent studies suggested that the substrate preference of FTO might depend on its cellular localization which varies between cell types. In the nucleus, FTO preferably targets m^6A whereas cytosolic FTO demethylates especially m^6Am (40, 41). Thus, special attention is needed in FTO research to distinguish the m^6A- and m^6Am-specific effects of this demethylase (42). No readers of m^6Am have been described so far.

4 Pathogenesis of T2DM: the role of m^6A and m^6Am modifications

4.1 Genetic predisposition to T2DM

The development of T2DM is the result of interaction between environmental factors (e.g. unhealthy diet, sedentary lifestyle, stress) and a strong hereditary component (43). Currently, several hundreds of genetic variants were associated with T2DM, although mostly with only minor effects on disease development (44).

Numerous studies suggested that m^6A and m^6Am demethylase *FTO* is among the genes whose variants possess the highest genetic risk of T2DM (44). However, this link is still controversial with significant interethnic differences (45, 46). For instance, the common *FTO* rs9939609 variant was associated with T2DM in white American, Palestinian, Asian Indian, and obese Iraqi populations, but not in Bengalee Hindu, North Indian, nor Saudi populations (47–57). Also, other genetic polymorphisms in the *FTO* gene were identified as T2DM risk factors. Carriers of the *FTO* rs17817449 variant in the Czech-Slavonic and obese Iraqi populations were more susceptible to T2DM and chronic diabetic complications (44, 51, 58). In Iranian obese women, *FTO* variants rs763967273, rs759031579, rs141115189, rs9926289, rs76804286, and rs9939609 were all related to T2DM (59). On the contrary, African-Americans carrying the rs1421085 C allele were found to be protected against diabetes (54). The polymorphisms in *FTO* gene seem to regulate the expression level of FTO and its enzymatic function. Detrimental effects of high or low expression of FTO were already confirmed in experimental studies. For instance, it has been shown that FTO depletion activates inflammatory response, one of the main pathogenic features in T2DM patients (60).

Besides *FTO*, variants of *IGF2BP2*, an m^6A reader, were also associated with a significant risk of T2DM development, namely variant rs4402960 in Asian Indian Sikhs, Czechs, or Italians, and rs11705701 in the Chinese population (44, 55, 61, 62).

Although further studies are needed to unravel the complex polygenic background of T2DM, it seems to be clear that genetic polymorphisms in genes encoding epitranscriptomic regulators are associated both with T2DM and its complications.

4.2 Pancreatic islets

Pancreatic β-cell failure mediated by metabolic stress is the central event in the pathogenesis of T2DM (63). Although the mechanisms underlying β-cell dysfunction are still not fully understood, emerging data suggest an involvement of epigenetic modifications in the adaptation of β-cells to metabolic stress (64).

m^6A sequencing in dispersed islets from controls and T2DM patients revealed 6,078 differently methylated sites in 4,155 mRNAs and a higher number of sites with decreased levels of m^6A methylation in T2DM compared to controls. Gene ontology analysis of the m^6A methylome revealed that the genes affected in T2DM patients are involved in cell-cycle regulation, receptor signaling, insulin secretion, and pancreas development (65). The decreased total m^6A levels were observed in Langerhans islets of T2DM patients and also in islets of mice fed with a high-fat diet (a model mimicking T2DM phenotype). Similarly, high glucose conditions (state typical for T2DM) also resulted in lower methylation levels in non-diabetic human pancreatic islets as well as in mouse β-cell line (Min6) (66). Gene expression analysis in whole islets collected from healthy humans and patients with T2DM revealed a down-regulation of several m^6A regulators in diabetic individuals – methyltransferase *METTL14*, demethylases *FTO* and *ALKBH5*, and readers *YTHDF1* and *YTHDF3*. In addition to transcripts, protein levels of methyltransferases METTL3 and METTL14 were also decreased (65). The reduction of *FTO* gene expression and METTL3/14 protein levels in T2DM human islets was observed also in other studies (67–69). RNA-seq datasets (GSE153855; GSE153855) from T2DM and non-T2DM individuals revealed increased gene expression of readers *IGF2BP2-3* and decreased gene expression of writer *WTAP* and readers *YTHDF2-3*, *YTHDC1*, and *HNRNPC* (70–72). m^6A reader *IGF2BP2* was also up-regulated in β-cells obtained from cadaver pancreases of T2DM patients (73). The current knowledge of diabetic epitranscriptomic changes in human Langerhans islets is summarized in Figure 2. Overall, it seems that the whole epitranscriptomic machinery is attenuated in human diabetic islets. The only up-regulated genes *IGF2BP2-3* have also functions unrelated to epitranscriptomics, which might explain their opposite trend.

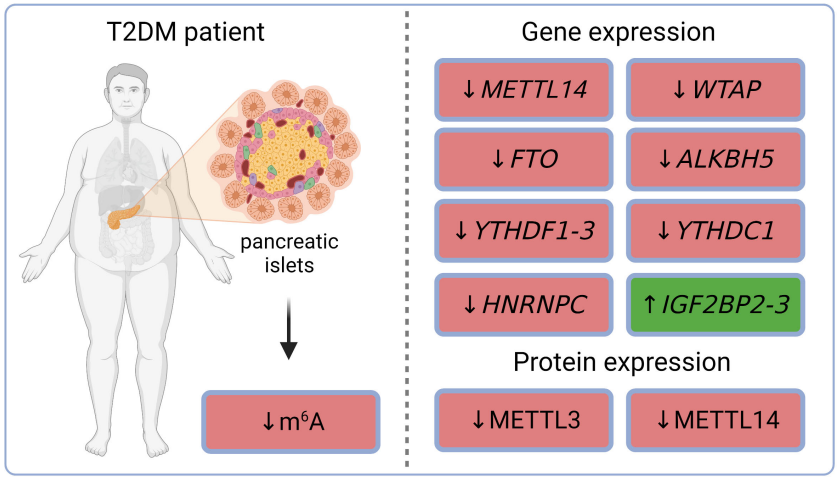

FIGURE 2
m^6A and m^6Am regulations in pancreatic islets of T2DM patients. ALKBH5, AlkB family member 5; FTO, fat mass and obesity-associated; HNRNPC, heterogeneous nuclear ribonucleoprotein C; IGF2BP2-3, insulin-like growth factor 2 mRNA binding proteins 2-3; m^6A, N^6-methyladenosine; METTL3, methyltransferase-like 3; METTL14, methyltransferase-like 14; T2DM, type 2 diabetes mellitus; WTAP, Willms' tumor 1-associating protein; YTHDC1, YTH domain-containing protein 1; YTHDF1-3, YTH domain-containing family proteins 1-3.

