Understanding the role of long non-coding RNAs in neuroblastoma development and progression

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Cover illustration: An RNA tale for pediatric cancer; discovery to mechanism.

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ABSTRACT

Neuroblastoma (NB), a common cancer of childhood, contributes to 15% of all pediatric cancer deaths. The improper neuronal differentiation of neural crest cells to mature neurons in the sympathetic nervous system leads to NB tumor formation. NB is an extremely heterogeneous disease and high-risk NB is very difficult to treat, with the incidence of relapse in 50% of cases despite of intensive chemotherapeutic treatment. Long non-coding RNAs (lncRNAs) are a class of biological molecules that are transcribed but not translated to any functional protein. The mechanism of functions for these lncRNAs are diverse and context-specific. De-regulation of lncRNAs has been proposed to play a critical role in cancer development and progression. The goal of the current thesis was to identify novel neuroblastoma-specific lncRNAs for better stratification of the disease and characterizing their functional role in greater details.

In the first study, we characterized differentially expressed lncRNAs between low-risk and high-risk NB tumors using transcriptome profiling. Among the differentially expressed lncRNAs, we chose a lncRNA, neuroblastoma associated transcript 1 (*NBAT1*), that maps to NB hotspot locus 6p22, which has been shown to harbor several NB-specific risk-associated SNPs. We showed that *NBAT1* is a tumor suppressor lncRNA and it carries out this tumor suppressor function through regulating cellular proliferation and differentiation. Consistent with its tumor suppressor properties, its higher expression in NB patients predicts a good prognosis. Mechanistically, *NBAT1* controls NB cell growth through epigenetically silencing cell proliferating

genes, as well as NB cell differentiation by repressing the neuron-restrictive silencer factor NRSF, also known as REST.

In the second study, we sought to investigate the functional connection between *NBAT1* and its sense partner *CASC15* lncRNA in NB development and progression. Like *NBAT1*, *CASC15* harbors NB-specific tumor suppressor properties and its higher expression in NB patients correlates with good clinical outcomes. We show that *CASC15*/*NBAT1* (6p22lncRNAs) promote cell differentiation by the specific regulatory interactions with *SOX9* and USP36 located on 17q, which is frequently gained in NB. We could show mechanistically that 6p22lncRNAs dictate *SOX9* expression by controlling CHD7 stability via modulating cellular localization of USP36, which is a deubiquitinase.

In the third and final study, we found that *NBAT1* is a p53 responsive lncRNA and regulates p53 subcellular localization. We observed that a decrease in *NBAT1* expression in NB cells results in resistance to genotoxic drugs, which in part occurs due to cytoplasmic p53 accumulation and concomitant loss of p53 dependent gene expression. Higher expression of the p53 exporter CRM1 in *NBAT1* depleted cells contributes to p53 cytoplasmic localization, while CRM1 inhibition in these cells restores p53 localization. We observed that combined inhibition of CRM1 and MDM2 sensitized aggressive NB cells with cytoplasmic p53, suggesting that this drug combination could be a potential therapeutic strategy for high-risk NB patients.

In summary, these findings highlight the regulatory role of lncRNAs in NB disease development.

Keywords: Neuroblastoma, Long non-coding RNAs, *NBAT1*, NRSF/REST, *CASC15,* 6p22lncRNAs, *SOX9,* USP36*,* CHD7, p53, CRM1, MDM2.

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SAMMANFATTNING PÅ SVENSKA

Neuroblastom (NB), en vanlig cancer i barndomen, bidrar till 15% av barnmortalitet från cancer. Felaktig neuronal differentiering av neurallist celler till mogna nervceller i det sympatiska nervsystemet leder till NB-tumörbildning. NB är en extremt heterogen sjukdom och högrisk NB är mycket svår att behandla, med förekomst av återfall i 50% av fallen trots intensiv kemoterapeutisk behandling. Långa icke-kodande RNA (lncRNA) är en klass av biologiska molekyler som transkriberas men inte översätts till något funktionellt protein. Funktionsmekanismerna för dessa lncRNA är mångfaldiga och kontextspecifika. Avreglering av lncRNA har föreslagits spela en kritisk roll i cancerutveckling och progression. Målet med den aktuella avhandlingen var att identifiera nya neuroblastom-specifika lncRNA för bättre stratifiering av sjukdomen och karakterisera deras funktionella roll mer detaljerat.

I den första studien har vi karaktäriserat differentiellt uttryckta lncRNA mellan lågrisk- och högrisk NB-tumörer med hjälp av transkriptom profilering. Bland de differentiellt uttryckta lncRNA har vi valt ett lncRNA, neuroblastoma ssocierat transkript 1 (*NBAT1*), som ligger i NB hotspot locus, 6p22, som har visat sig innehålla flera NB-specifika riskassocierade SNP. Vi har visat att *NBAT1* är en tumörsuppressor lncRNA och den utför denna tumörsuppressor funktion genom att reglera cell proliferation och differentiering. I överensstämmelse med dess tumörsuppressor egenskaper förutspår dess högre uttryck hos NB-patienter god prognos. Mekaniskt kontrollerar *NBAT1* NB-cell proliferation genom epigenetiskt tystande cell proliferationsgener, och NB-cell differentiering genom att hämma den neuron-restriktiv tystnadsfaktorn NRSF, även känd som REST.

I den andra studien eftersträvade vi att undersöka den funktionella kopplingen mellan *NBAT1* och dess senspartner *CASC15* lncRNA i NB-utveckling och progression. Liksom *NBAT1*, har *CASC15* NB-specifika tumörsuppressor egenskaper och dess högre uttryck hos NB-patienter korrelerar med ett bra kliniskt utfall. Vi visar att *CASC15* / *NBAT1* (6p22 lncRNA) främjar cell differentiering genom specifika reglerande interaktioner med SOX9 och USP36 lokaliserade på 17q, vilken ofta tilltar i NB. Vi kunde visa mekanistiskt att 6p22 lncRNA dikterar SOX9-uttryck genom att kontrollera CHD7-stabilitet via modulering av cellulär lokalisering av USP36, som är ett deubikitinas.

I den tredje och sista studien fann vi att *NBAT1* är ett p53-responsivt lncRNA och reglerar den subcellulära lokaliseringen av p53. Vi observerade att en

minskning av *NBAT1*-uttryck i NB-celler resulterar i resistens mot genotoxiska läkemedel, som delvis inträffar på grund av cytoplasmisk p53-ansamling och samtidig förlust av p53-beroende genuttryck. Högre expression av p53-exportör CRM1 i *NBAT1*-reducerade celler, bidrar till p53-cytoplasmisk lokalisering medan CRM1-hämning i dessa celler återställer p53 nukleär lokalisering. Vi observerade att en kombinerad hämning av CRM1 och MDM2, sensibiliserade aggressiva NB-celler med cytoplasmisk p53, vilket tyder på att denna läkemedelskombination kan vara en potentiell terapeutisk strategi för högrisk NB-patienter.

Sammanfattningsvis belyser dessa fynd den reglerande rollen för lncRNA i NB-sjukdomens utveckling.

Nyckelord: Neuroblastoma, långa icke-kodande RNA, *NBAT1*, NRSF / REST, *CASC15*, kromosom 6p22, SOX9, USP36, CHD7, p53, CRM1, MDM2.

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LIST OF PAPERS

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

I. Pandey GK*, **Mitra S***, Subhash S, Hertwig F, Kanduri M, Mishra K, Fransson S, Ganeshram A, Mondal T, Bandaru S, Ostensson M, Akyürek LM, Abrahamsson J, Pfeifer S, Larsson E, Shi L, Peng Z, Fischer M, Martinsson T, Hedborg F, Kogner P, Kanduri C. (2014). The risk-associated long noncoding RNA *NBAT1* controls neuroblastoma progression by regulating cell proliferation and neuronal differentiation. *Cancer Cell, 26*(5), 722-737. doi:10.1016/j.ccell.2014.09.014 (* Co-first author)

II. Mondal T, Juvvuna PK, Kirkeby A, **Mitra S**, Kosalai ST, Traxler L, Hertwig F, Wernig-Zorc S, Miranda C, Deland L, Volland R, Bartenhagen C, Bartsch D, Bandaru S, Engesser A, Subhash S, Martinsson T, Carén H, Akyürek LM, Kurian L, Kanduri M, Huarte M, Kogner P, Fischer M, Kanduri C. (2018). Sense-Antisense lncRNA Pair Encoded by Locus 6p22.3 Determines Neuroblastoma Susceptibility via the USP36-CHD7-SOX9 Regulatory Axis. *Cancer Cell, 33*(3), 417-434 e417. doi:10.1016/j.ccell.2018.01.020

III. **Mitra S**, Muralidharan SV, Di Marco M, Juvvuna PK, Kosalai ST, Huarte M, Mondal T, Kanduri C. A p53 responsive lncRNA *NBAT1* determines chemotherapeutic response in neuroblastoma through regulating p53 sub-cellular distribution (Manuscript)

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IV. Meryet-Figuiere M, Alaei-Mahabadi B, Ali MM, **Mitra S**, Subhash S, Pandey GK, Larsson E, Kanduri C. (2014). Temporal separation of replication and transcription during S-phase progression. *Cell Cycle, 13*(20), 3241-3248. doi:10.4161/15384101.2014.953876

V. Mondal T, Subhash S, Vaid R, Enroth S, Uday S, Reinius B, **Mitra S**, Mohammed A, James AR, Hoberg E, Moustakas A, Gyllensten U, Jones SJ, Gustafsson CM, Sims AH, Westerlund F, Gorab E, Kanduri C. (2015). MEG3 long noncoding RNA regulates the TGF-beta pathway genes through formation of RNA-DNA triplex structures. *Nat Commun, 6*, 7743. doi:10.1038/ncomms8743

CONTENT

ABBREVIATIONS

NB	Neuroblastoma
NBAT1	Neuroblastoma associated transcript 1
ALK	Anaplastic Lymphoma Kinase
TP53INP1	TP53 inducible nuclear protein 1
IGFSF4	Immunoglobulin superfamily 4
REST	RE1-Silencing Transcription factor
INSS	International NB staging system
CCHS	Congenital central hypoventilation syndrome
iPS	Induced pluripotent stem cell
GWAS	Genome-wide association studies
SNPs	Single-nucleotide polymorphisms
USP36	Ubiquitin-Specific Protease 36
NC	Neural crest
NT	Neural tube
NCCs	NC cells
RA	Retinoic acid
BMP	Bone morphogenic protein
NP	Neural plate
NPB	Neural plate border
FGF	Fibroblast growth factor

TGFβ	Transforming growth factor-beta
ChIP	Chromatin immunoprecipitation
EMT	Epithelial-to-mesenchymal transition
SA	Sympathoadrenal
TH	Tyrosine hydroxylase
CAM	Chick chorioallantoic membrane
ATRA	All-*trans*-Retinoic acid
RA	Retinoic acid
dβh	Dopamine-*β*-hydroxylase
ENCODE	The Encyclopedia of DNA Element
ncRNA	Non-coding RNA
tRNAs	Transfer RNAs
rRNAs	Ribosomal RNAs
miRNAs	MicroRNAs
snRNAs	Small nuclear RNAs
siRNAs	Short-interfering RNAs
snoRNAs	Small nucleolar RNAs
piRNAs	Piwi-interacting RNAs
exRNAs	Extracellular RNAs
lncRNAs	long non-coding RNAs
circRNAs	Circular RNAs

ceRNAs	Competing endogenous RNAs
RNA-seq	RNA-sequencing
Linc-RNAs	Long intergenic non-coding RNAs
CAGE-seq	Cap analysis of gene expression
PRC2	Polycomb repressor complex2
UPS	Ubiquitin-proteasome system
DUBs	Deubiquitinating enzymes
USPs	Ubiquitin-specific proteases
DLBCL	Diffuse large B cell lymphoma
CRC	Colorectal cancer
NSCLC	Non-small-cell lung carcinoma
EOC	Epithelial ovarian cancer
DDR	DNA damage response
ESCC	Esophageal squamous cell carcinoma
FFPE	Formalin-Fixed Paraffin-Embedded (tissue)
eQTL	Expression quantitative trait loci

1 INTRODUCTION

1.1 Neuroblastoma

Neuroblastoma (NB) is the most commonly diagnosed malignancy in the first years of life. The appearance of the neural crest derived NB tumors of the sympathetic nervous system is due to improper neuronal differentiation and causes 15 % of all childhood cancer mortality (*1-3*). Around 90 % of NB tumors are diagnosed by 5 years age of children with a median diagnosis age of 22 months (*4, 5*). NB is an extra cranial solid tumor that commonly occurs in adrenal medulla but can form in other places where sympathetic nervous tissue is present including paraspinal sympathetic ganglia in the chest, abdominal tissue, neck region, and liver (*6-8*). Many contributing parameters like the age of the patient, histology of the tumor, stage of the disease, DNA ploidy, chromosomal alteration, status of the *MYCN* oncogene and also NB tumor differentiation status are used for predicting disease outcome and treatment (*9*). Depending on the clinical stratification, NB tumors have been classified in several different risk-categories. For risk assessment the International NB Staging System (INSS) took the initiative to stage NB in a detailed manner and the main five stages are stage 1, stage 2 (A&B), stage 3, stage 4 & 4S. Stage 1 and 2 are designated as an early stage, which is generally localized, non-metastatic tumors and responds to radiation and chemotherapy. On the other hand stage 3 and 4 are designated as high-risk or advanced stage NB tumors with metastatic behavior and that are often resistant to chemotherapy treatment. Stage 4S is the 5th category of NB tumors where patients go through spontaneous regression of NB tumors without treatment (*1, 3, 10-12*). In a broader sense, NB tumors are divided into three risk groups (low, intermediate and high-risk) based on age, histology and MYCN status (*10*).

1.1.1 Historical background of neuroblastoma

NB was first described by the German physician Rudolf Virchow in 1864 and he classified the tumors he found in the abdomen of children as a glioma (*13*). In 1891, Felix Marchand first observed the features of the developing tumors in the sympathetic nervous system of an adrenal medulla that lies over the kidneys (*14, 15*). NB was again characterized by William Pepper in 1901, as it is prone to liver metastasis compared to bone metastasis in children (*16*). In 1910, James Homer Wright illustrated these tumors as a collection of an immature, primitive form of undifferentiated nerve cells which are neurocytes or neuroblasts and for this reason he named the tumors as neurocytoma or neuroblastoma (*17*). Wright also noted the round clumps of cells that formed

in bone marrow and these are now commonly referred to as "HomerWright pseudorosettes" (*17*). In 1927, Cushing and Wolbach described that not all NBs were metastatic. Some tumors were classified as malignant and they spread rapidly to various organs in the body like liver, skin, bone and bone marrow, other tumors resolved by themselves without treatment (*18*). Ganglioneuromas are a rare kind of tumor that turned into a non-malignant masses, which may spontaneously regress and this transformation from cancerous to noncancerous form is rare in babies over 6 months of age (*14, 19*). In 1957, Mason noted the risk assessment marker cathecholamines, which is a hormone and produced in high quantities by the high-risk NB tumors. The presence of the malignancy can be detected by encountering the presence of catecholamines in the urine of children with NB (*20*).

1.1.2 Genetic alterations and chromosomal aberrations in neuroblastoma

High-risk tumors are highly aggressive and harbor a variety of non-random chromosomal alterations such as the *MYCN* oncogene amplification, 1p, 11q deletion, 17q gain etc (*21*). The high-risk tumors have a high chance of recurrence, even after treatment with the most intensive multimodal therapies and show an unfavorable outcome. Low-risk NB tumors are associated with hyperploidy or whole chromosomal aberration and they are prognostically favorable and have a high chance of regression (*22, 23*). Besides the chromosomal alterations, some NB are associated with a number of recurrent point mutations in the kinase domain of ALK (Anaplastic Lymphoma Kinase) receptor tyrosine kinase gene locus, which is centromeric to the *MYCN* locus (*24, 25*). Another prognostic marker of NB has been shown in many studies, is neurotrophin receptors that recognize neurotrophins (hormone-like chemicals that help nerve cells maturation). NB with more neurotrophin receptors especially the nerve growth factor called *TrkA*, has a better prognosis (*26, 27*).

Tumor cell ploidy

Tumor cell ploidy (DNA index) serves as a strong and important prognostic marker for NB patients. Hyperdiploid DNA content generally correlates with less aggressive lower stage and MYCN non-amplified tumors with favorable disease behavior. Near-diploid (and near-tetraploid) tumors tend to correlate with more aggressive malignant tumors. Patients who are younger than one year old are more suitable for prediction of this correlation but it is not significant for patients who are 18 to 24 months of age of patients (*28, 29*).

