The roles of RNA m6A modification in disease mechanisms of high-risk neuroblastoma

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Cover illustration: This cover design features an artistic representation of RNA, decorated with roses to represent the m6A modifications. The vibrant pinks and lush greens reflect a complex interplay between molecular biology and neuroblastoma progression, while the neural network patterns subtly depicted in the background emphasize the neurobiological context of the research. This visual illustrates the thesis's focus on the importance of m6A modifications in shaping the molecular dynamics of neuroblastoma.

By Ketan Thombare

SVANENMÄRKET

Trycksak
3041 0234

Dedicated to my family and friends

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ABSTRACT

Epitranscriptomics involves covalent modifications of RNA bases, with N^6-methyladenosine (m6A) being one of the most abundant and functionally characterized. This modification plays a key role in RNA metabolism, cellular differentiation, and DNA damage response. Although m6A has been implicated in various cancers, its role in neuroblastoma (NB) tumorigenesis remains largely unexplored. High-risk NB tumors are often resistant to standard therapies, underscoring the need for novel treatment approaches. In many high-risk NBs, the *MYCN* oncogene is amplified, promoting an undifferentiated cell state associated with poor prognosis. Telomere maintenance is also predictive of survival in NB, with some tumors maintaining long telomeres through a telomerase-independent process called alternative lengthening of telomeres (ALT), which is associated with disease persistence and frequent relapses.

In this thesis, we optimized an m6A RNA-immunoprecipitation followed by sequencing (m6A-RIP-seq) method, allowing us to create the first m6A profile of NB tumors using low-input RNA samples. Applying m6A-RIP-seq in ALT-positive NB tumors, we uncovered an essential role for m6A-modified TERRA RNA in telomere maintenance. Our results suggest that m6A modification of TERRA RNA promotes its localization to telomeres in an hnRNPA2B1-dependent manner, forming condensate-like structures critical for TERRA's function in ALT-positive NB. Furthermore, targeting the m6A

methyltransferase METTL3, either alone or in combination with other therapeutic agents, led to significant telomere damage in ALT-positive NB cells.

Additionally, we developed a novel MYCN-driven NB model by differentiating human embryonic stem cells into trunk neural crest cells (tNCCs) and then into sympathetic neurons. MYCN overexpression in this model recreated the undifferentiated state characteristic of NB. Using this model, we found that MYCN and m6A jointly regulate the progression from tNCCs to sympathoadrenal progenitor cells. MYCN overexpression disrupted the expression of m6A-related genes, contributing to an undifferentiated cell state as observed in NB. Analysis of the m6A profile in MYCN-driven NB tumors revealed that m6A regulates key NB-specific genes. Inhibiting METTL3 reversed the undifferentiated state induced by MYCN and synergized with chemotherapy to reduce tumor volume in MYCN patient-derived xenografts *in vivo*.

In conclusion, our findings suggest that targeting the m6A epitranscriptome could provide a promising therapeutic strategy for high-risk NB, particularly in cases driven by ALT or *MYCN* amplification.

SAMMANFATTNING PÅ SVENSKA

Epitranskriptomik handlar om hur kemiska modifieringar av baserna i cellens RNA påverkar dess funktion. N^6-metyl-modifiering av RNA-basen adenosin (m6A) är en av den vanligast förekommande och bäst karakteriserade modifieringen. Denna modifiering spelar en viktig roll i cellens differentiering, RNA-metabolism och svar på DNA-skador. m6A har även kopplats till olika cancerformer, men dess roll i neuroblastom (NB) är till stor del fortfarande okänd. Hög-risk NB tumörer är ofta resistenta mot standardterapier, vilket understryker behovet av nya behandlingsmetoder. I många hög-risk NB är *MYCN*-onkogenen amplifierad, vilket främjar odifferentierade cancerceller och medför dålig prognos. Underhåll av kromosomernas telomerer är en annan prediktor för överlevnad vid NB, där vissa tumörer upprätthåller långa telomerer genom en process som är oberoende av telomeras, så kallad alternativ längdökning av telomerer (ALT), vilket också är associerat med sämre behandlingseffekt och ökad risk för återfall.

I denna studie har vi optimerat en metod för m6A RNA-immunoprecipitering följt av sekvensering (m6A-RIP-seq), vilket har möjliggjort den första kartläggningen av m6A-profiler från NB-tumörer med liten mängd RNA. Genom att tillämpa m6A-RIP-seq i ALT-positiva NB-tumörer, har vi funnit en viktig roll för m6A-modifierat TERRA RNA i tumörcellernas telomerunderhåll. Våra resultat tyder på att denna m6A-modifiering främjar lokalisering av TERRA RNA till telomerer på ett hnRNPA2B1-beroende sätt. Detta leder till kondenserade strukturer som är avgörande för TERRA RNAts funktion i ALT-positiv NB. Hämmande av m6A metyltransferas METTL3, enskilt eller i kombination med andra läkemedel, skadade telomererna i ALT-positiva NB tumörceller.

Vi utvecklade även en ny cellulär NB-modell genom att differentiera humana embryonala stamceller till trunk neural crest cells (tNCCs) och vidare till sympatiska nervceller. MYCN-överuttryck i dessa celler återskapade det odifferentierade tillstånd som är karakteristiskt för NB. Genom att studera denna modell fann vi att MYCN och m6A gemensamt reglerar övergången från tNCCs till sympato-adrenala progenitorceller. Överuttryck av MYCN störde uttrycket av m6A-relaterade gener, vilket bidrog till ett odifferentierat celltillstånd som observerats i NB. Analys av m6A-profilen i MYCN-drivna NB-tumörer visade att m6A reglerar viktiga NB-specifika gener. Hämning av

METTL3 återställde det odifferentierade tillståndet och minskade tumörvolymen *in vivo* i möss xenograftade med MYCN-postiva tumörceller från NB-patienter, i synergi med kemoterapi.

Sammanfattningsvis tyder våra fynd på att behandling riktad mot m6A epitranskriptomet är en möjlig terapeutisk strategi för hög-risk NB, särskilt i fall som drivs av ALT eller *MYCN*-amplifiering.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Vaid R*, **Thombare K***, Mendez A, Burgos-Panadero R, Djos A, Jachimowicz D, Lundberg KI, Bartenhagen C, Kumar N, Tummler C, Sihlbom C, Fransson S, Johnsen, JI, Kogner P, Martinsson T, Fischer M, and Mondal T. METTL3 drives telomere targeting of TERRA lncRNA through m6A-dependent R-loop formation: a therapeutic target for ALT-positive neuroblastoma. *Nucleic Acids Res, 2024*. doi.org/10.1093/nar/gkad1242.

II. **Thombare K***, Vaid R*, Das S and Mondal T. Targeting m6A-mediated TERRA RNA condensates as a therapeutic strategy for ALT-positive neuroblastoma. *Manuscript*.

III. **Thombare K***, Vaid R*, Pucci P*, Lundberg KI, Ayyalusamy R, Baig MH, Mendez A, Burgos-Panadero R, Höppner S, Bartenhagen C, Sjövall D, Rehan AA, Nale SD, Djos A, Martinsson T, Jaako P, Dong JJ, Kogner P, Johnsen JI, Fischer M, Turner SD and Mondal T. METTL3/MYCN cooperation drives neural crest differentiation and provides therapeutic vulnerability in neuroblastoma. *The EMBO Journal, 2024*. doi.org/10.1038/s44318-024-00299-8.

IV. Vaid R*, **Thombare K***, Ayyalusamy R, Fischer M and Mondal T. Deciphering m6A-modified gene signatures in neuroblastoma subtypes: from identification to functional characterization. *Manuscript*.

*Equal contribution

Publications not included in this thesis:

I. Vaid R, Mendez A, **Thombare K**, Burgos-Panadero R, Robinot R, Fonseca BF, Gandasi NR, Ringlander J, Baig MH, Dong JJ, Cho JY, Reinius B, Chakrabarti, LA, Nystrom K and Mondal T. Global loss of cellular m6A RNA methylation following infection with different SARS-CoV-2 variants. *Genome Res, 2023*. doi.org/10.1101/gr.276407.121.

II. Djos A, **Thombare K**, Vaid R, Gaarder J, Umapathy G, Reinsbach SE, Georgantzi K, Stenman J, Caren H, Ek T, Mondal T, Kogner P, Martinsson T and Fransson S. Telomere Maintenance Mechanisms in a Cohort of High-Risk Neuroblastoma Tumors and Its Relation to Genomic Variants in the TERT and ATRX Genes. *Cancers (Basel), 2023*. 15 doi.org:10.3390/cancers15245732.

III. Sharma T, Mondal T, Khan S, Churqui MP, Nystrom K, **Thombare K**, Baig MH and Dong JJ. Identifying novel inhibitors targeting Exportin-1 for the potential treatment of COVID-19. *Arch Microbiol, 2024*. doi.org/10.1007/s00203-023-03761-z.

CONTENTS

ABBREVIATIONS

ADRN	Adrenergic
ALKBH5	AlkB Homolog 5
ALT	Alternative Lengthening of Telomeres
ARID1A	AT-rich Interaction Domain 1A
ARID1B	AT-rich Interaction Domain 1B
ASCL1	Achaete-Scute Family bHLH Transcription Factor 1
A-to-I	Adenosine-to-Inosine Editing
ATRX	Alpha Thalassemia/Mental Retardation Syndrome X-
BARD1	BRACA1 Associated RING Domain 1
BMP	Bone Morphogenetic Proteins
carRNA	Chromosome-Associated Regulatory RNA
CCR4-NOT	Complex Carbon Catabolite Repression-Negative On
CD	Circular Dichroism
CHCs	Chromaffin Cells
DART-m6A-seq	Deamination-Aided RNA Tagging for m6A Sequencing
DNA	Deoxyribonucleic Acid
DRS	Direct RNA sequencing
eIF3	Eukaryotic Translation Initiation Factor 3
ELISA	Enzyme-Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assays
EMT	Epithelial-to-Mesenchymal Transition
FGF	Fibroblast Growth Factor
FMRP	Fragile X Mental Retardation Protein
FOS	FOS Proto-Oncogene
FTO	Fat Mass and Obesity-Associated Protein
G4	G-Quadruplex
GATA2	GATA Binding Protein 2
GATA3	GATA Binding Protein 3
GLORI	Nitrite-Mediated Deamination
H3K36me3	Trimethylation at Lysine 36 of Histone H3
H3K9me2	Dimethylation at Lysine 9 of Histone H3
H3S10	Phosphorylation of Histone 3 at Serine 10
HAND2	Heart and Neural Crest Derivatives Expressed 2
hESCs	Human Embryonic Stem Cells
hnRNPA2B1	Heterogeneous Nuclear Ribonucleoprotein A2/B1
IAPs	Intracisternal A Particles

IDR	Intrinsically Disordered Regions
IDRF	Image-Defined Risk Factors
IGF2BP3	Insulin-Like Growth Factor 2 mRNA-Binding Protein 3
ISL1	Islet 1
JUN	JUN Proto-Oncogene
KAP1	KRAB-Associated Protein 1 (also known as TRIM28)
KIF1B	Kinesin Family Member 1B
LINE-1	Long Interspersed Element 1
LLPS	Liquid-Liquid Phase Separation
LMO1	LIM Domain Only 1
lncRNA	Long Non-Coding RNA
M	Metastatic disease
m1A	N^1-methyladenosine
m5C	5-methylcytidine
m6A	N^6-methyladenosine
m6A-label-seq	m6A Labeling Sequencing
m6Am	N^6,2′-O-dimethyladenosine
m6A-RIP-seq	m6A RNA Immunoprecipitation Sequencing
m6A-SAC-seq	m6A Selective Alkylation of RNA Sequencing
m6A-SEAL-seq	m6A-Sequencing Using Enzyme-Linked Immunosorbent
m7G	N^7-Methylguanosine
MALAT1	Malignant Brain Tumor 1
MAML3	MAML1-Associated Protein 3
MazF	MazF Endoribonuclease (RNA Endonuclease)
Mazter-seq	MazF-Mediated RNA Sequencing
MEFs	Mouse Embryonic Fibroblasts
MEIS2	MEIS Homeobox 2
MES	Mesenchymal
MeRIP-seq	Methylated RNA Immunoprecipitation Sequencing
mESCs	Mouse Embryonic Stem Cells
METTL14	Methyltransferase-Like 14
METTL3	Methyltransferase-Like 3
MIBG	Meta-Iodobenzylguanidine
MS	Mass Spectrometry
MYCN	Myc Oncogene Family Member N
NB	Neuroblastoma
NCCs	Neural Crest Cells
NEXT	Nuclear Exosome Targeting Complex

NFKB	Nuclear Factor Kappa B
NRAS	Neuroblastoma RAS Viral Oncogene Homolog
NRG1	Neuregulin 1
NXF1	Nuclear Export Factor 1
ONT	Oxford Nanopore Technologies
PDX	Patient-Derived Xenografts
PHOX2A	Paired Like Homeobox 2A
PHOX2B	Paired Like Homeobox 2B
PRC2	Polycomb Repressive Complex 2
PRRX1	Paired Related Homeobox 1
RBP	RNA-Binding Protein
rG4	RNA G-Quadruplex
RNA	Ribonucleic Acid
R-loops	RNA-DNA Hybrids
RRM	RNA Recognition Motif
RUNX1	RUNX Family Transcription Factor 1
S	Special NB in infants under one year
SAM	S-Adenosylmethionine
SCPs	Schwann Cell Precursors
SDF1	Stromal Cell-Derived Factor 1
SGS	Second-Generation Sequencing
SN	Sympathetic Neurons
SNAI2	Snail Family Transcriptional Repressor 2
SNPCs	Sympathetic Neuron Precursors
SRSF10	Serine/Arginine-Rich Splicing Factor 10
SRSF3	Serine/Arginine-Rich Splicing Factor 3
TadA	T adenine Deaminase (RNA Editing Enzyme)
TBX2	T-Box 2
TERRA	Telomeric Repeat-Containing RNA
TERT	Telomerase Reverse Transcriptase
TFs	Transcription Factors
TGS	Third-Generation Sequencing
TMM	Telomere Maintenance Mechanisms
tNCCs	Trunk Neural Crest Cells
TWIST1	Twist Family BHLH Transcription Factor 1
VIRMA	Viable RNA Methylation
WNT	Wingless-Related Integration Site Family Proteins
WTAP	Wilms Tumor 1-Associating Protein

XIST	X-inactive Specific Transcript
YTHDCs	YTH Domain Containing Proteins
YTHDF1	YTH Domain Family Member 1
YTHDF3	YTH Domain Family Member 3
YTHDFs	YTH Domain Family Proteins
Ψ	Pseudouridine

DEFINITIONS

Epigenetic	Changes in gene expression or cellular phenotype that do not involve alterations to the underlying DNA sequence.
Epitranscriptomic	The study of chemical modifications on RNA molecules that affect their stability, translation, and function.
G4 (G-quadruplex)	A four-stranded structure formed in nucleic acids when guanine-rich sequences fold back on themselves.
Homologous recombination	A DNA repair mechanism that uses a similar DNA sequence as a template to fix double-strand breaks.
lncRNA (Long non-coding RNA)	A type of RNA longer than 200 nucleotides that does not code for proteins but plays roles in regulating gene expression.
m6A Switch	A mechanism by which m6A RNA modification regulates RNA functions by altering interactions with RNA-binding proteins.
R-loop	A three-stranded structure formed when RNA hybridizes with a DNA template strand, displacing the complementary DNA strand.
Transcription	The process of synthesizing RNA from a DNA template, allowing genetic information to be converted into functional products.
Translation	The process of synthesizing proteins from mRNA templates, occurring at ribosomes in the cytoplasm.