In contrast to these results, Bornaque et al. (66) showed that high glucose concentrations in Min6 cells increased mRNA expression of important m^6A regulators – methyltransferase *Mettl3* and demethylases *Fto* and *Alkbh5*. Glucose treatment also induced a shift in the subcellular protein localization of METTL3 and ALKBH5 (66). Overexpression of *FTO* in Min6 cells promoted the production of reactive oxygen species (ROS) and led to NF-κB activation, which resulted in the inhibition of insulin secretion (74). These differences between a specific mouse cell line and heterogeneous human islets might be explained by interspecies variation or islet heterogeneity.

MTC specifically regulates the postnatal functional maturation of β-cells. Mice with deletion of *Mettl3/14* in $Ngn3^+$ endocrine progenitor cells developed hyperglycemia and hypoinsulinemia 2 weeks after birth. This study also showed that *Mettl3/14* deletion silenced the expression of important transcription factors, such as *Mafa*, *Nkx6-1*, or *Pdx1* (69). Other studies using mouse models with β-cell-specific deletions of MTC subunits (*Mettl3*, *Mettl14*, *Wtap*) also pointed out the importance of MTC in maintaining β-cell function. Deletion of either subunit resulted in decreased m^6A levels (65, 70, 75). METTL3 deficiency led to β-cell failure and hyperglycemia (75). METTL14-deficient mice exhibited decreased β-cell mass, reduced insulin secretion, and glucose intolerance (65, 76, 77). Deficiency of WTAP was associated with a reduction of METTL3 levels and resulted in severe hyperglycemia and β-cell failure. Overexpression of *Mettl3* in β-cells partially prevented the negative effects of WTAP deficiency (70). Comparing *Mettl3*-βKO and *Wtap*-βKO mice revealed down-regulation of β-cell-specific transcription factors (such as *Mafa*, *Nkx6-1*, *Pdx1*, *Neurod1*, or *Foxa2*) and insulin secretion-related genes (such as *Ins1*, *Ins2*, *Brsk2*, *Cacna1c*, *Doc2b*, *Ffar1*, *G6pc2*, *Gck*, *Gipr*, *Hadh*, *Ica1*, *Nnat*, *Park7*, *Pclo*, *Selenot*, *Serp1*, *Slc30a8*, *Stxbp51*, *Sytl4*, *Trpm2*, *Ucn3*, and *Uqcc2*) (70). Besides methyltransferases, also β-cell-specific deletion of reader *Ythdc1* resulted in β-cell failure and diabetes (71, 78). This was likely due to the decreased gene expression of β-cell-specific transcription factors (such as *Mafa*, *Nkx6-1*, *Neurod1*, and *Hmgn3*) and insulin-related genes (such as *Ins1*, *Ins2*, *Gck*, *G6pc2*, *Sytl4*, *Doc2b*, *Pclo*, *Cacna1c*, *Slc30a8*, *Ffar1*, *Gipr*, *Nnat*, and *Selenot*). Transcription factor MAFA decreased dramatically also on protein level in *Ythdc1*-βKO islets (71). Yang et al. suggested that YTHDC1 may regulate mRNA splicing and export to modulate glucose metabolism in β-cells by interacting with serine/arginine-rich splicing factor 3 (SRSF3) and cleavage and polyadenylation specific factor 6 (CPSF6) (78).

These data indicate that m^6A/m^6Am epitranscriptomic machinery vastly affects the biology of pancreatic β-cells and plays a role in the induction of diabetic phenotype. However, the data are still fragmental, and more studies covering more m^6A/m^6Am regulators are needed to elucidate the exact role of epitranscriptomic regulations in the diabetic pancreas.

4.3 Heart

Cardiovascular disease (CVD) is a common comorbidity and a major cause of mortality among people with T2DM. More than 30% of all T2DM patients are affected by CVD (79). Cardiac dysfunction observed in patients with diabetes that occurs in the absence of other cardiovascular risk factors (such as hypertension, coronary artery disease, or valvular disease) is referred to as diabetic cardiomyopathy (DCM) (80). This condition is characterized by cardiac diastolic dysfunction and later by heart failure (HF) and cardiac death. It is estimated that the risk of HF is 2-3 times higher in individuals with T2DM and that approximately 12% of diabetic patients eventually develop severe HF often leading to death (81). The epitranscriptomic modifications, including m^6A, are known to play various roles in the physiology and pathophysiology of the cardiovascular system (20, 82–85). Recent studies have shown that

changes in m^{6}A methylation also contribute to HF progression (86–90). However, the role of cardiac m^{6}A and m^{6}Am machinery is not well-characterized in T2DM.

Altered cardiac m^{6}A patterns were detected in db/db mice (model of T2DM and DCM). The differentially methylated transcripts were linked mainly to cardiac fibrosis, myocardial hypertrophy, and myocardial energy metabolism (91). The higher total m^{6}A mass in DCM was associated with the down-regulation of demethylase FTO on both gene and protein levels, while levels of METTL3, METTL14, and ALKBH5 were stable (91). Interestingly, mice with T1DM-induced DCM (C57BL/6 mice injected with streptozotocin) exhibited a different dysregulation of epitranscriptomic machinery (Figure 3). Total m^{6}A levels in the hearts of these mice were decreased. This was linked with an increase of ALKBH5 in the cardiomyocytes of DCM mice and subsequent activation of the Hippo signaling pathway through a YTHDF2-dependent action (92). These results suggest that the two types of diabetes might affect the epitranscriptomic background of DCM differently. It has been reported already that T1DM and T2DM might affect the heart in a different way and result in dissimilar DCM phenotype. This was explained mainly by the different myocardial insulin action (insulin deficiency in T1DM vs insulin resistance and hyperinsulinemia in T2DM) and thus distinct signaling downstream of the insulin receptor (93). Therefore, the contradictory epitranscriptomic results may be explained by the different phenotype between the two types of diabetes. However, further research is needed to resolve this issue.