MYCN amplification

Currently amplification of the *MYCN* gene on chromosome 2p24 remains the best- characterized genetic marker to stratify risk in NB. In 1983, *MYCN* was first identified in human NB which is homologous to v-*myc* and distinc from the *MYC* gene (*30, 31*). Amplification of the *MYCN* gene accounts for approximately 20-30 % of all NB cases and possesses a strong correlation with poor prognosis in NB patients (*1, 3, 22, 32, 33*). Around 50 % of NB patients diagnosed with metastasis (*3*) with a significant spread to bone marrow (70%), bone (55%), lymph nodes (30%), liver (30%), and brain (18%) (*34*). This invasive and metastatic behaviour of NB cells correlates with the *MYCN* gene amplification and expression (*35, 36*). *MYCN* acts as a master regulator that involves numerous cellular processes and contributes to the different hallmarks of cancers. *MYCN* contributes to sustaining growth by promoting proliferation and cell cycle progression, for example, *MYCN*-amplified NBs failed to arrest in G_1-phase in response to DNA damage via TP53 inducible nuclear protein 1 (TP53INP1) downregulation (*37-39*). MYCN represses genes which leads to differentiation of NB cells (*40*). MYCN also promotes maintaining a stem-like state of self-renewal by blocking the differentiation pathway and promoting the expression of pluripotency factors (*41*). MYCN also is shown to be involved in reprogramming fibroblasts into iPS cells, substituting MYC (*42*). MYCN can also regulate apoptosis and angiogenesis (*43*). MYCN is overexpressed in other cancers like glioblastoma, retinoblastoma and small cell lung carcinoma (SCLC) as in NB (*44-46*). To understand NB tumorigenesis, several MYCN transgenic mice have been developed, as overexpression of MYCN contributes to tumor progression interacting with other oncogenes partners (*47, 48*).

1p deletion

Segmental chromosomal loss of the distal short arm of chromosome 1 (1p) accounts for 25-35 % of NB tumors (*1-3*). 1p loss usually correlates with *MYCN* amplification and poor patient survival (*10*). Bader and colleagues investigated the importance of chromosome 1 in NB by transferring the normal portions of chromosome 1 short arm (1p) into the NB cell line. This 1p transfer to the NB cell line led to the differentiation and suppression of proliferation and tumorigenicity (*49*). The association of the loss of 1p chromosome with high-risk NB can be justified by the presence of tumor suppressor genes in this region. Several tumor suppressor genes *CHD5, CAMTA1, KIF1B, CASZ1* and *mir-34A* have been identified in the 1p-deleted region. Introduction of *CHD5, CAMTA1, KIF1B* and *mir*-34A genes were able to reduce cell proliferation and led to apoptosis (*50, 51*).

11q deletion

Loss of the long arm of chromosome 11 (11q deletion) is another frequent chromosomal alteration, noticed in high-risk NB and it accounts for 30-40 % of NB tumors (*10*). Interestingly this is inversely correlated with MYCN amplification (*52*). 11q deletion is associated with poor prognosis and decreased probable survival ability in NB patients (*10*). Transfer of chromosome 11 to the NB cell line also can induce differentiation, similar to chromosome 1 (*49*). Several tumor suppressor genes are located in this region, for example, immunoglobulin superfamily 4 (IGFSF4), a tumor suppressor in lung cancer/cell adhesion molecule 1 (TSLC1/ CADM1) (*53, 54*). TSLC1 gene transfer into TSLC1 low expressed NB, leads to reduced proliferation of NB cells (*54*).

17q gain

Another important chromosomal alteration in NB is the gain of the distal part of chromosomal arm 17q. 17q gain is associated with stage 4 metastatic NB significantly and it accounts for 40-50 % of NB patients which is more frequent than 1p deletion, 11q deletion and MYCN amplification (*10*). The gain of 17q correlates with MYCN amplification and leads to poor prognosis of NB patients (*1, 3, 10, 55*). Genes like PPMID, Survivin, NM23A are located in this region (*10*). Survivin, which is an apoptotic inhibitor correlates with the unfavourable outcome and its frequent overexpression has been observed in NB patients (*10, 56*). Another gene SOX9 is also located in 17q region and SOX9 overexpression has been shown to perturb differentiation in NB cells and the knocking down of SOX9 can rescue the differentiation phenotype which suggests 17q gain associated with SOX9 overexpression and poor outcome in NB (*57*).

Anaplastic Lymphoma Kinase (ALK) amplification - cause for both sporadic and familial neuroblastoma

Hereditary predisposition in NB is rare, found in 1-2 % of NB cases. *ALK* acts as a predisposition gene for NBs (*25, 58*). Amplification of an *ALK* gene can promote ALK activation and correlates with the poor survival of the NB patients (*59, 60*). Other than amplification or overexpression of an *ALK* gene, point mutations in *ALK* have also been observed in both familial and sporadic NB patients (*24, 25, 58, 61*). ALK has been previously considered as an orphan receptor, but a recent study shows ALK has more than two ligands, heparin and members of the FAM150 protein family (*62, 63*). *ALK* and *MYCN* can be co-amplified in high-risk NB as they share similar locations on 2p (*64*). *ALK* expression is limited to neural tissues. In one mouse model (controlled by the dopamine β-hydroxylase (*Dbh*) promoter), it has been shown that gain-of-

function mutation in *ALK* leads to NB tumor formation but requires constant co-expression of *MYCN* (using the tyrosine hydroxylase (*TH*) promoter) and this observation has also been validated in a Zebrafish model (*48, 64*). This co-operation of *ALK* and *MYCN* can be explained due to ALK-mediated activation of phosphoinositide 3-kinase (PI3K) signaling (*48, 64*). Most of the point mutations of an *ALK* gene are reported within the ALK kinase domain and occurred in 7-9 % of NB patients (*65*). The two most frequently observed hot-spot mutations in the ALK kinase domain are mutations in ALK-F1174 and ALK-R1275, which account for 70-80 % of all mutant cases (*24, 25, 58, 61*). It has been reported recently that activating ALK point mutations (F1174L/S, R1275, Y1278S and L1196M) is frequently observed in 30-40 % of relapsed NB cases and determines the sensitivity towards ALK inhibitors (*66-68*). Transient siRNA knockdown or inhibition of ALK in NB cells leads to decreased cell proliferation (*25, 69*). Targeting ALK and its downstream target can be a therapeutic strategy for ALK-positive NB patients.

PHOX2B – a hereditary predisposition in neuroblastoma

PHOX2B (which encodes paired mesoderm homeobox protein 2B and is a master regulator of neural crest development) has been identified as a first bonafide gene that can predispose in NB while mutated with a single copy in the germline (*70, 71*). This germline mutation of the *PHOX2B* gene could highlight the complex genetics of NB.

The *PHOX2B* gene can regulate autonomic nervous system development (*72*) and children with congenital central hypoventilation syndrome (CCHS) which has a neural crest origin, are more prone to get specific mutation and neuroblastic tumor development (*73, 74*). Interestingly *PHOX2B* has been shown as a key regulator of NB differentiation and stemness maintenance. A higher *PHOX2B* expression level correlates with NB cell proliferation and self-renewal and retinoic acid-induced neuronal differentiation can downregulate *PHOX2B* expression and thereby can inhibit tumorigenicity by suppressing the self-renewal capacity of NB cells (*75*).

Additionally, *PHOX2B* mutations also account for about 2% of sporadic NB patients (*76*). Together this indicates that *PHOX2B* might serve as a potential therapeutic target in NB patients.

Other genomic rearrangements

Using high throughput whole-genome sequencing, genomic survey of NB tumors reveals a loss of function genetic alteration of *ATRX* (encoding the RNA helicase) in approximately 10% of patients, promoter rearrangement of *TERT* (encoding telomerase reverse transcriptase) which promotes enhancer hijacking, in approximately 25 % of NB tumors approximately (*77, 78*).

Amplification of *LIN28B* occurs rarely in NB but overexpression of polymorphic alleles within the *LIN28B* (encoding lin-28 homolog B) locus was identified to have a strong association with high-risk NB (*79*). In NB cells and mice (under the control of the *Dbh* promoter), *LIN28B* misexpression leads to the accumulation of high levels of N-MYC and poor prognosis (*80*). Moreover, other amplifications and focal gain show enrichment of other target genes of N-MYC (*81*).

1.1.3 Neuroblastoma and genome-wide association studies (GWAS)

Over the last 10 years, genome-wide association studies (GWAS) have evolved into a convenient tool for investigating the structure of common human genetic variation or the overall genetic architecture of human disease. GWAS studies revealed NB is associated with common polymorphic alleles that provide complexity to the disease. 12 highly significant genetic associations have been identified and validated to date (*82*). Though each association consists of moderate individual effect to initiate a disease, there is also a possible cooperation of multiple associations in an individual patient which can promote malignant transformation during neurodevelopment (*83*). Many GWAS associated NB susceptibility genes have been shown to act as a potential oncogene or tumor- suppressive gene in a disease context (*84*). Barr EK et al described the identifications of GWAS associated genes in a detailed manner (*83*), which I will briefly discuss in this thesis (Figure 1).

In 2008, the first successful GWAS in NB was conducted using 464,934 single-nucleotide polymorphisms (SNPs) and a cohort, where 1032 NB patients compared with 2043 healthy controls of European descent (*85*). Interestingly three variants on chromosome 6p22 were identified by this study, which mapped to the long non-coding RNAs (lncRNAs) *NBAT1* (*CASC14*) and *CASC15*. The most statistically significant SNP was rs6939340, which shows strong association with MYCN amplified or stage 4 NB tumors. It has also been shown decreased expression of *NBAT1* correlates with poor survival, cell proliferation and tumor growth (*86*). The sense-antisense lincRNA pair - *NBAT1* and *CASC15*, harboring the disease-associated SNP rs6939340 have been mechanistically investigated to understand the significance of this genetic predisposition. By modulating the Ubiquitin-Specific Protease 36 (USP36) localization, decreased expression of *CASC15* and *NBAT1* leads to the stability of CHD7, a chromatin remodeler. CHD7 maintain an un-differentiated cellular state by inducing SOX9 expression and leads to NB tumor formation (*57*). Hence, trait associated lncRNAs *NBAT1* and *CASC15* act as tumor suppressor lncRNAs in NB development and can be considered using for NB risk assessment.

The next GWAS analysis conducted with the same cohort but using only 397 high-risk patients from the original 1032 patient cohort, who were compared to the same 2043 healthy controls (*87*). Several new risk SNPs were identified on chromosome 2q35,which were located in the intronic region of the *BARD1* gene, moreover, this risk SNPs were validated in an independent Italian cohort as well (*88*). *BARD1* isoforms have high oncogenic activity and it can lead to neoplastic transformation of mouse fibroblasts (*89*). Additionally, *BARD1* interacts with AURKA, which actively stabilizes MYCN protein and AURKA induces the growth of MYCN-amplified NB cell lines suggested by *in vitro* analysis and the AURKA inhibitor alisertib (MLN8237) was able to inhibit the growth of NB xenografts (*90*).

After three years from the first GWAS study, the cohort of genotyped NB patients increased and by comparing 1627 NB patients with 3254 controls, risk variants in *CASC15* and *BARD1* confirmed and additionally identified a new locus on 11p15.4 mapping to the *LMO1* gene (*91*). The newly identified GWAS SNPs associated with the *LMO1* gene can predict clinical outcome of NB patients and higher *LMO1* expression contributes to the growth of NB cells (*91*). Increased *LMO1* synergizes with MYCN In a zebrafish model of NB and promotes tumorigenesis (*92*). With the advancement of the GWAS studies, the susceptibility SNPs have been identified in other genes like DUSP12, HSD17B12, DDX4, IL31RA by comparing 574 low-risk NB cases with 1722 control cases (*93*).

Diskin et al. conducted a GWAS in 2012, comparing a discovery cohort of 2101 patients to 4202 European controls and besides confirming the previous findings, identified new risk variants in *HACE1* and *LIN28B* that were associated with the development of NB (79). Furthermore, predisposition SNPs in *HACE1* and *LIN28B* were confirmed in an African American cohort for the first time (79). *HACE1* has been noted as a tumor suppressor in many cancers including NB (94) and *LIN28B* generally remains overexpressed in high-risk NB and elevates MYCN expression and stabilization by both inhibiting the miRNA let-7, and increasing RAN and AURKA expression (80). Whole exome sequencing and whole genome sequencing of blood leukocyte from 240 matched tumor and normal pairs also revealed germline pathogenic variants in *ALK, CHEK2, PINK1, TP53* and *BARD1* genes (95). Taken together GWAS studies can contribute to the clinically approachable biology in NB.

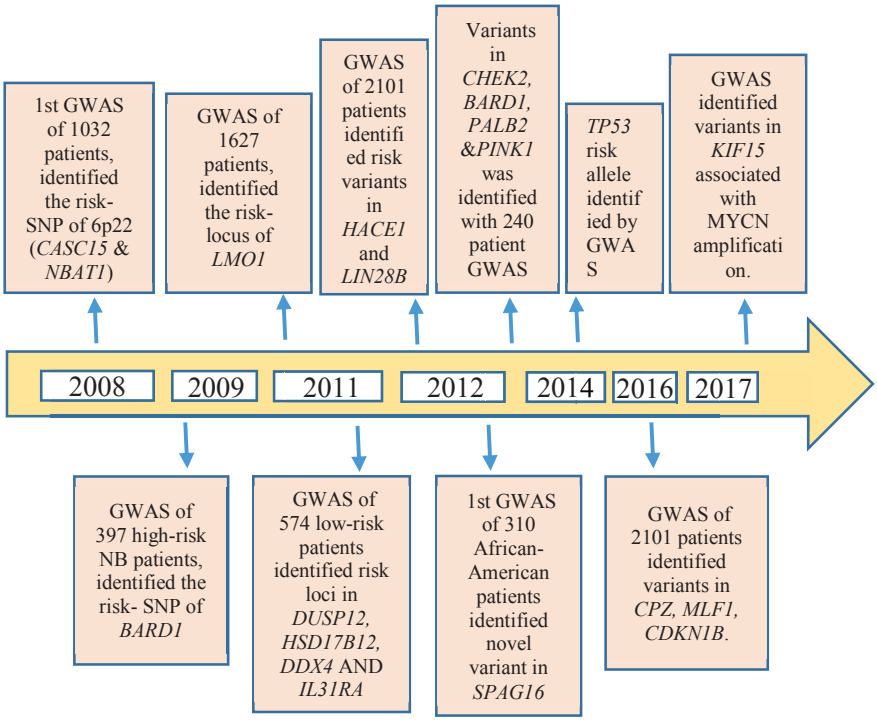

Figure 1. Timeline of identified genetic variation in NB. GWAS, genome-wide association studies. ***Modified from Barr EK et al; Children; 2018:5(9)119.***

1.1.4 Neuroblastoma – a disease of differentiation

NB arises from the neural crest (NC) cells, a transient population of embryonic cells in the developing sympathetic nervous system (*3*). The NC cells are generated at the dorsal edge of the neural tube (NT) of the vertebrate embryo under the guidance of some NC specific gene regulation. Further NC cells (NCCs) undergo an epithelial-to-mesenchymal transition (EMT) and migrate ventrally close to the neural tube and begin to differentiate to a plethora of cell-types and tissues in response to local signaling modifications (*96*) . Some of the NC cells differentiate into neurons of the sympathetic ganglion and sympathetic ganglia like adrenal chromaffin cells (the catecholamine-secreting cells of the adrenal medulla) as mentioned in Figure 2 (*97*). Other adjacent neural crest-derived cells differentiate into Schwann cells (associated with neural axons and line the ventral roots of the spinal cord, chromaffin cells of

the adrenal medulla and melanocytes), and satellite cells (associated with neural cell bodies) (*98*). NB s and NB cell lines possess immature sympathetic neurons (sometimes called N-type cells) and Schwann cells (S-type cells) like cells. Patients with a higher proportion of Schwann cells in a tumor have a rare MYCN amplification and better outcome (*99*). On the other hand, MYCN amplification is present in immature neuronal NB cells in patient tumors and leads to malignancy as *MYCN* functions to maintain the pluripotent, proliferative state and to prevent differentiation of neuronal crest (*43*) (Figure 2).

