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Mitochondrial metabolic enzymes as RNA Binding Proteins: Exploring the RNA binding landscape of MDH2 and ATP5A1

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“Curiouser and Curiouser”

-Alice (Lewis Carroll), Alice in Wonderland

Contributions

I hereby declare that the work presented in this thesis has not been submitted for any other degree or professional qualification, and that it is the result of my own independent work. Experiments, figures and contributions by others have been clearly marked throughout the thesis. eCLIP libraries were sequenced at EMBL Genecore facility. Primary analysis of the eCLIP data to identify MDH2 bound targets was performed by Dr. Thileepan Sekaran using tools developed by Dr. Thomas Schwarzl together with Dr. Sudeep Sahadevan. For the proteomic datasets, the samples were analyzed at EMBL Proteomics Core Facility. Dr Per Haberkrant and Dr. Jennifer Schwarz from the facility performed mass spectrometry analysis, peptide identification and count. Dr. Frank Stein analysed the proteomic datasets to identify differentially changing proteins. Dr. Aindrila Chatterjee and Prof. Dr. Matthias Hentze supervised this project.

Summary

RNA-protein interactions critically regulate almost every facet of cellular biological processes. Beyond the canonical RNA binding proteins (RBPs) regulating the lifecycle of RNA, systematic analyses of RNA bound proteomes have revealed several unconventional RBPs. Among them, enzymes of intermediary metabolism draw attention in their potential to connect cellular energy metabolism with RNA regulation. Some metabolic enzymes function similarly to canonical RBPs, altering the expression of their target RNAs. Recent evidence also points to RNA as a regulatory molecule, controlling the metabolic function of these enzymes (e.g., glycolytic enzyme ENO1). RNA-metabolic enzyme interactions can thus reciprocally link changes in gene expression with metabolic changes. In this thesis, I explore the RNA binding function of two mitochondrial metabolic enzymes- malate dehydrogenase 2 (MDH2) and ATP synthase subunit alpha (ATP5A1).

I primarily focused on characterizing MDH2 as an RNA binding protein, first verifying its RNA binding activity biochemically with orthogonal approaches. MDH2 binds mainly cytosolic RNAs- particularly tRNAs. MDH2 binds RNA predominantly outside the mitochondria, its compartment of function. Based on indications from in-vitro RNA-binding assays, the concentration of MDH2's substrates and cofactors (particularly malate and NAD^+) can direct this preference. Furthermore, MDH2's RNA binding alters with cellular metabolic state. MDH2 binds less RNA in hepatocyte-like cells with increased mitochondrial respiration compared to a proliferative cancer state. Thus, we find that MDH2's RNA binding function is an active, compartment-specific process that may have regulatory consequences on protein and/or RNA.

ATP5A1-RNA associations were characterized in-vitro, focusing on ATP5A1's propensity to bind TOP-mRNAs. Towards understanding regulatory consequences of these interactions, I assayed the effects of ATP5A1 depletion on nascent protein synthesis. A fraction of ATP5A1-bound targets is affected in protein synthesis, the mechanisms and implications of which need to be further explored. Evaluation of ATP5A1's protein interactors revealed a network of cytosolic translation factors and components of RNP complexes in addition to established mitochondrial interactions. These proteins can localise close to the mitochondrial outer membrane, the site where

Summary

ATP5A1 begins its mitochondrial import and where ATP5A1 interacts with RNA. Taken together, I discuss how ATP5A1's RNA and protein interactions could be coordinated spatially with resulting functional effects on ATP5A1-bound RNAs as well as ATP5A1's import into the mitochondria.

Together, this study explores the RNA binding functions of two mitochondrial metabolic proteins providing insights into the identity of RNAs associated with them, the location of their association and potential regulation of their RNA binding function.

Zusammenfassung

RNA-Protein-Wechselwirkungen sind für fast alle Aspekte zellulärer biologischer Prozesse von entscheidender Bedeutung. Neben den kanonischen RNA-bindenden Proteinen (RBP), die den Lebenszyklus der RNA regulieren, hat die systematische Analyse des RNA-gebundenen Proteoms unkonventionelle RBP in der RNA-Biologie aufgedeckt. Besonders Enzyme des intermediären Stoffwechsels scheinen dabei eine wichtige Rolle zu spielen, da sie das Potenzial haben, den zellulären Energiestoffwechsel mit der RNA-Regulation zu verbinden. Einige Stoffwechselenzyme funktionieren ähnlich wie kanonische RBP und verändern die Expression ihrer Ziel-RNAs. Jüngste Erkenntnisse zeigen, dass RNA als regulatorisches Molekül die Stoffwechselfunktion dieser Enzyme steuern kann (z. B. des glykolytischen Enzyms ENO1). Die Wechselwirkungen zwischen RNA und Stoffwechselenzymen können somit Veränderungen der Genexpression mit Veränderungen des Stoffwechsels verknüpfen. In dieser Arbeit untersuche ich die RNA-Bindungsfunktion von zwei mitochondrialen Stoffwechselenzymen - Malatdehydrogenase 2 (MDH2) und ATP-Synthase-Untereinheit alpha (ATP5A1).

In dieser Arbeit habe ich MDH2 als RNA-bindendes Protein charakterisiert, indem ich zunächst seine RNA-Bindungsaktivität mit orthogonalen Ansätzen biochemisch verifiziert habe. MDH2 bindet hauptsächlich zytosolische RNAs - insbesondere tRNAs. Die Bindung von RNA durch MDH2 findet überwiegend außerhalb der Mitochondrien, dem Funktionskompartiment von MDH2, statt. In-vitro-Bindungstests deuten darauf hin, dass die unterschiedliche Zusammensetzung der Metaboliten im Zytosol und in den Mitochondrien (insbesondere im Hinblick auf die Substrate und Kofaktoren von MDH2) für diese Präferenz verantwortlich sein könnten. Darüber hinaus ändert sich die RNA-Bindung von MDH2 mit dem zellulären Stoffwechselzustand, vor allem unter Bedingungen, bei denen die mitochondriale Funktion beeinträchtigt ist. Zusammenfassend lässt sich sagen, dass die RNA-Bindung von MDH2 ein aktiver, kompartimentspezifischer Prozess ist, der regulatorische Auswirkungen auf Protein und/oder RNA haben kann.

ATP5A1-RNA-Assoziationen wurden in-vitro charakterisiert, wobei der Schwerpunkt auf der Analyse der TOP-mRNA Bindeaktivität von ATP5A1 lag. Um die regulatorischen Konsequenzen dieser Interaktionen zu verstehen, habe ich die

Auswirkungen einer ATP5A1-Depletion auf die Proteinsynthese untersucht. Ein Teil der ATP5A1-gebundenen Ziel-RNA weist eine Veränderung in der Proteinsynthese auf. Die Mechanismen und Auswirkungen dieser Interaktion müssen noch weiter erforscht werden. Die Auswertung der Proteininteraktoren von ATP5A1 zeigte ein Netzwerk von zytosolischen Translationsfaktoren und Komponenten von RNP-Komplexen auf, die zusätzlich zu den bekannten mitochondrialen Proteininteraktoren gebunden werden. Diese Proteine sind in der Nähe der äußeren Mitochondrienmembran lokalisiert, dem Ort, an dem ATP5A1 seinen mitochondrialen Import beginnt und wo ATP5A1 mit RNA interagiert. Zusammengenommen scheinen die RNA- und Proteininteraktionen von ATP5A1 räumlich koordiniert zu sein, was funktionelle Auswirkungen auf ATP5A1-gebundene RNAs und den Import von ATP5A1 in die Mitochondrien hat.

Insgesamt bietet diese Studie über die RNA-Bindungsfunktionen mitochondrialer Stoffwechselproteine neue Einblicke in die Koordination von Stoffwechselfunktionen und RNA-Regulation in verschiedenen zellulären Kompartimenten.

Table of contents

Contributions	vii
----------------------------	------------

Summary.....	ix
---------------------	-----------

Zusammenfassung	xi
------------------------------	-----------

Table of contents	xiii
--------------------------------	-------------

Acknowledgements	xvii
-------------------------------	-------------

Part I Introduction

Chapter 1: Introduction	1
--------------------------------------	----------

1.1	The RNA world	1
1.1.1	Different flavors of RNA	1
1.2	RNA-protein interactions.....	2
1.2.1	How do proteins find RNA? Mechanisms and regulations of protein-RNA interaction.....	2
1.2.2	RNA interacts with proteins- what happens next?	3
1.2.3	Proteome-wide RNA interactome captures.....	4
1.3	Cellular energy metabolism and RNP complexes	6
1.3.1	Energy metabolism: the crux of life.....	6
1.3.2	Interplay between metabolic networks and RNP complex formation	11
1.3.3	Illustrations of metabolic enzymes functioning as RBPs.....	12
1.4	Diving deeper: Mitochondrial metabolism and its regulation	14
1.4.1	A Central organelle - structure, function and composition of mitochondria	14
1.4.2	Mechanism of protein import into the mitochondria	15
1.4.3	Mitochondrial bioenergetics	19
1.4.4	RNA binding proteins regulating mitochondrial function	22
1.5	Aims and structure of the thesis.....	26

Part II Results and Discussion

Chapter 2: RNA binding activity of MDH2	31
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2.1	Introduction to MDH2	31
2.2	Objectives	35
2.3	Results.....	35

Table of contents

2.3.1	Mass spectrometry-based RNA Binding Protein Identification attributes RNA binding function to MDH2	35
2.3.2	MDH2 is a bona-fide RNA binding protein (RBP)	38
2.3.3	Identification of RNAs bound by MDH2	41
2.3.4	MDH2 binds tRNAs in-vitro	48
2.3.5	Subcellular location of MDH2's RNA binding	52
2.3.6	MDH2's canonical mitochondrial targeting signal is dispensable for RNA binding	54
2.3.7	Effect of a cryptic mitochondrial targeting signal on MDH2 import	56
2.3.8	Preliminary results: How cytosolic constriction affects MDH2's RNA binding?	58
2.3.9	MDH2's substrates and co-factors impacts MDH2-RNA associations	60
2.3.10	RNA binding of MDH2 is responsive to changes in the NAD level in the cell	62
2.3.11	RNA binding of MDH2 is sensitive to cellular state with altered metabolic fluxes	65
2.3.12	Mode of MDH2 binding to RNA	67
2.3.13	Preliminary experiments towards identifying MDH2's RNA binding region	70
2.4	Discussion.....	72
2.4.1	MDH2 preferentially binds cytosolic RNAs, specifically tRNAs	72
2.4.2	MDH2's RNA binding typically occurs outside of the mitochondria	73
2.4.3	MDH2 RNA binding is sensitive to altered mitochondrial bioenergetics	74
Chapter 3: RNA binding activity of ATP5A1		77
3.1	Introduction to ATP5A1	77
3.2	Objectives	80
3.3	Results.....	80
3.3.1	In-vitro characterization of ATP5A's binding preference	80
3.3.2	Effect of ATP5A1 depletion on nascent protein synthesis	87
3.3.3	Interactors of ATP5A1	92
3.4	Discussion.....	96
3.4.1	ATP5A1 forms a nexus of interactions between mRNAs and proteins involved in cytosolic translation.....	96
3.4.2	Altered protein synthesis after ATP5A1 depletion.....	98
 Part III Conclusions and Outlook		
Chapter 4: Conclusions and outlook.....		103
4.1	MDH2 as an RNA binding protein	103
4.2	ATP5A1's RNA binding activity	104
4.3	Broader perspectives.....	105

Synopsis of main findings.....	107
4.4 MDH2 as an RNA binding metabolic protein	107
4.5 ATP5A1 as an RNA binding metabolic protein	108
 Part IV Materials and Methods	
 Chapter 5: Materials and Methods	111
5.1 Materials	111
5.1.1 Antibodies	111
5.1.2 Enzymes	111
5.1.3 Kits	112
5.1.4 Chemicals/Reagents	112
5.1.5 Consumables	115
5.1.6 Buffers and solutions	115
5.1.7 Equipment	117
5.1.8 Software	118
5.1.9 Plasmids	118
5.1.10 Oligos	118
5.2 Methods	121
5.2.1 Bacterial transformation.....	121
5.2.2 Plasmid preparation.....	122
5.2.3 Polymerase chain reaction (PCR)	122
5.2.4 Agarose gel electrophoresis	122
5.2.5 Cloning based on restriction digestion and ligation.....	122
5.2.6 Site directed mutagenesis.....	123
5.2.7 DNA and RNA amount measurement	123
5.2.8 Preparation of cell lysates	124
5.2.9 Protein quantification.....	124
5.2.10 SDS-PAGE and western blotting.....	124
5.2.11 Total protein staining	125
5.2.12 Silver staining.....	125
5.2.13 Protein purification.....	125
5.2.14 Synthesis and purification of RNAs.....	126
5.2.15 Electromobility shift experiments	127
5.2.16 Cell culture	127
5.2.17 Cell passaging	128
5.2.18 Stable cell line generation and maintenance	128
5.2.19 Cryopreservation	128
5.2.20 Treatment of cells with NAD-level modulators.....	129

Table of contents

5.2.21	NAD (H) quantification	129
5.2.22	Differentiation of Huh7 cells	129
5.2.23	Plasmid transfections	129
5.2.24	siRNA transfections	129
5.2.25	Crosslinking cells with ultra-violet (UV) light	130
5.2.26	Immunoprecipitation of proteins (endogenous and tagged proteins).....	130
5.2.27	Co-immunoprecipitation	130
5.2.28	Polynucleotide Kinase assay	131
5.2.29	Complex Capture	131
5.2.30	Mitochondrial fractionation and crosslinking	132
5.2.31	Immunofluorescence (IF) and Microscopy	133
5.2.32	Seahorse assay.....	133
5.2.33	MDH2 enzyme assay	133
5.2.34	QuanCAT	134
5.2.35	Sample preparation and acquisition of mass spectrometry data	135
5.2.36	Bioinformatic analysis of mass-spectrometry data	139
5.2.37	eCLIP library preparation	139
5.2.38	eCLIP Sequencing.....	143
5.2.39	eCLIP data analysis.....	143
5.2.40	Statistical analyses	144

Part V Supplementary data

Supplementary data.....	147
Supplementary table.....	154
References.....	157
List of abbreviations	175
List of figures.....	177
List of tables	179

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Part I

Chapter 1: Introduction

Chapter 1: Introduction

1.1 The RNA world

How did life begin? One of the best supported origin story heroes ribonucleic acids (RNA). The theory highlights the ability of RNA to function as a catalyst (enzyme) and replicate itself (Bartel and Szostak, 1993; Gilbert, 1986; Noller, 1992); Saito (2022) - the basis for more complex systems based on proteins to evolve.

Even though proteins have taken over many primordial functions of RNAs, RNA still remains one of the key factors in active gene regulation. In addition to serving as the intermediate between the deoxyribonucleic acid (DNA) and proteins, RNA can adopt intricate structures helping to catalyze fundamental processes like protein synthesis and splicing (Mandal and Breaker, 2004). Complex RNA folds in riboswitches bind to specific metabolites and in response, regulate downstream protein synthesis (Wilson and Lilley, 2015). The myriad of different ways by which RNA performs transcriptional as well as posttranscriptional regulation are being actively researched and constantly expanding (Yao et al., 2019).

1.1.1 Different flavors of RNA

Broadly, RNAs are classified based on their ability to code for a protein as “coding” or “noncoding” RNAs (ncRNAs). In eukaryotes, coding RNAs or messenger RNAs (mRNAs) are post-transcriptionally modified at the 5’ end (capping) and 3’ end (polyadenylation) to provide stability and promote translation. Introns in the mRNA are removed by the splicing machinery which also includes RNA components like small nuclear RNAs (snRNAs). ncRNAs like tRNAs and rRNAs are important for cellular translation. tRNAs are cloverleaf structured RNAs that are essential for translation (Wang and Farhana, 2022). Fragments of tRNAs have been discovered to have new roles in transcriptional regulation as well (Shen et al., 2018). rRNAs are structural constituents of ribosomes. They act as a protein scaffold and catalyze the reactions of translation in the ribosome (Wang and Farhana, 2022). Small nucleolar RNAs (snoRNAs) direct functional modifications of rRNAs. Other noncoding RNAs include circular RNAs (circRNAs), microRNAs (miRNAs), piwi interacting RNAs

(piRNAs), long non-coding RNAs (lncRNAs), enhancer RNAs (eRNAs) and many more (Cech and Steitz, 2014; Ebert and Sharp, 2012; Malone and Hannon, 2009) (Fig 1.1). These RNAs can differentially regulate gene expression at the epigenetic, transcriptional and translational levels.

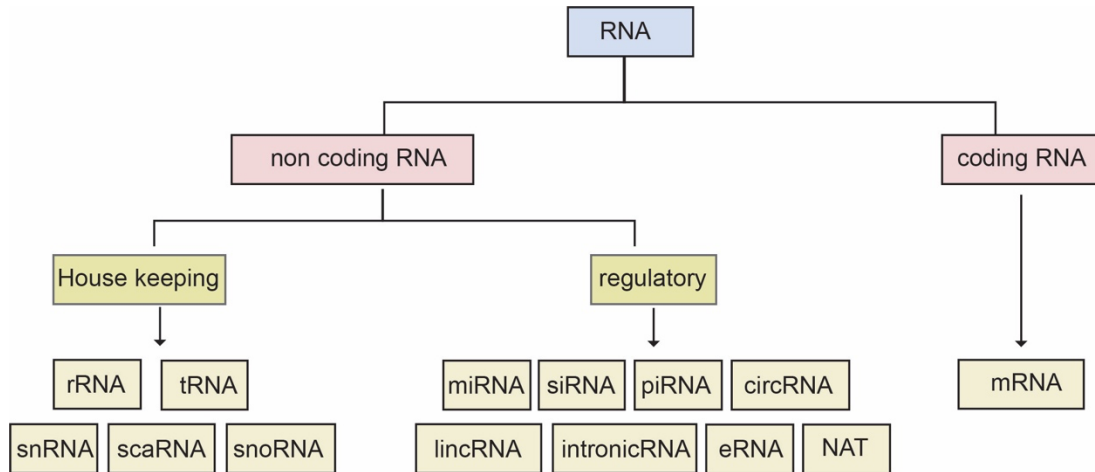


Figure 1.1. Different types of RNA in eukaryotes. RNA is broadly classified as coding and noncoding, each divided into subtypes based on cellular functions. The RNA classification list depicted here is not exhaustive and includes numerous RNA subcategories (Micallef and Baron, 2021). Figure redrawn and adapted from (Bhatti et al., 2021).

1.2 RNA-protein interactions

1.2.1 How do proteins find RNA? Mechanisms and regulations of protein-RNA interaction

RNA exists in cells in functional association with proteins that often exert stabilizing, remodeling, modifying, guiding and localizing effects on the RNA. How do all these proteins specifically find and bind their RNA targets in the complex cellular mesh? Both the binding parties bring some selectivity to this interaction. Several distinct RNA binding domains are present in canonical RNA binding proteins (RBP) that preferentially bind different parts of the RNA- the base, the 2'OH or the phosphodiester backbone, alone or in combination (Corley et al., 2020). On the other hand, RNA can fold into complex structures which perfectly fit into a protein binding pocket, as is the case for the IRP1/aconitase protein binding the iron responsive element (IRE, an RNA stem-loop structure) (Walden et al., 2006) or the 40S ribosomal subunit with the HCV IRES (Hepatitis C virus internal ribosome entry site) (Quade et

al., 2015). Alternately, recognition of features like the 5' m7G (7-methyl-guanosine) cap and 3' polyA tail allow proteins to interact broadly with RNAs (Mangus et al., 2003; Topisirovic et al., 2011). In some instances, disordered regions in proteins fold into stabilized conformations on interaction with their targets (Yang et al., 2019). Intermolecular interactions between RNA as well as proteins serve as scaffolding to recruit RBPs and their target RNAs to higher order structures like, phase separated condensates (Campos-Melo et al., 2021; Mittag and Parker, 2018).

Modifications - of protein (England et al., 2022; Lovci et al., 2016) as well as RNA (Lewis et al., 2017) can be defining factors dictating RNA-protein interactions. In addition, RNA-protein interactions are also regulated by ligands (Fe in the case of IRP1-IRE interactions) (Hentze et al., 2004) and specific metabolites (ATP in the case of helicases which stabilizes RNA-bound form) (Linder and Jankowsky, 2011).

To sum up, RNA-protein interactions are coordinated in cells in a context-specific manner determined by features on RNA and proteins. In addition, interactions with other molecules- proteins, RNA, ligands and metabolites- can regulate formation of RNA-protein complexes.

1.2.2 RNA interacts with proteins- what happens next?

RNA-protein interactions can have regulatory effects on both the protein and the bound RNA. RBPs can coordinate multiple aspects of gene-expression, binding RNA to direct its fate at different stages of its lifecycle (Rissland, 2017). In eukaryotes, post-transcriptional processing of RNA and its modifications (e.g., 5' capping and 3' polyadenylation) involves several RBPs. Once it is processed and exported out of nucleus, mRNAs associate with RBPs that control its stability and translation. RBPs can regulate a broad spectrum of mRNA by recognizing features like the m7G cap and polyA tail or specific mRNAs by binding to distinct motifs. Translation initiation factors like eukaryotic initiation factor 4E (eIF4E), eIF4G and polyA binding protein (PABP) are key examples of the first category of RBPs that show broad RNA association. Specific RBPs come into play when there is a need to respond or adapt to stimuli with gene expression changes. When an RNA needs to be degraded, protective RBPs bound to it are displaced by RNA-destabilizing proteins. They remove protective features of the RNA such as the m7G cap and the polyA tail. Then, nucleases

like Xrn1 recognize and hydrolyze decapped and deadenylated RNA (Glisovic et al., 2008; Mitchell and Parker, 2014; Muller-McNicoll and Neugebauer, 2013).

Recognition of specific motifs in the 5'/3' UTR of the transcripts by specific RBPs allow their selective regulation in response to changes in cellular environments (Meyuhas and Kahan, 2015; Szostak and Gebauer, 2013). Some of these effects are also aided by sequestration into membrane-less compartments to aid a fast response to entry in and out of stressful conditions. RBPs influence the cellular localization of the RNA, enabling localized translation of protein at or near their site of function and/or generation of protein gradients (Martin and Ephrussi, 2009).

On the other hand, RNA can also regulate protein function, directly or indirectly affecting their localization or cellular interactions. dsRNA binding by protein kinase R (PKR) leads to its dimerization and activation. Activated PKR can inhibit protein synthesis in cells by phosphorylating translation initiation factor eIF2 α (Balachandran and Barber, 2007). RNA can sequester proteins and other interacting RNAs into granules/ phase separated entities, thus regulating their function. For example, lncRNA NEAT1 sequesters proteins in phase separated bodies (paraspeckles) regulating transcription (Clemson et al., 2009; Hirose et al., 2014). RNA interactions can also bridge proteins, causing the effector protein to exert its effect on the target protein. The ubiquitination activity of some E3 ligases is enhanced by RNA binding. This is the case for TRIM25, in a mechanism that possibly involve RNA binding-induced structural changes (Choudhury et al., 2017). p62, an autophagy regulator is also critically regulated by RNA. p62 needs to multimerize for its autophagic function and RNA binding inhibits this multimerization acting as a regulator of autophagy (Horos et al., 2019).

1.2.3 Proteome-wide RNA interactome captures

As RNA-protein associations have critical cellular regulatory roles, there has been concerted efforts to identify all the RNA binding proteins from a cell. In these efforts, RBPs are identified by quantitative mass spectrometry after specific or en masse purification of RNAs together with associated RBPs (Grawe et al., 2021; Hentze et al., 2018). Due to the inherent transient and unstable nature of the RNA-protein interactions, most methods rely on ultraviolet (UV) light to crosslink RNA to the protein. The ribonucleoprotein (RNP) complex is thus preserved through subsequent

purification steps. The first global RNA interactome captures (RIC) crosslinked RBPs to RNA with UV light at 254 nm or to RNAs labeled with a photoactivatable uridine analog 4-thiouridine (4SU) with UV light irradiation at 365nm. Following crosslinking, these studies selectively purified mRNA binding proteins (Baltz et al., 2012; Castello et al., 2012). For this, polyadenylated mRNA-protein complexes were enriched using oligo-dT coupled beads.

Subsequent studies have expanded the catalogue of RBPs by purifying total RNA-associated protein complexes using diverse methods. This strategy extends RBP-identification to organisms lacking polyA modifications like *Escherichia coli* and RBPs binding RNAs lacking a polyA tail (Shchepachev et al., 2019). Silica-based solid phase extraction of nucleic acids co-purified crosslinked protein-nucleic acid complexes, providing a new method for the identification of RBPs associated with total-RNA (Asencio et al., 2018). For example, total RNA-associated protein purification (TRAPP) (Shchepachev et al., 2019) uses silica-mediated pulldown of total RNA-associated protein, whereas other methods exploit altered physiochemical properties of RNP complexes in organic phase separation (orthogonal organic phase separation (OOPS) (Queiroz et al., 2019), protein-crosslinked RNA extraction (XRNAX) (Trendel et al., 2019), phenol toluol extraction (Ptex) (Urdaneta et al., 2019)). Another strategy metabolically labels total RNA with alkynyl uridine analog, followed by UV crosslinking and click chemistry conjugation with azide-biotin. RNA-protein complexes are enriched by streptavidin purification (CARIC) (Huang et al., 2018).

At present, 54 datasets profiling RBPs from bacteria to humans provide a valuable resource to mine undiscovered biology (Caudron-Herger et al., 2021; Gebauer et al., 2021; Schwarzl et al., 2022a). In human (cell line) studies, ~1900 RBPs have been identified (Hentze et al., 2018). These studies recurrently identify canonical RBPs with well-characterized RNA binding domains like RRM/ KH/ Dead box helicase. However, about half of the RBPs identified so far have no known RNA binding domain or motif, emphasizing the myriad of RNA-binding mechanisms waiting to be explored (Hentze et al., 2018). Enzymes, especially those functioning in intermediary metabolism are enriched among these non-canonical RBPs, hinting at complex connections between RNA and metabolism (Fig 1.2 illustrates some of the metabolic proteins in glycolytic and mitochondrial energy metabolism attributed with RNA binding function). Several RNA binding metabolic enzymes have a nucleotide binding

domain which can potentially provide and RNA binding interface (Castello et al., 2015). This indicates a potential regulation of RNA binding function of metabolic enzymes in coordination with their enzymatic function. Investigating how, why and when metabolic enzymes bind RNA can thus reveal novel insights into their cellular function.

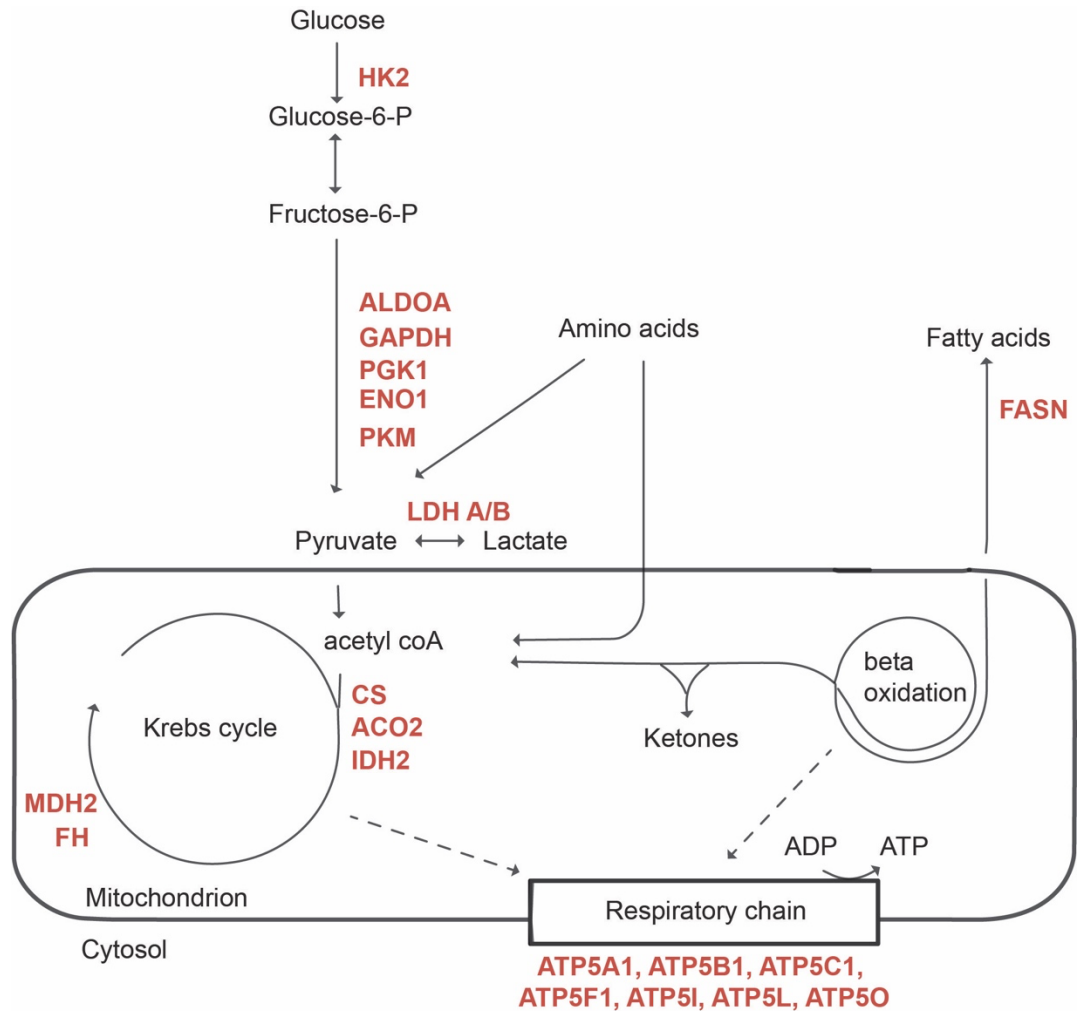


Figure 1.2. Metabolic enzymes as RNA binding proteins. Simplified scheme of cellular energy metabolism with metabolic proteins identified as RNA binding proteins in humans highlighted. Figures redrawn and adapted from (Perez-Perri et al., 2018) with information of RNA binding from (Castello et al., 2015; Schwarzl et al., 2022a).

1.3 Cellular energy metabolism and RNP complexes

1.3.1 Energy metabolism: the crux of life

Energy metabolism is crucial to maintain cellular homeostasis, coordinating the anabolic and catabolic processes that consume and generate ATP respectively. It

allows cells/organisms to successfully adapt to different environmental cues, balancing proliferation and differentiation. Dysregulation of metabolic pathways contributes to pathologies, including diabetes, non-alcoholic fatty liver disease (NAFLD), cardiovascular events and oncogenesis (Hirschey et al., 2015; Lillich et al., 2020). The relationship between diseases like cancer and metabolism is reciprocal and complex, with extensive rewiring of cellular metabolism as cancer cells adapt to their metabolic environment. The “Warburg effect” in cancer cells describe their increased reliance on glycolysis even in the presence of oxygen (Warburg, 1924). Now, we know that this observation does not result from defective mitochondrial respiration as originally envisaged by Otto Warburg (Schiliro and Firestein, 2021). Rather, cancer cells have an active tricarboxylic acid (TCA) cycle to meet the biosynthetic demands of the cell (Anderson et al., 2018). Increased aerobic glycolysis also fuels the pentose phosphate pathway (PPP) for lipid and nucleotide biosynthesis and contributes to antioxidant responses required for uncontrolled proliferation (Jiang et al., 2014).

Compared to more proliferative cells such as in cancer and primed stem cells, differentiated cells show increase reliance on the mitochondrial respiration for energy production (Tsogtbaatar et al., 2020). Altered metabolism also actively controls differentiation processes through the depletion/accumulation of different metabolic substrates/cofactors (acetyl CoA, ATP, NADH, S-adenosylmethionine, alpha-ketoglutarate etc.). These metabolic substrates are needed for the cellular transcriptional, epigenetic remodeling that accompanies differentiation (Tyurin-Kuzmin et al., 2020). The effect of metabolism in cellular remodeling is not restricted to differentiation, but rather to all cellular processes that respond to nutritional status/uptake and metabolic state or other stimuli (e.g., immune response, hypoxic response) (Metallo and Vander Heiden, 2013; Rabhi et al., 2017).

A complex system of enzymes organized into different compartments and clusters drives the energy metabolism in the cell. The energy costs of enzymatic reactions together with the concentration of reactants and substrates attribute directionality to the chemical reactions in our body. The latter also directly regulate metabolic enzyme activity in concert with other allosteric modulators. Sometimes, reactions in pathways are coupled together by channeling the products of one reaction to promote the subsequent reactions. This pathway organization helps to overcome the high energy barrier of some reactions and aids in the coordinated regulation of different metabolic pathways (Judge and Dodd, 2020; Nelson, 2005).

At an organism level, metabolic pathways are synchronized across various tissue types in organs depending their capacity to utilize and store different energy sources (Alberts, 2013). They are coordinately regulated based on our fasting/feeding/activity status and nutrient availability. The main energy sources utilized in these pathways are glucose, fatty acids, ketones, amino acids, and lactate. The body stores energy in the form of glycogen and triglycerides (polymer of glucose and fatty acids respectively) and breaks them down as required. When these sources run out, proteins are also mobilized as amino acids for energy release. Cardiac tissue mainly derives energy from fatty acids, as does the adipose tissue, liver and skeletal muscles in resting state (Kodde et al., 2007). The triglycerides are predominantly stored in the adipose tissue. Brain exclusively depends on glucose for energy, with minor contributions from ketone bodies. A constant supply of glucose in the bloodstream (5.5 mmol/L) ensures energy supply to brain, blood cells lacking mitochondria and skeletal muscles in O_2 deficiency during active exercise. This requires inter-organ energy communication to prioritize selective metabolic utilization and signal the liver to stimulate gluconeogenesis when the glycogen stores are depleted. Liver thus acts as the hub of glucose metabolism coordinating glycogenolysis, glycogenesis (break down and synthesis of glycogen) and glycolysis, gluconeogenesis (break down and synthesis of glucose) (Cahill, 2006; Judge and Dodd, 2020).

Different metabolites/ energy sources are selectively processed in compartmentalized metabolic pathways. ATP is produced directly as the end-product of some reactions, while others transfer the electrons to molecules like NAD^+ paying off in ATP at specialized downstream steps. The major cellular catabolic pathways are organized as follows:

(i) Glycolysis – Glucose is oxidized to two molecules of pyruvate producing ATP in a series of 10 enzymatic steps. It occurs in the cytoplasm and doesn't require O_2 . On glucose oxidation, NAD^+ – the cell's crucial electron acceptor – gets reduced to NADH. NAD^+ needs to be recycled from NADH for continued glycolysis. This can be coordinated either within the cytosol in O_2 deficient conditions or with the mitochondria in aerobic conditions. In addition to energy production, glycolytic intermediates like Glucose-6-phosphate can be redirected for nucleotide synthesis in the pentose phosphate pathway (PPP). Although inefficient, glycolysis is critical to meet the energy demands of cell when O_2 is limiting. Depending on the O_2 availability,

its end-products are then passed on to the mitochondria as acetyl CoA for efficient extraction of energy (Fig 1.3).

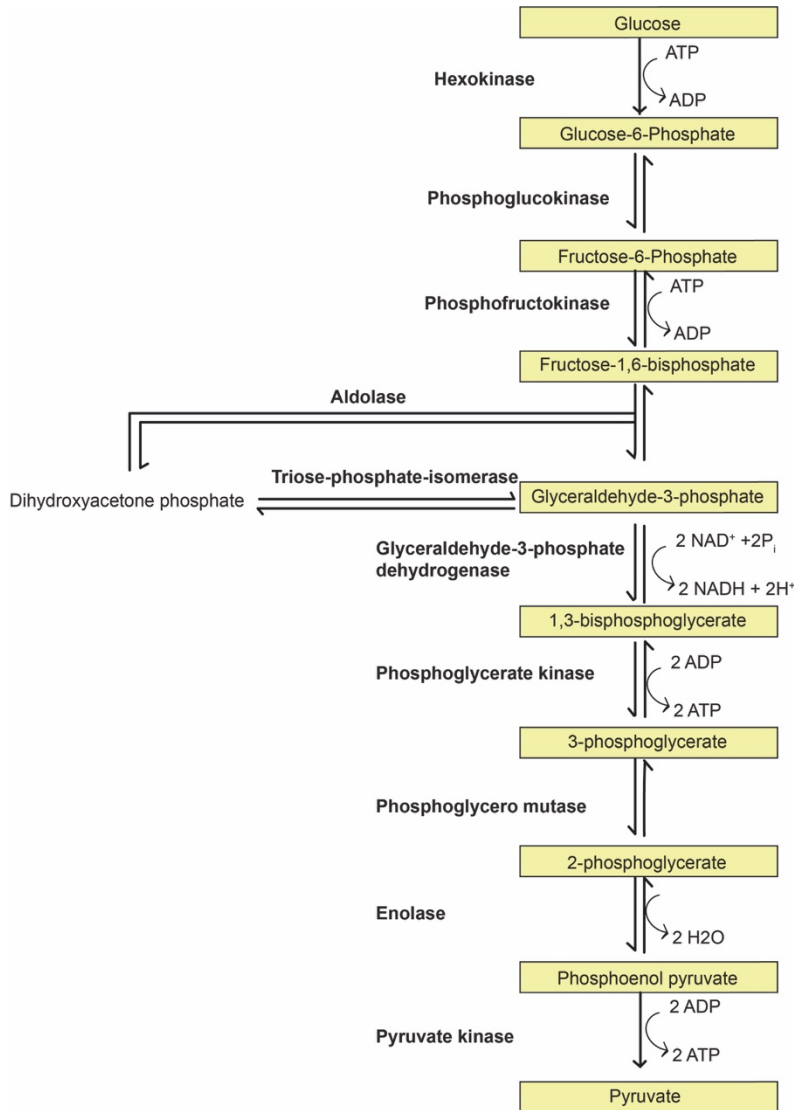


Figure 1.3. Reactions of glycolysis. In a series of sequential steps, one molecule of glucose is converted into two molecules of pyruvate, resulting in the production of 2 ATP (net). Redrawn based on (Alberts, 2022).

(ii) Tricarboxylic Acid (TCA) cycle/Krebs cycle – TCA cycle functions in the mitochondrial matrix to harness energy from acetyl CoA into reducing equivalents stored in NADH as well as FADH₂ (Fig 1.4). Apart from glucose, break-down of fatty acids also supplies acetyl CoA to the TCA cycle. Amino acids have several entry points into TCA cycle as well. Glutamine for example, is a major nutrient source for cultured cancer cells which produces energy by TCA cycling. TCA metabolites are withdrawn from the cycle and redirected for biosynthetic needs making TCA cycle central to cellular energy pathways (Martinez-Reyes and Chandel, 2020). The TCA cycle will be discussed in more detail in section 1.4.3.

(iii) Oxidative phosphorylation (OXPHOS) – The “payout” phase of the mitochondrial oxidative metabolism, OXPHOS occurs in the inner mitochondrial membrane (IMM). Here, a chain of electron acceptors establishes a proton gradient across the IMM coupled with the reoxidation of NADH and FADH₂. Driven by this proton motive force, the ATP-synthase complex catalyzes ATP production (Fig 1.4). The ATP synthase complex will be discussed in further detail in section 1.4.3.

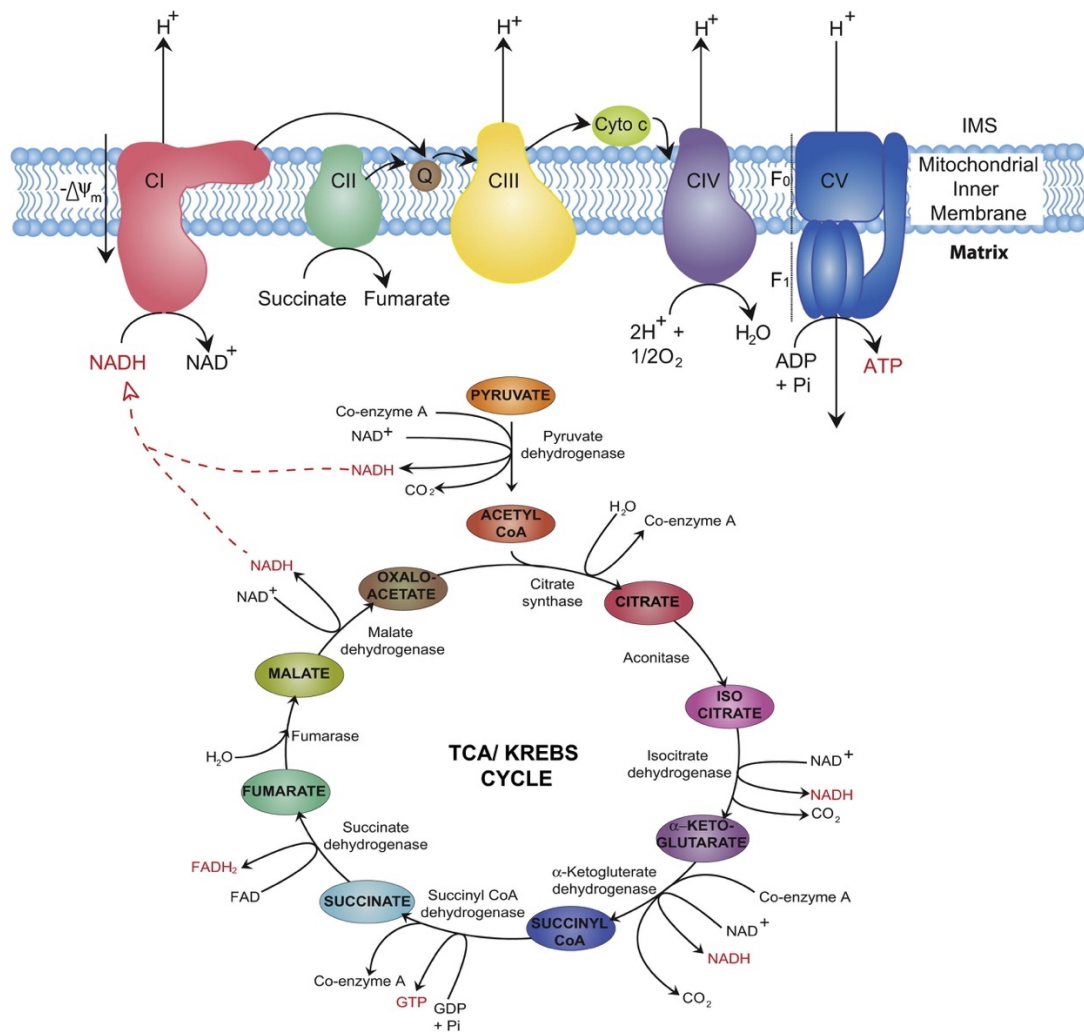


Figure 1.4. Steps of Mitochondrial TCA cycle and oxidative phosphorylation. The TCA cycle occurs in mitochondrial matrix to oxidise acetyl CoA (and other metabolites). The reducing power of NADH and FADH₂ is used by the ETC to pump protons into intermembrane space. The proton gradient is utilized by ATP synthase complex to produce ATP. Image adapted from (Osellame et al., 2012). Distributed under Creative Commons Attribution 4.0 International License.

In addition to the ATP producing pathways mentioned above, the pentose phosphate pathway also importantly contributes to glucose metabolism. It branches off from the first step of glycolysis to generate ribose 5-phosphate and NADPH. Ribose-5-phosphate

is essential for nucleic acid synthesis. NADPH contributes towards biosynthesis of nucleotides, fatty acids and amino acids in addition to contributing towards cellular antioxidant response (Ge et al., 2020).

1.3.2 Interplay between metabolic networks and RNP complex formation

Interconnections between cellular metabolism and RNA-protein interactions are being increasingly recognised, with mutually directed branches extending between metabolic adaptations and epi/post-transcriptional regulations.

Cellular metabolic networks are fine-tuned in response to nutrient availability, cell identity and function as well as diverse environmental stressors. This requires regulation of metabolic enzymes, with regard to their expression and function by several RBPs (Burkhart et al., 2013; Desvergne et al., 2006). Metabolic enzymes can be regulated transcriptionally (for e.g., by the nuclear hormone receptors (NHR)) in response to feeding and circadian cycles (Hong et al., 2014). Post-transcriptional effects including modulation of translation and localization also shape the metabolic proteome as we will see in more detail in the context of mitochondria (Chubukov et al., 2012; Wang et al., 2017).

On the other hand, metabolic conditions determine the rate of energy intensive processes like global protein translation. In response to changes in the nutrient environment, global changes in protein synthesis are simultaneously coordinated with selective regulation of metabolic enzymes. By modulating the post-transcriptional regulatory landscape and/or sequestering mRNAs into granules for storage or degradation, cells can respond rapidly to stimuli. (Lee and Tu, 2017). The mTOR pathway is a prominent example of a master regulatory pathway that responds to nutrient status. In this pathway, metabolic cues prompt phosphorylation-induced signaling cascades to regulate cellular translation. Several RNA binding proteins are regulated by these signals, thus tuning the degradation or translation of their bound mRNAs. Liquid-liquid phase separation driven formation of RNA granules (e.g., stress granules, P bodies) are also influenced by cellular energy status (Wang et al., 2022).

These granules are enriched in RBPs as well as their specific RNA targets (Tian et al., 2020).

RNA-RBP interactions can also be modulated by RNA modifications. Some of these modifications (e.g., methylation, thiolation) are specifically dependent on metabolic factors like S-adenosylmethionine (SAM) and amino acids like methionine and cysteine whose production is energy intensive. Isotype specific modifications of tRNAs with specific anticodons thus occur only in energy replete conditions. These modified tRNAs specifically increase translation of required cellular factors enriched in codons recognized by these tRNAs. Thus, cellular response to nutrient environment integrates availability of metabolites with translational output through RNA modifications (Helm and Alfonzo, 2014).

Energy metabolism and metabolism of RNA, including its interaction with RBPs are thus intricately connected. Coordinated regulation of these pathways in turn ensures a harmonized adaptation to different stimuli.

1.3.3 Illustrations of metabolic enzymes functioning as RBPs

Beyond the effects of cellular energy status and metabolites on RBP-RNA interactions in the cell, metabolic enzymes can also directly bind RNA. RNA interactome captures in mouse cardiomyocytes identified around 73 metabolic enzymes as RNA-binders (Liao et al., 2016). For the large part, the RNA-binding function of metabolic enzymes is shrouded in mystery. However, few well-studied RNA-binding metabolic enzymes serve as a guide to the RNA binding potential of metabolic enzymes.

IRP1 is a prominent example of metabolic enzyme involved in RNA binding. IRP1 requires an iron-sulphur cluster in its active site for its enzymatic function (Muckenthaler et al., 2017). In iron-replete conditions, this cytosolic aconitase functions as an enzyme converting citrate to isocitrate. As an apoprotein lacking an iron-sulphur cluster in iron-deficient cells, IRP1 binds to IRE elements in RNA. IRP1-IRE interactions stabilize the RNA or blocks its translation depending on the site bound (Casey et al., 1988; Constable et al., 1992; Hentze and Argos, 1991; Hentze et al., 2010).

GAPDH is another example of a metabolic enzyme with an alternate RNA binding function. The nucleotide-binding Rossmann fold of GAPDH is suggested to mediate

ITS RNA interactions. It preferentially binds AU rich regions in the 3' UTR of targets, regulating mRNA stability in some cases. GAPDH binds a diverse set of RNAs ranging from tRNAs, rRNAs, mRNAs and viral RNAs. However, a comprehensive transcriptome wide profiling of GAPDH-RNA interactions has not yet been performed (Dollenmaier and Weitz, 2003; Nagy and Rigby, 1995; Singh and Green, 1993; Zhou et al., 2008). When GAPDH puts on its RNA binding hat depends on cellular energy metabolism. GAPDH switches between RNA regulation and enzymatic function, with its glycolytic function taking precedence over RNA binding function. This switch is possibly mediated by using a single interface (the Rossman fold) for RNA binding and NAD⁺ binding for its enzymatic activity (Nagy et al., 2000). Thus, for both IRP1 and GAPDH, the metabolic and RNA-binding functions are well-separated and mutually exclusive.

Inosine 5'-monophosphate dehydrogenase 1 (IMPDH1), pyruvate kinase M2 (PKM2) and 17 β -hydroxysteroid dehydrogenase 10 (HSD17B10), thymidine synthase (TYMS) are further examples of enzymes “moonlighting” as RNA binding proteins. HSD17B10 is a mitochondrial protein that processes mitochondrial tRNAs (Beckmann et al., 2015). IMPDH1 regulates translational efficiency of rhodopsin mRNA and its dysfunction is implicated in retinitis pigmentosa (McGrew and Hedstrom, 2012). PKM2 acts as a translational activator of bound mRNAs (Li et al., 2020; Simsek et al., 2017), whereas TYMS binds and inhibits translation of its own RNA in a negative feedback loop (Chu et al., 1991).

However, recent studies highlight the potential of RNA to regulate the enzyme's function. For example, RNA-association inhibits the catalytic function of metabolic enzyme, enolase1 (ENO1) affecting processes like cellular differentiation closely linked to metabolic state (Huppertz et al., 2022). Similar RNA inhibition of catalytic activity is observed for the cytosolic serine hydroxymethyltransferase 1 (SHMT1). SHMT1 also regulates expression of its mitochondrial isoform SHMT2, binding to its 5'UTR (Guiducci et al., 2019). These examples illustrate how RNA-metabolic enzyme interactions can affect both the protein and the RNA. We can visualize these effects concurrently or dictated in specific directions by additional cellular context.

1.4 Diving deeper: Mitochondrial metabolism and its regulation

1.4.1 A Central organelle - structure, function and composition of mitochondria

The mitochondrion derives its name from the Greek words *mito*, meaning thread and *chondron*, meaning grain (Benda, 1898; Liesa et al., 2009). These morphological features of mitochondria make it one of the most sophisticated cell organelles and contributes to its function. These structures fascinated Richard Altmann who extensively described it in 1890s, calling them bio-blasts (Altmann, 1894). From these early descriptions, mitochondrial research has advanced to place mitochondria as one of the master regulators of cellular health (O'Rourke, 2010). Beyond being the “cellular powerhouse” coordinating energy production, mitochondria direct stress responses, immunity, apoptosis, autophagy and cell differentiation (Giacomello et al., 2020; Shen et al., 2022). Mitochondria form metabolic-state dependent contacts with other cellular organelles like the endoplasmic reticulum (ER) and lysosomes. These interfaces guide mitochondrial dynamics like fission and fusion (Shen et al., 2022).

Mitochondria have an endosymbiotic origin and are closely coupled with the formation of the first eukaryotes with well-defined compartments (Roger et al., 2017). This origin is reflected in the presence of a mitochondrial genome and its classic double membrane structure. The outer and inner membranes of mitochondria (OMM and IMM) are separated by an intermembrane space. Beyond the inner membrane lies the matrix. In contrast to the relatively permeable outer membrane, the inner membrane houses dedicated transporters for ions and other molecules. Invaginations of the inner membrane called cristae contain components of electron transport chain and oxidative phosphorylation whereas most enzymes of the TCA cycle are present in the mitochondrial matrix (McCarron et al., 2013). Mitochondrial respiration contributes to the formation of a proton gradient/membrane potential across the IMM. This membrane potential in turn directs mitochondrial processes of ATP production, protein import, uptake of ions like Ca^{2+} etc (Osellame et al., 2012).

Mitochondrial morphology varies across cell types and growth conditions, ranging from long filaments or rods to spheres and ovoids. It also forms elongated interconnected networks across the cell, implicated in efficient energy production and cellular signaling. The structure of mitochondria is determined by the balance between

its fission and fusion as well as the biogenesis and remodeling of cristae (Giacomello et al., 2020). These mitochondrial dynamics alter mitochondrial architecture to match cellular energy demands as well to maintain mitochondrial health (Liesa and Shirihai, 2013).