Most of the studies dealing with m^{6}A/m^{6}Am regulations in DCM have been executed on T1DM animal models. Pyroptosis, a type of proinflammatory cell death, is tightly involved in DCM progression. Methyltransferase METTL14 was down-regulated in the hearts of rats with DCM (T1DM-induced) and enhancement of its expression inhibited pyroptosis in myocardial tissues and improved systolic function (increased fractional shortening and ejection fraction) via down-regulation of lncRNA *Tincr*. The expression of *Tincr* was regulated in a YTHDF2-dependent manner (94). Peng et al. (95) reported that lncRNA *Airn* ameliorated diabetes-induced (T1DM) cardiac dysfunction caused by cardiac fibrosis. Their data showed that *Airn* binds to m^{6}A reader IGF2BP2 and protects it from ubiquitin-proteasome-dependent degradation, leading to an m^{6}A-dependent stabilization of *p53* mRNA by IGF2BP2 and subsequent reduction in cardiac fibrosis (95).

Despite the limited amount of data available, it is becoming evident that epitranscriptomic dysregulations in diabetic cardiac tissue might have a significant effect on the function of the heart. However, the exact role of m^{6}A and m^{6}Am in DCM induced by each type of T2DM is yet to be deciphered.

4.4 Kidneys

Diabetic nephropathy (DN), also known as diabetic kidney disease, is a prevalent microvascular complication of T2DM often leading to end-stage renal disease, a life-threatening condition (96). According to the International Diabetes Federation reports, up to 40% of diabetic patients might develop DN (97).

Xu et al. reported, that human kidney 2 (HK-2) cells stimulated with high glucose decreased total m^{6}A methylation level and also methyltransferases METTL3 and METTL14 (98). Interestingly, Jiang et al. observed increased m^{6}A modification in diabetic mice which was caused by elevated levels of METTL3. They also found increased METTL3 levels in renal biopsies from DN patients. Further experiments showed that METTL3 exerted pro-inflammatory and pro-apoptotic effects in an IGF2BP2-dependent manner and that targeting METTL3 alleviated the DN injury (99). A negative effect of METTL3 in DN was reported also by Tang et al. (100). METTL14 was also highly expressed in the kidneys of DN patients and HRGEC (high glucose-induced human renal glomerular endothelial cells). METTL14 worsened renal injury and inflammation was reported in db/db mice (101). Lu et al. also reported high levels of METTL14 in renal biopsy samples from

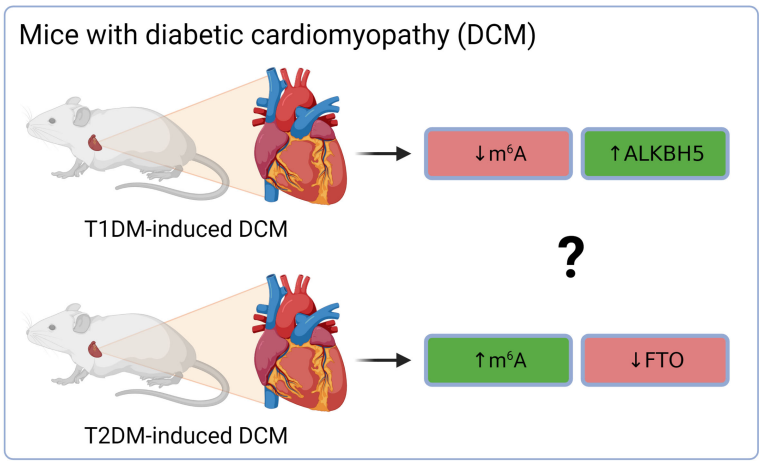

FIGURE 3
Different epitranscriptomic regulations in DCM on T1DM and T2DM mouse hearts. ALKBH5, AlkB family member 5; FTO, fat mass and obesity-associated; m^{6}A, N^6-methyladenosine; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

patients with glomerulosclerosis and DN. Mice with podocyte-specific METTL14 deletion were then associated with improved glomerular function and alleviated podocyte injury compared to wild-type nephropathic mice (102). Also the third component of the MTC – WTAP – was reported to induce pyroptosis and inflammation in high glucose-treated HK-2 cells (103). Besides the methyltransferases, FTO was described to promote the progression of DN (104). However, several SNPs in the *FTO* gene were associated with a significantly lower risk of nephropathy in T2DM patients (62). Urine levels of m^6A were decreased in patients with T2DM and even more with DN (105).

The existing data indicate that m^6A machinery is affected in DN and that its dysregulation has a negative outcome on the progression of the pathology.

4.5 Liver

Liver disease ranks among notable causes of death in T2DM patients (106). Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease and is strongly associated with T2DM (107–109). The prevalence of this comorbidity among T2DM patients reaches up to 70% (110). It has been described that NAFLD is promoted by m^6A modification dysregulation (111–116). Moreover, liver tissues from T2DM patients and mice on HFD showed elevated levels of m^6A and also METTL3. Hepatocyte-specific knockout of *Mettl3* in mice then led to improved insulin sensitivity and decreased fatty acid synthesis (117). Jiang et al. also reported that baicalin – a flavonoid glycoside used in traditional Chinese medicine – suppressed T2DM-induced liver tumor progression in a METT3/m^6A-dependent manner (118).

4.6 Eyes

Chronic exposure to hyperglycemia affects the microvasculature, eventually leading to diabetic retinopathy (DR), the main cause of blindness in the developed world. It has been described that m^6A modification is regulated by various risk factors associated with DR, such as inflammation, oxidative stress, angiogenesis, or glucose and lipid metabolism (119). *FTO* polymorphism (rs8050136) was associated with a higher risk of DR (120). In retinal pigment epithelium (RPE) cells, high-glucose conditions down-regulated the expression of METTL3 on both transcript and protein levels. Further experiments showed that METTL3 overexpression alleviated the cytotoxic effects of high-glucose on RPE cells, while METTL3 depletion had the opposite effect (121). Conversely, diabetic stress-induced up-regulation of METTL3 and subsequent increase of m^6A levels in human retinal pericytes and also mouse retinas. Specific depletion of METTL3 in pericytes suppressed diabetes-induced pericyte dysfunction and vascular complication *in vivo* (122). A recent study showed down-regulation of METTL3 in vitreous humor samples from patients with DR, a mouse model of DR, and also high glucose-induced human retinal microvascular endothelial cells (123).

