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FUNCTIONAL ANALYSIS OF LONG NON-CODING RNAS IN CANCER

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Functional analysis of long non-coding RNAs in cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

For years the functional role of noncoding RNAs was greatly underestimated. Whole genome RNA sequencing projects that unraveled pervasive transcription emitting from large parts of the human genome changed those perspectives and prompted scientists to further look into the involvement of noncoding RNAs (ncRNAs) in development and disease. To date, we have significantly advanced our understanding of ncRNAs, however only a fraction of them has been functionally characterized. The subject of this thesis is to investigate the functional role of long noncoding RNAs (lncRNAs) and their involvement in cancer, particularly in cutaneous malignant melanoma.

Previously, the tumor suppressor gene *PTEN* has been reported to be post-transcriptionally regulated by its pseudogene. In **paper I**, we investigated an antisense RNA (*PTENpg1 asRNA*) that is transcribed from the *PTEN* pseudogene (*PTENpg1*). We uncovered various *PTENpg1 asRNA* isoforms and designated two of them as α and β. The role of the *PTENpg1 asRNA β* isoform is to form an RNA:RNA duplex with the *PTENpg1* transcript. This interaction stabilizes and assists the *PTENpg1* transcript out to the cytoplasm. On the other hand, the *PTENpg1 asRNA α* has a very different function, namely mediating epigenetic changes by recruiting EZH2 and DNMT3a to the *PTEN* promoter.

In **paper II**, we further sought out to understand the recruitment of *PTENpg1 asRNA α* to the *PTEN* promoter. We observed promoter-associated/5´UTR transcript emitting from *PTEN*, which binds and facilitates the recruitment of *PTENpg1 asRNA α* to the *PTEN* promoter. In return, *PTENpg1 asRNA* recruits DNMT3a to the promoter, which leads to epigenetic silencing of *PTEN*.

In **paper III** we investigated the role of *PTENpg1 asRNA* in vemurafenib resistance of melanoma. We observed increased *PTENpg1 asRNA* expression and consequently low PTEN levels caused by enrichment of EZH2 and H3K27me3 at the *PTEN* promoter. Further, we found that C/EBPβ transcriptionally induced *PTENpg1 asRNA* in vemurafenib-resistant melanoma cell lines. In addition, manipulation of key components of the *PTENpg1 asRNA* network caused re-sensitization of the resistant melanoma cells to vemurafenib, and high *PTENpg1 asRNA* expression was found to correlate with shorter survival in melanoma patients.

In **paper IV**, we investigated the effect of the *C/EBPβ* antisense (*C/EBPβ-AS*) transcript on transcriptional regulation of C/EBPβ. We found that C/EBPβ auto-regulates its own and also regulates *C/EBPβ-AS* expression. In return, *C/EBPβ-AS* inhibits C/EBPβ positive feedback loop by modulating epigenetic changes at the *C/EBPβ* promoter in melanoma cell lines. Interestingly, knockdown of *C/EBPβ-AS* caused re-sensitization to vemurafenib.

This thesis highlights the dynamics of lncRNAs in epigenetic silencing and their involvement in cancer and therapy resistance.

LIST OF SCIENTIFIC PAPERS

I. Johnsson P, Ackley A*, **Vidarsdottir L***, Lui WO, Corcoran M, Grandér D, Morris KV. A pseudogene long-noncoding-RNA network regulates *PTEN* transcription and translation in human cells. *Nature structural & molecular biology* 2013 Apr; 20(4): 440–446.

II. Lister N*, Shevchenko G*, Walshe JL, Groen J, Johnsson P, **Vidarsdóttir L**, Grandér D, Ataide SF, Morris KV. The molecular dynamics of long noncoding RNA control of transcription in PTEN and its pseudogene. Proc Natl Acad Sci USA 2017 Sep 12; 114(37): 9942-9947

III. **Vidarsdottir L**, Azimi A, Rahmanto AS, Petri A, Kauppinen S, Ingvar C, Jönsson G, Olsson H, Stolt MF, Tuominen R, Sangfelt O, Hansson J, Egyházi-Brage S, Grandér D, Johnsson P. *PTENpg1* antisense RNA mediates *PTEN* suppression in vemurafenib resistance and predicts clinical outcome in melanoma patients. Manuscript.