INTRODUCTION

Neuroblastoma

Neuroblastoma (NB) is the most common extracranial solid tumor in children. It is responsible for 15% of childhood cancer-related deaths[1-4]. The majority of cases, approximately 90%, are diagnosed in patients under the age of 10 years, with 40% identified in infancy[5]. NB is highly heterogeneous and characterized by distinctive but variable biological and clinical features. The disease phenotype is strongly affected by age and ethnicity. The median age of diagnosis is 18 months, and younger patients generally have a better prognosis than older ones[5,6]. Although NB is rare in adolescents and adults, making up less than 5% of cases, it often presents as a slow-growing but lethal tumor[7]. Moreover, individuals of African descent are more likely to develop malignant forms of the disease compared to those of European descent[8]. NB is more frequently observed in boys than in girls, although the underlying genetic and epigenetic factors contributing to this disparity remain unidentified[9].

Classification and prognostic markers

Once NB has been diagnosed, an assessment of the extent to which cancer has spread is carried out, known as staging. This assessment is essential in determining an effective treatment strategy. Staging gives some idea about the amount of cancer in the body and helps categorize a patient into a risk group that will help predict the response to the treatment and survival outcomes. Other than staging, important prognostic markers are used to predict the treatment outcomes and enable better predictions than that provided by staging alone. In general, two major systems are in use for the staging of NB- the International Neuroblastoma Risk Group Staging System (INRGSS) and the International Neuroblastoma Staging System (INSS)[4,10].

The INRGSS was developed to determine a child's stage and risk group before the start of treatment[10,11]. It uses imaging techniques such as CT or MRI scans and MIBG scans, as well as clinical examinations and biopsies. This system helps predict tumor resectability- that is, how much of it can be surgically removed- by defining image-defined risk factors (IDRFs) that could result in difficulties during surgery. The NB tumor is classified into four stages by INRGSS, which include L1-where the tumor is localized to one side of the body without IDRFs; L2-tumors are confined to one side of the body but

involve at least one IDRF; M-metastatic disease to distant body parts; and MS-a metastatic disease in children under 18 months, but the spread of the disease is limited to skin, liver, and/or bone marrow[6,10,12].

In contrast, the INSS is based on the result of a surgical procedure[10,11]. Thus, it cannot be applied to children who have not undergone surgery or who are not surgical candidates. The stages in this system include the following: Stage 1-Localized cancer entirely excised via surgery; Stage 2A-residual tumor on one side of the body with cancerous involvement of the lymph nodes on that side of the body; Stage 2B-tumor present with affected lymph nodes nearby; Stage 3-a cancer diffused across the midline or into the nearby lymph nodes on the opposite side; Stage 4-distant diffusion to multiple organs; and Stage 4S-a special NB in infants under one year with limited diffusion into certain organs without extensive involvement of the bone marrow. Also, the term "recurrent" describes cancer that returns subsequent to treatment[13-15].

Understanding NB prognosis is much more than staging alone; this is why prognostic markers are relevant to the child's outlook for cure. The most common prognostic factors include age, tumor histology, DNA ploidy, *MYCN* gene amplification, chromosome changes, neurotrophin receptors, and serum levels of certain substances[16]. Younger patients, especially those below 12 -18 months, usually have a better prognosis than older children[4]. The tumors of favorable histology are composed of cells that appear more normal and are well differentiated, usually indicating a better prognosis and treatment outcome. On the other hand, in unfavorable histology, there is a higher proportion of immature cells, which is linked to more aggressive behavior and will generally give a worse prognosis[4].

DNA ploidy, which gives information on the quantity of DNA in tumor cells, further refines prognosis. Tumors containing extra DNA, known as hyperdiploid tumors, are generally related to good outcomes, particularly for younger children[16,17]. *MYCN* gene amplification, which is associated with aggressive tumor behavior, especially correlates with more difficult treatment circumstances[18]. Chromosomal changes such as deletions on chromosomes 1 and 11 or gains on chromosome 17 provide further important information regarding the prognosis[4,19,20]. **Figure 1** provides an overview of clinical and molecular characteristics across 208 untreated NB cases. This figure highlights cases with *MYCN* amplification, ALT, and other high-risk features, which are essential for risk stratification and prognostic assessment[21].
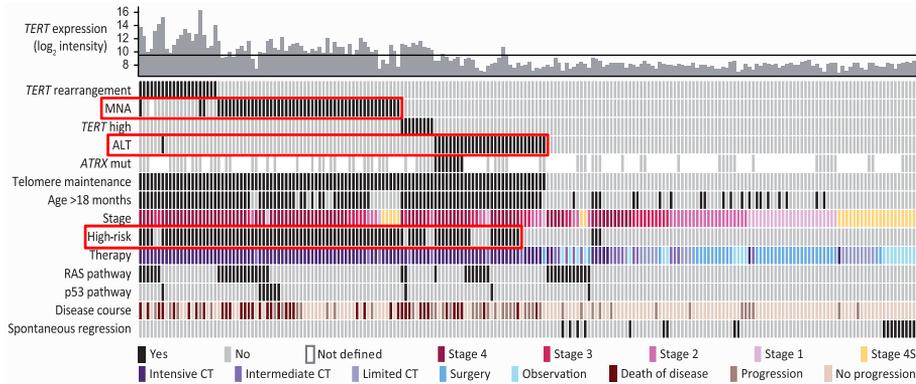
Figure 1. *Clinical and molecular characteristics across 208 untreated neuroblastoma cases, displayed in left-to-right order. Red boxes highlight MNA, ALT, and high-risk cases, while the black line in the top panel indicates the TERT expression threshold. CT, chemotherapy; MNA, MYCN amplification. Adapted from Ackermann et al., Science, 2018, with permission from AAAS[21].*

Outcomes also depend on the expression of neurotrophin receptors such as TrkA; high levels are associated with a good prognosis[22]. Some serum markers provide additional prognostic information. For instance, in children with NB, elevated blood levels of ferritin and lactate dehydrogenase (LDH) indicate poor outcomes[23,24]. These prognostic markers give further details about the complexity of NB so that clinicians can make decisions to optimize treatments appropriately.

Doctors divide children with NB into risk groups based on how likely it is that their cancer will come back after treatment. Risk groups are critical in predicting the possibility of a cure in children with NB diagnoses and help guide the intensity of treatment. Classifications are based on cancer stage and several prognostic factors, such as age and tumor characteristics. The children in the low-risk group often require limited interventions, such as surgery alone, and have a 5-year survival rate exceeding 95%, while rates for those in the intermediate-risk group range from 90% to 95%, with high-risk children facing an approximate 50% determined survival rate[25].

Genetics

Chromosomal aberrations significantly add to the clinical heterogeneity of NB. Generally, tumors in low-risk patients have whole chromosomal gains and frequently harbor hyperdiploid tumor cells. On the other hand, segmental

chromosomal gains or losses are more frequent in high-risk tumors (**Figure 1**)[26-29]. This profile is notably displayed by high-risk NB with segmental chromosome 17q gains, hemizygous losses on chromosomes 1p and 11q, and somatic amplification of the *MYCN* oncogene[4]. Moreover, high-risk cases can also show rearrangements in the 5p15.33 chromosomal region, very close to the telomerase reverse transcriptase gene (*TERT*)[30,31]. Despite all these complex genetic alterations, NB tends to have an overall low number of somatic mutations, with no single mutation characterized as a universal driving force of the disease[32-34].

Most NB cases occur spontaneously, with familial cases representing approximately 1-2% of the total cases[4]. Studies have shown that germline mutations of genes, including *PHOX2B*, *ALK*, and *KIF1B*, confer susceptibility to familial NB. Mutations in *PHOX2B* are detected in 6-10% of familial NB and about 2% of individuals who sporadically develop the disease[33-35]. Among the most frequent mutations affecting the *ALK* gene, missense mutations (such as R1192P, R1275Q, and G1128A) significantly contribute to familial NB cases and are present in about 10-12% of sporadic cases[36-38]. Additionally, germline mutations in *KIF1B* increase the risk of developing neuronal tumors, including NB and pheochromocytoma[39,40].

Other recurrent genetic changes include mutations in the genes *LIN28B*, *ATRX*, *ARID1A/1B*, *BARD1*, *PTPN11*, *LMO1*, *NRAS*, and *TP53*[32-34,41-47]. Furthermore, inter- and intra-tumorigenic heterogeneity adds additional complexity to the clinical scenario[38,41,42,48]. Tumors with the same phenotypic and morphological characteristics can respond differently to therapy, given their unique molecular profiles. It thus becomes fundamentally essential to characterize the molecular landscape in NB patients, especially in high-risk cases, to enable targeted therapies that address specific genetic aberrations in addition to conventional therapeutic approaches[49].

Plasticity

NB carries significant genetic heterogeneity that influences clinical outcomes. In a few high-risk patients, especially those with stage 4S disease, spontaneous regression of the tumor cells is observed, whereas the majority of patients with NB at diagnosis present with disseminated disease[4,12]. The mechanisms of such regression are poorly understood but suggested factors include epigenetic regulation, neurotrophin deprivation, loss of telomerase activity, and immune responses[50]. Although NBs generally bear low mutational burdens compared

to adult cancers, they are nonetheless able to exhibit intra-tumor heterogeneity, often with distinct relapse-specific evolutionary trajectories and increased mutational burdens, including RAS-MAPK and Hippo-Yap pathways[41,42,51].

A recent finding demonstrated that there are two cell types present in NB tumors- undifferentiated mesenchymal cells (MES-type) and committed adrenergic cells (ADRN-type)[52,53]. These cellular entities exhibit divergent transcriptomic profiles, with MES-type cells sharing characteristics with human neural crest-derived cell lines and, as such, are more primitive compared to ADRN-type cells[52]. The complexity of NB is further evidenced by the fact that these cells express identical genetic abnormalities yet have different transcriptomic signatures[52].

One of the salient features of NB is the plasticity of these cell types in the form of interconversion between ADRN and MES states via epigenetic reprogramming[54,55]. This plasticity depends upon the action of transcriptional networks and regulatory elements, including super-enhancers (SEs), driving critical gene expression[56]. Among them, the transcription factors (TFs) specific to ADRN include the following: ASCL1, GATA2, GATA3, HAND2, ISL1, LMO1, MEIS2, MYCN, PHOX2A, PHOX2B, TBX2, the tyrosine kinase receptor ALK; the enzyme DBH and TH involved in catecholamine metabolism. On the other hand, TFs that are expressed in MES cells include the following: MAML3, NFKB, PRRX1, SNAI2, TWIST1, RUNX1, JUN, and FOS, among others[52-58]. Moreover, high-risk NBs often contain a higher percentage of MES cells associated with poor prognosis, which can retain an ADRN state indicative of marked plasticity within the tumor[52,53,55,59]. Such regulatory complexity requires tailored therapeutic strategies since the coexistence of both cell types with their particular gene expression profiles may affect treatment responses and disease progression.

Neuroblastoma origin and neural crest differentiation

NB is a neuroendocrine neoplasm originating from sympathoadrenal cells during the fetal development of the sympathetic nervous system (SNS). The sympathoadrenal lineage originates from multipotent migratory neural crest cells (NCCs), which are a transitory population of cells localized in the dorsal region of the neural tube (**Figure 2**)[60,61]. Following the closure of the neural tube, NCCs undergo an epithelial-to-mesenchymal transition (EMT) that allows for their migration toward a variety of target tissues, in particular toward the side of the aorta[62]. This initial migration of undifferentiated NCCs depends

upon chemoattractive signals. It is directed by growth factors and morphogens, including fibroblast growth factor (FGF), Wingless-related integration site family proteins (WNT), bone morphogenetic proteins (BMP), and retinoic acid (RA)[63-65]. These signals help in specifying the anteroposterior and dorsoventral identities of NCC[65].

NCCs have been further divided into cranial, cardiac, vagal, trunk, and sacral based on the migratory pathways and differentiation capabilities mediated by extrinsic signals provided by the surrounding tissues. Due to this multi-lineage competence, the NCCs are frequently referred to as the "fourth germ layer[66]." They possess the capability to differentiate into several cell types, such as neurons, glial cells, Schwann cells, melanocytes, and chromaffin cells of the adrenal medulla[67]. Such wide differentiation potential forms the basis for heterogeneity in NB that may arise from a variety of neural crest elements. However, most NB cases occur in the abdomen and adrenal medulla, while relatively fewer cases occur in the paraspinal sympathetic ganglia[2].

The initial early migratory stage of NCCs is SOX10+, guided by the chemoattractive signals from the dorsal aorta through BMP4 and BMP7, which induce the expression of the chemokines such as stromal cell-derived factor 1 (SDF1) and neuregulin 1 (NRG1)[68]. Receptors for these ligands, including C-X-C motif chemokine receptor 4 (CXCR4) and epidermal growth factor receptor (EGFR), facilitate the directed migration of SOX10+ early NCC toward the dorsal aorta. Upon reaching the vicinity of the dorsal aorta, migrating NCC are no longer called SOX10+ early NCC but are instead known as sympathoadrenal precursor cells (SAPs). In the dorsoventral split, SAPs commit to further differentiation influenced by their microenvironment to forming either sympathetic neuron precursors (SNPCs) or Schwann cell precursors (SCPs)[69,70].