The human mitochondrial genome codes for around 22 RNAs including 13 tRNAs, 2 rRNAs and 10 protein coding genes (Friedman and Nunnari, 2014). The 10 mRNAs code for select components of the OXPHOS machinery which function together with proteins imported from the cytosol. In fact, majority of ~1500 proteins functioning in the human mitochondria are transcribed from the nuclear genome with a localization signal that targets it to the mitochondria (Zhao and Zou, 2021). Thus, mitochondrial function requires close communication and coordination with cytosolic factors.

The protein composition and mass of mitochondria is dictated by cellular needs and nutrient conditions. In yeast for example, mitochondria form 9% of the total cellular protein mass in fermenting conditions; this proportion increases to 20% in respiring conditions. Alteration in energy production proteins contributes to majority of this increase (Morgenstern et al., 2017). This rapid adaptation to the environment is brought about by gene expression changes in concert with increased mitochondrial import. The mitochondrial import machinery has a large buffering capacity regulated by posttranslational modifications of translocase components (Pfanner et al., 2019). I will expand on the mitochondrial protein import machinery in the following sections.

1.4.2 Mechanism of protein import into the mitochondria

Mitochondrial precursor proteins from the cytosol can be targeted to the appropriate sub-compartments of mitochondria depending on their specific targeting signals. Various mechanisms of protein targeting to the mitochondria is considered, occurring at different stages in the protein lifecycle. In some cases, mitochondrial-protein mRNAs are already in the vicinity of mitochondria indicating co-translational import (Verner, 1993; Williams et al., 2014). Post-translational import is suspected in other cases (Bykov et al., 2020).

Most of the information about mitochondrial import comes from research in yeast, with the basic principles being conserved across species. Mitochondrial precursors targeted to the mitochondria possess two kinds of signals - N-terminal presequences and internal targeting sequences - directing them to different mitochondrial

compartments (Fig 1.5). N-terminal presequences determine import majorly to the mitochondrial matrix as described in detail below.

Mitochondrial import of most cytosolic precursors begins at the translocase of the outer membrane (TOM) complex present in the outer mitochondrial membrane (OMM). Here, the protein targeting signals are recognized by receptors in the TOM complex. The TOM complex consists of 7 proteins, which includes 3 receptor proteins (Tom20, Tom 22, Tom70), the membrane channel (Tom 40) and three smaller proteins (Tom5, Tom6, Tom7) (Kiebler et al., 1990; Shiota et al., 2015). The Tom20 receptor binds precursors with presequences, while proteins targeted to the mitochondrial membranes are recognized by Tom70 (Brix et al., 1997). The translocation pores in the TOM channel are made by β -barrel segments of Tom40 (Hill et al., 1998; Hohn et al., 2018). Tom 22 is required for the stability of the multicomponent TOM complex, in addition to recruiting Tom 20 and Tom 70 to the TOM channel (van Wilpe et al., 1999). After passing through the channel, different pathways branch off downstream to direct the proteins to their place of function (Grevel et al., 2019).

The presence of hydrophobic elements in the internal targeting sequence engages the carrier pathway (Tim22 complex) that dictates import into the IMM. Proteins targeted to the intermembrane space are characterized by cysteine motifs that are utilized by the mitochondrial intermembrane space import and assembly (MIA) for import. The mitochondrial outer membranes contain two types of proteins, each integrated by a different mechanism. β -Barrel proteins possess multiple beta strands that is inserted into the OMM by the sorting and assembly machinery (SAM). Alpha-helical proteins targeted to the OMM can have alpha-helical transmembrane segments that span the membrane once (either at the N-terminus or C-terminus) or multiple times. The import of alpha-helical proteins is exceptionally not mediated by the Tom40 channel, but involves the mitochondrial import (MIM) channel (Wiedemann and Pfanner, 2017).

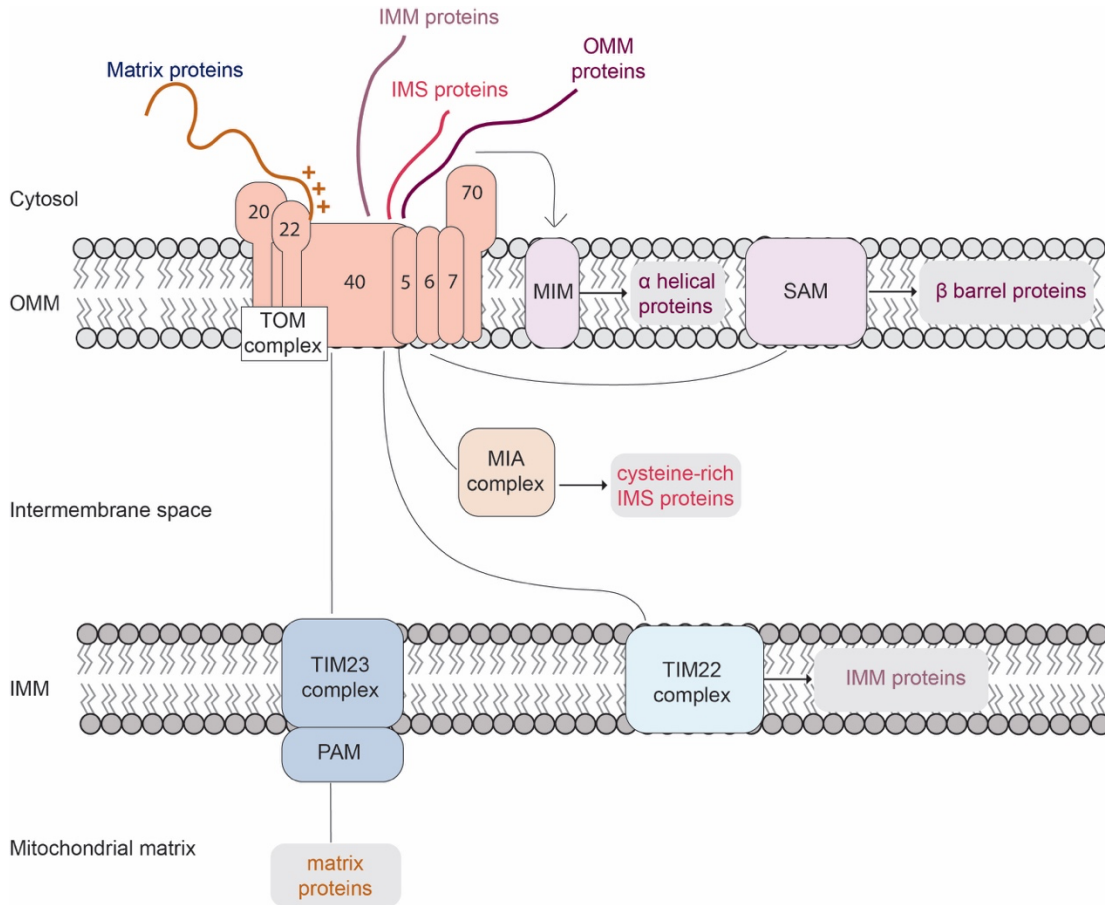


Figure 1.5. Mitochondrial protein import pathways. The diverse targeting signals of nuclear encoded mitochondrial proteins are recognized by the receptors of the TOM complex. Different pathways (SAM, MIM, TIM23, TIM22) downstream of recognition guide the proteins to their functional location. Redrawn based on figures from (Bauer et al., 2000; Kunova et al., 2022).

1.4.2.1 Targeting to the mitochondrial matrix

Proteins with presequences form the majority of mitochondrial proteins and are mostly destined for the mitochondrial matrix, with some proteins being targeted to inner membrane and intermembrane space. Presequences contain positively charged amino acids and form amphipathic alpha-helices that predominantly range between 15-55 amino acids (Vogtle et al., 2009). The presequence is recognized by Tom 20/Tom 22 through hydrophobic interactions and the protein is further passed into the membrane channel (Abe et al., 2000). The pre-protein then traverses the intermembrane space, where it is bound by the translocase of the inner membrane 23 (TIM23) complex. This complex consists of the proteins Tim 23, Tim 17, Tim 21 and Tim 50 (Mgm2) (Fig 1.6).

TIM complex together with the presequence translocase-associated motor (PAM) completes the translocation of the matrix proteins. Components of the TOM and TIM complex interact in the IMS, mediating the handover of the precursor protein (Chacinska et al., 2010; Dekker et al., 1997). The negative membrane potential on the matrix side drives the entry of the positively charged presequences of the pre-proteins across the inner membrane (Martin et al., 1991; Turakhiya et al., 2016).

For complete translocation of the precursor into the matrix, mtHsp70 component of the PAM complex is important. mtHsp70 is a mitochondrial matrix chaperon which serves as an ATP driven motor to import proteins into the matrix. It also helps fold the pre-protein into its mature form in the matrix. Since the import is an energy intensive process, the required ATP is supplied by localizing respiratory chain complexes to the vicinity of the TIM channel (Mick et al., 2012; Sinha et al., 2014).

In the matrix, the pre-protein is further processed to cleave off the targeting signal and finally folded into a mature functional form. Mitochondrial processing peptidase (MPP) in the matrix cleaves off the presequences (Hawlitsek et al., 1988). Depending on the residue at the N-terminal of the cleaved protein, it is further processed by peptidases Icp55 and Oct1. This results in a stabilizing N-terminal residue for achieving the final conformational state of the proteins (Vogtle et al., 2011; Vogtle et al., 2009) (Fig 1.6).

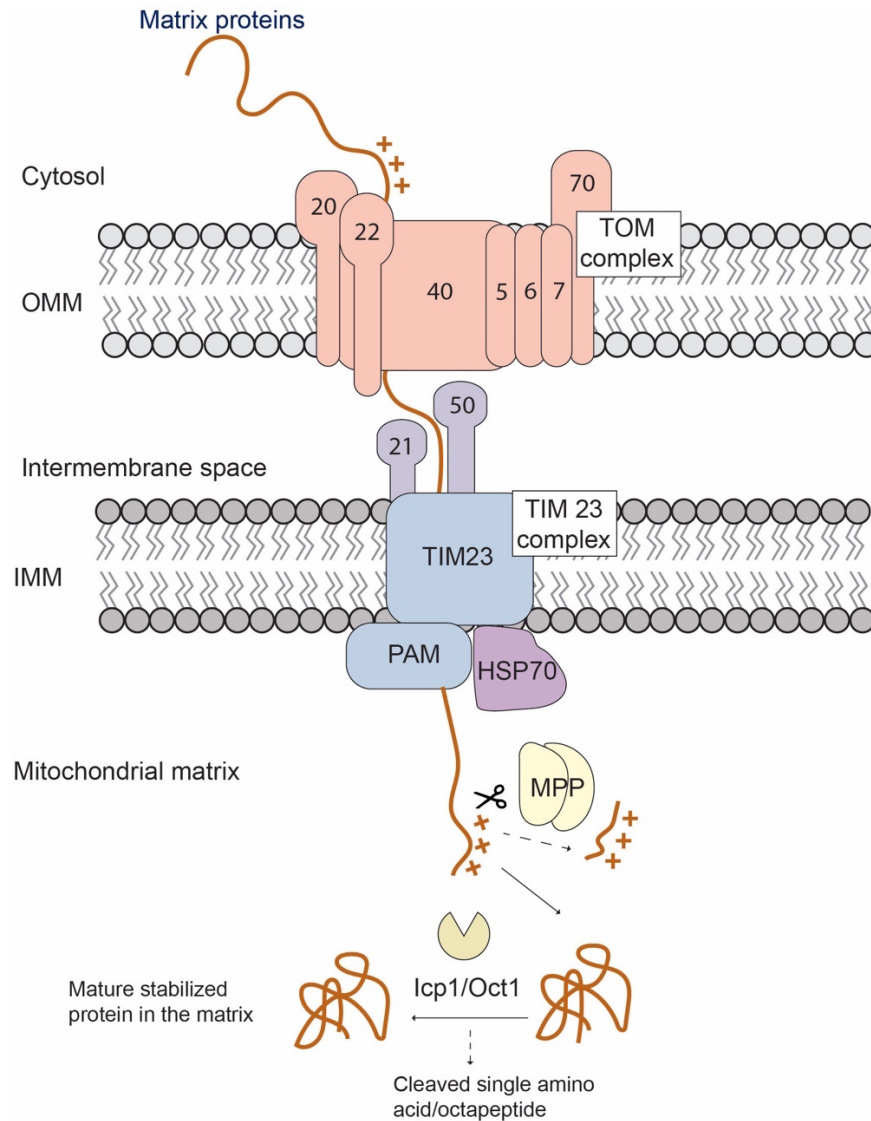


Figure 1.6. Protein import into the mitochondrial matrix. Matrix-destined proteins have an N-terminal amphipathic helix sequence recognized by Tom 20/22 receptors. The inner membrane TIM23 complex together with the PAM complex mediates matrix import. The targeting signal is cleaved off in the mitochondrial matrix and the protein is folded into its functional form. Redrawn based on figures from (Becker et al., 2012).

1.4.3 Mitochondrial bioenergetics

Mitochondria are the site of TCA and OXPHOS, producing bulk of cellular energy through aerobic respiration.

1.4.3.1 TCA cycle

TCA cycle was first described in pigeon muscle by Hans Krebs, giving it the moniker-Krebs cycle (Krebs and Johnson, 1937). The TCA cycle starts by forming citrate by

condensing oxaloacetate (OAA) and acetyl CoA. The citrate is sequentially converted to cis-aconitate, isocitrate, alpha-ketoglutarate (α -KG), succinyl-CoA, succinate, fumarate, malate and oxaloacetate which contributes to citrate formation, thus forming a complete cycle (Choi et al., 2021; Sanchez-Garcia et al., 2021). The TCA cycle is thus in constant flux with metabolites entering the cycle at multiple points and its intermediates feeding into biosynthetic pathways. Beyond energy production and biosynthesis, mitochondrial metabolites also contribute to cell signaling pathways (Martinez-Reyes and Chandel, 2020) (Fig 1.7).

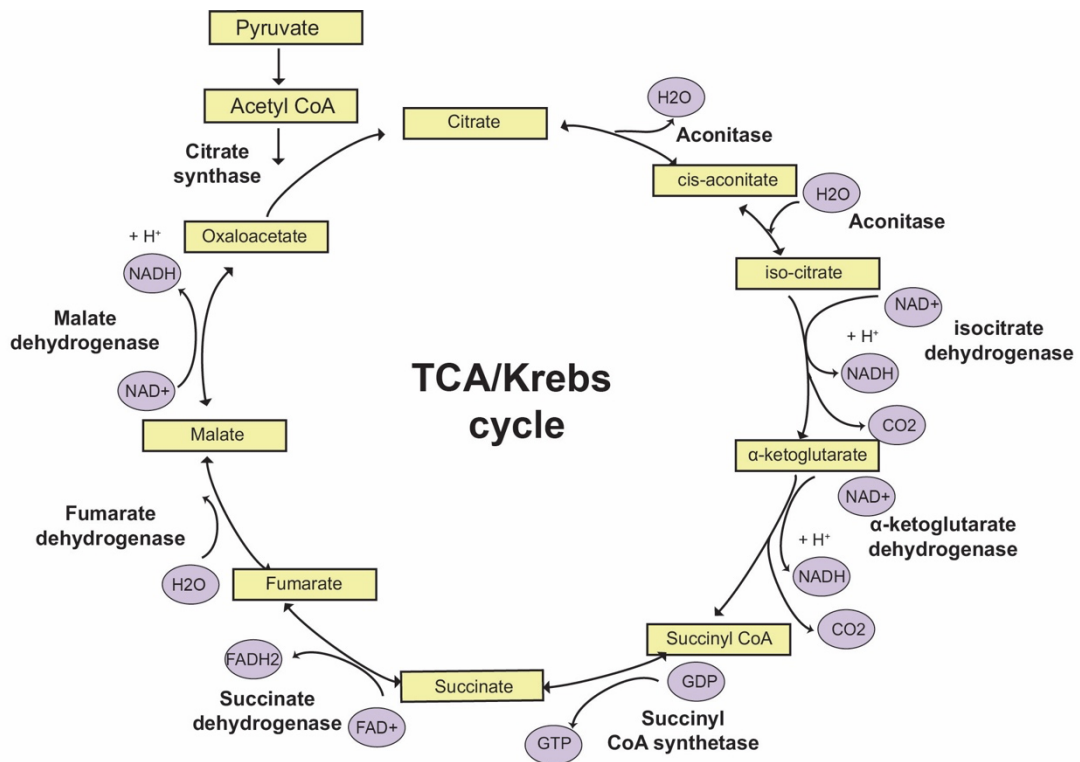


Figure 1.7. Mitochondrial tricarboxylic acid cycle. Schematic representation of the reactions of the mitochondrial TCA cycle.

In addition to energy production, TCA cycle supplies intermediates for biosynthesis of nucleic acids, lipids and proteins. The cycle is replenished by multiple anaplerotic reactions, for example the conversion of pyruvate to OAA and glutamine to α -KG (Owen et al., 2002). TCA metabolites are also involved in cell signaling - directing post-translational modifications like acetylation (acetyl CoA), chromatin methylation dynamics (α -KG, fumarate, succinate), hypoxic response (α -KG, fumarate, succinate) and many more (Martinez-Reyes and Chandel, 2020).

The TCA regulation of cells is intricate, with the relative use of different nutrient sources adjusted according to availability. A key recent example is TCA rewiring concurrent with cell cycle dynamics, with glucose and glutamine being used to feed TCA in the G1 and S phase respectively (Ahn et al., 2017). Cells control the activity of TCA cycle enzymes through feedback, allosteric, transcriptional modulations and post-translational modifications (Haddad and Mohiuddin, 2022). Substrates and products directly regulate TCA enzymatic activity, stimulating and inhibiting their enzymatic activity respectively. In addition, NAD^+/NADH and ADP/ATP exert allosteric effects on these enzymes to signal abundant energy supply and readjustment of the TCA flux accordingly (Krebs, 1970). Selective modifications of enzymes like phosphorylation, acetylation, succinylation, malonylation alter their enzymatic activity. Depending on the metabolic requirements, components of the TCA cycle can also be transcriptionally controlled by factors like PPARs, PGC1- α and HIF1- α (Karwi et al., 2019).

1.4.3.2 OXPHOS

Embedded in the IMM, OXPHOS machinery consists of the electron transport chain (ETC) and the ATP-synthase complex. The ETC is a series of protein complexes named I-IV (Fig 1.8). Electrons from NADH enter the ETC at Complex I (NADH:ubiquinone oxidoreductase) while ETC links with TCA cycle in Complex II (succinate dehydrogenase) to accept the FADH_2 electrons. Complex I electron transfer is coupled with proton export from matrix into the intermembrane space. Electrons from both Complex I and II are transferred sequentially to Complex III (Q-cytochrome c oxidoreductase) and IV (cytochrome c oxidase) further increasing the proton gradient. In this process, Complex IV also bind O_2 converting it to water. The proton gradient generated between the matrix and intermembrane space across the IMM gives rise to the mitochondrial membrane potential ($\Delta\Psi$). This potential is harnessed by the final component of the machinery- the ATP synthase complex/ Complex V. This molecular motor comprises a transmembrane rotor (F_0 component) and a catalytic stalk (F_1 component). The turning of the F_0 rotor channels protons back into the matrix, coupled with the catalytic production of ATP from the F_1 component (Nolfi-Donagan et al., 2020).

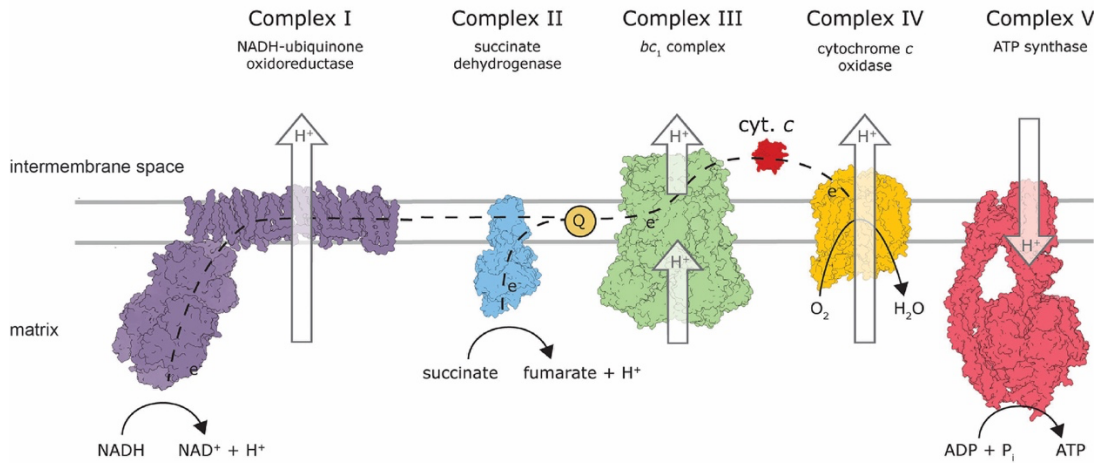


Figure 1.8. Mitochondrial electron transport chain and the ATP synthase complex. Electron transport is coupled to proton export across the inner membrane. The proton gradient is harnessed by the mitochondrial ATP synthase complex to produce ATP. Image adapted from (Brzezinski et al., 2021).

1.4.4 RNA binding proteins regulating mitochondrial function

Coordinated communication with the cytosol ensures homeostatic functioning of mitochondria. Expression of mitochondrial genes need to be coordinated with expression and import of nuclear encoded cytosolic proteins. RNA binding proteins play a major role in this coordination, with functions in mitochondrial gene expression and regulation of cytosolic lifecycle of mitochondrial proteins.

1.4.4.1 Nuclear encoded RBPs functioning in the mitochondria

The human mitochondrial genome of 16.5 kb produces polycistronic transcripts that are further processed, stabilized and matured, translated by a gamut of nuclear encoded RNA binding proteins. They function together with the mitochondrial encoded RNA components, the 2 MT-rRNAs and 22 MT-tRNAs (Pearce et al., 2017; Taanman, 1999). These RBPs function as (i) RNA polymerase and transcription factors (e.g., POLRMT, TFAM, TF2B) (ii) precursor RNA processors including endonucleases (e.g., RNaseP, ELAC2) and other factors (FASTK-family of proteins). Endonucleolytic processing of the polycistronic transcript releases the MT-mRNAs and MT-rRNAs from their flanking MT-tRNAs (Sanchez et al., 2011). (iii) RNA modifying and maturing factors (for e.g. mediating methylation and pseudouridylation of MT-rRNAs and MT-tRNAs (Bohnsack and Sloan, 2018), polyA tailing of MT-

mRNAs with MT-PolyA polymerase) (iv) Mitochondrial translation machinery including mitochondrial ribosome proteins and assembly factors (v) amino acyl tRNA synthetases (Kummer and Ban, 2021). Mitochondrial RNA processing, maturation and assembly of the mitoribosome occurs in phase separated granules in the mitochondrial called MRG (Mitochondrial RNA granules) where the RBPs and interacting RNAs are enriched (Xavier and Martinou, 2021).

1.4.4.2 Organellar cross-talk- cytosolic RNP complexes that regulate mitochondrial function

Mitochondrial biology requires close coordination between the nuclear transcribed mitochondrial proteins and the mitochondrially transcribed proteins. Mechanisms beyond transcriptional regulation bring about a fast and dynamic response of energy metabolism proteins to metabolic cues. Cellular RBPs mediate this regulation across compartments, with diverse factors present in mitochondria and cytosol. Some cytosolic RBPs coordinate lifecycle of mitochondrial mRNAs from their nuclear biogenesis to mitochondrial import. Nuclear paraspeckle formation by lncRNA NEAT1 in concert with the RBP NONO sequesters mitochondrial mRNAs away from cytosol, preventing their translation in conditions of mitochondrial stress (Wang et al., 2018). This nuclear retention also happens in concert with the cellular feeding dynamics (Benegiamo et al., 2018). Aberrant pre-mRNA processing of mitochondrial transcripts by a mutant PABPN1 and subsequent degradation by Smg leads to mitochondrial dysfunction (Chartier et al., 2015; Vest et al., 2017). This implies the importance of coordinated regulation of mitochondrial mRNAs at basal state by RBPs for mitochondrial health.

Puf3p is an RBP extensively studied in *Saccharomyces cerevisiae* for its multifaceted roles as the cells shift from fermentation to mitochondrial respiration. From a mitochondrial mRNA decay factor during fermentation, Puf3p flips roles to promote localized mitochondrial mRNA translation near mitochondria (Schatton and Rugarli, 2018). This is mediated by Puf3p phosphorylation, which affects Puf3p's function as well as subcellular localization (Lee and Tu, 2015). Targeting mitochondrial mRNAs to mitochondrial membrane (where localized ribosomes translate it) is now being considered as a general principle of regulation. The mechanisms of this process are still being investigated. Some known factors involved in this regulation include COPI vesicles which transport mRNAs to mitochondrial proximity and the LARP1-mTORC1 axis. The latter also regulates mitochondrial mRNAs in addition to selective

mitochondrial localization. RBPs like CLUH selectively bind to mitochondrial protein mRNAs and protect it from degradation (Schatton and Rugarli, 2018). CLUH organizes these mitochondrial mRNAs into granules for effective translation, and engages with different transcripts depending on the cell requirements (Pla-Martin et al., 2020). RNA granules like P-bodies are also observed in the vicinity of mitochondria. The role of these granules in the transportation of mitochondrial targets and potential storage in an inactive state for ready mobilization in mitochondrial biogenesis is being explored (Schatton and Rugarli, 2018).

In addition to well-characterized RBPs, proximity labelling methods like APEX-PS have uncovered novel RBPs associated with OMM with impact on mitochondrial function (Qin et al., 2021). Beyond direct regulation of mitochondrial RNAs, RNA binding proteins also factor in mitochondrial dynamics, controlling fission, fusion and morphology (Ravanidis and Doxakis, 2020). RNAs also directly contribute to mitochondrial regulation. For example, several ncRNAs have also been implicated in mitochondrial dynamics affecting the expression of proteins like FIS1 and DRP1 through miRNA mediated regulation (Lin et al., 2020).

1.4.4.3 Mitochondrial metabolic enzymes as RNA binding proteins

So far, we have explored the role of RBPs in regulating mitochondrial function. We also illustrated examples of metabolic enzymes functioning as RBPs, either regulating the fate of bound RNAs or RNA regulating the function of the protein. In the newly discovered panel of metabolic enzymes binding RNA, several mitochondrial metabolic enzymes are prominent. The list includes enzymes functioning in the TCA cycle as well as mitochondrial OXPHOS. They are found to bind RNA in multiple species, from *Escherichia coli* to humans (Caudron-Herger et al., 2021; Schwarzl et al., 2022a).

Preliminary characterizations of some mitochondrial RNA binding enzymes hint at potential physiological significance of these RNA interactions. Glutamate dehydrogenase (GDH) (catalyzing the reversible interconversion of glutamate to α -KG and ammonia with the reduction of NAD(P)^+ to NAD(P)H) have been found to bind the nuclear encoded COX mRNA, with the regulatory consequences still unknown (Preiss et al., 1993; Preiss et al., 1995). Enzymes involved in fatty acid oxidation such as enoyl CoA hydratase, thiolase and hydroxyacyl-CoA dehydrogenase

trifunctional multienzyme complex subunit beta (HADHB) are also bi-functional RNA-binding proteins (Adams et al., 2003; Brennan et al., 1999; Nanbu et al., 1993).

In particular, most enzymes of the TCA cycle including citrate synthase (CS), isocitrate dehydrogenase (IDH2), aconitase (ACO2), succinyl CoA ligase, succinate dehydrogenase (SDH), malate dehydrogenase (MDH2) and fumarate hydratase (FH) have been found to bind RNA (Caudron-Herger et al., 2021; Ciesla, 2006; Schwarzl et al., 2022a). Majority of them still remain unexplored as to the physiological impact of RNA binding. ACO2 is the mitochondrial isoform of IRP1- a famous example of metabolic enzyme as a regulatory RBP. Reports on the RNA binding activity of IDH2 in yeast implicates it in the translational regulation of mitochondrial mRNAs (de Jong et al., 2000; Dekker et al., 1992; Elzinga et al., 1993).

Of the enzymes in ETC, subunits of Complex I, Complex II (SDH) and Complex IV bind RNA (Mullari et al., 2017; Schwarzl et al., 2022a). Several components of the ATP synthase complex (ATP5A1, ATP5B1, ATP5C1, ATP5F1, ATP5I, ATP5L, ATP5O, ATP6V0C) are also RNA binding proteins (Caudron-Herger et al., 2021; Schwarzl et al., 2022a).

RNA binding of some these enzymes is affected by metabolites in-vitro, in particular, the metabolites involved in the catalytic steps of these enzymes. For example, IDH2's RNA binding is sensitive to isocitrate and AMP whereas GTP and NADH in combination inhibits the RNA binding of GDH (Ciesla, 2006). These examples hint at the complex biology of mitochondrial metabolic enzymes beyond their enzymatic function and how they are coordinated potentially through metabolites.

Mitochondrial metabolic enzymes need to be investigated in further detail with regard to the functional effects of their RNA interactions. Given the cytosolic origins of these mitochondrial proteins, it is also important to consider the coordination of the RNA-binding function between these compartments. The possible consequences on either the protein or RNA at various stages in their lifecycle need to be considered. This can be post-transcriptional/translational regulation of the bound RNAs by the enzymes as well as functional and localization regulation of the protein by the RNA. Either way, RNA-associations of mitochondrial enzymes have the potential to influence mitochondrial/cytosolic function or their cross-organelle coordinated functions.

In this study, we aim to expand our knowledge in this regard by investigating two mitochondrial metabolic enzymes binding RNA. The first one is MDH2, a member of

the TCA cycle. The second one is ATP5A1, a mitochondrial matrix component of the ATP-synthase complex. MDH2 and ATP5A1 will be introduced in detail in Part II of the thesis, with Chapter 2 dedicated to the RNA binding activity of MDH2 and Chapter 3 to the RNA binding activity of ATP5A1.

1.5 Aims and structure of the thesis

RNA binding proteins in the cell can regulate the lifecycle of the RNAs they bind to. In addition, RNAs can functionally direct the fate of the proteins they interact with. Identification of metabolic enzymes with RNA binding function have added a complex layer to the interconnections between cellular energy metabolism and gene regulation. In particular, interactions of nuclear encoded mitochondrial enzymes with RNA warrant further exploration. Their potential to coordinate crosstalk between metabolic outcomes and gene expression change- possibly across cellular compartments- has not been studied intensively. My doctoral research aims to bridge this gap, characterizing the RNA binding functions of mitochondrial metabolic enzymes: Malate dehydrogenase 2 (MDH2) and ATP-synthase subunit alpha (ATP5A1).

The primary focus of this thesis was to functionally characterize malate dehydrogenase 2 as an RNA binding protein. **Chapter 2** expands on my efforts in this direction. I aimed to first verify MDH2's RNA binding activity and identify its in-vivo targets using eCLIP. Given MDH2's cytoplasmic transit before mitochondrial import, I investigated the subcellular location of RNA binding. In addition, I explored contributions of MDH2's mitochondrial targeting signal towards binding RNA. I characterized MDH2's RNA binding function in-vitro, assessing how its substrates and co-factors affect interactions with RNA. Finally, I try to correlate MDH2's RNA binding function with its metabolic function to reveal underlying principles of its functional coordination.

I also collaborated with Dr. Aindrila Chatterjee from our lab, in a project to understand the RNA-binding function of ATP-synthase subunit alpha which is covered in **Chapter 3**. Here, my focus was to determine regulatory effects of ATP5A1 exerted on the RNAs bound. First, I aimed to verify the RNA-binding preferences of ATP5A1 (identified by eCLIP) in-vitro. I also explored the effect of ATP5A1 depletion on

global protein synthesis, querying in particular the ATP5A1-bound RNA targets. To gain a mechanistic understanding of how the RNA binding could be coordinated in cells, I identified cellular protein interactors of ATP5A1.

Chapter 2 and 3 are structured in 4 parts – introduction, objectives and results followed by a discussion.

Part II

Results and Discussion

Chapter 2: RNA binding activity of MDH2

Chapter 3: RNA binding activity of ATP5A1

Chapter 2: RNA binding activity of MDH2

2.1 Introduction to MDH2

Malate dehydrogenase 2 (MDH2) is an enzyme in the Krebs cycle which catalyzes the reaction of malate to oxaloacetate with the simultaneous reduction of NAD^+ to NADH. One of the isozymes of the malate dehydrogenase family of enzymes (Musrati et al., 1998), MDH2 is localized to the inner matrix of mitochondrion. In addition to its role in Krebs cycle, MDH2 contributes to other metabolic processes like gluconeogenesis, amino acid synthesis, glyoxylate cycle due to the multiple reactions that consume or create malate and oxaloacetate (Goward and Nicholls, 1994). MDH2 is also a component of the Malate-Aspartate Shuttle (MAS) which transports metabolites and reducing equivalents from cytoplasm across the mitochondrial membrane (Berg et al., 2002). Malate-aspartate shuttle relies on having different compartmentalized pools of malate dehydrogenases and glutamate-oxaloacetate aminotransferases and uses two sets of antiporters to transport malate and aspartate across the mitochondrial membrane. In essence, oxaloacetate is reduced to malate by MDH1 (malate dehydrogenase 1) in the cytoplasm using NADH as a cofactor. Malate is then shuttled across the mitochondria, where it is converted back to oxaloacetate by MDH2 with the concomitant reduction of NAD^+ to NADH in the mitochondria (LaNoue et al., 1974; Lanoue and Schoolwerth, 1979). In doing so, it effectively transfers the electrons from cytoplasmic NADH (which cannot cross the mitochondrial inner membrane) into the mitochondria and replenishes the cytoplasmic pools of NAD^+ for glycolysis to continue (Barron et al., 1998). This also helps to maintain the relative ratios of NAD^+/NADH in the cytosol and mitochondria. The cycle is completed when oxaloacetate pools in cytoplasm are replenished using aspartate as an intermediate. Depending on the concentrations of metabolites across the two compartments, the shuttle can also proceed in the opposite direction (Bremer and Davis, 1975). Malate aspartate shuttle contributes towards the coordinated regulation of metabolism across different cellular compartments (Safer, 1975) (Fig 2.1).

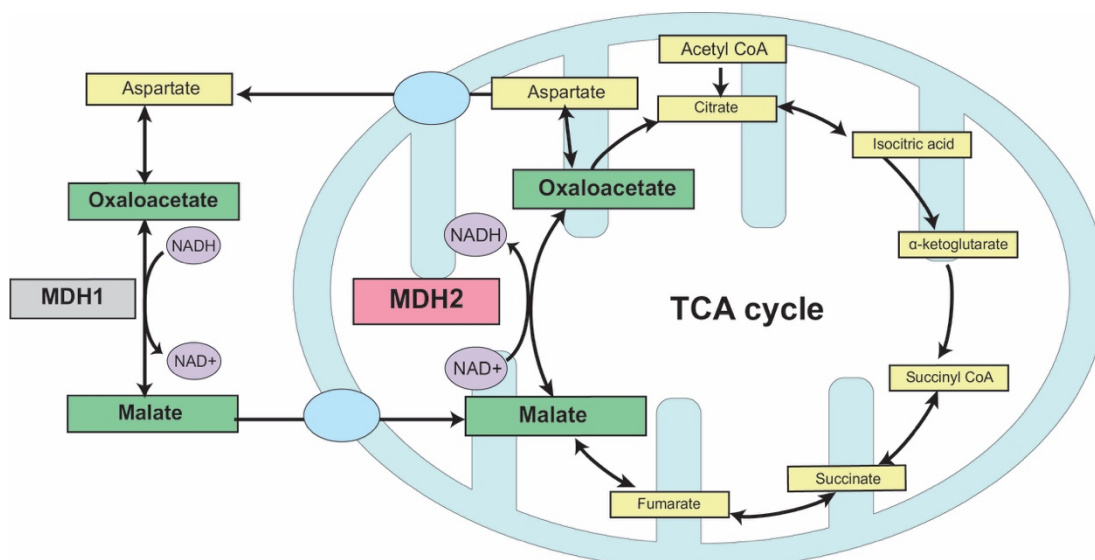


Figure 2.1. MDH2, a mitochondrial metabolic enzyme. MDH2 functions as a metabolic enzyme catalysing interconversions of malate and oxaloacetate using NAD⁺ as a cofactor. It is part of the TCA cycle as well as the malate aspartate shuttle.

Given its central role in metabolism, the enzymatic activity of MDH2 has been extensively studied. MDH2 functions as a homodimer (Devenyi et al., 1966), with each subunit weighing around 34 kDa. The disassociation of MDH2 into functionally inactive monomers is dependent on enzyme concentration, pH and association with the cofactors (Bleile et al., 1977; Shore and Chakrabarti, 1976). Though the reaction catalyzed by MDH2 is reversible, in the mitochondria it proceeds in favor of oxidation of malate. The low concentration of oxaloacetate in mitochondria maintained by its rapid utilization in the TCA cycle, the ease of malate transport into the mitochondria, NAD⁺/NADH ratio in the mitochondria and the allosteric activation by citrate pushes the reaction in this direction (Mullinax et al., 1982b).

Malate dehydrogenases belong to a superfamily of proteins called 2-hydroxy acid dehydrogenases (Birktoft et al., 1982), which also includes lactate dehydrogenases (LDH). Structurally, the major domains of MDH2 are the LDH-N terminal domain and LDH-C terminal domain. The NAD binding site maps to the N-terminal domain, with the substrate binding to the C-terminal domain and the active site is formed at the junction of the two domains. There is a characteristic invariant His-Asp pair at the active site of this family of enzymes that acts as a proton-relay system (Birktoft and Banaszak, 1983; Musrati et al., 1998). Moreover, a flexible loop in malate dehydrogenases closes over the active site to bring about the ideal conformation for

catalysis (Musrati et al., 1998). As is the case for other dinucleotide binding enzymes, GAPDH, LDH, PGK- to name a few, MDH2 has a Rossmann-like-fold (consisting of alternating beta strands and alpha helical segments) in the N-terminus mediating the binding to NAD^+ (Bellamacina, 1996; Rao and Rossmann, 1973) (Fig 2.2).

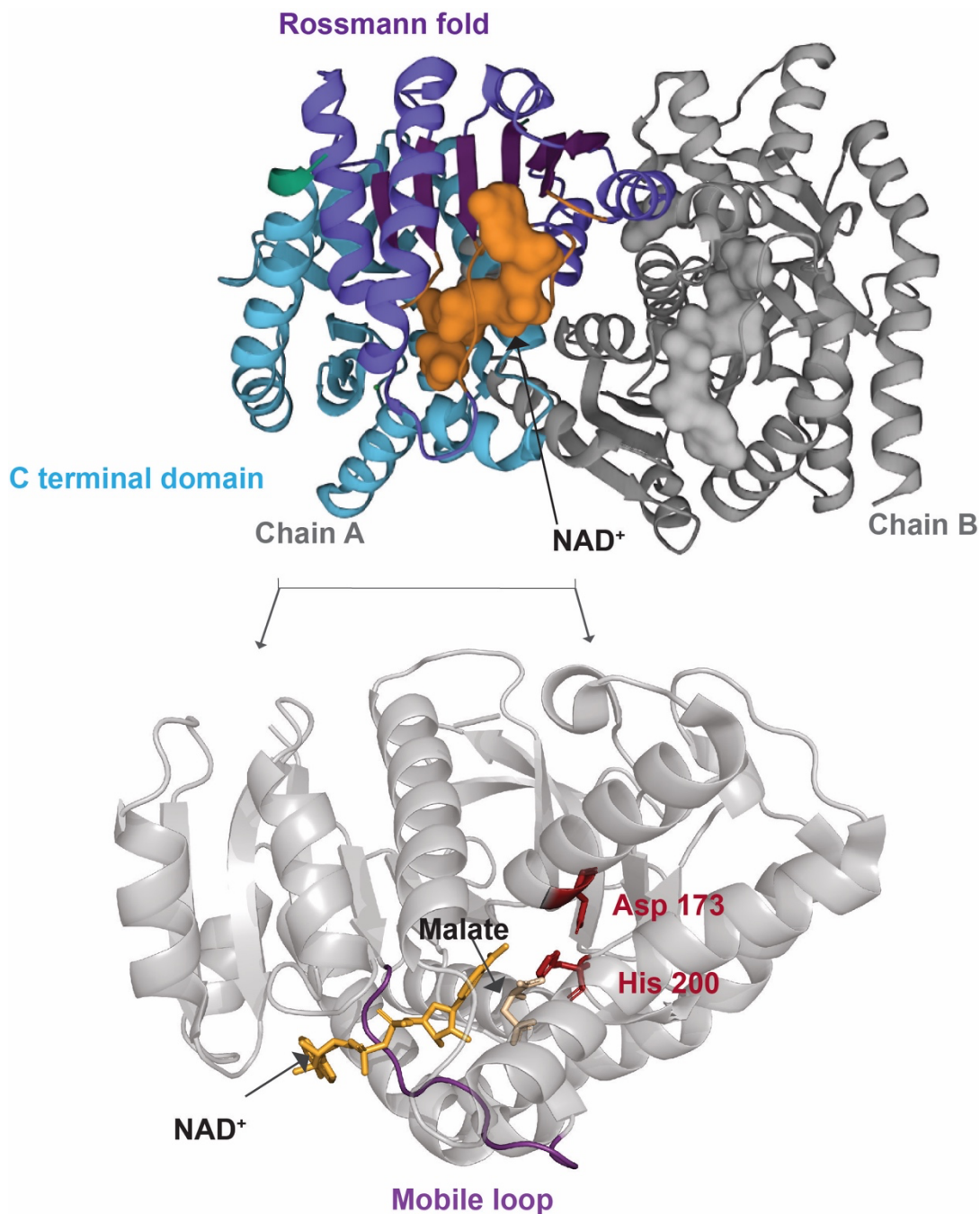


Figure 2.2. Structural features of human MDH2. Structure of MDH2 dimer, with the domains in one monomer highlighted. The N-terminal domain containing the Rossmann is coloured in purple, the alpha helices is in a lighter shade and the beta strands are marked in a darker shade. The C-terminal domain is coloured in blue. Close-up image of the MDH2 monomer showing the NAD^+ and malate bound (coloured in orange and salmon respectively). The His-Asp pair important for proton relay in the catalytic activity is highlighted in red. The mobile loop important for catalysis is highlighted in violet (PDB structure 2DFD).

While its catalytic activity has been examined in detail, a relatively novel aspect of MDH2's function has surfaced in recent years, which is its RNA binding ability. MDH2 has been identified as an RNA binding protein in several of the RNA interactome capture studies performed across several different model systems- mainly human and mouse cell culture lines, yeast, flies, worms and plants (reviewd in Table 1 in the following section) (Baltz et al., 2012; Beckmann et al., 2015; Castello et al., 2012; Liao et al., 2016; Marondedze et al., 2016; Matia-Gonzalez et al., 2015; Perez-Perri et al., 2018; Wessels et al., 2016).

MDH2 features prominently among the metabolic enzymes identified in RNA interactome captures, especially as a part of the group of enzymes having nucleotide binding domains like the Rossman fold (Castello et al., 2015). RNA binding activities have been demonstrated for several enzymes with an NAD binding Rossman fold, notably for GAPDH (Nagy et al., 2000), LDH (Pioli et al., 2002), UDP- glucose dehydrogenase (Barbas et al., 2013), supporting the ability of this domain to mediate binding to RNA. However, few studies have focused on MDH2's RNA binding function. MDH2 was shown to bind to the 3' UTR of SCN1A mRNA coding for a voltage gated ion channel, affecting its stability. The mechanism of this regulation is unidentified (Chen et al., 2017). In addition, MDH2 was co-purified with the lncRNA AC020978, a regulator of non-small cell lung cancer. MDH2-AC020978 axis was proposed as an activator of PI3K/AKT/mTOR signaling pathway to promote tumorigenesis (Xu et al., 2021). Another lncRNA GAS5 is implicated in regulating TCA cycle by disrupting the formation of MDH2-FH-CS metabolon complex (fumarate hydratase: FH, citrate synthase: CS) (Sang et al., 2021). These studies present the tip of the iceberg with regard to the mechanisms and consequences of MDH's RNA binding function. While aforementioned studies have focused on MDH2's binding to specific RNA targets, no comprehensive analysis of MDH2's total RNA binding has been done yet. Studying the principles of MDH2's RNA binding together with an unbiased transcriptome-wide screen for MDH2's RNA targets can reveal broader themes underlying MDH2's RNA binding function.

The relatively unexplored RNA binding activity of MDH2 opens up new questions about the role and regulation of this well characterized metabolic enzyme, which remains to be answered. Dysregulation of MDH2 has been linked to different tumors

like pheochromocytoma/paraganglioma (Cascon et al., 2015), with reports of MDH2 mediating drug resistance in several cancers (Liu et al., 2013; Lo et al., 2015). Mutations in MDH2 has been reported to cause early onset severe encephalopathy (Ait-El-Mkadem et al., 2017) where the loss of function of the enzyme leads to severe neurological manifestations in children. The lack of a strong respiratory chain deficiency in this model also raises the possibility of an unexplored role for MDH2, in addition to Krebs cycle defects. Therefore, understanding the RNA binding activity of MDH2 is necessary to understand the critical role of this enzyme in various biological contexts. Moreover, by exploring MDH2's RNA binding activity in detail, we aim to reveal possible general principles of interplay of gene regulation and metabolism, which can also be extended to the study of RNA binding function of other enzymes.

2.2 Objectives

This study aims to explore the RNA binding activity of MDH2 in detail. The various facets of this include:

1. Validation of the RNA binding activity of MDH2
2. Identification and validation of cellular RNA targets bound by MDH2: eCLIP analysis
3. Where does MDH2 bind RNA?
4. How is the RNA binding activity correlated with MDH2's metabolic function?
5. Elucidation of mode of binding of MDH2 to RNA
 - a. Which residues in MDH2 are important for RNA binding?
6. What is the broader physiological role of MDH2 binding to RNA in a cell?

2.3 Results

2.3.1 Mass spectrometry-based RNA Binding Protein Identification attributes RNA binding function to MDH2

MDH2, a mitochondrial TCA cycle enzyme, is a key player in coordinating cellular metabolism between mitochondria and cytosol. It features prominently among

metabolic enzymes that have qualified as RNA binding proteins (RBPs) in recent proteome-wide cellular RBP-catalogues. From the initial proteome-wide studies in 2012 (Baltz et al., 2012; Castello et al., 2012), the RBP-profiling field has expanded-employing diverse strategies for RNA selection, preservation and purification of ribonucleoprotein complexes (RNP) across different cell-types and organisms (recently reviewed (Smith et al., 2021)). I reviewed MDH2's RNA binding function in 54 datasets (Caudron-Herger et al., 2021; Schwarzl et al., 2022a) available till 2020 (Table 1). MDH2 is identified as a putative RBP in multiple datasets utilising diverse cell/tissue types and purification strategies (Table 1, supplementary table 1). MDH2's RNA binding function is conserved from bacteria to humans (Table 1). MDH2 shows remarkable sequence conservation across species, especially in residues proximal to NAD⁺ and malate- MDH2's substrate binding sites (Fig 2.3, supplementary figure 1). The NAD⁺ binding region of MDH2 contains Rossmann-fold, a sequence feature implicated in RNA binding for other enzymes like GAPDH and LDH (Barbas et al., 2013; Nagy et al., 2000; Pioli et al., 2002), a possibility that can extend to MDH2 as well.

MDH2 has been detected both in Poly-A and total RNA- based interactomes (Table 1, supplementary table 1), hinting at its capacity to bind different classes of RNAs. Thus, MDH2 has emerged as a putative RNA binding protein across different human cell lines and species.

Species	Model	Studies	RNA pulldown/ Strategy
<i>Homo sapiens</i>	HEK293, Huh7, HeLa, MCF7, Jurkat	(Backlund et al., 2020; Baltz et al., 2012; Bao et al., 2018; Beckmann et al., 2015; Castello et al., 2012; Castello et al., 2016; Garcia-Moreno et al., 2019; Huang et al., 2018; Mullari et al., 2017; Perez-Perri et al., 2018; Trendel et al., 2019; Urdaneta et al., 2019)	Oligo(dT)/LNA-pulldown, Trizol extraction, phenol toluol extraction, 5-ethynyluridine (EU) incorporation-Streptavidin pulldown
<i>Mus musculus</i>	Mouse embryonic stem cells, HL1 (cardiomyocytes)	(Bao et al., 2018; He et al., 2016; Liao et al., 2016; Mallam et al., 2019)	5-ethynyluridine (EU) incorporation-oligo(dT)-pulldown,

			oligo(dT) -pulldown, Differential fractionation $\pm$ RNase
<i>Saccharomyces cerevisiae</i>	BY4741 strain	(Matia-Gonzalez et al., 2015; Scherrer et al., 2010; Shchepachev et al., 2019)	Silica beads purification of RNA, oligo(dT)-pulldown, Microarray analysis
<i>Drosophila melanogaster</i>	Pre- and post-MZT embryos, S2 cells	(Panhale et al., 2019; Wessels et al., 2016)	Oligo(dT)-pulldown
<i>Arabidopsis thaliana</i>	Ecotype Columbia (Col-0) cell suspension culture	(Marondedze et al., 2016)	Oligo(dT)-pulldown
<i>Caenorhabditis elegans</i>	Young adult N2, mixed stage animals	(Esmailie et al., 2019; Matia-Gonzalez et al., 2015)	Oligo(dT)-pulldown
<i>Escherichia coli</i>	MG1655 strain	(Queiroz et al., 2019; Shchepachev et al., 2019)	Orthogonal organic phase separation, silica beads purification of RNA
<i>Salmonella typhimurium</i>	Salmonella typhimurium SL 1344	(Urdaneta et al., 2019)	Phenol toluol extraction

Table 1. Catalogue of studies identifying MDH2 as an RNA binding protein.

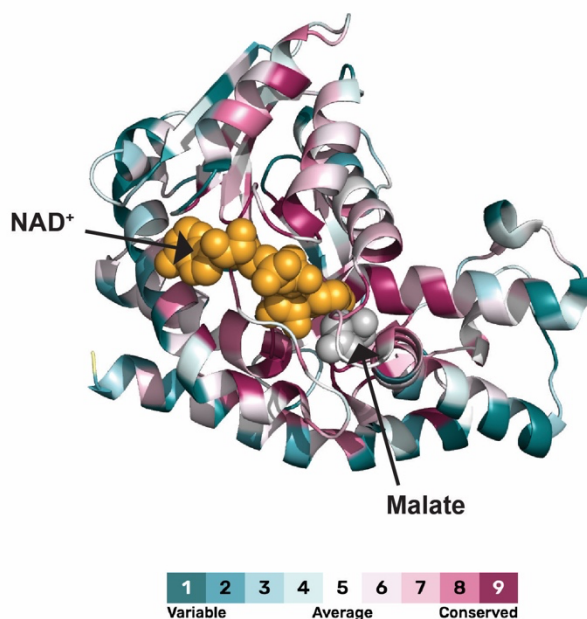


Figure 2.3. Sequence conservation of MDH2. The sequence conservation scores calculated by alignment of MDH across species is mapped on to human mitochondrial MDH2 (PDB ID-

2DFD). Analysis was performed using the CONSURF tool (Ashkenazy et al., 2016). NAD is highlighted in orange spheres and malate is coloured in grey spheres. MDH2 is coloured according to sequence conservation scores as indicated in the figure.

2.3.2 MDH2 is a bona-fide RNA binding protein (RBP)

In order to verify the RNA binding activity of MDH2, I used two orthogonal biochemical approaches. All experiments were performed on human HCC cell line Huh7 at 90-95 % confluency, ensuring above 90% viability.

First, I used the classical radioactive polynucleotide kinase (PNK) assay (Fig 2.4 A). Here, cells are first irradiated with ultraviolet light (UV 254 nm, 150mJ/cm² energy) to induce covalent crosslinks between proteins and the nucleic acids bound to them. This preserves the interaction through the downstream purification procedures in the assay. MDH2 is immunoprecipitated from the crosslinked cell lysates after limited RNaseA treatment. Sensitivity to RNase digestion would indicate that the co-purified complex is RNA. 5' end of the copurified RNAs is labelled with radioactive γ -³²P-ATP for visualization. This is followed by SDS-PAGE, transfer to a nitrocellulose membrane and autoradiography. PNK assay for MDH2 revealed RNase sensitive autoradiograph signals which condensed to a band slightly above the expected protein molecular weight (Fig 2.4 B). This indicates a positive RNA-protein interaction. The modest upward shift in the MDH2 migration could be due to the short RNA fragment it protects from digestion. At minimal RNase digestion, the purified complexes

migrate in a smear indicating a diversity in the length of the RNAs bound. Identical assay performed with isotype matched IgG serves as the negative control.