NCCs can form multiple tissues or organs of an adult organism. NCCs consists of two major populations of cells– cranial NCCs and trunk NCCs (*100*). Trunk NCCs are originated from the caudal region of an embryo and migrate with the help of different pathways (*100*). The differentiation potential of trunk NCCs is not only dependent on their initial genetic multipotency but also on the surrounding microenvironments, they face during migration (*101, 102*). Retinoic acid (RA) treatment induces differentiation of immature neuron-like cells into more mature neurons *in vitro* (*103*) but the possibility to differentiate into Schwann cells has not yet been studied therapeutically. Overall NB can be perceived because of the failure of neural crest cell differentiation, so understanding the mechanisms by which the key factors and pathways that regulate normal neural crest differentiation can help in identifying novel therapeutic targets and prevention as described by *Tomolonis et al*. (*104*). I have summarized some of the key pathways related to NC development and differentiation below.

Regulation of NC development and differentiation through signaling pathways of bone morphogenic protein (BMP), Wingless/Int (WNT), Fibroblast growth factor (FGF), and Notch/Delta

Neural crest Induction begins with neural tube development during gastrulation. The primary neural tube consists of non-neural ectoderm and neural plate (NP) tissues with the junction called the neural plate border (NPB). Modulation of the genes within this NPB region leads to NC induction (*105*). The major interconnected signaling pathways related to this process are bone morphogenic protein (BMP), Wingless/Int (WNT), Fibroblast growth factor (FGF), and Notch/Delta signaling. These signaling molecules trigger the transcription factors that allow for NC specification.

BMP is a protein derived from the transforming growth factor-beta (TGFβ) family that is secreted by neighboring non-neural ectoderm of the primitive neural tube. The Smad family of transcription factors are activated by BMP

signaling and that leads to growth and differentiation-related gene transcriptional activation (*106*). Combination therapy using BMP-6 and retinoic acid derivatives led to synergistic differentiation of IMR32 NB cell lines into dopaminergic neurons as noticed by elevated expression of tyrosine hydroxylase, neuronal maturation in morphology, and inability to resume cell division (*107*). Furthermore, in mouse NB cell line neuro2a, the addition of BMP leads to decrease in inhibitors of differentiation and increasing neuronal-specific factors which leads to differentiation (*108*) (Figure 2).

Wnt is another key-ligand that involves in NC induction by controlling β-catenin signaling. Wnt secreted by neighboring non-neural ectoderm, binds to Frizzled receptors expressed on the cells of the NPB, and leads to the induction of β-catenin genes. Canonical Wnt signaling is both sufficient and necessary for NC induction (Figure 2). In NB, the role of the Wnt pathway in tumorigenesis is controversial. The decreased Wnt-5 expression could be reversed by retinoic acid differentiation therapy and this has been shown using an IGR-N-91 xenograft model and cell lines derived from the primary tumors (*109*). On the contrary, it has been shown that activation of canonical Wnt signaling through β-catenin in NB cell line SK-N-SH led to *in vitro* doxorubicin resistance (*110*). These two studies suggests in one way Wnt signaling promotes differentiation while others propose high Wnt activity leads to drug resistance as well.

Fibroblast growth factor (FGF) is another important molecule that binds and signals through receptor tyrosine kinases, which are also known as fibroblast growth factor receptors (FGFRs). FGFR signaling activates many downstream pathways involved in proliferation and survival, including Ras/ERK and Akt/mTOR (*111*). FGF signaling through FGFR4 activates STAT3 (signal transducer and activator of transcription 3) and leads to expression of NC border genes and NC specifiers, while the loss of FGFR4 prevents this induction, has been shown in a study (*112*). In NB cell lines, primary patient samples and xenograft mouse; STAT3 expression is associated with NB Cancer stem cells (CSCs)-like cell maintenance with increased tumorigenicity and chemoresistance (*113*). Moreover, use of STAT3 inhibitor Stattic led to decreased tumorigenicity, metastasis and chemoresistance in NB cell lines and xenograft tumor models (*114*).

Notch pathway proteins are transmembrane signaling molecules and play a critical role in the maintenance of cells in a proliferative state with blocked differentiation. In NB cell line SH-SY5Y, Notch1 signaling inhibition led to neuronal differentiation via a JNK-CRT mediated pathway and correspondingly treatment of Notch inhibitors (γ-secretase inhibitors, GSIs) in

NB xenograft mice led to the suppression of tumor progression (*115*). Furthermore, the treatment with a combination of GSIs and retinoic acid derivatives of SH-SY5Y and IMR-32 human NB cell lines led to synergistic complete cell growth arrest, decreased cell mortality and promote neuronal differentiation (*116*).

c-Myc and N-Myc dependent regulation

c-Myc and N-Myc play an important role by directly modulating a gene network involved in pluripotency maintenance (*117*). c-Myc is activated by LIF/STAT3 signaling and maintains pluripotency (*118*). Myc is well implicated for maintenance of pluripotent like signature as it is one of the four Yamanaka transcription factors that can reprogram terminally differentiated cells into pluripotent stem cells and it acts via epigenetic modulation in early reprogramming process (*119, 120*). MYCN amplification, the strongest indicator of the high-risk status of NB and poor survival, leads to N-Myc protein upregulation in 50 % of high-risk NB tumors (*43*). MYCN amplified tumors basically consist of undifferentiated or poorly differentiated neuroblasts, regulated by N-Myc and other downstream signaling pathways (*32*). Chromatin Immuno Precipitation assay (ChIP-assay) after conditional expression of N-Myc in human NB cell line Tet21N, reveals that N-Myc can regulate some critical embryonic stem cell factors associated genes like LIN28B, LIF, KLF2 (*41*).

SOX9 regulation of NC survival

NC specifier genes have a late onset and they can trigger NC cells to initiate an EMT by assimilating the migratory phenotype. The key regulatory transcription factors involved in this transitional program are Sox9, Sox10, Twist1, FoxD3 and Snail2 (*96, 121*). Sox9 regulates the initiation of the EMT program by Ap2 signaling and promotes NC survival by cell cycle arrest and apoptotic inhibition via Snail2 regulation (*96, 122*). The lncRNAs *CASC15* and *NBAT1* have been shown to promote differentiation in NB cells by regulating SOX9 expression via interacting with a deubiquitinase USP36 (*57*).

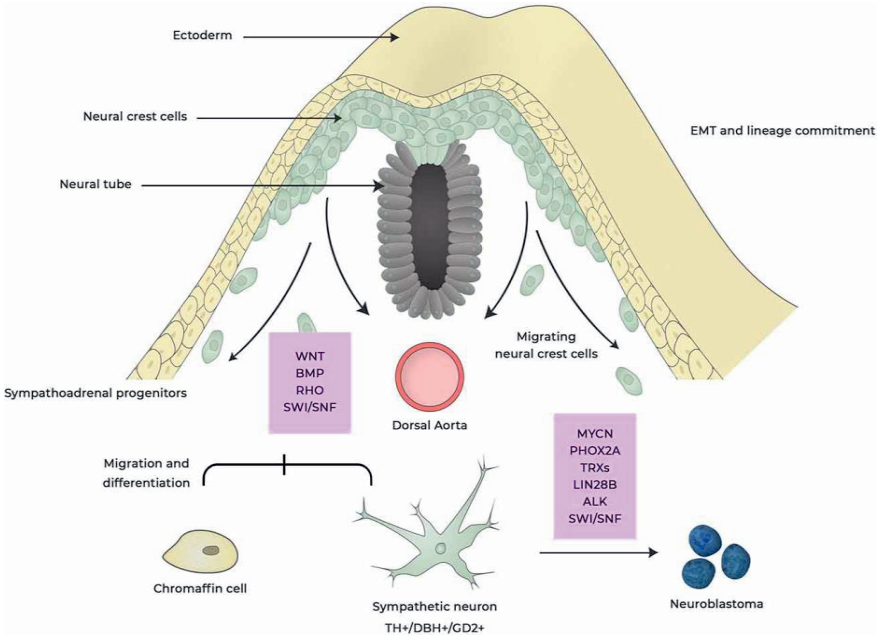

Figure 2. NB is a disease of differentiation. Neural crest derived cells undergo epithelial-to-mesenchymal transition (EMT) and are then subjected to migration and differentiation to wide range of cell types to form different anatomical structures. A complex network of signaling and transcriptional changes regulate this process. Deregulation of any of these events can trigger the changes in the properties of the migrating neural crest cells that leads to NB tumor formation. ***Adapted from Johnsen JI et al; Front Mol Neuroscience; 2019; 12: 9.***

PHOX2B regulation of NB tumorigenesis

NB typically arises from sympathoadrenal (SA) progenitor cells which give rise to the sympathetic nervous system in the adrenal medulla (chromaffin cells) and paraspinal ganglia (*97, 123*). NC cells migrate from their origin after going through the EMT and destined to become SA precursors cells. SA precursors cells ventrally migrate from the neural tube and aggregate at the dorsal aorta and form the primitive sympathetic ganglia (*124*). The well-known transcription factor PHOX2B (paired-like homeobox 2b) is expressed almost in all noradrenergic neurons and plays a critical role in the neurogenesis in the autonomic nervous system. Loss of PHOX2B caused reduced expression of markers of autonomic neural crest development and lineage except for MASH-1, in SA progenitors at the dorsal aorta and primitive adrenal gland (*72, 125*).

In NB patients PHOX2B expression is very common and it is used as a prognostic marker in patients after chemotherapy treatment to detect minimal residual disease (*126*). PHOX2B was identified as a key mediator of NB pathogenesis using a TH-MYCN mouse model, which correlates with MYCN expression and higher PHOX2B induces the proliferation of undifferentiated neuronal progenitor cells (*127*). Retinoic acid (RA)-induced neuronal differentiation can downregulate PHOX2B expression followed by suppression of the self-renewal capacity of NB cells and inhibition of tumorigenicity, and this once again confirmed PHOX2B has a key regulatory role in NB cell differentiation and stemness maintenance (*75*) (Figure 2).

1.1.5 Status of p53 in Neuroblastoma

Most stage 4 NB patients over 1 year of age remain chemo- and radiotherapy sensitive initially, but they gradually acquire resistance in relapsed tumors except a few who become long term survivors (*128*). p53 is a well-investigated tumor suppressor gene and it inhibits tumorigenicity by initiating cell cycle arrest, DNA damage repair and apoptosis in response to cellular stress signals like DNA damage or alkylating agents. Deregulation of the p53 tumor suppressor pathway has been a major cause for accumulating resistance to cytotoxic therapy in many tumor cells (*129*). Interestingly, in 60% of all human cancers approximately, p53 remains mutated but in NB tumors, p53 mutations are very rare, only 1.9% of NBs (4 out 208 tested NB tumors) have been reported by whole genome sequencing (*130, 131*) possessing p53 mutation. In the absence of mutation, p53 functional inactivation can happen in different ways and affects the p53 downstream function. The functional inactivation of p53 is believed to occur due to other mechanisms, for example, viral inactivation, MDM2 amplification and deletion of the INK4a-ARF gene encoding p14ARF (*132*) or altered subcellular localization of p53. Preferential cytoplasmic accumulation of wild type p53 with loss of nuclear p53 has been identified in several studies, which leads to functional inactivation of p53 in undifferentiated high-risk NB tumors (*133-135*). Cytoplasmic localization of wild type p53 in 96% (30 among 31 cases) of tested undifferentiated NB tumors was observed with 4.5 to 8 fold increase of p53 protein level over normal and no MDM2 gene amplification. Whereas 14 differentiated tumors showed no abnormalities of p53, suggesting p53 cytoplasmic localization is the cause for functional inactivation of p53 in undifferentiated NB tumors (*134*). Subsequently, the aberrant cytoplasmic localization of wild type p53 in NB tumors associated with elevated Thr-55 phosphorylation of P53, which induces p53 and CRM1 (nuclear export factor) interaction and that leads to cytoplasmic export of p53. Inhibition of Thr-55 phosphorylation can restore the p53 nuclear localization in NB cells (*136*). However, the p53 subcellular

localization in NB remains controversial. The above examples of investigations support the aberrant cytoplasmic localization of p53 as a cause of p53 functional inactivation in high-risk NB. But on the contrary, others support p53 accumulated in nuclear compartment and remain functional regardless of differentiation status of NB tumors (*137*) or despite cytoplasmic localization, p53 signal transduction pathway remains intact (*138*). Hence understanding the different mechanisms related to p53 irregular function in NB, may contribute to develop alternative therapeutic strategy.

1.1.6 Neuroblastoma disease models

NB causes 15% of pediatric death, this necessitates the advancement of novel therapeutic approaches for battling against NB (*139*). The development of advanced therapeutic strategies in NB is dependent on the *in vitro* and *in vivo* disease model system to pilot the experimental-therapies. Cell culture and xenograft represent preclinical models for developing new therapeutic approaches to treat NB tumors. Development of the new disease models can answer many unaddressed questions, like how a segmental chromosomal alteration leads to NB tumor formation or how gene epistasis contribute to developing disease phenotype, or the recurrent mutations in the genes in a particular chromosomal locus have a concerted effect on NB cancer development. The ability to understand how acquired mutation, chromosomal copy number changes or loss/gain of gene segments contribute to NB development demands NB disease models. Disease models can be also used to evaluate and identify novel drugs that can target responsible pathways which contribute to NB.

In vitro disease model

Cultured cancer cells are widely used as an important *in vitro* model for identifying the disease biomarkers and testing anti-cancer compounds pre-clinically. One of the most used NB cell lines is SH-SY5Y (non-MYCN amplified), which is sub-cloned from its original parental cell line SK-N-SH (*140*). The importance of this cell line is it possesses many characteristics like dopaminergic neurons as it express tyrosine hydroxylase (TH) and dopamine-β-hydroxylase as well as the dopamine transporter, on the other hand, SH-SY5Y can be differentiated into fully mature neurons with the help of various differentiation triggering agents (for example RA). A number of other NB model cell lines have been used in research. They are able to proliferate *in vitro* and remain undifferentiated and also capable of differentiation into neuronal cells depending on their risk-trait. For example, IMR32 (*141*), Kelly (*142*), NB69 (*143*), SK-N-DZ, SK-N-BE2, SKNAS (*144*) and SHEP are used as *in*

vitro cell models of NB. Neuro2a cells are a fast-growing mouse NB cell line, used as an *in vitro* NB model system.

In vivo disease model

Preclinical *in vivo* models of NB are essential for developing new therapeutic strategies and testing new chemotherapeutic drug combinations for clinical trials. The **TH-MYCN mouse model** is the most widely used transgenic model for preclinical testing of NB. This NB -prone mouse model resembles many of the characteristics of human MYCN amplified NB, established by driving MYCN expression using the rat *Th* promoter containing a strong enhancer from the rabbit β-globin gene (*47*). TH-MYCN mouse model has been used to understand role of p53 in neuroblastoma development and chemotherapeutic resistance (*145*). Another transgenic mouse model in NB is LSL-*MYCN*; Dbh-iCre with Cre-conditional induction of *MYCN* in dopamine β-hydroxylase-expressing cells. Approximately >75% of these mice also can develop neuroblastic tumors, regardless of strain background and are used for developing novel targeted therapy (*146*).

Another *in vivo* NB model is the **chick chorioallantoic membrane** (CAM) xenograft model which has been used for many years to support the NB tumor growth and for testing new therapies in pre-clinical trials. The differentiation status of NB tumors, which is formed on the chick CAM, can be analyzed by observing the changes in gene expression, proliferation and cell morphology (*147*). A microenvironment-driven pro-metastatic switch for NB has been identified by making several stable NB cells expressing fluorescent markers and grafted them into the sympatho-adrenal crest level of HH14 chick embryos and analyzed grafted embryo at HH25 in transverse slices and 3D imaging and tracking the movement of NB cells (*102*). Promoting NB differentiation with the all-*trans*-Retinoic acid (ATRA) is well described and this effect was recapitulated in the chick embryo NB model (*148, 149*), therefore this model provides a rapid, cost-effective *in vivo* model in NB for selecting promising drugs for preclinical analysis.

The zebrafish model system also emerges as an invaluable model system in NB. Presently this exciting *in vivo* model system is also in use for high-throughput drug screening as it provides a visual assessment of both drug efficacy and drug toxicity (*150*). The first zebrafish model of NB was generated by Zhu et al. by driving the expression of *MYCN* using dopamine-*β*-hydroxylase (*dβh*) driven promoter and in this model overexpression of mutant ALK act in synergistic manner to induce tumor formation (*64*). This zebrafish model system has become an essential tool for the study of ALK and MYCN-driven NB, which is complementary to *in vitro* cell culture-based drug

screens (*68, 151*). As only 20-30% of NBs harbor *MYCN* amplification, the zebrafish model of other important drivers of high-risk NB has to be developed.