The multipotent SCPs differentiate into glial cells or chromaffin cells through a transient bridge cell population[70]. Bridge cells are an intermediate population derived from the SCPs that are essential for the passage of SCPs into mature chromaffin cells in the adrenal medulla[59,71]. Chromaffin cells are neuroendocrine cells specialized in secreting catecholamines, which are extremely important for the body's response to stress. For example, mouse lineage-tracing studies indicate that about 80% of chromaffin cells originate in the adrenal medulla from late-migratory NCC-derived SCPs, whereas the remaining 20% are derived from SAPs[70].
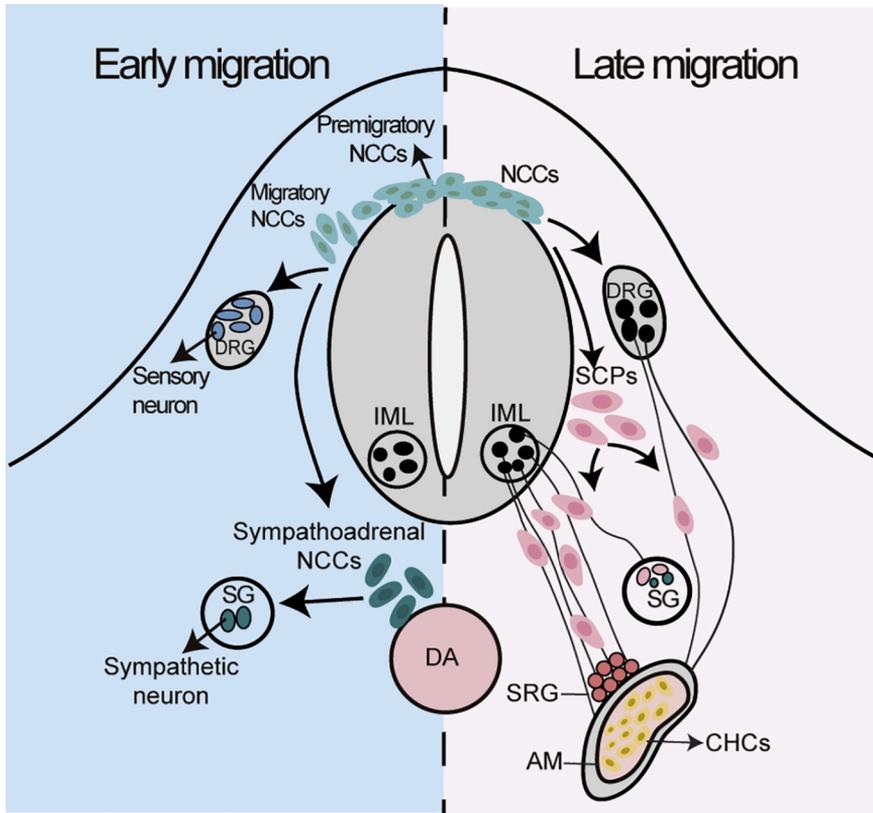
Figure 2. *Diagram illustrating the development and migration of neural crest cell (NCC) derivatives. Abbreviations: AM, adrenal medulla; CHCs, chromaffin cells; DA, dorsal aorta; DRG, dorsal root ganglia; IML, intermediolateral cell column; NCCs, neural crest cells; SCPs, Schwann cell precursors; SG, sympathetic ganglion; SRG, suprarenal sympathetic ganglion. Adapted from Rui Dong et al., Cancer Cell, 2020, with permission from Elsevier[61].*

The SNS originates from trunk NCCs (tNCCs), which form the SAPs that further differentiate into sympathetic neurons (SN) and chromaffin cells (CHCs) with catecholaminergic properties[72,73]. The delineation of these pathways is of utmost importance in understanding the cell of origin for NB. The earliest described cell of origin for NB to date is the SAP, which does not receive or inadequately respond to differentiation signals[59,74,75]. High levels of MYCN play an important role in the early development of NCC migration and proliferation[76]. However, during terminal differentiation, when NCCs differentiate into neural or chromaffin cells within the sympathoadrenal lineage, there is a progressive decline in the expression levels of MYCN[76]. Such down-regulation in MYCN expression is a prerequisite for the proper

terminal differentiation of these cells. If the expression of MYCN continues through this maturation phase, it could lead to the acquisition of paraneoplastic lesions that contribute to the development of NB[62,76,77].

A number of similarities between NB and developing sympathoadrenal neuroblasts (also named sympathoblasts) have identified single-cell RNA-sequencing (scRNA-seq). Low-risk NBs resemble committed neuroblasts, whereas *MYCN*-amplified tumors display mesenchymal characteristics that resemble undifferentiated neuroblasts and bridge cells[71]. These observations reinforce that neuroblasts are cells of origin for NB. More specifically, undifferentiated cells from high-risk NB are similar to the progenitor populations found in the postnatal adrenal gland, while low-risk NBs are similar to postnatal chromaffin cells and committed fetal neuroblasts. These findings explain the heterogeneity in NB, confirming the hypothesis that NB originates from multiple cell types arrested at distinct stages of differentiation. High-risk NB appears to originate either from rapidly cycling progenitor cells that undergo a tumorigenic transformation at some point in development or from cells that fail to differentiate postnatally due to mutations or chromosomal changes[65].

Further support for a prenatal origin of NB comes from expression profile analyses indicating similarities between human fetal adrenal neuroblasts and NB cells. A distinctive feature of childhood cancer is the initial hyperplasia of precursor cells that undergo programmed cell death, mimicking physiological organogenesis where excess cells are produced. In a subset of NB patients experiencing spontaneous regression of the disease without treatment, the incidence of NB precancerous lesions is much greater than the clinically diagnosed disease[78].

Chromosomal aberrations linked to the pathogenesis of NB seem to arise in the first trimester of pregnancy, thereby linking genetic events to the prognosis of the disease[79]. Since tumors with longer evolutionary histories are usually more aggressive, it hence indicates that genetic progression is associated with malignancy. Clonal tracing studies have confirmed that NBs originate during embryogenesis, with dormant metastatic clones potentially present at diagnosis. Moreover, with time, disease-defining loci such as *ATRX* and *TERT* show an increased number of variations, thereby further complicating the genetic landscape of NB[80].

Although we have a better overall understanding of the origin of NB, the exact timing and cell type are yet to be defined and remain active areas of research. Furthermore, the switching between adrenergic and mesenchymal cellular states, among other cellular states, reveals the plasticity of NB cells and their adaptation to microenvironment contexts, a characteristic that contributes to heterogeneity[52].

In summary, the origin of NB is intimately related to the developmental pathways controlling NCC migration and differentiation into SAPs. There is a strong interplay between genetic and developmental mechanisms in the arising and heterogeneity of NB, and it forms a basis for the use of targeted therapies considering its biology.

Neuroblastoma and telomere maintenance

A defining characteristic of malignant tumor cells is their ability for unlimited growth, called replicative immortality[81]. It becomes possible because of the activation of molecular mechanisms providing telomere stability-specialized heterochromatic structures capping the ends of eukaryotic chromosomes[82]. Telomeres consist of non-coding, repetitive DNA sequences, very often as short hexameric elements (TTAGGG in vertebrates)[83,84]. Owing to the end replication crisis, telomeres shorten progressively during each division in normal somatic cells. Eventually, when there is a complete loss of chromosomal protection at the telomeres, a DNA damage response would, instead, be initiated, leading to senescence or cell death[81,84]. In contrast, cancer cells bypass these restrictions due to the presence of telomere maintenance mechanisms (TMM), which activates telomerase-positive, reverse transcriptase enzymes that extend telomeres, and another pathway called Alternative Lengthening of Telomeres (ALT)[21,85-88].

Telomere biology plays an important role in NB[21,30,31,89-95]. NB represents a clinical spectrum, from spontaneous regression in low-risk disease to aggressive disease progression in high-risk disease, with almost 50% mortality despite intensive therapy[4,96]. This diversity has puzzled researchers for a long time, leading to extensive research into the molecular mechanisms that govern these diverse phenotypes. Recent studies have emphasized the role of TMM in NB pathogenesis, where active mechanisms have been attributed to high-risk cases, with its absence associated with favorable outcomes seen in low-risk tumors (**Figure 1**)[21,86].

Telomerase activation is one of the major driving forces underlying NB progression, especially the high-risk type[31]. Amplification of the *MYCN* proto-oncogene, present in approximately 20% of NBs at diagnosis, is clinically associated with adverse outcomes[97-99]. MYCN directly binds to the *TERT* gene promoter, which leads to increased expression of the TERT, crucial for telomerase activity[4,30,31,96,100]. This process leads to the replicative immortality of NB cells and escapes senescence and apoptosis. Somatic genomic rearrangements involving the TERT locus have also been identified in many high-risk NBs, which activate the telomerase expression as a result of the translocation of strong enhancer elements to the *TERT* gene[30,31]. This disruption of normal regulatory mechanisms results in significantly higher TERT levels that are associated with the aggressive behavior of tumors. In contrast, a lack of telomerase activity generally characterizes good clinical behavior in low-risk NBs and points to a key role of telomerase activation in malignant transformation[21,31,101].

Besides telomerase, ALT is one of the most important mechanisms in the maintenance of telomeres present in approximately 22% of high-risk NBs[86,102]. ALT is defined by the presence of ALT-associated promyelocytic leukemia bodies (APBs), subnuclear structures involved in homologous recombination processes necessary for telomere elongation without using telomerase[103,104]. This process allows the cancer cells to maintain telomere length and achieve replicative immortality, similar to the activation of telomerase, thereby evading cellular consequences of telomere shortening. So far, ALT has been associated with genomic alterations, notably mutations in the *ATRX* gene, which encodes a chromatin remodeler important for maintaining proper structure at telomeres and is required for regulating accessibility within telomeric DNA[30,31,34,105].

The loss of ATRX function disrupts normal telomere maintenance, promoting ALT activation because of the facilitation of alternative repair pathways[106,107]. Further, the ALT pathway is associated with replication stress at the telomeres and double-strand breaks that promote the generation of extrachromosomal telomeric DNA as C-circles[108,109]. TERRA (telomeric repeat-containing RNA) is also implicated in ALT; it is believed to play a role in the regulation of telomere length and in the recruitment of the repair machinery required for maintaining telomere integrity[110]. The presence of TERRA at telomeres is thought to contribute to the formation of APBs and the activation of recombination processes. TERRA is highly expressed in ALT-positive

(ALT+) tumors compared to non-ALT cases[85,102]. Moreover, ALT is characterized by unique histone modifications, which include H3K9me3, which represents heterochromatin formation and telomere clustering[102,111].

The clear presence of TMM in NBs has important translational implications for NB management since TMM can serve as biomarkers for risk stratification. High-risk NBs invariably exhibit active TMM, whereas low-risk tumors lack these mechanisms[21,112]. This distinction may guide treatment intensity and improve patient outcomes. However, the assessment of TMM has yet to be integrated into routine clinical practice due to the absence of standardized testing protocols and the complexity of the tests.

Targeting TMM is highly promising, especially in the context of telomerase-active tumors, wherein direct telomerase inhibitors such as imetelstat have been capable of demonstrating preclinical efficacy that has been difficult to translate clinically due to associated toxicities[113]. In contrast, the targeting of ALT+ tumors remains even more complex and requires novel therapeutic strategies. Although ALT+ NBs might progress more slowly compared to telomerase-active tumors, they usually respond inadequately to conventional therapies[34,86,114-116]. Thus, the clinical management of such cancers is quite challenging.

The dependence of ALT+ tumors on homologous recombination repair mechanisms for their immortality highlights the need for developing therapeutic strategies specifically aimed at targeting TMM[117,118]. Understanding TMM in NB would be critical for elucidating its pathogenesis and improving clinical outcomes.

Preclinical models in NB research

Preclinical disease models are valuable in biomedical research, including drug discovery, diagnostic tests, and the study of disease mechanisms. While these experimental systems are essential, no model is perfect, and their clinical relevance has long been one of the major obstacles to cancer research[119,120]. In NB, which is known for its heterogeneous nature, such shortcomings are strongly noticeable.

Classical approaches, such as traditional 2D cell cultures and laboratory animals, have been in wide use for many decades[119]. Although NB cell lines are well established, such as SH-SY5Y and SK-N-BE(2), which are easily accessible and reproducible, are susceptible to problems with cross-

contamination, which can make research unreliable[121]. Besides that, 2D cultures cannot mimic the complicated interactions within tumors and provide substantial gaps in understanding tumor development. The limitations of current models are particularly evident in NB because most of the present models do not represent the complex biology and clinical heterogeneity of this disease, hence making the study of tumor initiation, tumor progression, and therapeutic response very difficult[122].

To overcome these problems, researchers have adopted more sophisticated, physiologically relevant 3D culture methodologies such as spheroids and organoids. Such cultures will enable better modeling of the tumor microenvironment and studies on cell-cell interaction, response to drugs, and tumor progression[122]. Recently, conditional reprogramming has become a promising approach that allows the expansion of patient-derived cells by preserving their genetic and epigenetic profiles. This strategy reinforces personalized medicine approaches by allowing researchers the capability to test therapeutic responses in a more individual-specific manner[123].

Among all the systems, *in vivo* murine systems have played an essential role in NB studies. For instance, the TH-MYCN model is a genetically engineered mouse model that effectively models genetic alterations common in human NB and gives insights into tumor initiation and progression[124]. While these models have proven to be very useful, they often fail to recapitulate metastatic behavior and can take quite some time to develop tumors[122]. The Patient-derived xenografts (PDX) models have gained traction because they preserve much of the complexity and heterogeneity of human tumors, allowing assessments of therapeutic efficacy in a more clinically relevant setting. However, the drawbacks are that PDX models are resource-intensive and suffer from the disadvantages of engraftment success and availability of tissues[122,125,126].

Emerging models, such as humanized mice and zebrafish, provide additional avenues for studying NB. Humanized mice incorporate human immune components, making them valuable for exploring immune interactions in tumor progression[127-129]. Zebrafish offer a cost-effective and rapid system for studying genetic mechanisms and drug responses due to their transparent embryos. Furthermore, chick embryos have become a valuable tool in the study of NB because they provide opportunities for investigations of the interaction between tumor cells and the local microenvironment. Although this model

does not entirely replicate human pathophysiology, the possibility of following tumor growth and behavior in real-time allows an in-depth understanding of cellular and molecular driving NB development[130,131].

In summary, while traditional models, including mouse and zebrafish systems, have provided valuable insights into NB biology, there remains a significant gap in the availability of more relevant human models. This limitation is particularly noteworthy in the context of understanding NB initiation and progression. This thesis seeks to tackle this gap by establishing a NB model through the differentiation of human embryonic stem cells (hESCs) into sympathetic neurons (SN), using conditional MYCN overexpression to induce cellular transformation. This approach not only provides an opportunity to investigate the developmental aspects of NB but also highlights the ongoing need for improved models to effectively understand the complexities of the disease.

RNA modifications

Changes in gene expression are among the primary reasons for tumor development. Numerous regulatory layers actively modulate gene expression at the DNA, RNA, and protein levels in addition to changes in DNA sequence. These layers include epigenetic, transcriptional, epitranscriptomic, and translational processes. Epitranscriptomics is a new layer of regulation in gene expression and refers to chemical modifications to RNA molecules without changing the nucleotide sequence. It is a variable and dynamic process[132].

