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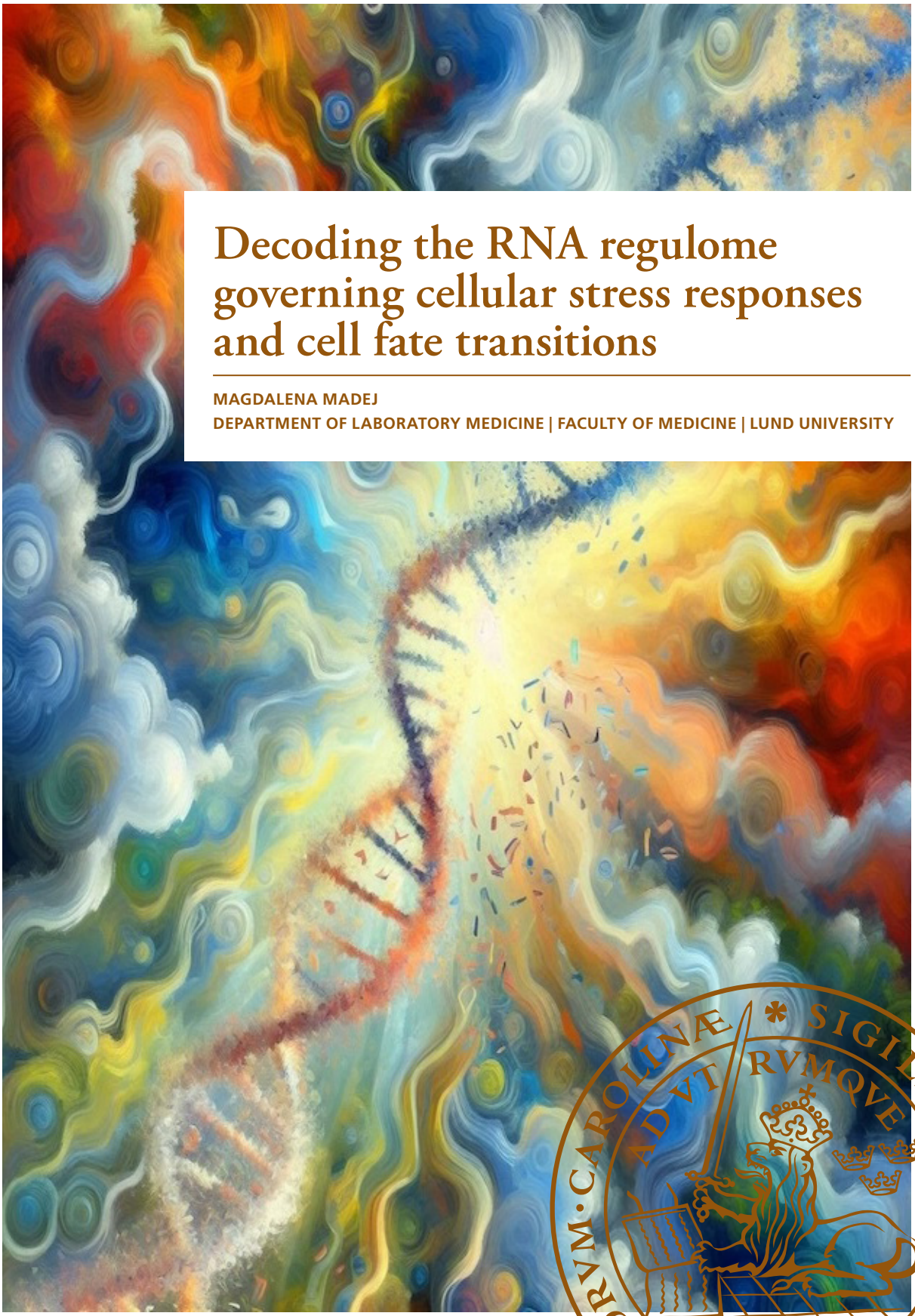

Decoding the RNA regulome governing cellular stress responses and cell fate transitions

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DOCTORAL DISSERTATION

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Title: Decoding the RNA regulome governing cellular stress responses and cell fate transitions

Abstract:

RNA modifying enzymes play a pivotal role in reshaping gene expression programs in response to diverse intracellular and extracellular cues, thereby maintaining cellular homeostasis. When dysregulated, these enzymes are increasingly implicated in the onset and progression of various diseases. While traditionally recognised for their catalytic functions, mounting evidence suggests that many RNA modifying enzymes exhibit additional non-catalytic “moonlighting” activities. However, the full extent of their multifunctionality remains poorly understood.

In the first study, I defined a previously unrecognized role for pseudouridine synthase 10 (PUS10) in regulating the innate immune response. Using in vitro and in vivo model systems, I demonstrated that PUS10 deficiency leads to upregulation of interferon-stimulated genes (ISGs). Mechanistically, PUS10 utilizes a specific subset of tRNA-derived small RNAs (tdRs) to regulate translation and suppress the expression of endogenous retroelements. PUS10 loss triggers activation of the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway, likely via RNA-DNA hybrids accumulation. Furthermore, I uncovered a transcriptional signature associated with PUS10 that correlates with autoimmune disease. Together, these findings identify PUS10 as a novel regulator of viral mimicry and immune balance.

Based on prior evidence, I discovered that PUS10 depletion affects DNA damage response (DDR) signalling pathways, sensitizes cells to DNA damage-induced cell death and disrupts cell cycle progression. PUS10 appears to influence DNA repair pathway choice by suppressing error-prone non-homologous end joining (NHEJ) and favouring high-fidelity homologous recombination (HR). Beyond genome maintenance, PUS10 modulates cell fate transitions by constraining oncogene-driven transformation and suppressing somatic reprogramming efficiency.

In a separate collaborative study, I defined how another pseudouridine synthase, PUS7, modifies a stem-cell enriched subset of tdRs to regulate translation via interaction with PABPC1, with implications for haematopoiesis and leukaemogenesis.

Finally, I contributed to research uncovering a role for RNA demethylase ALKBH5 in modulating translation of the splicing factor SF3B1. This regulation directs splicing of DNA repair and epigenetic regulators during oncogenic transformation, ultimately influencing genome integrity and leukaemia progression in vivo.

Collectively, these studies reveal novel roles for RNA modifying enzymes in coordinating cellular responses to genotoxic, immune, and oncogenic stress, highlighting their importance in regulating translation, inflammation, genome stability, and cell plasticity across health and disease.

Key words: RNA, tRNA-derived small RNAs (tDRs), RNA modifications, pseudouridine, pseudouridine synthase (PUS), transposable elements (TEs), inflammation, autoimmune diseases, cancer, DNA damage response (DDR), haematopoietic stem cells (HSCs), induced pluripotent stem cells (iPSCs)

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On the cover: Impressionism-inspired depiction of genome disruption and dysregulation alongside molecular guardians that preserve proper cellular functions. The partially disintegrating DNA double helix represents loss of genomic integrity and deregulation events, such as transposable element activation. Surrounding spiral motifs symbolize RNA molecules involved in maintaining cellular homeostasis. This image was generated using DALL·E via Bing Image Creator.

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To my family

Success is not final, failure is not fatal:
it is the courage to continue that counts.
Winston Churchill

Table of Contents

Abstract

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In the first study, I defined a previously unrecognized role for pseudouridine synthase 10 (PUS10) in regulating the innate immune response. Using *in vitro* and *in vivo* model systems, I demonstrated that PUS10 deficiency leads to upregulation of interferon-stimulated genes (ISGs). Mechanistically, PUS10 utilizes a specific subset of tRNA-derived small RNAs (tdRs) to regulate translation and suppress the expression of endogenous retroelements. PUS10 loss triggers activation of the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway, likely via RNA-DNA hybrids accumulation. Furthermore, I uncovered a transcriptional signature associated with PUS10 that correlates with autoimmune disease. Together, these findings identify PUS10 as a novel regulator of viral mimicry and immune balance.

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Finally, I contributed to research uncovering a role for RNA demethylase ALKBH5 in modulating translation of the splicing factor SF3B1. This regulation directs splicing of DNA repair and epigenetic regulators during oncogenic transformation, ultimately influencing genome integrity and leukaemia progression *in vivo*.

Collectively, these studies reveal novel roles for RNA modifying enzymes in coordinating cellular responses to genotoxic, immune, and oncogenic stress, highlighting their importance in regulating translation, inflammation, genome stability, and cell plasticity across health and disease.

Abbreviations

AGS	Aicardi-Goutières syndrome
ANG	Angiogenin
AS	Alternative splicing
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
CDC25	Cell division cycle 25
cDNA	Complementary DNA
cGAMP	Cyclic GMP–AMP
cGAS	Cyclic GMP–AMP synthase
ChiP	Chromatin immunoprecipitation
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
DDR	DNA damage response
DDRNA	DNA damage response RNA
DNA	Deoxyribonucleic acid
DSB	Double strand break
dsRNA	Double-stranded RNA
EN	Endonuclease
ER	Endoplasmic reticulum
ERV	Endogenous retrovirus
ES	Exon skipping
ESC	Embryonic stem cell
GMP	Guanosine monophosphate
GWAS	Genome-wide association studies
H2AX	Histone H2A variant X
HIV-1	Human immunodeficiency virus type-1
HJ	Holliday junction
hm^5C	5-hydroxymethylcytosine
HR	Homologous recombination
HSC	Haematopoietic stem cell

HSPCs	Haematopoietic stem and progenitor cells
IBD	Inflammatory bowel disease
iCLIP	Individual-nucleotide resolution UV crosslinking and immunoprecipitation
IFN	Interferon
IKK	IκB Kinase
iPSC	Induced pluripotent stem cell
IRF	Interferon regulatory factor
ISGs	Interferon-stimulated genes
KD	Knock-down
KO	Knock-out
KRAB-ZFP	Krüppel-associated box protein containing zinc-fingers
LINE	Long interspersed nuclear element
lncRNA	Long non-coding RNA
LTR	Long terminal repeat
m^5C	5-methylcytosine
MAVS	Mitochondrial antiviral-signalling protein
MDA5	Melanoma differentiation-associated protein 5
MDC1	Mediator of DNA damage checkpoint 1
MERVL	Murine endogenous retrovirus-L
METTL3	Methyltransferase-like 3
miRNA	microRNA
MRN	MRE11-RAD50-NBS1
mRNA	messenger RNA
MS	Mass spectrometry
mTOG	5’ terminal oligoguanine motif
NBS1	Nibrin
NHEJ	Non-homologous end joining
nt	nucleotide
ORF	Open reading frame
ORF2p	ORF2 protein
PBS	Primer binding site
PES	Pyrimidine enriched sequences
Pol	Polymerase
Poly I:C	Polyinosinic:polycytidylic acid
PIKK	Phosphatidylinositol 3-kinase-like kinase
PUS	Pseudouridine synthase
RIG-1	Retinoic acid-inducible gene I

RNA	Ribonucleic acid
RNAi	RNA interference
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT	Reverse transcriptase
SF	Splicing factor
SINE	Short interspersed nuclear element
SLE	Systemic lupus erythematosus
ssDNA	Single-stranded DNA
STING	Stimulator of interferon genes
TBK1	TANK-binding kinase 1
tDR	tRNA-derived small RNA
TE	Transposable element
TPRT	Target-primed reverse transcription
tRF	tRNA-derived fragment
tRNA	Transfer RNA
WT	Wild-type
Ψ	Pseudouridine

Popular summary

This thesis invites readers into the fascinating world of RNA, where seemingly unrelated cellular events like protein production, genome protection, and immune defence are orchestrated by RNA modifying enzymes to safeguard our health.

The first tale revolves around pseudouridine synthase 10, PUS10, an RNA modifying enzyme, that plays a surprising role in regulating inflammation. Instead of modifying RNA, PUS10 empowers small fragments of transfer RNA (tRNA) to silence remnants of ancient viral infections embedded in the genome. When this control fails, these so-called "fossil viruses" reawaken, triggering immune responses that can drive chronic inflammation and autoimmune diseases such as lupus and inflammatory bowel disease (IBD). As the story unfolds, the reader learns how RNA biology intersects with immune regulation, why these viral elements – once dismissed as junk DNA – still matter, and how these insights may guide future diagnostics and RNA-based therapies for autoimmunity and inflammation-linked cancer.

In a preview of the sequel, PUS10 reappears – this time as a guardian of the genome integrity. Our DNA is constantly at risk, whether from everyday replication errors or damage from sunlight, smoking, or alcohol. To defend against these threats, cells launch an emergency system known as the DNA damage response. PUS10 steps in here again, contributing to the cell's ability to repair its genome. Intriguingly, PUS10 also seems to influence whether cells become cancerous or, in a very different context, whether they can be reprogrammed back into a pluripotent state with the potential to become any cell type in the body. How PUS10 governs these seemingly opposing fates remains a mystery, and the extent to which these diverse functions are interconnected is a question that still awaits an answer.

Another side story centres on a different member of the pseuduridine synthase family, PUS7. This character plays a more traditional role by modifying small fragments of tRNA, which in turn regulate the translation (protein production) of a specific group of messenger RNAs (mRNAs). When this system becomes dysregulated, it disrupts the formation and function of blood cells in patients with a myelodysplastic syndrome (MDS) subtype that carries a high risk of progression into acute myeloid leukaemia (AML).

The final chapter introduces another key player implicated in the increased risk of leukaemic transformation in MDS – the splicing factor SF3B1. Alternative splicing,

the process by which cells generate different versions of protein from a single gene, is essential for protein diversity. SF3B1 functions as a master regulator of the splicing of genes involved in DNA repair and regulation. Interestingly, the production of SF3B1 is regulated at the protein-making stage by a chemical modification, methylation, present on its mRNA. This modification is removed by the RNA demethylase enzyme ALKBH5. When this control is disrupted, it leads to abnormal splicing of certain mRNAs that can contribute to leukaemia development.

Overall, this thesis reveals unexpected and multifaceted roles for RNA modifying enzymes in protein production, safeguarding genome integrity, and regulating inflammation, uncovering molecular stories that shape human health and disease.

Streszczenie popularnonaukowe

Rozprawa ta zabiera czytelnika w fascynujący świat RNA, gdzie z pozoru odrębne procesy zachodzące w komórkach takie jak produkcja białek, ochrona materiału genetycznego przed uszkodzeniami czy odpowiedź immunologiczna są precyzyjnie regulowane przez enzymy modyfikujące RNA, które wspólnie czuwają nad zdrowiem organizmu.

Pierwsza historia skupia się na niespodziewanej roli jednego z enzymów modyfikujących RNA – syntazy pseudourydyny 10 (PUS10) – która reguluje odpowiedź immunologiczną w sposób niezależny od swojej aktywności enzymatycznej. Zamiast bezpośrednio modyfikować RNA, PUS10 wykorzystuje małe fragmenty transferowego RNA (tRNA), aby uciszyć pozostałości dawnych infekcji wirusowych zapisane w genomie. Gdy ta kontrola zawodzi, tak zwane „skamieniałości wirusowe" budzą się do życia, uruchamiając reakcję układu odpornościowego, która może prowadzić do przewległego stanu zapalnego i rozwoju chorób autoimmunologicznych, takich jak toczeń czy nieswoiste zapalenia jelit. W miarę rozwoju tej opowieści, czytelnik odkrywa jak biologia RNA splata się z regulacją odporności, dlaczego fragmenty pochodzenia wirusowego, kiedyś uznawane za zbędne „śmieciowe DNA", wciąż mają znaczenie, oraz w jaki sposób ta wiedza może przyczynić się do tworzenia nowych narzędzi diagnostycznych i terapii RNA w leczeniu chorób autoimmunologicznych i nowotworów związanych ze stanem zapalnym.