Despite these conflicting data on METTL3 expression, it seems to be clear that epitranscriptomic regulations are affected in DR, but the exact role of m^6A in the pathogenesis remains to be elucidated in the future.

4.7 Skin

Dysregulation of autophagy is a contributing factor for delayed wound healing in diabetic skin. YTHDC1, an m^6A reader, has been described as a modulator of autophagy in diabetic keratinocytes which regulates the mRNA stability of an autophagy receptor (124). Interestingly, YTHDC1 interacted and cooperated with ELAVL1 (ELAV-like RNA binding protein 1), a well-established RNA stabilizer also linked to m^6A methylation. It has been described previously that loss of m^6A methylation enhances ELAVL1 RNA binding to increase RNA stability (125).

4.8 Blood

Decreased m^6A methylation levels were detected in RNA isolated from the peripheral blood of T2DM patients and also diabetic rats (126, 127). In accordance with these results, significantly higher gene expression of *FTO* (and not *ALKBH5*) in peripheral blood from T2DM patients was detected (126). However, Onalan et al. (127) observed an up-regulated expression of both demethylases in venous blood samples from T2DM patients. The increased expression of FTO on both gene and protein levels was later confirmed by another study which pointed out the correlation between high FTO levels and T2DM severity (128). The gene expression of *FTO* was also up-regulated in white blood cells from T2DM patients compared to healthy individuals and the expression level of *FTO* was positively correlated with fasting glucose concentration (129). Besides erasers, *METTL3* mRNA was down-regulated in serum samples from T2DM patients (121). Progressively higher T2DM risk was associated with low serum IGF2BP3 levels (72).

Taken together, the content of m^6A or its regulators in the peripheral blood may serve as novel potential biomarkers of T2DM in the future (126).

4.9 Treatment of T2DM: the role of m^6A and m^6Am modifications

Metformin is the first-line therapy for the treatment of T2DM, yet its molecular mechanisms of action are not fully understood (130, 131). The main effect of metformin treatment is inhibition of hepatic gluconeogenesis. At the molecular level, several mechanisms have been proposed to explain this phenomenon, such as inhibition of mitochondrial complex I activity, activation of AMPK, or increase in hepatocellular redox state due to inhibition of GPD2 (glycerol-3-phosphate dehydrogenase 2). The secondary effects of metformin treatment include an increase in muscle

glucose uptake, a decrease in intestinal glucose absorption, and a change in the composition of the gut microbiome (130).

According to recent studies, metformin also affects epitranscriptomic regulations, including m^{6}A machinery. Metformin was shown to reduce m^{6}A methylation via the down-regulation of methyltransferase METTL3 in breast cancer cells (132). In hepatocellular carcinoma, metformin treatment was associated with METTL3 inhibition (133). Metformin also attenuated multiple myeloma cell proliferation and encouraged apoptosis by suppressing METTL3-mediated m^{6}A methylation of its targets (134). Surprisingly, METTL3 expression was up-regulated after metformin treatment in adenocarcinoma cells (135). YTHDC2, a key m^{6}A reader, is an important target of metformin in preventing the progression of vascular smooth muscle cell (VSMC) dysfunction under high glucose, a simulation of VSMC dysfunction caused by T2DM (136). Recently, Liao et al. (137) showed that metformin combats obesity by targeting FTO in an m^{6}A-YTHDF2-dependent manner. This study suggests that metformin inhibited the protein expression of FTO, resulting in higher m^{6}A methylation in mRNAs of crucial cell cycle regulators. The binding of YTHDF2 to modified transcripts then triggered mRNA decay and subsequent decrease of protein expression. In consequence, the mitotic clonal expansion process was blocked and adipogenesis was inhibited.

This fragmentary information suggests that metformin may both decrease and increase m^{6}A methylation and that the target tissue or cell type may be the determining factor. However, *in vivo* studies focusing on the epitranscriptomic effect of metformin are needed to decipher this phenomenon, as the *in vivo* and *in vitro* response may also differ, especially if the primary target of metformin treatment is the liver. Despite these ambiguities, the association between epitranscriptomics and metformin is revealing itself, however, the role of m^{6}A modification in the treatment of diabetes remains unclear.