IV. **Vidarsdottir L**, Fernandes R, Zachariadis V, Das I, Edsbäcker E, Azimi A, Hansson J, Egyházi-Brage S, Johnsson P, Grandér D. C/EBPβ-AS inhibits transcription of C/EBPβ through a negative feedback loop. Manuscript.

* Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

AGO	Argonaute
AKT	Protein kinase B
AIRN	Antisense of insulin–like growth factor 2 receptor non-protein coding RNA
asRNA	Antisense RNA
bp	Base pair
BRAF	V-Raf murine sarcoma viral oncogene homolog B
bZIP	Basic leucine zipper protein
C/EBPBα	CCAAT/enhancer-binding protein alpha
CAGE	Cap-analysis of gene expression
CBX4	Chromobox homolog 4
ChIP	Chromatin immunoprecipitation
CMM	Cutaneous malignant melanoma
CTCF	CCCTC-binding factor
EED	Embryonic ectoderm development
ENCODE	Encyclopedia of DNA elements
Endo-siRNA	Endogenous small interfering RNAs
ES	Embryonic stem
EST	Expressed sequence tags
exRNA	Extracellular RNA
EZH2	Enhancer of zeste homolog 2
FACS	Fluorescence-activated cell sorting
FANTOM	Functional Annotation Of Mouse
GAS5	Growth arrest specific 5
GRO-seq	Global run-on-sequencing
H3K27me3	Histone 3 lysine 27 trimethylation
H3K4me3	Histone 3 lysine 4 trimethylation
HOTAIR	HOX transcript antisense RNA
Igf2r	Insulin-like growth factor 2 receptor
kb	Kilobase

lncRNA	Long noncoding RNA
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
MAPK	Mitogen-activated protein kinase
MENβ	Multiple endocrine neoplasia beta
mESC	Murine embryonic stem cells
miRNA	MicroRNA
mRNA	Messenger RNA
ncRNA	noncoding RNA
nt	Nucleotides
ODN	Phosphorothioate oligodeoxynucleotides
NEAT1	Nuclear enriched abundant transcript 1
PcG	Polycomb group proteins
PDK	3-phosphoinositide-dependent kinase
PI3K	Phosphatidylinositol-4,5-bisphosphate 3 kinase
piRNAs	Piwi-interacting RNAs
polII	Polymerase II
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressor complex 2
pri-miRNA	Primary miRNA
PtdIns(3,4,5)P_3	Phosphatidylinositol (3,4,5)-trisphosphate
PTEN	Phosphatase and tensin homolog
PTENpg1	PTEN pseudogene 1
RACE	Rapid amplification of cDNA ends
RIP	RNA immunoprecipitation
RISC	RNA induced silencing complex
RT	Reverse transcription
scaRNA	Small Cajal body-specific RNA
siRNA	Small-interfering RNA
shRNA	Short hairpin RNA
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA

SUZ12	Suppressor of zeste 12
TF	Transcription factors
tiRNA	Tiny promoter-associated RNAs or transcription initiation RNA
TOP1	Topoisomerase I
TSS	Transcription start sites
UCSC	University of California, Santa Cruz
UTR	Untranslated region
UV	Ultraviolet light
XCI	X-chromosome inactivation
Xist	X-inactive specific transcript

INTRODUCTION

The existence of an "RNA world" suggesting that life was solely based on RNAs had emerged long before sequencing of the human genome[1]. In spite of strong evidence for central role of RNAs in early life it was generally believed that RNAs in humans were merely intermediates between DNA and proteins. This dogma, however, changed when human sequencing projects reported that about 80% of the human genome was transcribed and only less than 1% of these transcripts would give rise to proteins[2,3]. This contradicted previous presumptions about the vast majority of the genome to be inert and not transcribed[4,5].