W zapowiedzi kolejnego rozdziału tej historii PUS10 powraca – tym razem jako strażnik integralności genomu. DNA jest nieustannie narażone na uszkodzenia, zarówno w wyniku codziennego kopiowania materiału genetycznego, jak i działania czynników zewnętrznych takich jak promieniowanie UV, palenie czy alkohol. Aby temu przeciwdziałać, komórki uruchamiają system alarmowy zwany odpowiedzią na uszkodzenia DNA. I tu znów wkracza PUS10, wpływając na zdolność komórki do naprawy genomu. Co ciekawe, PUS10 wydaje się również mieć wpływ na to, czy komórka stanie się nowotworowa lub, w innym jeszcze kontekście, czy uda się ją zreprogramować do stanu pluripotencjalnego, w którym może przekształcić się w dowolny typ komórki w organizmie. W jaki sposób PUS10 reguluje te pozornie przeciwstawne losy komórek, wciąż pozostaje zagadką. Również to, czy wszystkie te funkcje są ze sobą powiązane, jest pytaniem, na które nauka dopiero szuka odpowiedzi.

Kolejna historia skupia się na innym członku rodziny syntetaz pseudourydyny – PUS7. Ten bohater pełni bardziej tradycyjną rolę, modyfikując małe fragmenty tRNA, które z kolei regulują translację (produkcję białek) określonej grupy matrycowego RNA (mRNA). Gdy ten system ulega rozregulowania, zaburza to powstawanie i funkcjonowanie komórek krwi u pacjentów cierpiących na podtyp zespołu mielodysplastycznego, który wiąże się z wysokim ryzykiem przejścia w ostrą białaczkę szpikową.

Ostatni rozdział wprowadza kolejnego gracza zaangażowanego w rozwój białaczek u pacjentów z zespołami mielodysplastycznymi – czynnik splicingowy SF3B1. Alternatywny splicing to proces, w którym komórki wytwarzają różne izoformy białek na podstawie pojedynczego genu – mechanizm niezbędny dla zapewnienia różnorodności białek w organizmie. SF3B1 pełni rolę głównego regulatora splicingu genów odpowiedzialnych za naprawę i regulację DNA. Co istotne, produkcja samego SF3B1 jest regulowana już na etapie syntezy białka poprzez chemiczną modyfikację – metylację – obecną na jego mRNA. Modyfikacja ta jest usuwana przez enzym demetylujący RNA – ALKBH5. Gdy ten mechanizm kontroli zostaje zaburzony, dochodzi do nieprawidłowego splicingu wybranych mRNA, co może sprzyjać rozwojowi białaczki.

Podsumowując, lektura tej rozprawy pozwala odkryć zaskakujące i wielowymiarowe role enzymów modyfikujących RNA – w produkcji białek, ochronie integralności genomu oraz regulacji odpowiedzi immunologicznej – opowieściach molekularnych, które definiują nasze zdrowie i choroby.

Populärvetenskaplig sammanfattning

Denna avhandling tar med läsaren in i RNA:s fascinerande värld, där till synes orelaterade cellfunktioner som proteinproduktion, skydd av arvsmassan och immunförsvar samordnas av RNA-modifierande enzymer för att upprätthålla vår hälsa.

Den första berättelsen kretsar kring pseudouridinsyntas 10, PUS10, ett RNA-modifierande enzym som spelar en oväntad roll i regleringen av inflammation. Istället för att modifiera RNA direkt, möjliggör PUS10 för små fragment av tRNA (transfer-RNA) att tysta ned rester av gamla virusinfektioner som finns inbäddade i vårt genom. När denna kontroll förloras väcks dessa så kallade "fossila virus" till liv igen, vilket aktiverar immunförsvaret och kan leda till kronisk inflammation och autoimmuna sjukdomar som lupus och inflammatoriska tarmsjukdomar. Genom berättelsens gång får läsaren insikt i hur RNA-biologi och immunreglering hänger ihop, varför dessa virusrester – tidigare avfärdade som skräp-DNA – fortfarande har betydelse, och hur denna kunskap kan bidra till framtidens diagnoser och RNA-baserade behandlingar mot autoimmunitet och inflammation.

I nästa kapitel återvänder PUS10 – denna gång som väktare av genomets integritet. Vårt DNA utsätts ständigt för risker, exempelvis vid celldelning eller vid påverkan från solljus, rökning eller alkohol. För att bemöta dessa hot aktiverar cellen ett akut reparationssystem, känt som DNA-skaderesponsen. Här spelar PUS10 återigen en roll genom att bidra till reparation av DNA. Fascinerande nog verkar PUS10 även påverka huruvida en cell utvecklas till en cancercell eller – i ett helt annat sammanhang – om den kan omprogrammeras till ett pluripotent tillstånd, där den kan utvecklas till vilken celltyp som helst i kroppen. Hur PUS10 styr dessa till synes motsatta öden är fortfarande oklart, och om dess olika funktioner hänger ihop återstår att undersöka.

En annan delberättelse fokuserar på ett annat enzym i samma familj PUS7. Den här karaktären spelar en mer traditionell roll genom att modifiera små fragment av tRNA, som i sin tur reglerar translationen (proteinsyntes) av en specifik grupp budbärar-RNA (mRNA). När detta system störs påverkas bildningen och funktionen av blodceller hos patienter med myelodysplastiskt syndrom (MDS), särskilt i den undergrupp som har hög risk att utvecklas till akut myeloisk leukemi (AML).

I det sista kapitlet introduceras ännu en viktig aktör i leukemiutvecklingen vid MDS – splicingsfaktorn SF3B1. Alternativ splitsning, processen där cellen skapar olika

proteinvarianter från samma gen, är avgörande för proteinmångfalden. SF3B1 fungerar som en central reglerare för splitsningen av gener som styr DNA-reparation och reglering. Intressant nog regleras produktionen av SF3B1-protein på själva proteinsyntesnivån genom en kemisk modifiering – metylation – som finns på dess mRNA. Denna modifiering tas bort av enzymet ALKBH5, som fungerar som en RNA-demetylas. När denna kontrollmekanism störs leder det till onormal splitsning av vissa mRNA, vilket i sin tur kan bidra till utvecklingen av leukemi.

Sammanfattningsvis avslöjar denna avhandling oväntade och mångfacetterade roller för RNA-modifierande enzymer i regleringen av proteinproduktion, genomstabilitet och inflammation – molekylära berättelser som formar vår hälsa och sjukdomsutveckling.

Aim of the thesis

The overarching goal of this thesis is to decode the RNA regulome governed by RNA modifying enzymes underpinning cellular stress responses and cell fate transitions, with particular emphasis on the pseudouridine synthases PUS7 and PUS10, and the RNA demethylase ALKBH5. By elucidating how these enzymes reshape gene expression programs in response to genotoxic, inflammatory, and oncogenic stress, this work aims to uncover their roles in maintaining genome integrity, regulating translation, and controlling immune signalling and cell plasticity.

To achieve this, the thesis is divided into the following specific aims:

1) To delineate the role of PUS10 in modulating retrotransposon-driven inflammation through transfer RNA (tRNA)-derived small RNAs (tDRs) and explore the implications of its dysregulation in autoimmune diseases and cancer (Paper I).

2) To investigate how PUS10 loss affects various cellular processes, such as maintenance of genomic integrity and cell fate determination (Paper II).

3) To examine how PUS7-mediated pseudouridylation of small tRNA-fragments (tRFs) containing 5' terminal oligoguanine motif (mTOG) regulates translation in stem cells and its impact on haematopoiesis and leukaemogenesis (Paper III).

4) To determine the molecular control of SF3B1 and the consequences of its dysregulation on genome-wide splicing and leukaemogenesis (Paper IV).

Through comprehensive investigations of select enzymes – PUS7, PUS10 and ALKBH5 – this thesis seeks to understand how RNA modifying enzymes impact the molecular mechanisms of stem cell biology and differentiation, immune regulation, and disease pathogenesis, providing valuable insights that could guide the development of novel therapeutic strategies.

List of Papers

Articles included in this thesis

Paper I

Madej, M., Ngoc, P.C.T., Muthukumar, S., Konturek-Ciesla, A., Tucciarone, S., Germanos, A., Ashworth, C., Kotarsky, K., Ghosh, S., Fan, Z., Fritz, H., Pascual-Gonzalez, I., Huerta, A., Guzzi, N., Colazzo, A., Beneventi, G., Lee, H.M., Ciesla, M., Douse, C., Kato, H., Swaminathan, V., Agace, W.W., Castellanos-Rubio, A., Salomoni, P., Bryder, D., and Bellodi, C. (2025). PUS10-induced tRNA fragmentation impacts retrotransposon-driven inflammation. Cell Rep 44, 115735. 10.1016/j.celrep.2025.115735.

Paper II (manuscript)

Madej, M., H. Finnan, C. Pires, N. Arh, A. Konturek-Cieśla, M. Cieśla, S. Muthukumar, CF Pereira, and C. Bellodi. (2025). PUS10 drives DNA repair pathway choice and cell fate plasticity in physiological and oncogenic contexts.

Paper III

Guzzi, N., Muthukumar, S., Ciesla, M., Todisco, G., Ngoc, P.C.T., **Madej, M.**, Munita, R., Fazio, S., Ekstrom, S., Mortera-Blanco, T., Jansson, M., Nannya, Y., Cazzola, M., Ogawa, S., Malcovati, L., Hellstrom-Lindberg, E., Dimitriou, M., and Bellodi, C. (2022). Pseudouridine-modified tRNA fragments repress aberrant protein synthesis and predict leukaemic progression in myelodysplastic syndrome. Nat Cell Biol 24, 299-306. 10.1038/s41556-022-00852-9.

Paper IV

Ciesla, M., Ngoc, P.C.T., Muthukumar, S., Todisco, G., **Madej, M.**, Fritz, H., Dimitriou, M., Incarnato, D., Hellstrom-Lindberg, E., and Bellodi, C. (2023). m(6)A-driven SF3B1 translation control steers splicing to direct genome integrity and leukemogenesis. Mol Cell 83, 1165-1179 e1111. 10.1016/j.molcel.2023.02.024.

Articles not included in this thesis

Beneventi, G., Munita, R., Cao Thi Ngoc, P., Madej, M., Ciesla, M., Muthukumar, S., Krogh, N., Nielsen, H., Swaminathan, V., and Bellodi, C. (2021). The small Cajal body-specific RNA 15 (SCARNA15) directs p53 and redox homeostasis via selective splicing in cancer cells. NAR Cancer 3, zcab026. 10.1093/narcan/zcab026.

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Diecke, S., Madej, M., Ambrosini, C., Neumann, K., Aygar, S., Org, T., Healy L., Stachelscheid, H., David, L., Battle-Morera, L., and Johansson, PA. (2025). Gene Editing in hPSCs: Core Facility Recommendations for Experimental Design and Post-Editing (manuscript)

Pietraszek-Gremplewicz, K., Kozakowska, M., Bronisz-Budzynska, I., Ciesla, M., Mucha, O., Podkalicka, P., Madej, M., Glowniak, U., Szade, K., Stepniewski, J., Jez, M., Andrysiak, K., Bukowska-Strakova, K., Kaminska, A., Kostera-Pruszczyk, A., Jozkowicz, A., Loboda, A., and Dulak, J. (2018). Heme Oxygenase-1 Influences Satellite Cells and Progression of Duchenne Muscular Dystrophy in Mice. Antioxid Redox Signal 29, 128-148. 10.1089/ars.2017.7435.

Author's contribution to the papers

The following section outlines my specific contributions to each of the included studies, in accordance with the thesis template guidelines.

Paper I

This project was conceptualised together with my supervisor, and I executed most of the experimental work presented in this manuscript under his supervision. I generated the *Pus10*-knockout (KO) mouse model, including plasmid preparation, coordination with the core facility, and the establishment of a genotyping strategy. I handled animal care, performed Kaplan-Meier survival analysis, collected tissues, and isolated mouse embryonic fibroblasts (MEFs), which I subsequently characterised.

I initiated and coordinated the collaboration for analyses of the haematopoietic system, including transplantation and high molecular weight polyinosine-polycytidylic acid (Poly I:C HMW) treatment, and was responsible for associated *in vivo* experiments, some conducted with the support of a collaborator.

I independently prepared RNA and small RNA sequencing libraries, and carried out individual-nucleotide resolution UV crosslinking and immunoprecipitation (iCLIP) experiments together with a colleague. I generated CUT&RUN samples and processed them jointly with a collaborator. I coordinated and performed HSPC isolation and FACS-isolation alongside a collaborator, independently prepared RNA, assessed RNA quality, and arranged international shipment. I oversaw bioinformatic analyses, including troubleshooting and iterative communication. I established various laboratory protocols, including those for detecting RNA-DNA hybrids, and performed several of the *in vitro* experiments presented in this paper.

Additional analyses, including body weight measurements and global protein synthesis, were originally performed by me during the initial study phase. Although I was not directly involved in the subsequent revision process per agreement with the Medical Faculty of the Lund University, I understand that some raw data and resources generated by me were utilised for the final revisions.

I analysed data, coordinated with selected collaborators, and wrote the initial manuscript draft.

Paper II

This project was conceptualised together with my supervisor, and I executed most of the experimental work presented in this manuscript under his supervision. I generated and maintained the relevant cellular models, performed MEF isolation in collaboration with a partner group for reprogramming experiments, and characterised induced pluripotent stem cells (iPSCs) either independently or in collaboration with a student under my supervision. I established and optimised key protocols in the lab, particularly those related to the DNA damage response.

The electrophoretic mobility shift assay (EMSA) was performed in collaboration with a postdoctoral researcher from the group. Reprogramming efficiency experiments were carried out by a collaborator.

I analysed data, coordinated with collaborators, and wrote the initial manuscript draft.

Paper III and Paper IV

In these collaborative studies, I provided support in the preparation of molecular reagents, biological tools, and experimental models, and performed selected experimental analyses.

Introduction

Science is like a puzzle of infinite shape and scale,
pieced together by many people across the globe.

Katalin Karikó

The critical contribution of pseudouridine to the life-saving efficacy of mRNA COVID-19 vaccines, recognized by the 2023 Nobel Prize in Physiology or Medicine awarded to Katalin Karikó and Drew Weissman for their groundbreaking findings concerning nucleoside base modification and mRNA interactions with immune system, draws collective attention to intricate world of RNA modifications. While these modifications have long been studied as individual chemical marks, they are now increasingly appreciated as components of a broader regulatory system orchestrated by RNA modifying enzymes. Beyond their site-specific catalytic activities, such as pseudouridylation and methylation, many of these enzymes also exert non-catalytic "moonlighting" functions that reprogram gene expression and shape diverse cellular outcomes.

RNA modifications

Since the first discovery of naturally occurring chemical modifications, such as 5-methyl-cytidine (m^5C) and pseudouridine (Ψ), of RNA, called "epitranscriptome", more than 60 years ago, over 170 RNA modifications have been described to date.[1-3] These modifications extend to all four nucleobases in RNA: adenine (A), cytosine (C), guanine (G) and uridine (U). RNA modifications influence multiple layers of gene regulation, including transcriptional, post-transcriptional, and translational processes, by impacting RNA metabolism through the modulation of RNA structure, stability and RNA-protein interactions. Among the most prevalent modifications widespread on coding and non-coding RNAs are methylation, pseudouridylation, and adenosine-to-inosine (A-to-I) editing. The effect of RNA modifications on RNA metabolism is thoroughly investigated, particularly in the context of the three key types of RNA involved in protein synthesis – messenger RNA (mRNA), tRNA, and ribosomal RNA (rRNA). Modifications of these RNA

occurs co- or post-transcriptionally and, by bridging transcription with translation, influence cell function. The role of the epitranscriptome in modulating cell fates has been extensively studied in cellular responses to external stress stimuli, including DNA damage, oxidative stress, and chemotherapeutic drugs.[4-6] Additionally, RNA modifications are crucial in facilitating the ability of cells to adapt to rapidly changing microenvironments, a requirement for precise cell fate transitions, such as somatic cell reprogramming and stem cell differentiation.[7] Intriguingly, an aberrant epitranscriptome has been identified as a causative factor in various human diseases, including neurological and metabolic disorders, and cancer.[5,7] This thesis focuses on elucidating the molecular and cellular functions of RNA pseudouridylation and methylation, shedding light on how their dysregulation may be implicated in human health and disease.