Drosophila melanogaster also remains another important genetic model for NB to identify promising drug compound and to understand mode of action of NB disease causing gene like ALK. In a study, transgenic Drosophila were generated with ectopic expressions of different human ALK mutants, expressed in the Drosophila eye to define the role of 3 classes of ALK mutations in NB and their ligand dependency was characterized (*152*). *Drosophila* ALK model has been also successfully used to check the efficacy of ALK inhibitor Briganitib (*153*).

1.1.7 Neuroblastoma treatment strategies

A multidisciplinary team of doctors treat a NB patient and used to make decisions of treatment strategies and recommendations depending on several factors, for example:-

- The size and location of the tumor
- Malignancy of the tumor, whether the tumor is spread
- The risk-group classification of the tumor
- Possible side effects
- Preferences of the family
- Overall child health and other conditions

Patients under observation

Small infant group (0-6 month) with localized NB, do not need any treatment, not even surgery. Generally, these children with small tumors remain under close observation, physical examination and laboratory tests. The majority of these tumors regress by their own without further treatment except in the instance of tumor growth, when surgery with or without chemotherapy is recommended. In most of the cases these group of patients survive. (*154*).

Surgery

Surgery is a process of a tumor and some surrounding healthy tissue removal. The surgical oncologists remove the entire tumor when the cancer has not spread but mostly NB is diagnosed after the cancer has spread. So as much of the tumor as possible is removed during surgery, followed by chemotherapy

and radiation therapy to destroy the remaining cancer cells. Also a biopsy is usually done to detect the tumor type (*154*).

Chemotherapy

Chemotherapy is the particular drug or combination of drugs to destroy cancer cells, by reducing the ability of cell growth. Most NB patients will need to have chemotherapy either for primary treatment or for shrinking of the tumor before surgery or destroying cancer cells after surgery. Chemotherapy is chosen based on the NB risk group. Intermediate-risk group children often receive Carboplatin, Cyclophosphamide, Doxorubicin or Etoposide (*155, 156*). On the other hand high-risk group children often receive Busulfan, Cisplatin, Cyclophosphamide, Cytokines, Etoposide, Topotecan, Vincristine or Melphalan (*155, 156*). There are dose-dependent and variable side effects from patient to patient. However, in recent years the cure rate has not significantly improved (*156*).

Radiation therapy

Sometimes radiation therapy, which is high-energy x-rays or other particles, is used in NB patients to destroy cancer cells. External-beam radiation therapy from a machine outside the body is the most common type of radiation treatment. There are severe side effects of radiation therapy. Sometimes radiation therapy can hinder the normal growth and development of a child's brain and the testicles in boys and ovaries in girls, so other treatment strategies are opted for first (*157*).

Retinoic Acid Therapy

NB is a disease for poorly differentiating tumor cells, so induction of the differentiation should reduce the growth and proliferation in these cancer cells. It is evident from several scientific reports that 13-cis retinoic acid induces differentiation and reduces cancer cell proliferation in NB cells (*35, 86*). Generally, high-risk patients are also treated with RA and children with treatment reported to have improved event-free survival and reduced toxicity (*158, 159*).

Immunotherapy

Immunotherapy is designed for boosting the body's natural defense system to fight against cancer. The compounds used in immunotherapy made either by the body or in the laboratory, to target the abnormal antigen formed during cancer development and restores the immune system function. NB cells express a high level of sialic acid and gangliosides such as a compound called

disialoganglioside (G_{D2}) on their cell surface (*160*). This agent is required for cell adhesion, migration and metastasis (*161*). Immunotherapy using anti-monoclonal antibody directed against G_{D2} has been used for treating NB patients. The clinical trial testing has been conducted with anti-GD2 monoclonal antibody therapy (dinutuximab) combined with cytokines and retinoid therapy versus only RA therapy in high-risk NB patients. Dinutuximab (Unituxin) was approved as a first-line therapy for high-risk patients by the U.S. Food and Drug Administration (FDA) in 2015 (*162*).

Targeted delivery of radionuclides

A radionuclide named I-metaiodobenzylguanidine (^{131}I-MIBG) also has been implicated as a therapy of NB, which helps to increase the cure rate of stage 3 NB patients and improve the response (measured by considering the tumor volume, numbers of metastatic lesions and level of urine catecholamines) of stage 4 NB patients, but long term toxicity is a risk factor for this treatment (*163*).

Bone marrow or stem cell transplantation

Diseased bone marrow can be replaced by highly specialized cells called hematopoietic stem cells, which are blood-forming cells and found both in bloodstream and in the bone marrow. This transplantation can destroy the cancer cells in the bone marrow, blood and other body parts and allow them to create healthy bone marrow with the blood stem cell replacement. This transplantation process is called bone marrow transplantation, but recently it has been more often called stem cell transplantation, though not actual bone marrow tissue is transplanted, but rather blood stem cells. Depending on the source of the replacement of blood stem cells, there are two different kinds of transplantation, allogeneic (ALLO) and autologous (AUTO). In high-risk NB, AUTO transplants are often used. Different combinations of high dose chemotherapy are usually opted prior to the transplantation (*154*).

Targeting MYCN and ALK

MYCN status in NB patients remains a valid and most important prognostic marker for NB diagnosis (*1, 3*), which indicates targeting MYCN expression in high-risk NB patients could be beneficial for treatment. As drugs cannot directly bind to the DNA binding domain of MYCN due to the lack of the appropriate binding motifs (*164*), targeting MYCN indirectly to regulate its activity has recently become a widely accepted approach. For example, Aurora kinase A/B inhibitors, inhibitors of MYCN/MAX interaction, BET

bromodomain family members inhibitors P13K/AKT/m TOR inhibitor, ALK inhibitors are used as indirect targeting of MYCN (*159, 165, 166*). In recent studies it has been shown that ALK can transcriptionally regulate MYCN via AKT/ERK5 pathway, indicates that targeting ALK and its downstream targets in ALK- positive NB cell lines might be a potential therapeutic target by controlling MYCN expression in NB patients (*165, 166*). There are several ALK inhibitors are in use and have been developed and explored in clinical trials in recent years, for example Crizotinib, Ceritinib, Brigatinib, Loratinib(*167, 168*).

Tyrosine kinase inhibitors

It has been shown a small molecule inhibitor of Trk tyrosine kinase, CEP-701 can inhibit the growth in NB *in vivo* and a phase I trial is ongoing clinically (*169*). There are few more tyrosine kinase inhibitors, which are in clinical trials as well for example epidermal growth factor receptor (*3, 170*).

1.2 Non-coding RNA

The emerging evidences from high-throughput genomic and transcriptomic studies suggest that the portion of the genome which has the protein-coding potential is more or less constant across the evolutionary ladder, while the non-coding portion of the genome which is transcribed into RNA but does not code for any protein has expanded significantly, indicating that the complexity of developmental processes in higher organisms is probably due to expansion of regulatory potential of the non-coding portion of the genome. Only a minor portion (2%) of the pervasively transcribed eukaryotic genome accounts for the protein-coding RNA, the major portion (98%) of the transcribed genome is non-protein coding which includes both intergenic and intronic sequences, as shown by the high-throughput RNA-sequencing studies in recent years (*171-173*). Simultaneously the Encyclopedia of DNA Element (ENCODE) analysis reveals that 80 % of the genome has a “biochemical function”, which indicates that the non-coding portion of the genome is not junk as was previously thought before (*174*).

1.2.1 Non-coding genome and organismal complexity

The evolution of organismal complexity in higher eukaryotes has been a long-raised and fascinating question. Recent high-throughput studies helped us to visualize the developing genomic complexity to the multi-cellular higher eukaryotes based on the number of protein-coding genes. *Burkholderia xenovorans*, a free-living bacterium contains 8602 protein- coding genes (*175*), while the number of estimated protein-coding genes in humans is in the range of 20,000-25,000 which almost similar number protein-coding genes as present in the nematode (*176*), *Caenorhabditis elegans* with around 19,735 protein-coding genes (*177*). These observations bring us to the 'G-value' paradox, which depicts that the number of the protein coding genes has not changed much from the worm with only 1000 cells to the human with diverse cell types (*175*). Thus, there are some different multilayered mechanisms present in the higher organisms to efficiently control the complex system. The Organismal complexity is partly explainable by the presence of alternative splicing, RNA editing, trans splicing, alternative promoter uses but the enormous genetic changes that can contribute to the cellular complexity of higher eukaryotes, are largely unexplored. On the other hand as the non-coding portion of the genome has increased significantly in the higher complex organism (*178*), this part of the genome has gained attention recently and might be a contributor to genomic complexity as well. Previously most of the non-protein coding portions of the genome were thought to be junk or selfish DNA, with the exception of some regulatory regions like insulators and enhancers (*179*). But with the advancement of high-throughput studies it has been clear that major part of the non-protein coding genome transcribes RNAs that called non-coding RNA (ncRNA) (*171, 172*), which lack coding capacity. Along with that in the mammalian genome, a large number of protein-coding genes have the antisense counterparts, indicated by FANTOM3 consortium and also intronic regions of protein coding genes are transcribed as well to generate the intronic transcripts (*180*). All these contributes to a complex transcriptional output (*181*).

After understanding the intriguing complexity of the human transcriptome, the next and obvious question is how does this massive non-coding transcriptional output beside the protein-coding genes contribute to the higher organismal complexity? To elucidate this question, there are many investigations reported over the years that suggest the non-coding portion of the genome has a diverse and significant role in bringing cellular complexity. Interestingly, a subset of ncRNAs has been implicated in epigenetically regulated biological phenomena like X chromosome inactivation in female mammals (*182, 183*), genomic imprinting (*184-187*), homeobox gene regulation (*188*) and maintenance of

pluripotency (*189-191*). On the other hand many ncRNAs, such as *PCAT-1, MALAT-1, HOTAIR and ANRIL* have been implicated in tumor initiation and progression in several cancers (*192-196*). Therefore, there is a clear indication that evaluating and characterizing that non-coding region of the genome could solve the puzzle about organismal complexity partially by explaining complex phenotypes in higher organisms.

1.2.2 Classes of non-coding RNAs (ncRNAs)

Distinct classes of the ncRNAs have been identified using high throughput approaches in divergent tissue types and distinct developmental stages in the eukaryotic genome. The ncRNAs are mainly comprised of major two categories, ncRNAs of the first category are highly abundant and housekeeping in nature such as transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) and the second category ncRNAs are regulatory in nature. Regulatory ncRNAs can be classified again in two different subtypes according to their size – small non-coding RNAs and long non-coding RNAs. Small non-coding RNAs are microRNAs (miRNAs), small nuclear RNAs (snRNAs), short-interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs), piwi-interacting RNAs (piRNAs) and extracellular RNAs (exRNAs), which are highly conserved and involved in transcriptional and post-transcriptional silencing of genes. Whereas long non-coding RNAs (lncRNAs) which are more than 200nt in length, moderately conserved and can be sub-divided into circular RNAs (circRNAs) and linear lncRNAs such as antisence, intergenic, intronic, competing endogenous RNAs (ceRNAs) etc. LncRNAs regulate gene expression through a plethora of mechanisms and contribute to crucial biological functions, among which a small portion has been identified and understood to date (*197, 198*) for example genomic imprinting, cellular differentiation, pluripotency maintenance, RNA maturation and transport, sponging miRNA and gene regulation, X-inactivation and signal transduction (*173, 199-203*). The accumulating evidence indicates that lncRNA has an extensive role in the eukaryotic gene regulatory network and understanding and uncovering their significant functional roles in greater detail can enable us to better understand the genomic complexity in the higher organism (*204*). In the current thesis, our interest is to identify and characterize lncRNAs in NB tumor development and progression.

1.2.3 Long non-coding RNAs (lncRNAs)

The current thesis is focused on long non-coding RNAs (lncRNAs) subtype of ncRNAs. lncRNAs are more than 200 nucleotides in length and lack protein-coding potential. lncRNAs are transcribed by RNA polymerase II as other protein-coding genes, capped, polyadenylated mostly and spliced frequently (*205-207*). lncRNAs are localized in both nuclear and cytoplasmic compartments and found in almost all tissue types but in the brain and central nervous system, the abundant expression of lncRNAs has been noticed (*208*). According to the comparative studies lncRNAs have lesser but longer exons generally compared to exons of protein-coding genes (*209*). LncRNAs usually overlap with more repetitive RNAs than mRNAs (*210*) and they are less efficiently spliced and possess less transcription factors and histone marks binding to the promoter region in comparison with mRNA (*211*).

Identification of long non-coding RNAs

By analyzing the full-length cDNA libraries in the mouse, the first long non-coding RNAs were identified (*212*). With the advancement of sensitivity of genome tiling arrays, many more lncRNAs were annotated, revealing that the number of non-coding sequences is significantly larger than protein-coding sequences in human genome. Many other approaches gave sensitivity to lncRNAs detection such as tilling microarrays combining with genome-wide chromatin maps using H3K4me3-H3K36me3 active histone marks detected 1600 long intergenic non-coding RNAs (lincRNAs) (*205, 206*). Cap analysis of gene expression (CAGE-seq) (*207*) provided high-resolution mapping of precise transcription start sites where as poly(A)-position profiling by sequencing (3P-seq) helped to map the 3´end of the transcript precisely (*213*). Combining both the CAGE-seq & 3P-seq have further contributed to the annotations of novel and stable lncRNA transcripts. According to the latest version of GENCODE analysis, human genome comprised of 58,288 genes in total and of which 19,836 gene transcripts code for protein and the rest amount of genes are non-coding transcripts among them 15,778 are lncRNA genes only, indicating that the number of protein-coding genes are almost similar to the number of lncRNAs in human genome (*214, 215*).

Evolutionary perspective and conservation of lncRNA

lncRNAs are not that conserved and evolved more rapidly than protein-coding genes. Whole-genome alignment of human and mouse lncRNAs

revealed that the exonic region and promoter regions of lncRNAs are evolved much more slowly or are more conserved than intronic regions of lncRNAs and intergenic regions of the genome (*206, 209, 216*). Strikingly, despite any sequence homology in genomic alignment-based estimation, many lncRNAs possess a conserved genomic organization including exon-intron structure (*210*). These phenomena suggest that these lncRNAs might have conserved sequence-based function but current available tools are not sufficient to identify this sequence conservation. For example, *Malat1,* which is a known, frequently, expressed, nuclear-localized, single-exon transcript and thought to be mammalian specific according to its genomic alignment conservation. But recently orthologues of *Malat1* have been identified in mammals, fish and frogs containing the same length, exon-intron structure and expression pattern. Though except in the 3' terminal part, which has a 70 base homology region there are no significant sequence conservation of mammalian *Malat1* with its fish orthologue (*210*). Overall, it suggests that it is inaccurate to predict the functionality of lncRNAs considering their sequence conservation.

lncRNA subtypes

lncRNAs can be categorized and divided into several classes depending on their position in the genome, distance from protein-coding genes, functional diversity or different approach of processing such transcripts. Some categories of lncRNAs are described in Figure 3. Intronic sense and antisense lncRNAs are transcribed from introns of a protein-coding gene with the same and opposite directions to the protein-coding gene transcription respectively (*217*). Long intergenic RNAs (lincRNAs) transcribed from the intergenic region of the genome or a separate transcriptional unit from protein-coding gene (*217*). Whereas enhancer lncRNAs transcribe from an enhancer region having a capability to enhance the protein-coding gene transcription on the same locus (*218*). Divergent lncRNAs are originated from bidirectional promoter where lncRNAs share the promoter of protein-coding genes and might have a cis-regulatory function on the protein-coding genes. On the other hand, circular lncRNA produced from long primary transcripts only but in an unusual RNA processing pathway that gives the RNA circular structure (*218*).

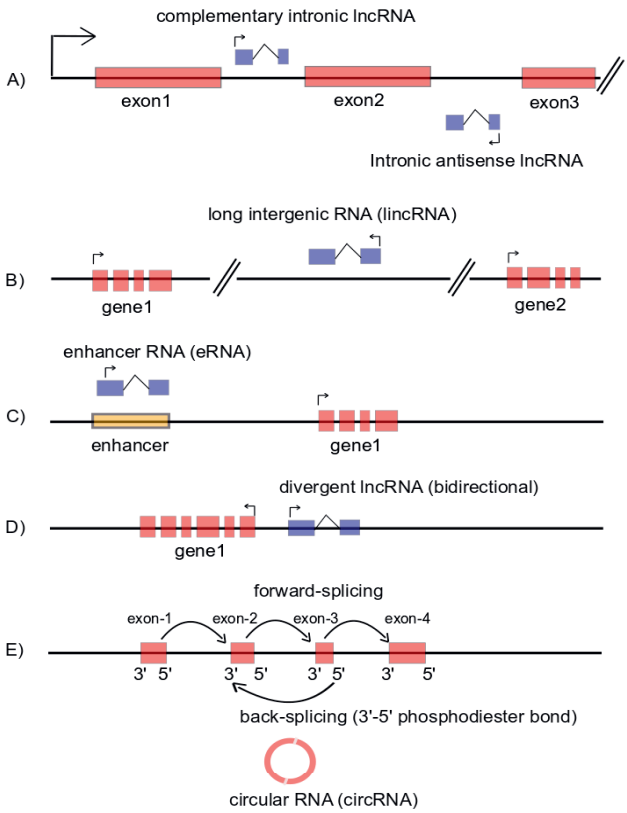

Figure 3. The subtypes of long non-coding RNAs (lncRNAs): A) Complimentary intronic and intronic antisense lncRNAs. B) Long intergenic RNA (lincRNA) C) Enhancer RNA (eRNA) D) bidirectional divergent lncRNA D) Circular RNA (circRNA) generated for back splicing of exons.