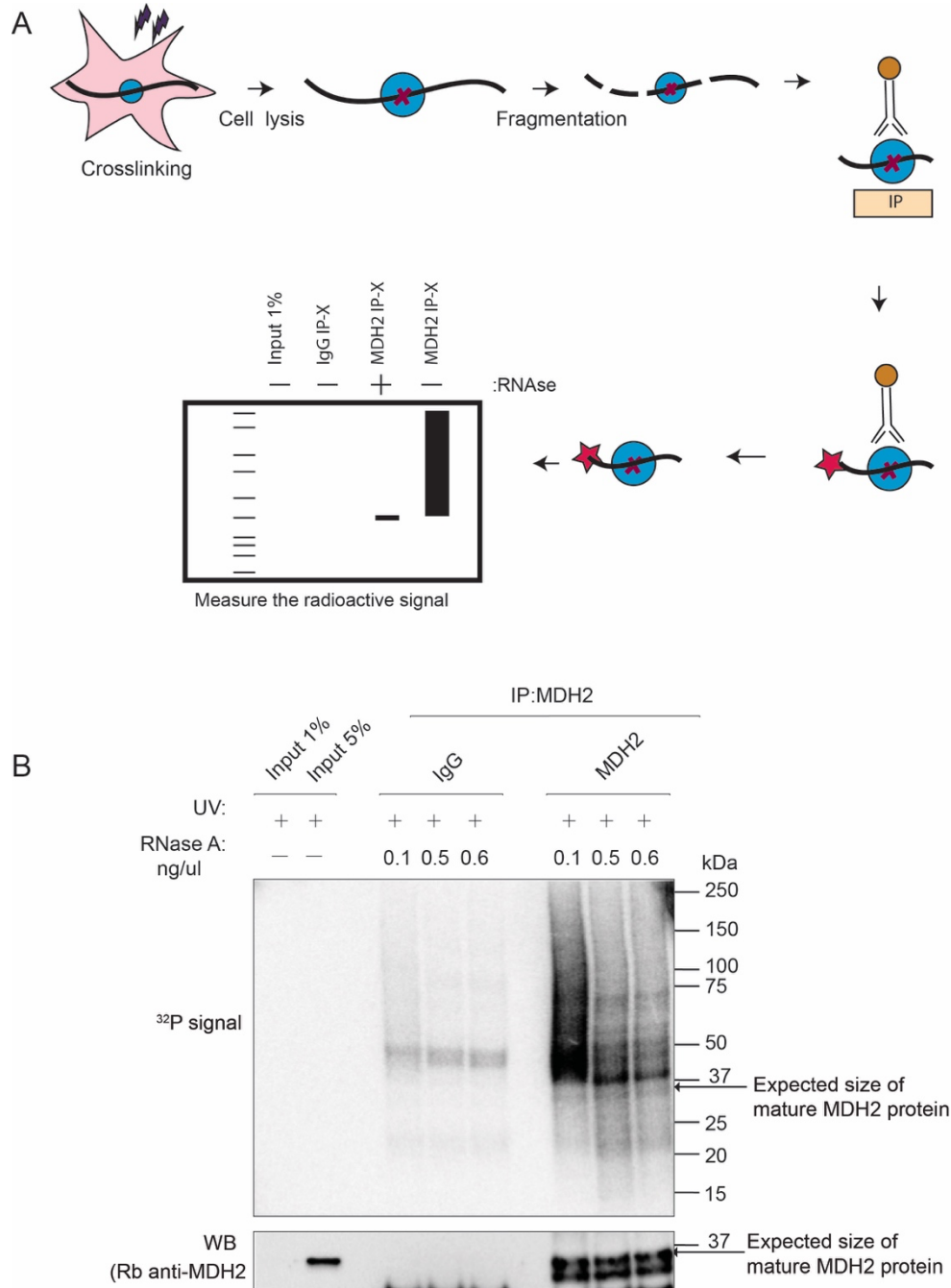


Figure 2.4. PNK assay validating MDH2's RNA binding. (A) Scheme of the PNK experiment. (B) Representative PNK assay. Top panel shows the phosphor image to visualize co-purified RNA, bottom panel shows IP efficiency. Black arrows indicate the expected molecular weight of the protein.

Next, I used a recently developed technique called ‘Complex Capture’ or ‘2C’ (Asencio et al., 2018) (Fig 2.5 A). This method exploits the fact that UV (254 nm, 150mJ/cm² energy) mediated covalent crosslinking of RNAs and proteins (Budowsky et al., 1986; Pashev et al., 1991; Shetlar, 1980) are preserved during RNA purification. In 2C, we utilise silica columns to purify such covalently linked proteins along with RNA. The eluates are treated with RNase I to digest the RNA from the RNA-protein (RNP) complexes, aiding the visualization of the protein around the expected molecular weight. The eluates are then analysed using western blot with antibodies specific for MDH2. Detection of a band specific for MDH2, demonstrates that MDH2 interacts with RNA. To confirm this retention was specific and RNA dependent, samples were treated with RNase I prior to a second round of 2C. In this case, specific MDH2 protein bands were absent, whereas the control samples without RNase I treatment showed specific bands (Fig 2.5 B). MDH2’s behaviour in 2C resembles that of DHX9 and hnRNPc- two canonical RNA binding proteins. Moreover, beta actin (with no identified RNA binding function) is absent in 2C eluates, serving as a specificity control (Fig 2.5 B). This leads us to conclude that it is specific MDH2-RNA interaction that mediates its retention in silica column.

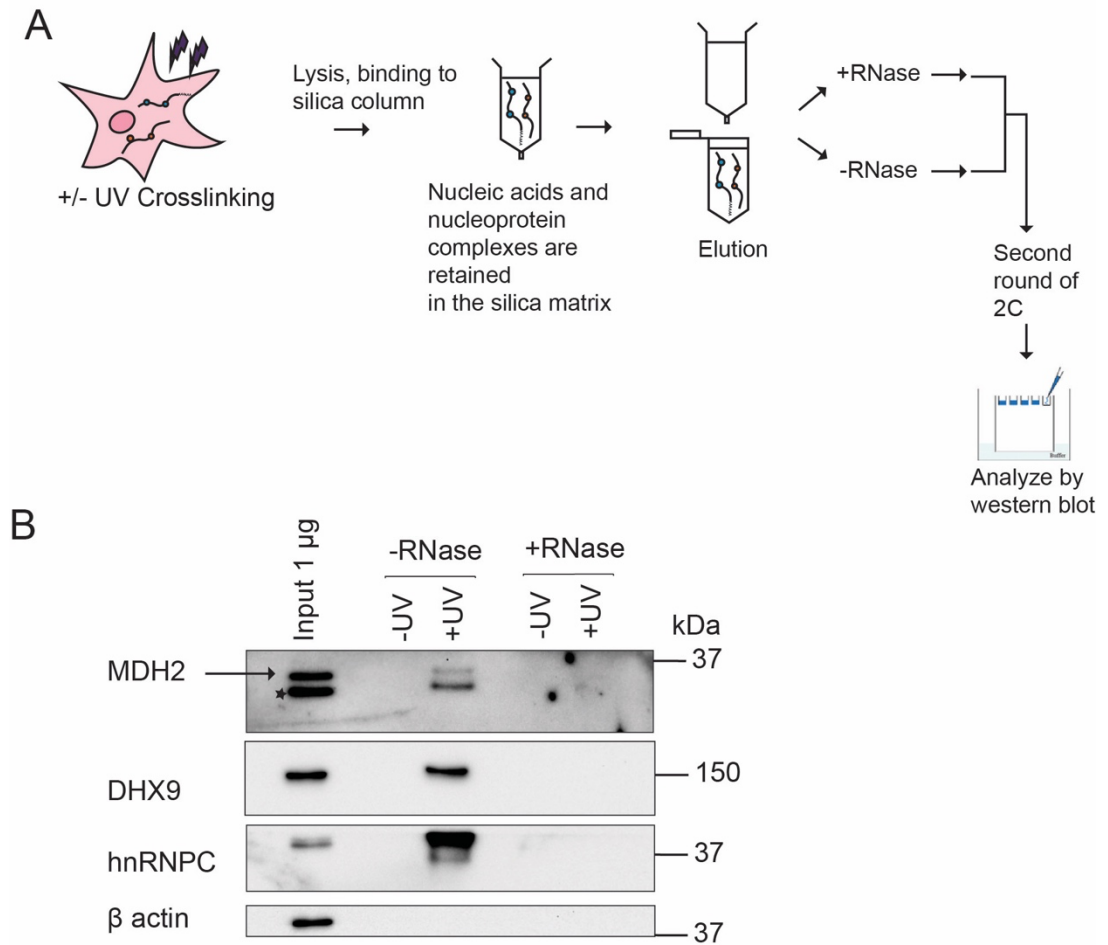


Figure 2.5. 2C assay for MDH2's RNA binding. (A) Scheme of experiment (B) Representative 2C assay. Western blot showing specific signal for MDH2 in “–RNase” treatment, which is eliminated with 2nd round of 2C performed after RNase treatment (“+RNase”). DHX9, hnRNP C were used positive controls and beta actin as the negative control. Black arrow indicates expected molecular weight of the MDH2 protein. * shows residual signal from previous western blot probing.

In conclusion, using two orthogonal approaches I could verify the RNA association of MDH2.

2.3.3 Identification of RNAs bound by MDH2

Next, I used eCLIP-seq (enhanced crosslinking and immuno precipitation) for unbiased identification of the RNA binding partners of MDH2. In eCLIP (Van Nostrand et al., 2016), UV crosslinking is used to preserve native RNP interactions, followed by RNA fragmentation and immunoprecipitation (IP) of the protein of interest. After reverse transcription of the RNAs pulled down by the protein, adaptors are ligated to the cDNA for library preparation (libraries for MDH2 eCLIP are shown

in Supplementary figure 2). Samples are then analysed by next-generation sequencing (Fig 2.6). An in-house developed bioinformatic package ‘DEWSeq’ (Sahadevan et al., 2022; Sahadevan, 2019; Schwarzl et al., 2022c) was used for data analysis. Dr. Thileepan Sekaran performed primary data analysis to identify MDH2-bound targets. Comparison against appropriate size-matched input (SMI) was used to identify target RNAs. The SMI approach normalizes against background RNAs, showing similar gel migration pattern and abundance. In addition, a control immunoprecipitation experiment performed with isotype matched IgG was used to eliminate RNAs pulled down non-specifically. Three replicates each of MDH2 IP (~9.1 million uniquely mapped reads/sample), SMI (~4.3 million uniquely mapped reads/sample) and IgG IP (~4.3 million uniquely mapped reads/sample) was generated. In a principal component analysis, replicates of each type (SMI/MDH2 IP/IgG IP) clustered together, whereas the different sample types showed variation along the first two principal components (Supplementary figure 2).

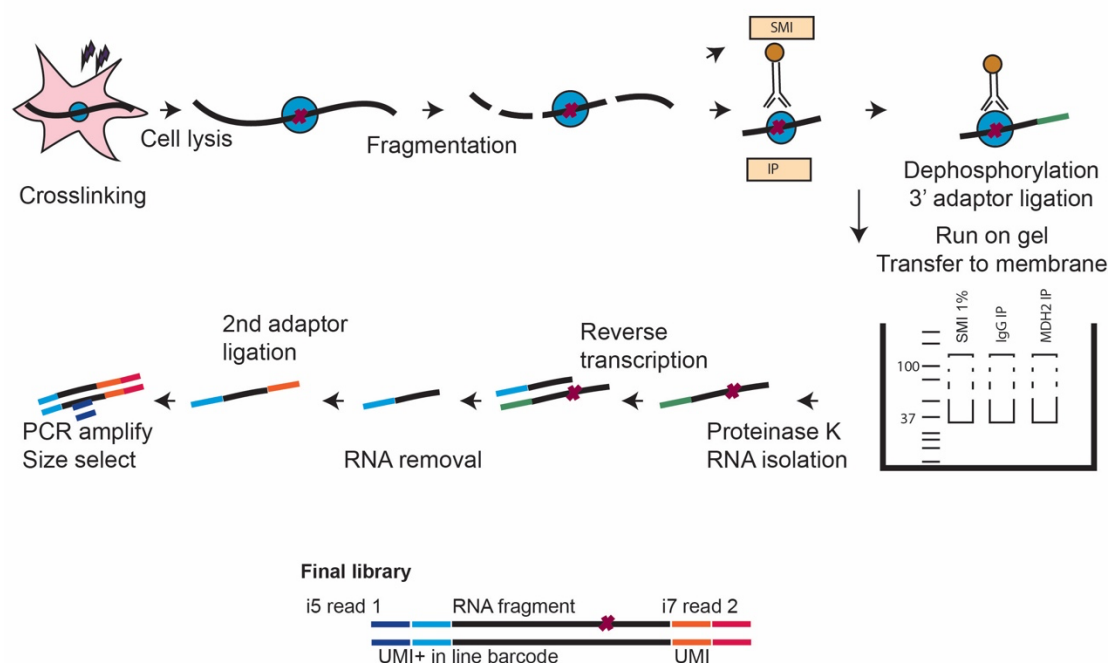


Figure 2.6. eCLIP experimental scheme.

MDH2’s primary enzymatic function is in the mitochondrial matrix. Transcribed from the nuclear genome, MDH2 is translated in cytosol as a precursor protein before import into the mitochondrial matrix. To avoid a-priori assumptions about MDH2’s RNA binding preferences, eCLIP was performed from whole-cell lysates instead of isolated

mitochondria. In total, 121 high confidence RNAs bound to MDH2 were identified, from different target classes (Fig 2.7). To our surprise, MDH2 showed a preference for cytosolic RNAs (transcribed in nucleus) over the highly abundant MT-RNAs (transcribed from mitochondrial DNA) present in the locale of the mature protein.

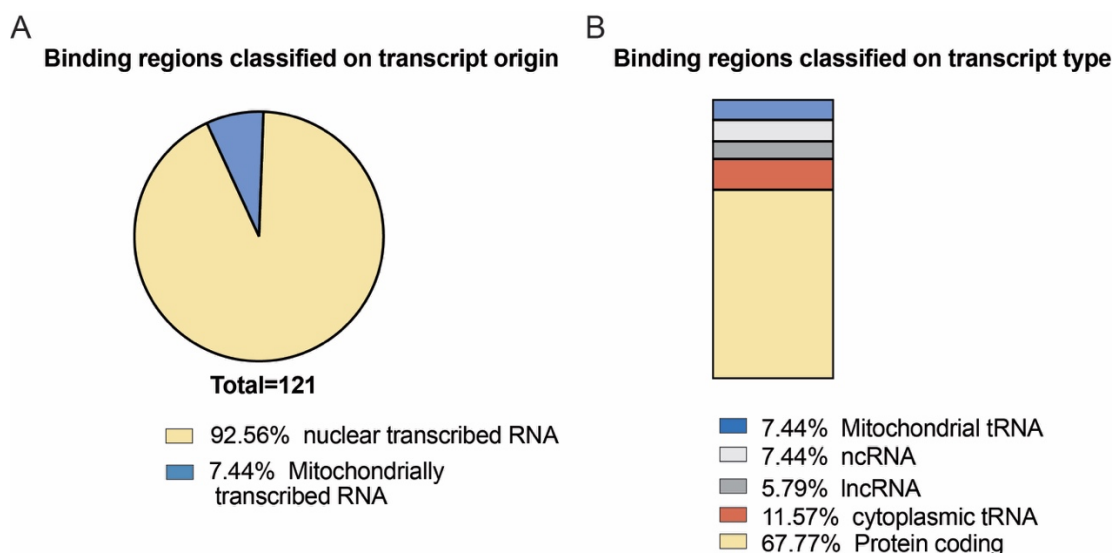


Figure 2.7. MDH2 binds cytosolic RNAs (A) Distribution of RNA targets based on transcript origin (B) Distribution of RNA targets based on transcript type.

Amongst mitochondrial RNAs, 9 MT-tRNAs were identified to bind MDH2 (out of 22 MT-tRNAs expressed in humans) (Fig 2.7 A-B, Fig 2.8 A). The preference for tRNAs was conserved across compartments, with specific cytosolic tRNAs contributing to the bulk of the MDH2 crosslinking sites detected by eCLIP (Fig 2.8 A, B). Cytosolic tRNAs stand out in their high enrichment over the SMI control (Fig 2.8 A) in comparison to MT-tRNAs. In addition, MDH2 binds to a variety of cytosolic mRNAs and ncRNAs, which trail behind tRNAs in their crosslinking intensity (Fig 2.8 A, B).

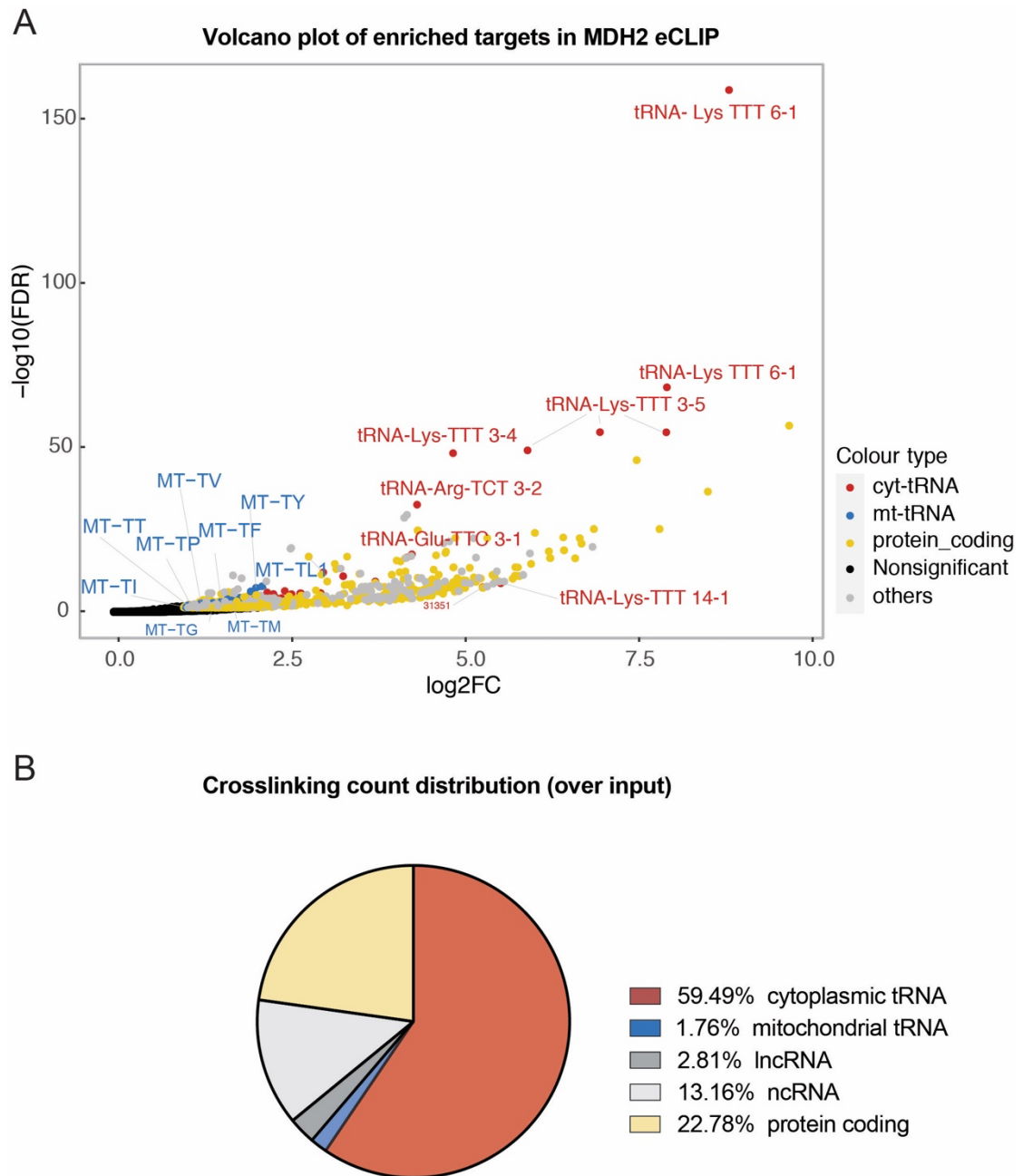


Figure 2.8. MDH2 prefers to bind tRNAs (A) Volcano plot showing the enrichment of different targets over the SMI. Targets with $\log_2FC \geq 1$ and $FDR \leq 0.05$ are considered significant. These targets are coloured according to class (B) Enrichment of MDH2 crosslinking counts over SMI across RNA types.

The cytosolic tRNAs bound by MDH2 distribute mainly to two isodecoder classes - Arg TCT and Lys TTT. For these cytosolic tRNAs (select representative candidates shown in Fig 2.9 A, B), the binding sites are not uniformly distributed along the tRNA. Crosslinking sites are present selectively on 3' half of the tRNAs (Fig 2.9 A, B). Within this region, enrichment sites for tRNAs differ. For the Arg TCT tRNA class, the

crosslinking sites map variably to the T-loop and the isoacceptor stem of the tRNA. Arg TCT 3-2 for example has more crosslinking sites at the T-loop compared to isoacceptor stem than Arg TCT 1-1 and vice-versa (Fig 2.9 A, C). For Lys TTT tRNAs, the MDH2 crosslinking sites extend to the anticodon loop where they are particularly enriched (Fig 2.9 B, D).

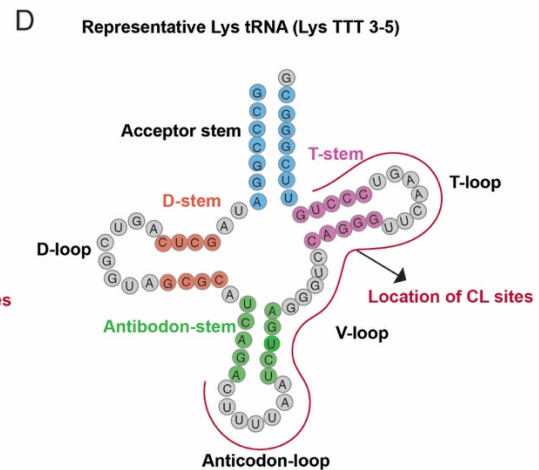
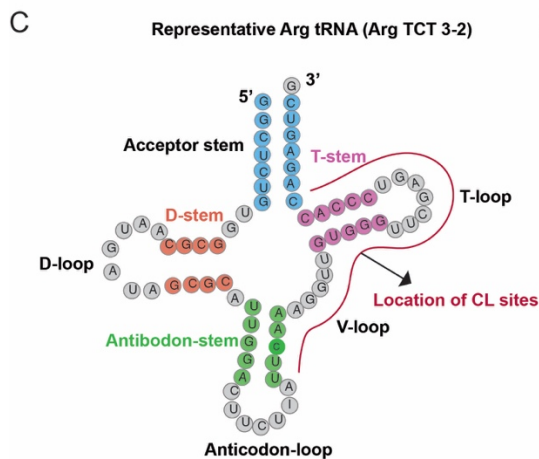
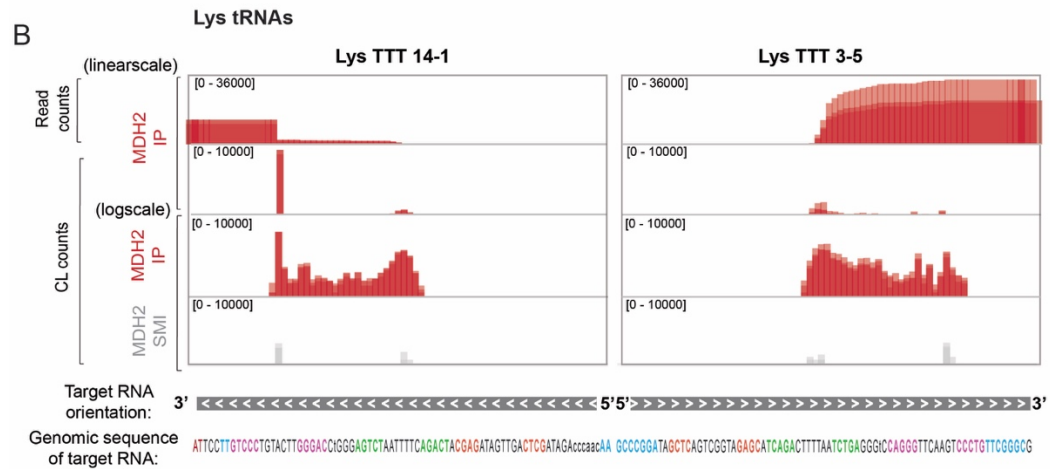
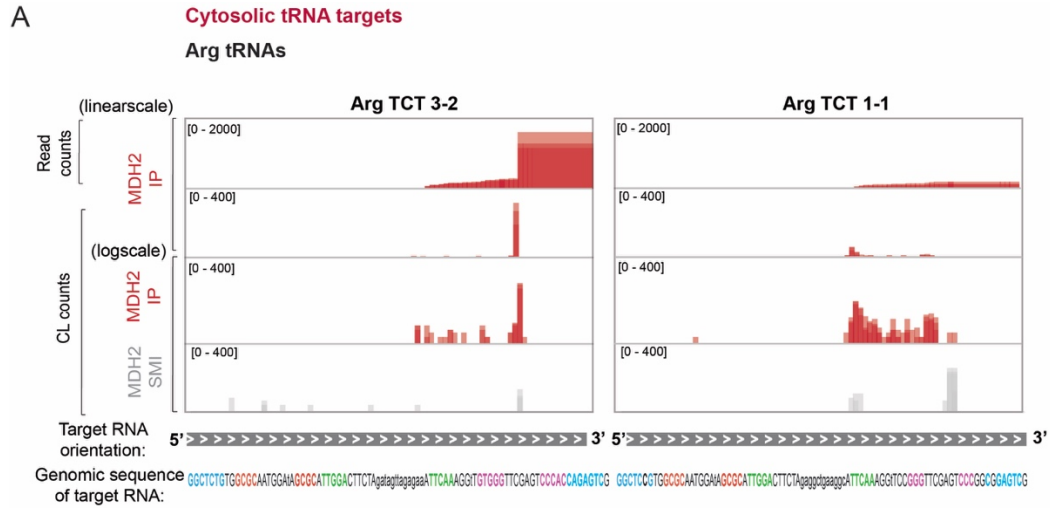


Figure 2.9. MDH2 crosslinking sites on select tRNA targets. (A) IGV (Integrative Genomics viewer) view of readcounts and crosslinking counts mapped on to the genomic sequence of tRNAs Arg TCT 3-2 and Arg TCT 1-1. Read counts are shown only for MDH2 IP, highlighted in red. Crosslinking counts are highlighted for both MDH2 IP (red) and SMI (grey). Crosslinking counts are shown in both linear and log scale to visualise binding site distribution over the tRNA length, considering the disparity in crosslinking counts along this sequence. Genomic sequence is highlighted according to structural elements in tRNA as shown in illustration in C, D. *Blue* =isoacceptor stem, *Orange*=D-stem, *Green* =anticodon stem, *purple*=T-stem. Orientation of target RNA indicates the 5' and 3' end of RNA. (B) IGV view of readcounts and crosslinking counts mapped on to the genomic sequence of tRNAs Lys TTT 14-1 and Lys TTT 3-5. Labelling as above (A). (C) Arg TCT 3-2 (UCU antibodon) 2D structure is shown with different arms coloured according to type. The distribution of crosslinking sites is indicated by the red trace. (D) Lys TTT 3-5 (UUU antibodon) 2D structure is shown with different arms coloured according to type. The distribution of crosslinking sites is indicated by the red trace.

It is well known that tRNAs frequently contain heavily modified nucleotides, which can induce premature reverse transcriptase (RT) termination or RT-stops. eCLIP uses similar RT-stops at UV induced protein-RNA crosslink sites to obtain single-nucleotide resolved binding information. Our eCLIP analysis counts all RT-stops as crosslinking-induced, interpreting them as RBP binding sites, including possible contributions from modification-induced RT-stops. Therefore, we need further assessment to distinguish between MDH2's genuine preference for these tRNA sites as opposed to modification-induced RT-stops. In addition, tRNAs can be cleaved *in-vivo* to generate tRNA derived fragments (tRFs) and tRNA halves, with functions in coordinating gene expression and stress responses (Shen et al., 2018). MDH2's target tRNAs can also generate smaller fragments (Kumar et al., 2015; Pliatsika et al., 2018). However, contribution of modification induced RT-stops should be deconvolved to correctly interpret the binding site information. At present, our eCLIP analysis pipeline cannot distinguish whether MDH2 binds cognate whole tRNAs at specific sites over tRFs derived from them.

MDH2's binding sites on MT-tRNAs (representative candidates shown in Fig 2.10 A) fall preferentially on their anticodon loop. In some cases, (for e.g., MT-TM) other open regions like D-loop also capture crosslinked MDH2. MDH2 binds specific mRNAs, (Fig 2.10 B) albeit with reduced frequency compared to tRNAs (comparing CL counts on RNAs which are indicated in figure 2.9, 2.10). Differential abundance of these classes of RNA- tRNAs have higher copy numbers over mRNAs (Deng et al., 2022; Johnson et al., 1974; Palazzo and Lee, 2015)-could determine MDH2's crosslinking

frequency. This doesn't preclude potential context-specific regulatory effects of MDH2 on the target mRNAs.

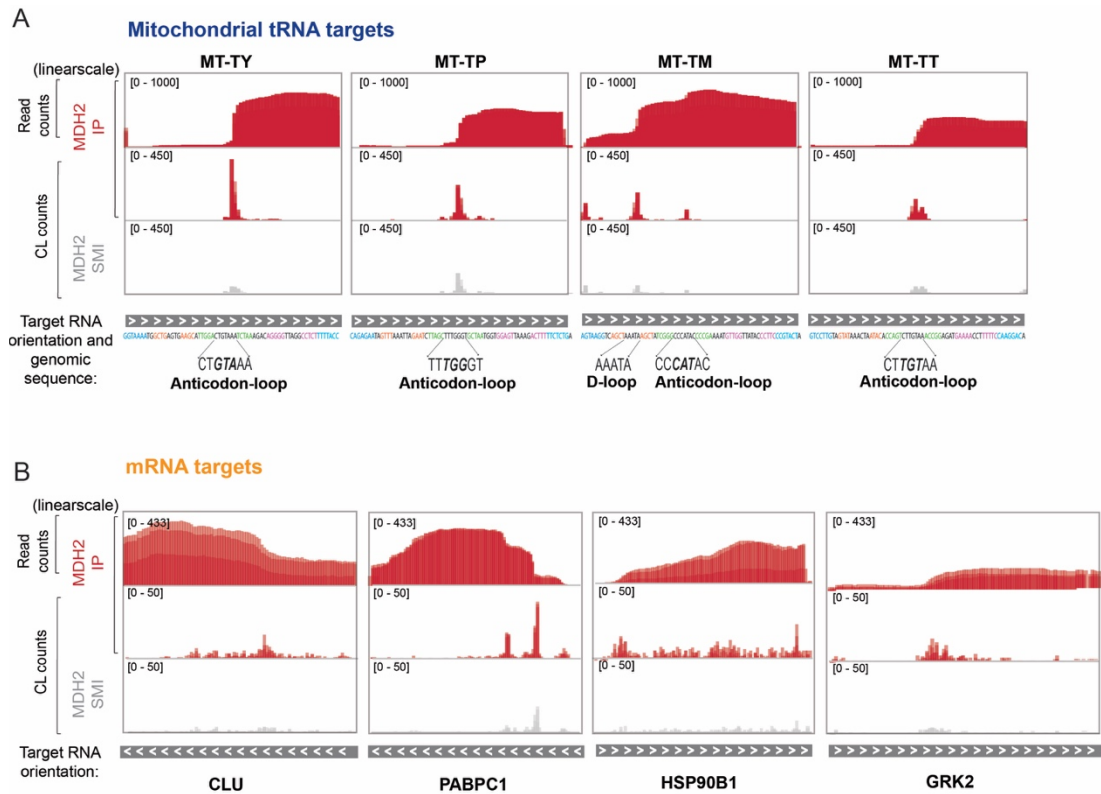


Figure 2.10. MDH2 crosslinking sites on MT-tRNAs and mRNAs (A) IGV view of readcounts and crosslinking counts mapped on to the genomic sequence of tRNAs MT-TY, MT-TP, MT-TM, MT-TT. Read counts are shown only for MDH2 IP, highlighted in red. Crosslinking counts are highlighted for both MDH2 IP (red) and SMI (grey). Orientation of target RNA indicates the 5' and 3' end of RNA. Genomic sequence is highlighted according to structural elements in tRNA as shown in illustration in 3.7B. Blue =isoacceptor stem, Orange=D-stem, Green=anticodon stem, purple=T-stem. (B) IGV view of readcounts and crosslinking counts mapped on to the genomic sequence of mRNAs CLU, PABPC1, HSP90B1, GRK2. Labelling as above (A).

To sum up, we find that MDH2 binds cytosolic RNAs- tRNAs, mRNAs, ncRNAs- preferentially over mitochondrial RNAs- tRNAs. MDH2 binding events on tRNAs are enriched over other classes of RNA.

MDH2's preferential binding to cytosolic RNAs raised an important question about the subcellular locale of MDH2-RNA association and its physiological consequences. Mitochondrial DNA encodes the minimal set 22 tRNAs required for its organellar translation (Gustafsson et al., 2016; Suzuki et al., 2020). Although many organisms including yeast can functionally import select cytosolic tRNAs (as reviewed in

(Bhattacharyya and Adhya, 2004), studies in human mitochondria have only tested artificial import of select yeast tRNAs (Entelis et al., 2001; Rubio et al., 2008). Till date, there is no proof of specific cytoplasmic t-RNAs enriched in MDH2-eCLIP dataset being imported into human mitochondria (Mercer et al., 2011), raising the possibility of MDH2-RNA association being a cytosolic event in addition to mitochondrial. To further investigate this aspect, I first verified the eCLIP data in-vitro and then explored MDH2's RNA binding preferences in subcellular compartments.

2.3.4 MDH2 binds tRNAs in-vitro

We used fluorescent RNA electrophoretic mobility shift assays (EMSA) to assess MDH2's affinity for its target RNAs over non-targets, in-vitro. For this, I expressed and purified full length human MDH2 (with a C-terminal V5-StrepII tag) from *Escherichia coli*- Rosetta (DE3) strain, using Streptactin beads (methods section 5.2.13) (Fig 2.11 A-B). MDH2 has a N-terminal mitochondrial targeting signal (MTS) which is cleaved after mitochondrial import in-vivo to form the mature protein. Considering a potential cytosolic location of RNA-binding, where MDH2 has an intact MTS, we included MTS sequence in the protein purification construct. StrepII tag was chosen for protein purification because of its small, inert nature and low background in Strep-pulldowns. I analysed the purity of the Strep-pulldown using SDS-PAGE, staining for the total protein and selectively for MDH2 using specific antibodies via western blot. Protein stain analysis shows full length tagged MDH2 at the expected size (39.3 kDa), as well as a band at the size of the expected MDH2 dimer (above 75kDa). There are additional bands below 37 kDa and 75 kDa (Fig 2.11 B). Processed forms of MDH2, where the MTS has been cleaved off, could migrate similarly. The mitochondrial processing peptidase (MPP) has bacterial homologs that could cleave off the targeting signal (Caspari and Lafontaine, 2021). We are planning to further perform mass spectrometry analysis to clarify this possibility as well identify contaminant proteins that may be co-purifying. Western blot analysis with human MDH2 specific antibodies confirm MDH2's enrichment in the purified protein. The antibody also specifically recognizes the band above 75kDa, confirming MDH2 dimerization (Fig 2.11 C).

Cytosolic tRNAs were one of the strongest enriched hits in eCLIP. So, I chose an exemplary tRNA- Arg-TCT 3-2, for further in-vitro analysis. To characterize the

binding preference in detail, I designed RNA oligos covering different regions of the target tRNA, on the basis of eCLIP crosslinking coverage. Probe 1 and 2 spanned 3' and 5' ends of the tRNA (Arg-TCT 3-2) respectively (Fig 2.11 D). These oligos were fluorescently labelled at 3' end with Cy5 and were commercially synthesized with HPLC grade purification.

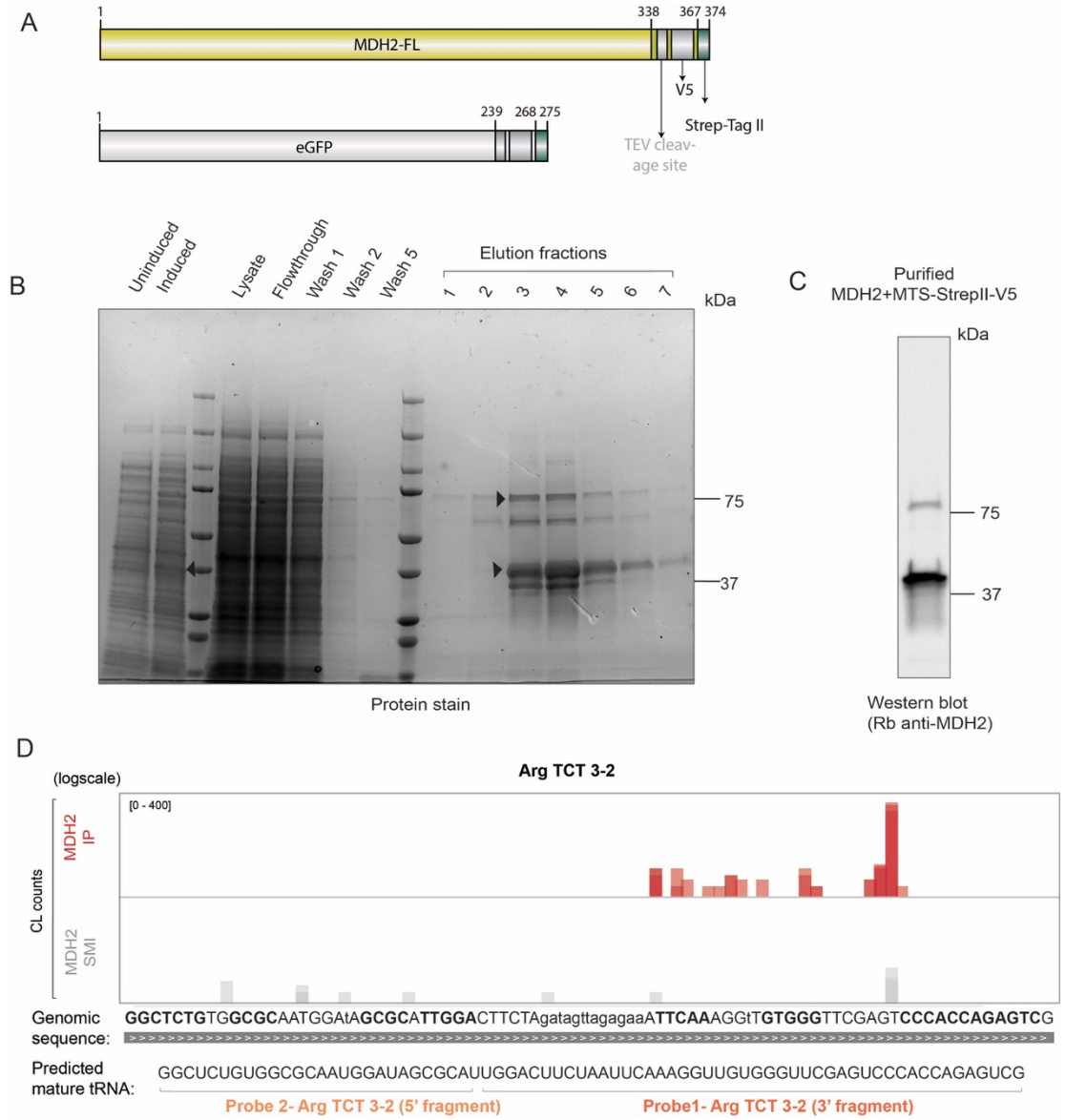


Figure 2.11. MDH2 protein purification and probe design for EMSA (A) Schematic of constructs used for protein purification (B) Gel run after protein purification and visualised with a protein stain. Arrows mark the expected size of tagged MDH2 and a potential dimer (C) Western blot performed for purified MDH2. (D) MDH2 binding sites on Arg TCT 3-2 tRNA and the probes designed to test binding.

EMSA analysis confirmed MDH2's interaction with both Arg-TCT 3-2 tRNA fragments. Addition of increasing amounts of purified MDH2 protein (0.18 μ M - 5.9 μ M) to the tRNA fragments (fixed at 10 nM concentration) showed a concentration dependent increment in formation of higher molecular weight complexes (Fig 2.12 A), suggestive of RNP-complexes. Without added protein, RNA-probe 1 resolves into multiple bands during electrophoresis, indicating presence of alternate structural conformations or RNA multimers (Fig 2.12 A, lane 2) in the assay conditions. In the same gel running conditions, RNA-probe 2 shows a single band (lane 10). When an estimated 0.73 μ M MDH2 protein is added to the RNA, the higher order structures start to be significantly enriched over the free species (Fig 2.12 A, lanes 5 and 13). The super shifted bands observed could reflect MDH2 multimers bound to RNA. Majority of probe 2 migrates with retarded mobility on titrating with 1.48 μ M MDH2, whereas for probe 1 it is about 5.9 μ M (Fig 2.12 A, lanes 6-8 and 14-16). This indicates MDH2's preference for probe 2 (5' Arg-TCT 3-2 RNA fragment) over probe 1 (3' Arg-TCT 3-2 RNA fragment).

Given the MDH2's higher affinity to the 5' Arg-TCT 3-2 RNA fragment, we used this fragment as the target probe for further characterization. To analyse non-specific binding by the retained tag (V5-StrepII), eGFP-V5-StrepII protein was tested in binding assays with target RNA (Supplementary figure 3 shows eGFP protein purification). eGFP-V5-StrepII showed minimal RNA-binding as evidenced by unchanged RNA migration after binding reactions with up to 5.9 μ M eGFP protein (Figure 2.12 B, lanes 1-10). Next, I compared MDH2's affinity for the target RNA (probe 2) directly against a non-specific RNA sequence based on the 5'UTR of RPS23. In eCLIP, MDH2 IP does not have enriched binding sites on this sequence compared to SMI (Supplementary fig 3). Therefore, a sequence modified from 5' UTR of RPS23 was used as a non-specific fragment (Supplementary fig 3). As observed before, free probe 2 RNA entirely shifts to a migration pattern characteristic of RNP complexes on titrating with 0.18 μ M - 5.9 μ M MDH2 protein. Addition of MDH2 also shifts the mobility of the non-specific RNA, visibly with the addition of >0.73 μ M MDH2 protein. Here, in contrast to the target probe 2, higher molecular weight complexes do not increase in intensity with increased MDH2 in the binding reaction, saturating out at around 2 μ M (Fig 2.12 B, lanes 19-26). This indicates that MDH2 binding to probe 2 is specific in comparison to the lower RNA binding to non-specific RNA fragment.

K_d values were estimated by quantifying the fraction of bound RNA in the RNP-complex (Fig 2.12 C). The differences observed in MDH2's preference to the RNA fragments tested were reflected in their relative K_d values. Notably, MDH2 binds the 5' tRNA half of Arg TCT 3-2 more efficiently than 3' half hosting the crosslinking sites (K_d 5' Arg TCT 3-2 = $0.948 \pm 0.331 \mu\text{M}$, K_d 3' Arg TCT 3-2 = $4.06 \pm 3.24 \mu\text{M}$). Extensive analysis of different eCLIP datasets (published and unpublished) suggests that eCLIP crosslinking sites on RNA serve as a marker for proximal authentic protein binding sites and may not consistently coincide (Schwarzl et al., 2022c). Thus, MDH2's in-vitro preference for 5' half of Arg TCT 3-2 over the eCLIP enriched 3' fragment may be indicative of true binding site preferences in the cell that needs to be confirmed by other assays.

MDH2 binds the non-specific RNA fragment with $K_d = 2.775 \pm 1.88 \mu\text{M}$, lower than that of the target RNA fragment (probe 2). By including competing non-specific fragments in the binding reactions, specificity estimates for target RNA fragments can be refined in future experiments.

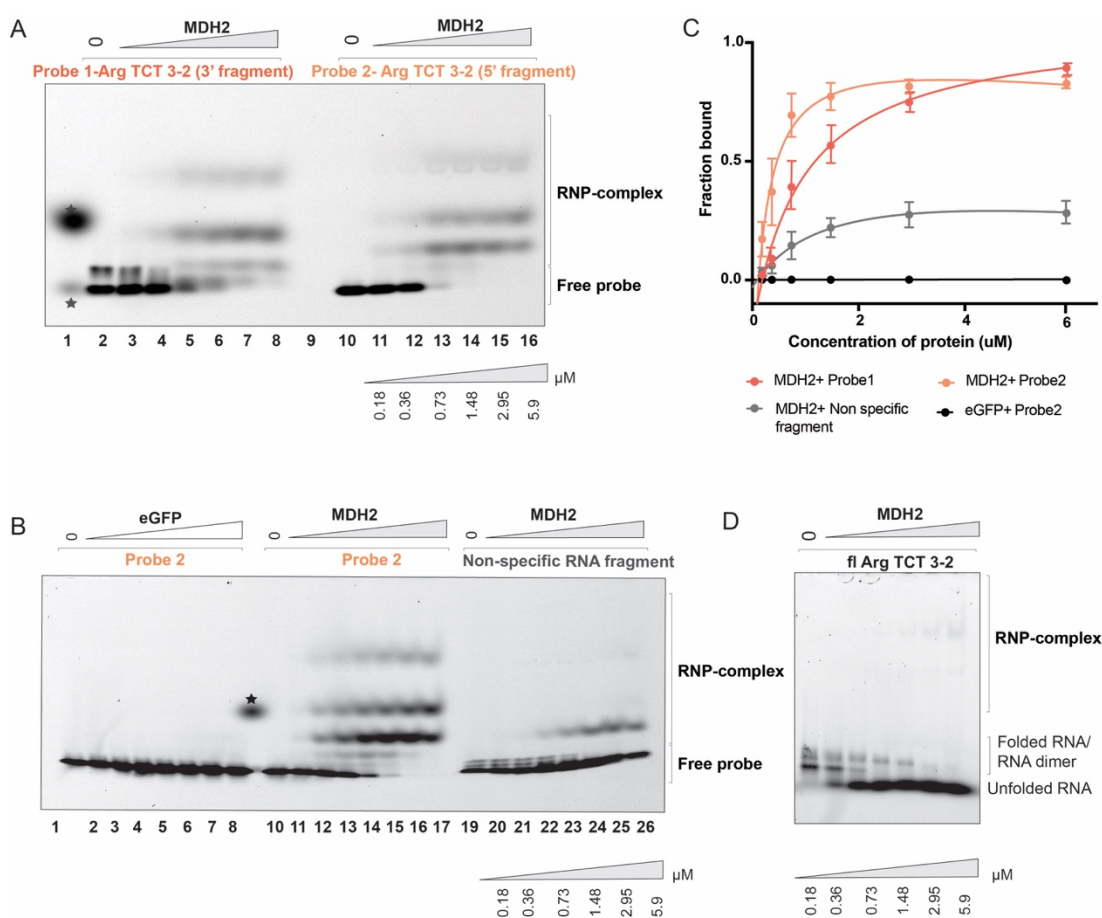


Figure 2.12. In-vitro characterisation of MDH2's RNA binding (A) Representative EMSA assay showing MDH2 binding to Arg TCT 3-2 tRNA fragments (Probe 1 and 2) (B) Representative EMSA assay assaying specificity of MDH2's binding. Binding of eGFP and MDH2 to Arg TCT 3-2 5' fragment and MDH2 with a non specific RNA fragment is shown (C) Binding curves plotted to estimate MDH2's affinity (K_d values) towards the RNA fragments. Data reported as mean $\pm$ S.D. number of experiments, $n=3-5$. (D) EMSA assay showing MDH2 binding with flArg TCT 3-2 tRNA.

Next, I assessed MDH2's interaction with full length Arg TCT 3-2 tRNA. tRNA was in-vitro transcribed incorporating a fluorescein UTP label. The full length tRNA was also bound by MDH2, albeit to a lesser extent than the short RNA fragments (Fig 2.12 D). Curiously, this RNA showed a downward migratory shift in a protein concentration dependent manner. The protein could thus potentially unfold the RNA or reduce intermolecular interactions. Further experiments to understand the structural states of the tRNA and its dependency on the presence of MDH2 can clarify this observation.

In addition, I have validated MDH2's binding to Lys TTT 3-5, another top cytosolic tRNA binder. Lys TTT 3-5 RNA fragment used for the in-vitro assay mapped the 3' end of this tRNA, which contained eCLIP crosslinking sites for MDH2 (Supplementary figure 4).

Taken together, in-vitro assays confirm MDH2's capability to directly bind select cytosolic tRNAs as evidenced by the eCLIP data. This substantiates the possibility that MDH2's RNA binding activity could happen outside the mitochondria.

2.3.5 Subcellular location of MDH2's RNA binding

MDH2 is synthesised as a precursor protein (pre-protein) in cytosol with a mitochondrial targeting signal (MTS). The MTS is cleaved off from the mature protein after import into the mitochondrial matrix (Aziz et al., 1981), the site of TCA cycle. To analyse the subcellular locale of MDH2's RNA binding, I performed comparative PNK assays on whole cell and mitochondrially enriched fractionations (Fig 2.13 A). In line with its well-known localisation in the mitochondrial matrix, we observed higher MDH2 protein and IP signals in the mitochondrially enriched sample (Fig 2.13 B-C). However, the PNK signal intensity from the mitochondrial fraction was much less than the whole cell fraction (Fig 2.13 C-D). This information, in addition to MDH2's preference for cytosolic tRNAs, indicate that MDH2-RNA interactions seem to preferentially occur outside the mitochondria.