Pseudouridylation

Pseudouridine is the most abundant RNA modification, commonly referred to "the fifth ribonucleoside".[8,9] Development of a methodology facilitating mapping of Ψ with single-nucleotide resolution using primer extension in 1993 has led to identification of Ψ locations. Briefly, the method relies on the formation of stable adducts between Ψ and N-cyclohexyl-N'-β-(4-methylmorpholinium) ethylcarbodiimide p-tosylate (CMCT) that block reverse transcriptase. Such truncated cDNA is sequenced to reveal Ψ sites.[10] Adaptation of the technique to achieve compatibility with Illumina next-generation sequencing (NGS) platform has largely facilitated mapping of Ψ across the transcriptome.[11-13] Another approach to study this RNA modification is based on synthesis of a modified carbodiimide that enables chemical coupling of biotin to Ψ and subsequent sequencing of enriched Ψ-containing RNAs.[14] More recently, a direct RNA sequencing platform developed by Oxford Nanopore Technologies (ONT), which enables detection of various RNA modifications, including pseudouridine, has emerged as an alternative to the conventional NGS-based technologies. Here, identification of RNA modifications with single nucleotide resolution relies on systemic base-calling errors, with pseudouridine manifesting as uridine-to-cytosine mismatches.[15] Lastly, the development of "PRAISE", a method for the selective chemical labelling of Ψ through bisulfite/sulfite treatment and subsequent detection of deletion signatures during reverse transcription, allows for quantitative transcriptome-wide assessment of Ψ sites with single-base resolution.[16] Despite rapid technological advances in quantitative profiling of Ψ, more studies are needed to functionally elucidate the biological role of pseudouridylation. Ψ is installed in a RNA-dependent manner by dyskerin (DKC1) or RNA-independent mechanisms by stand-alone pseudouridine synthases (PUSes). Ψ affects functional properties of RNAs, thus impacting fundamental cellular processes such as protein synthesis and splicing.[17]

Stand-alone PUSes

Stand-alone PUS enzymes recognise a uridine to be modified within a specific sequence context and/or structural motif of the target RNA. Despite the low sequence similarity the thirteen human PUSes can be classified into six families – TruA, TruB, TruD, RluA, RsuA and Pus10. The common denominator among all the PUS enzymes is the structure of the catalytic domain and the presence of a conserved catalytic aspartate (Asp) residue in the active site. These enzymes target either a single uridine or multiple uridines across various RNA species, such as tRNA and rRNA, in the nucleus, cytoplasm and mitochondria.[17] While PUS proteins have primarily been studied for their enzymatic functions, they may also possess non-enzymatic roles, as exemplified by PUS10.[18]

PUS10

Present only in eukaryotes and archaea, PUS10 lacks sequence homology to other PUSes and therefore belongs to a family on its own.[19] Orthologs of PUS10 catalyse the conversion of uridine to pseudouridine at positions 54 and 55 in specific tRNAs such as $tRNA^{Lys3}$, which serves as a primer for the reverse transcription of human immunodeficiency virus type-1 (HIV-1).[20-23] This dual role in basic RNA biology and viral life cycle exemplifies the functional versatility that will be explored thought this thesis.

In recent years several studies have emerged revealing potential biological functions of PUS10. Notably, RNA interference (RNAi) library screening identified PUS10 as a modulator of TRAIL-induced apoptosis. Specifically, PUS10 was found to be cleaved by caspase-3 and caspase-8, thereby facilitating cytochrome c release from mitochondria, either directly or indirectly.[24,25] Subsequent analysis demonstrated that caspase-3 mediates the translocation of PUS10 from the nucleus to the mitochondria during the early stages of apoptosis initiation. Although this translocation, combined with the release of mitochondrial contents, has been proposed to contribute to a positive feedback loop that amplifies caspase-3 activity, it remains unclear whether this role depends on PUS10's enzymatic function or reflects a non-catalytic, "moonlighting" mechanism.[26]

Moreover, PUS10 regulates diverse biological processes, partly depending on its cellular location, with some functions requiring its catalytic activity and others occurring independently of it. In the nucleus, PUS10 plays a role in microRNA (miRNA) biogenesis through direct interaction with pri-miRNA and the DROSHA-DGCR8 complex, without involvement of its pseudouridine synthase activity. In contrast, in the cytoplasm, PUS10 pseudouridylates tRNAs and contributes to the regulation of cell growth.[18]

A recent study reported downregulation of PUS10 in metastatic renal cell carcinoma (RCC), with low expression correlating with poor prognosis. Repressed by hypoxia

and HIF-1A, PUS10 inhibits the migration of cancer cells. PUS10 affects nuclear distribution protein C (NUDC)/Cofilin1-dependent cytoskeleton organization through regulation of miR-194-5p maturation, independently of its pseudouridine synthase activity.[27]

Additionally, the PUS10 locus has been identified as part of a shared susceptibility region for the development of celiac disease (CeD) and ulcerative colitis (UC) through genome-wide association studies (GWAS).[28,29] These findings suggest that PUS10 may be crucial for understanding the underlying mechanisms of specific autoimmune diseases.

At the organismal level, PUS10 expression increases in aged haematopoietic stem and progenitor cells (HSPCs). Independently of its catalytic activity, overexpression of PUS10 reduces the reconstitution capacity of HSPCs and inhibits their expansion *in vitro*. Loss of PUS10 disrupts haematopoietic balance and impairs haematopoietic stem cell (HSC) function in aged, but not young mice. Mechanistically, PUS10 is ubiquitinated by the CRL4-DCAF1 complex and its upregulation correlates with a decline of the CRL4-DCAF1 complex with age.[25]

Altogether, the current state of knowledge positions PUS10 not merely as a traditional tRNA-modifying enzyme, but as a multifaceted regulator involved in a wide range of cellular processes – many of which occur independently of its catalytic activity. PUS10 exemplifies a growing class of RNA modifying enzymes that adopt broader regulatory roles, spanning translation control, microRNA biogenesis, with implications in haematopoietic function, and disease susceptibility, often through non-canonical, moonlighting mechanisms. These diverse activities challenge the conventional view of RNA modifying enzymes as purely enzymatic proteins and prompt deeper exploration of both their catalytic and non-catalytic contributions to regulation of cellular processes.

Regulatory roles of tRNA and its derivatives

As early as 1954, Francis Crick proposed the existence of a small molecule essential for cellular information flow in his ‘adaptor hypothesis’ – a prediction that was experimentally confirmed just four years later.[30-32] Extensive research has since elucidated how tRNAs mediate protein synthesis by bridging codons in mRNA with their corresponding amino acid, a process enabled by specific recognition through aminoacyl-tRNA synthetases and decoding via anticodon loops.[33-37] tRNA stands out as the most abundant non-coding RNA, accounting for up to 15 % of total RNA content in an eukaryotic cell.[38] Remarkably, human nuclear-encoded tRNAs carry an average of 11 to 13 chemical modifications per molecule, making them the most highly modified class of RNA.[2,39] This is of essence of their functional activity as pseudouridylation catalysed by PUSes enhances tRNA structural stability and

contributes to translation accuracy.[40,41] For decades, tRNAs were viewed almost exclusively through the lens of translation; however growing evidence highlight their involvement in modulation of cellular responses to environmental and physiological cues. Beyond their canonical role, tRNAs contribute to antiviral defence and virus–host interactions. Certain tRNA-derived fragments bind directly to interferon-stimulated proteins such as interferon induced protein with tetratricopeptide repeats 5 (IFIT5) and Schlafen 11 (SLFN11), modulating innate immune responses.[42,43] Conversely, various retroviruses, including HIV-1, and retrotransposons, a class of mobile genetic elements embedded within vertebrate genomes, exploit host tRNAs as primers for reverse transcription.[44] Notably, pseudouridine at position 54 (Ψ54) has been detected in several of these tRNAs, further underscoring the intersection between tRNA biology and RNA modifying enzymes, with potential implications for viral replication, host–pathogen interactions, and regulation of transposable element (TE) activity.[44-46]

Previously disregarded as byproducts of tRNA degradation, tDRs have recently emerged as a distinct class of small regulatory RNAs with diverse biological functions, including gene silencing, epigenetic modulation, regulation of ribosome biogenesis, and translation efficiency.[8] Intriguingly, the abundance of tDRs do not necessarily correspond to that of the parent tRNA, and their generation can be triggered by specific conditions such as transition between developmental stages, stress responses, and viral infection.[47-49] Based on the cleavage site along the mature tRNA sequence and their length, ranging from 10 to 45 bases, tDRs are classified into two major categories: tRNA halves, also referred to as tRNA-derived stress induced RNAs (tiRNAs), and tRFs.[50] tiRNAs are derived from mature tRNAs by angiogenin (ANG) in response to stress stimuli.[51-54] tRFs are generated from mature or pre-tRNAs and classified into tRF-1, tRF-3 (3'-tRF), tRF-5 (5'-tRF), and internal tRF (i-tRF), depending on the cleavage site, by various enzymes.[55,56] tRF-1 fragments originate from the 3′ trailer sequences of pre-tRNAs and are produced by RNaseZ/ELAC2.[55] In contrast, tRF-3s are formed when the TΨC loop at the 3′ end of mature tRNAs is cleaved by enzymes such as Dicer, ANG, or other RNase A family members.[57] Finally, tRF-5s result from cleavage of the D-loop or stem region at the 5′ end of mature tRNAs, primarily mediated by Dicer (Figure 1).[58] Noteworthy is the modulation of tDR by RNA modifications such as PUS7-mediated Ψ that promotes tRNA cleavage under specific conditions.[59]

Although many studies indicate that RNA modifications protect tRNAs from cleavage, emerging evidence suggests that certain modifications may instead facilitate the generation of tDRs.[60] A notable example is the role of PUS7 in promoting generation of specific 5′-tRFs containing terminal oligoguanine (TOG) motifs. In stem cells, PUS7-catalysed pseudouridylation at position U8 of tRNA appears to enhance the production of TOG-containing tDRs, suggesting that Ψ8 may promote selective tRNA cleavage in this context.

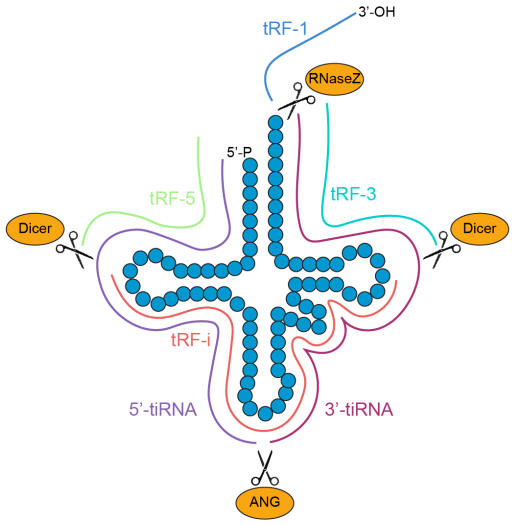

Figure 1 Classification and biogenesis of tRNA-derived small RNAs (tDRs). Based on cleavage position and length, tDRs are divided into tRNA halves (tiRNAs) and tRFs. tiRNAs are generated from mature tRNAs under stress by angiogenin (ANG). tRFs are derived from pre- or mature tRNAs and classified into tRF-1, tRF-3, tRF-5, and internal tRF (tRF-i), depending on the cleavage site. Known processing enzymes include Dicer, ANG, and RNaseZ/ELAC2. Adapted with modifications from [61]. Illustration partially created using BioRender.

Recent research has implicated tDRs in a broad spectrum of molecular functions, including mRNA stabilisation, translational control, gene silencing, and suppression of TEs.[61] Dysregulation of tDR biogenesis and function has been associated with cancer development and progression, altered immune response, and impaired developmental processes.[60,61] Notably, tDRs accumulate under physiological conditions, as illustrated by the enrichment of specific tDRs in mature sperm, where they are involved in paternal intergenerational inheritance alongside DNA.[62,63] Mechanistically, tDRs exhibit diverse modes of action: they may act in RNAi-like pathways, interact directly with RNA-binding proteins (RBS) and other RNA species to regulate their activity and localization or form higher-order structure, including G-quadruplexes, that influence translation initiation and stress granules assembly.[60] Further development of experimental approaches targeting tRFs, focusing on their sequence, post-transcriptional modification, and structural properties, will be crucial for uncovering the full extent of their biological functions.

Transposable elements

Ahead of her time, American geneticist Barbara McClintock perceived the genome a "a highly sensitive organ of the cell that monitors genomic activities and corrects common errors, senses unusual and unexpected events, and responds to them, often

by restructuring the genome", a perspective that paved the way for her revolutionary discovery of mobile genetic elements capable of transposition in the mid-1940s.[64-66] TEs typically range in length from 100 to 10 000 base pairs, occasionally extending to greater sizes.[67] Nearly all eukaryotic genomes examined to date, harbor a substantial proportion of TEs with their abundance generally correlating to genome size.[68] TEs are extremely diverse across species and often species-specific.[69] They constitute up to half of the genetic content in humans and most mammals.[69-71] Although disregarded as "junk DNA" for many years, the expression and mobility of TEs have now been recognized as both a threat to host fitness and a crucial source of evolutionary novelty.