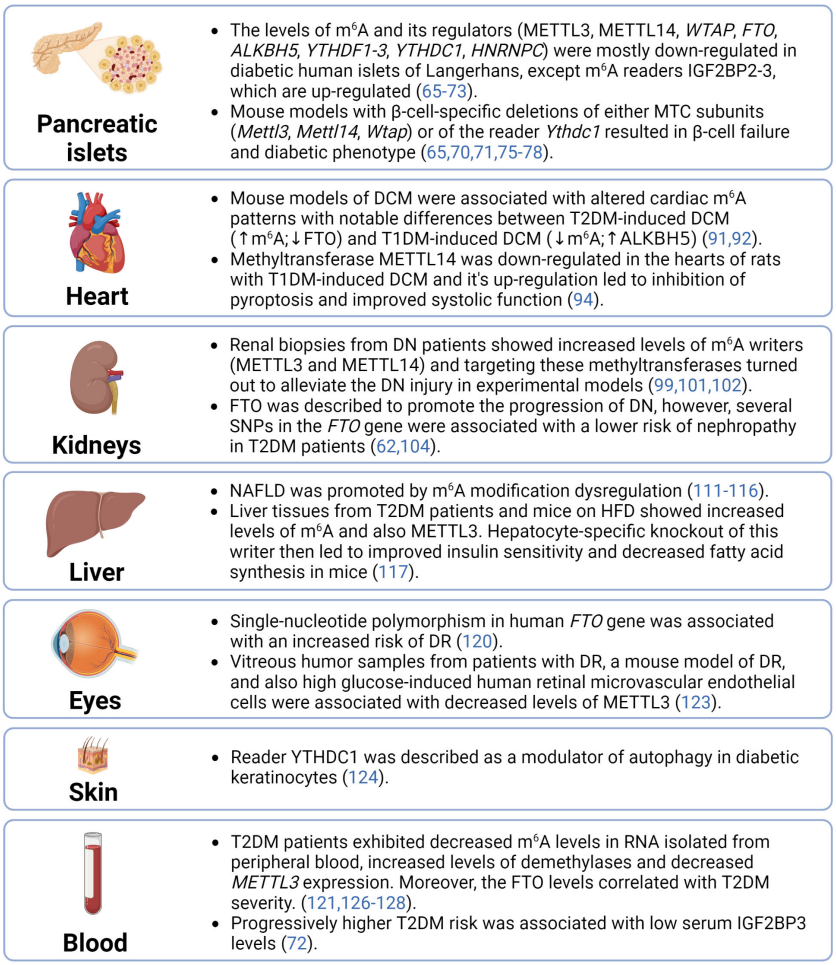

FIGURE 4
Summary of epitranscriptomic regulations in diabetic tissues. ALKBH5, AlkB family member 5; DCM, diabetic cardiomyopathy; DN, diabetic nephrophathy; DR, diabetic retinopathy; FTO, fat mass and obesity-associated; HFD, high-fat diet; HNRNPC, heterogeneous nuclear ribonucleoprotein C; IGF2BP2-3, insulin-like growth factor 2 mRNA binding proteins 2-3; m^{6}A, N^6-methyladenosine; METTL14, methyltransferase-like 14; METTL3, methyltransferase-like 3; MTC, multicomponent methyltransferase complex; NAFLD, non-alcoholic fatty liver disease; SNPs, single-nucleotide polymorphisms; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; WTAP, Willms' tumor 1-associating protein; YTHDC1, YTH domain-containing protein 1; YTHDF1-3, YTH domain-containing family proteins 1-3.

5 Conclusion and perspectives

The significant role of epitranscriptomics in cellular physiology and pathophysiology has been widely accepted by the scientific community in the past few years. However, despite the increased interest of researchers in RNA modifications, the complex epitranscriptomic regulations are still not fully understood. Our review focused on two of the most prevalent modifications – m^6A and m^6Am – in the pathogenesis of T2DM. The fragmental current knowledge indicates that diabetic tissues are associated with the dysregulation of epitranscriptomic machinery (summarized in Figure 4). However, it is essential to correctly distinguish whether these dysregulations contribute to the development of the disease or are merely a consequence of it. Several studies already showed that a deficiency of epitranscriptomic regulators can promote the pathological conditions typical for T2DM. Thus, targeting the epitranscriptomic regulations might have future applications in the clinic and consequently reduce the morbidity and mortality of T2DM patients.

Author contributions

DB and SB drafted the article, LP-H and MH provided substantive revisions. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Charles University Grant Agency (grant number GA UK 243423) to SB; the Czech Science Foundation (grant number 19-04790Y) to MH; the Czech Science Foundation (grant number 22-11439S) to LP-H; and the project National Institute for Research of Metabolic and Cardiovascular Diseases (Programme EXCELES, ID project No. LX22NPO5104) – Funded by the European Union – Next Generation EU.

Acknowledgments

Figures were created with BioRender.com.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Attachment VII

REVIEW

Epitranscriptomic Regulations in the Heart

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Received October 25, 2023
Accepted March 6, 2024

Summary

RNA modifications affect key stages of the RNA life cycle, including splicing, export, decay, and translation. Epitranscriptomic regulations therefore significantly influence cellular physiology and pathophysiology. Here, we selected some of the most abundant modifications and reviewed their roles in the heart and in cardiovascular diseases: N^6-methyladenosine (m^6A), N^6,2`-O-dimethyladenosine (m^6Am), N^1-methyladenosine (m^1A), pseudouridine (Ψ), 5-methylcytidine (m^5C), and inosine (I). Dysregulation of epitranscriptomic machinery affecting these modifications vastly changes the cardiac phenotype and is linked with many cardiovascular diseases such as myocardial infarction, cardiomyopathies, or heart failure. Thus, a deeper understanding of these epitranscriptomic changes and their regulatory mechanisms can enhance our knowledge of the molecular underpinnings of prevalent cardiac diseases, potentially paving the way for novel therapeutic strategies.

Keywords

Epitranscriptomics • RNA modifications • Epigenetics • m^6A • RNA • Heart

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Introduction

The original central dogma of molecular biology states that DNA is transcribed into RNA, which is subsequently translated into proteins [1]. However, the whole process is under the control of epigenetic mechanisms. Epigenetic mechanisms involve chemical modifications to the DNA itself, to the proteins that package DNA into chromatin (histones), or to the RNA molecules transcribed from the DNA (Fig. 1). Importantly, the epigenome is responsive to various environmental factors (diet, stress, exposure to toxins, etc.) and can produce heritable phenotypic changes without altering the DNA sequence [2,3].

RNA modifications are specifically known as the epitranscriptome. The research field of epitranscriptomics is rapidly developing. Currently, over 170 chemical RNA modifications are known (common RNA modifications overviewed in Fig. 2) [4]. The largest number of modifications with the widest chemical diversity is present in tRNA; however, various modifications also occur in other RNA types, including mRNA [5]. These modifications may be either irreversible or reversible [6]. Epitranscriptomic regulators can be described according to their function as writers (addition of the epitranscriptomic mark), erasers (removal of the epitranscriptomic mark), and readers (binding to the modified nucleotide). Dynamic regulation of epitranscriptomic modifications can affect key stages of the RNA life cycle, including splicing, export, decay, and translation [7,8].

Remodeling of the cardiac epitranscriptome has been described in several physiological as well as pathological states. This review summarizes the current knowledge and gaps about RNA modifications in cardiac biology and cardiovascular diseases (CVDs). A better understanding of epitranscriptomic regulations in the healthy and diseased heart opens the door for clinically relevant discoveries in the future.