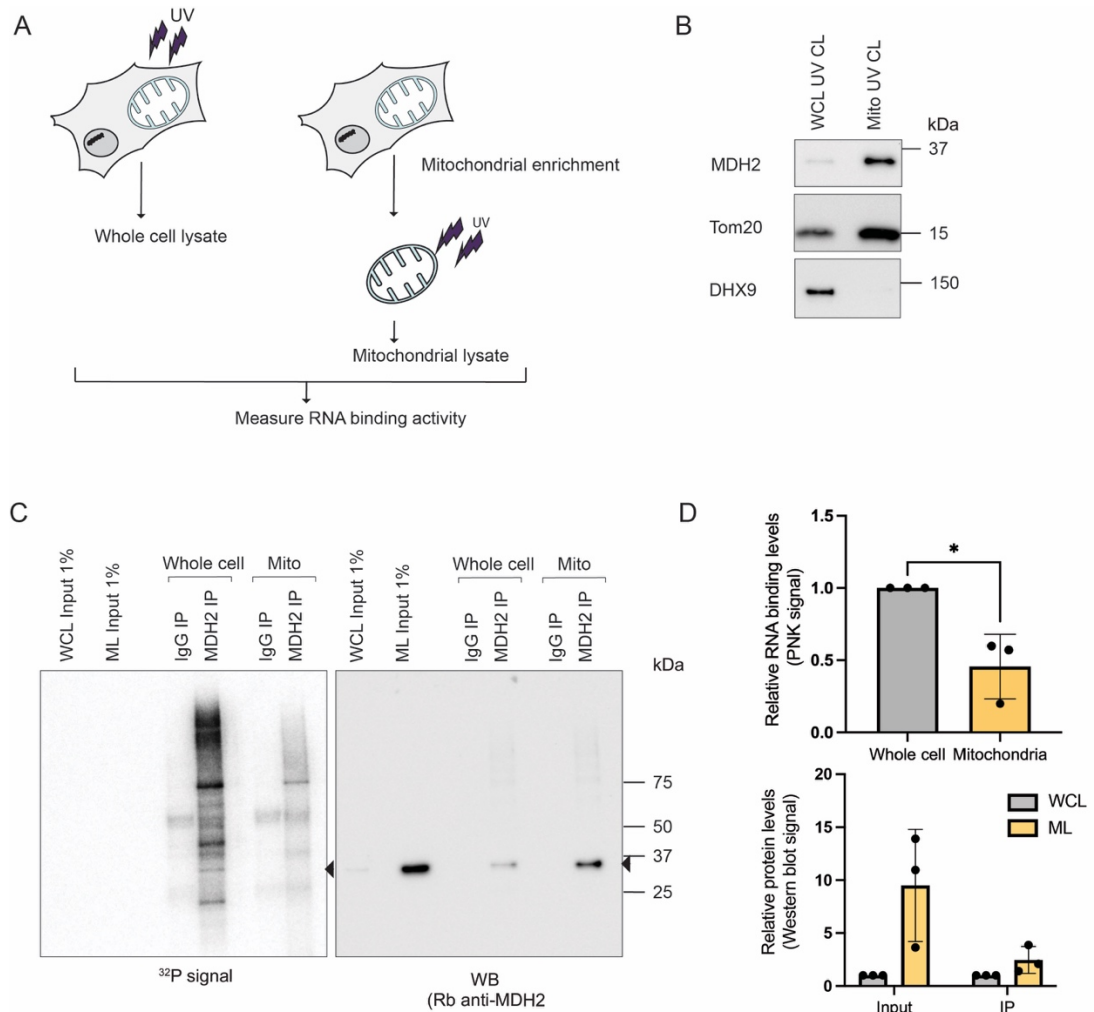


Figure 2.13. MDH2 binds RNA outside of mitochondria. (A) Experimental scheme (B) Western blot showing mitochondrial enrichment (C) PNK assay for MDH2 reveals increased RNA signal from whole cell lysates compared to mitochondrially enriched fractions. Black arrows indicate expected molecular weight of mature MDH2 (D) Quantification of PNK signal and IP efficiency. Data reported as mean $\pm$ S.D. number of experiments, $n=3$, $*=p<0.05$, Unpaired two-tailed t -test.

Enhanced cytosolic RNA binding of MDH2 has several facets that warrant exploration:

- (i) MDH2 exists as a pre-protein outside of the mitochondria. Does MDH2 pre-protein bind RNA with higher affinity than the mature protein?
- (ii) Is mitochondrial targeting required for RNA binding? Even in the cytosol, RNA-MDH2 interactions can be restricted to a specific niche and context. Importance of mitochondrial targeting can clarify if MDH2's RNA associations happen selectively in mitochondrial proximity, potentially linked with the protein import or malate-aspartate shuttle function.

(iii) What is the effect of the metabolic environment on MDH2's RNA binding? The selectively permeable mitochondrial membrane forms a barrier between the cytosol and mitochondria. The resulting distinct biochemical organization of the compartments (Alberts, 2022; Pan et al., 2018; Vo et al., 2004), specifically metabolite concentrations, can determine where MDH2 binds RNA. In the mitochondria, preferential binding of MDH2's substrates/ cofactors/ allosteric regulators might block MDH2's RNA binding interface(s). Furthermore, RNA binding can be impeded by MDH2's selective associations with other mitochondrial metabolic enzymes or itself in case of dimerization.

In the following sections, I examine some of these possibilities.

2.3.6 MDH2's canonical mitochondrial targeting signal is dispensable for RNA binding

The presence of canonical mitochondrial targeting signal (MTS) (1-24 aa) at the N-terminus distinguishes the cytosolic MDH2 pre-protein from the processed MDH2 in mitochondria. This sequence mediates mitochondrial targeting in homologous rat/bovine MDH2 sequences and is conserved in humans, predicting similar import mechanisms (Aziz et al., 1981; Birktoft and Banaszak, 1983; Sztul et al., 1988). To probe the significance of this peptide, I generated a mutant, MDH2 Δ 2-24, lacking the predicted MTS stretch (Fig 2.14 A). This mutant was expected to translate as a potentially cytosolic form of mature MDH2. wtMDH2 and MDH2 Δ 2-24 constructs were cloned with a 3xFLAG,HA tag at the C-terminus. RNA binding was compared with PNK assays after transient transfections. Consistent with the mitochondrial processing of the wtMDH2 to remove the MTS, wtMDH2 and MDH2 Δ 2-24 runs at similar molecular weight in the western blot (Fig 2.14 B). Deletion of canonical MTS (MDH2 Δ 2-24) does not impair MDH2's RNA association (Fig 2.14 B-C), showing PNK signal comparable to wtMDH2. Thus, amino acids 2-24 (predicted MTS) does not determine MDH2's preferential RNA binding in the cytosol.

Next, I investigated the localization of MDH2 Δ 2-24 using confocal immunofluorescence imaging. After overexpression of tagged MDH2 constructs, I co-stained the cells for FLAG tag and Tom70 which marks the OMM (outer mitochondrial membrane). MDH2 Δ 2-24 signal followed the Tom70 staining pattern, indicating mitochondrial targeting (Fig 2.14 D). This unexpected observation raised

the possibility of a buried cryptic targeting signal in MDH2. Importantly, the dispensability of the MDH2 (MDH1 in yeast) N-terminal presequence has been previously described in yeast (Thompson and McAlister-Henn, 1989). Although superfluous for yeast in-cellulo import, the deletion of N-terminal MDH presequence leads to impaired import in-vitro in this study. A follow-up study in yeast (Small and McAlister-Henn, 1997) identified a second cryptic MDH1/2 targeting information next to the N-terminal-presequence, mediating import in the absence of presequence. This redundant MDH targeting mechanism could be conserved in humans, considering the similarity of mitochondrial MDH across species (Supplementary figure 1).

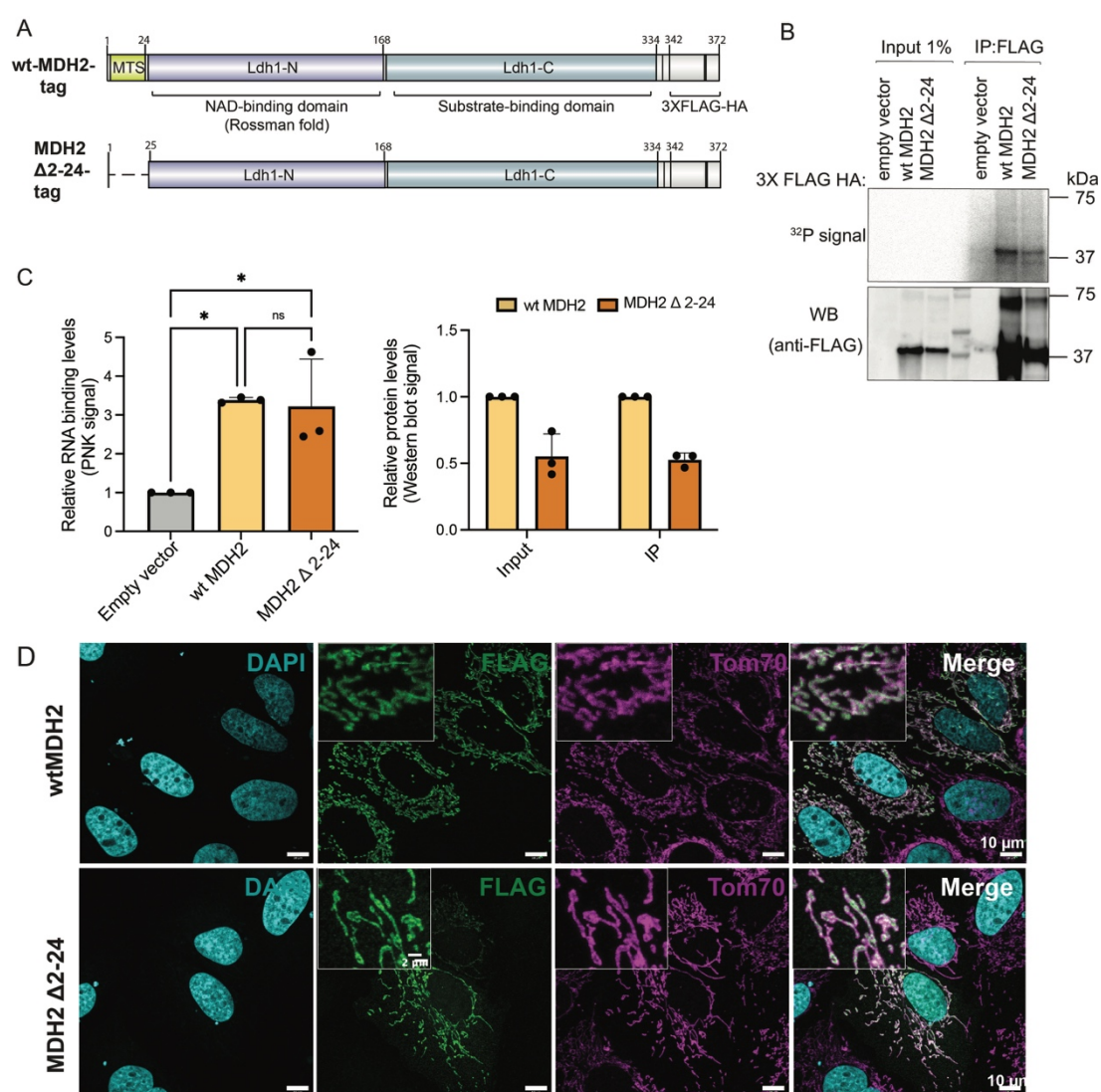


Figure 2.14. Canonical MTS is dispensable for RNA binding. (A) Scheme of constructs (B) PNK assay to compare RNA binding of wtMDH2 and MDH2 Δ2-24 (C) Quantification of PNK signal, protein expression and IP efficiency (D) Confocal immunofluorescence images showing mitochondrial co-localisation of MDH2 Δ2-24. Images are maximum intensity projections of Z- stack images. Data reported as mean ± S.D, number of experiments, n=3, *= $p < 0.05$, Ordinary one way ANOVA with Tukey's multiple comparison test correction.

While the intrinsic effect of canonical MTS on RNA binding is minimal, the question of whether mitochondrial targeting is required for RNA binding still remains open. Assessing the RNA binding of an import-defective version of MDH2 can address this. Towards this, I aimed to find the sequence of MDH2 necessary for import.

2.3.7 Effect of a cryptic mitochondrial targeting signal on MDH2 import

To evaluate potential cryptic MTS in human MDH2, I used two MTS prediction softwares – Mitoprot-II and iMLP (Boos et al., 2018; Claros, 1995). Mitoprot-II predicts the propensity of a N-terminal stretch of amino acids to be targeted to the mitochondria. iMLP instead uses deep learning to predict internal MTS-like signals that aid in import. iMLP software outputs propensity scores for each residue and values above 0 suggest stretches of sequences with iMTS properties. Comparative analysis was performed for wtMDH2 and MDH2 Δ 2-24 sequences with these tools.

Mitoprot-II confirmed the presence of a canonical N-terminal amphipathic alpha helix in wtMDH2. In line with mitochondrial pre-protein processing, peptide cleavage sites for MPP and Oct1 are recognized. For MDH2 Δ 2-24, Mitoprot-II analysis revealed redundant targeting information relevant for mitochondrial import. MDH2 Δ 2-24 has a Tom20 recognition motif (1-5aa) at the N-terminus, followed by an amphipathic helix (13-22) and MPP cleavage site (aa 32) (Fig 2.15 A).

As expected, iMLP software outputs high non-zero propensity scores for the N-terminal amino acid stretch (1-32 aa) of wtMDH2. The N terminus of MDH2 Δ 2-24 (1-10aa) also retains a non-zero propensity to be an MTS-like sequence, albeit lower than that of wtMDH2. In addition, both wtMDH2 and MDH2 Δ 2-24 possess an internal stretch (222-251) which has iMTS-like properties (Fig 2.15 A).

To analyse how these potential MTS stretches may affect import, I created two additional variants of MDH2 (i) MDH2 Δ 2-53, further deleting the predicted second cryptic MTS at N-terminus of MDH2 Δ 2-24 (ii) MDH2 Δ 2-24, 222-251 to study the added effect of iMTS-L (222-251) stretch to assist import (Fig 2.15 B). Immunofluorescence staining of these constructs are shown in Fig 2.16. MDH2 Δ 2-53 shows diffuse cytoplasmic staining suggesting impaired localization to the

mitochondria. MDH2 Δ 2-24, 222-251 shows more erratic localization across cells, co-staining with Tom70 in some cases and diffuse staining in others.

Thus, in the absence of the canonical MTS (1-24 aa), an internal cryptic targeting information contained in aa 24-53 mediates MDH2's mitochondrial import. The cytosolic mislocalization of the MDH2 Δ 2-53 makes it a useful tool to analyse the effect of mitochondrial targeting on MDH2's RNA binding.

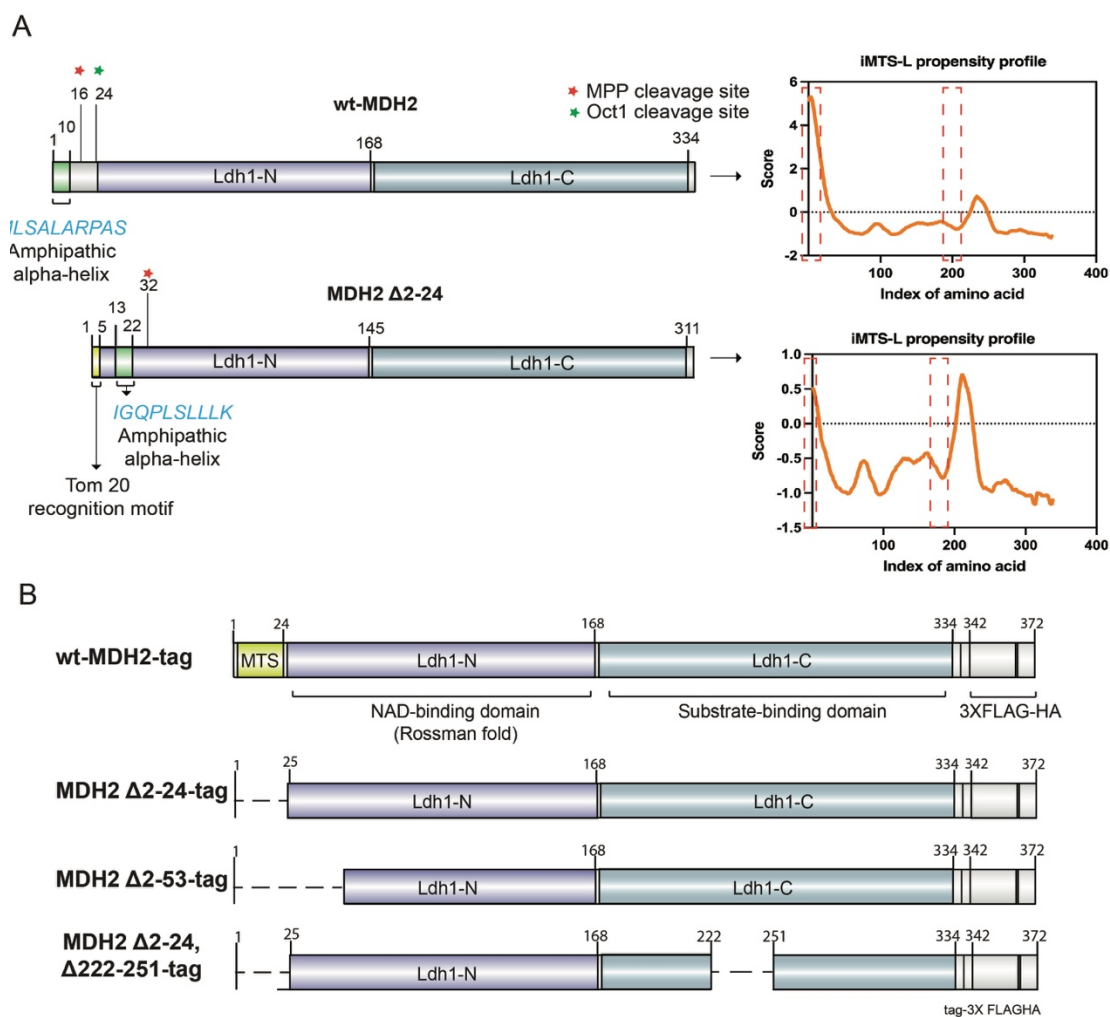


Figure 2.15. Effect of cryptic targeting signal on MDH2's mitochondrial import (A) Analysis of cryptic targeting signals in wtMDH2 and MDH2 Δ 2-24 using Mitoprot-II and iMLP. (B) Design of deletions to probe MDH2 localisation.

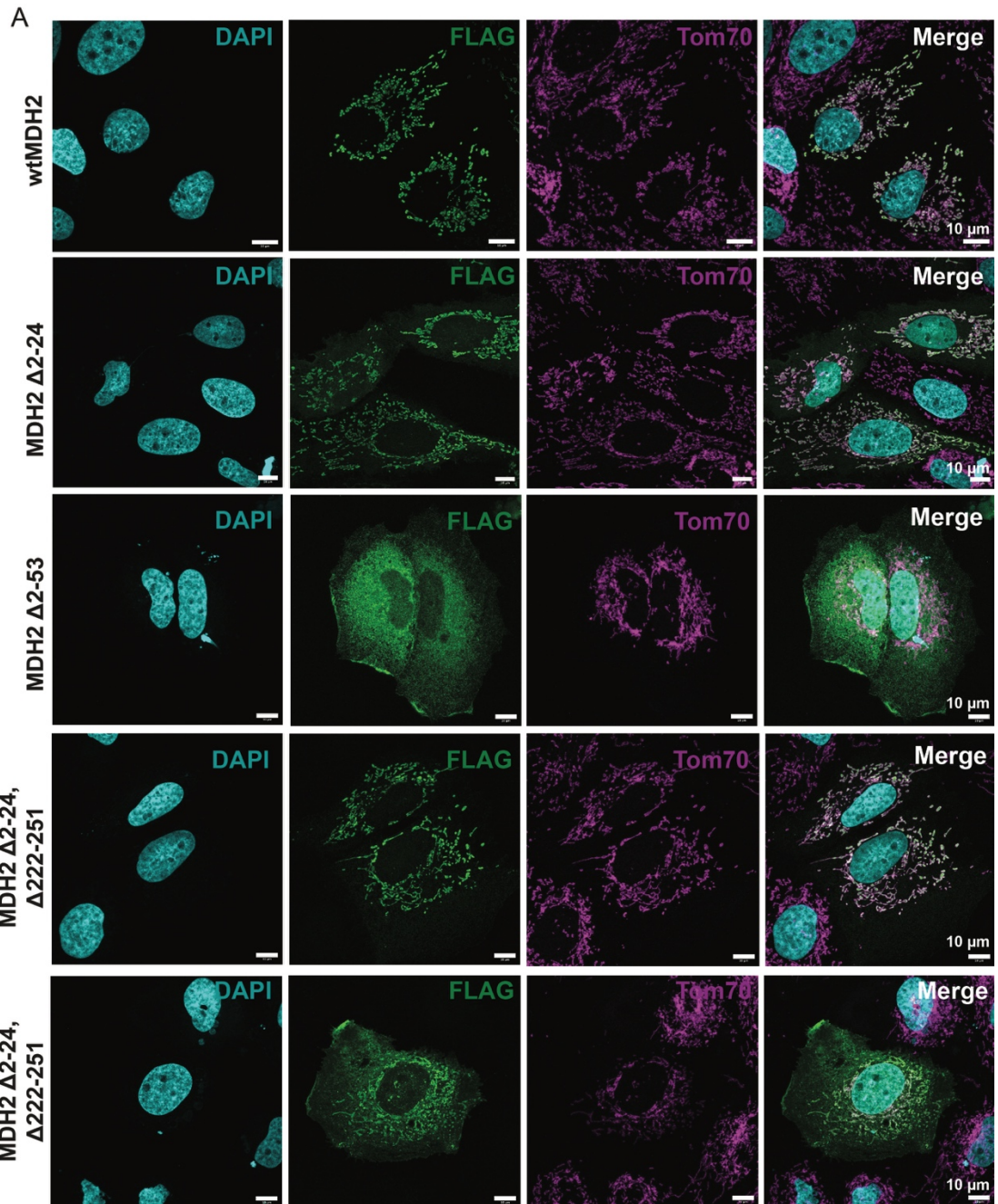


Figure 2.16. *MDH2 Δ 2-53 shows diffuse cytoplasmic staining.* Confocal immunofluorescence images showing localisation of the deletion constructs. MDH2 Δ 2-53 shows cytoplasmic staining in contrast to the wtMDH2 and MDH2 Δ 2-24 which follows the staining pattern of mitochondrial membrane protein, Tom70. MDH2 Δ 2-24, 222-251 shows both mitochondrial and cytoplasmic staining in cells sampled. Images are maximum intensity projections of Z- stack images.

2.3.8 Preliminary results: How cytosolic constriction affects MDH2's RNA binding?

To accurately assess the effect of localization on MDH's RNA binding, I transiently expressed MDH2 $\Delta 2$ -53, MDH2 $\Delta 2$ -24 and wtMDH2 in cells depleted of endogenous MDH2 with specific siRNAs. This prevents MDH2 variants copurifying RNA through interactions with the endogenous MDH2. siRNA knockdown has been standardised as shown in Supplementary figure 5. In the western blot, as described above, wtMDH2 and MDH2 $\Delta 2$ -24 migrates at the same size, a sign of similar pre-protein processing. MDH2 $\Delta 2$ -53 migrates below processed wtMDH2 and MDH2 $\Delta 2$ -24 (Fig 2.17 bottom panel). This indicates that in MDH2 $\Delta 2$ -24, use of internal redundant targeting information next to the canonical MTS (1-24) is not coupled to further mitochondrial processing at the subsequent MPP cleavage site predicted.

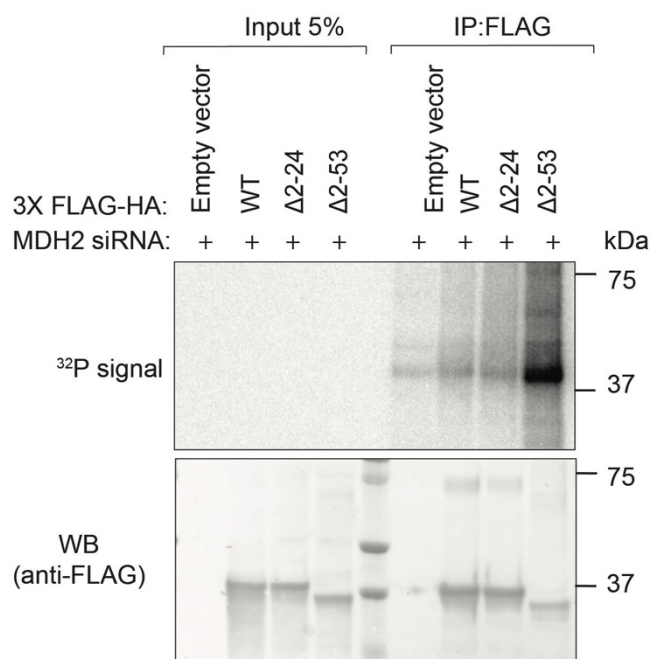


Figure 2.17. PNK assay for mislocalised MDH2. The ³²P signal shows increased intensity for MDH2 $\Delta 2$ -53, which is mislocalised to the cytoplasm. The western blot image is a colorimetric image taken after antibody probing.

MDH2 $\Delta 2$ -53 shows increased signal in the PNK assay than wtMDH2 suggesting that restriction to cytosol can enhance MDH2's RNA binding. However, the lower molecular weight of MDH2 $\Delta 2$ -53 is not reflected in the migration pattern of MDH2 $\Delta 2$ -53 -RNA complex, which is similar to that of wtMDH2-RNA complex (Fig 2.17). This discrepancy warrants further investigation. Although higher bulk PNK signal can indicate enhanced RNA binding, further validation experiments using techniques like RNA-PLA or RIP-qPCRs are needed to confirm this observation.

These preliminary results hint at the dispensability of mitochondrial targeting for MDH2's RNA binding. Furthermore, MDH2 Δ 2-24, lacking the pre-protein-specific peptide sequence shows comparable RNA binding to wtMDH2. Taken together, inherent encoded information in MDH2 may not dictate the subcellular location of RNA binding. As posited above, the metabolic environment of subcellular compartments is an important consideration. In-vitro assays in the following section try to address this aspect.

2.3.9 MDH2's substrates and co-factors impacts MDH2-RNA associations

In the mitochondria, MDH2 preferentially binds to malate and NAD^+ as substrates, forming oxaloacetate (OAA) and NADH (Fig 2.18 A). Though energetically unfavourable, the high concentration of NAD^+ in the mitochondria coupled with the rapid consumption of OAA favour the production of OAA and NADH. At high concentrations, malate and OAA, together with citrate can also allosterically inhibit the backward reaction (Eo et al., 2022; Mullinax et al., 1982a; Zhelev et al., 2022). I used EMSA analysis to dissect the effect of these metabolites on MDH2's RNA binding behaviour. For this, MDH2's binding to the 5' Arg-TCT 3-2 RNA fragment was assessed in the presence of an increasing titration of metabolite concentrations.

Addition of MDH2's metabolic substrates, malate and oxaloacetate, lead to biphasic changes in MDH2's in-vitro RNA binding. Both metabolites facilitated RNA binding till a threshold concentration - 0.5 mM and 1 mM for malate and OAA respectively. At higher concentrations, MDH2 substrates efficiently competed with RNA binding (Fig 2.18 B-C). Malate and OAA bind MDH2 in similar structural conformations which can explain their similar effects on RNA binding. However, oxidized and reduced forms of MDH2's cofactor (NAD^+/NADH) exert disparate effects on RNP complex formation. Similar to MDH2 substrates, lower concentrations of NAD^+ promoted RNA binding. The trend flips at 1 mM NAD^+ leading to gradual dissociation of MDH2-RNA complexes. In contrast, NADH shows comparatively minimal effect on RNA binding (Fig 2.18 D-E).

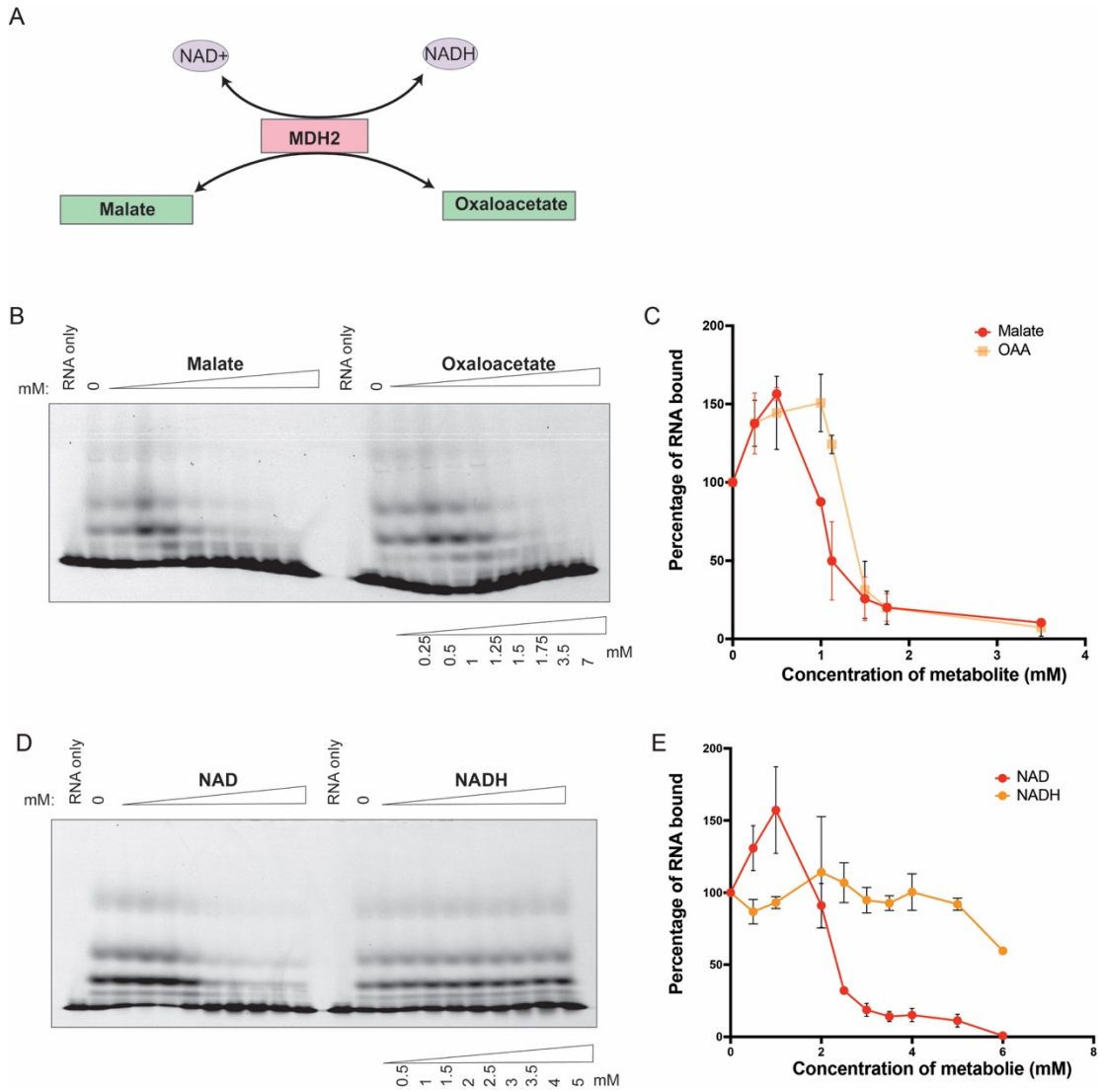


Figure 2.18. MDH2's reactant metabolites regulate RNA binding in-vitro. (A) Scheme of MDH2's reaction with the substrates and co-factors highlighted (B) Representative EMSA for competition of malate and oxaloacetate with MDH2's RNA binding (C) Quantification of the effect of substrates on percentage of RNA bound by MDH2 (D) Representative EMSA for competition of NAD⁺ and NADH with MDH2's RNA binding (E) Quantification of the effect of cofactors on percentage of RNA bound by MDH2. Data reported as mean $\pm$ S.D, number of experiments, $n=3$

In order to put this finding in context, I reviewed metabolite concentrations relevant for MDH2's enzymatic activity. A recent study (Dasika et al., 2015) compared the functional effects of subcellular physiological metabolite concentrations on cytosolic (MDH1) and mitochondrial (MDH2) MDH proteins. Mitochondrial matrix conditions relevant for MDH activity were simulated by [NADH] = 0.8 mM, [NAD] = 5.8 mM, [MAL] = 5 mM, and [OAA] = 0.3 μ M. MDH2 activity dominates over MDH1 activity in these conditions, while cytosolic conditions [NADH] = 0.01 mM, [NAD] = 0.99

mM, [MAL] = 0.5 mM, and [OAA] = 5 μ M favour MDH1 activity. While these estimations may very likely vary from the absolute concentrations in vivo, they can serve as a guide for comparative estimation of metabolite concentrations. This in turn, can help understand how metabolites in the local environment can affect RNA binding. In this case, the cytosolic conditions, specifically for NAD⁺ (0.99 mM) and malate (0.5 mM), align well with the critical concentrations for these metabolites in the EMSA (Fig 2.18 B-E). Specifically, we see that below ~0.5 mM malate and 1 mM NAD⁺, RNA binding is facilitated. Since it is the elevated local concentration of NAD⁺ and malate in the mitochondria that drive MDH2's bioenergetics, they could be the relevant metabolites regulating MDH2's RNA binding function in-cellulo.

Our data indicates that the cytosolic environment may favour MDH2's RNA binding over its metabolic function, where the concentrations of relevant metabolites are not high enough to displace or compete with RNAs that are bound. Rather, at this concentration range, they facilitate RNA binding. In the mitochondria, however, higher concentration of NAD⁺ and malate may inhibit the RNA binding and enhance MDH2's enzymatic activity. Therefore, compartmentalized metabolic concentrations could be one of the determinants of MDH2's preferential cytosolic RNA associations.

2.3.10 RNA binding of MDH2 is responsive to changes in the NAD level in the cell

MDH2's RNA binding could be sensitive to cellular NAD⁺ levels, as indicated by in-vitro EMSAs. To investigate the impact of NAD⁺ on the RNA binding activity of MDH2 in-cellulo, we manipulated cellular NAD(H) levels (Grudzien-Nogalska et al., 2018). The salvage pathway being the major source of NAD⁺ in the cells (Yang and Sauve, 2016), we used a specific inhibitor, FK866, against the rate-limiting enzyme in this pathway, NAMPT (Fig 2.19 A). Treatment with 5 nM FK866 for 48 hours substantially reduced the total cellular NAD(H) content (Fig 2.19 C). To increase NAD levels, we supplemented the cells with NAM, a precursor of the NAD⁺ biosynthesis. NAM treatment resulted in a modest increase in NAD(H) content of the cell after 48 hours (Fig 2.19 A-C).

When NAD⁺ levels are reduced, MDH2 shows increased binding to RNA compared to the steady state. This is in line with the effect of NAD⁺ on MDH2's RNA binding

in-vitro (section 2.3.9). The increase in NAD levels do not significantly alter the RNA binding of MDH2 in the conditions we have tested (Fig 2.19 D-E).

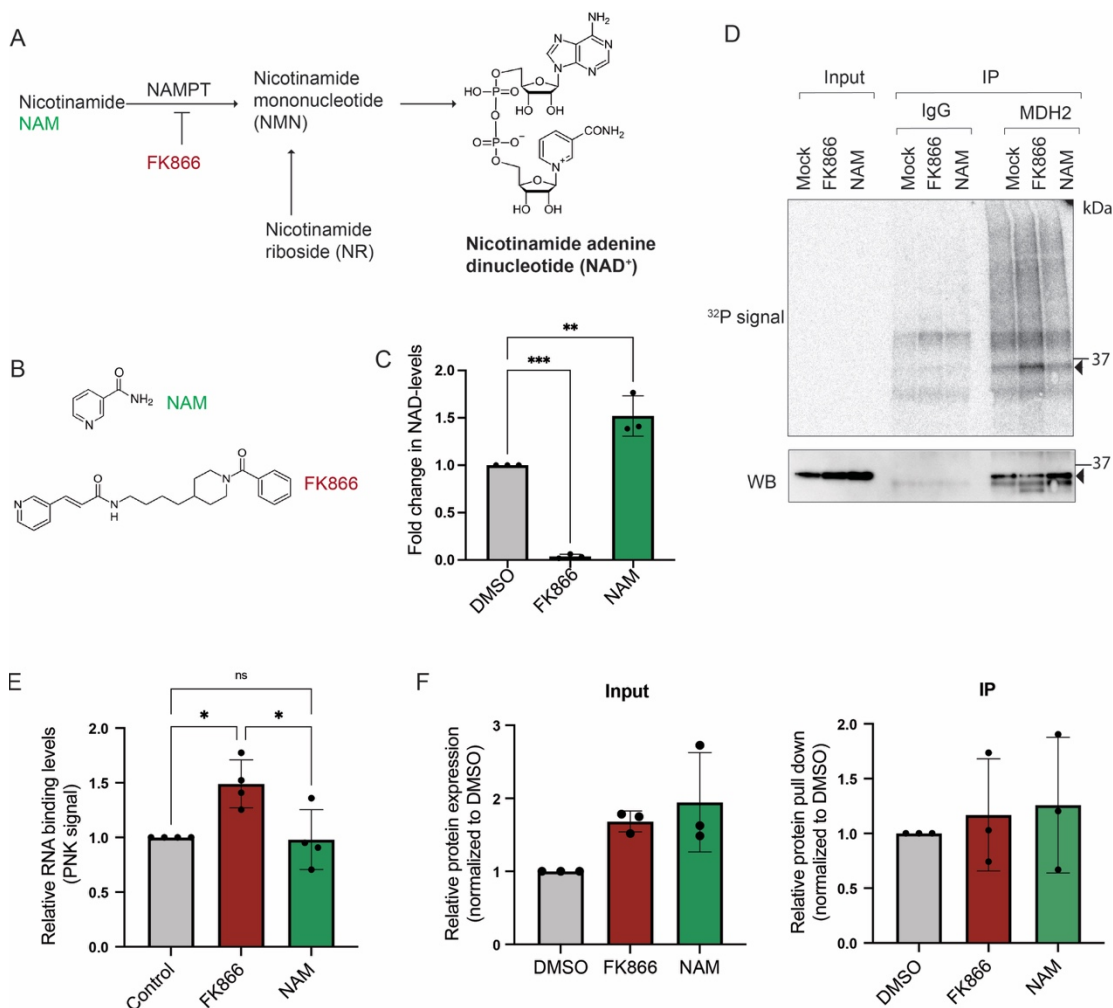


Figure 2.19. NAD modulation alters MDH2's RNA binding levels. (A) NAD salvage synthesis pathway with the targeted steps highlighted. (B) Chemical structure of NAM and FK866 (C) NAD(H) levels measured after 48h of NAM and FK866 treatment (D) Representative PNK assay showing changes in MDH2's RNA binding after NAD modulation. Black arrows indicate expected molecular weight of mature MDH2 (E) Quantification of the changes in MDH2's RNA binding after treatment (F) Quantification of the total MDH2 protein amount used in the PNK assay and amount immunoprecipitated. Data reported as mean $\pm$ S.D, number of experiments, $n=3$, $*=p<0.05$, $**=p<0.01$, $***=p<0.001$, Ordinary one way ANOVA with Tukey's multiple comparison test correction.

As reduction in NAD(H) levels is known to affect cellular OXPHOS, we measured respiratory status of these cells. Basal OCR was elevated on FK866 treatment, possibly as a compensatory mechanism to fuel cellular processes in NAD⁺ limiting conditions (Fig 2.20 A-B). In reduced NAD(H) state, NAD(H) independent complex-II mediated respiration could drive the compensatory increase in basal OCR. The spare and

maximum respiratory capacity is severely diminished underlining the decreased ETC capacity when NAD(H) levels are down (Fig 2.20 B). The spare respiratory capacity is a measure of a cell's reserve energetic potential to respond to stress. Here, cellular adaptation to the stress of reduced NAD(H) levels might explain the reduction in the respiratory capacity. Although here we haven't analysed the TCA flux specifically, reduced metabolite flow into TCA and mitochondrial dehydrogenase activity has been observed on FK866 treatment (Hasmann and Schemainda, 2003; Oyarzun et al., 2015; Tan et al., 2015).

Taken together, reduction in cellular NAD⁺ -MDH2's cofactor- levels lead to altered mitochondrial respiration which is coupled with an increase in MDH2-RNA interactions.

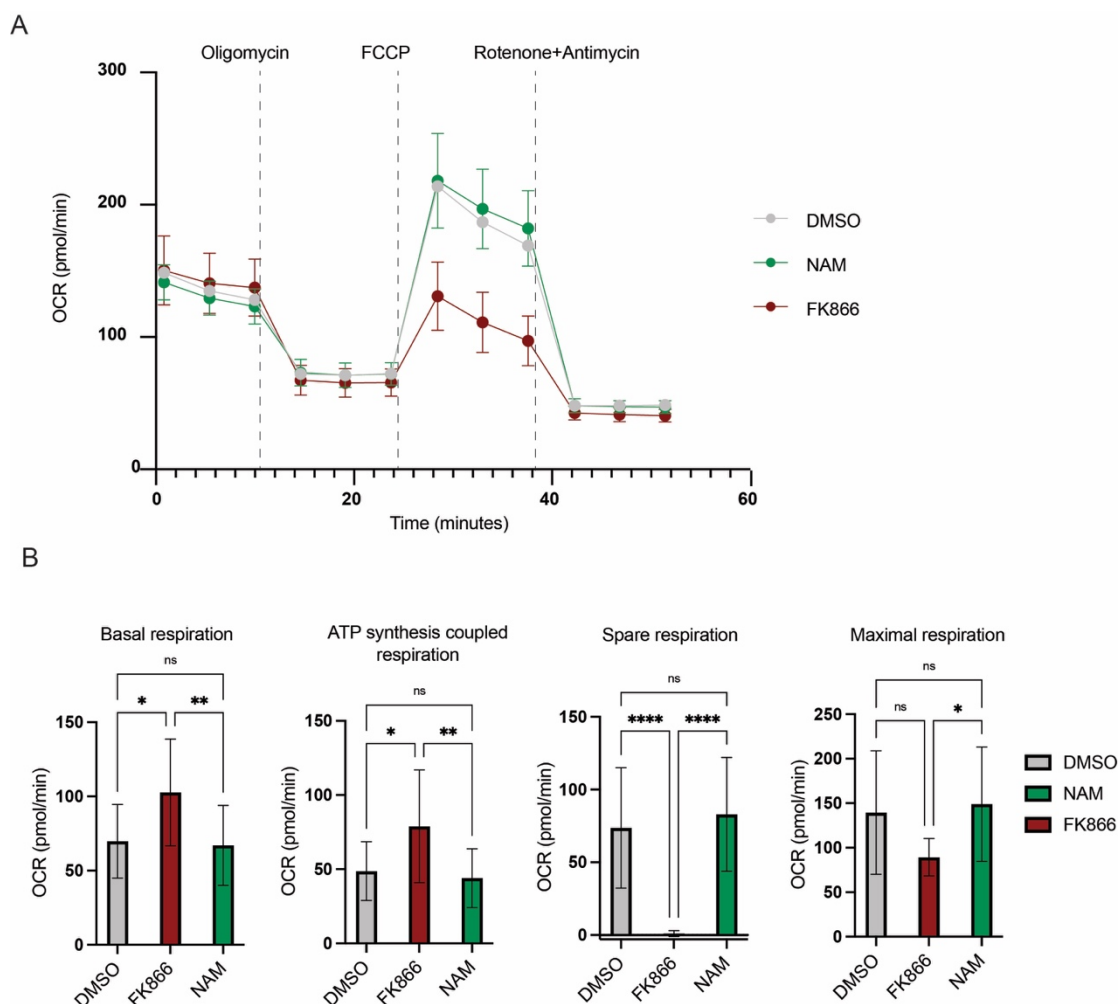


Figure 2.20. NAD modulation impacts cellular respiration. (A) Mitochondrial respiration measured after 48h of NAM/FK866 treatment (B) Basal, ATP-coupled, spare and maximal respiration calculated for of NAM/FK866 treated cells. Data reported as mean $\pm$ S.D, number

of experiments, $n=3$, $*=p<0.05$, $**=p<0.01$, $****=p<0.0001$,. Ordinary one way ANOVA with Tukey's multiple comparison test correction.

2.3.11 RNA binding of MDH2 is sensitive to cellular state with altered metabolic fluxes

To deconvolute the effects of cellular metabolic state on MDH2's RNA binding, we sought to explore a more physiological system with well-defined changes in TCA cycle and respiratory usage. Hepatocarcinoma cells (HCCs) (like Huh7 used in this study) show an altered metabolic usage from primary hepatocytes, with a shift to increased use of glycolysis. In addition, TCA metabolic rewiring in HCCs fuel biosynthetic processes required to maintain cell proliferation (Tenen et al., 2021; Todisco et al., 2019). Culturing Huh7 cells in human serum, restores a hepatocyte-like metabolic profile (Steenbergen et al., 2018). I assayed MDH2's RNA binding function in this more physiological system. For this, I cultured Huh7 (human liver cancer line) cells in 2% human serum (HS). Cells cultured in HS (>23 days) differentiate to be more hepatocyte-like. These cells exhibit increased respiration, reduced glycolytic usage, enhanced lipid and glycogen storage as well as increased ketogenesis/glycogenesis (Fig 2.21 A). After HS adaption for >40 days, we independently confirmed the increased mitochondrial bioenergetics by measuring OCR (Fig 2.21 B-C).

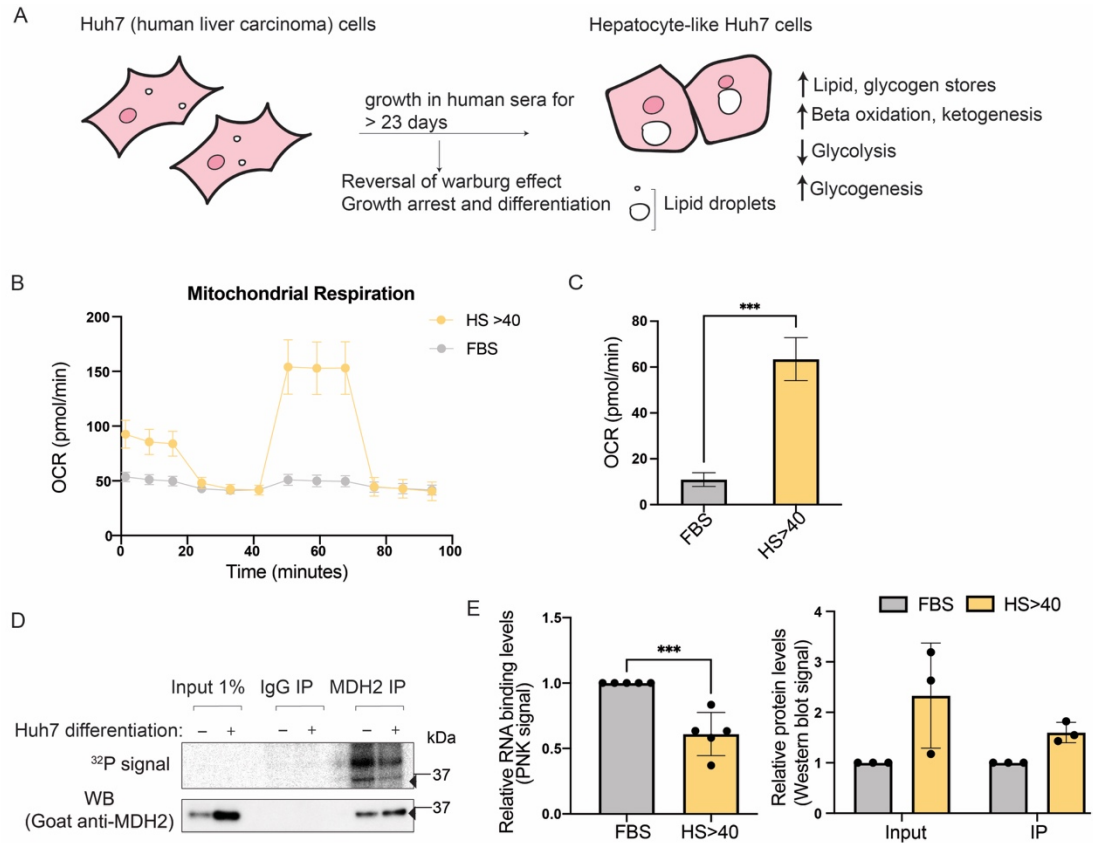


Figure 2.21. MDH2's RNA binding changes in cellular states with altered mitochondrial bioenergetics (A) Metabolic and phenotypic changes on differentiation of Huh7 to be more hepatocyte-like by adaptive growth in HS (B) Mitochondrial respiration in Huh7 cells adapted to HS >40 days in comparison to those grown in FBS (C) Quantification of the increased basal respiration rate in Huh7 cells. Data reported as mean $\pm$ SEM, number of experiments, $n=3$, ***= $p<0.001$, unpaired t -test (D) Representative PNK assay showing RNA binding changes in HS adapted Huh7 cells. (E) Quantification of the PNK assay and relative MDH2 protein amounts used in the assay. Data reported as mean $\pm$ S.D, number of experiments, $n=3$, ***= $p<0.001$, unpaired t -test.

Comparison of MDH2's RNA binding ability reveals a reduction in the hepatocyte-like state, even as the total MDH2 protein levels go up (Fig 2.21 D-E). This indicates that MDH2-RNA interaction is an active process depending on cellular metabolic state/ glycolytic flux instead of being a passive outcome of altered MDH2 levels.

Overall, we find that RNA binding of MDH2 is sensitive to cellular metabolic state. These point to the idea of a coordinated regulation of its function in the mitochondria and its RNA binding in the cytosol. RNA-binding thus establishes itself as an actively regulated facet of MDH2's cellular function rather than a passive process. The outcomes of this interaction are still to be defined, including effects on both MDH2 and the bound RNA. We need more precise tools to interrogate this aspect, for

example, an RNA-binding deficient version of MDH2 that can be employed to extract downstream consequences of impaired RNA binding. In the following section, I describe our efforts in this direction.

2.3.12 Mode of MDH2 binding to RNA

To have a mechanistic understanding of the MDH2-RNA interaction, we wanted to investigate the MDH2 residues critical for RNA binding. I first compiled putative RNA binding peptides of MDH2, informed by available proteomic RBD-map data sets (Castello et al., 2016; He et al., 2016; Horos et al., 2019; Liao et al., 2016) (Fig 2.22 A). These studies pointed to an extended region in MDH2 (52-104 aa) that comprised a consensus RNA binding peptide in the N-terminal domain of MDH2. When mapped onto the available 3D structure of human MDH2 (PDB ID- 2DFD), the RNA binding region was spatially proximal to the NAD binding pocket (Fig 2.22 B). Potential RNA binding residues in this peptide were selected for testing, based on the following criteria: (i) Solvent accessibility; (ii) amino acid polarity and (iii) theoretical predictions based on the observed frequency of various amino acids at RNA-protein contact sites (Terribilini et al., 2006). Based on this, four residues: H70, K74, K78, K105 were selected for downstream mutational studies (Fig 2.22 B). These residues were substituted with alanine, to remove the specific side chains without disrupting the protein backbone conformation. H70 and K74 mutations were made in the same construct. The mutated sequences were cloned into a pCDNA3.1 vector under the control of a CMV promoter and modified to have a 3xFLAG,HA tag at the C-terminal of the protein. In addition, these constructs were made resistant to siRNA knockdown using synonymous mutations.

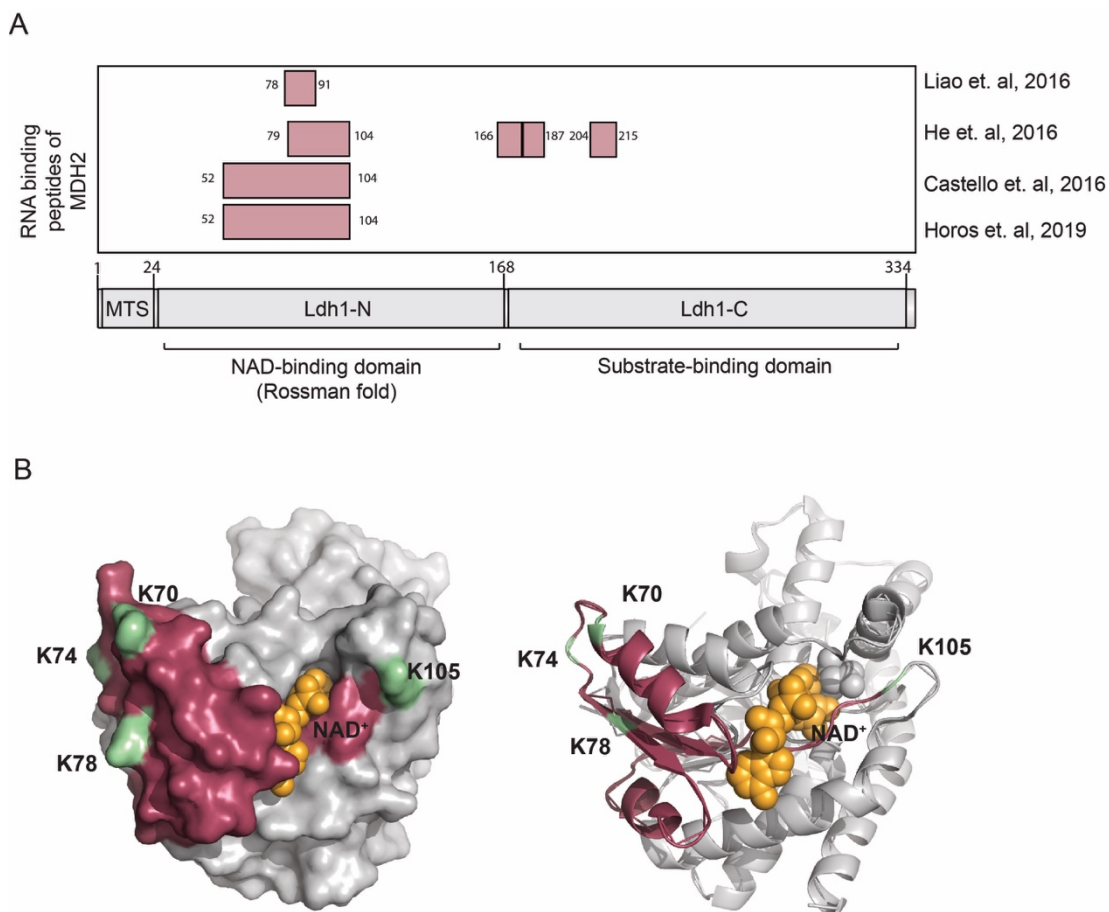


Figure 2.22. RNA binding regions of MDH2 mapped by RBD map (A) RNA binding peptides identified by RBD map for MDH2 mapped on to the MDH2 protein domains (B) Extended 52-104 amino acid region of MDH2 identified by RBDmap highlighted (red) on the 3D structure of MDH2 (PDB:2DFD) and the residues selected for mutational analysis highlighted in green. Structures were visualised in PyMOL.