Types of RNA modifications

RNA modifications were first identified in the 1950s; however, their functional importance has been largely unexamined for many years[133]. With the recent development of high-throughput sequencing and sensitive detection methods, more than 170 RNA modifications have so far been identified in a number of RNA species, such as mRNA, tRNA, rRNA, and non-coding RNAs[134]. Some of the RNA modifications in mRNAs include N^6-methyladenosine (m6A), N^6,2′-O-dimethyladenosine (m6Am), adenosine-to-inosine (A-to-I) editing, pseudouridine (Ψ), 5-methylcytidine (m5C), N^1-methyladenosine (m1A) and N^7-methylguanosine (m7G) (**Figure 3**)[135-139]. N^6-methyladenosine (m6A) is the most abundant and widely studied. Although m6A was discovered in the 1970s, renewed interest in this modification emerged following recent technological advancements that allowed for comprehensive mapping of its

distribution throughout the transcriptome alongside the crucial finding of its reversible nature[140-145].
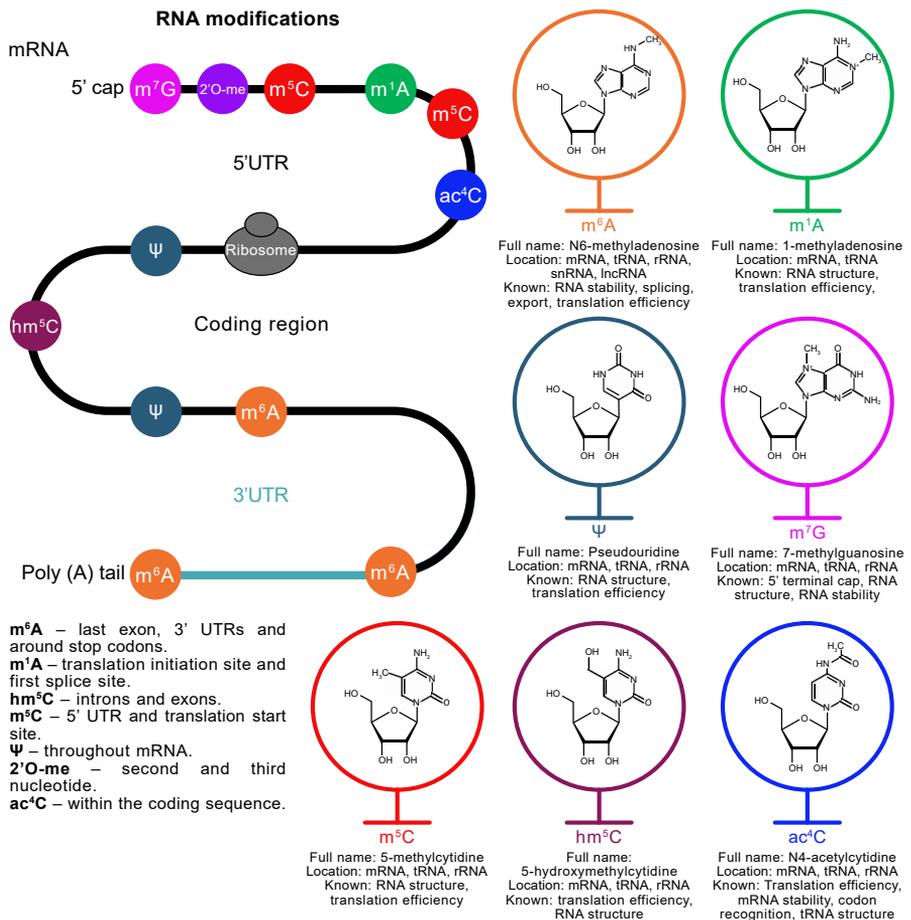

Figure 3. *Overview of common RNA modifications and their functions, including locations on mRNA and roles in RNA fate and metabolism. Adapted from an original figure by Abcam, with permission[137-139].*

The current thesis will mainly focus on the m6A RNA modification. It plays a critical role in various biological processes, including cell growth, signal transduction, and carcinogenesis[146-148]. It influences RNA fate through mechanisms such as splicing, mRNA localization, and degradation[146-148]. As a dynamic and reversible epigenetic mark, m6A is added to RNA by methyltransferases (referred to as "writers"), including METTL3, METTL14, WTAP, and VIRMA. Conversely, demethylases like FTO and ALKBH5 act

as "erasers," removing this modification, while RNA-binding proteins (RBPs) such as IGF2BPs, YTHDCs, and YTHDFs function as "readers," recognizing m6A sites[132,146-148].

Molecular consequences of the m6A modification

At the molecular level, m6A modification is a critical factor in almost all of the processes of RNA metabolism concerning mRNA (**Figure 4**)[132]. This modification affects mRNA expression, splicing, nuclear export, translation efficiency, and stability of RNA[132,140,141,144,149-156]. The impact of m6A on these processes generally appears to be mediated through RBPs. Nevertheless, the specific factors that determine whether an m6A-modified RNA will be regulated at splicing, export, stability, or translation and whether it may be affected at multiple levels remain uncertain. While the prime focus here is mRNA, it is essential to acknowledge that m6A also impacts other RNA types, including miRNAs and circular RNAs, though to a lesser extent[132].
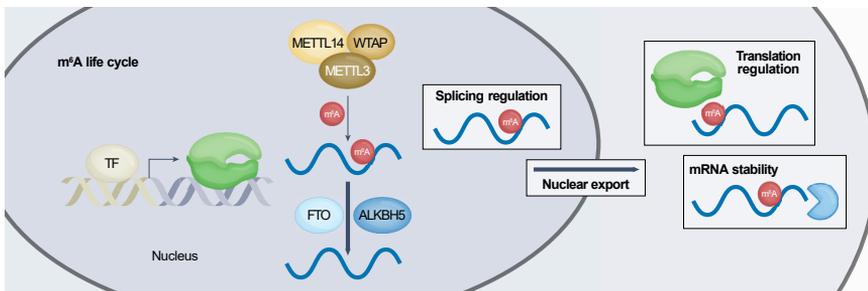

Figure 4. Regulation of m6A modification by the METTL3/METTL14/WTAP complex and demethylases (FTO, ALKBH5), with roles in mRNA localization, splicing, export, stability, and translation. Adapted from Boulias, K., Greer, E.L., Nat Rev Genet, 2023, with permission from Springer Nature[132].

Pre-mRNA splicing

The pre-mRNA splicing process is controlled by the m6A modification either by direct interactions with the nuclear reader YTHDC1 or by an "m6A switch" mediated through heterogeneous nuclear ribonucleoproteins (hnRNPs). It has been shown that YTHDC1 binds m6A-modified mRNA to either promote splicing by interacting with the splicing factor SRSF3, promoting exon inclusion, or repressing SRSF10 to enable exon skipping[132,149,157,158]. For example, studies in human cell lines revealed that YTHDC1 mediates a set of splicing pathways that are critical for the alternative splicing of genes involved in oncogenesis, further illustrating the tremendous impact of m6A on mRNA

processing. Besides, the m6A switch would influence local RNA structure, increase the binding of splicing factors, and favor the retention of exons[149]. These interactions reflect the complexity of RNA processing, and thus, further research is required to shed light on specific splicing pathways regulated by these interactions and how other splicing factors interact with m6A-modified transcripts.

Nuclear export

The interaction with nuclear export factors by m6A-binding proteins controls the subcellular localization of modified mRNAs. It has been reported that YTHDC1 interacts with export proteins such as SRSF3 and NXF1, while FMRP binds to CRM1 to facilitate the nuclear export of m6A-modified transcripts[132,159-161]. Furthermore, studies have demonstrated that disrupting these interactions causes the retention of modified mRNAs within the nucleus, thereby impeding their translation. This modification appears to facilitate mRNA shuttling into the cytoplasm for translation through a variety of pathways, but further research will be required to define the shades of nuclear export specificity. Understanding how modifications of m6A influence the dynamics of RNA localization may provide insight into regulatory mechanisms that drive gene expression and cell response.

RNA stability

The stability of RNA is significantly influenced by m6A modification. Initial reports indicate that m6A-modified mRNA is less stable compared to unmodified ones, leading to low abundance and translation potential[162]. Some studies documented that m6A-modified RNAs are specifically degraded by the recruitment of CCR4-NOT deadenylase complex using YTHDF2 for mRNA decay[132,163]. On the other hand, the binding proteins, like IGF2BPs, have a stabilizing effect on the m6A-containing transcripts, suggesting that interactions are complex and finely tuned to decide their fates of degradation or stabilization[164]. Such a delicate balance between degradation and stability can massively alter the expression levels of genes regulated by this modification, with a view toward underlining the importance of such processes within cellular dynamics and responses to stress.

Translation efficiency

Depending on the context, m6A modifications can either promote or inhibit translation. When located in the 5' UTR, m6A can enhance cap-independent

translation during stress response by recruiting eukaryotic translation initiation factor 3 (eIF3), which further facilitates the binding of the translation initiation complex[132,152]. On the contrary, m6A in the 3′ UTR promotes cap-dependent translation by interacting with YTHDF1 or YTHDF3. Indeed, it has been reported that the incorporation of m6A in the 3′ UTR is positively associated with increased translational efficiencies upon exposure to specific stresses[132,154,165]. However, m6A can hinder translation by interfering with tRNA interactions, thereby slowing down the translation elongation process[166]. The combination of these two functions of m6A modifications shows how translational efficiencies may be subtly tuned in response to cellular conditions, a process critical for adaptive cellular responses to changes in environmental conditions.

Genomic consequences of the m6A modification

The past decade has revealed that m6A plays an essential role not only in the regulation of RNA dynamics and metabolism but also contributes to epigenetic regulation of gene expression. It contributes to the organization of genomic architecture and the preservation of genomic stability. m6A exerts its influence on genomic stability and function through various species of RNAs, including nascent pre-mRNA transcripts, long non-coding RNAs (lncRNAs), chromosome-associated regulatory RNAs (carRNAs), and R-loops (**Figure 5A-E**)[132].

Chromatin crosstalk

Several mapping studies identified significant overlap between the m6A marks on pre-mRNAs and regions enriched for histone modifications, particularly trimethylation at lysine 36 of histone H3 (H3K36me3). The presence of H3K36me3 allows it to recruit the m6A methyltransferase (METTL3/14) complex to actively transcribed regions, allowing for co-transcriptional deposition of m6A (**Figure 5A**)[167]. Another proposed mechanism by which m6A epigenetically regulates gene expression is by triggering the erasure of repressive histone marks, such as H3 dimethylated at lysine 9 (H3K9me2), through the recruitment of demethylases[168]. This coordinated interplay between m6A and chromatin states is a good example of how epitranscriptomic changes reshuffle the transcriptional landscape, increasing flexibility and precision in controlling gene expression.

lncRNA regulation: XIST and MALAT1

The regulatory functions of m6A modifications extend to lncRNAs, which have a substantial influence on the function of these RNAs and their participation in critical biological processes[140]. Notably, XIST, the major driving factor of X-chromosome inactivation, is highly modified with m6A (**Figure 5B**)[141,169].
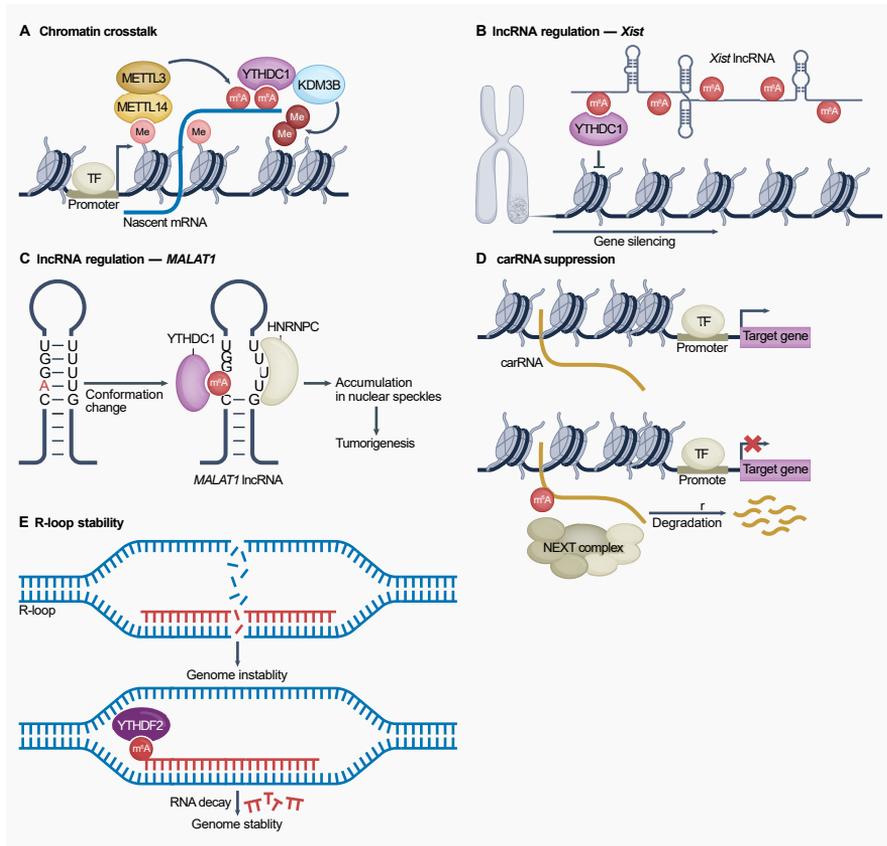

Figure 5. *Summary of m6A's roles in chromatin and RNA regulation. (A) m6A and chromatin modifications reinforce active transcription regions; (B) m6A promotes gene silencing through Xist lncRNA; (C) m6A on MALAT1 affects nuclear structure; (D) m6A causes carRNA degradation to repress gene expression; (E) m6A degrades RNA hybrids, preventing genome instability. Adapted from Boulias, K., Greer, E.L., Nat Rev Genet, 2023, with permission from Springer Nature[132].*

The interactions between m6A-modified XIST and the YTHDC1 protein play a crucial role in silencing genes on the inactivated X chromosome[170,171]. This interaction thus points toward the importance of m6A in controlling the functions of lncRNA during developmental processes. Similarly, MALAT1 is a lncRNA associated with oncogenic processes and, like many others,

undergoes the m6A modification. These modifications increase the stability and localization of MALAT1, allowing it to execute its functions related to gene expression regulation and, consequently, the development of cancer (**Figure 5C**)[132,172,173]. The involvement of m6A in these lncRNAs shows the broader implications of this modification in gene regulatory networks.

carRNA suppression

It has been shown that modification of m6A is a key factor in the regulation of carRNAs, including enhancer RNAs and repeat RNAs[174,175]. Recent studies have found that the m6A methyltransferase complex is involved in the degradation of a subset of carRNAs essential for maintaining chromatin stability and the regulation of gene expression. The m6A-modification of long interspersed element 1 (LINE-1) repeat RNAs promotes the association of the nuclear exosome targeting complex (NEXT) to facilitate their degradation, thereby preventing aberrant transcriptional activation that may cause genomic instability (**Figure 5D**)[132,174]. This regulation underlines the role of m6A in repressing the expression of repetitive elements, which is essential for maintaining genomic integrity and cellular homeostasis.

R-loop stability

R-loops form co-transcriptionally when the RNA strand hybridizes with the template DNA. R-loops play critical roles both in the regulation of transcription and in the maintenance of genome stability (**Figure 5E**)[173]. A recent report implicates the m6A modification in regulating R-loop dynamics. In human pluripotent stem cells, m6A is deposited on R-loops[176]. Depletion of the m6A-binding protein YTHDF2 results in the accumulation of these structures, leading to increased DNA damage marked by γH2AX[176]. This observation suggests that m6A modifications are critical for the timely resolution of R-loops and prevent their accumulation with consequent genomic instability. Furthermore, evidence has also shown that m6A affects the process of DNA damage repair protein recruitment, thus indicating its dual role in maintaining genomic stability through the management of R-loops[177,178].