1.2.4 lncRNA 'mode of action'

lncRNAs possess many important roles in biological development and disease. They are involved in various mechanisms like epigenetic modification of gene expression, chromatin remodeling and also gene-regulation in transcriptional and post-transcriptional levels. The broad landscape of the different mode of actions of lncRNAs can be narrowed down in 4 major categories of actions as described by several studies (*201,*

219) and these are lncRNA mediated decoy, scaffold, guide and acting as enhancer RNAs (Figure 4).

Decoys

This mechanism of action is mainly titrating away several DNA binding proteins like transcription factors and prevents their access to the target regions of DNA. For example, *Gas5* lncRNA binds to the DNA-binding domain of glucocorticoid receptor and prevents glucocorticoid receptor binding to DNA. During growth factor starvation, the elevated level of *Gas5* lncRNA helps in a decoy to release the receptors from DNA and prevent the transcription of the metabolic genes (*220*). Another example the decoy mechanism of lncRNA is the lncRNA *PANDA* which decoys the transcription factor NF-YA and prevents its binding to the DNA and transactivation of several apoptotic related genes and finally block p53 mediated apoptosis (*221*).

The decoy function of lncRNA can be explainable in another aspect; lncRNA can sequester miRNAs as well and prevent miRNA mediated silencing of mRNA expression via acting as a sponge or competing for endogenous RNAs (ceRNAs). For example, lncRNA *SNHG7* directly interacting and sponging miR-216b prevents miRNA mediated gene degradation of GALNT1 and EMT markers (E-cadherin and Vimentin) which leads to higher proliferation, migration and invasion in colorectal cancer (*222*). Another example of ceRNA is lnc-MD1, which sponges mir-133 and regulate mir-133 dependent muscle specific transcription factor MEF2C (*223*).

Scaffold

Another important mode of lncRNA function is it can act as scaffold, where RNA acts as adaptor and binds to two or more protein partners and form an RNA-protein complex and regulate biological functions. For example, *TERRA* or the telomerase RNA *TERC* modulates telomerase function by scaffolding or assembling the telomerase complex (*224*). *HOTAIR* lncRNA, which regulates the gene expression by binding to both polycomb repressor complex 2 (PRC2) and LSD1-coREST complex (*225*). *Kcnq1ot1* is another example of the scaffold, which promotes the transcriptionally silenced state by the two silencing histone marks H3K27me3 and H3K9me3 via interacting with the PRC2 and G9a both (*185*). This RNA-scaffold mechanism has been shown in many lncRNAs

studies, gaining its functional importance in lncRNA mediated gene regulation.

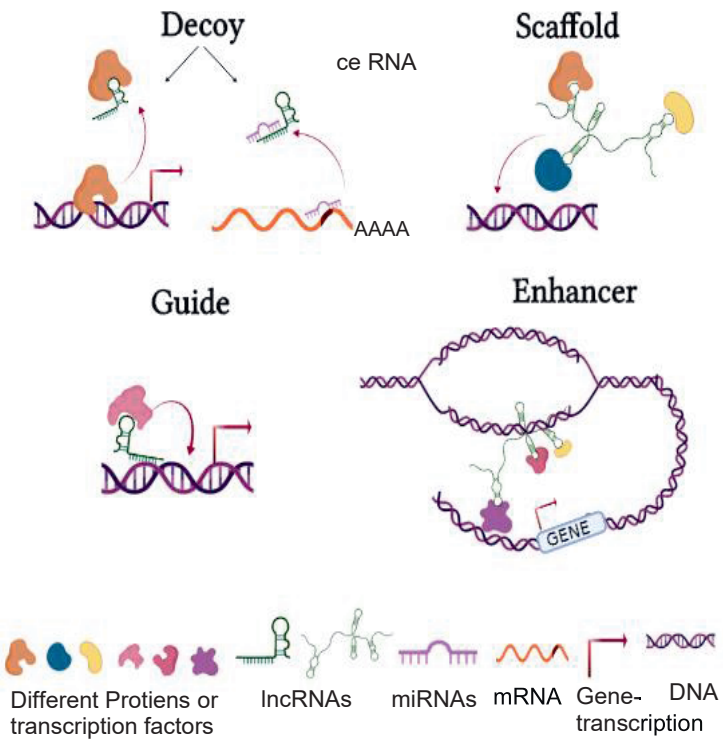

Figure 4. The 'mode of action' of the lncRNAs: Four major kinds of functions of lncRNAs - Decoy, Scaffold, Guide and Enhancer are represented in the figure. ***Concept taken from John L Rinn et al; Annu Rev Biochem; 2012; 81:145-66***

Guide

This mechanism is required for appropriate localization of specific protein complexes. *HOTAIR* lncRNA is an example of guide lncRNA. *HOTAIR* lncRNA derived from HOXC locus and in *trans* it guides PRC2 complex to HOXD locus. *HOTAIR* lncRNA regulate gene expressions in *trans* in breast cancer cells leading to invasiveness and metastasis (*226*). *LincRNA-p21* gets induced by p53 during DNA damage and then binds to hnRNP-K and guides this protein to bind to the specific promoter for the regulation of p53 mediated gene apoptosis (*227*).

Enhancer RNAs

Enhancer RNAs are the lncRNA transcripts, which originate from enhancer region and enhancer transcription is mostly bidirectional. Some of the enhancer RNAs may lack a poly A-tail and have very low copy number (*228*). These enhancer RNAs can modulate the expression of neighboring genes by associating with the promoter-enhancer complex. For example, *HOTTIP*, an enhancer like lncRNA interacts with WDR5 protein, maintains the H3K4me3 active histone mark, and leads to gene activation. After encoding from 5' region of *HOXA* gene cluster a chromosomal looping of *HOXA* gene brings enhancer like *HOTTIP* lncRNA to the proximity to the multiple *HOXA* genes (*229*).

1.2.5 LncRNA and chromatin interaction

Among the plethora of functions of lncRNA, I will be highlighting the chromatin interaction of lncRNAs in the current thesis. Before I describe the chromatin interacting lncRNAs, I will provide a small summary how chromatin marks control gene expression. Histones are the major component of chromatin, which wrapped around the DNA. The histone tails can have different modifications. Like tri-methylation at 4th lysine of Histone H3 tail (H3K4me3) over gene promoter or at tri-methylation of H3 at 36th position of lysine (H3K36me3) over gene body leads to transcriptional activation. Histone modifications can also dictate gene repression, for example tri-methylation of H3 tail at lysine 9 (H3K9me3) leads to gene silencing. Histone modification of chromatin is established by chromatin modifying enzymes and for specific chromatin modifications the chromatin modifiers also varies (*230*). Chromatin interaction is a well-known mechanism adopted by lncRNAs as they are able to interact with chromatin-modifying enzymes and target these specific epigenetic modifiers to target gene loci in *cis* or *trans*.

Some of the cis-regulatory lncRNAs are *Xist/RepA, Airn* and *Kcnq1ot1*, they have been shown to be associated with repressive complex PRC2 to regulate gene expression in an allele specific manner. Imprinted lncRNAs-*Airn* and *Kcnq1ot1* expressed from one parental allele and by interacting with chromatin modifiers and recruiting them over the repressed gene promoters leads to formation of silent chromatin (*185, 231*). *RepA* is transcribed from the repeat element present within the *Xist* gene and it promotes the formation of the transient heterochromatic structure by EZH2 interaction over the *Xist* promoter of the future inactive X chromosome which is important for *Xist* induction and spreading of *Xist* lncRNA over

the inactive X-chromosome (*232*). The repressive chromatin complexes can be recruited by some specific protein binding domain or regions or motifs in lncRNA such as a GC rich double stem-loop structure (28nt) in *RepA* lncRNA interacts directly with EZH2 (*232*). These functional regions or structural motifs of lncRNA can act as a scaffold and recruit interacting protein partners and guide these complexes for efficient targeting of the repressive epigenetic machinery.

One of the first described lncRNA mediated epigenetic regulation in *trans* can be exemplified by *HOTAIR* intergenic lncRNA transcribed from *HOXC* locus and it can mediate gene regulation of *HOXD* locus in *trans* via interacting and recruiting PRC2 protein complex like EZH2 and SUZ12 (*188*). *HOTAIR* lncRNA also has been shown to possess two RNA domains, one domain which at the 5' end, interacts with PRC2 and another domain at the 3' end interacts with LSD1, an H3K4me3 demethylase (*225*). In the first study of this thesis, we have shown that *NBAT1* lncRNA suppresses its target genes related to cell proliferation and migration via interacting with EZH2 and establishing H3K27me3 inactive modification at the promoters of some of these genes in a *trans*-regulatory manner (*86*).

1.2.6 LncRNA mediated protein stability

Ubiquitination is a most important post-translational modification where ubiquitin is added to the target protein sequences and reduces protein stability via degradation. The highly conserved protein Ubiquitin is consists of 76 amino acid residues and universally found in all eukaryotic tissues (*233*). The process of ubiquitination can be divided into two groups depending on the numbers of ubiquitin molecules linked to protein; monoubiquitination or polyubiquitination. Polyubiquitin chains formed via conjugating the monomers with either several lysine residues or with N-terminal methionine residue and produce various ubiquitin signals (*234*). The C-terminal glycine of ubiquitin attaches to the Lysine residue of target proteins by an isopeptide bond as a post-transcriptional modification and marked as degradation signal for the target proteins (*233, 235, 236*). There is a series of enzymatic events with the sequential function of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) which together build the degradation mechanism of the ubiquitin-proteasome system (UPS). A covalent bond transfer with ubiquitin occurs at each enzymatic step to finally the target protein (*237, 238*). Ubiquitination possesses many other important roles besides proteasomal degradation in several vital cellular mechanisms, such as DNA repair, cell cycle regulation and gene

expression. Interestingly there is a group of proteins called Deubiquitinating enzymes (DUBs), which play an antagonistic role by removing monoubiquitin or polyubiquitin chains from target proteins and reverse the fate of protein stability. In human, there are almost 102 putative DUB genes, which categorized mainly into two types cysteine proteases and metalloproteases. Metalloproteases are consisting of large number (58) of ubiquitin-specific proteases (USPs). This ubiquitination and deubiquitination mechanisms can fine-tune the stability of the proteins in the cellular system.

Presently, there are many pieces of evidence to support the idea that lncRNA can mediate the ubiquitination process and modulate the ubiquitin-proteasome system with a role in human cancer. For example, *HOTAIR* lncRNA binds to E3 ubiquitin ligases Mex3b and Dzip3 and then binds to their target proteins Snurportin-1 and Ataxin-1 respectively, finally form a scaffold with all of them and target them to the proteasome machinery by enhancing target-substrate ubiquitination followed by degradation of target proteins tested in HeLa and human fibroblast cells (*239*). Another example of *HOTAIR* lncRNA driven ubiquitination has been shown in prostate cancer. *HOTAIR* can bind to the androgen receptor (AR) and stabilize it via blocking the binding of MDM2 (E3 ubiquitin ligase) to AR and prevent its ubiquitination followed by protein degradation (*240*). *NDRG1-OT1* is another hypoxia-responsive lncRNA, which is responsible for inhibition of both *NDRG1* mRNA expression and NDRG1 protein levels. *NDRG1-OT1* destabilizes NDRG1 by ubiquitin-mediated proteolysis, shown in the breast cancer cells (*241*). In our 2nd study of this thesis, we identified that low expression of 6p22 derived lncRNAs *CASC15* and *NBAT1* contributes to *SOX9* upregulation by regulating cellular localization of USP36, a DUB (*57*). These above observations suggest that lncRNAs have an important role in the regulation of protein stability in two different ways either by modulating ubiquitination of protein via E3-ubiquitin ligases followed by proteasome degradation or by modulating de- ubiquitination of a protein by regulating function of DUBs to restore the stability of ubiquitinated proteins.

1.2.7 LncRNA regulation in cancer

With all the above discussions, it is quite clear that lncRNAs are the crucial fine tuners in the regulation and maintenance of the cellular state. Now in this section, I would like to discuss the direct implication of lncRNAs in a disease like cancer. The emerging evidence in last few years strengthened the concept that lncRNA function in both tumor suppression or in

tumorigenesis. The regulatory lncRNAs in different types of cancers are listed in table.1, with their functional role and interactive protein partners (Table.1).

Tumor suppressor lncRNAs

lncRNAs can fine-tune the *p53* tumor suppressor pathway by actively participating in the pathway or they can act as tumor suppressors by themselves often in several types of cancers. *p53* is an important tumor suppressor gene and its function is well studied in cancer research. *p53* can activate numbers of genes related to cell cycle arrest and apoptosis and many lncRNAs are also involved in this *p53* mediated gene regulatory pathways in cancers (*242*).

Maternally expressed gene 3 (***MEG3***) lncRNA is reported to possess tumor suppression characteristic in different cancers, as it can stimulate *p53* mediated transactivation by directly binding to the *p53* DNA binding domain (*243*). *MEG3* has an opposite correlation with tumor sizes and metastasis in cervical tumors (*244*) and similarly, *MEG3* downregulation also leads to autophagy and increased cell proliferation in bladder cancer (*245*). In hepatocellular carcinoma cells *MEG3* lncRNA inhibits cell growth via sponging *miR-9-5*p and upregulate *SOX11* (*246*). Additionally in glioma, overexpression of *MEG3* also cause reduced proliferation, migration and invasion by regulating the *miR-96-5p*/MTSS1 signaling pathway (*247*). Overall *MEG3* lncRNA represents a tumor suppressor lncRNA in various cancers.

Another well-studied *p53*-regulated lncRNA is ***lincRNA-p21***, which is a co-activator of *p21* expression as it controls *p21* in *cis*. In diffuse large B cell lymphoma (DLBCL) cell lines, over activation of *lincRNA-p21* causes decreased cell proliferation and cell cycle progression and higher expression of *lincRNA-p21* leads to progression-free and overall survival in DLBCL patients as well (*248*). Consistently in colorectal cancer (CRC) *lincRNA-p21* enhances the sensitivity to radiation therapy by promoting apoptosis and decreased level of *lincRNA-p21* correlated with CRC disease progression (*249, 250*).

Another tumor suppressor lncRNA has been investigated in Non-small-cell lung carcinoma (NSCLC) is ***PANDAR***. Decreased expression of *PANDAR* correlates with higher overall mortality in NSCLC patients. Overexpression of *PANDAR* could inhibit the proliferation of NSCLC cells by inducing apoptosis via targeting *p53* (*251*).

There are many other lncRNAs, account for possessing a tumor suppressor role. For example, ***GAS5*** lncRNA low expression was shown to strongly correlate with gastric cancer metastasis and unfavorable outcome. The overexpression of *GAS5* represses gastric cancer cell invasion and migration through stabilizing *p53* (*252*).

Oncogenic LncRNAs

Besides the tumor suppressor lncRNAs, there are many lncRNAs whose de-regulation can promote cell growth and metastasis in different types of cancers; they are described as oncogenic lncRNAs.

The metastasis-associated lung adenocarcinoma transcript 1 ***(MALAT1)***, also known as *NEAT2*, is a well-investigated highly conserved oncogenic lncRNA. *MALATI* knock out mice develop normally and suggesting *MALAT1* is not essential for early development (*253*). Depletion of *MALAT1* showed a reduction in cell proliferation and tumor progression in breast cancer cells (*254*). *MALAT*1 induces EMT transition switch via the PI3K/AKT pathway in epithelial ovarian cancer (EOC) (*255*). Overall these studies indicate the oncogenic role of *MALAT1* and raise a possibility that *MALAT1* inhibition as therapeutic option.