Classification of TEs

Based on their mode of transposition, TEs can be categorized into two major classes: DNA transposons and retrotransposons. Furthermore, each of these classes may be subdivided into orders depending on the specific mechanism of replication and chromosomal integration.[72] DNA transposons mobilize either through a 'cut and paste' mechanism involving excision and insertion of a DNA intermediate, or a 'peel and paste' replicative mechanism that entails a circular DNA intermediate, as exemplified by *Helitrons*.[73] While DNA transposons are active in plants and lower animals, they have undergone inactivation in mammals, with the notable exception of bats.[74] In contrast, retrotransposons (also known as endogenous retroelements [EREs]), are found in all higher species.[69,75] However, their activity significantly differs between rodents and humans.[74] Retrotransposons, commonly referred to as 'copy and paste' elements, replicate through RNA intermediates that are reverse transcribed back into a DNA copy prior to integration into the genome. They are further classified into 1) long terminal repeats (LTR) elements; 2) 'target-primed' non-LTR elements, and 3) relatively unexplored, Tyrosine Recombinase (YR) – mobilized elements. Autonomous LTR elements consist of full or partial sequences encoding *gag*, *pol* and *env*, flanked by LTRs. They are generally transcribed as a single polycistronic RNA by polymerase (Pol) II from a promoter within 5' LTR. These RNAs are then translated into proteins forming a cytoplasmic viral-like particle. Within this structure, retrotranscription is initiated by a tRNA or a tRF paired with a 5' end of RNA, known as the primer binding site (PBS). Subsequently, cDNA is integrated into the host genome in a similar manner to the 'cut and paste' mechanism.[69] Most of the LTR-containing retroelements in vertebrates belong to the endogenous retrovirus (ERV) superfamily. While LTR retroelements activity causes 15% spontaneous germ-line mutations in laboratory mice strains, active source elements in humans remains unknown.[74] Non-LTR retrotransposons, classified as long and short interspersed nuclear elements (LINEs and SINEs, respectively), lack LTRs. LINEs are comprised of two open reading frames (ORFs). Although ORF1 may be dispensable or absent in certain LINEs, in TEs such as those within L1 superfamily, which are highly active in humans and mice, it forms

oligomeric chaperone involved in the recognition and nuclear import of the RNA template. The ORF2 protein (ORF2p) exhibits endonuclease (EN) and reverse transcriptase (RT) activities, crucial for target-primed reverse transcription (TPRT), alongside with three additional domains whose functions remains to be fully elucidated.[69,76] L1 is transcribed into mRNA by Pol II and exported to the cytoplasm. Following translation, L1 RNA, ORF1p, and ORF2p form a ribonucleoprotein (RNP) complex that is imported into the nucleus. Canonically, integration of a new copy of L1 into the host genome occurs through a mechanism known as TPRT. This process begins with the cleavage of DNA by the L1 EN, which preferentially targets a 5'-TT/AAAA-3' site. Subsequently, the L1 RNA undergoes reverse transcription, primed by the liberated 3'-OH genomic DNA. Finally, the newly synthesized cDNA is integrated into the host genome. Interestingly, reverse transcription frequently terminates prematurely leading to 5'-truncation. Since the internal Pol II promoter is usually located within the 5' end, such truncation results in the cessation of further propagation of the newly inserted L1 copy.[69,74] Alternatively, reverse transcription is initiated in an EN-independent manner, at pre-existing DNA lesion.[77] SINEs are non-autonomous retroelements mostly derived from noncoding genes such as tRNAs. Devoid of protein coding sequences, they are transcribed by RNA Pol III and mobilize themselves by exploiting the machinery utilized by LINEs (Figure 2).[69,74]

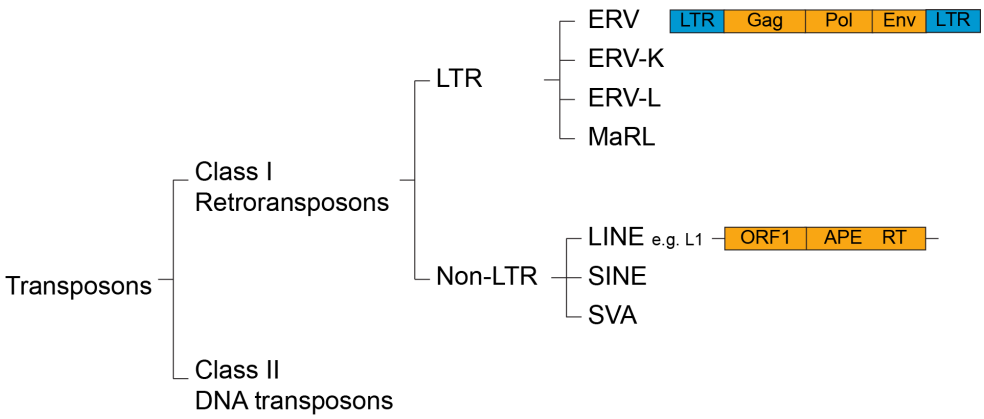

Figure 2 Schematic overview illustrating the classification of transposable elements (TEs). Adapted with modifications from [78] and [79]. APE, apurinic endonuclease; ERV, endogenous retrovirus; LINE; long interspersed nuclear element; LTR, long terminal repeat; MaRL, mammalian-apparent LTR retrotransposon; ORF, open reading frame; RT, reverse transcriptase; SINE; short interspersed nuclear element; SVA, SINE, VNTR (a variable number of tandem repeat), and Alu.

Regulatory activities of TEs

Aside from their harmful effects on the host, TEs are becoming recognized as a potent source of genome innovation. Over the course of evolution, TEs have been

co-opted by the host to regulate genes crucial for organismal development and propagation. Specifically, TEs serve as a substantial reservoir of tissue-specific and alternative promoters, regulatory elements such as enhancers and insulators, as well as non-coding RNAs, including miRNAs and long non-coding RNAs (lncRNAs), involved in gene expression modulation. However, dysfunctional TE-derived regulatory sequences may contribute to development of cancer and inflammatory diseases.[80]

Active TEs

Although constituting a significant proportion of the human genome, only a minority of TEs retain their ability to retrotranspose. Specifically, in addition to LINE-1 elements, distinctive mobility is exhibited by Alu and SINE-VNTR-Alu (SVA) elements that rely on LINE machinery. Intriguingly, recent studies indicate that human ERVs possess the capacity to generate active elements, as exemplified by ERV-K, the youngest element of the human ERV superfamily. ERV-K exhibits transcriptional and translational activity, producing viral particles under specific conditions.[81] In the murine genome, the most active elements belong to the LINE (LINE-1 superfamily), SINE (B1 and B2 superfamilies) and LTR (IAP, ETn, and MLV superfamilies) orders (Figure 3).[78]

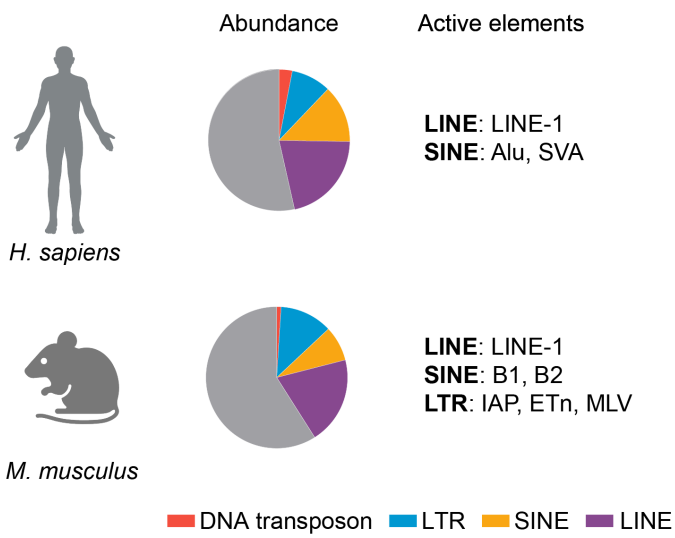

Figure 3 Genomic abundance and composition of transposable elements (TEs) in human and mouse genomes. Adapted from original RepeatMasker-based annotations as presented by [78]. LINEs and SINEs dominate both genomes, with varying contributions from LTRs and DNA transposons. LINE; long interspersed nuclear element; LTR, long terminal repeat; SINE; long interspersed nuclear element. Illustration partially created with BioRender.

Mechanisms to control TEs activity

TE activity poses a persistent threat to the host by inducing genome instability and perturbing genes or their regulatory networks, with more than 120 insertions being associated with human diseases.[78] Apart from insertional mutagenesis, TEs may exert harmful effects by interfering with host mRNA transcription and processing through the de-repression of TE loci, the potential cytotoxicity of TE-encoded peptides, and activation of innate immune response via cytosolic TE-derived nucleic acids.[79,82] Consequently, various defence mechanisms have evolved in mammalian cells aimed at repressing TE expression. These mechanisms encompass transcriptional repression, RNA degradation, and translational control.[78,83]

Due to the prevalence of TE expression in the germline and early embryos, where a permissive chromatin environment facilitates TE mobility, effective control of TEs becomes particularly crucial. Within the germline genome, P-element induced Wimpy testis (PIWI) proteins and their associated PIWI-interacting RNAs constitute the primary mode of TE repression, a mechanism conserved across animal species. In mammals, piRNA pathways are indispensable for recognizing TE sequences without prior information and initiating *de novo* transcriptional silencing in male germ cells. Nevertheless, the precise mechanistic underpinnings remain to be fully elucidated.[83,84] Additionally, another class of small non-coding RNAs, small interfering RNAs plays a pivotal role in TE silencing. When associated with Argonaute proteins, small interfering RNAs (siRNAs) target TEs through complementary base pairing, thereby mediating their repression at the transcriptional level or promoting their post-transcriptional degradation.[85]

Remarkably, the largest class of DNA-binding transcription factors in mouse and humans, Krüppel-associated box proteins containing zinc-fingers (KRAB-ZFPs), have emerged as key players in silencing TEs in a sequence-specific manner during early embryogenesis and in adult tissues.[78,79] KRAB-ZFPs are thought to co-evolve with TEs in an "arms race model" as a strategic response mechanism to counteract TE invasion. Through the interaction with the transcriptional regulator, TRIM28, KRAB-ZFPs facilitate the recruitment of a chromatin-modifying machinery, ultimately fostering formation of heterochromatin and deposition of *de novo* DNA methylation.[86]

Furthermore, TEs are maintained in a repressive state by replication-dependent passive DNA methylation, predominantly mediated by DNA methyltransferase (DNMT) enzymes, with the 5-methylcytosine (m^5C) modification likely representing the most common strategy in higher eukaryotes. Furthermore, m^5C can undergo oxidation yielding 5-hydroxymethylcytosine (hm^5C), 5-formylcytosine (f^5C), and 5-carboxylcytosine (ca^5C). The oxidation is catalysed by the ten-eleven translocation–J-binding protein (TET–JBP) family of enzymes, thereby underscoring their role as additional regulators of TEs.[78] Interestingly, besides small

RNAs pathways and DNA modifications, tRFs, splicing surveillance, and RNA modifications have all been implicated as modulators of TEs expression.[85]

LTR-retroelements and tRNA fragments

LTR-retrotransposons and -viruses predominantly employ intact, fully structured host tRNA molecules as primers for reverse transcription, notwithstanding the limited *in vitro* evidence suggesting use of shorter DNA and RNA fragments for priming.[87] Intriguingly, 3'-tRFs have been reported to suppress retroelements activity through two distinct mechanisms depending on their length, by targeting PBS. Specifically, 22 nucleotide (nt) 3'-tRFs exert post-transcriptionally silencing effects on coding-competent, autonomous LTR-retroelements, subsequently diminishing their RNA and retroviral protein levels. Conversely, 18 nt 3'-tRFs blocks reverse transcription of the coding and non-coding, non-autonomous LTR-retroelements.[88] Additionally, 5'-tRF of glycine tRNA (5'-tRF-Gly-GCC) plays a noteworthy role in transgenerational regulation by repressing genes associated with the LTR-retroelement MERVL during mouse preimplantation embryo development.[89] Subsequent investigations have uncovered that 5'-tRF-Gly-GCC impacts global chromatin organization by controlling ncRNAs biogenesis rather than engaging in sequence-specific suppression of genes driven by MERVL LTR-promoter activity.[90] Despite significant advances in understanding the role of tRFs in modulating TEs, their interaction with other regulatory mechanisms in diverse biological contexts remains to be fully determined.

TE-triggered innate immune response

Emerging evidence suggests that nucleic acids derived from dysregulated TEs are recognized as infectious, eliciting a robust induction of the innate immune response that serves as a non-specific frontline defence mechanism against various pathogens, including viruses. Upon the loss of epigenetic silencing and subsequent transcriptional activation, TE-derived transcripts are transported into the cytoplasm. There, TE-derived nucleic acids or newly translated proteins are sensed by pattern recognition receptors (PPRs), leading to the activation of inflammatory response mediators.[79] Specifically, DNA or RNA-DNA hybrids originating from aberrant TE expression trigger the cyclic GMP–AMP synthase (cGAS)-stimulator of interferon genes (STING) signalling cascade, that is canonically associated with cellular recognition of bacterial and viral components.[91,92] To prevent potential misidentification of endogenous DNA as foreign, PRRs are sequestered within intracellular compartments typically devoid of nucleic acids.[93] However, recent findings suggest the presence of cGAS within the nucleus, operating similarly to its cytoplasmic counterpart depending on ligand availability.[93,94] Nevertheless, the precise molecular mechanisms governing these processes remain incompletely

understood. When DNA ligands bind to cGAS, surpassing a certain signalling threshold, cGAS becomes enzymatically activated, producing 2′3′ cyclic GMP–AMP (cGAMP).[93,95] This cGAMP then binds to STING dimers on the endoplasmic reticulum (ER) membrane, causing structural changes and STING oligomerization.[96,97] Following detachment from the ER membrane and interaction with trafficking factors, the STING oligomer is loaded into coatomer protein complex II (COPII) vesicles.[98] While in transit through the ER–Golgi intermediate compartment (ERGIC) and Golgi apparatus, STING activates TANK-binding kinase 1 (TBK1), leading to TBK1 autophosphorylation. Subsequent phosphorylation of STING at Ser366 by TBK1 facilitates the recruitment of interferon regulatory factor 3 (IRF3).[99] Phosphorylation of IRF3 by TBK1 results in its dimerization, translocation to the nucleus, and induction of type I interferons (IFN) and interferon-stimulated genes (ISGs), among others, ultimately activating the cellular antiviral programme.[93,95,100]

Alternatively, the transcriptional activation of type I IFN and subsequent antiviral response is triggered through detection of both viral and host-derived RNAs by primarily cytosolic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs).[101] RIG-I recognizes short double-stranded RNAs (dsRNAs; <300 bp), whereas MDA5 (melanoma differentiation-associated protein 5) detects larger dsRNAs (>1000 bp) originating from genomic RNA or transcription intermediates.[102,103] Additionally, the third member of the RLR family, laboratory of genetics and physiology 2 (LGP2), lacks structural domains necessary for independent signal-transducing activity and instead regulates the function of RIG-1 and MDA5.[104,105] Upon ligand binding to RIG-1 or MDA5 and their oligomerization, RLRs associate with mitochondrial antiviral-signalling protein (MAVS). Subsequently, MAVS activates TBK1 and IκB kinase-ε (IKKε), which in turn trigger activation of IRF3 and interferon regulatory factor 7 (IRF7), leading finally to the transcriptional induction of ISGs and the initiation of antiviral response.[103]

TEs and human diseases

The most overt causative association between TEs and pathological conditions arises from the disruption of genes resulting from germline insertions. However, it is noteworthy that aberrant expression of TEs also contributes to the development of disease states.[106]

Numerous studies have reported hypomethylation of the L1 promoter and the consequent overexpression of L1 and ORF1p in various cancers such as ovarian, lung, and gastrointestinal track tumours. Elevated levels of L1-derived RNA and protein correlate with characteristics indicative of aggressive cancer, including high-grade malignancy, advanced disease stages, and poorer patient outcomes in terms of survival. Given it potential clinical relevance, L1 has emerged as a promising marker for neoplasia.[107,108] Despite evidence of somatically acquired

insertions of TEs in tumours, they are often regarded as passenger events rather than significant drivers of cancer development.[106] Additionally, current knowledge indicates that L1 expression may induce cytotoxicity and DNA damage, making it less likely to directly confer a growth advantage to cells. However, there remains a possibility that L1-induced DNA damage could lead to cellular transformation or oncogene-induced senescence.[106]

In addition to the extensively studied members of the LINE family, the expression of SINE and ERV elements have been observed in cancerous cells following treatment with DNA-demethylating agents. Such treatment elicits an innate immune response through the viral defence pathway, thereby enhancing the clinical anti-tumour efficacy. This effect is mediated by the cytosolic sensing of dsRNA, derived at least partially from ERV genes.[109,110] Consequently, targeting TEs holds particular promise for immunotherapies.

Interestingly, innate immune activation through MDA5 triggered by increased expression of TEs, mainly ERVs and LINEs, is crucial for exit from quiescence and subsequent haematopoietic regeneration following chemotherapy in HSCs.[111] Altogether, TE-driven signalling has emerged as a pivotal cellular response to diverse physiological and pathological challenges.