Detection methods

Over the last decade, various methods have been developed to identify sites of m6A modification and determine the level of such modification on mRNA. These methods can be broadly categorized into three types, depending on whether they use sequencing-independent biochemical approaches or second-

generation sequencing (SGS)-dependent and third-generation sequencing (TGS)-dependent approaches. Sequencing-independent approaches typically quantify m6A by enzyme-linked immunosorbent assay (ELISA) or digestion followed either by slot blot or quantitative PCR (qPCR). However, most of these techniques have only been limited to quantifying the global m6A on mRNA or measuring m6A at a single site[179].
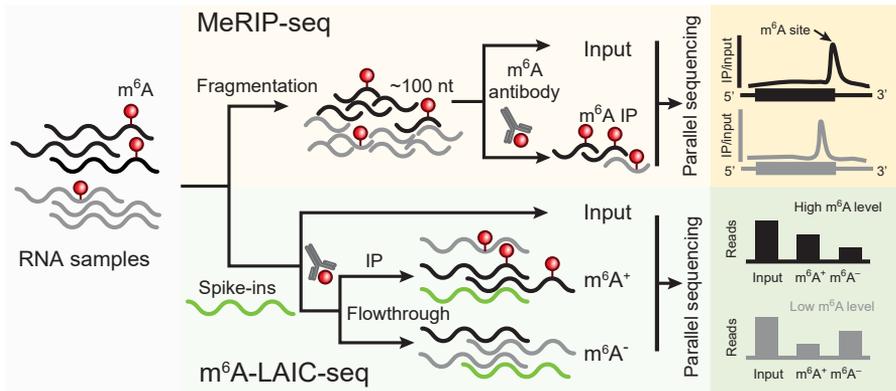

Figure 6. *The diagram depicts the workflow for MeRIP-seq. The RNA sample is chemically fragmented to obtain RNA fragments of ~100 nucleotides, which are then immunoprecipitated using an anti-m6A antibody. The enriched RNA is eluted for library preparation, with a portion of the fragmented RNA serving as an input control, followed by high-throughput sequencing and data analysis. In contrast, m6A-LAIC-seq labels RNA to identify m6A sites before fragmentation, allowing for targeted enrichment of m6A-containing RNA[180].*

Various high-throughput sequencing strategies for transcriptomic m6A profiling have recently emerged, thanks to the advent of NGS and TGS technologies that have really revolutionized life sciences. Most of these methods involve capturing the m6A sites through approaches that can be further divided into two groups: antibody-dependent and antibody-independent approaches. The pioneering approach, MeRIP-seq, employed an anti-m6A antibody[140] and was followed by the development of a wave of methods that depend on antibodies, including m6A-LAIC-seq (**Figure 6**). Although these methods have provided many valuable insights, they are limited by variability regarding RNA sequences and secondary structures recognized by the antibodies and the absence of high-resolution information on stoichiometry[181-184]. In order to overcome these challenges, a variety of antibody-free approaches have been developed, including Direct RNA Sequencing (DRS) using the Oxford Nanopore Technology (ONT) platform.

Table 1. *Summary of techniques for profiling m6A modifications, data compiled from Yang et al., Front Cell Dev Biol, 2024; Zhang et al., Exp Mol Med, 2022; and Wu et al., Front Cell Dev Biol, 2023[179,185,186].*

Technique	Dependence/ Sequencing	Resolution/ Quantification	Pros/cons
DART-m6A-seq	APOBEC1- YTH protein/ Yes	Single base/ No	Low requirement of initial RNA. Affected by vector transfection. Cover a limited proportion of m6A sites.
GLORI	Glyoxal G protection, nitrite-mediated A deamination/ No	1 nt/ Yes	High accuracy; possible RNA degradation;economic; no context bias.
LC/MS	- No	Total m6A level/ Yes	Analyzes hydrolyzed nucleosides to quantify m6A and other modifications.
m6ACE	UV254; exonuclease XRN1/ Yes	Single base/ Yes	Directly map transcriptome-wide m6A.
m6A-CLIP/ miCLIP	m6A antibody; UV254/ Yes	Single base/ No	An indirect way to analyze m6A positions. Low crosslinking yield.
m6A-ELISA	m6A antibody/ No	-/ Yes	Detects absolute and relative amounts of m6A using a m6A antibody.
m6A-label-seq	a6A antibody/ Yes	Single base/ No	Work in cultured cell lines. Low incorporation efficacy of a6A.
m6A-LAIC-seq	m6A antibody; ERCC RNA Spike-In Mix/ Yes	Gene level/ Yes	Cannot obtain m6A positions and fractions.
MAZTER-seq	MazF/ Yes	Single base/ Yes	Cover a limited proportion of m6A sites (16%–25%).
MeRIP-seq/m6A-RIP-seq	m6A antibody/ Yes	100–200 nt/ No	RNA immunoprecipitation method for m6A enrichment.
m6A-SAC-seq	MjDim1/ Yes	Single base/ Yes	Low requirement of initial RNA.
m6A-SEAL	FTO/ Yes	100–200 nt/ No	Full commercialization. Expand the application for m6A imaging.
Mazter-seq	MazF/ Yes	Single base/ Yes	Identifies m6A sites; uses MazF endonuclease.

ONT	Oxford Nanopore Technologies/ Yes	Variable/ Yes	Real-time sequencing; long reads; portable technology.
PacBio	Pacific Biosciences/ Yes	Variable/ Yes	High-resolution sequencing; long reads; full-length transcripts.

Although this thesis will focus on MeRIP-seq and DRS using the ONT platform, it is important to note that there are many additional methods in the field. **Table 1** provides an overview of various detection methods, as discussed in detail by many recent studies[179,185].

MeRIP-seq

MeRIP-seq (also named m6A-RIP-seq) is the most widely used method for transcriptome-wide mapping of m6A RNA modifications. This technique relies on an antibody specific to m6A, applied to fragmented RNA molecules. The enriched RNA fragments are then used to prepare RNA-seq libraries using standard methods (**Figure 6**). Theoretically, read coverage will be higher in m6A sites as sequencing reads aligned to the reference genome will pile up around these modified sites[140,141].

MeRIP-seq has the advantages of stability, convenience, quickness, and not being very expensive. Thus, it is suitable for large-scale experiments[187]. However, it has a high background noise level and needs a higher quantity of starting RNA[179]. Other limitations include misrecognizing other modifications having a structure similar to m6A, like m6Am, by using anti-m6A antibodies. Notably, this approach has limited power in detecting low-resolution m6A sites since it preferentially detects regions of higher methylation. The ongoing optimization of MeRIP-seq protocols and analysis pipelines will eventually overcome these limitations and further our understanding of m6A dynamics in cancer development and progression.

ONT Direct RNA Sequencing

Recently, direct RNA sequencing (DRS) by Oxford Nanopore Technologies (ONT) has emerged as a potent tool for the detection of m6A modifications due to its long sequencing reads and simple library preparation. Probably the most distinctive advantage of DRS lies in its direct sequencing of the native RNA molecules, thus permitting the detection of RNA modifications at single-nucleotide resolution. As these modified and unmodified ribonucleotides exhibit different signal profiles during sequencing, analysis of these signals

enables the detection of m6A sites without any additional preprocessing of RNA samples. Since each consecutive 5-mer nucleotide sequence passing through the nanopore generates a unique ionic current, this pattern enables the identification of the specific nucleotides, whether modified or unmodified, as they transit through the pore[179,185]. The possibility brought about by DRS has made it widely applicable in the profiling of a wide variety of RNA modifications, including those occurring in RNA viruses[188-191].

While extraction of RNA modification information from the DRS reads remains a challenge, development in computational analysis techniques has been created to solve it. The research started to look into the base call error rate and raw signal patterns- the "squiggles"-to improve the accuracy of the detection of m6A[192,193]. Compared with other sequencing methods, DRS has a comparatively high error rate; however, it provides the opportunity to analyze entire mRNA molecules directly. This direct analysis of the entire mRNA is an advantage for observing m6A modifications in specifically spliced isoforms and correlating these modifications with other features of transcripts. Extensive iterative measurements of the signal are often required to calculate error frequencies, which can, in turn, reduce the accuracy of detecting m6A sites, especially those with low abundance. Further research to make these techniques more sensitive and accurate needs to go into the establishment of proper control groups, algorithmic refinement, and engineering of nanopore proteins specifically designed for m6A detection.

Main molecular players in the thesis

MYCN

NB continues to be one of the most challenging areas in pediatric oncology, particularly high-risk NB. Amplification of the *MYCN* gene characterizes an aggressive form of NB and is present in almost half of the high-risk tumors[97,99,124]. This amplification is associated with advanced disease stages and elevated mortality rates, establishing MYCN as an important biomarker in NB prognosis[4,97,99]. MYCN is a MYC family of TFs that has an important function in regulating different cellular processes, including proliferation, differentiation, and apoptosis[194]. Therefore, *MYC* genes have been implicated in tumorigenesis.

The *MYCN* gene, located on chromosome 2p24.3, is a TF essential in cellular functions[97]. MYCN possesses the characteristic basic helix-loop-helix domain

involved in dimerization with a partner protein, MAX. MYCN performs this action through dimerization with the targeted DNA sequences called E-boxes in the promoters of target genes[195,196]. High levels of MYCN expression strongly correlate with high cell proliferation rates and a low rate of apoptosis, which are indicative of its strong oncogenic potentials. Experimental models have documented that the conditional expression of MYCN in neural crest cells is sufficient to induce neuroblastic tumors, thus confirming its driving role in NB development[124,197].

While the function of MYCN largely has been a transcriptional activator, recent findings have elicited a much wider role that puts it in resolving transcription-replication conflicts. The transcription-replication conflict is the incidence when the moving transcription apparatus collides with certain obstacles, such as DNA damage or R-loops that eventually block the RNA polymerase II (RNAPII) complex, hindering further transcription elongation[198]. Accordingly, the MYCN protein interacts with topoisomerases I and II as part of a complex known as a "topoisome." Interactions between MYCN and these proteins modulate supercoiling and torsional stress associated with transcription and replication[199].

Besides the association with topoisomerases, MYCN collaborates with Aurora-A, a kinase that phosphorylates histone 3 at serine 10 (H3S10). This specific phosphorylation event at H3S10 promotes the release of RNAPII from chromatin, which allows the transcriptional activation of target genes and thus enables MYCN to orchestrate transcriptional dynamics in response to growth signals[200,201]. This mechanism is highly relevant because MYCN regulates transcriptional elongation, enhancing not only oncogene expression but also maintaining the efficiency of the transcription apparatus under cellular stress.

Furthermore, MYCN plays a critical role in RNA processing. It interacts with the deubiquitinating enzyme USP11 and the breast cancer susceptibility gene protein BRCA1, allowing the recruitment of the mRNA decapping machinery, including DCP1A, to resolve R-loops[202]. The resolution of these structures by MYCN allows for continuous transcription by releasing RNAPII from chromatin, preventing transcriptional stress that might cause genomic instability. It also mediates recruitment of the RNA exosome complex to the transcription start sites where MYCN degrades the exposed 3′ ends of RNAs that arise through stalled and backtracked RNAPII[203]. This process is an

important means of maintaining RNA homeostasis, ensuring the defective transcripts do not build up and interfere with cellular function.

MYCN also interacts with high molecular weight complexes, including RBPs, such as the nuclear exosome targeting (NEXT) complex[204]. In these contexts, MYCN provides an RNA-binding subunit that augments the RNA exosome processing of intronic transcripts to ensure efficient RNA maturation and avoid the accumulation of unprocessed RNA species.

Recent advances in epitranscriptomics have revealed that m6A modifications regulate MYCN expression and function in NB. The m6A reader IGF2BP3 was identified as an essential factor for NB cell proliferation and was significantly overexpressed in *MYCN*-amplified tumors. Depletion of IGF2BP3 strongly reduced MYCN expression and inhibited tumor growth because its RNA stability was controlled by m6A modifications in generating a feedback loop that increases MYCN expression[205]. Moreover, MYCN-activated KAP1 (TRIM28) can stabilize the *MYCN* mRNA by complexing with the m6A reader YTHDC1 and methyltransferase METTL3. KAP1 depletion reduced MYCN stability and suppressed tumor progression by regulating networks of MYCN action and pointing out new therapeutic approaches in targeting the m6A pathway for the treatment of MYCN-driven malignancies[206].

Targeting MYCN networks in NB is considered to have a few promising strategies as these proteins are often considered "undruggable[207]." Aurora-A inhibitors have been shown to disrupt MYCN stabilization, leading to its degradation[208,209]. Clinical trials are showing the efficiency of Aurora-A inhibitors in combination with irinotecan and temozolomide[210]. Moreover, BET inhibitors appear effective in targeting oncogenic signaling pathways dependent on MYCN[211]. Apart from this, downstream signaling pathways, such as cyclin-dependent kinases, which are crucial for cell cycle regulation, have shown promise, with several reports, especially of CDK4/6 inhibitors in the context of *MYCN*-amplified cases[212].

Taken together, MYCN coordinates transcription and replication in NB. Its various interactions with proteins dynamically facilitate both the activation of transcription and the resolution of transcription-related stresses critical to the stability of the genome and tumorigenesis. Strategies targeting disrupted interaction of MYCN can be combined with the targeting of pathways regulating MYCN for new therapeutic options in high-risk NB. However, there

is a gap in crucial findings regarding how MYCN determines RNA metabolism or what the wider implications of m6A modification are. This knowledge will be a critical element in the development of efficient interventions against MYCN-driven malignancies and deepen our understanding of the molecular mechanisms underlying NB progression.

METTL3/14 complex

The *METTL3* gene (methyltransferase-like 3) and *METTL14* (methyltransferase-like 14) encode proteins of a stable heterodimeric complex that catalyze the addition of N6-methyladenosine (m6A) to nuclear RNAs, representing the most common internal modification within eukaryotic mRNA[213,214]. In the METTL3/14 complex, METTL3 is the catalytic subunit, while METTL14 confers substrate recognition and stimulates the enzymatic activity of METTL3[213,215]. Together, they install the m6A marks on mRNA at specific DRACH sequence motifs (D = A/G/U, R = A/G, H = A/C/U), thus setting them as central modifiers in controlling gene expression across diverse biological contexts[140,141].

It is interesting that despite the DRACH motif appearing with a frequency of ~57 nucleotides in mRNA, only a minority of such potential sites are methylated[140,141]. This observation thus begs the question of site- and transcript-specific selectivity in m6A modification. Even though DRACH motifs are highly abundant, only certain transcripts undergo m6A modification, and this remains a very poorly understood basis for such selective methylation. Accordingly, one proposed mechanism is that the METTL3/14 writer complex is recruited to specific transcripts through TFs. In human embryonic stem cells (hESCs), METTL3/METTL14/WTAP have been shown to interact with SMAD2/3, a critical TGFβ-regulated TF. SMAD2/3 interaction with methyltransferase complex enables m6A methylation of TGFβ signaling-dependent methylation of transcripts[216]. The CEBPZ TF, on the other hand, recruits METTL3 to the promoters of certain genes in the case of AML cells to specifically methylate their transcripts[217].