To functionally characterize MDH2 mutants, I expressed the protein constructs in Huh7 cells with transient transfections. The mutants have comparable expression levels to the wildtype MDH2 (Fig 2.23 A, input). Since MDH2 needs to dimerize to be functionally active, we probed the interaction of the exogenous tagged versions of MDH2 with endogenous MDH2. Co-immunoprecipitation experiments show that MDH2 mutants pull down endogenous MDH2 comparably to wtMDH2. (Fig 2.23 A).

To further probe the functionality of MDH2 mutants, we measured their enzymatic activity. I set up an assay to measure the MDH2 mediated reduction of oxaloacetate to malate utilizing NADH. The reaction progress is measured by monitoring the decrease in the NADH absorbance (340 nm). This assay has been optimised to work with purified recombinant MDH2 as well proteins immunoprecipitated after overexpression

in Huh7 cells (Supplementary figure 6). The enzymatic activity assays revealed similar levels of functional activity for the MDH2 mutants and wtMDH2 (Fig 2.23 B).

Next, I assayed the RNA binding activity of the MDH2 variants after transient transfections with PNK assays. The mutants bind RNA to similar levels as the wtMDH2. The interaction of MDH2 variants with endogenous MDH2 (Fig 2.23 A) can mediate this positive RNA binding independent of their inherent binding ability. To dissect out the RNA binding contribution of endogenous protein from the transgene and decrease the possibility of transfection associated variability, I went on to generate doxycycline (DOX) inducible cell lines stably expressing the MDH2 variants. In these cell lines, I depleted endogenous MDH2 with siRNA and then induced transgene expression with 100 nM doxycycline. PNK assays in this setup revealed that the variants still bind RNA (Fig 2.23 D). The expression of wtMDH2 was lower than MDH2 variants in the inducible cell lines which complicated quantitative comparisons. Given that transient transfections with comparable protein expression of wtMDH2 and variants also confirmed the RNA binding ability of the mutants, I didn't pursue further experiments with the cell lines.

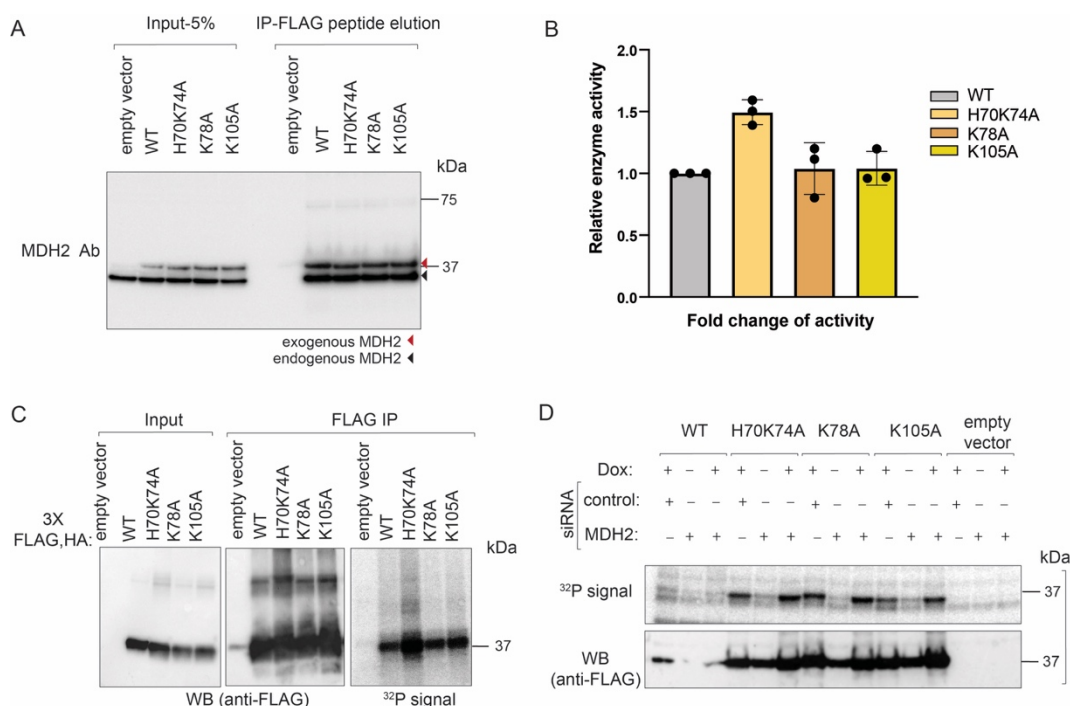


Figure 2.23. Characterisation of RNA binding mutants. (A) MDH2 mutants can interact with endogenous wtMDH2 as seen in co-immunoprecipitation assay (B) MDH2 mutants are enzymatically active (C) Transient transfection of MDH2 mutants followed by PNK assay (D) PNK assay after endogenous MDH2 knockdown in cells inducibly overexpressing MDH2 mutants. Data reported as mean $\pm$ S.D, number of experiments, $n=3$.

It is possible that these amino acid residues in isolation are not determinants of RNA binding. They might act in concert with other amino acids along the putative RNA-binding peptide and additional mutations could uncover these residues. Furthermore, substitutions to alanine at these positions might not be enough to disrupt the MDH2-RNA interface. Asparagine, glutamine, aspartic acid, and glutamic acid substitutions could have a stronger impact on the RNA-binding surface and hence be more revealing (Gray et al., 2017). After we have performed these experiments, new data has come out exploring new ways of detecting RNA binding peptides (Bae et al., 2020). For MDH2, this data points to possible RNA binding residues along the C-terminal substrate binding domain of the protein, which can also be targeted for further analysis.

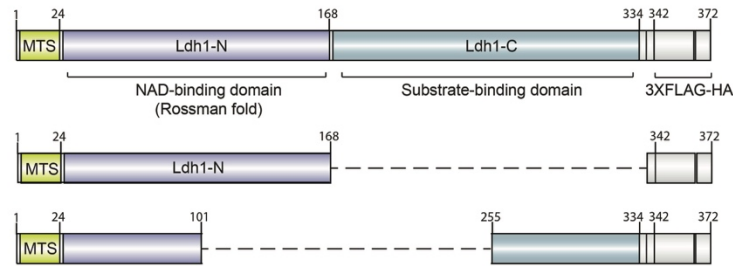
2.3.13 Preliminary experiments towards identifying MDH2's RNA binding region

To have a broader-scale understanding of MDH2's RNA binding function and to guide further targeted mutations, I undertook domain level mutational analysis. Given the significant impact of NAD⁺ on MDH2's RNA binding and the proximity of the MDH2's RBD peptide to its NAD binding pocket, the NAD-binding N-terminal domain (aa 1-168) seems most likely to mediate RNA binding. In favour of this hypothesis, several dehydrogenases like GAPDH and LDH bind RNA through their NAD⁺ binding Rossmann-Fold. To check if this domain in isolation is sufficient to bind RNA, I cloned the truncated MDH2 1-168 aa into pCDNA3.1 vector with a C-terminal 3x-FLAG,HA tag. In addition, I created a MDH2 Δ 101-255 construct to probe the importance of MDH2's enzymatic active site in RNA binding (scheme of constructs in Fig 2.24 A). The deleted stretch in MDH2 Δ 101-255 contains: (i) several key residues that mediate MDH2's interaction with malate (Asn124, His182, and Arg186 and Arg192) (Ugochukwu et al., 2006) (ii) a flexible loop that changes conformation on substrate/cofactor binding (Goward and Nicholls, 1994; McCue and Finzel, 2022) and (iii) the His-Asp (aa 200, 173) pair (Eo et al., 2022) involved in catalysis.

Immunofluorescence images after overexpression of these MDH2 constructs show co-staining with Tom70, indicating mitochondrial localisation (Fig 2.24 B). At present, I am optimizing RNA binding assays from these MDH2 domain deletion constructs.

These results can broadly inform further targeted mutational analysis to characterize MDH2's RNA binding.

A



B

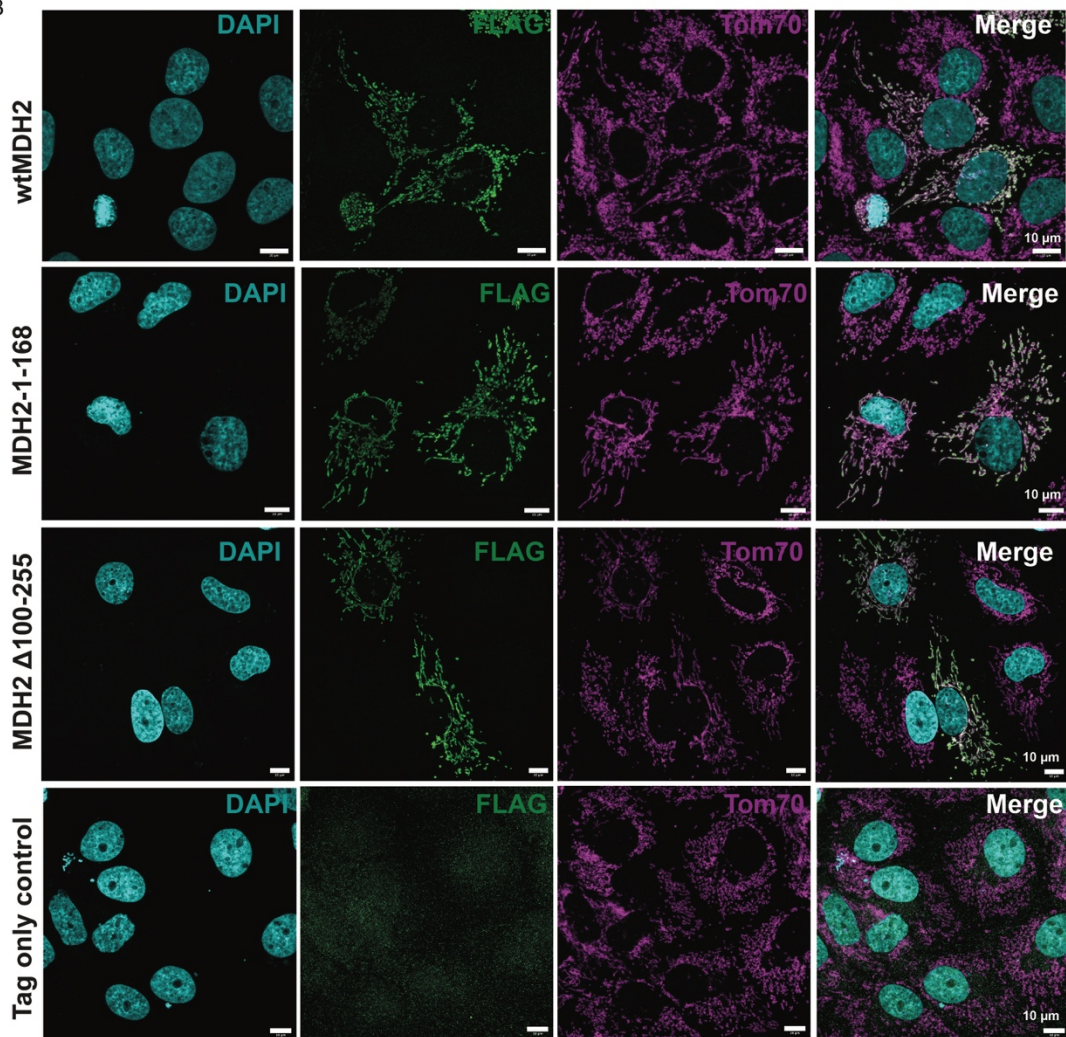


Figure 2.24. Characterisation of MDH2 domain truncations (A) Scheme of the MDH2 deletion constructs (B) Immunofluorescence panel showing localistaion of the MDH2 deletion constructs.

2.4 Discussion

In this part of my thesis, I investigated the RNA binding activity of MDH2 with regard to the RNA targets bound, the subcellular location of binding and factors/conditions that can affect MDH2's RNA binding. MDH2 binds cytosolic RNAs, including tRNAs predominantly outside the mitochondria. In-vitro experiments show that MDH2's RNA binding is sensitive to its co-factor NAD^+ and its substrates, malate and oxaloacetate. The sensitivity of MDH2's RNA binding to NAD (H) levels is also reflected in cellular experiments. In hepatocyte-like differentiated cells with altered mitochondrial respiration, MDH2's RNA binding activity is reduced- indicating interconnections between metabolic state and MDH2's RNA binding.

2.4.1 MDH2 preferentially binds cytosolic RNAs, specifically tRNAs

eCLIP analysis shows that MDH2 binds cytosolic RNA predominantly. The binding preference for cytosolic tRNAs in particular has several aspects that are open to further investigation. First, we need to untangle MDH2's bias for tRNA fragments (tRFs) vs full length tRNAs. In-vitro data points to a preference for the fragments which needs to be investigated in-vivo. Second, if MDH2 does indeed bind to full length tRNAs, aminoacylation status can be a discriminatory feature. If MDH2 can distinguish between the charged and uncharged versions of the tRNAs, this can be a potential mechanism for nutrient sensing that would define mitochondrial metabolism. In-vitro assays can clarify this preference. In addition, selective amino acid starvations can shed light on this by monitoring relative RNA binding in these conditions and subsequent analysis of MDH2's mitochondrial function.

What is the functional consequence of MDH2-RNA interactions? Several regulatory outcomes either on the protein or RNA can be envisaged, which might not be mutually exclusive.

(i) Given the diverse set of protein coding RNAs in MDH2's binding pool, MDH2 can have a hitherto unexplored role in the cytoplasm, controlling mRNA expression. There is precedence for alternate or moonlighting functions of metabolic enzymes. Glycolytic enzymes GAPDH and cytosolic aconitase (IRP1) bind mRNA UTRs (5' and 3') to regulate expression in addition to their metabolic functions.

(ii) Abundant RNAs, tRNAs in particular, might regulate MDH2's mitochondrial function depending on the cellular state and needs. For example, tRNA engagement can restrict MDH2's availability for mitochondrial function. Depending on MDH2's binding preference, the aminoacylation status or altered abundance of tRNAs in different conditions can thus exert modulatory effects. tRF/tRNA levels are regulated by physiological changes that require metabolic rewiring, such as in differentiation and cancer (Zeng et al., 2020a).

(iii) A scenario where (i) and (ii) could be coordinated can also be speculated. tRNA mediated sequestration can regulate MDH2's alternate function, as has been shown for YBX1. YBX1, a canonical RBP, binds tRFs, which sequester and thus prevent YBX1 from binding to and stabilising other-pro metastatic RNAs (Goodarzi et al., 2015).

These points regarding functional consequences remain speculative at present and await further experimental analysis for a mechanistic understanding of MDH2-RNA interactions.

2.4.2 MDH2's RNA binding typically occurs outside of the mitochondria

Fractionation assays confirm that the bulk of the RNA binding pool of MDH2 is outside the mitochondria. Since mature MDH2 is a predominantly mitochondrial matrix protein, compartment specificity of RNA association implies active cellular regulation. MDH2's N-terminal MTS, a distinguishing feature between the cytosolic pre-protein and mitochondrial mature form, does not determine selective RNA binding in the cytosol. Mitochondrial targeting may be dispensable for MDH2-RNA interactions (preliminary data), precluding selective RNA association only on the mitochondrial surface or vicinity where MDH2 might be locally concentrated.

We find that the metabolic environment in the different compartments can instruct MDH2's RNA association. We observed that presence of MDH2's specific substrates (malate/OAA) and cofactor (NAD^+) promotes MDH2-RNA associations at (lower) concentrations mimicking the cytosolic conditions in-vitro. At higher concentrations similar to the mitochondrial environment, RNA-MDH2 interaction is impaired. The effect of NAD^+ contrasts with the minimal effect of NADH at saturating concentrations. Competition of NAD^+ and substrates at higher concentrations may be indicative of overlapping binding sites for these metabolites and RNA. The mechanism for increased MDH2-RNA association at lower concentrations of metabolites needs to

be further explored. One possible explanation is that when their binding sites are occupied by RNA, metabolites bind to alternate allosteric sites that stabilize the RNA bound conformation.

MDH2's enzymatic mechanism involves conformational changes induced by sequential binding of the cofactor (NAD⁺/NADH) and the substrate (malate/OAA) (Eo et al., 2022; Minarik et al., 2002; Xu and Li, 2016). Therefore, MDH2 conformations where substrates/cofactors are independently bound can differ from those induced by their cooperative binding or the unoccupied conformation of the protein. Profiling changes in MDH2's RNA interaction in the presence of substrate/cofactor pairs (malate+NAD⁺/ OAA+NADH) can reveal more mechanistic details of this regulation. Furthermore, NAD⁺/NADH redox ratio, rather than absolute levels of NAD⁺ and/or NADH can dictate MDH2's RNA-binding dynamics in-vivo, avenues which need to be explored further.

In addition to direct regulation by metabolite concentrations, MDH2's dimerization and functional association with mitochondrial enzymes in-vivo can influence the context of its RNA binding. MDH2 forms a “metabolon” with fumarate hydratase and citrate synthase that increases the TCA flux in the direction of citrate synthesis (Bulutoglu et al., 2016; Morgunov and Srere, 1998; Zhang et al., 2017). If same or structurally close peptides are involved in RNA and protein association(s), the latter might be preferred in the mitochondrial context, thus precluding RNA binding.

Finally, there could be specific cytosolic factors that actively facilitate the MDH2-RNA association. Such factors can potentially also contribute to the compartment specific RNA binding displayed by MDH2. Taken together, the cytosolic environment may be conducive for MDH2's RNA association, while the mitochondrial environment favours MDH2's enzymatic activity.

2.4.3 MDH2 RNA binding is sensitive to altered mitochondrial bioenergetics

Regulation of the TCA cycle is complex. In addition to tight regulation with OXPHOS, TCA cycle can function independently of OXPHOS for anaplerotic functions depending on cell biosynthetic demand. MDH2's function in the TCA needs to be thus modulated in response to physiological changes that dictate metabolic reconfiguration. We discovered that cellular metabolic changes that affect MDH2's metabolic function also regulate MDH2-RNA interactions. Reduction in cellular NAD⁺ level elevates

basal respiration with concomitant decrease in maximal respiratory capacity. MDH2's RNA association is enhanced under these conditions. Cellular NAD level modulation can have multi-pronged effects on MDH2's RNA binding. As NAD^+ competes with RNA binding, the reduction in NAD levels can relieve an NAD^+ dependent inhibition on RNA binding. Prolonged FK866 treatment has been shown to decrease NAD^+ across all compartments- nucleus, cytoplasm and mitochondria (Chen et al., 2022). Under the assumption that mitochondrial NAD^+ levels are also reduced in our system, mitochondrial MDH2 pool could potentially bind mitochondrial RNA targets resulting in the observed increase in PNK signal.

The changes in mitochondrial bioenergetics on NAD^+ depletion could also exert a regulatory effect on MDH2's RNA binding. Protein import into the mitochondria is provisional to the energetic needs of the mitochondria (Tsuboi et al., 2020). If NAD^+ reduction diminishes the NAD^+ dependent steps of the TCA including that of MDH2, mitochondrial levels of MDH2 would potentially need to be recalibrated. Increase in MDH2's RNA binding on NAD^+ depletion can be part of this modulatory response, either as an active potentiator or a consequence of the modulation. In our experiments, we lack information about the regulatory effects directly on TCA. As basal OCR might not be a reliable indicator of the flux through different TCA arms (Schmidt et al., 2021), metabolic flux analysis can shed light on the changes in TCA. Insight on how NAD^+ modulation affects the compartmentalized pools of MDH2 is also lacking. Further experiments, for example, subcellular fractionation assays coupled with FK866 treatment can define significant changes in MDH2's cellular distribution. This would tell us if the increased RNA binding is contributed by elevated levels of MDH2 in cytosol on reduced cellular NAD^+ levels.

It is important to note that NAD^+ is also an important cofactor of a critical class of cellular deacetylases -sirtuins. Several mitochondrial enzymes including MDH2, are regulated by mitochondrial sirtuins (SIRT3/4/5) dependent deacetylation (Marcus and Andrabi, 2018; Parodi-Rullan et al., 2018). Thus, NAD^+ dependent effects on RNA binding can be due to direct alteration in cofactor levels or indirect effect of MDH2 acetylation.

We found that more physiological changes like cell differentiation affects MDH2-RNA association. Huh7 cells differentiated into hepatocyte-like state show increased respiration driven by glutaminolysis and substantial increase in beta oxidation. These

changes have also been shown to increase the TCA flux (Audano et al., 2019; Satapati et al., 2012). In hepatocyte-like cells with increased OXPHOS and TCA flux, MDH2 binds less RNA than in the more proliferative and glycolytic FBS-cultured cells. The shift to OXPHOS is accompanied by an increased abundance and enzymatic function of MDH2 in the mitochondria. This implies an inversely correlative relationship between MDH2's RNA binding function in the cytosol and enzymatic function in the mitochondria. This differential binding can be affected by: (i) altered cellular redox ratio and concentration of metabolites (ii) differential abundance of the tRNA/tRFs during cell differentiation/ cancer. This is a likely possibility as MDH2 bound tRNA isotypes are reported to be associated with pro-proliferation states (Gingold et al., 2014). (iii) Differential abundance of hitherto unexplored cytosolic factors that mediate RNA binding. As discussed in 2.4.1, the inverse correlation between MDH2's RNA binding and enzymatic functions can indicate a regulatory function of RNA on MDH2 and/or an alternate cytosolic function for MDH2 when the need for its primary enzymatic function is low.

Chapter 3: RNA binding activity of ATP5A1

3.1 Introduction to ATP5A1

ATP production in the mitochondria is vital for the survival/metabolic health of the cell. The F₁-F_o ATP synthase complex/Complex V/F-ATPase is a mitochondrial protein complex that synthesizes the majority of ATP in the cell, channeling the proton gradient across the mitochondrial membrane to a rotary motion of the complex (Neupane et al., 2019; Yoshida et al., 2001). It is composed of the F₁ subunit in the inner mitochondrial matrix and F_o subunit in the inner mitochondrial membrane. These subunits are connected by an asymmetric central and elongated peripheral stalk. The peripheral stalk connecting with the F₁ domain forms the stator part of the complex, whereas the central stalk with the F_o domain makes up the rotor (Fig 3.1). F₁ comprises of three copies of each of subunits α and β , and one each of subunits γ , δ and ϵ . The catalytic interface is between the α and β , both of which have the ability to bind ADP/ATP in a Mg²⁺ dependent manner (Ebanks et al., 2020). The F_o region has a central c-ring (made of 8 subunits in humans) which forms the proton conducting channel together with ATP6. In addition, it has subunits a, b, d, e, f, g, A6L and the oligomycin sensitivity-conferring protein (OSCP). When protons flow across the rotor, the c-ring rotates and this rotation is harnessed by the catalytic head through the γ subunit of the central stalk. The peripheral stalk holds the catalytic head in place preventing its rotation (Osellame et al., 2012; Song et al., 2018; Walker, 2013).

In addition to mitochondrial membranes, the ATP-synthase complex is present in bacteria as well as chloroplasts where they perform an energy transducing function as well. Although it can produce as well as hydrolyze ATP by reverse rotations, the hydrolysis function remains inhibited in most cases (except in some bacteria) (Walker, 2013). In the mitochondrial inner membrane, super/supra molecular organization of ATP synthases influences cristae formation with tight membrane curvatures (Devenish et al., 2008; Hahn et al., 2016).

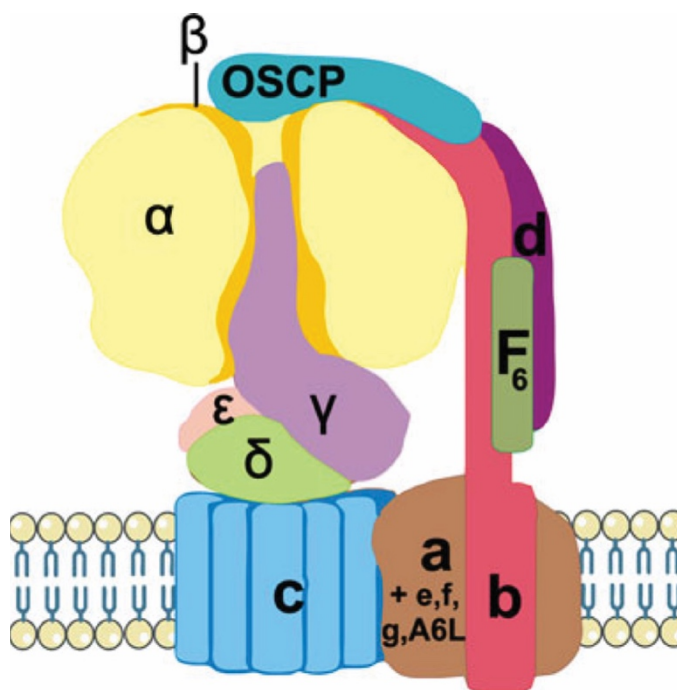


Figure 3.1. Structural organisation of the ATP synthase complex. The ATP synthase complex in humans consists of a F_o and F_1 component. The F_1 component consists of α , β , γ , δ and ϵ subunits. The F_o part consists of the c-ring, a, b, d, e, f, g, A6L, OSCP components. Image reused from (Jonckheere et al., 2012). Usage under License number-1300586-1 from copyright clearance center.

The human ATP synthase is composed of 29 proteins, 27 of which are transcribed from the nuclear genome. They are synthesized as precursors in the cytosol and imported into the mitochondria, where they are cleaved to their mature form. Only the F_o subunits, ATP6 (subunit a) and ATP8 (subunit A6L) are coded by the mitochondrial genome (Ruhle and Leister, 2015). Components of the mitochondrial ATP synthase complex have been found to have extra-mitochondrial regulatory functions. They act as cell-surface receptors binding ligands involved in innate immunity, lipoprotein metabolism, angiogenesis and nutrient uptake (Champagne et al., 2006; Chi and Pizzo, 2006; Devenish et al., 2008). ATP synthase complex can synthesize ATP at the cell surface (Moser et al., 2001), although the origin (mitochondrial export vs extra-mitochondrial assembly) and exact function of cell-surface ATP synthase are still being investigated. One hypothesis is that ATP synthase localization can generate niches with varying ATP, ADP, pH which can affect cell signaling and response to its environment (Chi and Pizzo, 2006).

The ATP synthase subunit α (coded by ATP5A1/ATP5F1A gene) is a nuclear encoded protein of the ATP synthase complex, a component of the F_1 complex. Though it does

not directly catalyze the formation of ATP, contacts of α subunit with the β subunit are essential for ATP generation. ATP5A1 has been found on the plasma membrane, where it is bound by angiostatin (Moser et al., 2001). Cytosolic accumulation of this subunit has been found together with tau protein aggregates in case of Alzheimer's disease (Sergeant et al., 2003). ATP5A1 mutations cause diseases like neonatal-onset mitochondriopathy (Lines et al., 2021) where its catalytic function is impaired. Reduced ATP5A1 expression correlates with progression of prostate cancer and clear cell renal cell carcinomas (Feichtinger et al., 2018; Yuan et al., 2018). In glioblastomas, ATP5A1 is upregulated with potential role in tumor progression (Xu and Li, 2016). Beyond a direct ATP synthesis function, alternate mechanisms of ATP5A1's involvement in these pathologies is under investigation.

ATP5A1 has been identified as an RNA binding protein by many studies (Backlund et al., 2020; Bao et al., 2018; Beckmann et al., 2015; Castello et al., 2012; Castello et al., 2016; Garcia-Moreno et al., 2019; He et al., 2016; Huang et al., 2018; Liao et al., 2016; Mallam et al., 2019; Mullari et al., 2017; Perez-Perri et al., 2018; Trendel et al., 2019). This aspect of ATP5A1's function has not been investigated in detail. One study posits potential role of ATP5A1's RNA binding in tumor progression (Song Ba et al., 2021).

ATP5A1 is being characterized with regard to its RNA binding function in our lab in a study led by Dr. Aindrila Chatterjee. I have collaborated in this study to understand the phenotypic implications of ATP5A1's RNA binding, particularly with regard to a possible "moonlighting" function of ATP5A1. So far, experiments in our lab have shown that ATP5A1 binds RNA in the vicinity of the outer mitochondrial membrane and that this binding is sensitive to metabolic cues. eCLIP analysis for ATP5A1 revealed a binding preference for predominantly protein coding mRNAs in their UTRs (untranslated regions). In particular, ATP5A1 binds mRNAs containing terminal oligopyrimidine (TOP) motifs in their 5'UTR. The TOP motif has been studied extensively in the context of translational regulation upon stress or metabolic cues (Fonseca et al., 2015; Miloslavski et al., 2014).

To probe potential regulatory effects of ATP5A1 on the RNA, transcriptomics analysis was performed by Dr. Aindrila Chatterjee after ATP5A1 depletion. It was observed that the levels of 5'UTR bound ATP5A1 targets go up, whereas the targets bound at the 3'UTR are downregulated. Given that ATP5A1 is poised to sense the energy status of the cell, it is possible that additional translational regulation of these targets can

occur, especially RNAs bound at the TOP motifs by ATP5A1. Furthermore, we have indications that RNA interactions influence ATP5A1's cellular fate. In-cellulo import assays (performed by Dr. Aindrila Chatterjee) show that mutating the RNA binding regions of ATP5A1 negatively impact its mitochondrial import.

In this section of the thesis, I expand on the analysis of potential translational regulation by ATP5A1, and probe the protein-binding partners of ATP5A1. Protein interactions of ATP5A1 can potentially mediate the functional impacts of ATP5A1-RNA interactions.

3.2 Objectives

In this study, I investigate ATP5A1's RNA binding activity with regard to ATP5A1's RNA binding preferences, how it could be coordinated in cells through protein effectors and regulatory functions of ATP5A1 on RNA. The main aspects are:

1. Validation of the ATP5A1's ability to bind TOP motif containing sequences in-vitro
2. Identification and validation of proteins interactors of ATP5A1
3. Effect of ATP5A1 depletion on translational efficiency with a focus on its target RNAs

3.3 Results

3.3.1 In-vitro characterization of ATP5A's binding preference

ATP5A1 target RNAs have been profiled by Dr. Aindrila Chatterjee in our lab, using eCLIP in Huh7 cells. This revealed ATP5A1's preference to bind specific mRNAs at a particular sequence in the 5' UTR – the TOP (terminal oligopyrimidine tract) motif. ATP5A1 shows enriched crosslinking sites on the majority of the 91 identified TOP-mRNAs, with 30 of the binding sites identified as statistically significant in the DEW-seq analysis pipeline. RPS6 and RPL13 are shown as representative examples of

ATP5A's binding pattern on TOP-mRNAs (Fig 3.2). Directly following the m7G cap structure in the RNA, the TOP motif comprises a stretch of 4-15 pyrimidines, invariably beginning with a C. TOP motif is a characteristic of mRNAs coding for the cellular translational machinery (Cockman et al., 2020), targeting them for translational control under conditions of stress. Several RBPs including La-related protein 1 (LARP1) of note, have been shown to bind the TOP motif and effect downstream translational regulation of TOP- mRNAs. Considering the hitherto unexplored RNA-binding activity of ATP5A1 and in light of its propensity to bind TOP-RNAs, we decided to characterize this binding in-vitro.

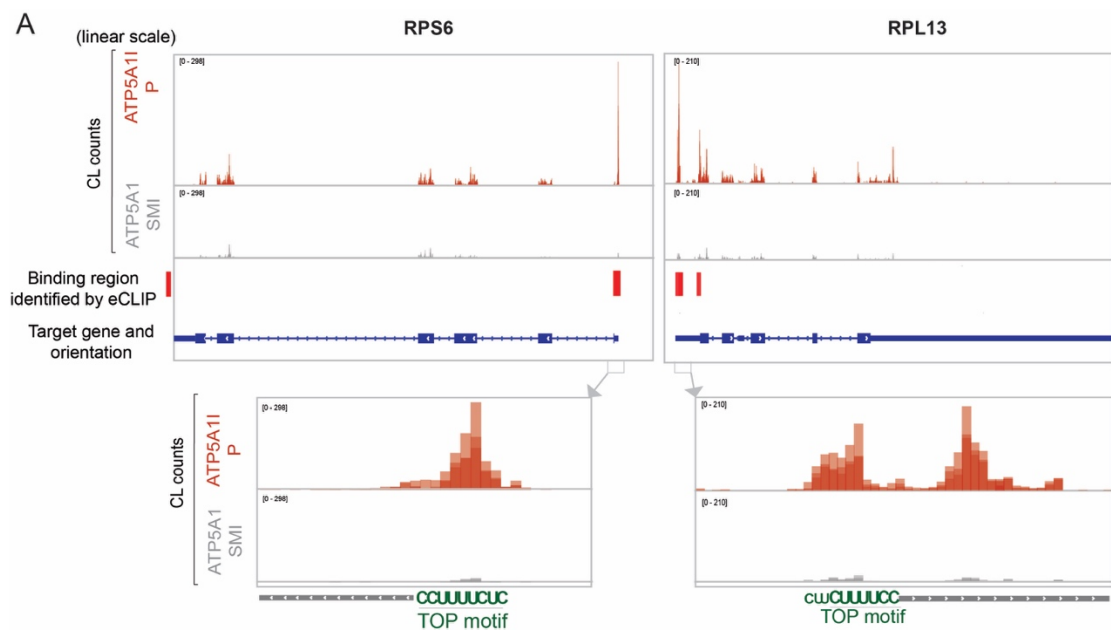


Figure 3.2. ATP5A1 binds to mRNAs with a 5' TOP motif. ATP5A1's crosslinking sites highlighted on representative TOP-mRNAs as visualised on IGV. Zoomed in view of the specific binding on the TOP motif is shown below.

Towards this, I used electromobility shift assays (EMSA) to estimate ATP5A1's affinity to RNA fragments containing TOP-motifs. Full length human ATP5A1 (flATP5A1-V5-StrepII) was expressed and purified from Escherichia coli Rosetta (DE3) strain as described in the methods section (5.3.13) (Fig 3.3 A). Western blot analysis (with two different antibodies) of the purified protein detected full length ATP5A1 (expected size 63.5 kDa) in the purified fraction, as well as smaller fragments (25-37 kDa) (Fig 3.3 B). This indicates possible formation of cleaved products from the flATP5A1. Curiously, size of the smaller fragment approximately matches that of

the nucleotide binding domain of ATP5A1 (192-415aa sequence ~ 30.5kDa) (Fig 3.3 C).

From the ATP5A1-bound RNAs detected by eCLIP, two TOP-mRNAs coding for ribosomal proteins- RPS6 and RPL13, were selected for EMSA analysis. The eCLIP-identified crosslink sites were used as a guide to demarcate the binding region of ATP5A1 on the TOP- mRNAs. Oligos (25-41 nt) spanning the binding site were chemically synthesized with 3' Cy5 fluorophore label to monitor the RNA binding of ATP5A1. A scrambled version of the sequence potentially destroys the enclosed TOP-motif and serves as a GC and size-matched control (Fig 3.3 D). Probing the folding patterns of these oligos using RNAfold (Lorenz et al., 2011) revealed a propensity to adopt secondary structures, which was more pronounced for RPS6 (Fig 3.3 E). At high concentrations, higher order multimers of these sequences are also predicted to form.

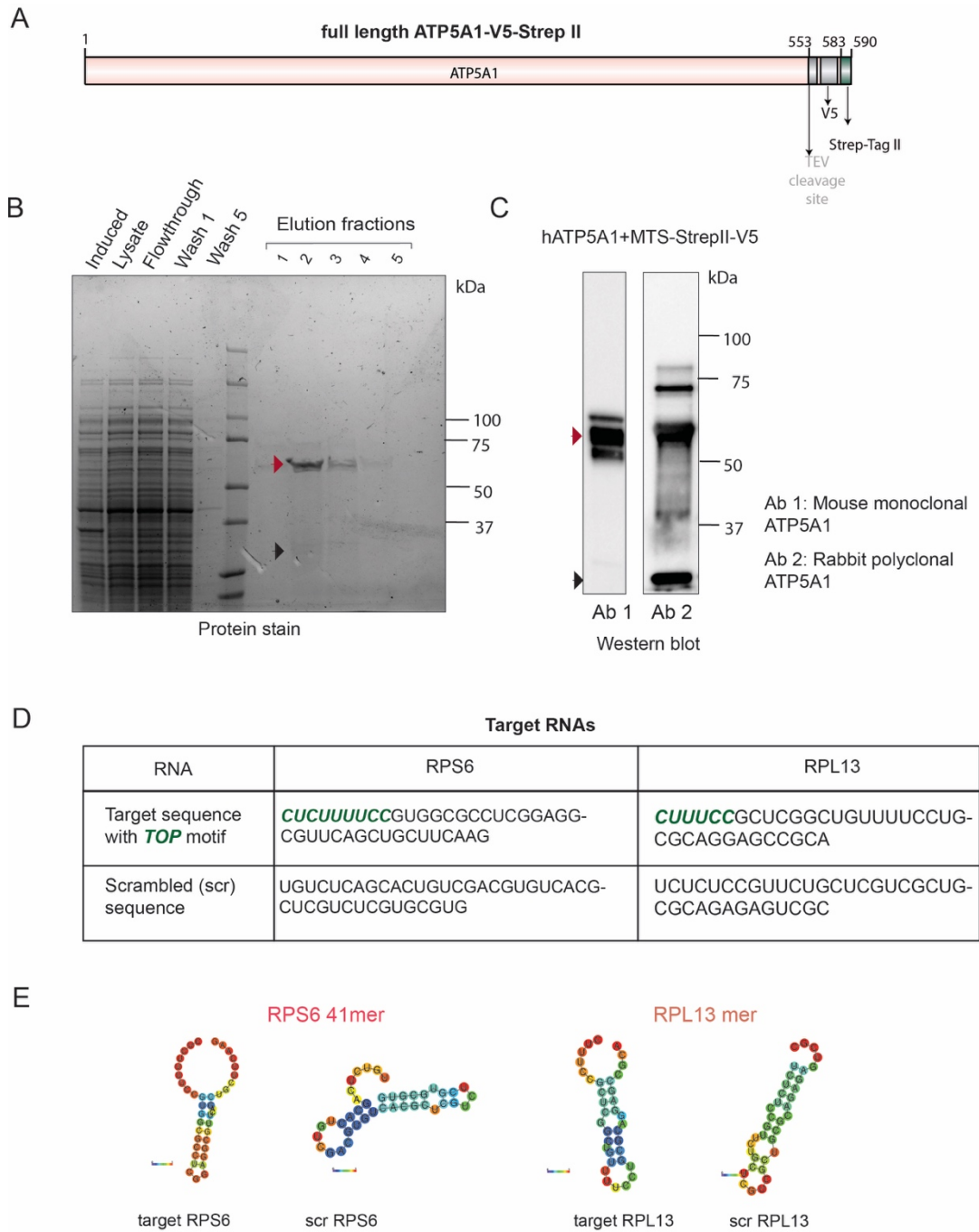


Figure 3.3. RNA targets and ATP5A1 protein used for EMSA assays (A) Sequence of RNA targets analysed in EMSAs with the TOP motif highlighted in green (B) Possible structural conformations of the RNA sequences as predicted by RNAfold (C) Scheme of ATP5A1 protein construct used for purification (D) Purified ATP5A1 visualised with protein stain and (E) western blot with specific antibodies.

In order to measure the affinity of ATP5A1 to the RNAs, I titrated increasing amounts of ATP5A1 (0.18-1.45 μ M) against a fixed amount of RNA (10 nM). Representative EMSAs are shown in Fig 3.4. The RPS6 target RNA fragment (Fig 3.4 A), in the absence of ATP5A1, resolves into multiple bands along the gel (Fig 3.4 A lane 1). This

could indicate different conformations and/or multimers as predicted by the in-silico analysis. The same is true for scrambled RPS6 RNA fragment (scrRPS6), which however shows a different migration pattern (Fig 3.4 A, lane 6). The comparative labelling intensities of the various RNA species also differ between target RPS6 and scrRPS6. Addition of ATP5A1 to the RNA alters its migration pattern, leading to the appearance of higher molecular weight bands. This indicates the formation of RNA-protein complexes (Fig 3.4 A, lanes 2-5), sensitive to levels of added protein. Scrambled RPS6 fragments are also bound by ATP5A1 at higher concentrations (Fig 3.4 A, lanes 7-10). A precise analysis of the binding affinity is complicated by these multiple shifted bands close to the free-RNA. In order to simplify the calculation of relative affinities and be consistent, I decided to consider all the bands above the fastest-migrating RNA band as an RNP complex (indicated by * in Fig 3.4A, C). Using the migration pattern of free RNA as a guide, I distinguished the free RNA fraction from the RNP-complex fraction. The fraction of RNA bound by the protein was calculated as follows:

$$\text{Fraction bound} = \frac{\text{Intensity of shifted bands (RNP complex)}}{\text{Intensity of the whole lane (Total RNA)}}$$

Similar observations were made for RPL13, with free RNA migrating in multiple bands (Fig 3.4 C lane 1, 6) and protein addition resulting in the appearance of multiple higher molecular weight complexes. Estimating the relative affinities by the quantification of fraction of RNA bound, I observed that ATP5A1 binds the TOP-motif containing target RPL13 and RPS6 with slightly increased affinity than their scrambled sequences. For a refined quantitative estimate of these affinities, more standardisation is required for reasons described below.

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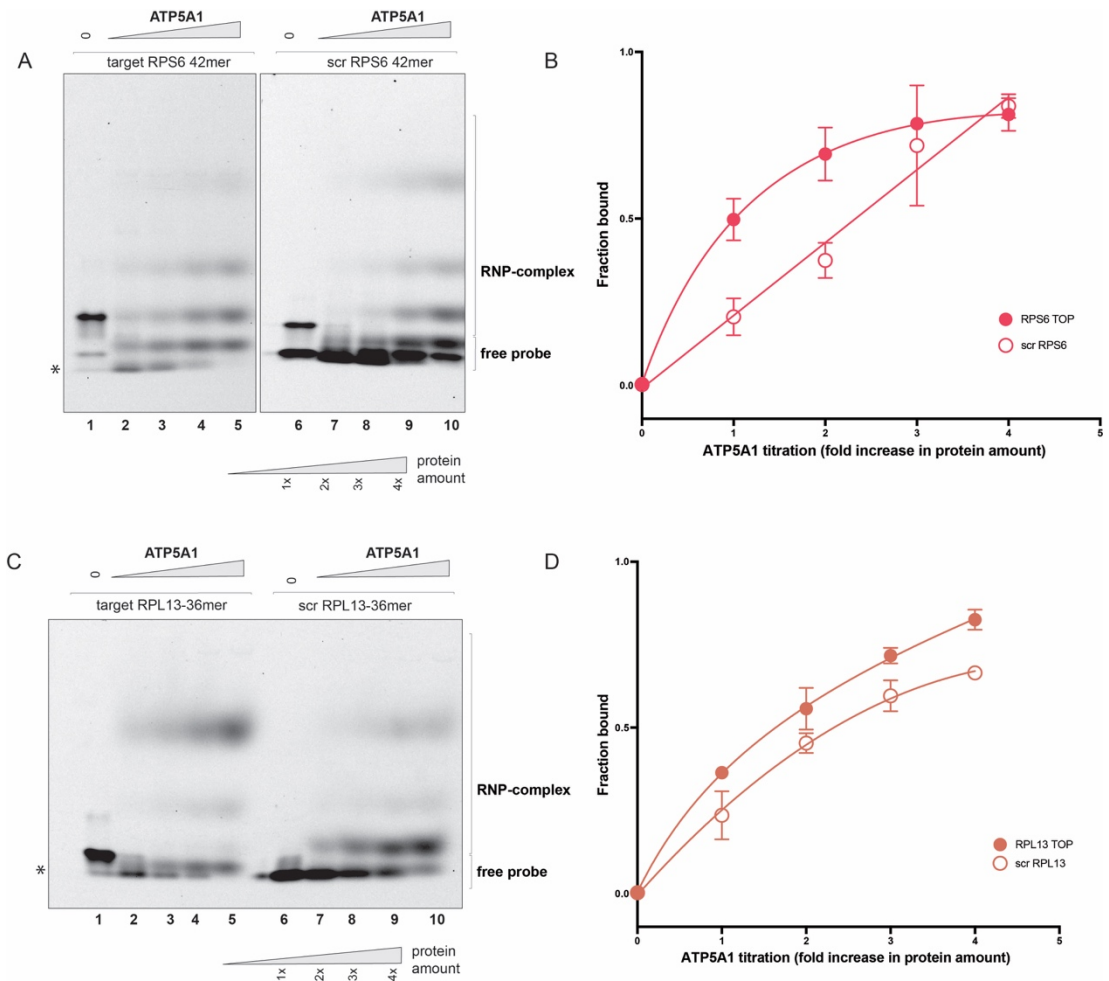


Figure 3.4. ATP5A1 binds TOP-motif sequences in-vitro. (A) EMSA assay with ATP5A1 and target RPS6 42mer, scrambled RPS6 42mer (B) Binding curves for ATP5A1-RPS6 interaction (C) EMSA assay with ATP5A1 and target RPL13 36mer, scrambled RPL13 36mer (D) Binding curves for ATP5A1-RPL13 interaction. Data reported as mean $\pm$ S.D, number of experiments, $n=3$. * indicates the fastest migrating RNA band, above which shifted bands were quantified.

Follow-up in-vitro experiments from the lab (from independent protein preparations) have further optimized the binding conditions improving ATP5A1's in-vitro binding specificity. Addition of EDTA as a metal ion chelator was found to improve the specificity of ATP5A1's RNA binding preference, indicating a possible regulatory role for metal ions in ATP5A1's binding (Fig 3.5, experiment performed by Dunja Ferring-Appel). Mg^{2+} could be the relevant ion here as Mg^{2+} is critical for ATP5A1's catalytic activity binding to ATP/ADP. Therefore, it is possible that an in-vivo regulation of ATP5A1's RNA binding involves these factors. Mass spectrometry analysis of ATP5A1 protein preparations used in the follow-up experiments have revealed co-purification of a large fraction of bacterial chaperon GroEL (CH60) migrating at the

same position with purified protein. The likely presence of high amounts of chaperones in the protein preparations make a quantitative K_d estimation inaccurate at present and I thus refrained from it, till further analysis. Additional measures of specificity can also be obtained by competition assays. Specifically, the binding of ATP5A1 to target RNA can be assayed in the presence of competing non-specific RNAs. Future optimized experiments can therefore be more informative about the specific and relative in-vitro binding affinities of ATP5A1 towards the RNAs tested.

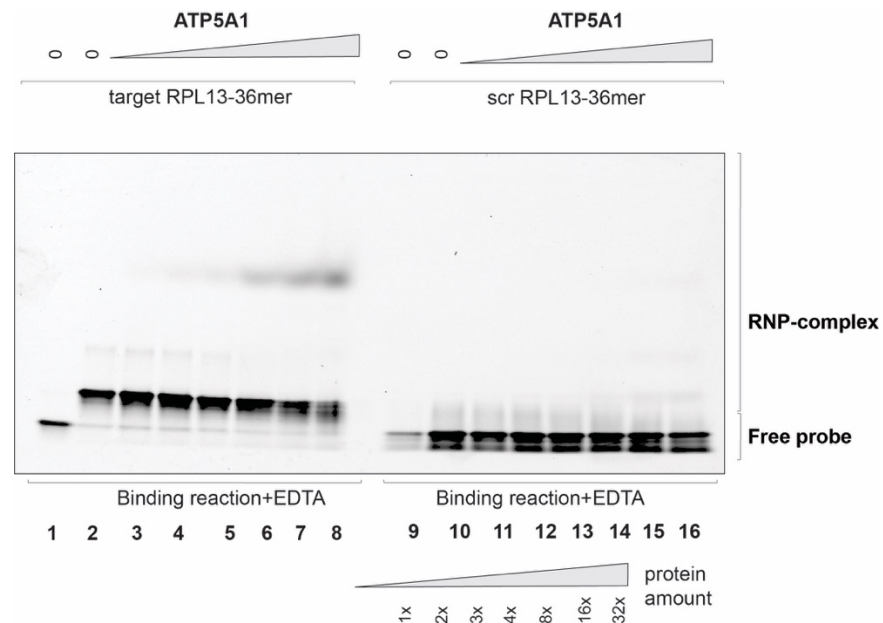


Figure 3.5. ATP5A1's in-vitro binding specificity can be improved by addition of ion chelator EDTA. Increasing amount of ATP5A1 was added to target RPL13 and scrambled RPL13, with specific RNP complexes seen for RPL13. Note: 1x amount of protein in experiments corresponding to fig 3.5 and fig 3.4 are not equal. Lane 1 and 2 are target RPL13 -36 mer without protein. RNA in lane 1 has been heated at 95°C before loading to resolve secondary structures. Lane 9 and 10 are scrambled RPL13 RNA treated similar to lane 1 and 2. Experiment was performed by Dunja Ferring-Appel.

To summarize, ATP5A's intrinsic capacity to bind TOP can be recapitulated with in-vitro experiments. We have made progress towards refining in-vitro binding conditions and estimating ATP5A1's binding affinities towards its cellular RNA targets. Additional cellular context can further determine the specificity and outcomes of ATP5A1-RNA interaction. Therefore, I next focused on the cellular outcomes of ATP5A1-RNA interactions and potential protein interactors of ATP5A1 that may mediate these outcomes.

3.3.2 Effect of ATP5A1 depletion on nascent protein synthesis

On ATP5A depletion, transcriptome analysis (performed by Dr. Aindrila Chatterjee) revealed that targets of ATP5A1 bound at their 5'UTR increase in abundance whereas those bound at 3'UTR are downregulated. We wanted to explore if these changes are amplified at the translational level as the role of ATP5A1 in translational regulation has not been explored so far. ATP5A1, on account of its ATP binding, is well poised to act as an energy sensor of the cell. Since translation needs to be recalibrated in an altered energy environment, it is feasible that ATP5A1 bridges this regulation. Towards examining this hypothesis, I decided to analyse how protein-synthesis rates are altered in the absence of ATP5A1.

Experimental set up was based on the established quantitative noncanonical amino acid tagging (QuaNCAT) method (Howden et al., 2013), as illustrated in Fig 3.6 A. ATP5A1 was efficiently depleted (80-90%) from cells at 96 hours following specific small interfering RNA (siRNA) treatment (Fig 3.6 B). After ATP5A1 depletion, I pulse-labelled (30 mins) the cells with a methionine analogue AHA (L-Azidohomoalanine) and stable-isotope-labelled amino acids (SILAC, lysine and arginine). This enables the downstream selective enrichment of newly synthesised proteins which have incorporated these labels. Using click chemistry, the AHA-modified proteins were covalently linked to alkyne-agarose beads and pulled down, followed by LC-MS analysis.