Due to their ability to elicit the interferon response in the same manner as upon viral infection, dysregulation of TEs has been proposed to be associated with a variety of inflammatory disorders.[112] A prominent example is Aicardi-Goutières syndrome (AGS) that manifests as severe brain dysfunction and malfunction of the immune system.[113] AGS arises from loss-of-function mutations in genes encoding proteins involved in nucleic acid sensing, such as three prime repair exonuclease 1 (TREX1), ribonuclease H2 endonuclease complex components, and adenosine deaminase acting on RNA 1 (ADAR1) responsible for dsRNA adenosine-to-inosine editing enzymes.[114-116] Conversely, gain-of-function mutations in *MDA5* results in hypersensitivity to Alu-derived dsRNA and, besides contributing to AGS, lead to a heritable form of systemic lupus erythematosus (SLE).[117] Several studies have identified accumulation of RTE-derived nucleic acids, namely dsRNA, RNA-DNA hybrids and extrachromosomal DNA as the trigger of the interferon response in AGS.[106] Notably, the administration of RT inhibitors (RTi) to AGS patients results in diminished expression levels of ISGs, indicating a promising therapeutic avenue for inflammatory conditions.[112]

Discovered decades ago, TEs were initially viewed as genomic parasites, occupying a significant portion of genomes. However, advancements in our understanding of TE biology have revealed their multifaceted roles in genome evolution, organismal development, and the consequences of their dysregulation in diseases. Despite these strides, significant knowledge gaps remain to be filled to fully address outstanding questions regarding the complex interactions between TEs and their host organisms.

DNA damage response

Every day, a human cell encounters up to 10^5 spontaneous DNA lesions.[118,119] DNA damage arises during DNA metabolism, is caused by reactive oxygen species (ROS), cellular metabolic bioproducts, or by exogenous agents such as UV light or X-rays.[120] Given the critical role of the genomic material in governing cellular functions, it is crucial to rapidly detect and accurately repair damaged DNA. Thus, the canonical DNA damage response (DDR) has emerged as a pivotal mechanism safeguarding genomic stability. The DDR constitutes a transduction signal cascade that senses DNA damage and robustly activates effector proteins. These proteins trigger transient cell cycle arrest to facilitate efficient removal of DNA lesions, apoptosis or cellular senescence in the case of severe damage. Specifically, the most deleterious type of damage, double-stranded DNA breaks (DSBs) is detected by the MRE11–RAD50–NBS1 (MRN) complex, which recruits the apical protein kinase ataxia-telangiectasia mutated (ATM), a member of the phosphatidylinositol 3-kinase-like kinase (PIKK) family, to the damaged site.[121] ATM phosphorylates the histone H2A variant X (H2AX), an event critical for the DDR initiation. This phosphorylation triggers a positive feedback loop, leading to the recruitment of additional ATM molecules and subsequent spread of γH2AX across the chromatin that further amplifies the DDR signalling.[122] Following the amplification of the signal by DNA damage mediators, MDC1 and 53BP1, ATM activates the protein kinase CHK2 through phosphorylation.[123-127] CHK2 then diffuses through the nucleus and triggers multiple signalling pathways that culminate in the activation of key effectors such as p53 and cell division cycle 25 (CDC25) phosphatases.[128-130]

Similarly, single-stranded DNA breaks (SSBs), coated by RPA, are recognized by another PIKK, ataxia telangiectasia and Rad3-related (ATR), and its interacting protein, ATRIP. Like ATM, ATR phosphorylates the histone H2AX nearest to the lesion.[131] The signal is further amplified by DNA damage mediators such as TOPBP1 and claspin, with claspin activating CHK1.[132,133] CHK1, like CHK2, subsequently triggers the activation of DDR effectors, including p53 and CDC25.[129,130]

DSB repair

Among all types of DNA lesions, DSBs represent the most severe threat to genomic integrity. DSBs can give rise to chromosomal aberrations, such as insertions, deletions, translocations, and copy number variations.[134,135] To ensure cell survival and prevent tumorigenesis, their timely and accurate repair is critical. DSBs are primarily resolved through two major pathways: the rapid, but error prone non-homologous end joining (NHEJ), and the slower, high-fidelity homologous recombination (HR), which uses an intact sequence from a sister chromatid or homologous chromosome as a repair template to restore the original DNA

sequence.[136] Repair pathway choice is influenced by cell cycle stage, with HR being predominantly active in S and G2 phases when a sister chromatid is available as a repair template.[137] However, even under these conditions, HR appears to be less efficient than NHEJ, which operates throughout the entire cell cycle.[136,137]

Once DSBs are sensed and H2AX becomes phosphorylated, a cascade of events unfolds at the site of damage. γH2AX enables the recruitment of MDC1, another target of PIKK family kinases, which facilitates the engagement of E3 ubiquitin ligases, including RNF8 and RNF168.[138-140] These ligases catalyse ubiquitylation of histone H1-linked and H2A that promotes accumulation of p53-binding protein (53BP1). The sustained presence of 53BP1 at the DSB favours NHEJ, whereas its displacement by BRCA1 and CtIP facilitates HR.[141-143]

53BP1 promotes NHEJ by recruitment of the DNA-PK complex, comprising DNA-PKcs and the Ku70/80 heterodimer, along with end-processing enzymes such as XRCC4 and LIG4, culminating in the ligation of broken DNA ends.[144-146]

In contrast, HR proceeds through the resection of DNA ends to generate single-strand overhangs, which are subsequently coated with RAD51, a recombinase that mediates homology search and strand invasion into the sister chromatid, enabling template-guided DNA repair.[147]

Traditionally, the DDR has been viewed as an exclusively protein-driven network. However, mounting evidence has revealed a critical involvement of RNA and RNA-associated processes in maintaining genome stability.

RNA in the DDR

An expanding body of evidence has demonstrated that various RNA species accumulate at DSB sites, where they actively regulate DNA repair. Among these, DICER- and DROSHA-dependent small RNAs, termed DNA damage response RNAs (DDRNAs), are generated at the site of damage and have been shown to be essential for the proper formation of DDR foci in an evolutionary conserved manner.[148] Upon DNA damage, RNA polymerase II (RNAPII) is recruited to the lesion through interaction with the MRN complex and initiates transcription of damage-induced long non-coding RNAs (dilncRNA). These transcripts are further processed by the RNAi machinery into DDRNAs. Both dilncRNAs and DDRNAs contribute to the assembly of DDR foci, promote the recruitment of 53BP1, and facilitate efficient DNA repair.[149]

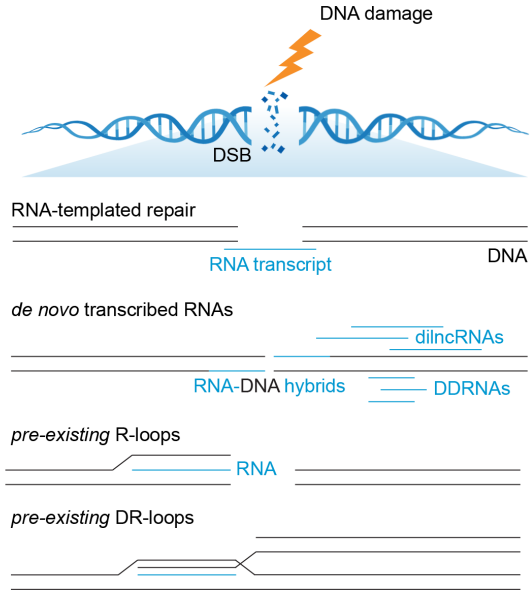

Figure 4 Diverse roles of RNA in DNA double-strand (DSB) repair. RNA molecules contribute to DSB repair through multiple mechanisms. De novo transcribed RNAs can form RNA/DNA hybrids at break sites, while damage-induced long non-coding RNAs (dilncRNAs) are processed into shorter DNA damage response RNAs (DDRNAs), both serving as scaffolds for recruitment of repair factors. Additionally, pre-existing RNAs can participate in repair by forming R-loops and double R-loops and DR-loops, or act as templates for RNA-templated DNA repair. Adapted with modifications from [6]. Illustration partially created with BioRender.

Intriguingly, endogenous RNA transcripts can also participate directly in HR though a mechanism known as RNA-templated DNA repair, in which RNA transcripts serve as a template for DNA synthesis.[150] Another study demonstrated that local transcription enhances HR through tethered RNA facilitating the formation of DR-loops composed of both DNA-DNA and DNA-RNA hybrids.[151] Conversely, the function of RNA within RNA-DNA hybrids formed at DNA break sites, denoted as break-induced RNA-DNA hybrids (BIRDHs), remains contentious, with ongoing debate whether they actively contribute to the DDR in a beneficial or detrimental manner or represent inadvertent byproducts of transcriptional activity (Figure 4).[152] Besides acting directly at sites of DNA damage, differential expression of mRNAs and lncRNAs also contributes to the broader cellular response to genotoxic stress.[6]

RNA modifications in the DDR

The first insight into the involvement of RNA modifications, specifically m^6a, in the DDR came from a study demonstrating methyltransferase-like 3 (METTL3)-dependent m^6A deposition on poly(A)+ transcripts, with the fat mass and obesity-associated protein (FTO) acting as a demethylase. Notably, METTL3 was shown to

be essential for the local recruitment of DNA polymerase κ (Pol κ) to DNA lesions. However, the global impact of m^6A modification on poly(A)+ transcripts during the DDR remains to be elucidated.[153] Similarly, reflecting the complex role of DSB-associated R-loops, recent reports suggest that m^6A modification of the RNA component of RNA-DNA hybrids may act as a double-edged sword, either promoting R-loop formation or facilitating their resolution.[6] As it is a highly dynamic process it may also act in a context-specific manner depending on the genomic locus, type of lesion, or specific DDR brunch activated and further research is indispensable to fill this gap.

Another RNA modification, m^5C, and its oxidation product hm^5C, have recently emerged as key regulators of DSB repair, with m^5C deposition on RNA-DNA hybrids promoting HR and influencing DNA repair pathway choice through modulation of R-loop dynamics.[154-156]

Recent advances in understanding the roles of m^6A and m^5C in the DDR have been facilitated by the integration of high-throughput sequencing approaches with established DNA damage assays, along with visualization of key repair components at damage sites using modification-specific antibodies. However, the lack of high-specificity antibodies for many other RNA modifications presents a major obstacle to their functional characterization. Emerging technologies such as Oxford Nanopore sequencing, combined with future tools designed to visualize RNA modifications at DNA lesions, hold the potential to revolutionize our understanding of how the epitranscriptome contributes to genome stability.

Challenges to haematopoietic stem cell integrity

HSCs sustain lifelong blood cell production by balancing two essential properties: long-term self-renewal and the ability to differentiate into all blood cell lineages.[157] Under homeostatic conditions, the majority of HSCs are quiescent entering the cell cycle sporadically to fulfil these functions before the exhaustion of their self-renewal ability.[158-160] With aging, the overall pool of HSCs tends to expand, even as subsets of these cells differentiate to sustain haematopoiesis.[161-163] However, aged HSCs show a decline in functionality, including reduced self-renewal capacity, impaired bone marrow homing and reconstitution, and increased bias toward myeloid lineage differentiation compared to their younger counterparts.[164,165] This deterioration is partially attributed to the cumulative effects of cellular proliferation, which can elevate ROS and other metabolic byproducts that induce DNA damage.[166] Additionally, persistent inflammation also contributes to the functional impairment of HSCs over time, whereas acute inflammatory stress transiently activates HSCs proliferation and differentiation, followed by a rapid return to homeostasis.

HSCs under inflammatory and genotoxic challenges

Under conditions such as infection, tissue injury, or autoimmunity, mature blood cells have long been recognized as both primary source of inflammatory cytokines and key effectors in the immune response. However, HSPCs are also highly responsive to inflammatory stimuli.[167,168] Repeated activation of HSCs by physiological stressors, such as chronic blood loss or administration of poly I:C that mimics viral infection by induction of the type I IFN response, has been demonstrated to compromise genomic integrity, ultimately leading to HSC attrition.[169] Notably, type I IFNs exert a dual effect on HSCs. Acute exposure transiently stimulates their proliferation due to a brief relaxation of quiescence-enforcing mechanisms. By contrast, chronic exposure promotes return to a quiescent state, which protects the cells from type I IFN-induced cytotoxicity. Nonetheless, when HSCs are reactivated, for example through transplantation or myeloablation, they become more susceptible to undergo p53-dependent apoptosis, underscoring quiescence as a critical safeguard of HSC integrity under sustained inflammatory stress.[170]

Growing evidence indicates that derepression of TEs, a hallmark of compromised genomic regulation, acts as a potent trigger of innate immune activation, impacting HSCs and linking inflammatory stress and genomic instability, ultimately contributing to stem cell dysfunction.[171] However, during development, inflammatory signalling induced by repetitive element transcripts via activation of RLRs supports the emergence of HSPCs.[172] Additionally, a recent study demonstrated that retrotransposon transcription activates the cGAS-STING, elevating interferon production and thereby promoting HSC activation and enhancing erythropoiesis under stress conditions.[173] Unlike the dynamic expression of TEs during HSPC development, quiescent HSCs display significantly lower TE levels than activated cells.[171] However, under stress conditions such as chemotherapy, elevated TE transcription activates MDA5 triggering an inflammatory response essential for HSC exit from quiescence, highlighting the critical role of TEs and inflammatory signalling in haematopoietic regeneration.[111]

Interestingly, genotoxic stress induced by ionizing radiation elevates LINE1 expression and retrotransposition contributing to persistent γH2AX foci and impaired HSC function.[174]

Maintaining genome integrity is essential for HSCs, as they are uniquely responsible for sustaining lifelong haematopoiesis through continuous self-renewal. Loss of ATM, a key DDR kinase, results in bone marrow failure due to compromised HSC self-renewal, largely driven by elevated oxidative stress.[175] Moreover, defects in DNA repair mechanisms impair HSC function during aging without necessarily depleting their reservoir.[176] Notably, reduced activity of DNA ligase IV, crucial for NHEJ repair of DSBs, leads to progressive HSC attrition over time, underscoring the dependency of HSCs on this pathway for maintaining function under

physiological stress.[177] Further studies have demonstrated that quiescent and proliferative HSCs employ distinct DNA repair strategies in response to ionizing radiation (IR). However, NHEJ-mediated repair in quiescent cells often results in persistent, mutagenic genomic rearrangements that may contribute to haematological abnormalities.[178]

Importantly, impaired DNA repair mechanisms are linked to various haematological disorders and chromosomal translocation represent a defining feature of many blood cancers.[179,180]

From HSC dysfunction to leukaemic transformation

Malignant transformation in leukaemia, as emphasized by Orkin and Zon, results from disruptions of key regulatory nodes in cellular networks, rather than random transcriptional changes.[157] One such early event is the acquisition of non-lethal mutations within the HSC compartment, which can give rise to mutant clones that gradually outcompete normal HSCs – a process observed in myelodysplastic syndromes (MDS) as well as acute and chronic myeloid leukaemias (AML and CML, respectively).[181-183] Major driver genes in MDS affect pathways such as DNA methylation, chromatin modification, RNA splicing, the cohesion complex, transcriptional regulation, cytokine and tyrosine kinase signalling, RAS signalling, cell cycle control, and DNA repair – many of which substantially overlap with those implicated in primary AML.[184] Disease progression in MDS is often marked by the stepwise acquisition of additional mutations, resulting in clonal evolution and increased blast counts over time, typically preceding leukaemic transformation. Ongoing research seeks to uncover how driver alterations functionally contribute to clonal advantage and promote positive selection.

Alternative splicing (AS) is a fundamental mechanism that expands genomic coding capacity by precisely excising introns and variably including or excluding exons, thereby generating multiple distinct mRNA isoforms from a single gene.[185] It is estimated that up to 95% of human genes undergo AS, establishing pivotal role in regulating nearly all aspects of cellular function.[186,187] Splicing is orchestrated by the spliceosome, a dynamic complex composed of five small nuclear ribonucleoprotein particles (snRNPs) and numerous auxiliary splicing factors (SFs) that coordinate splice site recognition and catalyse two sequential transesterification reactions.[185] Dysregulation of AS and mutations in SFs are implicated in diverse pathologies, including cancer such as MDS and AML.