LncRNA ***HOTAIR*** also possesses oncogenic characteristics like promoting invasion and metastasis, showed in different cancers by recruiting PRC2 or chromatin modifications (*226*). Interestingly in lung adenocarcinoma, *HOTAIR was* reported to contribute to chemoresistance to cisplatin treatment (*256*). Consistently in another study, *HOTAIR* expression was reported to be correlated with cisplatin resistance in NSCLC (*257*).

NORAD lncRNA is a crucial part of the DNA damage response (DDR) pathway and responsible for regulating chromosomal instability (*258*). In CRC tissues, esophageal squamous cell carcinoma (ESCC) and bladder cancer, the overexpression of *NORAD* have been found to contribute to tumor progression, hence serve as another oncogenic lncRNA (*259, 260*).

1.2.8 lncRNA as a disease biomarker

The lncRNAs are becoming promising as biomarkers for different diseases. As I have discussed above, diverse lncRNAs possess oncogenic or tumor suppressor roles, therefore, they could be used as biomarkers for disease diagnosis and for planning further therapeutic strategy, but further research is necessary. Though presently there are limited reports about lncRNAs which are regularly used as a validated biomarker for disease, I would like to discuss some of the few lncRNAs with a promise to develop into biomarkers for disease progression. The lncRNA *PCAT1* expression in the prostate cancer patient can be used to predict the disease outcome where higher expression of *PCAT1* correlates with aggressive disease suggesting this could develop as lncRNA biomarker (*192*). Interestingly, the lncRNA *DD3,* later named as prostate cancer antigen 3 (*PCA3*) acts as another specific prognostic biomarker in prostate cancer and can be detected in urine samples (*261*). *MALAT1* lncRNA has been used as a biomarker and detected in liquid biopsy of NSCLCs patients and also in Formalin-Fixed Paraffin-Embedded (FFPE) tissue specimens. *MALAT1* expression correlates with metastasis and EMT in NSCLC (*195, 262, 263*). In CRC tumor tissues as well, *MALAT1* serves as a potential predictor for tumor metastasis and prognosis symptoms (*264, 265*).

lncRNAs	Cancer types	Interactive protein partners	Functional roles	Reference
HOTAIR Oncogenic	Breast, CRC, gastric, lung, prostate cancer	PRC2	Chromatin remodeling, promote migration, invasion and metastasis and up-regulated in tumors.	*(226),(256), (257)*
MALAT1 Oncogenic	Lung, EOC,CRC, breast cancer	SFPQ	Promote tumor growth and metastasis, correlated with poor survival.	*(264), (253),(255) (254),*
NORAD Oncogenic	CRC, ESCC, bladder cancer	PUMILIO	Regulation of genomic stability. Up-regulated in tumors and contributes to tumorigenicity.	*(266),(258),(259),(260)*
HOTTIP Oncogenic	HCC		Upregulated in tumors along with HOXA13, associated with metastasis and survival.	*(267)*
PCAT1 Oncogenic	Prostate, CRC, HCC and gastric cancer.		High expression in tumors related to poor survival, migration, invasion, and metastasis.	*(268)*
MEG3 Tumor suppressor	Cervical, bladder cancer, HCC		Inhibition of tumor growth, cell proliferation, invasion and migration. Induce apoptosis.	*(243),(244),(245)*
LincRNA-P21 Tumor suppressor	Lung, DLBCL, CRC	HnRNP-K	Induce p53 mediated apoptosis. Reduction of cell proliferation, cell cycle progression & survival.	*(227),(248),(249)*
PANDAR Tumor suppressor	NSCLC		Inhibit proliferation, induce apoptosis via targeting p53	*(251)*
GAS5 Tumor suppressor	Gastric cancer	Glucocortic-oid receptor	Repression of cell invasion and migration through stabilizing p53. Titrate glucocorticoid receptors away from target genes.	*(252), (220)*
CASC15-S Tumor suppressor	Neuroblastoma		Induce neural growth and differentiation and suppression of tumor growth.	*(279)*

Table 1. The list of regulatory lncRNAs in cancer and their interactive protein partners and functional roles.

1.3 Long non-coding RNA – the rising player of neuroblastoma

Regardless of increased knowledge about NB, still we lack information on how different factors contribute to the high-risk NB disease progression. Also most importantly despite advancement in multi-disciplinary treatments for treating high-risk NB, the average percentage of patient survival is just 50% (*269*). This necessitates identifying the novel players and biomarkers to stratify the risk of NB and understanding the biology of NB disease progression in a better way. The lncRNAs have begun to be illuminated as a contributor to NB along with their role in different other cancers, in recent years. The deeper understanding of the regulatory mechanisms contribute in lncRNAs mediated NB tumor pathogenesis can provide novel perspective to the high-risk NB patient prognosis and future treatment strategies.

T-UCRs

Transcribed ultra-conserved regions (*T-UCRs*) are a group of lncRNAs which is 100% conserved between human, rat and mouse genomes. 481 *T-UCRs* were reported in a study by *Bejarno et al* in 2004 (*270*) and later these 481 *T-UCRs* found to be aberrantly expressed in human leukemia and other carcinomas (*271*). These observations were then taken forward to evaluate the role of *T-UCRs* in NB progression. *Scaruffi et al* (*272*) demonstrated that the upregulation of 15 *T-UCRs* was able to distinguish the survival outcome of the patients and predict the event-free survival (EFS) and overall survival (OS) in high-risk NB. 7 *T-UCR*s of the total 481 investigated *T-UCR*s were found to be overexpressed in *MYCN* amplified tumors and 3 of these lncRNAs were upregulated upon *MYCN* induction. The *T-UCRs* lncRNAs are linked with carcinogenic pathways, for example, cell cycle, replication, DNA repair, cell proliferation, differentiation and apoptosis. (*273*). Interestingly in a recent study 32 out of 481 *T-UCRs* were noted to show differential expression with retinoic acid (RA) induced neural differentiation (*274*). Downregulation of *T-UC.300A*, which is one of the 32 differentially expressed T-UCRs, was able to decline the proliferation and invasion in NB tumors. Functional analysis of other differentially expressed *T-UCRs* in NB tumors leave scope for a better understanding the importance and significance of *T-UCRs* in NB pathogenesis.

ncRAN

Yu et al first identified *ncRAN* lncRNA, which is differentially expressed in high-risk NB patients with 17q gain using cDNA microarray with 136 NB tumors (*275*). As 17q gain is the most frequent genetic alteration reported in NB and associated with a poor outcome (*276*), its transcriptional outputs need to be studied further, it may have role in determining disease phenotype. *ncRAN* lncRNA possesses an oncogenic property, as the overexpression of *ncRAN* is correlated with the aggressiveness of the high-risk NB (*277*). Though the mechanistic and functional role of *ncRAN* still needs to be investigated, this finding opens up the importance of analyzing the role of lncRNA transcripts that harbor in the chromosomal gain/loss regions related to NB stratification.

lncUSMycN

Recently *Liu and colleagues* reported a lncRNA *lncUSMycN*, which is located 14 kilobases upstream of the MYCN gene and maps to the frequently amplified chromosome 2p region. *lncUSMycN* is often co-amplified and overexpressed with the 130-kb amplicon containing *MYCN in MYCN* amplified NB cell line and its overexpression negatively correlates with patient overall and event-free survival. The transient/stable knockdown of *lncUSMycN* leads to decreased *MYCN* mRNA and its protein as well. *lncUSMycN* has an important role in *MYCN* RNA upregulation and cell proliferation in NB cells by interacting with its functional protein co-partner Nono, an RNA binding protein (*278*).

6p22 locus derived lncRNAs

Chromosome 6p22 locus harbors NB risk associated SNP, *rs6939340* where homozygous condition for 'G' allele is associated with aggressive NB (*85*). The SNP is located in the intronic region of two lncRNAs, *NBAT1* (neuroblastoma associated transcript1, *LOC729177 or CASC14)* and *LINC00340* (*CASC15*). These two lncRNAs' transcriptional activity correlates with the polymorphism *rs6939340,* which overlap with a putative enhancer. Both of the lncRNAs possess a tumor suppressor role in NB, which will be described in detail in this thesis. Another transcript of 6p22 locus is *CASC15-S, which* shares the promoter with *NBAT1* but transcribes in opposite direction, also act as a tumor suppressor for NB (*279*) . Any significant correlation was lacking between the risk genotype (*rs6939340)* and the *CASC15-S* transcript expression as verified by expression quantitative trait loci (eQTL) analysis (*279*). To address this

shortcoming orthogonal methodologies have been used by this study to fine map more SNPs which are associated with the 6p22 locus and identified another SNP which mapped to *rs9295534*, overlapped with an enhancer, located upstream of *CASC15-S*. Interestingly rs9295534 risk allele disrupts the enhancer function. Further, it has been shown the low expression of *CASC15-S* is associated with more aggressive NB by inducing proliferation and invasiveness of NB cells and substantial suppression of differentiation- specific proneural gene signatures that lead to decreased overall survival probability in NB (*279*).

Paupar

Another highly conserved lncRNA is *Paupar*, which is identified as a differentially expressed lncRNA transcript during mouse neuronal differentiation using N2A NB cells. *Paupar* controls a complex transcriptional network; in *cis*, it regulates expression of upstream gene *Pax6*, which is a known neural transcription factor. In *trans* regulation, *Paupar* regulates genes located on different chromosomes as well. *Paupar* knock-down disrupts the cell cycle profile and induces differentiation of N2A cells (*280*).

Other NB associated lncRNAs

In recent years, many other lncRNAs have been reported to have a significant role in NB disease progression. Six *MYCN* regulated lncRNAs were identified using a non-coding RNA microarray based screen and were characterized for their functional role in NB pathogenesis (*281*). Luciferase and ChIP assays demonstrated *MYCN* represses the promoter activity of *linc00467,* one of the six MYCN dependent lncRNAs, by directly binding to the promoter of *linc00467*. Further downregulation of *linc00467* led to reduced cell viability and elevated apoptosis via activating the tumor suppressor gene *DKK1* in NB cells. Apart from regulating lncRNA expression by direct binding, MYCN can also regulate lncRNA expression via activation of chromatin remodeling genes. It was reported in a recent study that *MYCN* activates *MALAT1* lncRNA via modulating the expression of its direct target genes *JMJD1A*, which is an H3K9-specific histone demethylase (*282*). Elevated *MYCN* expression was thought to induce higher JMJD1A levels and which in turn activates *MALAT1* by histone H3K9me3 demethylation at the *MALAT1* gene promoter. Moreover, it has been shown *MALAT1* can induce cell proliferation, cell invasion and cell migration of NB cell lines. JMJD1A

inhibitor DMOG treatment in NB cell lines demonstrates that MYCN target genes can be potential therapeutic option for NB treatment.

The higher expression of another lncRNA *SNHG16* was found to be positively associated with poor clinical outcomes and additionally silencing of this transcript leads to inhibition of cell proliferation and migration, and induced cell cycle arrest at the G0/G1 phase in NB SH-SY5Y cells (*283*).

RNA-seq analysis of *MIAT* lncRNA knockdown NB and glioblastoma cells reveals its oncogenic role and further characterization of *MIAT* function indicates that knockdown of *MIAT* can reduce survival and cell migration and promote basal apoptosis (*284*).

LncRNA *KCNQ1OT1* has been shown to induce apoptosis in NB cells by sponging the miR-296-5p so that it cannot bind to 3'UTR of its target apoptotic marker Bax (*285*).

2 AIM

The main objectives of the current thesis was to understand the role of long non-coding RNAs in pediatric cancer neuroblastoma (NB) development and progression. In all three papers of this thesis, we used NB risk-associated 6p22 locus as our model system to understand the specific roles of 6p22 locus derived lncRNAs in NB development and progression.

Paper I

- Identification of differentially expressed lncRNAs between low- and high-risk NB tumors.
- To functionally characterize the identified NB associated transcript 1 (*NBATI*) as a biomarker that could predict clinical outcome of NB.

Paper II

- Identification and functional characterization of *CASC15* as tumor suppressor lncRNA derived from 6p22 in NB.
- To characterize the protein partners of 6p22 derived lncRNAs that are involved in NB pathogenesis.
- To explain the functional regulatory axis of 6p22 derived lncRNAs which maintain an undifferentiated state in NB cells.

Paper III

- Identification of *NBAT1* lncRNA as a p53 responsive lncRNA which can regulate p53 subcellular localization.
- To understand the mechanism, how *NBAT1* lncRNA determines the genotoxic drug resistance in NB cells.
- Identification of a potential therapeutic approach for high-risk NB with defective p53 cellular localization.

3 MATERIALS AND METHODS

Patient and Tumor Material

The primary NB tumors were collected from surgery followed by a snap freeze and storing at -70° C for further analysis. In Paper I a set of 15 tumors we selected and extracted RNA from these tumors were sequenced - three low-risk tumors with numerical only genotype and 12 high-risk tumors with both 11q deletion (n = 6) & MYCN-amplification (n = 6). We used two cohorts of NB patients for further analysis in paper I. In cohort I, there were 106 Swedish and 2 German patients with ethical permit number (Dnr 2011/354), approved by Regional Ethical Review Board, Uppsala University, Uppsala. Cohort II consists of 498 patients and RNA-seq data from this cohort has been used (NB2004, 04-049; NB97, 9764; an ethical committee of the Medical Faculty of the University of Cologne). In Paper II along with cohort II, we extended the RNA-seq data from 59 Swedish patients who are part of cohort I.

Cell culture and differentiation

The human NB cell lines we used are SH-SY5Y, IMR32, SK-N-BE(2), SK-N-AS, SK-N-DZ, SK-N-F1, Kelly and SHEP for our studies and all of them were maintained in 37°C with 5% carbon dioxide. Dulbecco's modified Eagle's medium (DMEM) media supplemented with 10% fetal bovine serum (FBS) and antibiotics were used for culturing SH-SY5Y, SK-N-BE(2), SK-N-AS, SK-N-DZ, SK-N-F1 cell lines. For the IMR32 cell line, EMEM media supplemented with 10% FBS, 1% Glutamax, 1% Sodium pyruvate, 1% non-essential amino acids and antibiotics were used. SHEP and Kelly cell lines were cultured in RPMI media supplemented with 10% FBS, 1% glutamax and antibiotics.

For differentiation of SH-SY5Y cells, cells were seeded in a density of 60,000 cells/ml using 'pre-differentiation media' which is Dulbecco's modified Eagle's medium with F12 medium (1:1) (Gibco) supplemented with 10% heat-inactivated FBS, 2mM L-glutamine and antibiotics, 24h prior to differentiation induction. After 24 h, differentiation was induced by adding 10 mM all-trans retinoic acid (ATRA/RA, Sigma) along with "Differentiation medium" which is 1:1 mix of DMEM and F12 medium supplemented with only 1 % heat-inactivated FBS, 2 mM L-glutamine and antibiotic. Cells were supplied with fresh differentiation medium with RA every 3 days of the differentiation process. Cells were collected at desired time points. SK-N-BE(2), SK-N-AS, IMR32 cells were also differentiated. These cells were plated in their

respective culture media containing 10 % FBS, 1% glutamax and antibiotic in a density of 60,000 cells/ml and after 24 hours, differentiation media which is their respective culture media with 1% FBS, 1% Glutamax and antibiotic was added along with 10 mM RA to induce differentiation. Differentiation media containing 10 mM RA was changed every third day.

Immunofluorescence

NB cells were seeded on the glass coverslips, placed into the 24 well cell culture plate in a density of 50,000 to 60,000 cells/ml for differentiation experiments (paper I & paper II) and in a density of 140,000 cells/ml for genotoxic drug treatments followed by immunostaining experiments (paper III). Cells were incubated for 24 hours and attached to the coverslip. After 24h, the desired treatments has been started and at the different chosen time points cells on the coverslips were fixed for 15 minutes with 3.7% formaldehyde followed by 5 min washes (3 times) with phosphate-buffered saline (PBS). Next cells were permeabilized for 10 min with PBS containing 0.25% Triton X-100 (PBST) followed by 5 min washes (3 times) with PBS. Then using 1% Bovine Serum Albumin (BSA) in PBST, blocking was performed for 30 minutes. The slides were incubated with desired primary antibodies such as Tau (136400, Invitrogen), Tyrosine-hydroxylase (TH) (P21962, Invitrogen), p53 antibody (Millipore, 05-224), CRM1 antibody (CST, 46249S) for 1 hour at room temperate followed by 5 min washes (3 times) with PBS. Next coverslips were incubated with respective fluorescent tagged secondary antibodies for 1 hour at room temperature followed by 5 min washes (3 times) with PBS. Then the coverslips were mounted on a slide with DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) containing prolong gold anti-fade reagent mounting media (1972784, Invitrogen). Slides were kept at 4 ° C until microscopic images were taken.