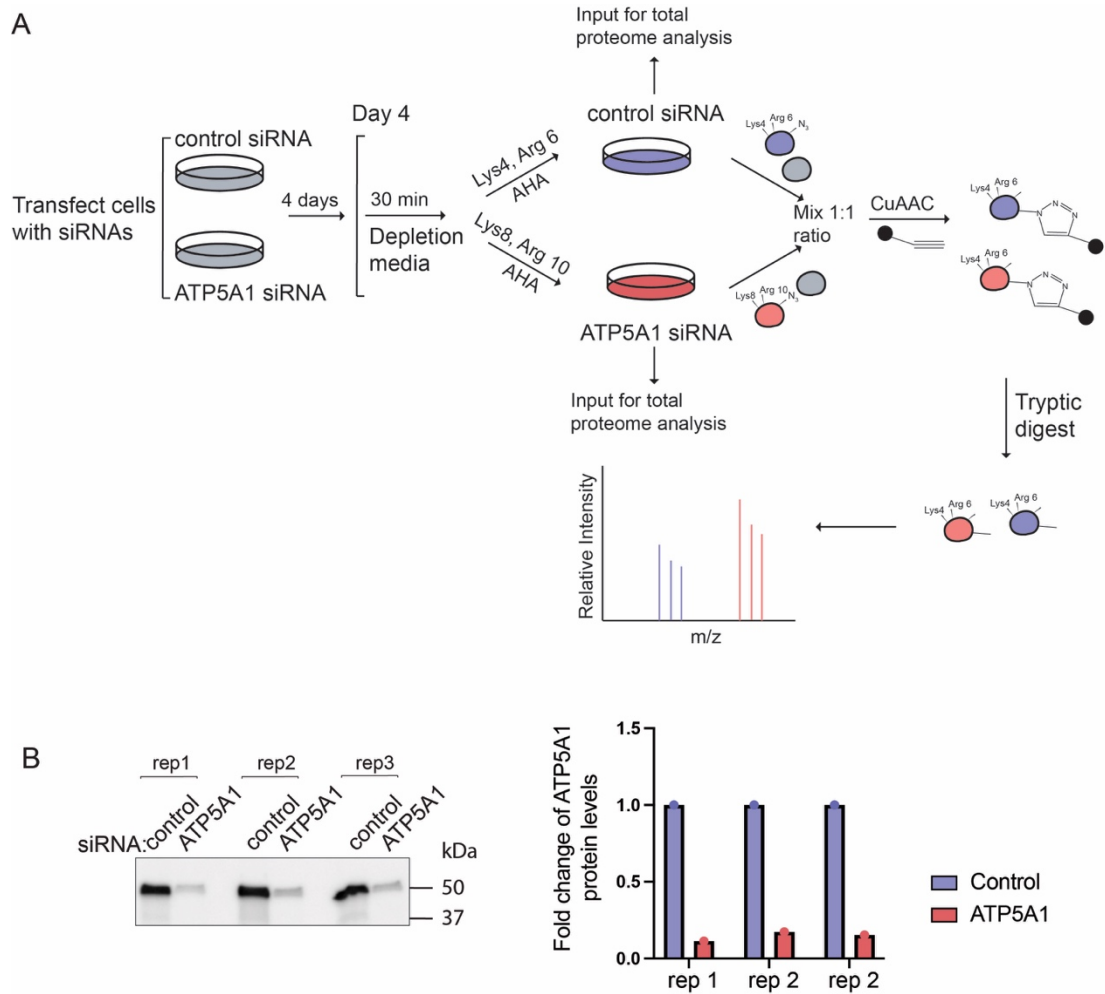
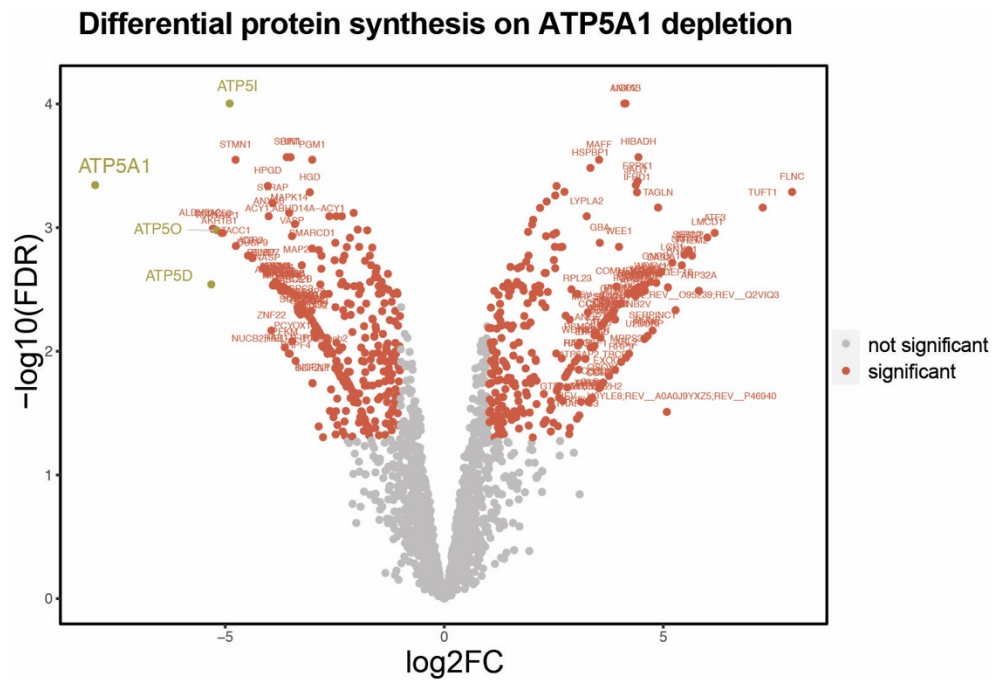


Figure 3.6. QuaNCAT experiment to measure effect of ATP5A1 depletion on nascent protein synthesis. (A) Experimental scheme to assess the effect of ATP5A1 on protein translation (B) Western blot to visualise ATP5A1 protein depletion with siRNAs.

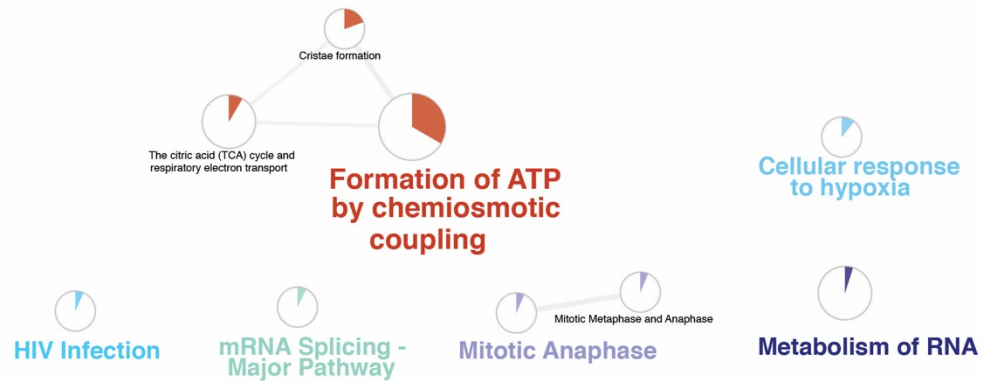
AHA pull down successfully enriched for newly synthesised proteins as evidenced by the SILAC incorporation (Supplementary figure 7). Around 2031 proteins were detected in the nascent proteome. After ~90% ATP5A1 depletion, 591 proteins were differentially synthesised ($\text{abs}(\log_2\text{FC}) > 1$ and 5% FDR). Of these, 284 proteins displayed increased synthesis, while 307 proteins were downregulated in protein synthesis (Fig 3.7). As expected, protein synthesis of ATP5A1 was maximally downregulated. Several components of the ATP-synthase complex (ATP5D, ATP5O, ATP5I, ATP5J, ATP5F1, ATP5B) show reduced synthesis, which can be a regulatory response to ATP5A1 depletion (Fig 3.7, Fig 3.8). In addition, translation of proteins associated with mitochondrial energy pathways (TCA cycle and mitochondrial ATP synthesis) are significantly downregulated- indicating impaired mitochondrial respiration. The cellular translational machinery (e.g., ribosomal 40S subunits,

translation initiation factors like eIF2, eIF3) is the principal pathway that is translationally upregulated in ATP5A1's absence. In the translational machinery, specific ribosomal subunits and translation elongation factors are upregulated. Inhibitors of the Wnt-beta catenin pathway involved in cell proliferation are also upregulated (Masckauchan et al., 2005; Zhang and Wang, 2020) (Fig 3.8). Considering a possible effect of ATP5A1 depletion on cellular energy levels, increased synthesis of energy consuming translation machinery is surprising. However, we do not observe a substantial decrease in Huh7 cell viability even on depletion of upto ~90% ATP5A1 with concomitant loss of the ATP synthase complex components. This could be explained by an increased dependence of FBS-cultured cells on glycolytic ATP rather than mitochondrial ATP synthesis. Thus, alternate mechanisms of ATP5A1 affecting altered cellular translation other than energy levels need to be considered. Beyond its well-known ATP synthesis role, ATP5A1 could have additional unexplored cellular functions that can affect cellular translation.



Enriched pathways with altered protein synthesis on ATP5A1 depletion

Downregulated pathways



Upregulated pathways

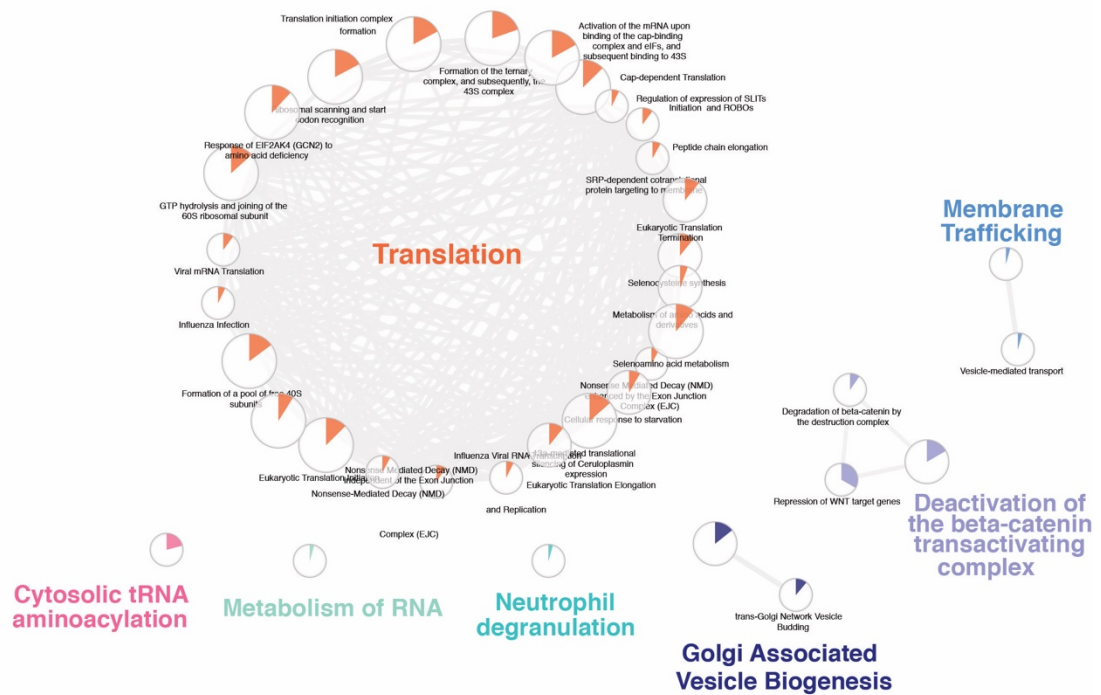


Figure 3.8. Deregulated pathways after ATP5A1 depletion analysed using the Reactome database and visualised in Cytoscape (CLUEGO), coloured according to functional classification. The thickness of lines connecting the pathways indicate strength of interaction (kappascore). Size of the the nodes reflect the significance of enrichment.

Global proteome changes were also analysed by LC-MS, using the input samples before AHA enrichment (Fig 3.9 A). Depletion of ATP5A1 did not have a dramatic impact on global protein abundance. The diminished levels of ATP-synthase subunits were mirrored in steady-state protein abundance, illustrating the tight co-regulation of the subunit protein-levels. Curiously, α -synuclein and sestrin 2 stand out (AMPK -

mTOR signalling) among the minor pool of upregulated proteins at steady state. Both interact with ATP5A1, and affect mitochondrial health (Kim et al., 2020; Ludtmann et al., 2018; Pennington et al., 2010; Ren et al., 2021). Correlating the changes in altered synthesis to the steady state, I confirmed that the majority of the targets altered in protein synthesis are maintained close to the steady state at the total protein level (Fig 3.9 B). This suggests active compensatory buffering mechanisms that maintain proteostasis in case of altered protein synthesis.

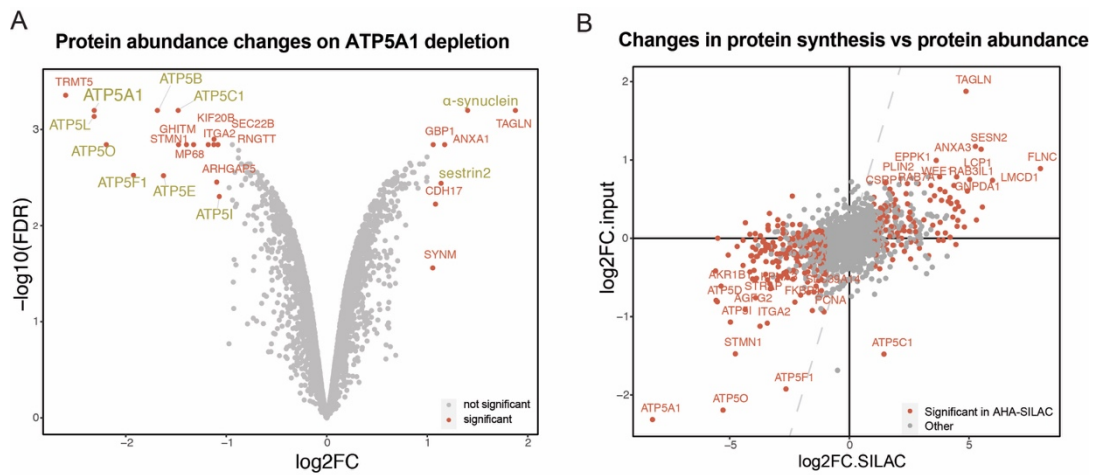


Figure 3.9. Global proteome is not altered significantly after ATP5A1 depletion (A) Volcano plot for global protein changes on ATP5A1 knockdown. Targets with $\log_2\text{FC} \geq 1$ or $\log_2\text{FC} \leq -1$ and $\text{FDR} \leq 0.05$ were considered significant. (B) Correlating the fold change in abundance of total protein in comparison to fold changes seen in synthesis. Proteins with altered translation rates are highlighted in orange.

I cross-referenced the ATP5A1-bound targets from eCLIP data with proteins showing altered synthesis to investigate effects possibly mediated by direct RNA binding (Fig 3.10). Only 57% of the ATP5A1 eCLIP targets are detected in the nascent proteome. 31% of the detected eCLIP-targets show altered protein synthesis on ATP5A1 depletion. I then correlated the binding sites of ATP5A1 on the RNA with the altered protein synthesis trends of these targets. Proteins coded by RNAs which were bound at the 5' UTR tended to be upregulated in synthesis, while those coded by RNAs bound at the 3' UTR are mostly downregulated (Fig 3.10 B). A similar pattern was observed at the RNA level in transcriptomic analysis performed by Dr. Aindrila Chatterjee in our lab. A striking example from this subset of ATP5A1 bound targets is LARP1, a well-studied TOP motif binder (Cassidy et al., 2019; Philippe et al., 2020). ATP5A1

depletion reduces LARP1 protein synthesis, the consequences of which haven't been explored yet.

In summary, translational efficiency of several pathways including components of the cellular translational machinery is altered in the absence of ATP5A1. These effects are buffered at the steady state levels, possibly due to cellular adaptation. A subset of RNAs bound by ATP5A1 are altered in protein synthesis. Whether these are direct effects mediated by ATP5A1's RNA binding remains to be investigated.

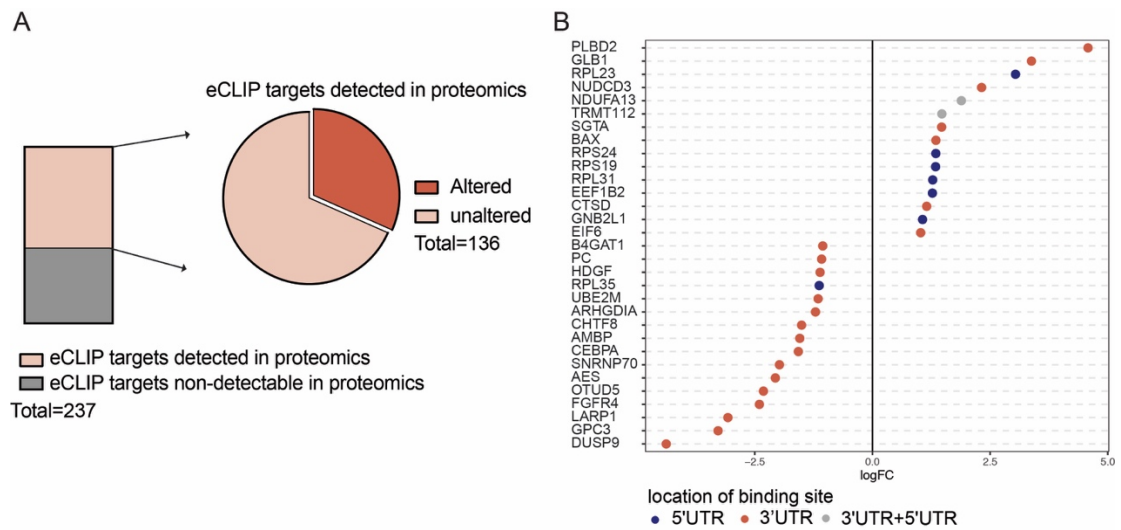


Figure 3.10. Change in translation of ATP5A1-bound RNAs (A) Overlap of the targets differentially synthesised with ATP5A1's eCLIP targets (B) logFC changes in eCLIP targets of ATP5A1 identified by QuaNCAT. The targets are coloured according to location of ATP5A1 binding sites on their corresponding mRNAs.

3.3.3 Interactors of ATP5A1

Towards a detailed mechanistic view of ATP5A1's RNA binding, I wanted to examine ATP5A1's interactions with other cellular proteins. ATP5A1-protein interactions can potentially dictate the outcome of ATP5A1-RNA binding events, as well as orchestrate the context and location of these events. To profile the binding partners of ATP5A1, we took a LC-MS based approach after immunoprecipitation under optimal buffer conditions (Fig 3.11 A, Supplementary figure 8). Immunoprecipitation with a control IgG was used to correct for non-specific binding. As expected, the components of the ATP-synthase complex were co-purified as some of the top binding partners (log2 fold change > 3) of ATP5A1, indicative of the data quality (Fig 3.11 B). Beyond its own

complex proteins, it interacts with other mitochondrial proteins including enzymes (e.g., pyruvate carboxylase, components of alpha-ketoglutarate dehydrogenase), chaperones (HSPA1/D9) and solute carrier proteins (phosphate transporter SLC25A3).

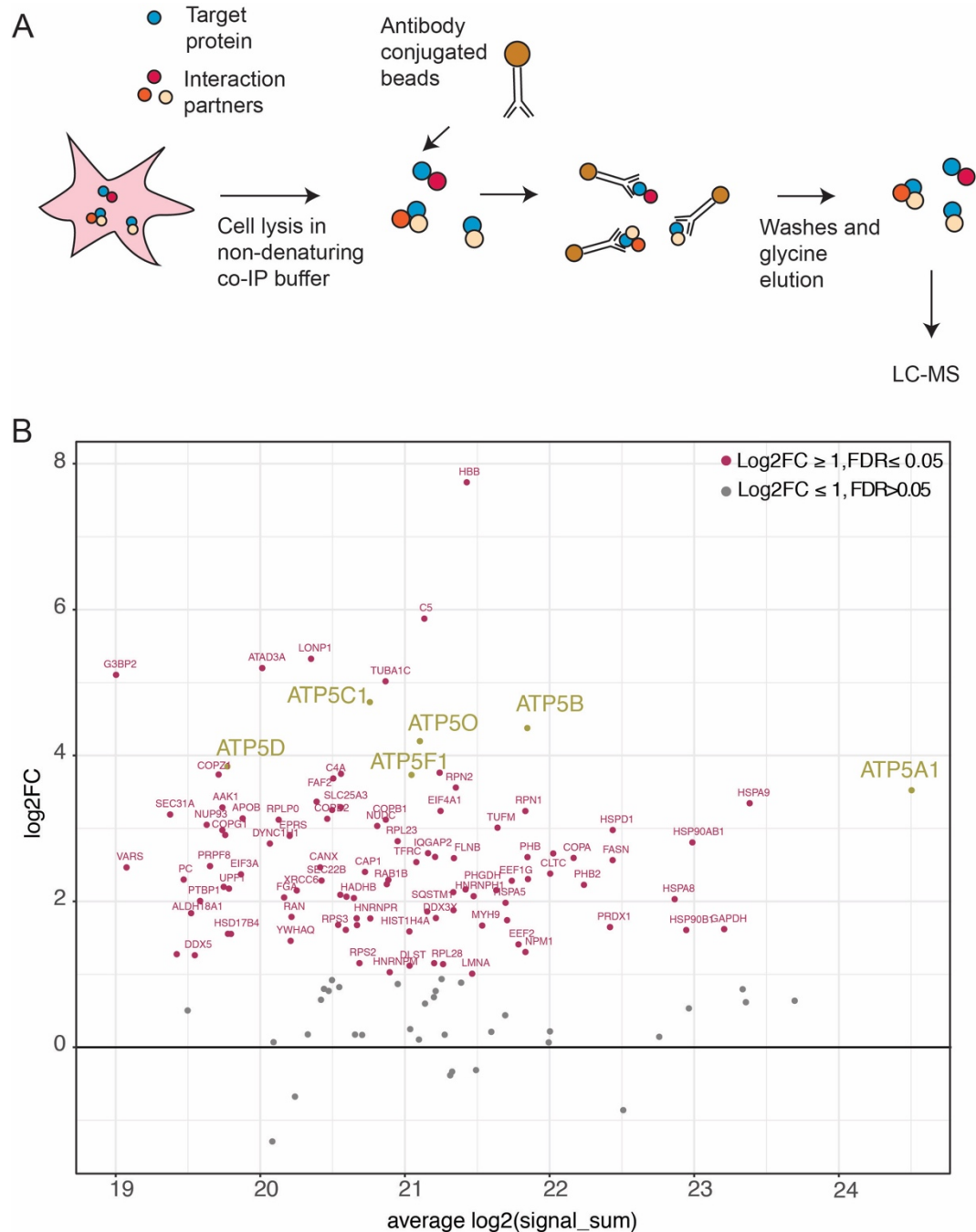


Figure 3.11. Identification of ATP5A1's protein interactors. (A) Experimental scheme (B) MA plot showing the log2FC of proteins copurified with ATP5A1 over a control IgG IP. X-axis indicates average log2 (summed signal intensity), a measure of protein abundance. Targets with log2FC ≥ 1 and FDR ≤ 0.05 are considered significant.

To our surprise, further analysis revealed that ATP5A1 shows extensive association with components of the cytoplasmic translational machinery as well as cytosolic RNP granule proteins (Fig 3.12). It binds components of both the large (60S) and small (40S) ribosomal subunits, as well as translational elongation factors. Several RBPs involved in stress granule/RNP complex assembly, like G3BP2 and IGF2BP1 are also co-purified with ATP5A1 (Zeng et al., 2020b). Interestingly, many components of the translational machinery and ribosomal proteins associated with ATP5A1 are also found in stress granules (eIF3A, eIF3I, eIF4A, RPS2, RPS3, RPS4, RPS11). Upf1, a well characterised RBP and resident of SGs, and dynein, which plays a role in the assembly and disassembly of stress granules (Loschi et al., 2009) were also found in ATP5A1 interactome.

Enriched pathways among ATP5A1 protein interactors

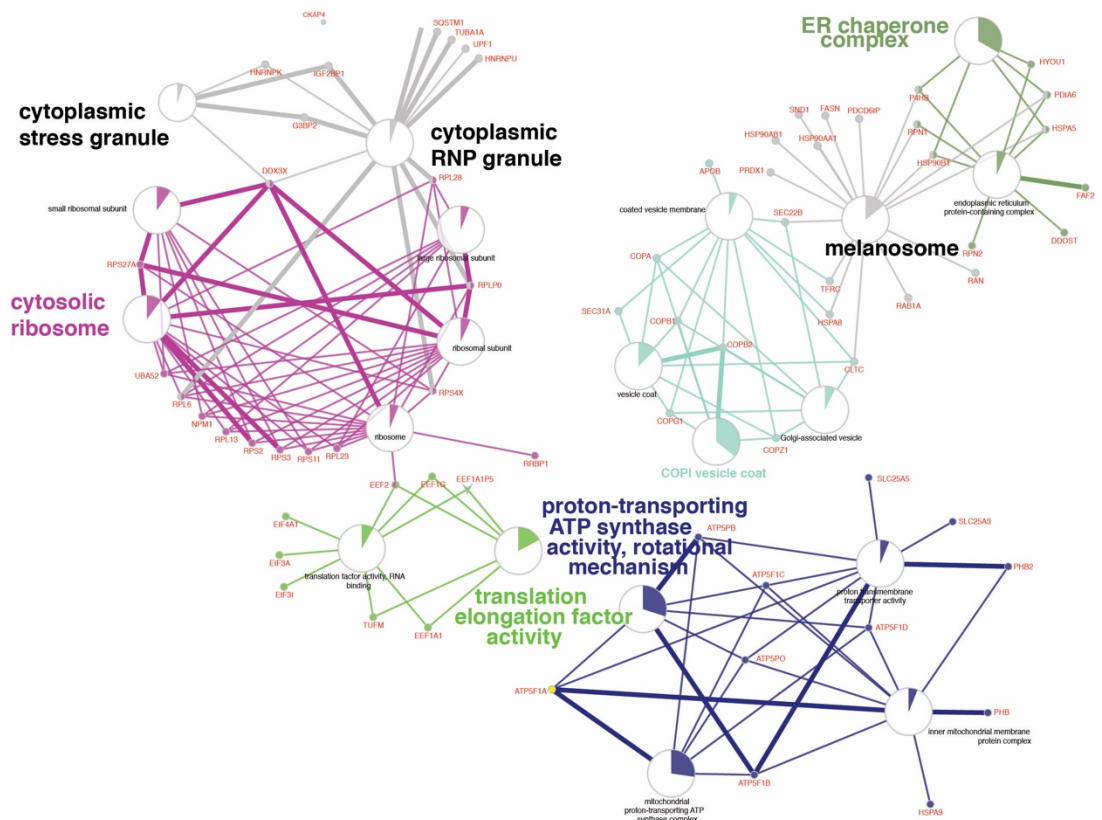


Figure 3.12. ATP5A1's interaction network extends beyond the mitochondria to cytosolic factors including translational machinery and RNP granules. Enriched pathways in ATP5A1 interactors visualised in Cytoscape- Figure made by Dr. Aindrila Chatterjee.

In light of ATP5A1's RNA binding tendency, we were particularly interested in further investigating the identified RNP granule proteins- G3BP2 and IGF2BP1 as bona fide

ATP5A1 binders. So far, we have not pursued ATP5A1's interactions with ribosomal proteins and translation factors, which can be potentially investigated in the future. I performed ATP5A1 immunoprecipitation (in similar conditions as the MS experiment) followed by western blot analysis of G3BP2 and IGF2BP1. I could confirm that both G3BP2 and IGF2BP1 are co-purified by ATP5A1, but not an isotype matched IgG control, ruling out non-specific binding (Fig 3.13). ATP5C1, a component of the ATP-synthase complex is used as a positive control for ATP5A1 interactions and nucleolin, with no known ATP5A1 association is used as a negative control. In addition, interactions of ATP5A1 with G3BP2 and IGF2BP1 have been independently confirmed by proximity ligation assays (performed by Dunja Ferring-Appel from our lab, data not shown). Thus, ATP5A1 association with RNP granule proteins like G3BP2 and IGF2BP1 are bona-fide cellular interactions with potential functional implications.

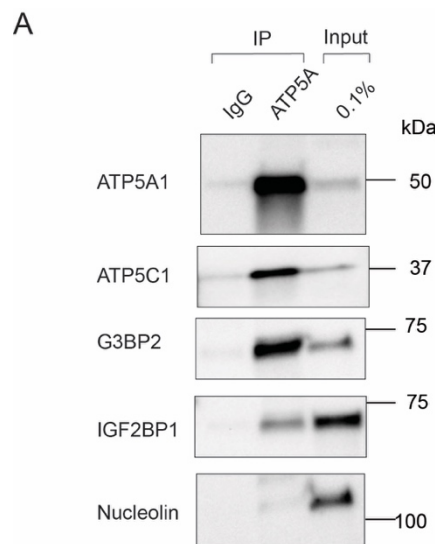


Figure 3.13. ATP5A1 co-purifies G3BP2 and IGF2BP1 in pulldown experiments. Validation of ATP5A1's interaction with G3BP2 and IGF2BP1, RNP granule proteins by co-immunoprecipitation.

We also investigated the RNA-dependency of these interactions by treating the cell-lysates with RNase prior to immunoprecipitations (Supplementary figure 8). ATP5A1's interactions are broadly insensitive to RNase treatment. Only two proteins IGF2BP1 and COPZ1 show reduced binding with ATP5A1 in the absence of RNA. On the other hand, we find that some ribosomal proteins show enhanced interactions

with ATP5A1 on RNase digestion. The implications of this observation are not clear now, but could be investigated in the future.

Taken together, ATP5A1's expansive association with translational machinery and components of RNA granules signifies a putative role of these associations in coordinating cytosolic RNA stability/ translation and mitochondrial ATP synthesis.

3.4 Discussion

In this part of my project, I examined ATP5A1's in-vivo RNA-binding preference using in-vitro experiments, confirming its ability to directly bind TOP-motifs. In addition, I explored how ATP5A1 affects global protein synthesis including those targets directly bound. To better understand the coordination of different functional aspects of ATP5A1, I profiled cellular protein interactors of ATP5A1. We found ATP5A1 associations with several key cytosolic factors (translational factors/ribosomal proteins/RNP granule proteins) in addition to its mitochondrial interaction partners.

3.4.1 ATP5A1 forms a nexus of interactions between mRNAs and proteins involved in cytosolic translation

Evaluating the molecular associations of ATP5A1 in the cell, we find that it shows frequent association with different facets of the cytosolic translation machinery, either by binding mRNAs encoding translation factors/ribosomes or by interacting with proteins directly. ATP5A1 binds mRNAs of cytosolic translation factors/ ribosomal proteins at their TOP-motifs (a well-known site of RNA regulation). This property has been characterized in-cellulo in our lab and confirmed by in-vitro EMSA assays as described in section 4.2.1. ATP5A1-RNA interactions selectively happen near the outer mitochondrial membrane (data not shown, Dr. Aindrila Chatterjee). On a protein level, ATP5A1 associates with multiple cytosolic ribosomal subunits and translation factors. ATP5A1 is translated in the cytosol and imported into the mitochondria. How these processes are coordinated between the cytosol and mitochondrial membrane interface is still unclear. Nuclear encoded mitochondrial precursor proteins can be imported post-translationally or co-translationally. In support of localised translation

near mitochondria, functional ribosomes have been found on the mitochondrial surface (Crowley and Payne, 1998; Gold et al., 2017). Though the exact mechanism of ATP5A1 import is still not fully understood, nascent ATP5A1 in any case, associates with the mitochondrial membrane for import into the mitochondria. ATP5A1-ribosome/translation factor association can be both a cause and/or consequence of this phenomenon.

As mentioned in section 4.2.3, some of these ribosomal proteins are also present in RNP granules, several components of which also interact with ATP5A1 (eg, G3BP2). Stress granules can also be present near mitochondrial membrane (Amen and Kaganovich, 2021), where the ATP5A1-RNA interaction takes place. Indeed, TOP-mRNAs can also localise to RNP/stress granules (Wilbertz et al., 2019). This sequestration of TOP-mRNA into granules is dynamic, metabolic state dependent and results in their translational regulation (Damgaard and Lykke-Andersen, 2011). RNP granules/stress granules (SG) are nucleated by the association of translationally regulated mRNAs with SG proteins like G3BP. Other components are recruited to these granules by a complex network of protein-protein and protein-RNA interaction (Ivanov et al., 2019). The composition and formation of RNP granules is also actively coordinated in response to cellular state. For example, they form in response to metabolic stressors, like the inhibition of glycolysis and mitochondrial respiration. This opens up a possible mechanism to tune ATP5A1's cytosolic RNA/protein interactions with its role in mitochondrial ATP synthesis.

Thus, we find that ATP5A1 and its cellular interactors -both proteins and RNAs- can be present near the mitochondria. It is possible that increased local concentration, (for e.g., in granules) can facilitate these interactions. The interacting protein factors can play a role in recruiting ATP5A1 to its target RNAs, which remains to be investigated. Studying the locality of ATP5A1- protein interactions (for e.g., G3BP2) in relation to ATP5A1-RNA interactions can clarify if indeed these components are brought together near the mitochondrial membrane. Proximity Ligation Assays (PLA) will be useful to assess this.

Do these interactions have physiological consequences? The effects of ATP5A1-RNA interactions can be multimodal. We are currently pursuing a contextual and reciprocal regulatory effect of ATP5A1-RNA association. On one hand, ATP5A1 can impact the fate of select target RNAs (RNA levels, localisation and translation), similar to the

regulatory effects of canonical RBPs. The potential of TOP-mRNAs to be translationally regulated underlines this hypothesis, which is discussed in the following section. In addition, a growing body of evidence attests to the regulatory potential of RNA on protein (Guiducci et al., 2019; Hentze et al., 2018; Horos et al., 2019; Huppertz et al., 2022). ATP5A1-RNA interactions can perhaps guide ATP5A1's function, predominantly affecting its import mitochondria. The site of these interactions, likely near the OMM, raises the possibility that these complexes can affect ATP5A1's entry into the mitochondria. These aspects are being independently investigated in our lab and in-cellulo experiments (Dr. Aindrila Chatterjee) indicate that RNA-binding deficient mutant of ATP5A1 impairs mitochondrial import.

3.4.2 Altered protein synthesis after ATP5A1 depletion

ATP5A1's role in de-novo cellular translation was assayed using QuaNCAT after ATP5A1 depletion. 591 proteins had altered protein synthesis in the absence of ATP5A1, while the global protein abundances were not dramatically altered. Due to the relatively short window of labelling (2h), the nascent pool of proteins that we detect is smaller compared to the total protein abundance. This can explain why only 58% of RNA targets directly bound by ATP5A1 are detected at protein level. Of this, ~32% is differentially regulated on ATP5A1 depletion. Targets upregulated on ATP5A1 depletion are frequently bound at 5' UTR and possess a TOP motif (except one which is downregulated). Transcripts that are bound at alternate sites, including 3'UTR show translational changes in both directions. We have to further investigate if these effects are indeed mediated by direct interactions or as a secondary response. It is important to note here that the depletion was done over the course of 96 h, which can lead to cellular adaptations. Use of RNA binding mutants of ATP5A1 and acute inhibition of ATP5A1's RNA binding activity can be illuminating here. ATP5A1 regulates a subset of its bound mRNAs, likely in a context-specific manner, which can be affected by additional cellular stimuli. For example, ATP5A1 depleted cells can be challenged in translation regulation in the context of stress and/physiological changes.

Whereas only a small pool of the directly bound targets are regulated translationally, the effects of ATP5A1 on global protein synthesis unveils a broader impact on cellular translational machinery. Potential changes in and effects of cellular energy levels on ATP5A1 depletion needs to be reconciled with these observations. It is possible that

this involves an alternate ATP-synthesis-independent function of ATP5A1 in the cytosol.

Part III

Chapter 4: Conclusions and Outlook

On RNA binding activity of MDH2 and ATP5A1

Chapter 4: Conclusions and outlook

Cellular metabolism maintains energy homeostasis and affect cellular signalling pathways through metabolites. Metabolic enzymes have to be therefore tightly regulated to match the changing energetic and biosynthetic demands of the cell (Chubukov et al., 2012; Metallo and Vander Heiden, 2013). A growing portfolio of metabolic enzymes have been identified to have a secondary ability to directly bind RNA, indicating a complex network connecting metabolism and gene regulation.

In this study, I looked at two mitochondrial proteins, MDH2 and ATP5A1, whose RNA binding activity has not been explored in detail. We investigated several aspects of this novel association, including possible regulatory outcomes and functional coordination strategies.

4.1 MDH2 as an RNA binding protein

I have validated MDH2's RNA binding activity and profiled RNAs bound by MDH2. MDH2 remarkably binds to cytosolic RNAs -especially tRNAs- primarily outside the mitochondria. On exploring how this compartmentalisation is regulated, I found the distinct metabolic environments of the compartments to be a potential driving factor. In the cytosol, active recruitment of MDH2 to its target RNAs can also mediate binding, a possibility I haven't studied here. MDH2's canonical MTS is dispensable for binding RNA, suggesting that mitochondrial targeting is not essential for RNA binding. Additionally, in conditions of altered mitochondrial metabolism, MDH2's RNA binding changes. For example, reduction of cellular NAD^+ (MDH2's co-factor) increased MDH2's RNA-association, simultaneously affecting cellular respiration. Thus, MDH2's RNA-binding activity could be inversely proportional to TCA function of MDH2. This is evidenced by MDH2's reduced RNA binding in differentiated cells, where TCA activity is higher. MDH2's RNA binding entertains two possibilities. First, MDH2 may perform a cytosolic RNA regulatory function when demand for its mitochondrial function is low. Second, RNA-association may actively regulate MDH2's availability to function in the mitochondria. These options can happen in concert and need not be mutually exclusive. Mechanistic consequences of MDH2-

RNA interactions have to be further studied to explore new avenues of regulation of this classical metabolic enzyme. RNA binding mutants can be further designed to probe the metabolic and regulatory consequences of MDH2-RNA interactions. Understanding how MDH2 binds RNA can help tease apart complex phenotypes caused by changes in MDH2's RNA binding activity and enzymatic function. This knowledge will aid in effective therapeutic targeting of MDH2 dysregulation in several diseases (Ait-El-Mkadem et al., 2017; Cascon et al., 2015; Liu et al., 2013; Lo et al., 2015).

4.2 ATP5A1's RNA binding activity

This part of the thesis contributed to ongoing research in the lab on ATP5A1's RNA binding activity (collaboration with Dr. Aindrila Chatterjee). I have characterized ATP5A1's RNA binding in-vitro, confirming its propensity to bind TOP-mRNAs. Evaluation of ATP5A1's protein interactions unveil a network of cytosolic translational factors and components of RNP complexes in addition to its known interactors in the mitochondria. Data from the lab (not shown) places the ATP5A1-RNA interactions near the outer mitochondrial membrane. Given that ATP5A1's protein interactors can also be in this locale, we imagine that RNA and protein interactions of ATP5A1 can be coordinated spatially. ATP5A1's possible recruitment to these interaction centres by the associated protein factors can be evaluated in the future. ATP5A1-RNA interactions can result in regulation of bound RNAs by ATP5A1 and a reciprocal effect of RNA on ATP5A1's function. In this thesis, I performed experiments to probe the regulatory potential of ATP5A1 towards bound RNAs. Since ATP5A1 binds translationally regulated TOP-mRNAs, we evaluated global translational efficiency on ATP5A1 depletion. A fraction of the ATP5A1 bound targets show altered protein synthesis in the absence of ATP5A1. Further experiments are required to estimate if these are indeed direct effects. Scoring this phenotype in conditions of stress -where TOP-mRNAs are translationally regulated- can be informative with regard to this regulatory potential.

4.3 Broader perspectives

Over the years, the RNA binding function attributed to enzymes of intermediary metabolism have opened new avenues to investigate interconnections between cellular bioenergetics and gene-expression changes. Early studies in this field identified metabolic enzymes as regulators of RNA expression - either of their own mRNAs /isoforms (e.g., TS, DHFR, SHMT), functionally related mRNAs (IRP1) or other RNAs (GAPDH) (Chu et al., 1991; Ercikan et al., 1993; Hentze et al., 2010; Liu et al., 2000; White and Garcin, 2016). Recent studies have also illustrated that metabolic enzymes can be directly regulated by RNA as is the case for SHMT1 and ENO1 (Guiducci et al., 2019; Huppertz et al., 2022).

This study, together with unpublished experiments from the lab, have found that mitochondrial enzymes MDH2 and ATP5A1 bind RNA outside the mitochondria, indicating the importance of cellular location in dictating their RNA-interactions. This could be a point of consideration in the study of other RNA binding mitochondrial metabolic enzymes. Some of them (e.g., glutamate dehydrogenase, enoyl coA hydratase, hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex β subunit (HADHB) have been co-purified with nuclear encoded mRNAs (Ciesla, 2006), highlighting the need for an unbiased analysis of where they bind their target RNAs.

Metabolite dependency of an enzyme's RNA binding might result in multifunctional outcomes. For enzymes like ENO1 and SHMT1, RNA interferes directly with enzymatic activity by hindering the binding of its substrates. In the case of MDH2, altered RNA binding dynamics in the presence of substrates/co-factor, might also serve to compartmentalize RNA binding. As this thesis illustrates in the case of MDH2 and based on indications from other enzymes like ENO1 and GAPDH, the RNA binding function of metabolic enzymes may be coordinated with processes altered in cellular metabolic energy status. This can ensure a contextual regulation of their enzymatic function and/or RNA regulatory functions. In fact, RNA binding of metabolic enzymes can be regulated by a combination of multiple factors including substrate/cofactor levels, protein posttranslational modifications such as phosphorylation/acetylation, enzyme multimerization and protein localisation. These factors can vary depending on metabolic state/requirements underlining the strong link between cellular metabolic

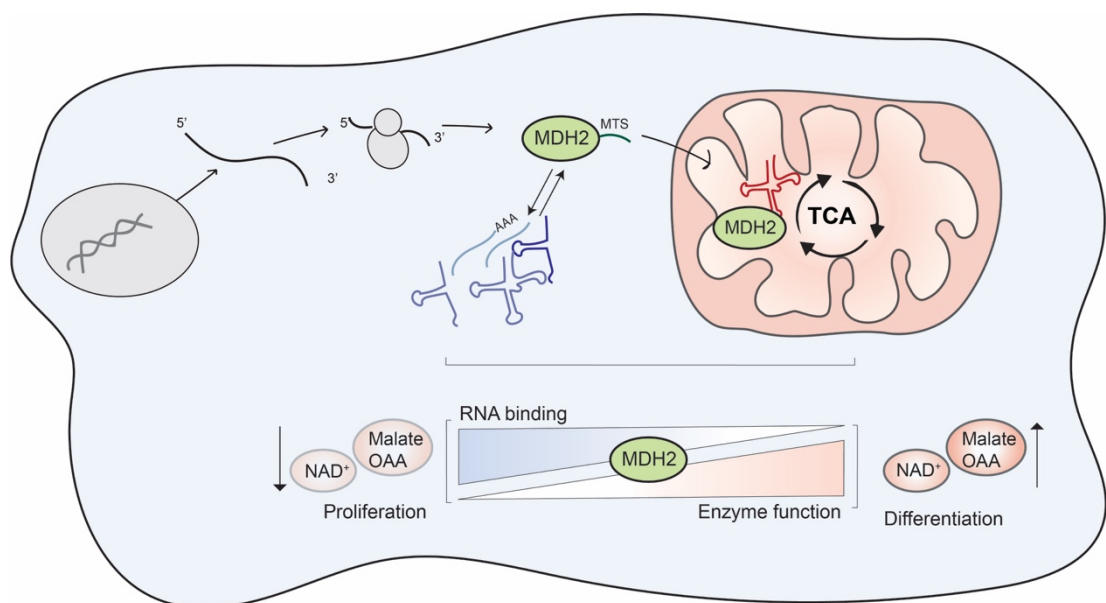
state and RNA-binding regulation of metabolic enzymes. Thus, where and how metabolic enzymes bind RNA could be dynamically determined by factors including environmental and growth stimuli that in turn dictates metabolic status.

So far, only a few of these metabolic enzymes have been analysed in depth with regard to their RNA binding function. While our current knowledge implies some common underlying principles of metabolic enzyme-RNA interactions, functional context-dependent consequences can differ from enzyme to enzyme, warranting a case-by-case exploration of the RNA binding function of each enzyme. Application of multi-omics approaches to study the RNA-targets, interaction partners of metabolic enzymes and their functional effects (both metabolic and gene expression changes) will bring us closer to having an integrated view of the RNA binding network of metabolic enzymes and its consequences. This can lead to a more comprehensive understanding of various cellular processes/states intricately linked to metabolism including cell differentiation, uncontrolled proliferation such as in cancer cells, cell-cycle regulation and nutrient uptake. In addition to transcriptional and post-transcriptional mechanisms to coordinately regulate metabolic enzymes in connected pathways, RNA interactions of these enzymes can be a regulatory factor for synchronized changes in metabolic activity.

Synopsis of main findings

4.4 MDH2 as an RNA binding metabolic protein

- MDH2 is identified as an RNA binding protein in several species including several human cell lines. I validated MDH2's RNA binding activity with orthogonal biochemical assays.
- MDH2 binds mainly cytosolic RNAs (tRNAs, mRNAs, ncRNAs) and some mitochondrial tRNAs as revealed by eCLIP.
- MDH2 binds RNA predominantly outside of the mitochondria. Mitochondrial targeting may be dispensable for RNA binding as indicated by mutational analysis of MDH2's targeting signals.
- MDH2's RNA binding is inhibited at high substrate (malate and OAA), cofactor (NAD^+) concentrations and stimulated at lower concentrations of these metabolites (in-vitro EMSA experiments).
- MDH2's RNA binding is sensitive to cellular metabolic state.
 - Decrease of cellular NAD(H) levels leads to enhanced MDH2-RNA interactions.
 - In hepatocyte-like differentiated cells, MDH2 binds less RNA than in more proliferative hepatocarcinoma cells. Differentiated cells have enhanced mitochondrial respiration pointing to an inverse correlation between MDH2's mitochondrial enzymatic function and RNA binding function.



4.5 ATP5A1 as an RNA binding metabolic protein

- Previous eCLIP analysis revealed ATP5A1's preference for TOP motif mRNAs. ATP5A1's ability to bind TOP motif containing RNA was confirmed in this study using in-vitro EMSA assays.
- Depletion of ATP5A1 alters the protein synthesis of a subset of ATP5A1's RNA interaction partners. In addition, a broader impact on the synthesis of cellular translational machinery components (ribosomal subunits, translation factors) and growth signalling pathways was observed.
- Mass spectrometry based ATP5A1 protein interactome analysis revealed ATP5A1's associations with components of cellular translational machinery (ribosomal subunits, translation factors) and components of RNP complexes/granules in addition to its known interactors in the mitochondria. These factors may be important for coordinating ATP5A1-RNA interactions and its consequences.