Somatic cell reprogramming

The groundbreaking discovery of induced pluripotent stem cells (iPSCs) has revolutionized biomedical sciences, beginning a new era in developmental biology, regenerative medicine, disease modelling, and pharmacology.[188] Less than two decades ago, the generation of cells resembling embryonic stem cells (ESCs) from somatic cells was achieved using a combination of four transcription factors - OCT4, SOX2, KLF4, and MYC – collectively known as *Yamanaka factors* after their discoverer.[189] Over the years, significant progress has been made in uncovering the molecular mechanisms that govern the reprogramming process, particularly those related to the regulation of pluripotency networks.[190] Core pluripotency factors, including OCT4, SOX2 and NANOG, define the pluripotent state and maintain it by binding promoters of actively transcribed genes involved in transcriptional regulation, signal transduction, and chromatin remodelling. Meanwhile, they suppress lineage-specific programs to prevent premature differentiation. Despite these advances, reprogramming remains relatively inefficient, underscoring the need to further elucidate molecular mechanisms and identify factors that can improve its efficiency.

Subsequent investigations into the molecular barriers to reprogramming, revealed critical roles for cellular stress response pathways. Suppression of the p53-p21 axis was shown to enhance iPSCs generation through increasing the number of cell divisions.[191] As a key guardian of genomic integrity, p53 coordinates transcription programs that respond to cellular stress and preserve homeostasis.[192] However, the overexpression of reprogramming factors, many of which are oncogenic markedly improves the efficiency of reprogramming in both murine and human systems.[193,194] Although beneficial for reprogramming efficiency, transient p53 suppression comes at a cost, increasing genomic instability and raising concerns about the safety and immunogenicity of the resulted iPSCs.[195]

Adding another layer of complexity, translational control, previously recognized as a potent regulator of pluripotency, has also been identified as a crucial modulator of the cellular reprogramming processs.[196] Eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs) negatively impact reprogramming efficiency, partially by promoting the translation of p21. Conversely, the simultaneous loss of p53 and 4E-BPs suppresses p21 transcription, while enhancing the translation of endogenous pluripotency-associated mRNAs such as *Sox2* and *Myc*. Under these conditions, reprogramming can be achieved using only exogenous *Oct4*, underscoring a pivotal role of translational regulation in facilitating efficient induction of pluripotency.

This thesis tells more than one story, an attempt to connect previously unlinked molecular players to understand what drives cellular physiology, how its disruption leads to pathology, and how malfunctioning cells affect the organism. Having

outlined the existing knowledge and unresolved questions, the following chapters aim to address some of these gaps, with the final discussion reflecting on emerging complexities and proposing new directions for future investigation.

Summary of results

Paper I

***Madej, M.**, Ngoc, P.C.T., Muthukumar, S., Konturek-Ciesla, A., Tucciarone, S., Germanos, A., Ashworth, C., Kotarsky, K., Ghosh, S., Fan, Z., Fritz, H., Pascual-Gonzalez, I., Huerta, A., Guzzi, N., Colazzo, A., Beneventi, G., Lee, H.M., Ciesla, M., Douse, C., Kato, H., Swaminathan, V., Agace, W.W., Castellanos-Rubio, A., Salomoni, P., Bryder, D., and Bellodi, C. (2025). PUS10-induced tRNA fragmentation impacts retrotransposon-driven inflammation. Cell Rep 44, 115735. 10.1016/j.celrep.2025.115735.*

The study uncovers a previously unrecognized role of PUS10, in suppressing transposon-driven inflammation. PUSs are RNA-modifying enzymes that catalyse the isomerization of uridine-to-pseudouridine, thereby fine-tuning RNA metabolism and they function across diverse physiological and pathological contexts. However, the mechanism by which PUSs shape gene expression programs under stress conditions remains incompletely understood. Among them, PUS10 has been previously implicated in haematopoiesis, TRAIL-induced apoptosis, and nuclear miRNA processing, a function surprisingly independent of its catalytic activity, as well as cytoplasmic tRNA pseudouridylation. Notably, GWAS have identified *PUS10* as a susceptibility locus for Crohn's disease and celiac disease, implicating it in the regulation of immune homeostasis.

Leveraging a *Pus10*-KO mouse model and transcriptomic profiling, we found that while PUS10 is dispensable for organismal development, its depletion triggers a robust upregulation of ISGs both *in vitro* and *in vivo*. Mechanistically, loss of PUS10 perturbs the abundance of specific tDRs, specifically downregulating a subset of 5'-derived tDRs (tdR-5) that directly interact with PUS10. Consistent with prior studies and our own previous work linking PUS enzymes and tdR regulation to translational control, we observed elevated *de novo* protein synthesis and enrichment of translational programs associated with inflammatory pathways. Given the emerging evidence that tdRs regulate ERVs and that their dysregulation can trigger a host immune response, we next examined ERV expression in the absence of PUS10. Indeed, PUS10 depletion led to marked ERV derepression, supporting the notion that PUS10 functions as a suppressor of TEs and implicating it as a modulator of a viral mimicry response.

As TE activation has been linked to the accumulation of cytosolic nucleic acids, we examined nucleic acid species in PUS10-deficient cells. While dsRNA levels remained unchanged, we observed a marked accumulation of RNA-DNA hybrids in both the nucleus and cytoplasm. This was accompanied by activation of the cGAS-STING pathway, a key cytosolic sensor of RNA-DNA hybrids. Notably, supplementation with tdR-5-GlyGCC – a tdR significantly downregulated upon PUS10 depletion – restored expression of the most upregulated ERV subfamilies, reduced RNA-DNA levels and attenuated cGAS-STING activation to baseline levels. These finding suggest that tdR-5-GlyGCC acts downstream of PUS10 to constrain TE-driven inflammation.

Building on previous findings that PUS10 loss impairs haematopoiesis and considering the exceptional sensitivity of HSCs to TE dysregulation and inflammation, we investigated the consequences of PUS10 depletion in the haematopoietic compartment. Transcriptomic profiling of Lineage-Sca-1+cKit+ (LSK) HSPCs from PUS10-deficient mice revealed upregulation of ERVs alongside robust activation of IFN-alpha and -gamma signalling pathways. To assess the functional relevance under stress, we subjected mice to competitive whole bone marrow (BM) transplantation (BMT). Remarkably, *Pus10*-KO cells exhibited enhanced donor reconstitution, particularly evident four weeks post-transplantation. Consistently, following acute inflammatory stress using serial injections of poly I:C to mimic viral infection, prior to BMT, we observed expansion of multiple HSPC populations in *Pus10*-KO mice. Together, these data identify PUS10 as a key modulator of HSC resilience to inflammatory stress.

Finally, we extended our investigation to human disease contexts and explored PUS10's contribution to autoinflammatory diseases, which are often characterized by aberrant activation of inflammatory pathways in response to self-derived nucleic acids. Using a PUS10-specific molecular gene signature, we identified a significant correlation between reduced PUS10 expression and chronic autoimmune conditions, including inflammatory bowel disease (IBD). These findings suggest that dysregulation of PUS10 may contribute to the initiation or exacerbation of autoimmune diseases.

Altogether, our results establish PUS10 as a previously unrecognized modulator of the TE-driven innate immune response, acting through a mechanism involving specific subsets of tdRs, exemplified by tdR-Gly-GCC. PUS10 dysfunction confers resilience to inflammatory cues in HSPCs within the murine haematopoietic system and correlates with gene expression programs in autoimmune conditions, underscoring its clinical relevance.

Key findings:

1. PUS10 depletion triggers an innate immune response driven by viral mimicry.

2. Loss of PUS10 perturbs tRF levels, leading to translation dysregulation and aberrant endogenous retroelements expression.
3. PUS10 depletion activates cGAS-STING-driven inflammation, likely via accumulation of RNA-DNA hybrids.
4. Altered PUS10 expression is linked to human autoimmune diseases, including IBD.

Paper II (manuscript)

***Madej, M.**, H. Finnan, C. Pires, N. Arh, A. Konturek-Cieśla, M. Cieśla, S. Muthukumar, CF Pereira, and C. Bellodi. (2025). PUS10 drives DNA repair pathway choice and cell fate plasticity in physiological and oncogenic contexts.*

Extending our previous findings that PUS10 constrains TE-driven inflammation via the cGAS-STING signalling pathway, likely mediated by the accumulation of RNA-DNA hybrids and dependent on a specific subset of td, we explored whether PUS10 also governs cellular adaptation to genotoxic and dynamic cell fate transitions.

Our earlier integrative transcriptomic and translatomic profiling uncovered PUS10-dependent gene expression programs co-regulated at the RNA and protein levels, particularly those associated with epithelial-to-mesenchymal transition (EMT). In parallel, we identified translation-specific signatures enriched for DNA repair, cell cycle progression, and E2F – hallmarks of genome surveillance and proliferative control. These findings led us to hypothesise that PUS10 safeguards genome integrity and orchestrates stress-responsive gene expression rewiring during processes such as oncogenic transformation and somatic reprogramming.

We found that PUS10 is rapidly upregulated following UV-induced DNA damage. Immunofluorescence analysis revealed that PUS10 re-localises into distinct nuclear bodies forming concentric structures around γH2AX-positive DNA damage foci. PUS10-deficient cells exhibited altered p53 activation kinetics, increased sensitivity to cell death, impaired cell cycle progression, and a trend toward elevated DNA damage as assessed by comet assay upon UV treatment. Importantly, several of these phenotypes were recapitulated in both human and murine cells, suggesting a conserved role for PUS10 in genome maintenance across species.

Structure-guided modelling and electrophoretic mobility shift assay (EMSA) confirmed that PUS10 binds single-stranded DNA (ssDNA) and displays higher affinity for Holliday junctions (HJ) – key intermediates of HR. In line with this, PUS10-deficient cells showed reduced levels of HR-associated proteins RAD51 and BRCA1. Complementary functional assays revealed that PUS10 suppresses error-

prone NHEJ, in a manner independent of its catalytic activity, and may facilitate high-fidelity HR-mediated repair of DSBs.

Beyond its role in maintaining genome integrity, we found that PUS10 may restrict oncogene-driven transformation, as *Pus10*-KO cells exhibited enhanced clonogenic survival following HRAS activation. Conversely, loss of PUS10 significantly increased the efficiency of somatic cell reprogramming, without affecting the pluripotency or differentiation potential of resulting iPSCs. Although the mechanistic underpinnings remain to be fully elucidated, our findings suggest that PUS10 may regulate cellular plasticity through the DDR modulation, translational control, or possibly a yet unidentified pathway.

Altogether, our preliminary data position PUS10 as a regulator of genome integrity and cell fate plasticity. These finding lay the foundation for future exploration into how PUS10 interfaces with broader stress adaptation pathways.

Key findings:

1. PUS10 deficiency sensitises cells to DNA damage-induced cell death and disrupts cell cycle progression.
2. PUS10 directly binds to ssDNA and exhibits higher affinity for HJs, a critical intermediate of HR.
3. PUS10 suppresses error-prone NHEJ and may facilitate high-fidelity HR-mediated DNA repair.
4. PUS10 may restrict oncogenic transformation following HRAS activation.
5. PUS10 enhances somatic cell reprogramming without compromising iPSC pluripotency or differentiation capacity.

Paper III

Guzzi, N., Muthukumar, S., Ciesla, M., Todisco, G., Ngoc, P.C.T., ***Madej, M.****, Munita, R., Fazio, S., Ekstrom, S., Mortera-Blanco, T., Jansson, M., Nannya, Y., Cazzola, M., Ogawa, S., Malcovati, L., Hellstrom-Lindberg, E., Dimitriou, M., and Bellodi, C. (2022). Pseudouridine-modified tRNA fragments repress aberrant protein synthesis and predict leukaemic progression in myelodysplastic syndrome. Nat Cell Biol 24, 299-306. 10.1038/s41556-022-00852-9.*

This study investigates the mechanistic basis of pseudouridylated tRF-mediated stem cell-associated translational repression during development and leukaemogenesis. tRFs are commonly dysregulated in cancer, yet their precise contribution to disease development remains incompletely understood. Extending prior work, this study expands on how PUS7-mediated Ψ of a stem-cell enriched

subset of small tRFs, characterized by a 5' terminal oligoguanine motif, termed mTOG, plays a critical role in repressing protein synthesis. This occurs through direct binding to polyadenylate-binding protein cytoplasmic 1 (PABPC1) and destabilization of the translation-initiation complex eIF4F. Such regulation significantly influences embryonic development and the growth and fate determination of HSCs. However, the precise molecular mechanism underlying this regulation and how its dysfunction contributes to leukaemogenesis remains elusive.

Building on previous data, this study demonstrates that mTOG interacts with PABC1 in a Ψ-dependent manner. The specific binding site was localised to the RRM3 domain of PABPC1, leading to allosteric remodelling or subsequent interactions with the RRM2 and RRM4 domains. Given that PABPC1 activity is regulated through interactions with PABP-interacting proteins 1 and 2 (PAIP1 and PAIP2, respectively), we examined how mTOG affects these interactions. The results show that mTOG-Ψ specifically disrupts the interaction between PABPC1 and PAIP1, but not with PAIP2. Furthermore, transcriptome-wide analysis of translation efficiency revealed that mRNAs containing 5' pyrimidine-enriched sequences (PES) sequences near the transcription start site are particularly sensitive to translation regulation by PUS7/mTOG.

Given the aberrant protein synthesis and impaired haematopoietic differentiation caused by PUS7/mTOG-mediated dysregulation of translation, along with the established association of PUS7 gene aberrations with the development of MDS and their high-risk progression to AML, this study further explored the implications of PUS7 dysfunction in leukaemogenesis. Indeed, low PUS7 expression and reduced mTOG levels, considered a direct readout of PUS7 activity, were found to be predictive of AML progression. Notably, mTOG levels surpassed PUS7 expression in predicting clinical outcomes, underscoring their significance as a superior biomarker of disease severity and progression.

Mechanistically, mTOG-Ψ, in conjunction with PAIP1, regulates global mRNA translation as well as the specific translation of 5' PES-enriched transcripts. Interestingly, treatment of malignant MDS-HSPCs with mTOG-Ψ resulted in improved differentiation and engraftment potential, highlighting the translational relevance of targeting this pathway.

Altogether, this study underscores the critical function of mTOG and PUS7-mediated pseudouridylation in haematopoietic differentiation and transformation. Importantly, mTOG-driven translation regulation governs MDS-to-AML progression, offering promising potential for therapeutic interventions in haemotological malignancies.

Key findings:

1. PUS7-mediated mTOG pseudouridylation regulates translation of 5'PES mRNA through direct interaction with PABPC1 and PAIP1.

2. Reduced mTOG levels predict MDS-to-AML progression and outperform PUS7 expression as a biomarker.
3. mTOG-Ψ treatment improves differentiation and engraftment of malignant MDS-HSPCs, highlighting therapeutic potential.