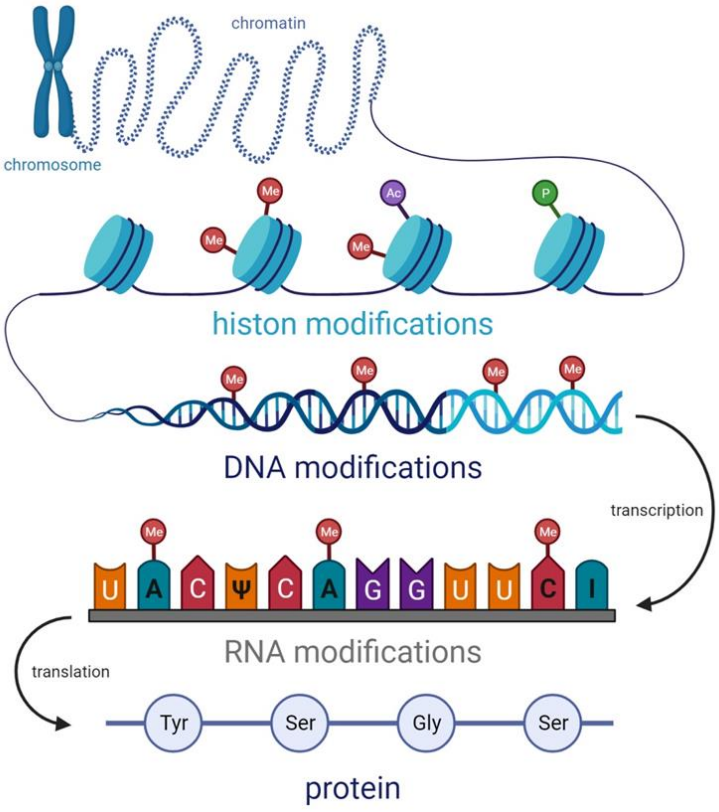

Fig. 1. Basic overview of epigenetic modifications

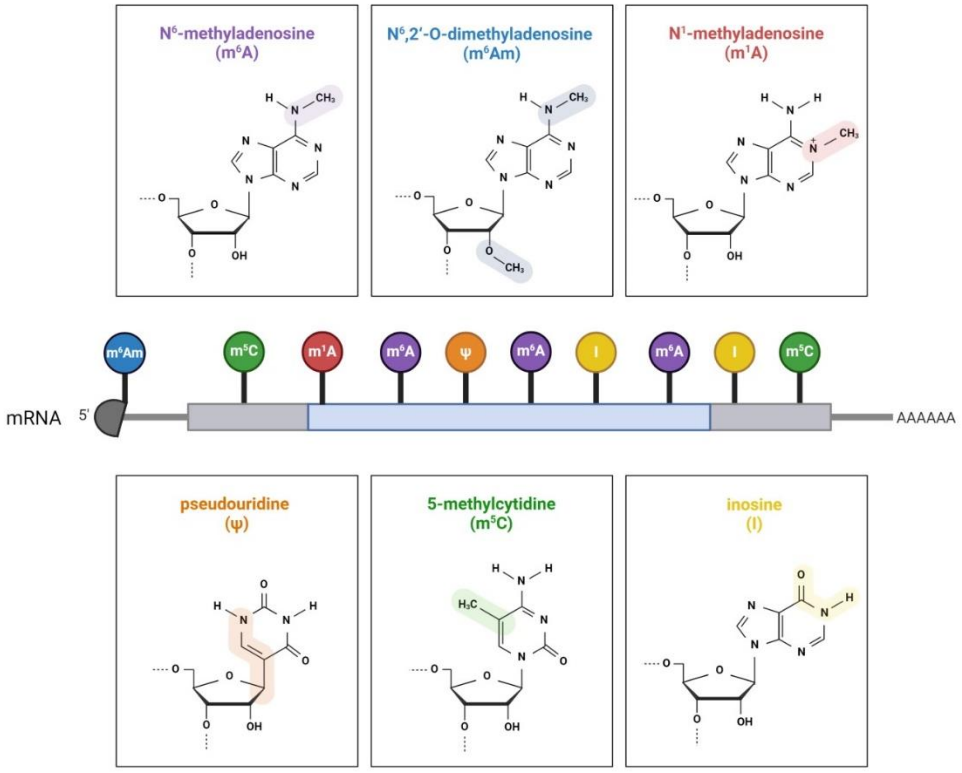

Fig. 2. Common RNA modifications

Common RNA modifications and their role in cardiac physiology

N^6-methyladenosine

N^6-methyladenosine (m^6A) is the most numerous modification in eukaryotic mRNA; however, it also occurs in other RNA types [9-12]. Multicomponent methyltransferase complex (MTC) is responsible for the deposition of the methyl group to adenosine, forming m^6A. The two main regulatory subunits of the MTC are methyltransferase-like 3 (METTL3) and methyltransferase-like 14 (METTL14). The catalytic function of the MTC is carried by METTL3 while METTL14 facilitates RNA binding [13,14]. The removal of the methyl group is mediated by two main demethylases. AlkB homolog 5 (ALKBH5) is the primary m^6A eraser [15]. Fat mass and obesity-associated protein (FTO) is not an m^6A-specific demethylase, however, m^6A is the preferable target of FTO in the nucleus [16-18]. There are many described m^6A readers. The most characterized include YTH domain-containing family proteins 1-3 (YTHDF1-3) and YTH domain-containing proteins 1-2 (YTHDC1-2). While readers YTHDF1-3 mediate primarily mRNA degradation, YTHDC1 regulates mRNA splicing and YTHDC2 promotes translation [19-25].

The heart is affected by m^6A already during its ontogenetic development as m^6A machinery regulates cardiomyocyte growth, proliferation, and differentiation [26-29]. Children born with a loss-of-function mutation in the *FTO* gene (m^6A demethylase) exhibited heart defects (ventricular septal defect, atrioventricular defect, patent ductus arteriosus), hypertrophic cardiomyopathy and died before 3 years of age [30]. Moreover, various gene variants of m^6A regulators were linked with CVDs, including myocardial infarction, acute coronary syndrome, increased risk of rejection in heart transplant patients, and sudden cardiac death [31-37]. It has been reported that m^6A also controls cardiac hypertrophy [38-40]. Dorn et al. [41] suggested that enhanced m^6A RNA methylation results in compensated cardiac hypertrophy, whereas diminished m^6A drives eccentric cardiomyocyte remodeling and dysfunction. Changes in m^6A methylation and dysregulation of m^6A machinery can contribute to the progression of heart failure [42-47]. Altered cardiac m^6A patterns were detected also in diabetic cardiomyopathy with distinct dysregulation of m^6A machinery in the two types of diabetes [48-50]. The heterogeneous role of m^6A modification in CVDs has been reviewed in several recent publications [51-60].