Part IV

Chapter 5: Materials and Methods

Chapter 5: Materials and Methods

5.1 Materials

5.1.1 Antibodies

Target	Species	Source	Dilution
MDH2	Rabbit	Proteintech (15462-1-AP)	1:1000 in WB
MDH2	Goat	Everest Bio (EB13027)	1:1000 in WB
FLAG	Mouse	Sigma-Aldrich (F1804)	1:2000 in WB 1:500 in IF
ATP5A1	Rabbit	Proteintech (14676-1-AP)	1:1000 in WB
ATP5A1	Mouse	Abcam (ab14748)	1:1000 in WB
ATP5C1	Rabbit	Proteintech (10910-1-AP)	1:1000 in WB
DHX9	Rabbit	Abcam (ab26271)	1:5000 in WB
Tom20	Rabbit	CST (42406)	1:1000 in WB
Tom70	Rabbit	Proteintech (14528-1-AP)	1:200 in IF
G3BP2	Rabbit	Proteintech (16276-1-AP)	1:1000 in WB
IGF2BP1	Rabbit	Proteintech (22803-1-AP)	1:1000 in WB

5.1.2 Enzymes

Enzyme	Source
EcoRI HF	New England Biolabs
XbaI HF	New England Biolabs
PacI HF	New England Biolabs
NotI HF	New England Biolabs
DpnI	New England Biolabs
T4 Polynucleotide Kinase	New England Biolabs
T4 RNA Ligase 1 (ssRNA Ligase), High Concentration	New England Biolabs
T7 polymerase	EMBL Pepcore
Proteinase K	Thermo Fisher Scientific, New England Biolabs
FastAP Thermosensitive Alkaline Phosphatase, 1 U/μl	Thermo Fisher Scientific

TURBO DNase, 2 U/μl	Thermo Fisher Scientific
RNase I, cloned, 100 U/μl	Thermo Fisher Scientific
RNase A	Thermo Fisher Scientific
RNase T1	Thermo Fisher Scientific

5.1.3 Kits

Name	Source
AffinityScript reverse transcriptase	Agilent
QIAprep Spin Miniprep Kit	QIAGEN
QIAGEN Plasmid Midi and Maxi Kit	QIAGEN
QIAquick PCR Purification Kit	QIAGEN
QIAquick Gel Extraction Kit	QIAGEN
Qiagen MinElute gel extraction kit	QIAGEN
Quick-RNA Miniprep	Zymo
SuperScript IV reverse transcriptase	Thermo Fisher Scientific
Phusion High Fidelity PCR kit	New England Biolabs
FastStart PCR master	Roche
Lipofectamine 3000	Thermo Fisher Scientific
Lipofectamine RNAiMAX reagent	Thermo Fisher Scientific
NAD/NADH Quantitation Kit	Sigma-Aldrich
MEGAscript™ T7 transcription kit	Invitrogen
Click-iT™ Protein Enrichment Kit for capture of azide modified proteins	Invitrogen
The Qubit® dsDNA HS Assay	Invitrogen

5.1.4 Chemicals/Reagents

Name	Source
Agencourt AMPure XP beads	Beckman Coulter
Agarose	Sigma-Aldrich
Aminoguanidine hydrochloride	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
BSA	Sigma-Aldrich

Blasticidin	Invivogen
Chloroacetamide	Sigma-Aldrich
Complete EDTA-free Protease Inhibitor Cocktail	Roche
CuSO ₄	Sigma-Aldrich
Dialyzed FBS	Thermo Fisher Scientific
Dithiothreitol	Biomol
DMSO	Sigma-Aldrich
DMEM 1g/L glucose	EMBL Kitchen
DMEM without methionine, arginine, lysine	AthenaES
1kb DNA ladder	New England Biolabs
100bp DNA ladder	New England Biolabs
DSP	Thermo Fisher Scientific
Dynabeads Protein G magnetic beads	Thermo Fisher Scientific
Dynabeads anti-rabbit magnetic beads	Thermo Fisher Scientific
Dynabeads MyOne Silane magnetic beads	Thermo Fisher Scientific
Ethidium Bromide	Sigma-Aldrich
ExoSAP-IT™ PCR Product Cleanup Reagent	Thermo Fisher Scientific
FLAG M2 beads	Sigma-Aldrich
FLAG peptide	Sigma-Aldrich
FK866	Sigma-Aldrich
Fluorescent RNA oligos	Sigma-Aldrich, IDT
Glycerol	Merck
HEPES	Biomol
Hydroxylamine solution	Sigma-Aldrich
Hygromycin	Invivogen
IPTG	Invitrogen
Iodoacetamide	Sigma-Aldrich
Immobilon Western HRP substrate	Millipore
Instant Blue stain	Abcam
Kanamycin	Sigma-Aldrich
KCl	Sigma-Aldrich
K ₂ HPO ₄	Sigma-Aldrich

KH ₂ PO ₄	Sigma-Aldrich
L-AHA	Jena Bioscience
L-glutamine	Thermo Fisher Scientific
Lysozyme	Thermo Fisher Scientific
Milk powder	Frema Reform
NAM	Sigma-Aldrich
Na-Acetate	Sigma-Aldrich
Na-Ascorbate	Sigma-Aldrich
Na ₂ HPO ₄	Sigma-Aldrich
NaH ₂ PO ₄	Sigma-Aldrich
NaCl	Merck
Na-deoxycholate	Sigma-Aldrich
(NH ₄)HCO ₃	Carl Roth
NP-40 (Igepal CA630)_	Sigma-Aldrich
NTP Set	Thermo Fischer Scientific
NuPAGE® LDS Sample Buffer	Thermo
OptiMEM	Gibco
Penicillin-Streptomycin Mix	Sigma-Aldrich
Pierce Anti-HA Magnetic Beads	Thermo Fischer Scientific
Protein ladder; Precision Plus Protein Dual Color	Bio-Rad
NucBlue Fixed Cell ReadyProbes Reagent	Thermo Fischer Scientific
Prediluted BSA standard	Thermo Fischer Scientific
ProLong Diamond	Thermo Fischer Scientific
RNasefree water	Ambion
RNasin Plus	Promega
RNasin	EMBL pepcore
SDS	Serva Elektrophoresis
Sera-Mag Beads	Thermo Fischer Scientific
Sequencing grade modified trypsin	Promega
SILAC amino acids (Lysine/Arginine)	Silantes
Strep-Tactin Superflow high-capacity resin	IBA lifesciences

SureBeads Protein A/Protein G magnetic beads	Bio-Rad
SyBR Gold	Invitrogen
SyBR Safe	Invitrogen
THPTA	Jena Bioscience
TMT6plex	Thermo Fisher Scientific
TMTpro	Thermo Fisher Scientific
Tris Base	Sigma-Aldrich
Trypsin	Thermo Fisher Scientific
Triton X-100	Merck
0.5M TCEP	Sigma-Aldrich
Tween20	Roth
Zeocin	Invivogen

5.1.5 Consumables

Name	Source
Amicon Ultra-0.5 Centrifugal Filter Unit	Merck millipore
Bio-Spin® P-6 Columns	Bio-Rad
Trans-Blot Turbo Mini 0.2 µm PVDF Transfer Packs	Bio-Rad
4-15% TGX gradient gels	Bio-Rad
Microplates	Greiner Bio-One
DNA/RNA LoBind Tubes	Eppendorf
Cell culture dishes	Nunc Thermo
Stericup Quick Release-HV Sterile Vacuum Filtration System	Merck Millipore
Cryogenic storage vials	Nunc
PCR tubes 0.2ml	Biozym
Reaction tubes 1.5ml/2ml	Eppendorf

5.1.6 Buffers and solutions

Name	Composition
Cell lysis and immunoprecipitation	

Co-IP lysis buffer	20mM HEPES, 150 mM NaCl, 2mM MgCl ₂ , 0.5% NP40
RIPA buffer	50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% Na-deoxycholate, fresh complete protease inhibitor cocktail
RIPA HS	50 mM Tris/HCl, pH 7.5, 500 mM NaCl, 1% NP40, 0.1% SDS, 0.5% Na-deoxycholate, fresh complete protease inhibitor cocktail
PNK buffer	50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.5% NP40, 10mM MgCl ₂ , fresh 5mM DTT, fresh complete protease inhibitor cocktail
TE buffer (for RNase digestion of 2C)	50 mM Tris HCl 2mM EDTA
SDS-PAGE and Western blot	
10X Laemelli running buffer	0.25M Tris base, 1.924 M Glycine, 0.034 M SDS
0.05% PBST	500 µl Tween20 in 1000ml PBS
Blocking buffer	5% milk in 0.05% PBST
Immunofluorescence (IF)	
Permeabilization buffer	0.2%Triton X-100 in PBS
IF blocking buffer	2.5% BSA in 0.05% Triton X-100 in PBS
IF wash buffer	0.05% Triton X-100 in PBS
Mitochondrial fractionation	
Buffer B	20 mM HEPES pH 7.4-7.6, 220 mM Mannitol, 70 mM Sucrose, 1 mM EDTA
Protein purification	
Buffer W	100 mM Tris/HCl, pH 8.0 150 mM NaCl, 1 mM EDTA
Buffer E (elution buffer)	100 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin
Buffer R (regeneration buffer)	100 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1 mM HABA (hydroxy-azophenyl-benzoic acid) pH 8.0

Protein storage buffer	10mM Tris, 150mM NaCl, 5% glycerol
In-vitro RNA binding assays	
EMSA binding buffer	10mM Tris-HCl pH 7.5, 150mM NaCl, 20% glycerol, 0.5mM DTT, 5mM MgCl ₂ , 10ng/ul BSA
10X EMSA gel running buffer	0.34M Tris-HCl, 0.66M HEPES, 1mM EDTA
10X Transcription Buffer	400 mM Tris pH 8.0, 380 mM MgCl ₂ , 10 mM Spermidine pH 7.4, 0.1% Triton X-100
eCLIP	
FastAP buffer	10 mM Tris-HCl pH 7.5, 5 mM MgCl ₂ , 100 mM KCl, 0.02% Triton X-100
Wash buffer	20 mM Tris-HCl pH 7.5, 10 mM MgCl ₂ , 0.2% Tween20
5X PNK buffer	350 mM Tris-HCl pH 6.5, 10 mM MgCl ₂ , Fresh 1 mM DTT
PK buffer	100 mM Tris-HCl pH 7.5, 50mM NaCl, 10 mM EDTA
Urea/ PK buffer	Dissolve 420 mg Urea in 500 µl PK buffer, then add PK buffer to final volume of 1mL

5.1.7 Equipment

Name	Source
Microplate reader	TECAN
Transblot semi dry blotting system	Bio-Rad
ChemiDoc imaging system	Bio-Rad
Spectrolinker XL-1500	Spectronics
Bioruptor Plus	Diagenode
Typhoon FLA 9500	GE healthcare
Nanodrop 2000 Spectrophotometer	Thermofischer Scientific

Pipettes	Gilson
FV3000 microscope	Olympus
PCR thermal cycler	Bio-Rad
SpeedVac Vacuum concentrator	Eppendorf
Seahorse XF analyzer	Agilent

5.1.8 Software

Name	Developer
Graphpad Prism 9	Graphpad
ImageLab	Bio-Rad
Cytoscape 3.9.1	Institute of systems biology
Endnote X 9.2	Clarivate Analytics
Fiji	Based on ImageJ from NIH
Adobe Illustrator CC	Adobe Systems
Integrative Genomics Viewer	Broad Institute
R Studio	R Studio, inc
Seahorse analytics	Agilent
Pymol 2.5.4	Schrödinger, Inc.

5.1.9 Plasmids

Name	Use
pOGG44	Stable inducible cell line generation
pCDNA-FRT-TO-3X-FLAG-HA wtMDH2 and variants	Mammalian expression vectors
petM22 V5-Strep II- eGFP	Protein purification construct
petM22 V5-Strep II- wtMDH2	Protein purification construct
petM22 V5-Strep II- wtATP5A1	Protein purification construct

5.1.10 Oligos

RNA oligos

Name	Sequence/ Source
MDH2 SMARTpoolsiRNAs	Dharmacon ON-TARGET plus(L-008439-01-0005)
eCLIP oligos	
RNA_X1A	/5Phos/rArUrArUrArGrGrNrNrNrNrNrArGrArUrCr GrGrArArGrArGrCrGrUrCrGrUrGrUrArG/3SpC3/
RNA_X1B	/5Phos/rArArUrArGrCrArNrNrNrNrNrArGrArUrCr GrGrArArGrArGrCrGrUrCrGrUrGrUrArG/3SpC3/
RNA_X2A	/5Phos/rArArGrUrArUrArNrNrNrNrNrArGrArUrCr GrGrArArGrArGrCrGrUrCrGrUrGrUrArG/3SpC3/
RNA_X2B	/5Phos/rArGrArArGrArUrNrNrNrNrNrArGrArUrCr GrGrArArGrArGrCrGrUrCrGrUrGrUrArG/3SpC3/
RiL19	/5phos/rArGrArUrCrGrGrArArGrArGrCrGrUrCrGr UrG/3SpC3/
EMSA oligos	
Lys TTT 3-5 probe	UGAGGGUCCAGGGUUCAAGUCCUGUUCGG GCG /3Cy5Sp/
Arg TCT 3-2 probe 2	GGCUCUGUGGCGCAAUGGAUAGCGCAUUGG /3Cy5Sp/
Arg TCT 3-2 probe 2	CUUCUAAUUCAAAGGUUGUGGGUUCGAGUC CCACCAGAGUCG /3Cy5Sp/
Non-specific RNA probe (mutRPS23 5' UTR)	AUAUUUAGCUCAGGCCCGUGGCGCC /3Cy5Sp/
RPL13 target probe	CUUUCCGCUCGGCUGUUUUCUGCGCAGGA GCCGCA /3Cy5Sp/
RPS6 target probe	CUCUUUUCGUGGGCGCCUCGGAGGCGUUCA GCUGCUUCAAG /3Cy5Sp/
RPL13 scrambled probe	UCUCUCCGUUCUGCUCGUCGUCGCGCAGAG AGUCGC /3Cy5Sp/
RPS6 scrambled probe	UGUCUCAGCACUGUCGACGUGUCACGCUCG UCUCGUGCGUG /3Cy5Sp/

DNA oligos

Name	Sequence
eCLIP oligos	
AR17:	ACACGACGCTCTTCCGA
rand3Tr3	/5Phos/NNNNNNNNNNAGATCGGAAGAGCACACGTCT G/3SpC3/
PCR_F_D501	AATGATACGGCGACCACCGAGATCTACACTATAGCC TACACTCTTTCCCTACACGACGCTCTTCCGATCT
PCR_F_D504	AATGATACGGCGACCACCGAGATCTACACGGCTCTG AACACTCTTTCCCTACACGACGCTCTTCCGATCT
PCR_F_D505	AATGATACGGCGACCACCGAGATCTACACAGGCGAA GACACTCTTTCCCTACACGACGCTCTTCCGATCT
PCR_F_D506	AATGATACGGCGACCACCGAGATCTACACTAATCTT AACACTCTTTCCCTACACGACGCTCTTCCGATCT
PCR_F_D507	AATGATACGGCGACCACCGAGATCTACACCAGGACG TACACTCTTTCCCTACACGACGCTCTTCCGATCT
PCR_R_D701	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTG ACTGGAGTTCAGACGTGTGCTCTTCCGATC
PCR_R_D703	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTG ACTGGAGTTCAGACGTGTGCTCTTCCGATC
PCR_R_D705	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC
PCR_R_D708	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC
PCR_R_D710	CAAGCAGAAGACGGCATACGAGATTTGCGGAGTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC
PCR_R_D711	CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTG ACTGGAGTTCAGACGTGTGCTCTTCCGATC
PCR_R_D712	CAAGCAGAAGACGGCATACGAGATCTATCGCTGTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC
Primers for cloning	
MDH2 Δ 2-24 Forward primer	ACCATGGCTAAAGTAGCTGTGCTAGGGGCCTCTGGA GG

MDH2 Δ 2-24 Reverse primer	GCTACTTTAGCCATGGTGGCTTACTTAATTAACAGCT TAAGTTTAAACGCTAG
MDH2 Δ 2-53 Forward primer	ACCATGACATTGTACGACATCGCGCACACACCCGGA
MDH2 Δ 2-53 Reverse primer	GTCGTACAATGTCATGGTGGCTTACTTAATTAACAGC TTAAGTTTAAACGCTAGAG
MDH2 Δ 101- 255 Forward primer	TATTCCGGCCCGCTTTGTCTTCTCCCTTGTGGATGCA ATGAATG
MDH2 Δ 101- 255 Reverse primer	GCGGGCCGGAATAACTACCACATCACAACCTTTCAG GC
MDH2 1-168 Forward primer	CAAAATCTTCGGCGCGGCCGCTGACTACAAGGACCA CG
MDH2 1-168 Reverse primer	CGCGCCGAAGATTTTGTGTTGGGGTTGTACACTCCATGC TT

5.2 Methods

General molecular biology methods

5.2.1 Bacterial transformation

Chemically competent *Escherichia coli* TOP10 or BL21 Rosetta strain cells were transformed with appropriate plasmids for cloning/ plasmid multiplication and protein purification respectively. Cells were incubated with the relevant DNA (100-300 ng) for 20 minutes on ice. This was followed by a heat shock of 30-35 seconds at 42°C and a 5 min incubation on ice. Transformed cells were grown in antibiotic-free LB medium for 45 min - 1 hour to allow the expression of the antibiotic-resistance gene from the plasmid. Transformed bacteria were selected by plating them on LB-Agar plates with the selection antibiotic and overnight growth (~16 hours) at 37°C.

5.2.2 Plasmid preparation

For plasmid amplification, bacterial cells were transformed with the plasmid of choice. Single bacterial colonies were picked and used to inoculate LB-media with the selection antibiotic (4 ml for miniprep/ 70 ml for midiprep/ 500 ml for maxiprep). The culture was grown overnight (~16 hours) at 37°C with rotation at 180 rpm. Plasmids were purified from the bacterial cultures using QIAGEN plasmid mini/midi/maxi prep kits according to manufacturer's instructions.

5.2.3 Polymerase chain reaction (PCR)

DNA was amplified in PCRs with the FastStart PCR mix (Roche) or Phusion polymerase (NEB). The latter was specifically used for site directed plasmid mutagenesis as mentioned below. For routine PCRs, 10-20ng DNA was mixed in a solution with final concentrations of 0.5 μ M of forward and reverse primer, 200 μ M dNTPs and 1X FastStart PCR mix. The PCR reaction was initially denatured at 95°C for 4 min, followed by 30 cycles of denaturation (95°C, 30 sec), primer annealing (55°C, 30 sec) and elongation (72°C, ~1kb/min).

5.2.4 Agarose gel electrophoresis

PCR products or restriction digestion products were resolved on 1% agarose gels in 1X TBE buffer at 100V for about 30 min -1 hour. SYBR-Gold/ SYBR- safe was added to the samples for visualization.

5.2.5 Cloning based on restriction digestion and ligation

For pCDNA 3.1 FRT-TO-3X FLAG-HA constructs, the parent plasmid containing the tags and selection marker was obtained from Dr. Aindrila Chatterjee. Sequences of wtMDH2, MDH2 H70K74A, MDH2 K78A, MDH2 K105A were ordered as gene fragments from Thermo Fisher Scientific with added restriction sites for PacI and NotI at the 5' end and 3' end respectively. The vector and inserts were digested with PacI and NotI according to the manufacturer's instructions at 37°C for 1 hour with rotation. The enzymes were heat inactivated according to the manufacturer's instructions. Digested vector DNA was resolved on agarose gels and purified from the gel with gel extraction kits (QIAGEN). Insert DNA was purified using PCR purification kits (QIAGEN) to remove digested nucleotides/small oligonucleotides.

Ligation reactions with the purified restriction digestion products were set up with 1 U T4 DNA ligase (NEB) in a 10 μ l reaction with final concentration of 1X T4 DNA

Ligase buffer. A 3:1 molar ratio of insert: vector was used. Reaction was performed at room temperature (~22°C) for 1 hour. 5 µl of the ligation reaction was used in bacterial transformations for clonal selection and amplification under selection for the appropriate antibiotic.

petM22-V5-StrepII protein purification constructs were prepared in a similar fashion using restriction sites for XbaI and EcoRI. Sequences for eGFP, human MDH2 and human ATP5A1 were purchased as gene fragments from IDT with the V5-StrepII tag sequence at the 3' end. XbaI and EcoRI sequences were included in the 5' end and 3' end of the sequences respectively. petM22 parent plasmid was gifted from EMBL PEPCore facility.

5.2.6 Site directed mutagenesis

Mutations, insertions and deletions of DNA were introduced into pCDNA 3.1 plasmids by PCR-based site directed mutagenesis using NEB Phusion polymerase. Long mutagenesis primers were designed to have minimum 18 bp complementarity with the template DNA on each side of the mutagenesis location (mutation/insertion/deletion). In case of insertions, the sequence was included in the primer in between the template-complementary sequences. The primer template overlap was kept higher than the primer-primer overlap by including non-overlapping overhangs in the forward and reverse primer. The PCR reaction contained 50 ng of the template plasmid, 0.5 µM of forward and reverse primer, 200 µM dNTPs and 1 U of Phusion polymerase in 1X HF buffer. The PCR reaction was initially denatured at 98°C for 3 min, followed by 18 cycles of denaturation (98°C, 30 sec), primer annealing (calculated according to the T_m of the extended primer, 1 min) and elongation (72°C, ~ 30sec/kb). After the PCR, the original template plasmid was digested with Dpn I by incubation at 37°C for 1 hour. The undigested PCR-amplified plasmid was purified using a PCR purification kit (QIAGEN) and eluted in 20 µl water. 5 µl of this product plasmid elution was transformed into Ecoli strains for clonal selection and amplification under selection for the appropriate antibiotic.

5.2.7 DNA and RNA amount measurement

Concentration of DNA/ RNA preparations were estimated by measuring absorption at 260 nm using a UV-Vis spectrophotometer (NanoDrop 2000).

General biochemical methods

5.2.8 Preparation of cell lysates

Cells were first washed with ice cold PBS. Cells were scraped after adding appropriate amount of lysis buffer and transferred to Eppendorf tubes. The lysate was incubated on ice for 15 minutes, followed by sonication using a Bioruptor Plus device. The lysates were sonicated for 10 cycles (sonication cycle: 30 sec ON, 30 sec OFF) and cleared by centrifugation at 10000 g for 15 min. The supernatant was collected, snap-frozen and stored at -80°C till further use.

5.2.9 Protein quantification

The total protein concentration was quantified using the Pierce 660 reagent in a microplate assay format, following the manufacturer's instructions. 150µl of the Pierce 660 reagent was added in each well. To generate a BSA standard curve, prediluted BSA standards were added to these wells in the range of 2-10 µg. 2-8 µl of the sample was pipetted into each well making sure the intensity of the colour formed was within the range of the BSA standard. Equal volume of the lysis buffer was also measured to control for the background absorbance. Absorbance at 660 nm was measured using a TECAN reader, after briefly shaking the contents of the plate and incubating at 25°C for 5 minutes for the colour to develop. After plotting a standard curve for BSA, the concentration of proteins in the sample lysate was estimated by extrapolation.

5.2.10 SDS-PAGE and western blotting

Protein samples were resolved according to molecular weight using SDS-PAGE. Samples were prepared for electrophoresis by addition of 4X NuPAGE loading buffer supplemented with TCEP as a reducing agent. The samples were heated at 70°C for 10 min. 4-15% Tris-Glycine (precast gradient gels) were used for protein separation with Laemmli buffer. Proteins were transferred to PVDF/nitrocellulose membranes using the Transblot semi dry blotting system (Bio-Rad). Transfer was done at 25V for 7 min. The membranes were incubated with the blocking buffer (5% milk in 0.05% PBST) for 30 min to 1 h at room temperature. This was followed by incubation with primary antibodies against the target protein at 4°C o/n or 3h at room temperature on a rotator. The primary antibody was diluted in blocking buffer. After primary antibody incubation, the membrane was washed 3 times with 0.05% PBST with 5-minute incubations. After washing off the unbound primary antibody, the membrane was incubated in HRP-conjugated secondary antibody diluted in the blocking buffer. The incubation was done at room temperature for 30 minutes. The 3X washes with 0.05%

PBST was repeated. For detection, Immobilon Western HRP substrate peroxide solution was used. The membranes were imaged using Bio-Rad ChemiDoc imaging system.

5.2.11 Total protein staining

After SDS-PAGE, total protein bands were visualized by incubating the gel with Instant Blue stain (Abcam) for a minimum of 30 min. The gel was washed with ddH₂O. (typically 3 times 20 min) until the unbound stain is washed away. The gel was then imaged using Bio-Rad ChemiDoc imaging system.

5.2.12 Silver staining

After SDS-page, samples were visualized with silver staining (e.g., co-immunoprecipitation samples to be analysed by mass-spectrometry). The gel was first fixed by incubation in a fixing solution containing 50% methanol and 5% acetic acid for 20 min. Then, the gel was washed sequentially in 50% ethanol and 30% ethanol for 5 min each, followed by a 10 min wash in water. The gel was incubated in freshly prepared 0.02% sodium sulphate solution. This was followed by 3 times 20 min washes of the gel with water. Then, silver solution (6 mM silver nitrate, 0.0185% formaldehyde) was added to the gel for 20 minutes. After this incubation, the gel was rinsed 3 times 20 min with water. To develop the stain, a developer solution (2% sodium carbonate (anhydrous), 0.0185% formaldehyde, 0.0004% sodium thiosulfate) was added to the gel till the desired intensity of bands was reached. The reaction was stopped by the addition of 5% acetic acid. The gel was stored in water till imaging.

5.2.13 Protein purification

The protein purification constructs (petM22) were expressed in BL21 Rosetta (DE3) cells with either autoinduction or addition of 1 mM IPTG. First, bacteria transformed with the respective constructs were inoculated in 25 ml LB media containing the selection antibiotic Kanamycin and grown overnight at 37°C. This serves as the preculture for the protein induction and expression.

For MDH2 and eGFP constructs, proteins were induced by growth in autoinduction media. In this case, the pre-culture was diluted 1:100 in Terrific Broth (TB) supplemented with 1.5% lactose, 0.05 % glucose, 2 mM MgSO₄ and grown at 37°C till the OD reached 0.7-0.8. The culture was then incubated at 18°C overnight with shaking at 180 rpm.

For ATP5A1, protein production was induced with IPTG. For this, the pre-culture was diluted in LB media and grown at 37°C till the OD reached 0.7-0.8. IPTG is added to a final concentration of 1 mM and flasks are transferred to an 18°C incubator for growth overnight.

The cells were lysed by sonication in Buffer W supplemented with complete EDTA free protease inhibitors and 250 µg/ml lysozyme. The cell suspension was sonicated for 7 minutes at 50% duty, 70% power on wet ice. After centrifugation at 15000g for 15 min to remove debris, the supernatant was transferred to a polypropylene column packed with Strep-Tactin Superflow high-capacity resin. As the lysate moves through the column with gravity flow, tagged proteins bind to the Streptactin beads. The unbound protein was washed off with Buffer W. The washes were repeated 5 times. The protein was eluted with Buffer E. The fractions with the highest concentration of the protein were pooled and dialyzed overnight with the Storage Buffer (see Table of buffers). The concentration of the samples was estimated by UV-Vis spectroscopy (absorbance at 280 nm) as well as against defined BSA standards in SDS-PAGE analysis. If the concentration of samples were too low, the volume of the samples was reduced using Amicon Ultra-0.5 Centrifugal Filter Unit for increased concentration. The purified protein was aliquoted and snap-frozen with liquid N₂. The single-use aliquots were stored at -80°C. The tag was left intact for in-vitro experiments.

5.2.14 Synthesis and purification of RNAs

Full length tRNAs were in-vitro transcribed from DNA oligos. In-vitro transcription was either performed with MEGAshortscript™ T7 transcription kit (Invitrogen) according to the manufacturer's instructions or using T7 polymerase produced at EMBL Pepcore facility. The ssDNA oligos were ordered from Sigma-Aldrich. The oligos contained cDNA sequence of the full length tRNAs with a T7 promoter sequence in reverse at the 5' end. The sequence of the T7 promoter was also ordered as a DNA oligo to prime the in-vitro transcription reaction. To facilitate detection of the in-vitro transcribed RNAs, the RNA was fluorescently labelled by including fluorescein conjugated UTP in the transcription reaction. The T7 promoter oligo was annealed to the template DNA in the T7 transcription buffer by heating at 90°C for 3 minutes and gradually cooling down to 5°C in stepwise increments. Following annealing, the transcription was initiated by adding 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.375 mM UTP, 0.125 mM fluorescein-UTP, 10 mM DTT and the T7 RNA

polymerase to the template in the T7 transcription buffer. The reaction was incubated at 37°C for 4 hours. The template DNA was digested with Turbo DNase after supplementing the reaction with CaCl₂. The DNase digestion at 37°C for 15 min was followed by ProteinaseK treatment at 37°C for 30 min. The reaction was stopped by adding 1/10 volume of 0.5 M EDTA to the reaction. In-vitro transcribed RNAs were precipitated from the reaction by overnight incubation with 1 ml 3 M sodium acetate and 3.5 volumes of ethanol at -20°C. The RNA is pelleted by centrifugation at 6500 rpm for 30 min and further purified by a preparative gel electrophoresis (7 M urea, 1X TBE, 6% polyacrylamide). RNA bands of the specific size were cut out from the gel, and incubated in 0.3 M Sodium Acetate, pH 5.2 solution at 4°C for an hour. After filtering the solution, the RNA was further precipitated with ethanol. The RNA pellet after centrifugation was washed with 80% ethanol. After resuspension in the appropriate amount of water, the RNA is stored at -80°C till further use. If there was predominantly only one band in the gel, transcription reactions were purified by Biospin6 Tris columns (Bio-Rad). For binding assays, the 25-35mers containing the binding sites of the target RNAs were ordered from Sigma-Aldrich/IDT with the 3' end fluorescently labelled with Cy3/Cy5. The RNA was reconstituted to 100 µM concentration in 10 mM Tris buffer with pH 8.5 and stored at -20°C in aliquots. 10 nM RNA was used for most binding experiments.

5.2.15 Electromobility shift experiments

The binding reaction was performed in EMSA binding buffer supplemented with 0.5 mM DTT, 0.01 µg/ul BSA, 5 mM MgCl₂ at 25°C for 20 minutes. The protein-RNA complexes were resolved by native polyacrylamide gel electrophoresis (4-15% Tris-Glycine gradient gels with 1X EMSA running buffer) at 100V for 75 min. The gel was imaged on Typhoon FLA-9500.

Cell culture methods

5.2.16 Cell culture

Huh7 cells were cultured in DMEM media supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, and 100 U/ml Penicillin-streptomycin. The cells were maintained in a 37°C incubator with 5% CO₂.

5.2.17 Cell passaging

In order to passage cells, the media from the cell culture dish was aspirated and cells were washed with PBS to remove any residual media. After trypsin treatment in the cell culture incubator (37°C with 5% CO₂) for 5 min, excess growth media (with FBS) was added to the plate to inactivate the trypsin. Cells were seeded in a plate with fresh media as required, usually in a 1:5 split ratio.

5.2.18 Stable cell line generation and maintenance

Parental Huh7 Flp-In TREx cell line (with zeocin and blasticidin antibiotic resistance) generated in (Horos et al., 2019) was used to make doxycycline inducible stable cell lines for MDH2 mutants. This was performed according to instructions from Thermo Fischer. Briefly, pOG44 plasmid and the pcDNA 3.1 FRT-TO-3X-FLAG-HA MDH2 constructs were co-transfected into the parental Huh7 Flp-In TREx cell line. The Flp-In recombinase expressed from the pOG44 plasmid mediates a homologous recombination between the FRT sites in the host cell line and in the pcDNA 3.1 FRT-TO plasmid. This inserts the gene of interest (GOI) with a hygromycin resistance gene into the parental cell line, disrupting the transcriptional unit for zeocin gene. Cell lines with stable integration of the GOI were selected by growth in hygromycin and blasticidin containing media.

Parental Huh7 Flp-In TREx cell line was maintained in DMEM media supplemented with 10% heat inactivated Tet-free FBS, 2 mM L-glutamine, 100 U/ml Penicillin-streptomycin, 5 µg/ml blasticidin and 100 µg/ml zeocin. Stable integrated Flp-In TREx cell lines were maintained in DMEM media supplemented with 5 µg/ml blasticidin and 200 µg/ml hygromycin.

5.2.19 Cryopreservation

To prevent genetic changes due to long-term cell culture, cryopreservation was used for long-term cell storage. For this, cells were trypsinized and pelleted by centrifugation at 500g for 5 min. After aspirating the supernatant, pellet was resuspended in a freezing mixture (5:4:1 ratio mixture of supplemented DMEM: FBS: DMSO). The concentration of cells in freezing mixture was typically $1-2 \times 10^6$ cells/ml. The mixture was aliquoted into cryo-storage vials and placed overnight in an isopropanol-containing cryo-freezing container at -80°C to allow slow-freezing of the cells. Cryogenic vials were then transferred to liquid nitrogen storage (-170°C) and thawed as needed.

5.2.20 Treatment of cells with NAD-level modulators

Cells were plated at 30-40% confluency on 15cm dish 24 hours before treatment. The next day, fresh media containing either 5 nM FK866, 5 mM NAM or DMSO was added to the cells. The treatment was performed for 48 hours before cell lysis and harvest. Cells were divided for NAD(H) measurement and PNK assays.

5.2.21 NAD (H) quantification

NAD (H) quantification was performed using the NAD/NADH quantification kit (Sigma-Aldrich) according to the manufacturer's instructions.

5.2.22 Differentiation of Huh7 cells

Huh7 cells were differentiated to be more hepatocyte-like according to a published study (Steenbergen et al., 2018). Briefly, cells were grown in DMEM base media supplemented with 2% human serum (HS) and 2 mM L-glutamine, 100 U/ml Penicillin-streptomycin. They stop dividing after about 7 days in HS culture. Before the 7-day mark they were transferred to collagen/poly-D-lysine coated plates to promote attachment. The cells showed morphological changes after 15 days of HS culture, including large lipid droplets, increased cell contacts and altered cell shape. Cell growth media was replenished every 2 days. HS-cultured cells have been maintained up to 70 days in culture for the set of experiments performed.

Transfections

5.2.23 Plasmid transfections

Cells were plated either on the same day (reverse method), or 16-20 hours before transfection (forward method). Transfections were performed with Lipofectamine 3000 reagent according to the manufacturer's instructions.

5.2.24 siRNA transfections

small interfering RNAs (siRNAs) were used to deplete the proteins. Cells were plated either on the same day (reverse method), or 16-20 hours before transfection (forward method). siRNAs were transfected into the cells with RNAiMax reagent according to the manufacturer's instructions. The plates were harvested 72-96 hours after knockdown.

Functional analysis methods

5.2.25 Crosslinking cells with ultra-violet (UV) light

Growth media was aspirated from the cell culture dish. Cells were washed with ice cold PBS two times. After aspirating all residual liquid from plates, the cells were irradiated with 150 mJ/cm² of UV light (254 nm) on ice.

5.2.26 Immunoprecipitation of proteins (endogenous and tagged proteins)

For purifying a target protein from cell lysates, specific binding to an antibody against the target protein immobilized on a solid support (in this case, magnetic particles) was utilized. The relevant antibody was first coupled to magnetic beads (Bio-Rad ProteinA/ ProteinG Surebeads /Dynabeads ProteinG/ Dynabeads Anti-Rabbit) by incubation at 4°C in 0.05% PBST for more than 2 hours. In addition to the specific antibody against the protein of interest, an isotype matched control antibody was also separately used in the immunoprecipitation. This accounted for non-specific interactions of factors with the antibody. For assaying proteins tagged with 3X-FLAG-HA, agarose FLAG M2 beads or Pierce magnetic anti-HA beads were used for pulldown. In order to remove proteins that non-specifically bind to beads, a preclearing step was performed, where the lysate was incubated with beads with no antibody present. Following this preincubation step, the beads were magnetized. Lysate was collected for further incubation with the antibody-immobilized magnetic beads. The antibody coupled beads and lysate mixture was incubated at 4°C for 2 hours with gentle agitation. Unbound proteins were washed away with the lysis buffer (with modifications as described for different applications below). For the immunoprecipitations of endogenous proteins, the proteins were eluted with 0.1 M glycine with pH 2.2 with shaking at room temperature for 5 min. The eluates were neutralized with 1.5 M tris pH 8.8. For the tagged constructs, 250 µg/ml 3X FLAG peptide was used for eluting the protein off the agarose beads. The purified proteins were used for downstream assays like western blot/ enzyme assays.

5.2.27 Co-immunoprecipitation

In order to identify the interactors of ATP5A1 I performed co-immunoprecipitation followed by mass spectrometry. For this, cells were grown to 80-90% confluency in 15 cm dishes. In some trial experiments, to better preserve the cellular protein-protein

interactions, the proteins were crosslinked with 2.5 mM DSP by incubation at room temperature (RT) For 7 min. The reaction was quenched with 0.25 M glycine, followed by cell lysis. For co-immunoprecipitation, the immunoprecipitation protocol is modified to use a non-denaturing co-IP lysis buffer for lysis and washes to better preserve the protein-protein interactions. After lysate-antibody-bead incubation, the beads were washed 3X with co-IP lysis buffer. After elution, the samples were prepared for western blot analysis or for mass spectrometry.

5.2.28 Polynucleotide Kinase assay

Cells were irradiated with UV light (254 nm) of 150 mJ/cm² energy on ice and lysed in the appropriate buffer. After sonication, centrifugation, and measurement of protein concentration, around 200-600 ug of total protein lysate was used for PNKs. Immunoprecipitation was performed as above. For endogenous IP, MDH2 rabbit antibody was coupled with Bio-Rad ProteinA Surebeads. For the MDH2 mutants tagged with 3X FLAG-HA at the C-terminus, agarose beads coupled with FLAG M2 antibody were used. The beads were washed with RIPA buffer, RIPA-HS buffer, PNK buffer. For labelling the RNA, the beads were incubated in the 30 µl T4 PNK reaction mix for 15 min at 37C. The reaction mix contained 0.3 µl of 0.1 µCi/µl γ-³²P ATP , 3 U of T4 PNK enzyme in 1X PNK reaction buffer. The beads were washed 4 times with the PNK buffer and proteins were eluted off the beads as described above. After addition of 4X loading buffer, the samples were boiled for 10 min at 70°C. The samples were separated using SDS-PAGE and transferred to a nitrocellulose membrane. After drying the membrane, it was exposed to a phosphor screen overnight. The signal was developed using a Typhoon FLA 9500 by scanning at 600 nm. After detection of the radioactive ³²P signal, the blots were used for detection with MDH2 antibody to visualize the pulldown efficiency.

5.2.29 Complex Capture

Complex capture/ 2C was performed according to a published protocol(Asencio et al., 2018).

Huh7 cells were grown to 80-90% confluency in 15cm dishes. Growth media was aspirated and cells were washed twice with ice-cold PBS. After making sure all the residual liquid is removed, the plates were irradiated with UV light (254 nm) of 150 mJ/cm² energy on ice. The cells from a 15cm dish were harvested by scraping with 500 µl of the RIPA lysis buffer supplemented with Complete EDTA-free protease

inhibitor. Following a 15 min incubation on ice, the cells were sonicated using Bioruptor Plus for 10 cycles (30sec on/30 sec off). The debris were removed by centrifuging the samples at 10000g for 15 min at 4°C. After protein measurement, 1 mg of samples were processed for 2C. A fraction of the input was set aside for western blot assessment. Components of the Zymo RNA mini kit was used for 2C as follows. After adding 4X volume of the RNA lysis buffer to the lysate, the mixture is incubated at 37°C for 5 min. Subsequently, 1X volume of 100% ethanol was added to the sample and mixed well. ZymoSpin III-CG column was used to capture nucleic acids, as well as nucleic acid-protein complexes. After washing the column with 400 µl of RNA Wash Buffer, DNA bound to the column is selectively digested with a DNaseI treatment at 37°C for 60 min. Following this, the columns are washed with 400 µl of RNA Prep Buffer, 700 µl RNA Wash Buffer, 400 µl RNA Wash Buffer respectively. Residual buffer in the column is removed by centrifugation at 12000g for 2min. The RNA-protein complexes are eluted from the column by incubating the column with water at 70°C for 5 min. This step is repeated twice for increased yield. The concentration of the eluted RNA is measured using a Nanodrop Spectrophotometer. Volumes of samples equivalent to 60 µg of RNA were then concentrated using a vacuum dryer/concentrator at 45°C. In a variation to assess RNA-dependency of silica retention, the eluates were divided into two sets. One set was treated with RNaseA-RNaseT1 mixture and the second set was left untreated in similar buffer conditions. The reaction was performed in Tris-EDTA buffer at 37°C for 2 hours followed by overnight incubation in the cold room. The samples were then processed for a second round of complex capture as described above. This was followed by western blot analysis of the eluates. In order to visualize the RNA-bound proteins using western blot at the expected molecular weight of the protein, the RNA in the samples (including the RNA which is crosslinked to the protein) were digested with RNaseI (Ambion). 2 µl of RNase I (Ambion) was added to the samples and incubated at 37°C for 60 min. After the addition of 4X loading dye supplemented with TCEP to the samples, they were heated at 70°C for 10 minutes. Samples were separated by SDS-PAGE and proteins of interest were visualized by western blot.

5.2.30 Mitochondrial fractionation and crosslinking

Cells were grown to 80-90% confluency in 7*15 cm² dishes. For harvest, cells were washed two times with ice-cold PBS followed by the addition of 3 ml of Buffer B (see Table of buffers) supplemented with 2 mg/ml BSA, complete EDTA-free protease

inhibitors and RNasin (RNase inhibitor). Cells were collected by gently scraping with rubber scrapers and allowed to swell by incubation at 4°C for 10 min. The cells were lysed with 20-25 strokes of a Dounce homogenizer. Nuclei was pelleted by centrifugation at 1000g for 10 min at 4°C. The supernatant was centrifuged at 15000g for 20 minutes at 4°C to pellet mitochondria. The mitochondrial pellet was washed 2 times with Buffer B, with the wash buffer removed by centrifugation for 20 minutes at 4°C. The pellet was resuspended in 100 µl of Buffer B and then irradiated with UV light after spreading on a glass bottom dish. The mitochondria were lysed by sonication after adding excess volume of RIPA buffer. The lysates were snap frozen and stored at -80°C till further use. Whole cell samples were processed at same time by crosslinking and lysis.

5.2.31 Immunofluorescence (IF) and Microscopy

Cells were grown to 60% confluency on coverslips and fixed with 4% paraformaldehyde. 0.2% Triton X-100 was used to permeabilize cells by incubation at room temperature (RT) for 10 minutes. Samples were blocked in the IF blocking buffer (2.5% BSA in 0.05% Triton X-100) for 30 minutes at room temperature. After blocking, samples were incubated with antibody diluted in IF blocking buffer for 1 hour at 37°C. Samples were washed 3 times in 0.05% Triton X-100 before incubating with the secondary antibody labelled with the fluorescent dye (also diluted in the blocking buffer) for 45 min to 1 hour at room temperature in the dark. After removing the antibody solution, Nucblue nuclear staining solution was added to the cells for 10 min. The samples were further washed two times before mounting on a glass slide using ProLong Diamond antifade mountant. The slides were left at room temperature to cure overnight before imaging and stored for long term at 4°C. Images were acquired on an Olympus 3000FV microscope with either a 60X objective/ 40X objective.

5.2.32 Seahorse assay

OCR and ECR was measured using a Seahorse XFe96 Analyzer following the manufacturer's instructions.

5.2.33 MDH2 enzyme assay

MDH2'S enzymatic activity was measured by monitoring oxaloacetate-dependent NADH oxidation. The final reaction mix contained 0.25 mM OAA, 0.125 mM NADH

in 0.1M potassium phosphate buffer, pH 7.4 in a reaction 200 µl volume. In case of immunoprecipitated samples, equal amounts of eluates were added to the reaction to measure activity. Decrease in NADH absorbance at 340 nm was measure in the TECAN multi-well plate reader. A standard curve with a titration of NADH concentrations was used to extrapolate the concentrations of NADH in the activity assays and thus measure MDH2's catalytic activity.

5.2.34 QuanCAT

In order to get quantitative estimates of protein synthesis changes, we combined a SILAC (incorporating medium/ heavy isotope labeled Lysine/Arginine in cells treated with the control/ ATP5A1 siRNA respectively) approach with pull-down of labelled nascent proteins. The cells were processed according to published protocol(Howden et al., 2013)

Transfection, labelling and lysis

Huh7 cells were transfected with control siRNAs/ ATP5A1 siRNAs following the reverse transfection protocol in 15 cm dishes. Experiment was performed in triplicate. After 96 hours, cells were depleted of methionine, lysine and arginine. For this, the cells were washed in warm PBS and incubated for 30 minutes in depletion medium (DMEM formulation without methionine, arginine and lysine) with 10% dialyzed FBS, L-glutamine and penicillin-streptomycin. Then, the cells were incubated in the same medium supplemented with 0.1 mM L-AHA and either 84 ug/ml [13C6, 15N4] L-arginine and 146 ug/ml [13C6, 15N2] L-lysine or 84 ug/ml [13C6]L-arginine and 146 ug/ml [4,4,5,5-D4]L-lysine for 2 hours. Afterwards, the cells were harvested by trypsinization and lysed in 770 µl Lysis buffer (8M Urea, 0.75M NaCl, 6.2% CHAPS in 300mM HEPES, pH 8). After 10-minute incubation on ice, the samples were sonicated for 10 cycles, 30sec on/off. The lysates were centrifuged for 10 minutes at 10000g. Control knockdown and ATP5A1 knockdown were mixed replicate-wise in equal amounts (770 µl+770 µl).

Click reaction, reduction, alkylation

200 µl of alkyne-agarose resin was prepared by washing in 2ml water. A CuSO₄-THPTA mastermix solution was prepared by adding 100 mM CuSO₄ (20 µl / reaction) to the (40 µl / reaction) 250mM THPTA. The mastermix was vortexed followed by a short centrifugation spin. To set up the click reaction, mixed lysates (made up to 1700

μl) were added to a 2 mL tube containing the 200 μl of washed resin slurry. To this, 60 μl of the CuSO₄-THPTA mix was added. 20 μl of the 1 M aminoguanidine hydrochloride solution and 20 μl 1M Na ascorbate was added to the lysate. The mixture was incubated for 2 hours at 40°C at 2000 rpm. The click reaction covalently links the AHA-modified nascent proteins to the alkyne agarose resin. After a short spin, the supernatant was removed and the beads were rinsed with water. 1 mL of SDS Wash Buffer and 20 μL of 0.5 M TCEP (10 mM final concentration) were added to the resin. After vortexing briefly to resuspend the resin, the reaction was incubated for 15 minutes at 70°C. The reaction was cooled to room temperature for 15 minutes. The supernatant was aspirated after a short spin to bring the resin to the bottom of the tube. 1 mL of iodoacetamine solution per enrichment reaction was prepared by dissolving 7.4 mg of iodoacetamide in 1 mL of SDS Wash Buffer. After adding iodoacetamine solution to the resin, the resin was vortexed briefly for resuspension, and incubated in the dark for 30 minutes.

Washes and trypsin digestion

The reaction was transferred to a spin column. 2 ml of SDS wash buffer was added to the column and allowed to flow to waste. This was repeated 5 times. SDS buffer wash was followed by 8 washes with 2 mL of 8 M urea/100 mM Tris, pH 8. After this, the column was washed with 2 mL of 20% acetonitrile 8 times. The resin was finally resuspended in trypsin digestion buffer (50mM ammonium bicarbonate) and transferred to a fresh tube. The column was rinsed with an additional 500 μl of digestion buffer, which was added to the transferred resin. The resin was pelleted by centrifugation for 5 minutes at 1000 rcf. The supernatant was removed, leaving approximately 200 μl of digestion buffer in the tube with the resin. 5 μl of 0.1 μg/μl trypsin was added to the resin slurry, vortexed briefly to mix the slurry and incubated at 37°C for overnight (~16 hours). The resin was pelleted by centrifugation for 5 minutes at 1000 rcf, and the trypsin-digested supernatant was transferred to a clean tube. 500 μl of water was added to the resin and the resin was pelleted again by centrifugation for 5 minutes at 1000 rcf. The supernatant from this step was added to the first batch of supernatant. These eluted samples were submitted to the EMBL mass-spectrometry facility together with 20 μg of the input lysates.

5.2.35 Sample preparation and acquisition of mass spectrometry data

QuanCAT

The samples were analyzed by mass spectrometry in the Proteomics Core facility as follows.

The nascent proteins after elution from the alkyne agarose beads by trypsin digestion was further processed at the Proteomics core facility. An in-solution tryptic digest was performed in a modified version of the Single-Pot Solid-Phase-enhanced Sample Preparation (SP3) protocol (Hughes et al., 2014; Moggridge et al., 2018). In total, 3 biological replicates were processed (n=3). Lysates were added to Sera-Mag Beads in 10 μ l 15% formic acid and 30 μ l of ethanol. The proteins were bound to beads by shaking for 15 min at room temperature. To remove SDS, 4 subsequent washes with 200 μ l of 70% ethanol was performed. Protein digestion was performed overnight at room temperature with 0.4 μ g of sequencing grade modified trypsin in 40 μ l Hepes/NaOH, pH 8.4 in the presence of 1.25 mM TCEP and 5 mM chloroacetamide. The eluates were separated from the beads which was washed with 10 μ l of an aqueous solution of 2% DMSO and the combined eluates were dried down.

Peptides were analyzed by LC-MS/MS on an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). First, peptides were separated using an Ultimate 3000 nano RSLC system (Dionex) equipped with a trapping cartridge (Precolumn C18 PepMap100, 5 mm, 300 μ m i.d., 5 μ m, 100 Å) and an analytical column (Acclaim PepMap 100. 75 $\times$ 50 cm C18, 3 mm, 100 Å) connected to a nanospray-Flex ion source. The peptides were loaded onto the trap column at 30 μ l per min using solvent A (0.1% formic acid). Using a gradient from 2 to 85% Solvent B (0.1% formic acid in acetonitrile), the peptides were eluted over 2 hours at 0.3 μ l per min (all solvents were of LC-MS grade). The Orbitrap Fusion Lumos was operated in positive ion mode with a spray voltage of 2.2 kV and capillary temperature of 275 °C. Full scan MS spectra with a mass range of 375–1200 m/z were acquired in profile mode using a resolution of 120,000 (maximum injections time of 50 ms, AGC target was set to 400% and a max injection time of 86 ms). Precursors were isolated using the quadrupole with a window of 1.2 m/z and fragmentation was triggered by HCD in fixed collision energy mode with fixed collision energy of 34%. MS2 spectra were acquired with the Orbitrap with a resolution of 30,000 and a max injection time of 86 ms.

Raw data was searched against the Uniprot human (UP0000005640, 191,419 entries, May 2016) proteome using MaxQuant 1.6.17.0. The default parameters were used with the following adjustments: Max. labeled was set to 3 with Medium labels Arg6 and

Heavy labels Arg10. The following variable modifications were considered: Met to AHA substitution (H(-3) C(-1) N(2) S(-1)), Met to Diaminobutyrate (H(-3) C(-1) N S(-1) O(-1)) and Met to Homoserine (H(-4) C(-1) S(-1)) (Cox and Mann, 2008).

The input samples were analyzed as follows. Lysates were subjected to an in-solution tryptic digest (see above) and eluted peptides were reconstituted in 10 μ l of H₂O and incubated for 1 h at room temperature with 80 μ g of TMT6plex label reagent dissolved in 4 μ l of acetonitrile. For quenching the excess TMT reagent, 4 μ l of an aqueous 5% hydroxylamine solution was added to the reaction. Peptides were reconstituted in 0.1 % formic acid and mixed to achieve a 1:1 ratio across all TMT-channels. The mixed peptide pool was purified by a reverse phase clean-up step (OASIS HLB 96-well μ Elution Plate, Waters #186001828BA). Peptides were subjected to an off-line fractionation under high pH conditions (Hughes et al., 2014). The resulting 12 fractions were then analyzed by LC-MS/MS on an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific).

For thos, peptides were first separated using an Ultimate 3000 nano RSLC system (Dionex) equipped with a trapping cartridge (Precolumn C18 PepMap100, 5 mm, 300 μ m i.d., 5 μ m, 100 Å) and an analytical column (Acclaim PepMap 100. 75 $\times$ 50 cm C18, 3 mm, 100 Å) connected to a nanospray-Flex ion source. The peptides were loaded onto the trap column at 30 μ l per min using solvent A (0.1% formic acid). Elution was carried out using a gradient from 2 to 38% Solvent B (0.1% formic acid in acetonitrile) over 60 min at 0.3 μ l per min (all solvents were of LC-MS grade). The Orbitrap Fusion Lumos was operated in positive ion mode with a spray voltage of 2.4 kV and capillary temperature of 275 °C. Full scan MS spectra with a mass range of 375–1500 m/z were acquired in profile mode using a resolution of 60,000 (maximum fill time of 50 ms; AGC Target was set to Standard) and a RF lens setting of 30%. Fragmentation was triggered for 3 s cycle time for peptide like features with charge states of 2–7 on the MS scan (data-dependent acquisition). Precursors were isolated using the quadrupole with a window of 0.7 m/z and fragmented with a normalized collision energy of 36%. Fragment mass spectra were acquired in profile mode and an orbitrap resolution of 15,000. Maximum fill time was set to 54 ms. AGC target was set to 200%. The dynamic exclusion was set to 60 s.

Acquired data were analyzed using IsobarQuant (Franken et al., 2015) and Mascot V2.4 (Matrix Science) using a reverse UniProt FASTA Homo sapiens database

(UP000005640) including common contaminants. The modifications considered were: Carbamidomethyl (C, fixed), TMT10plex (K, fixed), Acetyl (N-term, variable), Oxidation (M, variable) and TMT10plex (N-term, variable). The mass error tolerance for full scan MS spectra was set to 10 ppm and for MS/MS spectra to 0.02 Da. A maximum of 2 missed cleavages were allowed. A threshold of minimum of 2 unique peptides with a peptide length of at least seven amino acids and a false discovery rate below 0.01 were required on the peptide and protein level (Savitski et al., 2015)

Identification of ATP5A1 interactors by co-immunoprecipitation

Samples were first subjected to an in-solution tryptic digest as described above. Peptides were reconstituted in 10 µl of H₂O and incubated with 40 µg of TMTpro label reagent dissolved in 4 µl of acetonitrile for 1 h at room temperature. 4 µl of an aqueous 5% hydroxylamine solution was added to quench the excess TMT reagent. Peptides were mixed and purified by a reverse phase clean-up step (OASIS HLB 96-well µElution Plate, Waters #186001828BA). The samples were analyzed by LC-MS/MS on an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific).

For this, peptides were first separated using an Ultimate 3000 nano RSLC system (Dionex) equipped with a trapping cartridge (Precolumn C18 PepMap100, 5 mm, 300 µm i.d., 5 µm, 100 Å) and an analytical column (Acclaim PepMap 100. 75 × 50 cm C18, 3 mm, 100 Å) connected to a nanospray-Flex ion source. The samples were loaded onto the trap column at 30 µl per min using solvent A (0.1% formic acid). The elution was performed using a gradient from 2 to 40% Solvent B (0.1% formic acid in acetonitrile) over 2 hours at 0.3 µl per min (all solvents were of LC-MS grade). The Orbitrap Fusion Lumos was operated in positive ion mode with a spray voltage of 2.4 kV and capillary temperature of 275 °C. Full scan MS spectra with a mass range of 375–1500 m/z were acquired in profile mode using a resolution of 120,000 (maximum fill time of 50 ms or a maximum of 4e5 ions (AGC) and a RF lens setting of 30%. Fragmentation was triggered for 3 s cycle time for peptide like features with charge states of 2–7 on the MS scan (data-dependent acquisition). Precursors were isolated using the quadrupole with a window of 0.7 m/z and fragmented with a normalized collision energy of 38%. Fragment mass spectra were acquired in profile mode and a resolution of 30,000 in profile mode. Maximum fill time was set to 64 ms or an AGC target of 1e5 ions). The dynamic exclusion was set to 45 s.

Acquired data were analyzed using IsobarQuant (Franken et al., 2015) and Mascot V2.4 (Matrix Science) using a reverse UniProt FASTA Homo sapiens database (UP000005640) including common contaminants. The modifications considered were: Carbamidomethyl (C, fixed), TMT16plex (K, fixed), Acetyl (N-term, variable), Oxidation (M, variable) and TMT16plex (N-term, variable). The mass error tolerance for full scan MS spectra was set to 10 ppm and for MS/MS spectra to 0.02 Da. A maximum of 2 missed cleavages were allowed. A threshold of minimum of 2 unique peptides with a peptide length of at least seven amino acids and a false discovery rate below 0.01 were required on the peptide and protein level (Savitski et al., 2015).

5.2.36 Bioinformatic analysis of mass-spectrometry data

Measurements were performed by EMBL proteomic core facility and analysis was performed by Dr. Frank Stein from EMBL proteomic core facility.