Despite these insights, the regulation of most m6A-containing transcripts likely involves additional mechanisms that remain unidentified. Given these data, however, it is likely that the transcriptional regulation of most m6A-containing transcripts is due to other unidentified mechanisms. Indeed, factors such as promoter structure, the number of CpG islands, and chromatin modifications, including histone trimethylation, have been identified that

correlate with m6A levels, suggesting that chromatin marks may guide the METTL3/14 complex to specific transcripts[218]. For instance, interactions between METTL14 and H3K36me3 are suggested to enable co-transcriptional deposition of m6A, thereby linking mRNA methylation to the dynamics of chromatin[167].

In addition, co-transcriptional methylation appears to be guided by the recruitment of the METTL3/14 complex to regions of the transcript where RNAP II slows down or pauses during transcription[218,219] . Such pausing might favor m6A deposition near stop codons or long internal exons, where m6A enrichment commonly occurs. In addition, The RBPs RBM15/15B, for example, are close to the DRACH motifs, which can subsequently recruit the METTL3/14 complex and further facilitate m6A methylation at adjacent sites[170].

Another critical function of the METTL3/14 complex is that of maintaining the subtle balance between pluripotency and differentiation in ESCs. One study demonstrated that METTL3-dependent m6A methylation regulates the degradation of major pluripotency factors such as *NANOG*, *SOX2*, and *OCT4*[220]. Loss of METTL3 leads to sustained expression of these factors, which impairs cellular competency in exiting a pluripotent state and entering a lineage-specific differentiation pathway. *In vivo* studies with METTL3 KO mice showed that the loss of m6A had an embryonic lethal outcome wherein the embryos never differentiated the critical timing from embryonic day 3.5 to 6.5 to transition from a state of pluripotency into differentiation[220,221]. In the absence of METTL3, pluripotency circuits remain active; hence, there is a "hyper-pluripotent" state that blocks normal development[221].

Moreover, METTL3 is essential for maintaining transcriptional dormancy during paused pluripotency, a state in which ESCs can pause development while retaining the ability to resume growth[222]. Through m6A modification, METTL3 promotes global mRNA destabilization, which leads to the degradation of thousands of transcripts and helps maintain the paused state. A critical target of this process is the mRNA for the transcriptional amplifier MYCN, whose downregulation by METTL3 contributes to the suppression of nascent transcription. This dual action of mRNA destabilization and suppression of transcription allows for the transitions between the active and paused states in ESCs and places METTL3 as a major developmental timing regulator[222].

Further studies reproduced the phenotype by ablating METTL14, METTL3's partner in the m6A writer complex, underlining the co-dependency between the two proteins[223]. While the ablation of METTL3/14 does indeed allow maintenance of pluripotency even under naïve conditions, several reports have documented that depletion of METTL3/14 causes loss of self-renewal due to premature differentiation under the primed condition of differentiation[162]. It thus suggests that the role of METTL3 may be context-dependent, balancing pluripotency maintenance with timely promotion of differentiation through m6A-dependent regulation of key transcripts.

The function of METTL3 has further been extended from mRNA decay to the regulation of chromatin, where it has been implicated in the maintenance of heterochromatin in mouse embryonic stem cells (mESCs)[224]. METTL3 is enriched on pericentric heterochromatin regions, especially those containing endogenous retroviral elements, which include intracisternal A particles (IAPs). These regions are generally marked by the repressive histone modifications H3K9me3 and H4K20me3, which maintain the silencing of transposable elements and guard genome integrity. Loss of METTL3 removes these heterochromatic marks, which allows for the derepression of IAPs and compromises chromatin stability[224]. These data demonstrate that, in addition to mRNA, METTL3 contributes to the establishment of repressive chromatin, which is critical for genome integrity.

Interestingly, the RNA transcripts generated from these IAP regions are themselves m6A-modified by METTL3, which in turn recruits the m6A reader YTHDC1. This protein further assists the association of METTL3 with chromatin, creating a feedback loop that further reinforces heterochromatin stability. METTL3 is also associated with chromatin-modifying complexes containing SETDB1 and TRIM28 and is responsible for the H3K9me3 mark[224,225]. This observation further illustrates the complex role that METTL3 has in the coordination of m6A methylation with chromatin dynamics, from the transcriptomic level to the epigenetic level, in the maintenance of cellular homeostasis.

However, recent findings have revealed that alternative splicing of METTL3 may account for what was previously thought to be METTL3-independent m6A modifications[226]. Several studies reported residual m6A in cells where METTL3 had been knocked out, leading to speculation that other methyltransferases might be responsible for this remaining m6A[226-236].

However, it has been demonstrated that in most of these cases, residual m6A resulted from the expression of alternatively spliced METTL3 isoforms[226]. Such alternative isoforms, through CRISPR/Cas9-mediated mutations, bypass the intended knockouts by expressing catalytically active, albeit smaller, METTL3 proteins. METTL3 knockout in mESCs, for example, expresses hypomorphic alleles that result in the partial retention of m6A modifications. This finding points to the strict need to validate METTL3 knockouts in the studies of its functions in m6A biology, considering that these isoforms will indeed confound the interpretation of its role and functions.

The evidence further indicates that METTL3 is the major contributor to m6A in mRNA, accounting for over 95% of modification in diverse cell types, including mouse embryonic fibroblasts (MEFs) and mESCs[226]. The minimal residual m6A in those cells after METTL3 knockout most likely represented either technical artifacts or expression of noncanonical methyltransferases such as METTL16[226,237,238]. This explanation definitely shows the existence of "METTL3-independent" m6A marks and cements METTL3 as the major player in the deposition of m6A.

Beyond its role in development and chromatin regulation, METTL3 actually is fundamentally required for general RNA metabolism. The m6A modifications added through METTL3/14 subsequently regulate mRNA fates through recruiting m6A-binding proteins, including the YTH domain family of proteins. These could then guide the mRNAs either toward translation or degradation according to cellular needs. For example, the mRNAs modified by m6A and bound by YTHDF1 are usually directed to translation, while the mRNAs bound by YTHDF2 may be marked for decay[156,165,239]. Such a balance is critical in rapidly adjusting gene expression during cellular stress or differentiation, thus enabling the cells to adapt to environmental changes quickly.

Despite the critical functions of METTL3 in RNA and chromatin regulation, a number of important gaps exist in the broader mechanisms of METTL3. For example, it has not been determined yet through which means METTL3 is selectively recruited to certain chromatin regions or how it interacts with specific TFs to execute its functions. Although METTL3's m6A activity has been associated with chromatin states, interactions between m6A modifications and additional epigenetic modifications, such as DNA methylation, are still being explored[240]. In summary, further research is needed

on these aspects alone, such as how m6A methylation coordinates with chromatin and transcriptional regulation during developmental transitions.

Another area of active investigation is the therapeutic inhibition of METTL3. Considering the chemical nature of the reaction, numerous small molecule inhibitors of METTL3 have been identified, including STM2457, UZH1a, and UZH2-all competing with the cofactor S-adenosylmethionine (SAM) required for m6A methylation[241-243]. The application of these inhibitors in preclinical models reduces global m6A levels and disrupts m6A-mediated mRNA regulation, which has quite impressive effects in cancer models, wherein m6A marks promote oncogene expression. Importantly, STM2457 is under clinical study in patients with various advanced malignancies. Moreover, other inhibitors target the interaction of METTL3-METTL14 in order to interfere with the catalytic activity of the METTL3/14 complex, including Eltrombopag and CDIBA-4. These inhibitors severely weakened the proliferation of the cells in leukemia cell lines[244,245].

Interestingly, recent publications have clarified that its function in mRNA regulation mediated by METTL3 may not always depend on its methyltransferase activity alone. In some cancers, such as lung and gastric cancers, METTL3 exerts oncogenic functions through the m6A-independent enhancement of translation for a subset of mRNAs[246,247]. The observation has shifted the focus toward the development of new approaches capable of degrading METTL3 itself. For instance, the pursuit of PROTAC-based degradation strategies, such as using the small molecule WD6305-targets METTL3 for degradation, indirectly destabilizes METTL14 due to the interdependence within the complex[248]. This approach appears highly potent in malignancies where the cytoplasmic role of METTL3, promoting translation, supports tumor development.

TERRA

Telomeric Repeat-containing RNA (TERRA) is a lncRNA expressed from the subtelomeric regions that are recognized for carrying repetitive sequences of a telomeric nature[249,250]. It plays an important role in the maintenance of telomeres, which is required to protect genomic stability and prevent chromosomal deterioration[111,251-256]. TERRA expression is precisely regulated at each telomere. Promoter sequences located in the subtelomeric regions enable the binding of various TFs, driving TERRA expression. These subtelomeric promoters may vary in CpG content, offering an additional layer

of fine control over TERRA transcription[257,258]. Studies have proven that telomere length is a major determinant of TERRA expression, and critically short telomeres are able to trigger its transcription, which thus represents the cellular response under stressful conditions like oxidative stress, heat shock, and DNA-damaging agents[111,254,255,259,260]. The inability to initiate TERRA transcription under these conditions can cause telomere dysfunction, highlighting its key role in telomere stability.

A critical question in telomere biology is what drives TERRA expression from specific chromosome ends in response to cellular stress or other events and how this targeted expression impacts telomere integrity. Using genomic engineering to remove TERRA TF binding sites from a single chromosome end, it was noted that TERRA transcription was reduced specifically at the site. Interestingly, this led to activation of the DNA damage response only at telomeres lacking TERRA, underscoring a chromosome-specific role for TERRA in telomere protection[261]. Furthermore, TERRA molecules produced at one chromosome end can transiently localize to multiple telomeres in trans[262]. This finding suggests that a stable pool of TERRA may be sufficient to support telomere maintenance across various chromosome ends. It also raises an important question about how TERRA coordinates its association with multiple chromosome ends in trans to carry out its functions effectively.[261]. TERRA interacts with a number of key proteins implicated in important roles in telomere biology, including the RAD51 DNA recombinase and its partner RAD51AP1[262,263]. It is crucial in the formation of telomeric R-loops, the structural entity of homologous recombination, and the repair of DNA double-strand breaks. However, the dynamics of either TERRA R-loops or the involvement of specific nucleotides at telomeres are not well characterized. The available evidence suggests that TERRA R-loops have a preference for forming in cells with critically short and damaged telomeres, thus engaging the HDR machinery for repair. Although the exact pathway by which R-loop formation maintains telomeres has not been well understood, it has been suggested that TERRA R-loops may induce replication stress. This stress could promote homologous recombination, allowing elongation of the telomere. The recruitment of RAD51 and RAD51AP1 reinforces repair mechanisms that are important for telomere stability[262-264].

Similarly to its DNA counterpart, TERRA is also capable of folding into RNA G-quadruplex (rG4) structures *in vitro*. The use of circular dichroism and

electrophoretic mobility shift assays (EMSA), RNAs with TERRA-mimicking telomeric repeats have been proven to adopt a parallel G4 topology bot h in Na+ and K+ buffers, enabling intramolecular rG4s to form and fostering multimerization. This formation of rG4 might shield TERRA from RNase degradation[265-268]. Such an *in vivo* formation was confirmed in the colocalization of TERRA rG4 structures with telomeres in live HeLa cells[269]. Very recently, it was suggested that rG4 might form upon stressors, which opens some interesting ideas about the function of TERRA through rG4 formation during cellular stress. Beyond that, TERRA is involved in the process of heterochromatin formation at telomeres. The complex interactions, especially with specific chromatin-modifying complexes such as Polycomb Repressive Complex 2 (PRC2), establish the repressive state of chromatin at telomeres[270]. The interaction enables the deposition of repressive histone marks that are necessary for the maintenance of heterochromatin at telomeres and the correct capping and stability of telomeres to avoid inappropriate DNA repair processes that can result in the fusion or degradation of telomeres.

TERRA was first reported in 2007 and reshaped our understanding of telomeres, suggesting that the telomeres may themselves play an active role in the process of gene regulation[250]. A discovery emphasized the fact that the role of telomeres is not limited to structural functions but is extended into cellular processes like aging and carcinogenesis. Dysregulation of TERRA has been implicated in a wide range of cancers, most notably ALT-dependent cancers like NB, where high levels of TERRA reflect heightened telomere maintenance and poor prognosis[85,102,271,272]. This association has important implications for the development of targeted therapies. Considering the paramount functions of TERRA at telomeres and the implication in tumorigenesis, it has emerged as a candidate therapeutic target. Current research is focused on ways of inhibiting TERRA or its interactions with telomere-associated proteins. For example, small molecules that stabilize TERRA rG4 structures already show potential in decreasing telomerase activity and increasing DNA damage within cancer cell lines[273-275]. Further elucidation of the mechanistic functions of TERRA will thus be of benefit for informing strategies aimed at disrupting only the oncogenic processes involving this RNA, therefore improving treatment strategies against cancer.

A number of experimental techniques have explored the functions and mechanisms of TERRA. Techniques such as RNA sequencing have been quite

instrumental in the profiling of TERRA expression across different cell types and conditions, thus showing it as a stress response molecule. High-throughput sequencing was used to examine the levels of TERRA in cancerous versus non-cancerous tissues as a means of detailing its potential as a biomarker for telomere dysfunction[85,102,271,272]. CRISPR/Cas9 gene editing has been used in addition to making knockout models for the study of TERRA-specific functions[270,276]. In contrast, techniques like single-molecule fluorescence in situ hybridization (smFISH) have been used to image the localization of TERRA at the telomeres by using advanced imaging techniques[277]. So far, three different systems have been developed that visualize TERRA RNA; for instance, the MS2-GFP system that follows endogenous TERRA transcripts from a single telomere, CRISPR-dPspCas13b technology, which has been developed to track the dynamics of TERRA at the single-molecule level, and the PP7-GFP system that is used to investigate the association of TERRA with telomeres[262,277-279]. Besides those mentioned above, another system is the Clivia-tagged TERRA system, which has been used for real-time monitoring of the localization of TERRA in live cells, further investigating its dynamic features and functions[280,281]. In addition to these biochemical approaches, co-immunoprecipitation coupled with mass spectrometry has been performed to identify the proteins bound by TERRA and to characterize functional interactions[282,283]. Therefore, the proposed methodologies are highly relevant for elucidating the myriad roles of TERRA in maintaining genome stability and its implications in cancer biology.