Paper IV

Ciesla, M., Ngoc, P.C.T., Muthukumar, S., Todisco, G., ***Madej, M.****, Fritz, H., Dimitriou, M., Incarnato, D., Hellstrom-Lindberg, E., and Bellodi, C. (2023). m(6)A-driven SF3B1 translation control steers splicing to direct genome integrity and leukemogenesis. Mol Cell 83, 1165-1179 e1111. 10.1016/j.molcel.2023.02.024.*

This study explores the regulatory mechanisms of the wild-type (WT) SF3B subunit 1 (SF3B1) in cancer and consequences of its dysregulation in leukaemogenesis. AS is a fundamental process that enables generation of multiple mRNA isoforms from a single pre-mRNA, thereby allowing for cell type-specific protein diversity. This process is catalysed by the spliceosome, a multi-component RNP complex composed of small nuclear RNAs (snRNAs; U1, U2, U4, U5, and U6) and over a hundred associated splicing factors. Dysregulation of AS is widespread in cancer and frequently associated with mutations in genes encoding SFs. Nonetheless, even in the absence of such mutations, pervasive splicing defects are observed, indicating an involvement of additional regulatory mechanisms in malignancies. SF3B1, a core subunit of the U2 snRNP, is essential for pre-mRNA binding and branch-point sequence (BPS) recognition, and splicing fidelity. Previous studies have shown that oncogenic pathways driven by MYC, RAS and AKT/mTOR dysregulate SF3B1 translation, while recurrent SF3B1 mutations contribute to AS defects in various cancers, particularly in MDS. Notably, these mutations are typically heterozygous and mutually exclusive with other SF mutations, emphasizing the reliance of cancer cells on SF3B1-WT alleles. Furthermore, SF3B1 copy number loss impairs U2 snRNP function, leading to AS defects and vulnerabilities affecting cell growth. Despite these insights, the molecular regulation of SF3B1 in cancer and the consequences of its perturbation on AS and leukaemogenesis remain largely unexplored.

This study reveals an evolutionarily conserved, dynamic regulation of SF3B1-WT protein levels – decoupled from transcriptional changes, during the progression from MDS to AML. Analysis of patient-derived CD34+ HSPCs revealed that SF3B1 protein levels increase during MDS as compared to healthy controls and secondary AML. Similarly, the NUP98-HOXD13 (NHD13) transgenic mouse model, which faithfully recapitulates MDS and its progression to leukaemia, illustrates SF3B1 protein elevation in pre-leukaemic HSPCs, followed by a

subsequent decline upon transformation to AML. Importantly, SF3B1 downregulation accelerates leukaemic transformation *in vivo*.

Using primary human diploid fibroblasts (HDFs) to model the oncogenic response to MYC activation, an event associated with early MDS-to-AML progression, SF3B1 was shown to be rapidly upregulated at the translational level in a cap-dependent manner. Importantly, upon transformation induced by MYC and RAS oncogene overexpression, SF3B1 levels decline. Knockdown (KD) of SF3B1 in MYC-overexpressing HDFs revealed over 10,000 AS events (ASEs), with exon skipping (ES) as the most prevalent. Gene ontology (GO) enrichment analysis of overlapping ASEs and differentially expressed genes (DEGs) highlighted significant associations with the DDR pathway. Indeed, SF3B1 KD in MYC-overexpressing HDFs resulted in impaired DDR and repair, as evidenced by increased levels of DSBs and accumulation of γH2AX foci. Consistently, SF3B1-dependent ASEs associated with the DDR in HDFs significantly overlapped with datasets of human T cell acute lymphoblastic leukaemia (T-ALL) patients with downregulated SF3B1 and MDS patients with 5q deletion (del5q) who lack SF mutations. Transcriptomic analysis of pre-leukemic cKit$^+$-enriched *Sf3b1*-depleted HSPCs suggests a conserved role for SF3B1 in maintaining genome stability in MDS progression. Importantly, integration of transcriptomic analysis of SF3B1-depleted MYC-overexpressing HDFs with T-ALL datasets allowed establishment of a SF3B1-dependent gene signature (SF3B1-GS) that may predict clinical outcomes of AML patients.

Molecularly, SF3B1 translation following oncogenic stress is modulated by ALKBH5-mediated demethylation of the adenosine at position 88, close to the main ORF (mORF) within the SF3B1 5'UTR, favouring an alternative translation initiation site (TIS). Furthermore, loss of m^{6}A-mediated SF3B1 translation repression impaired leukaemic cell growth both *in vitro* and *in vivo*, induced their differentiation and reduced genomic instability following genotoxic stress.

Altogether, the results of this study highlight SF3B1 as a regulator of AS-mediated genome integrity programs upon oncogenic stress, impacting leukaemogenesis in both mouse and human models.

Key findings:

1. SF3B1 protein levels are dynamically regulated during the progression from MDS to AML.

2. SF3B1 KD leads to alternative splicing events, primarily ES, linked to DDR pathways during transformation.

3. ALKBH5-mediated demethylation of the SF3B1 5'UTR modulates its translation in response to oncogenic stress, impacting genome stability and leukaemogenesis.

Conclusions and future perspectives

Engaging in research, testing hypotheses, and addressing successive scientific questions often provides intellectual satisfaction. However, in many cases rather than leading to singular breakthroughs, these efforts contribute incrementally to a broader, evolving body of knowledge. Here, I aim to synthesise the key findings of my doctoral research and reflect on their potential to advance the state of knowledge in the fields of RNA and DNA biology, thereby shedding light on their implications for human health.

Collectively, the studies presented in this thesis illuminate how RNA modifying enzymes contribute to key cellular processes. Through their canonical catalytic functions or non-canonical "moonlighting" roles, they contribute to processes such as translational regulation, innate immune signalling, and genome maintenance, ultimately shaping cell identity and governing fate decisions under physiological and pathological conditions. This work positions post-transcriptional control as a critical interface connecting stem cell biology, inflammation, and cancer.

PUS10 as a regulatory node at the intersection of RNA biology, innate immunity, and transposon control

Our initial work identifies PUS10 as a previously unrecognized regulator of the innate immune response triggered at least in part by derepression of endogenous retroviruses and mediated by a specific subset of tDRs (Paper I). Loss of PUS10 leads to robust upregulation of ISGs and activation of the cGAS-STING pathway, likely through the accumulation of RNA-DNA hybrids, all uncoupled from its catalytic activity. Importantly, PUS10 dysfunction is associated with human autoimmune conditions, including SLE and IBD, underscoring its role in human immunity and positioning PUS10 as a critical modulator of viral mimicry.

Depletion of PUS10 selectively alters the abundance of several tDRs without affecting global levels of mature tRNAs. Among the most affected are 5′ tRNA-halves, with tDR-5-GlyGCC showing the most pronounced downregulation. Although we have not yet characterized RNA modifications on these fragments, emerging technologies such as direct RNA nanopore sequencing and Nano-tRNAseq could provide insights into their modification landscape and biogenesis.[197] Mapping these signatures will be critical to determine whether distinct chemical

marks co-occur on tDRs, potentially revealing a coordinated network of RNA modifying enzymes that regulate their processing, structure, and function in innate immunity and beyond.

Pulldown of tdR-5-GlyGCC in the absence of PUS10, coupled with liquid chromatography tandem mass spectrometry (LC-MS/MS), uncovered enrichment for RNA processing factors, suggesting that PUS10 may influence innate immune signalling by modulating tdR-protein interactions. Notably, prominent among the identified interactors are RNA-binding proteins, including USP39, a deubiquitinase involved in IFN signalling and genome stability; TARDBP, associated with R-loop biology and inflammatory signalling in neurodegeneration and IBD; and MOV10, a helicase that restricts retrotransposon mobility and regulates IRF3-driven antiviral response independently of RLR activation.[198-207] While these proteins represent intriguing candidates for further regulatory crosstalk, their precise roles in the PUS10-tdR axis remain to be experimentally validated.

CUT&RUN profiling revealed increased H3K4me3 deposition at transcription start sites (TSSs) of transcriptionally activated TEs, consistent with a permissive chromatin state. However, the upstream signals driving this chromatin remodelling remain undefined. Concurrently, PUS10 deficiency results in the accumulation of RNA-DNA hybrids in both nuclear and cytosolic compartments. The origin, composition, and functional relevance of these hybrids are unresolved and merit further investigation.

Future studies could leverage S9.6-based R-loop pulldown coupled with high-throughput sequencing to define the composition and genomic localization of these hybrids.[208] Dissecting their compartment-specific signatures – nuclear versus cytosolic – may reveal distinct mechanism of immune sensing, genome instability, or yet-uncharacterised cellular processes. Additionally, identifying R-loop interactors and mapping associated RNA modifications, given that RNA-DNA hybrids are known to be chemically modified, could uncover previously unappreciated pathways linking chromatin regulation, RNA metabolism, and innate immunity, while converging with broader stress responses, including those involved in genome surveillance as revealed in subsequent work.[209] Notably, our unpublished data indicates that PUS10 may directly bind DNA, therefore chromatin immunoprecipitation (ChiP)-seq using PUS10-specific antibody could reveal its genome-wide binding profile and clarify whether it plays a direct role in transcriptional regulation.

Our findings underscore a functional role for PUS10 in human immunity and malignancies associated with chronic inflammation. Paul Ehrlich's introduction of the concept of 'horror autotoxicus' over a century ago laid the foundation for understanding autoimmune diseases, wherein the immune system mistakenly targets the body's own tissues.[210] Despite significant progress in elucidating the molecular mechanisms underlying these conditions, there remains a critical need for

development of effective and affordable therapies aimed at treating and ultimately curing these devastating disorders. Here, we identify reduced PUS10 activity in patients with SLE and IBD, the latter of which confers increased risk for inflammation-driven malignancies such as colon cancer. Supporting a protective role for PUS10, its depletion sensitized mice to dextran sulfate sodium (DSS)-induced colitis, a murine mode of intestinal inflammation. Future work should delineate how PUS10 functions in disease-relevant cell types and whether its loss disrupts specific cellular or molecular programs. Human intestinal organoid systems, combined with patient-derived samples may provide a tractable platform for dissecting tissue-specific phenotypes and mechanistic pathways, with the potential to uncover predictive markers of disease progression or therapeutic response.[211]

Finally, several genes have been associated with variable susceptibility to viral infections including those encoding viral receptors and co-receptors, as well as factors involved in their modification, and components critical for innate and adaptive immunity.[212] Interestingly, preliminary data showing resistance to lentiviral transduction in PUS10-deficient cells open a new line of research into its role in antiviral defence, warranting dedicated investigation in virological models to determine whether PUS10 broadly modulates susceptibility to viral infection.

Collectively, these findings position PUS10 as a previously unappreciated regulator of the innate immune system, acting at the intersection of epitranscriptomic control, retroelement suppression, and nucleic acid sensing. By delineating its role in orchestrating antiviral defences and restraining autoimmunity, this study broadens our understanding of how RNA-mediated mechanisms and RNA modifying enzymes safeguard immune homeostasis, and how their dysregulation contributes to sustained inflammation and malignant transformation.

PUS10 modulates the DDR and repair

In the follow-up study (Paper II), we expanded on our previous findings that identified translationally upregulated mRNAs associated with DNA repair in PUS10-deficient cells (Paper I). We observed that PUS10 expression is rapidly upregulated following exposure to DNA damaging agents, and that its loss alters DDR outcomes, affecting cell cycle progression and sensitizing cells to DNA damage-induced cell death. Moreover, we provided evidence that PUS10 may influence DNA repair pathway choice, suppressing error-prone NHEJ while promoting high-fidelity HR. Guided by structural predictions that align a PUS10 domain with archaeal resolvases, we found that recombinant PUS10 binds ssDNA *in vitro*, with a notable preference for HJ structures – key intermediates in HR. Although preliminary, these findings suggest a novel function for PUS10 in genome maintenance and raise compelling questions about its role at the interface of RNA and possible RNA biology and DNA repair.

Prior studies indicate that PUS10 performs distinct functions based on its subcellular localization – a catalytically independent role in nuclear miRNA processing and catalytic activity-dependent modification of cytoplasmic tRNAs.[18] In the context of the DDR, several key questions remain unresolved. PUS10 has been identified as a target of ATM and ATR kinases, raising the possibility that it may be post-translationally modified upon genotoxic stress.[213] Whether PUS10 localizes to DNA damage sites and modulates nuclear programs, such as p53-driven transcription, or acts indirectly via translational control of DDR components remains to be elucidated.

To address this, immunoprecipitation-MS (IP-MS) following DNA damage may uncover DDR-related PUS10-interacting partners, while imaging approaches using co-localization with canonical DDR markers in engineered systems harbouring site-specific inducible lesions could reveal its spatial dynamics.[214] Furthermore, fluorescent tagging of endogenous PUS10 via CRISPR-Cas9 would enable live-cell tracking, providing temporal resolution of its recruitment relative to established repair factors.[215] ChIP at defined damage sites could provide additional confirmation of PUS10 presence at DNA lesions.

Nevertheless, an alternative model cannot be excluded, namely, that PUS10 modulates genome surveillance indirectly through regulation of RNA species involved in the DDR. To investigate this, we generated iCLIP-seq, RNA-seq, and small RNA-seq datasets in PUS10-deficient cells exposed to genotoxic stress. Comprehensive analysis of these datasets will enable the identification of RNA targets and molecular signatures of PUS10 activity following DNA damage. Integrating these findings with systems engineered to induce DNA damage at defined genomic loci will provide mechanistic insights into whether PUS10 acts directly at damaged sites, modulates the DDR pathway through RNA-mediated mechanisms, or engages in both modes of regulation.

Additionally, our prior work (Paper I) demonstrated that PUS10 loss leads to derepression of TEs and accumulation of RNA–DNA hybrids. Further investigation into the nature and origin of these hybrids may clarify whether TE dysregulation and hybrid formation intersect with PUS10 role in the DDR, or represent distinct molecular consequences. Notably, DNA damage is known to mobilize TEs, particularly retrotransposons, and RNA–DNA hybrids have been implicated in the DDR signalling and repair, although their functional relevance remains debated.[152,216] While some studies suggest they promote repair, others implicate them as drivers of genomic instability. Thus, future work should explore whether PUS10 modulates these processes through regulation of TE-derived or hybrid-associated transcripts.

Another intriguing possibility is that PUS10 influences the choice between distinct DNA repair pathways. Preliminary evidence suggests that PUS10 may suppress error-prone NHEJ and favour high-fidelity HR, yet this hypothesis requires further

validation using established reporter systems.[217] Moreover, since PUS10 translationally regulates genes involved in the G2-M checkpoint, it is essential to determine whether altered repair pathway usage reflects direct modulation of the DNA repair machinery or is a consequence of PUS10-dependent changes in cell cycle progression.

Together, these approaches aim to define the molecular framework linking PUS10 to genome maintenance. Building on these mechanistic insights, we further preliminary explored their broader medical relevance by discussing how PUS10's roles may influence disease contexts, such as cancer development, and potential therapeutic applications, particularly regarding its potential to enhance the safety and efficiency of iPSC generation.

Exploring the role of PUS10 in cancer

Disruptions in the DDR pathway can result in the accumulation of genomic instability, a key driver of cancer initiation and progression. Notably, chronic inflammation has been demonstrated to promote tumorigenesis, as exemplified by murine models of colon cancer, with persistent inflammatory signalling driving DNA damage through accumulation of reactive oxygen and nitrogen species.[218] Building on our earlier findings that PUS10 deficiency sensitizes mice to colitis, and that its activity is associated with human autoimmune diseases such as IBD, it is plausible that PUS10 may act as a molecular link between chronic inflammation and cancer. Given its emerging role in the regulation of genome integrity, further investigation into whether PUS10 loss exacerbates inflammation-induced DNA damage or disrupts repair fidelity could illuminate its potential function as a tumour suppressor. Understanding how PUS10 influences early transformation events under inflammatory stress may yield insights into the molecular underpinning of inflammation-associated malignancies.

Beyond inflammation-associated malignancies, it is also important to consider a broader role for PUS10 in cancer. Mutations in DDR genes such as BRCA1, BRCA2, ATM, and ATR underlie hereditary cancer predisposition syndromes and are frequently observed in tumours.[219-222] Along these lines, we have observed elevated PUS10 expression in a BRCA1-deficient breast cancer cell line, where its loss resulted in aberrant cell cycle progression and, over time, reduced viability. Even in a BRCA1-proficient cell line, PUS10 depletion altered the dynamic of DDR foci, consistent with its role in modulating DNA repair. These finding raise the possibility that PUS10 function intersects with BRCA1-regulated genome maintenance.