5.2.37 eCLIP library preparation

eCLIP (enhanced crosslinking and immunoprecipitation) was performed as described in (Van Nostrand et al., 2016) with modifications as described below. Technical replicates of UV-crosslinked lysates were collected and all samples were processed simultaneously for eCLIP. The lysates were treated with 1 U RNaseI and 2 U DNase at 37°C for exactly 5 mins followed by the addition of RNasinPlus (Promega) to inhibit the RNase. 1% of the input lysate was set aside to be used as the size matched input. MDH2 was immunoprecipitated from the 500 µg of lysates with 6µg of the antibody coupled to Protein A magnetic beads (Bio-Rad). Isotype matched IgG was used as a control. The beads were washed sequentially with RIPA-HS buffer, Wash buffer, FastAP buffer, performed as two sequential incubations with each buffer for 2 min with gentle rotation. In order to fully dephosphorylate the RNA and thus facilitate the ligation of the 3' linker in the following step, samples were treated with FastAP and T4 PNK (In the absence of ATP, T4 PNK enzyme utilizes its 3'end dephosphorylation ability). For this, FastAP reaction was performed in a mix of 100 µl with the FastAP buffer, 8 µl FastAP enzyme, 1 µl Turbo DNase and 2 µl RNase inhibitor at 37°C for 15 min. After the Fast-AP reaction mix was removed, the 300 µl T4 PNK reaction mix was added to the beads. For this, 7 µl T4 PNK enzyme was diluted in the PNK reaction buffer supplemented with 1 mM DTT, 1 µl Turbo DNase and 5 µl RNase inhibitor. The reaction was performed 37°C for 20 min. The beads were washed as follows: Wash buffer, RIPA-HS buffer (two times), Wash buffer (two

times), 500 µl Ligase Buffer and 300 µl Ligase buffer (three times) without DTT. Any residual liquid was removed from the beads after a short centrifugation. The 3' adapter (X1A) was ligated to the RNA-beads. Efficient ligation of the 3' adaptor is crucial for the library reparation as it serves as priming site for the downstream reverse transcription. The ligation was performed in a 30 µl volume with 3 µl 10X ligation buffer, 0.3 µl of 1M ATP, .8 µl of 100% DMSO, 9 µl of 50% PEG 8000, 0.4 µl of RNase inhibitor, 9 µl H₂O and 2.5 µl RNA ligase. The reaction was incubated at 22°C for 75 min with intermittent mixing. The beads were washed as follows: twice with 500 µl of Wash buffer, twice with 500 µl of RIPA-HS buffer, four times with 500 µl of Wash buffer. After resuspension in 100 µl Wash buffer, the beads were spun at 100 rpm for 1.5 min at 4°C and all residual liquid removed. For elution, the beads were resuspended in 20 µl 0.1M Glycine, pH 2 and incubated at room temperature for 5 min. 3 µl of 1.5 M Tris HCl, pH 8.5 was added to the elution for pH neutralization.

For the MDH2 eCLIP, samples were distributed as follows: 90 % for eCLIP library preparation, 10% for analytical western blot to calculate IP efficiency. NuPAGE sample buffer supplemented with TCEP was added to the eluates and heated at 70°C for 10 min. The samples were resolved on BIO-RAD- Criterion XT gels with MOPS buffer. The gel with samples for eCLIP library preparation was transferred on to a nitrocellulose membrane. To isolate RNA associated with MDH2, the region of membrane 37kDa to between 100kDa and 150kDa was cut (75kDa range). This allows the capture of even larger RNA-MDH2 complexes. The input samples and the control IgG samples were also cut out of the membrane at the same size range.

After fragmenting the membrane, the RNA was released from the membrane and the crosslinked protein by ProteinaseK digestion. This was performed in a 200 µl reaction mix with 40µl of the ProteinaseK enzyme by agitation at 37°C for 20 min. The reaction was supplemented with 200 µl Urea/ PK buffer, followed by incubation in Thermomixer at 1200 rpm, 37°C, for an additional 20 min.

For the purification of RNA from the reaction mix, 400 µl acid phenol/chloroform/isoamyl alcohol (pH 6.5, Sigma) was added to the reaction mix and incubated at 37°C for 5 min at 1200 rpm. After a brief spin, the supernatant was transferred to Phase lock gel HEAVY tube and incubated at 37°C for 5 min at 1200 rpm. In order to separate the aqueous phase from the phenol phase, the samples were centrifuged at 13,000g at room temperature for 15 min. The aqueous layer was

transferred to a new 5ml tube. The RNA was purified using the Zymo RNA Clean and Concentrator kit and eluted in 10 μ l volume.

Next, we processed the input samples in a similar manner to the IP samples for ligation of 3' linker and purification. The RNA from the input samples were dephosphorylated with FastAP treatment and T4 PNK treatment sequentially. For FastAP treatment, 2.5 μ l FastAP enzyme is added to 10 μ l of the input RNA together with 0.5 μ l RNase inhibitor in the FastAP reaction buffer. After incubation at 1,200 rpm, 37°C, 15 min, 75 μ l of the PNK reaction mix was added to the samples. The reaction mix consisted of 1 μ l RNase inhibitor, 1 μ l Turbo DNase, 7 μ l of PNK enzyme in the PNK buffer supplemented with fresh DTT. The reaction was allowed to proceed at 37°C for 20 min with agitation. MyONE silane beads were used to clean up the input RNA after ethanol precipitation and remove the excess the buffer components prior to 3' end linker ligation. 20 μ l of beads suspended in 300 μ l RLT buffer was allowed to bind to the input RNA sample. After addition of 10 μ l of 5 M NaCl and 615 μ l of 100% EtOH, the samples were incubated at room temperature for 15 min. The beads were washed thrice in 75% EtOH and all excess liquid was removed. RNA was eluted in 10 μ l H₂O and half of the elution was further processed for 3' linker ligation, the rest was kept aside as backup. The sample is supplemented with 13.5 μ l of Ligation mix containing 0.2 μ l 1M ATP, 0.3 μ l DMSO, 8 μ l 50% PEG8000, 0.2 μ l RNase inhibitor, 1.3 μ l RNA ligase in ligation buffer. Ligation was performed at room temperature for 75 min, with intermittent mixing. In order to exchange the buffer, the RNA in the reaction mix was again precipitated using the MyONE silane beads. For this round, 20 μ l of beads suspended in 61.6 μ l RLT buffer was added to the RNA sample with the subsequent addition of 61.6 μ l 100% EtOH. After incubation at room temperature for 15 min with intermittent mixing, the beads were washed thrice in 75% EtOH, and the RNA samples eluted in 10 μ l H₂O.

The RNA from the IP and input samples were reverse transcribed with Superscript IV enzyme using the AR 17 primer. (This was performed by first annealing 0.5 μ l of the primer with 10 μ l RNA at 65°C for 5 min. The reverse transcription mix was added to the pre-annealed primer-template mix and incubated at 23°C for 10 min, 55°C for 10 min followed by inactivation of the enzyme 80°C for 10 min.) Excess primers and nucleotides are hydrolysed by ExoSAP treatment by incubation with 3.5 μ l ExoSAP-IT at 37°C for 15 min. After addition of 0.1 μ l 0.5M EDTA, the RNA was removed

from the sample by addition of 3 μ l of 1M NaOH and incubated at 70°C for 12 min. The reaction was neutralized by addition of 3 μ l of 1M HCl.

The cDNA was precipitated on MyONE silane beads, with the addition of 10 μ l of beads suspended in 93 μ l RLT buffer and 111.6 μ l 100% EtOH to the sample. The beads were washed thrice in 80% EtOH. After air-drying the beads for 5 min, the cDNA was eluted in 5 μ l 5 mM Tris-HCl, pH 7.5. Without the removal of the eluted cDNA from the beads, the 5' linker (rand3Tr3 adaptor) was ligated to the cDNA. For this, 0.8 μ l of the adaptor was added together with 1 μ l DMSO to the samples and incubated at 75°C for 2 min, followed by incubation on ice. After the addition of 12.8 μ l of the ligation mix containing 0.2 μ l 1M ATP, 9 μ l 50% PEG8000, 1.5 μ l RNA ligase in ligation buffer, the samples were incubated at room temperature overnight with intermittent mixing. MyONE silane beads was used for precipitating the ligated cDNA by addition of 5 μ l of beads resuspended in 60 μ l to the sample and 60 μ l of 100% EtOH. 27 μ l 10 mM Tris-HCl, pH 7.5 was added to the beads for elution of the cDNA. PCR was performed to test the library quality and the samples visualized after resolving on a 6% Novex TBE gel.

Final PCR:

Separate 50 μ l PCR reaction was set up for each sample with 2X Q5 PCR mastermix, 2.5 μ l each of 20 μ M Forward and Reverse primer (D50x and D70x) in a PCR tube. A unique combination of D50x and D70x is used for each sample. 15 μ l of the IP/ input samples were added to the mix. The PCR cycling conditions were set up based on the test PCR. In total, 9 cycles of amplification were performed for input samples and 18 cycles for MDH2 IP samples:

98°C	30 sec	1 cycle
98°C	15 sec	6 cycles
68°C	30 sec	
72°C	40 sec	
98°C	15 sec	3 cycles for Input
72°C	60 sec	8 cycles for IP
72°C	60 sec	1 cycle

4°C	hold	
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SPRI cleanup and gel purification

The library was purified with SPRI beads, using 90 µl beads/ 50 µl PCR reaction. After a 10 min incubation at RT, the beads were washed 2 times with 75% EtOH. The library was eluted in 20 µl water and transferred to fresh tubes. The library was resolved on 3% low melting agarose gel at 95V for 50 minutes. SyBR-Safe was added to the samples for visualization. Under blue light, gel was cut between the 175-300bp region and transferred to a new tube. Library was purified from the gel using Qiagen MinElute gel extraction kit according to the manufacturer's instructions. The samples were eluted in 12.5 µl Buffer EB.

Quantification of library and pooling

The triplicates of each sample were mixed together in equal amounts and quantified using Qubit dsDNA HS Assay kit and Bionalyzer. The eCLIP libraries from different sample were pooled in equimolar ratios (35 fmoles) and SMI pooled together in equimolar ratios (24.5 fmoles).

5.2.38 eCLIP Sequencing

Samples were analysed on NextSeq 500. Paired end read was used with different cycle numbers for read 1 and read 2. 20 cycles were used for read 1, followed by 8 cycles for index 1. Then, read 2 was read with 8 cycles for the indexes and 130 cycles for read 2.

5.2.39 eCLIP data analysis

eCLIP statistical data analysis was performed by Dr. Thileepan Sekaran and Dr. Thomas Schwarzl. Fastqc was used for checking the quality of eCLIP data. Using umi-tools extract, the unique molecular identifier (UMI) was extracted from the reads and attached to the read name. After trimming the adaptors, reads shorter than 18 nucleotides were removed (cutadapt tool). STAR alignment of the trimmed reads to the human genome (GRCh38.v23 from GENCODE) was done on end-to-end alignment mode. UMI barcodes were used to remove PCR duplicated reads (umi-tools dedup). The tRNA coordinates from tRNAScan (Chan and Lowe, 2016) were included in the GENCODE GRCh38.v23 genome annotation. ht-seqclip (Sahadevan et al.,

2022; Sahadevan, 2019) suite was used to preprocess the annotation into windows of 50 nucleotides length, overlapping with a step-size of 20 nucleotides. The crosslink site (the -1 position before the read starts) was extracted using the same tool. The R/Bioconductor DEWSeq package was used to identify windows with significant enrichment ((log2 fold change >0.5, p-adjusted <0.1) in the IP samples over the SMI (Schwarzl et al., 2022b; Schwarzl et al., 2022c). Independent Hypothesis Weighting was used to correct for multiple hypotheses. Continuous overlapping significant windows were merged to identify binding regions. From these significant binding windows, relevant targets were further selected by thresholding the read counts (>12 in each replicate).

5.2.40 Statistical analyses

GraphPad Prism 9 was used for statistical analysis of experiments.

Part V

Supplementary data

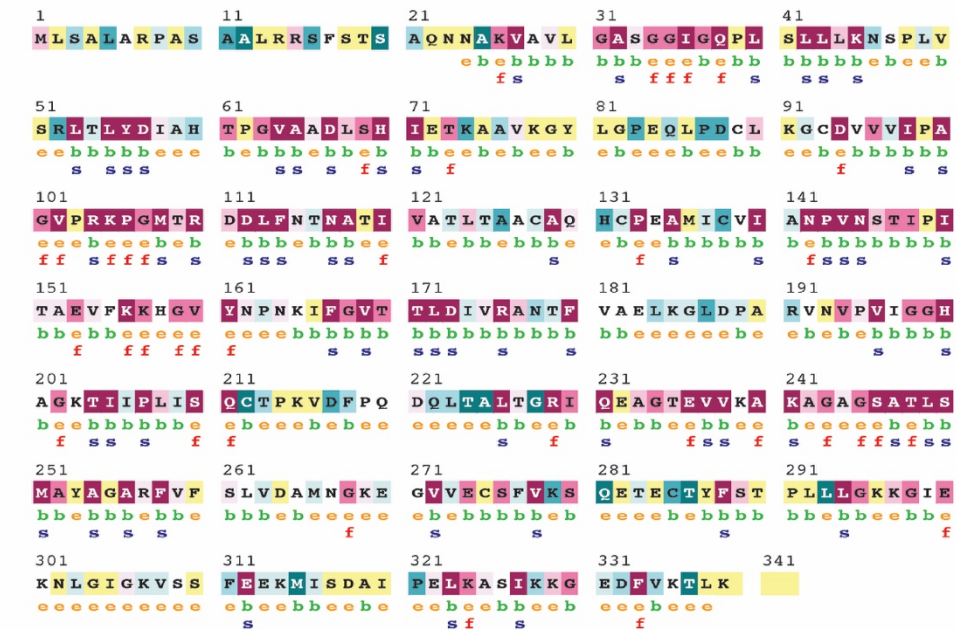
Supplementary data

A

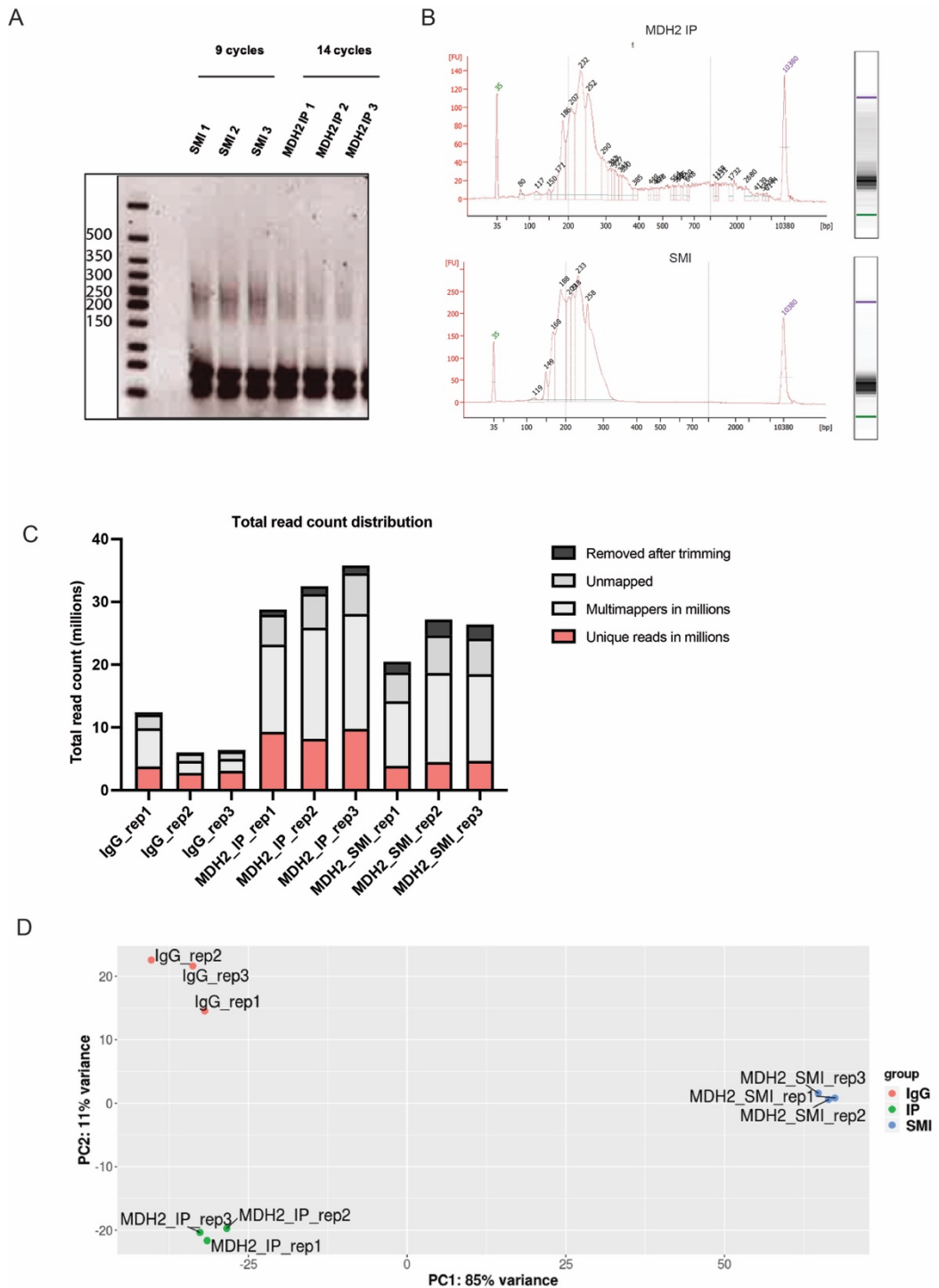
Percent Identity Matrix - created by Clustal2.1

1: sp	P17505	MDHM_YEAST	100.00	48.87	48.87	51.37	52.44	51.83	51.98	54.41	52.89
2: tr	A0A0H3NMB1	A0A0H3NMB1_SALTS	48.87	100.00	94.87	57.28	56.13	59.35	57.42	58.39	59.03
3: sp	P61889	MDH_ECOLI	48.87	94.87	100.00	57.28	55.48	57.74	56.77	58.39	58.39
4: sp	O02640	MDHM_CAEL	51.37	57.28	57.28	100.00	51.92	58.81	56.25	56.55	55.95
5: sp	Q9LKA3	MDHM2_ARATH	52.44	56.13	55.48	51.92	100.00	59.82	56.55	55.65	55.95
6: tr	Q9VEB1	Q9VEB1_DROME	51.83	59.35	57.74	58.81	59.82	100.00	58.63	59.23	59.82
7: tr	F6T7F0	F6T7F0_XENTR	51.98	57.42	56.77	56.25	56.55	58.63	100.00	84.02	83.73
8: sp	P40926	MDHM_HUMAN	54.41	58.39	58.39	56.55	55.65	59.23	84.02	100.00	94.97
9: sp	P08249	MDHM_MOUSE	52.89	59.03	58.39	55.95	55.95	59.82	83.73	94.97	100.00

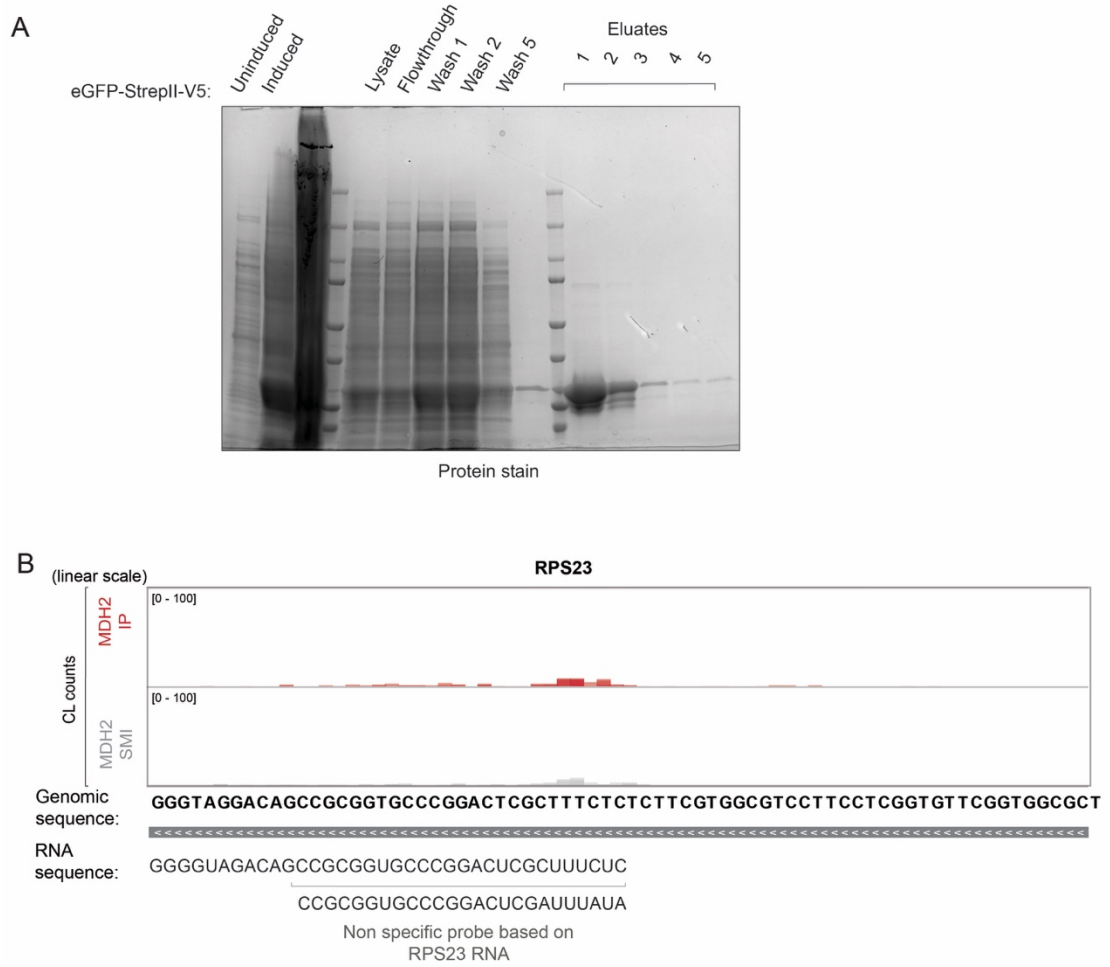
B



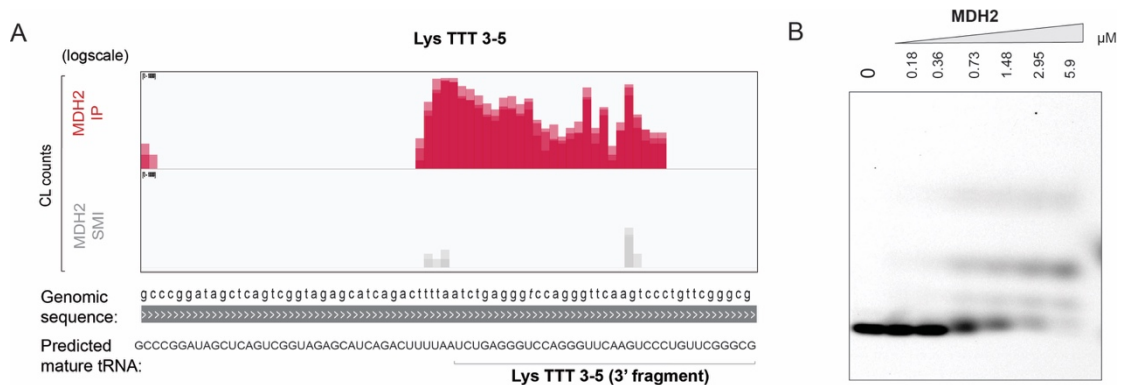
Supplementary figure 1. Sequence conservation analysis of MDH2 proteins from species with identified RNA binding property. (A) Percentage identity of MDH2 proteins across species with RNA binding property as calculated by ClustalW (B) Sequence conservation scores for MDH2 residues with the residue properties as indicated. The analysis was performed using the CONSURF tool using ClustalW alignment as input



Supplementary figure 2. Quality control steps for eCLIP. (A) Test PCR to visualise the efficiency of library preparation (B) Bioanalyzer traces of the MDH2 IP and SMI library (C) Read count distribution of samples (D) PCA plots of the samples to visualise key differences. Data analysis of the eCLIP data set to identify targets were performed by Dr. Thileepan Sekaran. PCA plot shown here was generated by Dr. Thileepan Sekaran.

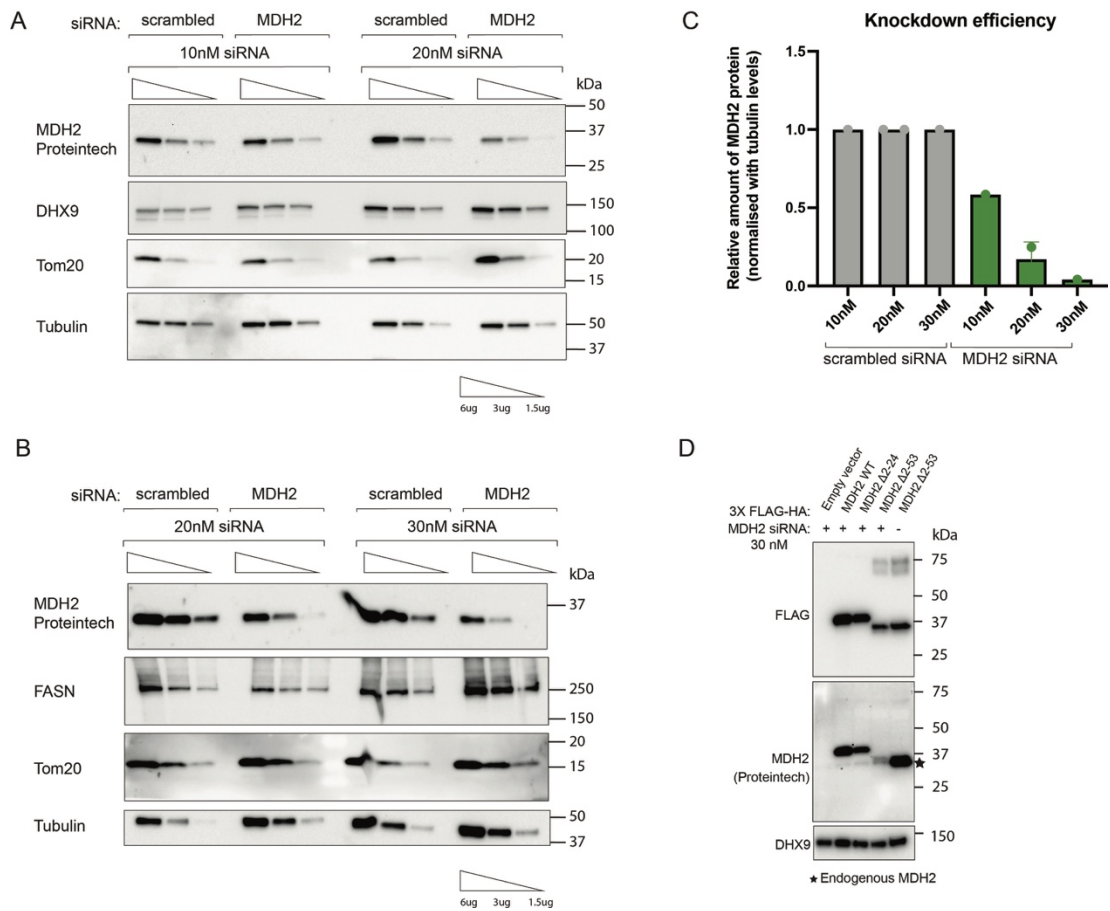


Supplementary figure 3. eGFP protein preparation and overview of MDH2 crosslinking RPS23 5'UTR (A) purified eGFP protein visualised after SDS-PAGE. (B) MDH2's crosslinking sites from eCLIP on the RPS23 5'UTR sequence. The nonspecific sequence used in MDH2 EMSA is highlighted below, which has three nucleotide variations from the original RPS23 sequence.



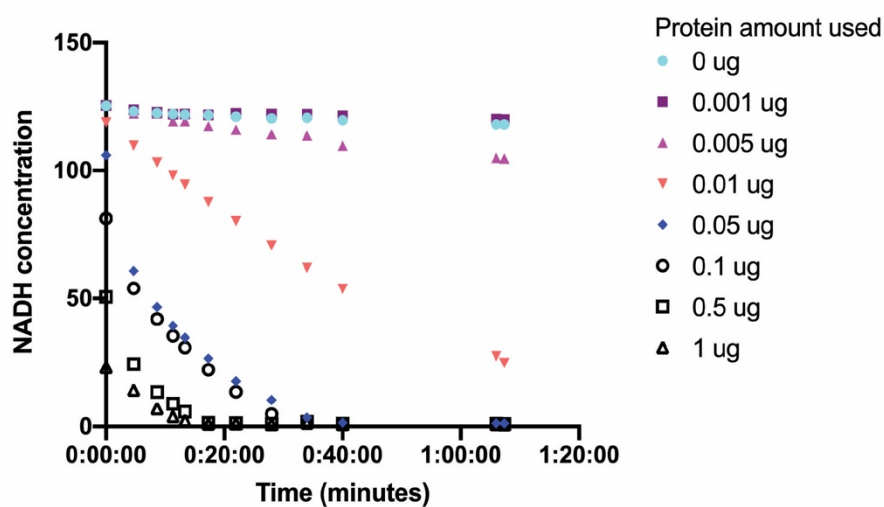
Supplementary figure 4. EMSA for MDH2's RNA target Lys TTT 3-5. (A) IGV view of readcounts and crosslinking counts mapped on to the genomic sequence of tRNA Lys TTT 3-5

as in figure 3.7. The probe is designed against the 3' end of the tRNA as highlighted. (B) Representative EMSA assay showing MDH2 binding to Lys TTT 3-5 fragment.

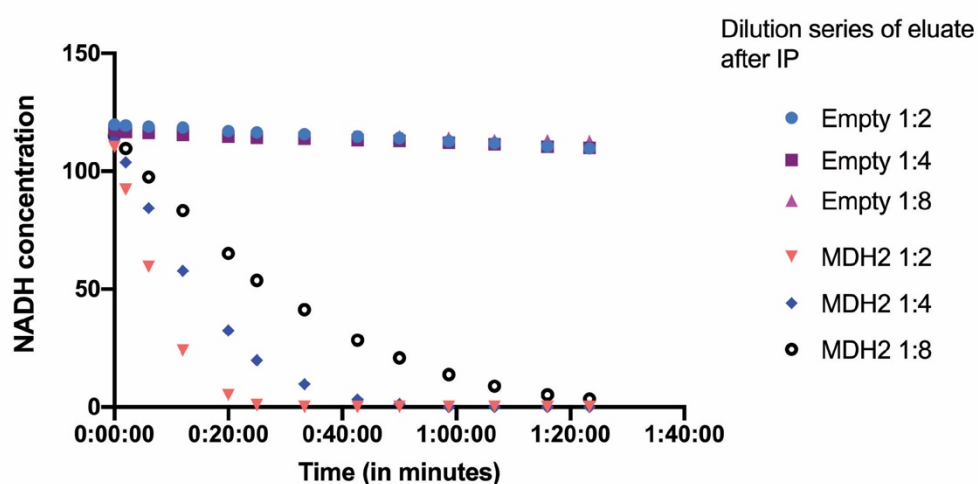


Supplementary figure 5. Standardisation of MDH2 depletion with siRNAs (A) siRNA mediated knockdown of MDH2, with 10 nM and 20 nM siRNA, using a scrambled siRNA sequence as control. DHX9, tubulin staining was used to control for equal protein loading in the western blot. Tom20 staining was used to monitor any potential effects on mitochondrial mass resulting from the knockdown. (B) Similar experiment to (A), but with 20 nM and 30 nM siRNA to assay if increased concentration of siRNAs can improve knockdown efficiency. (C) Quantification of MDH2 protein levels from western blots corresponding to (A) and (B). 30 nM MDH2 siRNA was used for further experiments due to increased efficiency of MDH2 depletion. (D) MDH2 protein levels assayed after MDH2 siRNA depletion and transient transfection of the tagged MDH2 constructs (corresponding to PNK experiment in section 2.3.8).

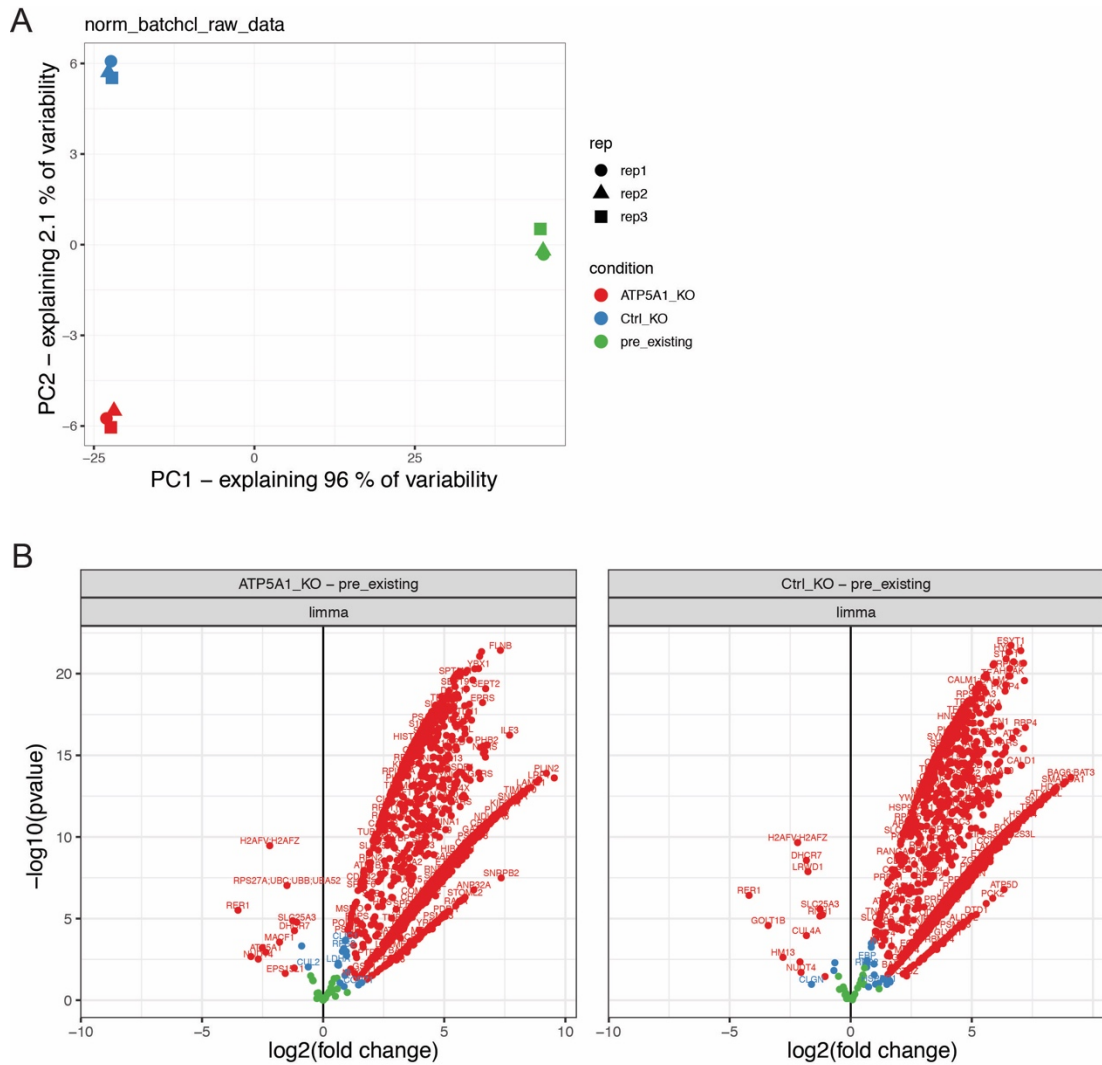
A Enzymatic activity of recombinant human MDH2



B Enzymatic activity of MDH2 after immunoprecipitation

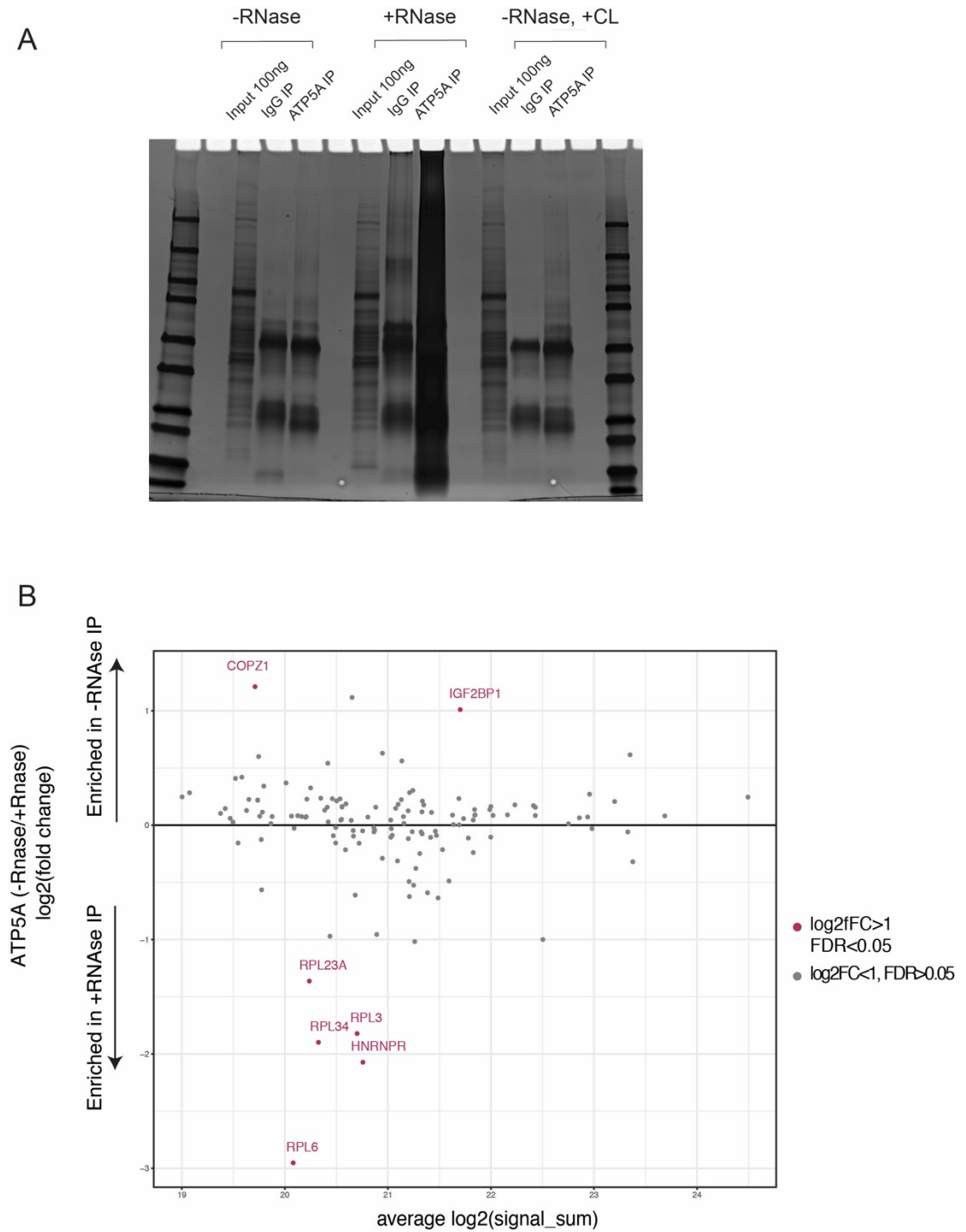


Supplementary figure 6. Standardisation of enzyme assay to measure MDH2 activity (A) Measurement of NADH reduction measured as a factor of time with a titration of recombinant human MDH2. (B) Measurement of NADH reduction measured as a factor of time with a titration of MDH2 immunoprecipitated from cells after overexpression.



Supplementary figure 7. Quality control plots for QuanCAT after ATP5A1 depletion. (A) PCA plot showing the clustering of sample replicates together (B) Volcano plot comparing the enrichment of labelled peptides in ATP5A1 depletion/ Control depletion versus the unlabelled peptides in the same samples. This serves as quality control for selective pulldown of nascent labeled peptides. The primary data analysis for mass spectrometry data was performed by Dr. Frank Stein from the EMBL proteomics core facility. The plots shown in this figure are made by Dr. Frank Stein.

Supplementary data



Supplementary figure 8. ATP5A1 co-IP mass spectrometry (A) Silver staining of the samples to visualise the input submitted for mass spectrometry after ATP5A1 pull down (B) Changes in the interactome on RNase treatment of the samples before ATP5A1 immunoprecipitation.

Supplementary table

Species	Studies	Methods	Name	Cell line	PolyA based	Non polyA based
<i>Homo sapiens</i>	(Backlund et al., 2020)	UV (254 nm) crosslinking-LNA-MS	eRIC	Huh7	Yes	No
	(Baltz et al., 2012)	UV (356 nm) crosslinking-Oligo(dT)-MS (4SU incorporation)	PAR-CLIP	HEK 293	Yes	No
	(Bao et al., 2018)	EU-Oligo(dT)-MS	RIC	HeLa	Yes	No
	(Beckmann et al., 2015)	UV (254 nm) crosslinking-Oligo(dT)-MS		Huh7	Yes	No
	(Castello et al., 2012)	UV (254 nm) crosslinking-Oligo(dT)-MS	RIC	HeLa	Yes	No
	(Castello et al., 2016)	UV (254 nm) crosslinking-Oligo(dT)-MS	RBD-map	HeLa	Yes	No
	(Garcia-Moreno et al., 2019)	UV (254 nm) crosslinking-Oligo(dT)-MS		HEK93	Yes	No
	(Huang et al., 2018)	EU/Steptavidin enrichment MS	CARIC	HeLa	No	Yes
	(Mullari et al., 2017)	UV (254 nm) crosslinking-Oligo(dT)-MS	pCLAP	HEK293	Yes	No
	(Perez-Perri et al., 2018)	UV (254 nm) crosslinking-Oligo(dT)/LNA-MS	RIC/eRIC	Jurkat	Yes	No

Supplementary data

	(Trendel et al., 2019)	UV (254 nm) crosslinking- Trizol extraction	XRNA X	MC7, HEK 293, HeLa	No	Yes
	(Urdaneta et al., 2019)	UV (254 nm) crosslinking- Phenol-Toluol extraction of RBPs	Ptex	HEK93	No	Yes
<i>Mus musculus</i>	(He et al., 2016)	UV (356 nm) crosslinking / MS (4SU incorporation)	RBR-ID	mESC	No	Yes
	(Liao et al., 2016)	UV (254 nm) crosslinking- Oligo(dT)- MS/lys-C digest	RIC/ RBD map	HL1	Yes	No
	(Liao et al., 2016) RBDmap					
	(Bao et al., 2018)	EU-Oligo(dT)- MS	RICK	mESC	Yes	No
	(Mallam et al., 2019)	RNase vs. control/SC-MS	DIF- FRAC	mESC	No	Yes
<i>Saccharomyces cerevisiae</i>	(Matia-Gonzalez et al., 2015)	UV (254 nm) crosslinking- Oligo(dT)-MS		BY4741	Yes	No
	(Scherrer et al., 2010)	Microarray- based		BY4741 / TAP- tagged strains	No	Yes
	(Shchepachev et al., 2019)	UV (254 nm) crosslinking- Total RNA- associated protein purification (254 nm)	TRAPP	BY4741	No	Yes

Supplementary data

<i>Drosophila melanogaster</i>	(Wessels et al., 2016)	UV (254 nm) UV (356 nm)/ crosslinking- Oligo(dT)-MS		Pre- and post-MZT embryos	Yes	No
	(Panhale et al., 2019)	UV (254 nm) crosslinking- Oligo(dT)-MS	CAPRI	S2 DRSC	No	Yes
	(Panhale et al., 2019)	FA- crosslinking- Oligo(dT)-MS	CAPRI	S2 DRSC	No	Yes
<i>Arabidopsis thaliana</i>	(Maronded ze et al., 2016)	UV (254 nm) crosslinking- Oligo(dT)-MS	mRBPo me capture	Col-0 cell suspensio n culture	Yes	No
<i>Caenorhabditis elegans</i>	(Matia-Gonzalez et al., 2015)	UV (254 nm) crosslinking- Oligo(dT)-MS		Mixed stage animals	Yes	No
	(Esmailie et al., 2019)	UV (254 nm) crosslinking- Oligo(dT)-MS		Young adult N2	Yes	No
<i>Escherichia coli</i>	(Queiroz et al., 2019)	UV (254 nm) crosslinking- Orthogonal organic phase separation	OOPS		No	Yes
	(Shchepach ev et al., 2019)	UV (254 nm) crosslinking- Total RNA- associated protein purification	TRAPP	MG1655	No	Yes
<i>Salmonella typhimurium</i>	(Urdaneta et al., 2019)	UV (254 nm) crosslinking- Phenol-Toluol extraction of RBPs	Ptex	Salmonella Typhimurium SL 1344	No	Yes

Supplementary table 1. Table of studies where MDH2 has been identified as an RNA binding protein with information about the methods

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List of abbreviations

RNA	ribonucleic acid
ncRNA	non coding RNA
DNA	deoxyribonucleic acid
RNP	ribonucleoprotein
m ⁷ G	7-methyl guanosine
polyA	polyadenosine
NAD	nucleotide adenosine diphosphate
IRE	Iron response element
IRP	Iron regulatory protein
RBP	RNA binding protein
HCV IRES	Hepatitis C virus internal ribosome entry site
ATP	Adenosine triphosphate
HSD17B10	17 β -hydroxysteroid dehydrogenase 10
KH	K-homology domain
RRM	RNA recognition motif
OMM	outer mitochondrial membrane
IMM	inner mitochondrial membrane
IMS	intermembrane space
MIA	mitochondrial intermembrane space import and assembly
SAM	sorting and assembly machinery
PAM	presequence translocase-associated motor
MPP	mitochondrial processing peptidase
IMPDH1	Inosine 5'-monophosphate dehydrogenase 1
TCA	Tricarboxylic Acid
PPP	pentose phosphate pathway
OXPHOS	Oxidative phosphorylation
OAA	oxaloacetate
α -KG	alpha-ketoglutarate
ETC	electron transport chain
MRG	Mitochondrial RNA granules
4-SU	4-thiouridine
OOPS	orthogonal organic phase separation
XRNAX	protein-crosslinked RNA extraction
Ptex	phenol toluol extraction
TRAPP	total RNA-associated protein purification
PKR	protein kinase R
OSCP	oligomycin sensitivity-conferring protein
UV	ultraviolet light
PNK	polynucleotide kinase
2C	Complex capture
eCLIP	enhanced CrossLinking and ImmunoPrecipitation

LARP	La-related protein
TOP	terminal oligopyrimidine tract
EMSA	electromobility shift assay
QuaNCAT	quantitative noncanonical amino acid tagging
SILAC	stable-isotope-labeled amino acids
AHA	L-Azidohomoalanine
siRNA	small interfering RNA
SG	stress granule
HCC	Hepatocarcinoma cells
MTS	mitochondrial targeting signal
LC-MS	Liquid chromatography-Mass spectrometry
GOI	Gene of interest
UMI	unique molecular identifier
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
RT	Reverse transcriptase

List of figures

Figure 1.1. Different types of RNA in eukaryotes.....	2
Figure 1.2. Metabolic enzymes as RNA binding proteins.....	6
Figure 1.3. Reactions of glycolysis.....	9
Figure 1.4. Steps of Mitochondrial TCA cycle and oxidative phosphorylation..	10
Figure 1.5. Mitochondrial protein import pathways.....	17
Figure 1.6. Protein import into the mitochondrial matrix.....	19
Figure 1.7. Mitochondrial tricarboxylic acid cycle.....	20
Figure 1.8. Mitochondrial electron transport chain and the ATP synthase complex.	22
Figure 2.1. MDH2, a mitochondrial metabolic enzyme.	32
Figure 2.2. Structural features of human MDH2.....	33
Figure 2.3. Sequence conservation of MDH2.	37
Figure 2.4. PNK assay validating MDH2's RNA binding.	39
Figure 2.5. 2C assay for MDH2's RNA binding.....	41
Figure 2.6. eCLIP experimental scheme.....	42
Figure 2.7. MDH2 binds cytosolic RNAs	43
Figure 2.8. MDH2 prefers to bind tRNAs	44
Figure 2.9. MDH2 crosslinking sites on select tRNA targets.....	46
Figure 2.10. MDH2 crosslinking sites on MT-tRNAs and mRNAs	47
Figure 2.11. MDH2 protein purification and probe design for EMSA.....	49
Figure 2.12. In-vitro characterisation of MDH2's RNA binding	52
Figure 2.13. MDH2 binds RNA outside of mitochondria.	53
Figure 2.14. Canonical MTS is dispensable for RNA binding.	55
Figure 2.15. Effect of cryptic targeting signal on MDH2's mitochondrial import	57
Figure 2.16. MDH2 Δ 2-53 shows diffuse cytoplasmic staining.....	58
Figure 2.17. PNK assay for mislocalised MDH2.	59
Figure 2.18. MDH2's reactant metabolites regulate RNA binding in-vitro.....	61
Figure 2.19. NAD modulation alters MDH2's RNA binding levels.	63
Figure 2.20. NAD modulation impacts cellular respiration.....	64
Figure 2.21. MDH2's RNA binding changes in cellular states with altered mitochondrial bioenergetics.....	66
Figure 2.22. RNA binding regions of MDH2 mapped by RBD map	68
Figure 2.23. Characterisation of RNA binding mutants.	69
Figure 2.24. Characterisation of MDH2 domain truncations	71

Figure 3.1. Structural organisation of the ATP synthase complex.	78
Figure 3.2. ATP5A1 binds to mRNAs with a 5' TOP motif.	81
Figure 3.3. RNA targets and ATP5A1 protein used for EMSA assays	83
Figure 3.4. ATP5A1 binds TOP-motif sequences in-vitro.	85
Figure 3.5.	86
Figure 3.6. QuanCAT experiment to measure effect of ATP5A1 depletion on nascent protein synthesis.	88
Figure 3.7. Altered protein synthesis after ATP5A1 depletion.	89
Figure 3.8. Deregulated pathways after ATP5A1 depletion.....	90
Figure 3.9. Global proteome is not altered significantly after ATP5A1 depletion....	91
Figure 3.10. Change in translation of ATP5A1-bound RNAs	92
Figure 3.11. Identification of ATP5A1's protein interactors.....	93
Figure 3.12. ATP5A1's interaction network extends beyond the mitochondria to cytosolic factors including translational machinery and RNP granules.	94
Figure 3.13. ATP5A1 co-purifies G3BP2 and IGF2BP1 in pulldown experiments..	95
Supplementary figure 1. Sequence conservation analysis of MDH2 proteins from species with identified RNA binding property.	147
Supplementary figure 2. Quality control steps for eCLIP.	148
Supplementary figure 3. eGFP protein preparation and overview of MDH2 crosslinking RPS23 5'UTR	149
Supplementary figure 4. EMSA for MDH2's RNA target Lys TTT 3-5.	149
Supplementary figure 5. Standardisation of MDH2 depletion with siRNAs	150
Supplementary figure 6. Standardisation of enzyme assay to measure MDH2 activity	151
Supplementary figure 7. Quality control plots for QuanCAT after ATP5A1 depelction.	152
Supplementary figure 8. ATP5A1 co-IP mass spectrometry.....	153

List of tables

Table 1. Catalogue of studies identifying MDH2 as an RNA binding protein	34
Table 2. Antibodies	103
Table 3. Enzymes	103
Table 4. Kits	104
Table 5. Chemicals/Reagents	104
Table 6. Consumables	107
Table 7. Buffers and Solutions	107
Table 8. Equipment	109
Table 9. Software	110
Table 10. Plasmids	110
Table 11. Oligos	110
Supplementary table 1. Table of studies where MDH2 has been identified as an RNA binding protein with information about the methods.....	156