Overall, TERRA functions as an integral player in telomere biology, from maintaining telomere integrity to regulating chromatin dynamics and participating in cellular signaling upon crisis. TERRA's interactions with the formation of R-loops, chromatin-modifying complexes, and innate immune pathways all underlie its importance both in normal conditions and in pathology. Further investigation of TERRA functions and regulatory mechanisms will be needed to unlock the full potential of this RNA as a therapeutic target in pursuit of improved treatment strategies against cancers that have hijacked ALT for telomere maintenance.

hnRNPA2B1

Heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2B1) is one of the members of the hnRNP A/B family, with various key roles played in RNA biology. Several splice variants are generated by the *hnRNPA2B1* gene,

including A2, A2b, B1, and B1b, each of which performs a different function due to minimal differences either in RNA-binding properties or intracellular distribution[284]. Despite the extremely high degree of sequence homology between hnRNPA2 and B1, their expression patterns are strikingly different among cell types, developmental stages, and environmental stimuli. Mechanistically, hnRNPA2B1 harbors a conserved RRM, which preferentially recognizes purine-rich sequences; RRM1 and RRM2 selectively recognize the AGG and UAG motifs, respectively[285]. This protein facilitates various functions of RNA processing, such as transcriptional regulation, mRNA transport, and alternative splicing[286-293]. However, its glycine-rich C-terminal domain (GRD) enhances protein-protein interactions[294]. The interaction of hnRNPA2B1 with specific RNA sequences and ssDNA molecules through the RNA binding for the maintenance of cellular RNA metabolism and function.

In the context of alternative splicing, hnRNPA2B1 is best known as a splicing regulatory factor that interacts with specific RNA sequences to control splicing outcomes. It does this either by the inclusion or exclusion of exons in target transcripts, thereby controlling the translation of functionally diverse protein isoforms[295-300]. It binds to sequences such as AUGGUA in viral RNAs, allowing viruses like HPV-16 to evade the immune response through the regulation of their splicing events[301]. Transport is an important process enabling the differential expression of certain proteins in well-defined subcellular compartments and their ectopic expression. In the central nervous system (CNS), axonal transport of myelin basic proteins (MBP) to axon-glia contact sites is a particularly good example of this transport. The splicing isoform hnRNPA2b shows very high expression in neural cells. Its function has also been important in assembling RNA transport particles[302].

Moreover, inhibition of hnRNPA2b function by isoform-specific antibodies disrupts particle assembly, underscoring the regulatory role of hnRNPA2B1 in mRNA transport. A2-mediated RNA transport involves recognition of the 21-nucleotide A2 response element (A2RE) by hnRNPA2 in the nucleus. A2RE elements further guide transport granules containing components of the translation machinery and molecular motors, like kinesins, along the microtubules toward specific cellular locations. This allows localized translation of RNA in an A2RE/hnRNPA2 dependent manner[303-306]. By adapting its ability to bind different RNA structures or motifs, hnRNPA2B1 can also use noncanonical transport pathways, which can influence dynamics

and efficiency in specific neuron contexts. This complex interplay of RNA binding, transport, and localized translation underlines the importance of hnRNPA2B1 in neuronal function and extends its general relevance to cellular RNA metabolism.

Beyond splicing, hnRNPA2B1 is involved in a number of other RNA processing events, including the regulation of mRNA stability, transport, and translation. Several RBPs and cofactors interact with hnRNPA2B1. Such interactions enable the assembly of ribonucleoprotein complexes that control gene expression at multiple levels. The m6A modification contributes significantly to the development of many biological processes and disease states, including cancer. hnRNPA2B1 is also recognized as one of the key readers of m6A by binding with RNAs at the m6A sites and promoting m6A-dependent primary microRNA processing events. However, some other studies have presented evidence that hnRNPA2B1 may not be a direct interactor of m6A sites but rather arrive at specific binding sites via a process termed the "m6A switch[149]." This report has hinted at a complex role of hnRNPA2B1 in m6A-mediated processes that still calls for active research. The ability to decipher the interaction of m6A modifications with hnRNPA2B1 will be an important milestone in unraveling the elaborated regulatory circuits operating in control of gene expression and RNA metabolism under normal physiological conditions and pathologies.

Among the diverse functions attributed to hnRNPA2B1, there is also involvement in cellular stress responses. Under stress, this protein becomes a component of stress granules sequestering mRNAs and thereby modulates mRNA translation and stability critical to maintaining cellular balance or adapting under adverse conditions[307].

In the context of cancer, upregulation of hnRNPA2B1 has been examined in a wide range of malignancies[308-314]. It has a facilitatory role in cell proliferation and survival and in metastasis. This protein controls several major signal transduction pathways, such as PI3K/Akt, Wnt/β-catenin, and MAPK/ERK, and there is a respective impact on the behavior of tumor cells and the therapeutic outcome[315-317]. For instance, in pancreatic cancer, it increases the interaction of KRAS with PI3K, thereby favoring the growth of cancer cells and chemoresistance[318].

Recent investigations have identified several features of hnRNPA2B1, which make this protein an attractive therapeutic target. Moreover, dietary flavonoids

are among the many compounds that have already shown the capability to modulate hnRNPA2B1 function and impact sensitivity in cancer cells against treatment. In addition, selective targeting of hnRNPA2B1 is possible with aptamers and small molecules, thus opening new avenues of therapeutic intervention[319,320].

Overall, the multifunctional RBP hnRNPA2B1 plays a critical role in RNA processing and fine-tuning gene expression, especially in cellular responses to stress, whose impact is by no means limited to cancer biology but extends to a wide array of physiological and pathological processes. The role played by hnRNPA2B1 in alternative splicing and RNA metabolism makes this protein relevant for maintaining cellular function and homeostasis, hence meriting further studies regarding the different roles it plays in health and disease.

AIMS

This doctoral thesis aims to investigate the role of METTL3-mediated m6A modification in NB. To better understand the disease's underlying mechanisms from a developmental perspective, we aimed to establish a humanized stem cell model of NB to address constraints in existing models. Our specific aims are as follows:

Paper I

- Characterize the m6A modification of TERRA.
- Investigate the role of m6A modification in TERRA pertaining to R-loop formation and telomere maintenance.
- Evaluate the therapeutic potential of METTL3 inhibition for ALT+ NB.

Paper II

- Investigate the role of m6A modification of TERRA in condensate formation.
- Investigate the role of m6A modification and G-quadruplex formation in TERRA condensates formation.
- Evaluate the potential of METTL3 inhibition for developing combination therapies against ALT+ NB.

Paper III

- Investigate the role of m6A modification in tNCC differentiation.
- Investigate the role of m6A modification inhibition in *MYCN*-amplified NB.
- Investigate the role of m6A modification in the regulation of *HOX* gene expression.
- Evaluate the therapeutic potential of METTL3 inhibition in *MYCN*-amplified NB.

Paper IV

- Profile the m6A epitranscriptome across three NB subtypes (ALT+, *MYCN*-amplified, and LR tumors).
- Identify common and subtype-specific m6A-modified gene signatures.

- Investigate the role of m6A-modified lncRNAs as potential regulators of NB subtype divergence.

METHODS

We employed a wide range of techniques and experimental approaches to conduct our investigation. Here, I elaborate on a few that have been used the most widely across all the studies.

hESC culture and differentiation

The human embryonic stem cell (hESC) line WA09 (H9) from Dr. Fredrik H. Sterky (Sahlgrenska University Hospital, Gothenburg) was cultured on Matrigel-coated plates with iPS-Brew XF media (Miltenyi). To differentiate hESC into trunk neural crest cells (tNCC), cells were dissociated with Accutase (Thermo Fisher) and induced into neuromesodermal progenitor cells (NMP) on day 3, progressing to tNCC (day 8), sympathoadrenal precursors (SAP, day 12), and sympathetic neurons (SN, day 18 onward) as described[321,322].

Briefly, hESCs were plated at 55,000 cells/cm² in NMP-inducing medium (1:1 DMEM/F12 and Neurobasal), supplemented with B27, N2, non-essential amino acids, Glutamax, CHIR99021 (3 µM), FGF2 (20 ng/ml), and ROCK inhibitor Y-27632 (10 µM) for the first day, followed by medium replacement without ROCK inhibitor. For tNCCs, NMPs were dissociated on Day 3 and plated at 30,000 cells/cm² in NC-inducing medium with DMEM/F12, N2, non-essential amino acids, Glutamax, SB431542 (2 µM), CHIR99021 (1 µM), DMH1 (1 µM), BMP4 (15 ng/ml), and Y-27632 (10 µM). The medium was replaced on Days 5 and 7 without ROCK inhibitor, and tNCCs were analyzed on Days 7 or 8. For differentiation of SAPs, Day 8 tNCCs were seeded at 200,000 cells/cm² in BrainPhys neuronal medium, supplemented with BMP4 (50 ng/ml), SHH (C24II) (50 ng/ml), and purmorphamine (1.5 µM), and cultured for 4 days (Day 12). For SN differentiation, Day 12 SAP cells were switched to BrainPhys neuronal medium with ascorbic acid (200 µM), NGF (10 ng/ml), BDNF (10 ng/ml), and GDNF (10 ng/ml).

MeRIP-seq

For papers I, II, and IV, MeRIP (m6A-RIP) was performed on NB cell lines, NB tumors, hESCs, and tNCCs. RNA samples (3-15 µg) were spiked with 10 ng of bacterial RNA before fragmentation using RNA Fragmentation Reagents (Thermo Fisher). The fragmented RNA was subsequently utilized for either RNA-seq or MeRIP-seq. For total RNA-seq, sequencing libraries were

prepared from 10 ng of the fragmented RNA using the SMARTer Stranded Total RNA-Seq Kit V2, Pico Input Mammalian (Takara Bio).

MeRIP was conducted on the fragmented RNA using an anti-m6A antibody (Synaptic Systems) according to the methodology previously described[323]. Both the input and MeRIP RNA were then prepared for sequencing using the same SMARTer kit. Finally, the libraries were single-end sequenced (1 × 88 bp) on the Illumina NextSeq 2000 platform, followed by data analysis to identify m6A modifications.

RNA-FISH

For papers I and II, TERRA RNA-FISH, cells were fixed in 4% formaldehyde for 10 min, washed twice with PBS, and stored in 70% ethanol at 4°C. Cells were then permeabilized with 0.25% Triton X-100 in PBS for 10 min and washed with PBS. Hybridization was performed at 37°C for 4 h with a TERRA probe ([TAACCC]$_7$-Atto488-3′) in a hybridization buffer containing 10% formamide. After washing with wash buffer A, cells were incubated with wash buffer B and mounted using ProLong Gold Antifade Mountant with DAPI (Invitrogen). Images were captured using an EVOS M7000 microscope.

Sequential immunofluorescence (IF) staining and RNA-FISH

For sequential immunofluorescence followed by RNA-FISH, cells were fixed and stored as described above. After permeabilization with Triton X-100, cells were blocked with 3% BSA in PBS-T for 30 min. Primary antibodies were incubated for 2 h, followed by washing and incubation with secondary antibodies for 1 h. After fixation and permeabilization, TERRA RNA-FISH was conducted, and imaging followed.

Simultaneous RNA-FISH and m⁶A IF staining

A modified protocol for RNA FISH was used to perform simultaneous TERR FISH and m⁶A IF staining[231]. Methanol (MeOH) fixation effectively preserves mRNAs and rRNAs but not short RNAs like tRNA and snRNA, which require stronger aldehyde-based fixation. Previous studies have shown the presence of m6A in well-folded 18S and 28S rRNA structures. Although MeOH denatures rRNA and exposes m6A sites, we included a refolding step to prevent anti-m6A antibodies from binding to rRNA m6A. While m6A in lncRNA and N6-2-O-dimethyladenosine (m6Am) constitute less than 5% of total m6A in mRNA, anti-m6A antibodies can still detect them.

For simultaneous TERRA RNA-FISH and m6A immunofluorescence, cells were fixed in methanol at -20°C for 15 min. After performing TERRA RNA-FISH, a refolding step was included during the washing to prevent anti-m6A antibody binding to rRNA m6A. Cells were then washed, permeabilized with Triton X-100, and blocked with BSA. Primary antibodies were incubated for 2 h, followed by secondary antibody incubation. Coverslips were washed, mounted, and imaged using an EVOS M7000 or a confocal microscope.

Proximity ligation assay

The Proximity Ligation Assay (PLA) detects protein-protein interactions at endogenous levels in situ with high sensitivity and specificity, allowing for detection at distances as small as 40 nm. It utilizes specialized antibodies that recognize the two target proteins, along with DNA primers covalently attached to these antibodies. PLA probes will only connect if both proteins are present in close proximity. Following hybridization, PCR amplification with fluorescent probes enables the detection of PLA spot signals via fluorescence microscopy.

For papers I and III, PLA was conducted using the Duolink PLA kit (Sigma) according to the manufacturer's protocol. A single antibody was used as a background control. Cells were fixed in 4% formaldehyde for 10 min and blocked for 1 h at 37°C. After incubation with primary antibodies for 1.5 h at room temperature, cells were treated with PLA probes for 1 h in a humidified chamber. Following three washes, a ligation-ligase solution was added and incubated for 30 min at 37°C. The slides were then incubated for 100 min in an amplification solution containing polymerase at 37°C in the dark. Washes were performed with PLA wash buffers A and B as instructed. Finally, cells were stained with Prolong Gold containing DAPI, coverslips were mounted, and fluorescence images were captured using a microscope.

In vivo tumorigenesis

For papers I and III, tumor xenografts were established by subcutaneously injecting 2-5 x 10^6 inducible control or METTL3 KD NB cells into the right dorsal flank of 5-week-old female nude mice (Crl:NU(NCr)-Foxn1nu) from Charles River in a 200 µL mixture of 1:3 Matrigel and PBS (n=4 per group). METTL3 KD was induced by adding 2 mg/mL doxycycline and 2% sucrose to the drinking water five days post-injection. Mice weight was monitored weekly, and tumor volume was measured every 2-3 days using a digital caliper, calculated with the formula Volume (mm^3) = (w^2 x l x π)/6, where w is the

width (shortest diameter), and l is length (longest diameter). Mice were sacrificed when tumors reached 1000 mm^3 or when weight loss exceeded 10% of initial weight. At the experiment's conclusion, tumors were collected, weighed, and processed for further analysis. All experiments adhered to the standards approved by the Institutional Ethical Committee of Animal Experimentation, Gothenburg, Sweden (ethical permit no. 3722/21).

In paper III, *in vivo* drug combination experiments were conducted using NSG mice from Charles River, housed in groups of 2-5 in individually ventilated cages with a 12 h light/dark cycle. All procedures adhered to UK Home Office license P4DBEFF63 under the Animals (Scientific Procedures) Act 1986 and received approval from the University of Cambridge Animal Welfare and Ethical Review Board (AWERB). COG-N-415x patient-derived xenograft (PDX) cells were obtained from the Children's Oncology Group. Cells were mixed with Matrigel (Corning) at a 1:2 ratio with PBS and injected into the left flank of NSG mice (3 x 10^5 cells in 300 μL) at 8 weeks of age. Tumor volumes were measured daily using the formula V = ab2/2. When tumors reached approximately 170 mm^3, mice were randomly assigned to four treatment groups (n=4-6 per group, balanced for sex) for 14 days, receiving intraperitoneal injections of vehicle (20% hydroxypropyl-beta cyclodextrin) daily, STM2457 (50 mg/kg), doxorubicin (0.2 mg/kg every three days), or a combination of both. Mice were euthanized at the end of treatment or when tumors reached 15 mm in any direction, whichever came first, without exceeding the maximal tumor size of 15-20 mm permitted by our Project Licence.