Altered m^6A levels in different CVDs might also serve as useful biomarkers. For instance, it has been described that patients with coronary artery disease (CAD) had significantly lower urine m^6A levels compared to healthy individuals [61].

Since cardiac m^6A machinery is dysregulated under many pathophysiological conditions, targeting m^6A modifiers can also induce cardioprotection. Several studies showed that demethylases FTO and ALKBH5 can protect cardiomyocytes against detrimental effects, such as treatment with cardiotoxic compounds or hypoxia/reoxygenation injury [43,62-69]. On the contrary, loss of METTL3 or METTL14 can alleviate myocardial injury and promote heart regeneration [70,71]. Thus, improving our knowledge of the m^6A regulations in the heart may lead to novel cardioprotective strategies using specific pharmacological activators or inhibitors targeting m^6A modifiers.

N^6,2'-O-dimethyladenosine

N^6,2'-O-dimethyladenosine (m^6Am) is formed by N^6-methylation of 2'-O-methyladenosine (Am). It has been described only in mRNA and snRNA [50,72]. This modification is present at the first transcribed nucleotide and forms the extended cap structure in at least 30-40% of all vertebrate mRNA [73,74]. Moreover, m^6Am is also present at the internal sites of snRNAs [17]. The formation of m^6Am in the cap is mediated by phosphorylated CTD interacting factor 1 (PCIF1), while methyl-transferase-like 4 (METTL4) is responsible for internal m^6Am formation [75-78]. The demethylation of m^6Am takes place mainly in the cytosol where it is mediated by FTO, the same eraser that targets m^6A in the nucleus [17,18,79,80]. There are currently no m^6Am readers mediating the biological functions of this modification described, but it is known that the presence of m^6Am in the cap structure markedly enhances mRNA stability (in mRNA cap) and splicing (in snRNA cap) [79,81].

The function of m^6Am modification in the heart is mostly unknown. There are several problems associated with m^6Am research: 1) many m^6A detection methods do not distinguish between m^6A and m^6Am; 2) FTO is not a specific eraser because it demethylates also m^6A and m^1A; 3) METTL4 can also catalyze 6mA methylation. Thus, the potential effect of m^6Am on cardiac function could be masked as m^6A in many studies [72]. Besides the non-specific demethylase FTO covered in the previous chapter, not much is known about the role of m^6Am and its

regulators in the heart. Publicly available RNA-seq datasets generated from human left ventricles of failing and non-failing hearts reported some degree of regulation of *METTL4* (down-regulation) and *PCIF1* (up-regulation) [72]. Besides that, we recently found that m^6Am writers were regulated also in cardioprotective interventions. METTL4 was decreased in the hearts of rats adapted to chronic hypoxia and PCIF1 was increased in the hearts of rats subjected to fasting [69,72].

N^1-methyladenosine

N^1-methyladenosine (m^1A) is found mainly in tRNA and rRNA, but less numerously also in mRNA [82-85]. The writer proteins responsible for m^1A methylation include tRNA methyltransferase 6 (TRMT6), TRMT61A, TRMT61B, TRMT10C or ribosomal RNA-processing protein 8 (RRP8; also known as NML) [86-90]. Demethylation of m^1A is catalyzed by erasers ALKBH1, and ALKBH3 [85,91-93]. Moreover, FTO (m^6A and m^6Am eraser) also works as a demethylase of m^1A in tRNA [17]. The m^1A modification affects the structure and stability of tRNA and rRNA and its presence in mRNA regulates translation [85,86,94-96].

So far, no association between m^1A and CVDs has been found [97]. Analysis of methylated nucleosides in urine that revealed altered m^6A levels in CAD patients did not find any changes in the case of m^1A [61].

Pseudouridine

Pseudouridine (Ψ), the C5-glycoside isomer of uridine (U), is the first discovered and overall the most prevalent RNA modification that has been identified in almost all known RNA types [98-100]. The conversion of U to Ψ is mediated by the diverse pseudouridine synthase (PUS) family [101]. So far, 13 members of PUSs have been described in eukaryotes [100]. The human homologs of PUSs include PUS1, PUS3, PUS7, PUS10, PUSL1, PUSL7, TRUB1-2 (TruB pseudouridine synthase 1-2), RPUSD1-4 (RNA pseudouridine synthase D1-4), and DKC1 (dyskerin pseudouridine synthase 1) [102]. The formation of Ψ is irreversible (unlike the aforementioned modifications) [103]. The only known Ψ reader is a yeast RNA helicase Prp5 interacting with snRNA [104,105]. The molecular functions of Ψ include stabilization of RNA conformations and destabilization of interactions with RNA-binding proteins; the most well-characterized function of Ψ in mRNA is the promotion of a stop codon read-through [100,106].

Plasma and urine levels of Ψ were linked to CVDs [107]. Patients with heart failure exhibited higher plasma concentrations of Ψ than healthy controls and this modification was suggested as a suitable biomarker for heart failure diagnosis [108-110]. Tetralogy of Fallot, the most common cyanotic congenital heart defect, is associated with decreased Ψ levels in ventricular myocardial tissues, which is under the control of small Cajal body-specific RNAs [111,112].

5-methylcytidine

5-methylcytidine (m^5C) is an abundant RNA modification present in a wide variety of RNA types. The writers responsible for the installation of m^5C in humans are NOL1/NOP2/SUN domain proteins 1-7 (NSUN1-7) and DNA methyltransferase homolog DNMT2 [113,114]. Ten-eleven translocation proteins 1-3 (TET1-3) and ALKBH1 are known as m^5C erasers. TET-mediated oxidation results in a formation of 5-hydroxymethylcytidine (hm^5C), while ALKBH1 is responsible for the oxidation of m^5C in mitochondrial tRNA generating 5-formylcytidine (f^5C) [115,116]. The readers of m^5C include Aly/REF export factor (ALYREF), which influences nuclear-cytoplasmic shuttling [117], and Y-box-binding protein 1 (YBX1), which preserves the stability of its target mRNA by recruiting ELAVL1 [118]. This modification is an important regulator of RNA export, ribosome assembly, translation, and RNA stability [113,119,120].