Immunoblot

Proteins were extracted from cultured cells with different treatments, using cell extraction buffer (Thermo Fischer Scientific, 1868573A) and complete protease inhibitor cocktail (Roche Diagnostics). A brief sonication (for better extraction of the chromatin-bound proteins) was performed, followed by spinning the lysate down at 13000 rpm for 20 min at 4°C, where the supernatant contains the protein of interest. Protein concentrations were measured by using the Pierce BCA Protein Assay Kit (Thermo Fischer Scientific). Lysates (20-30 mg) were resolved on 4-12% Bis-Tris Gel (NuPAGE, Invitrogen) followed by a transfer of proteins to a nitrocellulose membrane. Then the nitrocellulose membrane was blocked for 1 hour in 5%

skim milk (Cat#70166, SIGMA-ALDRICH) in 1X TBS-T (10 mM Tris-base pH 7.5, 150 mM NaCl, and 0.1% Tween) at room temperature. Membranes were then incubated with primary antibodies in the same blocking buffer against desired proteins in a particular ratio according to manufacturers' protocol for overnight at 4°C followed by 5 minutes (3 times) washes with 1X TBS-T. One hour incubation with secondary antibodies, with 5% skim milk made in 1X TBS-T was carried out, followed by 5 minutes (3 times) washes with 1X TBS-T. Using chemiluminescent Substrate (Cat#34080, Thermo Scientific) protein bands were visualized with the help of IMAGE BioRad 4.0 alpha software.

MTT, migration, invasion assay

The MTT assay was performed in NB cells with different conditions or after genotoxic drug treatments, using the Cell Titer 96 Non-Radioactive Cell Proliferation assay kit from Promega (G4000), according to the manufacturer's protocol.

The migration and invasion assay was performed with the help of biocoat invasion chamber (BD bioscience, 354480) following the manufacturer's instructions. In brief, cells were seeded in a density of 50,000-60,000 cells/ml in the upper chamber of the biocoat invasion chamber in respective media with 1 % serum (FBS) and the lower chamber was provided with 10 % FBS media which allow cells to migrate from the upper chamber to the lower chamber of the biocoat invasion chamber and then migrated cells were stained with SNABB-DIFF Kit (Labex AB, Sweden) and were counted under light microscopy.

Colony-forming assay (CFU)

10,000 cells were seeded per well in six well plates and were allowed to grow colonies for 10-12 days. Cells were then washed twice with PBS for 5 min followed by fixation with 100% Methanol for 5 min at room temperature.10 minute's incubation with 1% crystal violet solution was then performed after removing the fixation solution. The crystal violet solution was then removed and the plates immersed in tap water to carefully rinse off the crystal violet. After a short air-drying, images were taken.

Chromatin immune precipitation (ChIP)

Cells were fixed using 1% formaldehyde at RT for 10 minutes. Using 1X nuclei isolation buffer (40mM Tris-HCl pH7.5, 20mM MgCl2, 4% tritonX-100, 1.28M sucrose) nuclei were isolated from the formaldehyde cross-linked cells followed by resuspension in lysis buffer (0.1%SDS, 0.5% TritonX 100, 20mM Tris-HCl pH7.5, 150mM NaCl) with a ratio of 1ml lysis buffer per 10 million of cells. Then cells were sonicated (Bioruptor, around 30 cycles) to get chromatin fragments of about 500bp. Immunoprecipitation of chromatin using antibodies against the desired proteins was carried out for 4-5 hours at 4°C where chromatin was incubated with Protein A magnetic beads followed by washing and elution of the chromatin from the beads and then the DNA was purified and used for further analysis like ChIP quantitative-PCR.

4 RESULTS & DISCUSSION

4.1 The Risk-Associated Long Noncoding RNA *NBAT1* Controls Neuroblastoma Progression by Regulating Cell Proliferation and Neuronal Differentiation.

Neuroblastoma (NB) is an extra-cranial childhood malignancy of the sympathetic nervous system and accounts for nearly 6-10% of childhood cancers and 15% of cancer death in children. NB tumors are highly heterogeneous and depending on their clinical outcome, they can be divided into distinct clinical subtypes. Broadly, NBs can be classified into two subtypes – high-risk tumors (with *MYCN* oncogene amplification, 1p, 11q deletion and 17q gain) that are aggressive and has a high chance of recurrence and show unfavorable outcome. On the other hand, the prognostically favorable low-risk tumors (only with chromosomal copy number variation) with a high chance of regression. We aimed to characterize the mechanisms by which low-risk NB tumors undergo spontaneous tumor regression. Towards this main aim, we characterized differentially expressed transcripts between high- and low-risk NB, by sequencing transcriptomes of low-risk NB tumors and two high-risk subtypes; NBs harboring 11q deletion or *MYCN* amplification. We obtained 584, 756 and 70 differentially expressed transcripts in the comparisons between low-risk vs 11q deletion, MYCN and high-risk tumors respectively. We obtained 24 non-annotated lncRNAs from differential expression analysis of only lncRNAs between high and low-risk subtypes. We named them Cuff 1-24 and validated some of their expression in an independent cohort of 23 primary NB tumors. Some of these lncRNAs mapped to chromosomal loci that have been implicated in oncogenic (MYCN amplified region) and tumor suppressor functions (11q and 1p). This suggests that these differentially expressed lncRNAs can act as oncogenic or tumor-suppressing genes. We also did a mutational screening analysis of differentially expressed genes and found that 27.4% of them carried somatically acquired mutation or insertion and deletion.

We identified an annotated but uncharacterized lncRNA *LOC729177*, which showed differential expression between high and low-risk tumors, and we named this RNA *NBAT1* (NB associated transcript-1). We validated the expression of *NBAT1* in an independent cohort of 93 NB patients using qRT PCR analysis, and in RNA-seq data obtained from another independent clinical

cohort of 498 NB tumors. *NBAT1* is expressed at a lower level in high-risk tumors than low- risk tumors and its decreased expression correlates with poor prognosis in NB patients. We performed the multivariate Cox regression analysis on a large cohort of patients (n=498) and the result suggests that *NBAT1* can act as an independent prognostic marker for predicting event-free survival in NB patients. We also observed that a known high-risk associated SNP rs6939340 resides in the intron of *NBAT1* on chromosome 6p22. To investigate if there is any functional connection between NB risk associated SNP and *NBAT1* expression, we sequenced NB patients' genomic DNA from 51 high-risk primary tumors over the SNP and found that G/G genotype is associated with lower *NBAT1* expression in high-risk tumors. By analyzing DNA methylation of 5 CpG sites flanking the *NBAT1* promoter, we showed that the *NBAT*1 promoter was hyper-methylated in the high-risk NBs and under-methylated in low-risk NBs. These observations collectively indicate that both CpG methylation at the *NBAT1* promoter and the high-risk NB-associated SNP rs6939340 functionally contribute to the differential expression of *NBAT1*.

To understand the functional role of *NBAT1* in NB disease development, we downregulated *NBAT1* lncRNA in the SH-SY5Y cell line using siRNAs or sh-RNA. We found that the downregulation of *NBAT1* resulted in increased cell proliferation and migration in SH-SY5Y cells, which is consistent with *NBAT1* tumor suppressor properties. To identify the putative targets of *NBAT1*, we sequenced total RNA obtained from control, *NBAT1* depleted, and we found 1348 differentially expressed genes. Biological pathway analysis of the differentially expressed genes revealed that enrichment of biological processes such as cell migration, proliferation, neuronal differentiation, and nervous system development. To understand the significance of *NBAT1* target genes in *NBAT1* mediated cell proliferation and migration, we chose genes such as *SOX9, VCAN,* and *OSMR* with oncogenic properties. Downregulation of these genes in the *NBAT1* depleted cells reversed the high proliferative and invasive properties of *NBAT1* depleted cells.

We then addressed the mechanisms underlying the *NBAT1* dependent suppression of genes involved in oncogenic pathways. To this end, we performed RNA immunoprecipitation assay (RIP) using the EZH2 antibody and found that EZH2 specifically interacts with *NBAT1*. EZH2 is a member of the PRC2 complex, which is required for the maintenance of repressive mark H3K27me3. To further understand the functional connection between *NBAT1* and EZH2, we sequenced total RNA from *NBAT1* and EZH2 depleted SH-SY5Y cells and found a significant overlap of genes implicated in cell proliferation and cell migration. Interestingly, a large portion of the overlapped

genes was enriched with H3K27me3 modification and this repressive modification at the *NBAT1*/EZH2 target gene promoters was lost upon *NBAT1* downregulation. These results indicating that *NBAT1*/EZH2 functional interaction is critical for the repression of genes implicated cell proliferation and migration.

As we found neuronal differentiation as one of the top biological processes that are disturbed following *NBAT1* knockdown, we investigated the role of *NBAT1* in neuronal differentiation in SH-SY5Y cells. SH-SY5Y cells represent undifferentiated neural progenitors and they differentiate into neuronal cell types upon treatment with retinoic acid. We observed that *NBAT1* is significantly upregulated during retinoic acid (RA) induced neuronal differentiation of SH-SY5Y cells and its downregulation resulted in perturbed neuronal differentiation. Ectopic expression of *NBAT1* in NB cell lines with poor differentiation capacity such as SK-N-BE (2) and SK-N-AS, representing high-risk tumors, led to increased neuronal differentiation, and this observation is consistent with the pro-differentiation property of *NBAT1*.

We were next interested in investigating the reasons underlying the downregulation of neuronal lineage-specific genes upon *NBAT1* knockdown. We found that a neuronal-specific silencing factor *NRSF/ REST*, which represses the neuronal genes' expressions in non-neuronal tissues and embryonic stem cells, was upregulated following *NBAT1* depletion. We also found that this gene has an increased expression in MNA tumors in comparison to the non-MNA tumors in the R2 data set (A genomic analysis and visualization platform). Therefore, we sought to identify the connection between *NRSF/REST* and *NBAT1* during the neuronal differentiation of NB cells. Depletion of *REST* in the *NBAT1* knockdown SH-SY5Y cells, rescued the neuronal phenotype, indicating that *NBAT1* may control neuronal commitment via repressing the *NRSF/REST* pathway.

In summary, this study has identified several high-risk or low-risk NB specific lncRNAs, which could serve as potential biomarkers for disease assessment. Besides, our study has identified a novel NB-specific lncRNA *NBAT1*, whose higher expression predicts a good prognosis, and importantly, it acts as independent prognostic marker of event-free survival. Our functional investigations revealed that *NBAT1* is a tumor suppressor. It inhibits cell proliferation and migration by epigenetically repressing oncogenes and promotes neuronal differentiation via repressing *NRSF/REST* pathway (Figure 5).

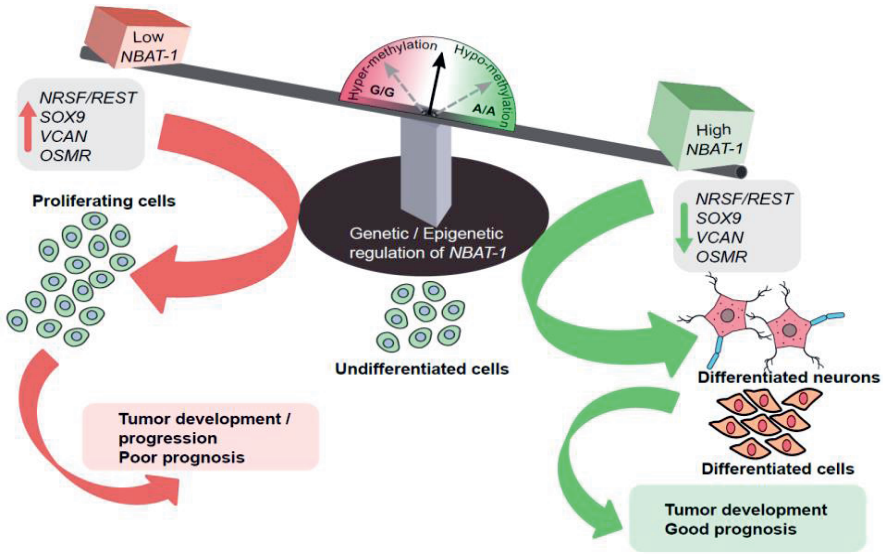

Figure 5. Proposed model explaining the tumor suppressor and neuronal differentiation properties of NBAT1. NBAT1 controls tumor progression by suppressing tumor-specific genes such as SOX9, VCAN, and OSMR via regulating chromatin structure epigenetically. On the other hand, high expression of NBAT1 promotes neuronal lineage commitment by suppressing NRSF/REST gene. ***Adapted from Pandey GK, Mitra S et al; Cancer Cell; 2014;*** *26(5):722-37.*

4.2 Sense-Antisense lncRNA Pair Encoded by Locus 6p22.3 Determines Neuroblastoma Susceptibility via the USP36-CHD7-SOX9 Regulatory Axis

Genome-wide association studies identified disease-associated SNPs but the role of such associations in the disease context has not been well understood. In many instances, the disease-associated SNPs overlap with lncRNA loci but again the functional connection between such SNP associated lncRNAs and disease has not been investigated in detail. Several SNPs located on the 6p22 locus are associated with aggressive NB and these SNPs overlap with 6p22 derived lncRNAs. In this paper II, we attempted to explore the role of 6p22 derived lncRNAs in NB pathogenesis.

In Paper I, we have characterized the role of 6p22 derived lncRNA *NBAT1* in NB pathogenesis. We have shown that *NBAT1* is expressed at a low level in high-risk tumor and this promotes the proliferation of the NB cells as well as prevents their differentiation. The 6p22 locus also contains another lncRNA named *CASC15* which is a sense partner to the antisense lncRNA *NBAT1*. *CASC15* lncRNA has 6 major isoforms *CASC15-001* to *CASC15-006*. Analysis of the RNA-seq data from 59 NB tumors from a Swedish cohort revealed that both *CASC15* and *NBAT1* expression is downregulated in high-risk NBs. Genotyping of 39 NB tumors for an SNP: rs6939340, associated with aggressive NB, revealed that *CASC15* and *NBAT1* transcripts were expressed at lower level in tumors with homozygous for the risk allele G (G/G) compared to the tumors homozygous for the non-risk allele (A/A) or heterozygous (A/G) allele, suggesting that the risk genotype (G/G) contributes to lower expression of 6p22 derived lncRNAs. Kaplan-Meier (KM) analysis revealed that *CASC15* lower expression predicts poor overall (OS) and event-free (EFS) survival in both the Swedish and German cohorts of NB tumors. Next, we wanted to explore in detail the role of different *CASC15* isoforms in NB disease development and progression. To address this, we analyzed RNA-seq data from 498 tumor dataset and found that among the *CASC15* isoforms, *CASC15-003* and *CASC15-004* were widely expressed and showed significant association with NB-specific clinical features such as age, risk (high/low) and MYCN status. In addition, higher expression of both *CASC15-003* and *CASC15-004* could predict good prognosis, suggesting that 6p22 locus derived lncRNAs harbor tumor suppressor functions. Consistent with this observation depletion of the *CASC15-003* and *CASC15-004* in SH-SY5Y cells resulted in increased cell proliferation, cell migration, and invasion property but the effect of the knockdown was more pronounced in the case of *CASC15-003* compared to *CASC15-004*. Consistent with *in vitro* cell culture data, xenografts derived from *CASC15-003* depleted cells, but not *CASC15-004* cells, had increased tumor growth. Furthermore, Gene Ontology (GO) analyses of transcriptomes of *CASC15-003* and *CASC15-004* depleted cells also revealed enrichment of biological pathways associated with tumor development and progression.

Next, we wanted to investigate if *CASC15* isoforms have any role in the neuronal differentiation of NB cells. The expression of *CASC15* isoforms was upregulated during RA mediated differentiation of NB cells. We observed that depletion of both the *CASC15* isoforms lead to perturbation in the neuronal differentiation but the effect was more evident in *CASC15-003* depleted cells compared to *CASC15-004*. To further reconfirm the role of *CASC15-003* in neuronal differentiation we deleted the *CASC15-003* promoter using CRISPR-Cas9. We obtained cells with heterozygous *CASC15-003* promoter deletions with a 40 to 60 % decrease in *CASC15-003* expression. These cells showed

perturbed neuronal differentiation following RA treatment. Gene expression analysis revealed that genes related to neuronal differentiation and stem cell maintenance were deregulated in *CASC15* isoforms depleted cells.