Ethical considerations

In this thesis, we investigated the role of m6A in neural crest differentiation and NB, with a strong emphasis on minimizing animal experiments. Following the principles of Replacement, Refinement, and Reduction (3Rs), we prioritized *in vitro* methods using hESCs to derive NCCs and establish an MYCN-driven NB model. This approach minimized animal use, provided insights specific to human physiology, and enabled validation of findings in patient samples. Animal studies were conducted only when *in vitro* findings required further validation, with all procedures adhering to guidelines from the respective local ethics committees, as detailed in the respective methods section.

Additionally, we followed ethical guidelines for both animal and human-derived cell models. In compliance with Swedish regulations on hESC derivation, human embryonic stem cells must be sourced from surplus IVF embryos, with the condition that any remaining embryo material is respectfully discarded. For this research, hESC lines were obtained from the U.S. National Stem Cell Bank at WiCell, ensuring adherence to these ethical standards. Given these considerations, there are no significant ethical concerns associated with the use of hESCs in this study, and the potential benefits of this research outweigh any associated risks.

Material Transfer Agreements (MTAs) were established to obtain cell lines and plasmids from institutions such as the Children's Oncology Group (COG), Addgene, and affiliated research laboratories. All data handling complies with European GDPR requirements, ensuring appropriate permissions for access and use of publicly available datasets and patient cohorts. Patient samples were provided anonymously by collaborators, with no identifying information, ensuring they cannot be traced back to individual patients. Furthermore, this research aligns with the ethical principles outlined in the Declaration of Helsinki.

RESULTS

Paper I

Understanding the molecular mechanisms that regulate telomere integrity and stability remains an important yet unresolved question in NB. Approximately one-third of high-risk NB cases rely on ALT, and 10-15 % of cancer cells utilize this mechanism. ALT-positive tumors show intrinsic DNA damage and depend on homologous recombination-dependent repair mechanisms to maintain telomere length. TERRA RNA is transcribed from telomeric ends and localizes on telomeres. R-loop structures are known to play a critical role in TERRA localization over telomere and in telomere maintenance. The exact molecular mechanisms governing TERRA function in ALT remain less understood, and if RNA modifications have any role in TERRA function, they are also unknown.

Our research aimed to elucidate the role of m6A modifications in TERRA lncRNA and their contribution to telomere stability and R-loop formation. We hypothesized that METTL3-mediated m6A modifications are crucial for TERRA's localization at telomeres and its function in maintaining telomere integrity. We demonstrated that m6A modifications are essential for TERRA's localization at telomeres, clarifying how ALT operates in NB. Using multiple experimental approaches, we established that m6A is enriched in TERRA, facilitating its interaction with RBPs such as hnRNPA2B1, particularly in R-loops within ALT-positive cells. Depletion of METTL3 led to the loss of TERRA RNA foci and a significant decrease in R-loop formation at telomeres, resulting in increased telomere damage, as evidenced by immunofluorescence staining for DNA damage markers such as γH2AX.

Our results suggest that m6A modifications in TERRA are pivotal for telomere maintenance through R-loop formation, which stabilizes telomeric DNA. This finding highlights the dual role of R-loops as regulatory elements and potentially detrimental structures, depending on their cellular context. While prior research has established that excessive R-loop formation can lead to genomic instability, a hallmark of cancer, the accumulation of R-loops upon METTL3 depletion underscores the specific contribution of m6A modifications in regulating this balance. This dynamic is particularly relevant in NB, where ALT mechanisms enable telomere elongation in the absence of telomerase activity, creating a unique vulnerability.

The interplay between m6A modifications, TERRA localization, R-loop formation, and telomere integrity suggests a potential therapeutic avenue. Targeting METTL3 could disrupt TERRA's localization and R-loop stability, leading to impaired telomere integrity and increased DNA damage in ALT-positive NB cells. Our *in vitro* studies demonstrate that administering METTL3 inhibitors diminishes telomere targeting and stability, underscoring METTL3 inhibition as a promising strategy for treating ALT-positive cancers. Furthermore, our findings support the hypothesis that modulating RNA modifications may enhance the efficacy of existing DNA-damaging therapies, potentially sensitizing these NB cells to treatment. However, further research is necessary to know if similar mechanisms operate in other ALT-positive malignancies and to assess the sensitivity of these cancer cells to METTL3 inhibition.

In conclusion, this study elucidates the mechanistic role of METTL3 in regulating TERRA lncRNA at telomeres and highlights the importance of m6A modifications in maintaining genomic integrity. Future research should explore the broader implications of RNA modifications on other long non-coding RNAs and their roles in cancer biology, particularly in the context of telomere dynamics and DNA damage responses. Understanding these interactions will be essential for developing therapies that target vulnerabilities in altered RNA modification pathways in NB.

Paper II

ALT+ NBs are a subset of high-risk tumors with poor prognosis and limited therapeutic options. These tumors exhibit high expression of TERRA RNAs, which are integral for maintaining telomere integrity by localizing to the telomeres and interacting with a number of proteins involved in the ALT process. Therefore, further understanding of the mechanisms of TERRA RNA condensate assembly may enable the design of novel, mechanism-based therapies specific to ALT+ NB.

Our findings unveil that TERRA RNA forms condensate-like foci in ALT+ cells sensitive to disruption by 1,6-Hexanediol treatment. Moreover, we identify that TERRA-interacting proteins are enriched with intrinsically disordered regions (IDR) that can drive liquid-liquid phase separation (LLPS). Furthermore, m6A modifications of TERRA RNA promote the formation of condensates mediated by hnRNPA2B1 *in vitro*. Notably, both RRMs and IDR domains of hnRNPA2B1 are required for the *in vivo* formation of TERRA RNA foci. Furthermore, simultaneous inhibition of METTL3 and ATM kinase synergistically impairs the viability of ALT+ NB cells.

In this study, we demonstrated that TERRA RNA undergoes condensate formation via interactions with IDR-containing proteins in a manner driven both by m6A modifications and G-quadruplex structures, which is essential for localization and functional role in ALT+ cells. Results emphasize that targeting TERRA condensates could represent a valuable therapeutic strategy in ALT+ NB and suggest that interference with this form of condensate formation could improve the action of treatments.

Future studies would be needed to further delineate the properties and dynamics of the TERRA condensates in ALT+ cells. These studies should also include identifying additional TERRA-interacting proteins, investigating other RNA modifications or structures modulating condensate assembly, developing combination therapy, and optimizing against TERRA condensates and associated pathways. Thus, preclinical evaluations of METTL3-ATM inhibitor combinations are justified in ALT+ NB models.

The results presented here open new perspectives for understanding ALT mechanisms and suggest possible selective therapies for high-risk NBs, which have limited therapeutic options. These insights may ultimately improve patient outcomes.

Paper III

Understanding the molecular mechanisms regulating neural crest differentiation in NB is important because disturbances in neural crest differentiation often result in aggressive tumor behavior and poor patient outcomes. NB, especially MYCN oncogene-amplified NB, also poses specific challenges concerning how genetic alterations affect cellular processes. The *MYCN* oncogene is a well-documented transcriptional regulator that is important in maintaining stem-like properties and preventing differentiation across multiple tumorigenic contexts. However, detailed mechanisms by which MYCN regulates RNA-modifying enzymes, such as METTL3, are not well understood.

Moreover, the m6A landscape during neural crest cell differentiation remains uncharacterized, which clearly points to a gap in our understanding of how such RNA modifications contribute to this critical process. We report here for the first time that MYCN mediates the recruitment of METTL3, a well-known m6A methyltransferase that modifies RNA and influences gene expression and cellular states. Considering the critical role of RNA modifications in controlling differentiation pathways, there is a need to define how MYCN-mediated recruitment of METTL3 alters the patterns of m6A modification to influence neural crest differentiation in NB. Understanding this relationship may provide novel approaches to targeted treatment for MYCN-driven NB and provide additional insights into the implications of epitranscriptomic modifications in cancer.

In this study, we employed a series of experiments to investigate the relationship between METTL3 and MYCN. We found that MYCN overexpression significantly increased the occupancy of METTL3 to gene loci associated with posterior *HOX* gene expression. This increase in METTL3 occupancy is associated with changes in m6A modification patterns promoting an undifferentiated state in NB cells overexpressing MYCN. Specifically, we found reduced expression of differentiation markers with increased cell proliferation, pointing toward the oncogenic role of MYCN via interaction with METTL3. These results establish an important connection between MYCN and METTL3, indicating that MYCN does not act purely as a transcriptional regulator but also modulates the post-transcriptional modifications important for the undifferentiated phenotype of NB cells.

These findings are important because they further confirm previous evidence on the close relationship between poor clinical outcomes in NB and *MYCN* amplification. The inhibition of differentiation while enhancing the degree of stemness by MYCN indicates an urgent need for innovative therapeutic strategies to target this pathway. Notably, our *in vivo* experiments showed that ablation of METTL3 significantly reduced tumor growth in *MYCN*-amplified NB cells within xenografted mice. Moreover, combination treatment using METTL3 inhibitor, together with conventional therapies such as doxorubicin, showed improved efficacy both *in vitro* and *in vivo*. These results prove that targeting METTL3 is very promising as a therapeutic intervention in NB, especially in tumors with *MYCN* amplification.

Overall, our results, for the first time, advance the understanding of the NB epitranscriptomic landscape by demonstrating that MYCN cooperates with METTL3 to establish an m6A epitranscriptomic signature that controls the expression of posterior *HOX* genes during neural crest cell differentiation. These results underpin a future role of targeting METTL3 as a novel therapeutic approach against high-risk NB, inducing differentiation and increasing sensitivity to chemotherapy. Further, our findings stress the need for deeper investigation into the wider implications of RNA modifications as a driver in NB differentiation processes and combination treatments of METTL3 inhibition with existing therapies, with the hope of improved patient outcomes.

Paper IV

In the first three papers of my thesis, I investigated the role of m6A RNA modification in MYCN- and ALT-driven NB tumors. These studies suggest that m6A modification plays a critical role in both tumor types. However, we did not previously present a comprehensive m6A profile of different NB subtypes. In this manuscript, we address this gap by performing m6A RNA-immunoprecipitation sequencing (m6A RIP-seq) across three key NB subtypes: ALT-positive (ALT+), *MYCN*-amplified (MNA), and low-risk (LR). One significant challenge in obtaining these profiles was the limited availability of tumor RNA from patient samples, which we overcame by optimizing a low-input m6A RIP-seq approach.

Our findings show widespread m6A modifications across NB tumor types, supporting our earlier observations on the role of m6A in NB. While there is some overlap in m6A modifications across tumor types, each subtype exhibits distinct m6A signatures. Genes modified by m6A were generally more structurally complex than non-m6A genes in NB tumors, indicating a role in fine-tuning gene expression. Additionally, tumor-specific m6A-modified genes were enriched with various TF motifs, suggesting a potential interaction between TF activity and m6A signatures. Integrating m6A modification data with gene expression analysis reveals that m6A may influence key biological features of NB. Notably, we also found that, alongside coding RNAs, lncRNAs were m6A-modified in a subtype-specific manner, suggesting that m6A-modified lncRNAs may contribute to the gene expression profiles unique to each subtype.

In Paper III, we demonstrated that MYCN can directly influence m6A-dependent gene expression by interacting with METTL3. Our comprehensive m6A profiling across NB subtypes indicates that *MYCN*-amplified tumors generally exhibit a higher number of m6A-modified coding RNAs and lncRNAs. To investigate this further, we conducted MYCN overexpression experiments in sympathoadrenergic progenitor (SAP) cells, where MYCN induction created an undifferentiated state resembling the NB phenotype. This induction led to a marked increase in m6A positivity, with the m6A-modified genes often overlapping with MNA-specific m6A signatures, suggesting that the m6A epitranscriptome may play a central role in MYCN-driven tumorigenesis.

We believe this study establishes a foundation for global epitranscriptomic studies in NB and will inspire further research into the role of RNA modifications in other cancers.

CONCLUSION

NB remains one of the most challenging pediatric cancers owing to its molecular heterogeneity and divergent clinical outcomes. This thesis conducts a thorough investigation of m6A modification, offering a comprehensive understanding of the manner in which m6A regulates gene expression and cellular differentiation, eventually affecting NB pathogenesis. Furthermore, we explore the complex role of m6A modification in shaping the biology of three distinct NB subtypes: ALT+, MNA, and LR tumors.

In paper I, we found that the m6A modification is vital for the function of lncRNA TERRA, which is predominantly expressed in ALT+ NB. We proved that the METTL3-driven m6A modification is needed for proper localization and function of TERRA at the telomeres to avoid DNA damage and maintain genomic stability. Additionally, we provide evidence supporting the targeting of METTL3 as a novel therapeutic strategy for the treatment of ALT+ tumors, which are known to follow a protracted disease course with frequent relapses.

Building upon these findings, we interrogated the biochemical dynamics of TERRA and demonstrated how m6A modification confers stability to TERRA and facilitates the formation of RNA condensates by interacting with RBPs such as hnRNPA2B1 in paper II. We provide insight into how such a regulatory mechanism supports ALT activity by elucidating how m6A promotes the LLPS of TERRA. Conclusively, this work identified that disruption of TERRA condensates by the inhibition of METTL3 could represent a promising combination therapy approach that augments the efficacy of existing treatments against ALT+ NB.

In paper III, we changed the focus to the differentiation of tNCC and illustrated how METTL3/MYCN reciprocally controls the expression of *HOX* genes during tNCC differentiation by fine-tuning m6A levels. We further showed that overexpression of the *MYCN* oncogene induces an undifferentiated state by downregulating these critical genes. This condition is reversible by METTL3 inhibition/depletion. The described connection between epitranscriptomics and differentiation outlines therapeutic possibilities for METTL3 inhibition in reversing the aggressive phenotypes driven by *MYCN* amplification.

In paper IV, we conducted extensive profiling of the m6A epitranscriptome across NB subtypes, including ALT+, *MYCN*-amplified, and LR tumors.

Common and subtype-specific m6A-modified gene signatures were identified, highlighting the widespread impact that m6A may have on the biological functions and clinical trajectories of these tumors. These findings were in line with the clinical features of aggressive subtypes, including DNA repair and chromosomal stability pathways enriched with m6A-modified genes. Modifications associated with cellular organization and development have been associated with LR NB. This differential regulation further establishes m6A as a critical player in the molecular heterogeneity of NB.

In summary, the research presented in these studies enhances our understanding of the complex molecular mechanisms underlying neuroblastoma. It highlights the important roles of RNA modifications, particularly m6A, in regulating gene expression, maintaining telomeres, and guiding cellular differentiation. These findings may also have broader implications, suggesting that similar mechanisms could be present in other cancers. By identifying potential therapeutic targets such as METTL3 and TERRA RNA dynamics, these studies lay the groundwork for developing new treatment strategies to improve outcomes for neuroblastoma patients. Future research should focus on exploring the epitranscriptomic landscape in neuroblastoma and other cancers to uncover new regulatory mechanisms and therapeutic targets. Investigating how RNA modifications interact with various signaling pathways and their effects on tumors will be essential.

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