In mammals, m^5C modification occurs more frequently in the myocardium and skeletal muscle compared to other organs. The enrichment of m^5C is especially present in mitochondrial-related genes, suggesting a particularly important function of m^5C in the high-energy demanding myocardium [121]. Indeed, specific inactivation of the methyltransferase NSUN4 in the heart caused cardiomyopathy with mitochondrial dysfunction [122]. Deficiency of methyltransferase *Dnmt2* gene in mice resulted in cardiac hypertrophy [123]. RNA binding protein and known m^5C reader YBX1 was also identified as a cardiac hypertrophy regulator [124,125]. NSUN2 was found to increase *Nrf2* expression by promoting m^5C methylation of its mRNA and enhancing its antioxidant stress effect, which attenuates doxorubicin-induced myocardial damage [126].

RNA editing

RNA editing includes nucleoside modifications such as adenosine deamination to inosine (A-to-I editing) or cytosine deamination to uridine (C-to-U editing), as

well as insertion and deletion of nucleotides [127,128]. Deamination of A to I is irreversible and it is performed by enzymes belonging to the adenosine deaminase acting on RNA (ADAR) family, which is represented by three ADAR orthologs (ADAR1-3) in mammals. ADAR1 and ADAR2 are widely expressed, while ADAR3 was detected only in the brain [129,130]. C-to-U editing is not as common as A-to-I editing [131]. The deamination of C to U is performed by a multiple-protein editosome, which includes the catalytic subunit apolipoprotein B mRNA editing enzyme catalytic subunit 1 (APOBEC1) and an RNA-binding protein APOBEC1 complementation factor (A1CF) [132]. RNA editing in protein-coding regions of mRNAs can result in the expression of functionally altered proteins while editing in microRNA (miRNA) precursors leads to reduced expression or altered function of mature miRNAs [133].

ADAR1 is an essential enzyme for normal embryonic cardiac growth and development [134]. Cardiomyocyte-specific deletion of *Adar1* in adult mice caused severe ventricular remodeling and spontaneous cardiac dysfunction associated with a significant rise in lethality [135]. ADAR1 was also shown to prevent autoinflammatory processes in the heart [136]. A-to-I RNA editing has been significantly increased among children with cyanotic congenital heart disease compared to acyanotic controls [137]. On the contrary, reduction of A-to-I editing and decreased levels of ADAR2 have been described in the failing human heart [138]. Strong down-regulation of ADAR2 and up-regulation of ADAR1 expression was observed in blood samples of patients with congenital heart disease. The decrease in ADAR2 levels was in line with its down-regulation in ventricular tissues of dilated cardiomyopathy patients. Thus, it has been suggested that ADAR2 activity might play a critical role in preventing cardiovascular disorders [139]. Indeed, Wu et al. [140] described that ADAR2 was up-regulated in the heart during exercise and that this enzyme protects the heart against myocardial infarction as well as doxorubicin-induced cardiotoxicity, supporting the hypothesis of the beneficial effect of ADAR2 on the heart. So far, RNA editing therapeutics have not been established for the treatment of CVDs, however, it is a prospective therapeutic approach that could be implemented in the near future [141].

Conclusion

CVDs remain the leading cause of death worldwide. The search for appropriate cardioprotective strategies is therefore of crucial importance. The significant role of epitranscriptomics in cellular physiology and pathophysiology has been already accepted by the scientific community in the past few years. However, the exact role of complex epitranscriptomic regulations in the heart and CVDs is still far from being understood. It is becoming clear that RNA modifications and their regulators play a vital role in the ontogenetic development of the heart. Many CVDs, such as myocardial infarction, cardiomyopathies, or heart failure, have been also associated with dysregulated epitranscriptomic machinery (Fig. 3). Most importantly, targeting the enzymes responsible for regulating the RNA modifications affected by these diseases proved to be beneficial for the heart. Thus, it is only a matter of time before targeting epitranscriptomic regulations becomes a part of clinical practice.

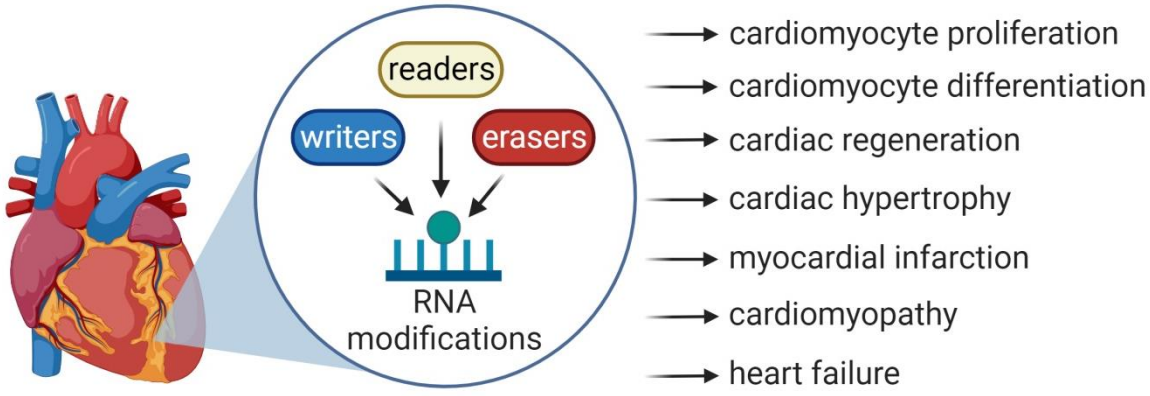

Fig. 3. Role of RNA modifications in the heart

Authors' contributions

B.D. drafted the article, K.F. and H.M. provided substantive revisions.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by the Czech Science Foundation under Grant (24-10497S) to H.M.; the Charles University Grant Agency under Grant (GA UK 668220) to B.D.; and the project National Institute for Research of Metabolic and Cardiovascular Diseases (Programme EXCELES, ID Project No. LX22NPO5104) to K.F. - Funded by the European Union – Next Generation EU.
Figures were created with BioRender.com.

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