In paper 1, we observed that the 6p22 locus derived *NBAT1* was critical for the differentiation of NB cells. Like *NBAT1*, *CASC15* isoforms, also derived from the 6p22 locus, have similar functions in neuronal differentiation, indicate that *NBAT1* and *CASC15* isoforms may functionally cooperate with each other to regulate the differentiation of NB cells. To identify genes that are critical for the neuronal differentiation phenotype, we performed a network analysis with deregulated genes after *CASC15-003* depletion. The gene network was enriched with pathways related to neural crest development and differentiation, stem cell maintenance and differentiation. This network included some critical NB associated genes like *PHOX2B*, *SOX11,* and *SOX9*. We compared the expression pattern of these key network genes in *CASC15-003* and *NBAT1* knockdown cells and found that the network genes showed similar expression patterns. These data indicate that *CASC15-003* and *NBAT1* may regulate common pathways associated with neuronal differentiation. This prompted us to check if these lncRNAs can functionally complement each other. To do so we overexpressed *NBAT1* lncRNA in the *CASC15-003* depleted cells and vice versa and found that both the lncRNAs were able to rescue the differentiation phenotype in the corresponding cell types suggesting *CASC15-003* and *NBAT1* can functionally complement each other for neuronal differentiation. We identified *SOX9* upregulation in the *CASC15-003* and *NBAT1* depleted cells as one of the key reasons for the lack of differentiation in these cell types and knockdown of *SOX9* could rescue the differentiation phenotype perturbed in *CASC15-003* and *NBAT1* depleted cells.

Next, we wanted to investigate the reason behind the upregulation of *SOX9* in the 6p22lncRNA depleted cells. To this end, we found that the expression of chromodomain helicase DNA binding protein 7 (CHD7), a known regulator of *SOX9* in neural crest cells, was upregulated at the protein level, but not at the mRNA level, in the *CASC15-003* and *NBAT1* depleted cells. Using chromatin immunoprecipitation (ChIP) we found that increased binding of CHD7 over the *SOX9* gene promoter and its distal neural crest enhancer in the lncRNA depleted cells, suggesting that higher level of CHD7 in these cells can upregulate *SOX9* transcription, which is evident in the transcriptomic analysis. We identified *CASC15-003* and *NBAT1* as post-transcriptional regulators of CHD7 protein and these two lncRNAs could contribute to determining the stability of CHD7 in NB cells. Overexpression of *CASC15-003* and *NBAT1* in NB cells led to a decrease in CHD7 protein stability and an increased level of CHD7 ubiquitination.

We were then interested in understanding the mechanism(s) by which *CASC15-003* and *NBAT1* regulate CDH7 stability. Chromatin isolation by RNA immunoprecipitation (ChIRP) of *CASC15-003* in NB cells revealed USP36, a deubiquitinase (DUB) enzyme, as one of the interacting partners. We validated the USP36 interaction with *NBAT1* as well, using ChIRP and RNA immunoprecipitation assays. We found USP36 was a regulator of CHD7 stability in NB cells via modulating the levels of CHD7 ubiquitination. Co-immunoprecipitation (Co-IP) experiment suggested that they interact physically in NB cells and are co-localized in the nucleolus. We next investigated how the USP36 association with *CASC15-003* and *NBAT1* controls its activity. We observed that USP36, which is preferentially localized to the nucleolus in normal cells, gets distributed throughout the nucleus following *CASC15-003* and *NBAT1* overexpression whereas their knockdown leads specific accumulation in the nucleolus. Nucleolar-specific localization of USP36 occurs via its interaction with nucleolar-specific protein NPM1 and overexpression of *CASC15-003* and *NBAT1* disturbs this interaction leading to USP36 distribution throughout the nucleus. Based on these observations, we propose that during differentiation of NB cells, upregulation of 6p22 derived lncRNAs (*CASC15-003* and *NBAT1*) displace the nucleolar localized USP36, thereby affecting USP36 and CDH7 interaction at the nucleolus, and causing CDH7 degradation. But in the *CASC15-003* and *NBAT1* depleted cells USP36 remains in the nucleolus. This leads to the removal of ubiquitin molecules from CHD7 by USP36 DUB activity, and CHD7 protein level therefore remains higher during differentiation. The higher levels of the CDH7 in the *CASC15-003* and *NBAT1* depleted cells maintain an undifferentiated state of NB cells by upregulating SOX9 expression.

Using locked nucleic acid (LNA) mediated transient knockdown of *CASC15-003* and *NBAT1* in NB cells, we have shown that these two lncRNAs act synergistically to regulate the differentiation of NB cells and the synergy between these lncRNAs was also evident at the level of USP36 cellular localization. Overall, these observations in paper II provides a new mode of lncRNA function that is the regulation of protein stability via controlling the cellular localization of a DUB (Figure 6).

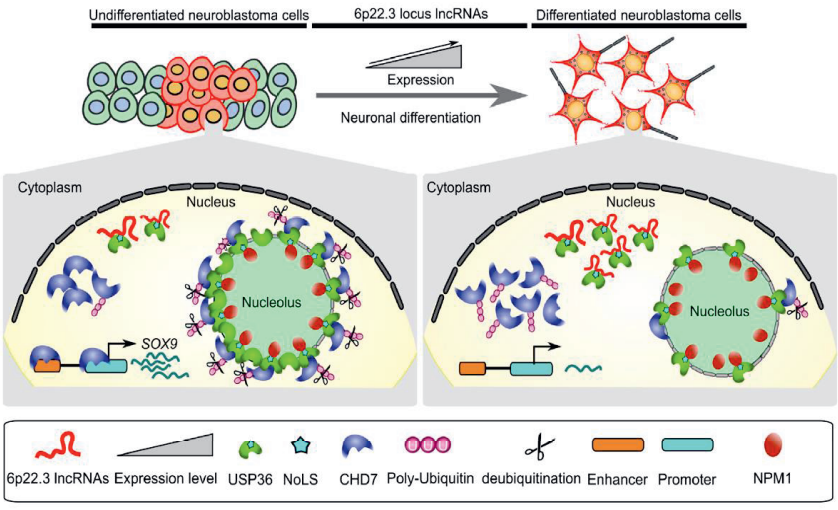

Figure 6. The proposed model depicts the role of 6p22lncRNAs during the NB cell differentiation through the USP36-SOX9-CHD7 regulatory axis. During the undifferentiated state, 6p22lncRNAs show reduced expressions and USP36 localized in the nucleolus by interacting with NPM1 through NoLS. CHD7 is co-localized with USP36 in the nucleolus and gets de-ubiquitinated and stabilized and leads to SOX9 upregulation to maintain the undifferentiated state. On the other hand, during differentiation, 6p22lncRNAs get upregulated and USP36 delocalized from the nucleolus as they interact and engaged with an elevated level of 6p22lncRNAs that leads to CHD7 ubiquitination and degradation and finally lower expression of SOX9, which leads to differentiation. ***Adapted from Mondal T et.al; Cancer Cell; 2018; Mar 12;33(3):417-434.e7.***

4.3 A p53 responsive lncRNA *NBAT1* determines chemotherapeutic response in neuroblastoma through regulating p53 sub-cellular distribution.

NB has been the most common and therapeutically challenging neoplasm during infancy. Despite multimodal therapy, including chemotherapy, immunotherapy, surgical removal of tumors followed by radiotherapy, high-risk NB tumors have a high chance of recurrence. Therefore, advancement in terms of prognostication as well as improved therapeutic opportunities is necessary for high-risk NBs. p53 is a well-studied tumor suppressor gene, involved in suppressing malignancy in a wide range of cancers, by promoting cell cycle arrest, DNA damage repair, and apoptosis in response to the cellular stress. Inactivation of the p53 transcription factor is a major cause of cancer

formation, and approximately, in 60% of all human cancers, p53 inactivation is caused by mutations in the p53 gene. Interestingly in NB tumors, p53 is rarely mutated, suggesting that p53 inactivation in NB tumors may be facilitated by mechanisms other than mutation, like altered intracellular localization of p53. Thus, understanding the alternative mechanisms, which contribute to p53 functional inactivation in NB, could contribute to developing new therapeutic approaches against NB.

Previously in the paper I of this thesis we identified *NBAT1* lncRNA, which is encoded from the 6p22, NB hotspot locus. *NBAT1* regulates tumor progression via its tumor suppressor activity. Low *NBAT1* expression is correlated with increased NB cell proliferation and poor prognosis. Based on this observation, we hypothesized that in high-risk patients, low *NBAT1* expression can be a causative factor for therapeutic resistance. We investigated in this paper, how the low *NBAT1* expression contributes to therapeutic resistance in NB tumors.

We observed that knockdown of *NBAT1* in NB cells provides resistance to genotoxic drugs whereas overexpression of *NBAT1* led to higher sensitivity to genotoxic drugs. We found a positive correlation between *NBAT1* level and sensitivity to MDM2 inhibitor Nutlin-3a treatment across different NB cell lines. Using quantitative RT-qPCR we observed that *NBAT1* expression was activated upon treatments with multiple genotoxic drugs. We verified *NBAT1* upregulation using RNA-seq data after treatment with Nultin-3a in SH-SY5Y cells. Loss and gain of function experiments of p53 combined with p53 ChIP-qPCR demonstrated that *NBAT1* is a direct target of p53. Moreover, *NBAT1* activation, due to genotoxic drugs, was also compromised in mutant p53 cell lines such as SK-N-AS and SK-N-BE2 cells. Taken together, these data indicate that *NBAT1* is a bonafide target of p53.

Gene Set Enrichment Analysis (GSEA) of the RNA-seq data of Nutlin-3a treated control and *NBAT1* depleted transcriptomes revealed enrichment of p53-regulated genes. Expression of well-known p53 regulated genes like *p21*, *MDM2* and *GADD45A* was compromised in Nutlin-3a treated *NBAT1* knockdown cells. This was further supported by ChIP-qPCR experiments wherein decreased enrichment of p53 was seen at the promoters in *NBAT1* depleted cells. Interestingly, decreased p53 promoter transcriptional activity was observed in p53 promoter controlled reporter constructs as well. These observations from the complementary experiments indicated that *NBAT1* depletion affects p53 mediated transcriptional activation under genotoxic drug treatments.

As *NBAT1* depletion affects p53 dependent gene expression and lends resistance to genotoxic drugs, the next important question arises how p53 dependent functions were disturbed in *NBAT1* depleted cells. Given the p53 mutations are rare in NB tumors, we thought that the subcellular localization of p53 might have altered in *NBAT1* depleted cells. To this end, we analyzed the localization of p53 in high- and low-risk NB tumors and we observed that in low-risk tumors p53 shows exclusive nuclear localization whereas in high-risk tumors p53 localization was nuclear-cytoplasmic. Supporting the p53 localization data in the NB tumor context, *NBAT1* depleted SH-SY5Y cells also showed preferential cytoplasmic localization compared to control cells. We also tested the effect of *NBAT1* overexpression on IMR32 cells where p53 is predominantly localized in the cytoplasm and found that *NBAT1* overexpression led to nuclear accumulation of p53 in IMR32 cells. Even ectopically expressed GFP tagged p53 (GFP-p53) was preferentially localized in the cytoplasmic compartment in the *NBAT1* depleted cells but GFP-p53 nuclear localization was recovered upon overexpression of *NBAT1*.

CRM1 is one of the well-known nuclear exporters, which transports p53 from the nucleus to the cytoplasm. We reasoned that altered cellular location of p53 in *NBAT1* depleted cells could be because of the de-regulation of the CRM1 function. To test this, we treated *NBAT1* depleted cells with CRM1 inhibitor leptomycin B (LMB) and LMB treatment could recover the nuclear localization of p53 in Nutlin-3a treated cells. We also observed that the CRM1 protein level was higher in *NBAT1* depleted cells. During genotoxic stress, p53 and CRM1 interaction are abolished, as detected by proximity ligation assay (PLA). This loss of p53-CRM1 interaction helps in the retention of p53 in the nucleus. In *NBAT1* depleted cells p53 and CRM1 interaction was still high after genotoxic stress, which correlates with cytoplasmic localization of p53. We also identified CRM1 is a target of USP36, a de-ubiquitinase (DUB) and CRM1 protein level positively correlated with the level of USP36, in NB cells. These observations suggesting that the higher CRM1 level in *NBAT1* depleted cells could be due to lower ubiquitination of CRM1 protein and this high level of CRM1 protein is responsible for p53 cytoplasmic localization in these cells (Figure 7).

Further, we used another CRM1 inhibitor Selinexor (KPT-330) which is less toxic than LMB. Selinexor alone and in combination with Nutlin-3a could also rescue the p53 nuclear localization in *NBAT1* depleted SH-SY5Y cells. The Selinexor and Nutlin-3a combination was also effective in inducing p53 nuclear localization in IMR32 cells than the individual drugs alone. Checking of apoptotic markers also indicates that apoptosis was induced more upon combination treatments in IMR32 cells. Interestingly mouse xenograft tumors

derived from IMR32 cells when treated with the Selinexor and Nutlin-3a combination were more effective in reducing the tumor size compared to individual drugs. Taken together we proposed to combine inhibition of CRM1 and MDM2 could be an effective therapeutic strategy for high-risk NB patients with lower *NBAT1* expression and cytoplasmic p53 localization.

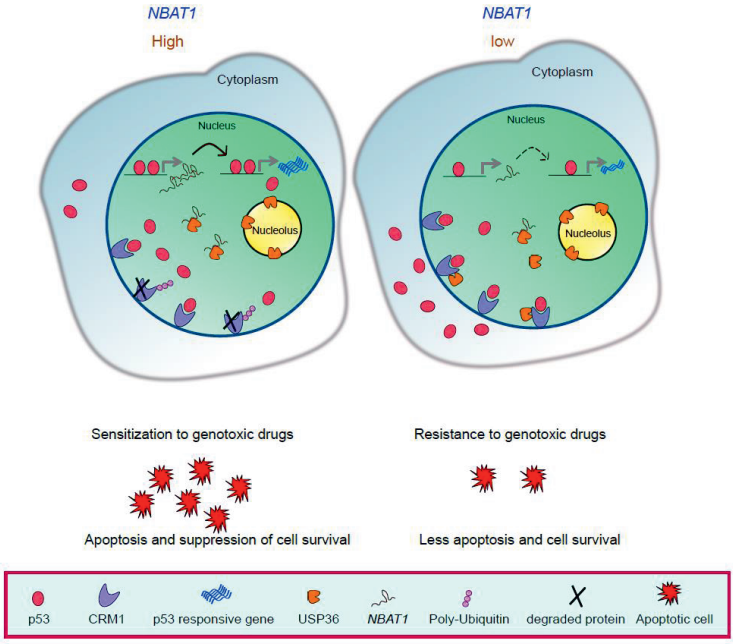

Figure 7.The proposed model depicts the mechanism of NBAT1 mediated p53 intra-cellular localization upon genotoxic drug treatment and the relation with drug-resistance.

5 CONCLUSION

The published articles and manuscript in this thesis has established the following conclusions.

Paper I

- We identified a differentially expressed lncRNA *NBAT1* between low- and high- risk NB tumors, which can serve as a biomarker for NB risk-assessment.
- A high-risk NB associated SNP on chromosome 6p22 and CpG methylation on *NBAT1* are two contributing factors for *NBAT1* differential expression.
- *NBAT1* depletion causes increase in cellular proliferation and invasion via epigenetically silencing of target genes.
- Loss of *NBAT1* affects neuronal differentiation via activating NRSF/REST that is a neuronal-specific transcription factor.

Paper II

- Sense/antisense lncRNA pair - *CASC15* and *NBAT1* encoded from trait-associated 6p22 locus show reduced expression in high-risk NB and have a tumor suppressor role.
- Decreased expression of the 6p22 derived lncRNAs are responsible for maintaining an undifferentiated state by regulating a common gene-regulatory network.
- 6p22 locus derived lncRNAs can regulate SOX9 expression via modulating USP36 (de-ubiquitinase) cellular localization which controls the stability of CHD7. CHD7, a chromatin remodeler, binds to SOX9 promoter and distal enhancer to induce SOX9 transcription.

Paper III

- 6p22 locus derived tumor suppressor lncRNA *NBAT1* is a p53 responsive lncRNA and has an ability to regulate the sub-cellular localization of p53.
- *NBAT1* depleted NB cells are resistant against genotoxic drugs via p53 cytoplasmic accumulation.
- In *NBAT1* depleted cells CRM1 function is altered and this cause the cytoplasmic localization of p53. Cytoplasmic

localization of p53 hinders p53 target genes activation during genotoxic drug treatment in *NBAT1* depleted cells.

- Inhibition of CRM1 can rescue p53 nuclear localization in *NBAT1* depleted cells.
- Combine inhibition of CRM1 and MDM2 was more effective in inducing p53 mediated gene expression and apoptosis in NB cells with cytoplasmic p53 suggesting this could be a possible therapeutic approach for high-risk NB.

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