Given this, it would be valuable to investigate how PUS10-deficient cancer cells respond to DNA damaging therapies, particularly those that exploit impaired repair capacity. Conventional chemotherapeutics such as cisplatin induce DSBs and

promote cell death, while targeted agents like the PARP inhibitor, Olaparib, more selectively impair DNA repair in BRCA1/2-deficient contexts.[223-226] Assessing whether PUS10 loss confers synthetic lethality in these settings could further inform treatment strategies for tumours with DDR deficiencies.

Interestingly, we also observed altered p53 activation dynamics in PUS10-deficient cells compared to controls. As p53 is a key tumour suppressor frequently mutated in cancer and central to the cellular response to genotoxic stress, further exploration of the PUS10-p53 axis in oncogenic contexts may reveal novel layers of genome surveillance and potential vulnerabilities.[227,228]

PUS10 as a barrier to somatic cell reprogramming

In Paper II, we found that loss of PUS10 enhances the efficiency of somatic cells reprogramming. PUS10-deficient MEFs gave rise to iPSCs at significantly higher frequencies that wild-type counterparts. Despite this increased reprogramming efficiency, the resulting iPSCs were comparable to controls in their expression of pluripotency markers and exhibited similar differentiation potential, suggesting that PUS10 loss facilitates reprogramming without compromising stem cell identity.

While these findings highlight PUS10 as a barrier to cell fate transitions, several important questions remain. The reported reprogramming was performed using classical Yamanaka factors delivered via lentiviral vectors. Given recent evidence that PUS10 modulates viral mimicry pathways and influences innate immune response, it is conceivable that the enhanced reprogramming efficiency observed in PUS10-deficient cells may reflect an altered response to lentiviral transduction. To disentangle this possibility, it will be informative to test alternative reprogramming strategies, such as delivery of modified synthetic mRNAs, which bypass antiviral sensing or purely chemical induction of pluripotency.[229,230] These approaches could clarify whether PUS10's effects are broadly applicable to cell fate transitions or restricted to vector-based reprogramming systems.

The underlying mechanisms by which PUS10 constrains reprogramming remain unresolved. Previous studies have demonstrated that pathways involved in the DDR, translation control, and interferon signalling critically shaped the pluripotency acquisition.[193,194,196,231] This thesis highlights PUS10's involvement in all these processes, yet how these activities converge to restrict reprogramming remains to be defined. Unbiased single-cell transcriptomics methodologies such as single-cell RNA-seq could be leveraged to resolve the heterogeneity of reprogrammed populations and identify gene regulatory networks perturbed in the absence of PUS10. Moreover, implementing single-cell trajectory inference, namely single-cell orientation tracing (SOT), may distinguish between successful and abortive reprogramming fates, revealing whether PUS10 modulates deterministic versus stochastic routes to pluripotency.[232] Given PUS10's function in regulating specific

RNA subsets, follow-up studies should also consider transcriptome-wide analysis of RNA-binding or RNA modification dynamics during reprogramming.

PUS7-modified tRFs suppress aberrant translation in MDS

Paper III builds upon our earlier discovery that PUS7 catalyzes pseudouridylation of a distinct class of tRFs, termed mTOGs, which regulate protein synthesis in ESCs and are essential for proper germ layer specification. We previously showed that PUS7-mediated mTOG-Ψ also modulates HSC fate, and that PUS7 dysfunction is prevalent in high-risk subtypes of MDS.[59] In this follow-up study, we delineated the molecular basis of mTOG-Ψ activity. We demonstrate that mTOG-Ψ directly interacts with RNA-binding protein PABPC1, impairing the recruitment of the translational co-activator PAIP1. This interaction selectively inhibits the translation of mRNAs bearing 5' PES, including those encoding components of the translational machinery that are frequently deregulated in cancer. Extending these mechanistic insights to clinical samples, we show that mTOG-Ψ dysregulation leads to aberrant translation of 5'PES transcripts in MDS-HSPCs and correlates with leukaemic progression and reduced patient survival. Together, these findings position mTOG-Ψ as a critical translational repressor and potential therapeutic target in MDS.

Several important avenues remain open for future investigation. First, while mTOG-Ψ-mediated translational repression has been characterized in the context of MDS, it would be valuable to explore whether similar pathways operate in other heamatological malignancies or solid tumours, particularly those with known alterations in translational control. Second, further work is needed to define the full repertoire of mTOG-Ψ targets and their functional relevance. Approaches such as iCLIP-seq could map transcriptome-wide interactions between PABPC1 and mTOG-Ψ, and determine whether translational repression is strictly dependent on 5' PES motifs or modulated by additional sequence or structural features. Likewise, elucidating how pseudouridylation affect mTOG structure, stability and protein-binding capacity may provide broader insights into how mRNA modifications regulate tRF function. Finally, given the association between PUS7/mTOG dysregulation and poor clinical outcomes in high-risk MDS, it would be of translational interest to restore or mimic mTOG-Ψ activity. Preclinical studies in murine models could assess whether introducing functional mTOG-Ψ or modulating their downstream targets is sufficient to restrain malignant progression.

m^{6}A-mediated control of SF3B1-driven splicing in genome integrity and leukaemogenesis

Paper IV expands on our prior work identifying a translationally regulated oncogenic program, driven by MYC, RAS, and AKT/mTOR signalling, that controls SF abundance, specifically targeting cancer-relevant SF3 complex.[233] Here, we demonstrate that SF3B1 regulates AS of mRNAs encoding DNA repair and epigenetic regulators in response to MYC activation, thereby safeguarding genome integrity. During MDS, reduced SF3B expression is associated with increased genomic instability. Mechanistically, SF3B1 translation is fine-tuned by ALKBH5-driven m^{6}A demethylation of its 5' UTR, which modulates translation initiation site selection under oncogenic stress.

While these findings reveal a novel mode of regulation for SF3B1-WT, they also set several directions for future investigation. It will be important to further dissect the roles of other RNA modifications in the regulation of SFs, to determine whether distinct modifications converge on common regulatory nodes or function in mutually exclusive manner. Such studies could uncover cooperative or antagonistic interactions between RNA modifications, revealing additional layers of post-transcriptional control that fine-tune SF abundance and activity under physiological and pathological conditions. Additionally, identifying the upstream regulators of RNA modifying enzymes could provide deeper insights into this regulatory network. Broadening this perspective, future studies should explore how cancer-associated deregulation of epitranscriptome alters spliceosome composition and function, ultimately driving tumorigenesis.

Concluding remarks

Together, the findings presented in Papers I and II position PUS10 as a multifunctional regulator at the intersection of RNA metabolism, immunity, genome stability, and cell fate control. By coordinating cellular responses to inflammatory and genotoxic stress, modulating TE expression, and acting as a barrier to somatic reprogramming, PUS10 emerges as a critical integrator of transcriptional and translational circuits that govern cellular identity. These insights expand our understanding of the diverse functions of RNA modifying enzymes and raise compelling questions about how distinct PUSes may differentially shape cellular plasticity and stress adaptation. Future work dissecting the combinatorial function of PUS family members may uncover whether they exhibit functional redundancy, cooperativity, or unique, non-overlapping functions in maintaining cellular homeostasis.

Paper III broadens our insights into another RNA modifying enzyme, PUS7, revealing its role in regulating translation through modification of tRFs and the impact of its dysregulation on leukaemic transformation in MDS. Meanwhile, Paper

IV uncovers a distinct regulatory mechanism in leukaemogenesis, highlighting m^6A as a crucial modulator of SF3B1 translation. This work further elucidates the complex interplay between RNA modifications, splicing, genome integrity, and cancer progression. Together, these studies underscore the expanding significance of RNA modifying enzymes in diverse aspects of cellular homeostasis and disease, paving the way for deeper understanding of pathological processes and development of innovative therapeutic strategies.

Selected methods

Comet assay

DNA damage was assessed using the Single Cell Gel Electrophoresis Assay Kit (Bio-Techne) following the manufacturer's instructions with protocol adaptations. Briefly, cells were suspended in 1% low-melting-temperature agarose (Lonza), pre-warmed to molten state, and promptly spread onto slides (CometSlide; Bio-Techne). For alkaline conditions, slides were subjected to overnight lysis at 4ºC, followed by incubation in Alkaline Unwinding Solutions. For neutral conditions, cells were lysed overnight at 4ºC. Electrophoresis was then carried for 45 minutes at 10 V at 4ºC using a horizontal electrophoresis chamber (Sub-Cell Model 96 Cell; Bio-Rad). DNA was stained with SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific). Images were obtained using a confocal fluorescent microscope (Zeiss 780 Confocal Laser-Scanning Inverted Microscope) and analysed by OpenComet or CometScore software.

CUT&RUN

CUT&RUN was carried out as previously described with some modifications.[234] In brief, 5×10^5 WT and *Pus10*-KO immortalised MEFs (MEFs-hT) were washed twice with wash buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM spermidine, 1× Roche cOmplete protease inhibitors), then immobilised on 10 µl ConA-coated magnetic beads (Bangs Laboratories), which were pre-activated in binding buffer (20 mM HEPES pH 7.9, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$), per sample. Following immobilisation, cells were resuspended in 50 µl buffer (20 mM HEPES pH 7.5, 0.15 M NaCl, 0.5 mM Spermidine, 1× Roche complete protease inhibitors, 0.05% w/v digitonin, 2 mM EDTA) supplemented with primary antibodies: goat anti-rabbit IgG (Abcam; #ab97047) or rabbit anti-H3K4me3 (Active motif; #39159), diluted 1:50. Samples were incubated overnight at 4 °C with rotation. Beads were then extensively washed with digitonin-containing buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM Spermidine, 1× Roche cOmplete protease inhibitors, 0.05% digitonin) and incubated with pA-MNase (a kind gift from Steve Henikoff) for 1 hr at 4 °C. After two additional washes, samples were resuspended

in 100 µl digitonin-containing buffer, cooled to 0–2°C and treated with 2 mM $CaCl_2$ for 30 min at 0 °C to activate genome cleavage. The reaction was halted by addition of 100 µl 2x stop buffer (0.35 M NaCl, 20 mM EDTA, 4mM EGTA, 0.05% digitonin, 50 ng/mL glycogen, 50 ng/mL RNase A), followed by vortexing and incubation at 37°C for 10 min to release cleaved fragments. Subsequently, cells and beads were centrifuged (16,000 × g, 5 min, 4°C) and the resulting supernatant was purified using a PCR clean-up kit (Macherey-Nagel). Libraries were prepared using the KAPA Hyperprep kit with unique dual-index adapters, pooled and sequenced on an Illumina Nextseq500 instrument.

iCLIP-seq

iCLIP-seq was performed as described elsewhere.[59] Briefly, 10×10^6 MEFs-hT were UV-crosslinked (200 mJ/cm^2) using a UVP crosslinker (Analytik Jena), harvested, and lysed in iCLIP lysis buffer (50 mM Tris HCl pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA) supplemented with protease inhibitors (Sigma Aldrich). Lysates were sonicated in three 10 s bursts at 20 W (Branson). Partial digestion of RNA and DNA was carried out using 5 U/ml RNase I (Thermo Fisher Scientific) and 1 U/mL TURBO DNase (Thermo Fisher Scientific) for 3 min at 37°C with shaking (1100 rpm). Samples were cooled on ice for 5 min, clarified by centrifugation (15000 rpm, 10 min, 4°C), and supernatants were collected. Protein-RNA complexes were immunoprecipated using pre-washed Protein A Dynabeads (Life Technologies), pre-coated with anti-PUS10 antibody. Beads were incubated with lysates for 2 h at 4°C with rotation, then washed with lysis buffer. A second Rnase I digestion was performed for 5 min at 37°C with shaking (1100 rpm). To terminate the reaction, high-salt buffer was added (50 mM Tris HCl, 1000 mM NaCl, 0.5% Triton X-100, 0.25% sodium deoxycholate, 1 M urea, 5 mM EDTA, 1 mM DTT). Beads were then washed twice with high-salt buffer at 4°C, once with PNK/Tween buffer (20 mM Tris HCl pH 7.4, 10 mM MgCl2, 0.2% Tween 20) and once with wash buffer (50 mM Tris/HCl pH 7.4, 10 mM MgCl2). Dephosphorylation was carried out in 20 µl PNK reaction mix (4 µl 5× PNK buffer pH 6.5 (350 mM Tris-HCl pH 6.5, 50 mM MgCl2, 5 mM DTT, 0.5 µL T4 PNK [NEB], 0.5 µL SUPERase-IN RNase Inhibitor [Thermo Fisher]) for 15 min at 37°C. Following an additional wash with high-salt buffer and two washes with wash buffer, samples were incubated overnight at 16°C with shaking in 20 µl of L3 adaptor ligation mix (2 µl 10× T4 RNA ligation mix [NEB], 1 µl T4 RNA ligase I [NEB], 0.5 µl SUPERase-IN RNase Inhibitor [Thermo Fisher Scientific], 1.5 pmol pre-adenylated L3 linker, 4 µl PEG400). The following day, beads were washed twice with high-salt buffer and once with wash buffer, then radiolabeled with 20 µl of T4 PNK mix containing 2 µl of 10X T4 PNK Buffer (NEB), 1 µl T4 PNK (NEB), 0.5 µL γ-32P-ATP (PerkinElmer) for 5 min at 37°C with shaking

(1100 rpm). After final washes with high-salt buffer (once) and PNK/Tween buffer (twice), complexes were eluted in 20 µl of 1.5× Nu-PAGE loading buffer (Thermo Fisher Scientific) and heated at 70°C for 10 min with shaking (1100 rpm). Following supernatant collection, 1 µl 1 M DTT was added and samples were denaturated at 3 min at 95°C. Complexes were resolved on a NuPAGE 4%–12% Bis-Tris gel (Thermo Fisher Scientific) and transferred to a 0.45 µm nitrocellulose membrane (GE Healthcare). Membranes regions corresponding to the protein-RNA complexes were excised and treated with PK buffer for 30 min at 37°C with shaking (1100 rpm), followed by addition of 7 M urea in PK buffer to stop the reaction. RNA was purified using Phase Lock Gel Heavy tube (VWR) and ethanol-precipitated. First-strand cDNA synthesis was performed using Superscript III (Thermo Fisher Scientific). The resulting cDNA was circularized with CircLigase II (Epicenter), annealed with 0.25 µM Cut oligo, and digested with BamHI (Thermo Fisher Scientific). Digested cDNA was ethanol-precipitated and resuspended in 21 µl of water. 1 µl of cDNA was used as a template for PCR amplification with Accuprime Supermix I for 18–21 PCR cycles. Sequencing libraries were prepared and run on the Illumina NextSeq 500 System using a single-read configuration.

Poly I:C treatment

Male and female WT and *Pus10*-KO mice, aged 11-20 weeks, were administered intraperitoneal (i.p.) injections of high molecular weight Poly I:C (Poly I:C HMW; InvivoGen) at a dose of 10 mg/kg for six consecutive days. Age- and sex-matched untreated mice were used as controls. One day after the final injection, mice were euthanised, bone marrow was harvested and used for subsequent transplantation experiment.

Use of AI tools

This thesis has been partly produced using the generative AI models. Specifically, DALL·E via Bing Image Creator was used to create a cover image for this thesis and ChatGPT (OpenAI, 2015-2025) was consulted for suggestions during the writing process, including brainstorming ideas, improving the logical flow of sections, and refining wording. I have processed the generated text and image and take full responsibility for the content. Additionally, the popular science summary was translated from English into Swedish with the help of ChatGPT and subsequently proofread by a native Swedish-speaking scientist.

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