In the 50s, the role of the very abundant ribosomal RNAs and transfer RNAs in protein synthesis became evident[6]. Although there has been evidence for other functional RNAs over the years, they received relatively little attention. In recent years publications about the function of noncoding RNAs (ncRNAs) have been accumulating providing us with a completely new insight into their complex regulatory functions. These publications have reported on various types of ncRNAs that participate in wide variety of physiological and developmental functions. One of the most well described groups of ncRNA is microRNAs (miRNAs), which can largely affect gene expression through a variety of mechanisms. Another emerging group of ncRNAs is long noncoding RNA (lncRNA) and their functions in regulating molecular processes are being gradually discovered.

One of the great surprises in the field of lncRNAs is the discovery of their role in the regulation of epigenetic processes[7]. Chromatin-modifying proteins can alter chromatin states and influence gene expression and lncRNAs have been shown to bind these proteins and guide them to gene specific loci[8-10].

Studies of lncRNAs are not only important for our understanding of the fundamental biological pathways, but also, and importantly, to understand and to seek cure of human diseases. In this thesis I pursue to understand functional roles of lncRNAs and their roles in cancer development and progression

BACKGROUND

In February 2001, the human genome sequence was published "head to head" by two different groups. They showed that the human genome comprises of approximately 30,000 genes and sequence similarities between individuals to be 99.9%[11,12]. Interestingly, a year later it was reported that the homology between mouse genes and humans was almost identical[13]. This was paradoxical to former belief that our genomes should reflect the superiority of humans, as proposed by Comings in 1972: "Being a little chauvinistic toward our own species, we like to think that man is surely one of the most complicated species on earth and thus needs just about the maximum number of genes"[14].

These recent exploratory efforts greatly extended our knowledge of our genomes. However, the human genome project only sequenced euchromatin. At this time it was common conception that ncRNAs were "junk" that our genome had accumulated throughout evolution. In spite of few reports revealing the biological function of ncRNAs, it has taken decades for ncRNA to gain recognition in the scientific community. The exception was transfer RNA and ribosomal RNA discovered in 1950 and also a few decades ago there were reports about functional RNAs, including RNAse P, small nuclear RNAs (snRNAs) and a RNA component of the signal recognition particle, 7SL [15]. In the 90s, the ncRNA X-inactive specific transcript (Xist) was discovered. Xist mediates the inactivation process of the X chromosome in placental mammals[16,17]. This was considered the exception rather than the rule due to the unique early developmental functions carried out by XIST in shutting down activity of a whole chromosome.

The transcriptional complexity and the landscape of the mouse and human genomes were elucidated by major sequencing efforts performed by the FANTOM (Functional Annotation Of Mouse) and the ENCODE (Encyclopedia of DNA elements) consortia. The backbone of the FANTOM projects is a technique called the CAGE (cap-analysis of gene expression) technique, which captures 5´capped RNAs transcribed by RNA polymerase II (polII)[18]. By using this technique, the FANTOM3 project showed that majority of the mouse genome is transcribed and the coverage of the transcripts is about 62.5% of the genome[3]. The aim of the ENCODE project was to explore and annotate all functional elements of the human genome by using high-throughput methods. The first pilot phase of the ENCODE project indicated a lot of pervasive transcription[19] and later they showed that approximately 60-75% of the human genome is transcribed into RNAs while only 1% will code for proteins[20]. It had become increasingly difficult to overlook all of the pervasive transcription in the human genome and the fact that non-coding transcripts in humans exceeds other species[21]. So maybe after all humans are more complex than other species. To date, predicted protein-coding genes in humans are estimated around 20,000 and lncRNAs are thought to account for about 10,000[22]. This prediction might however be underestimated. For example, one recent study overlaid RNA sequencing libraries from cell lines, tumor and normal tissues that showed that 68% of the transcribed genes were lncRNAs and 79% of them were previously unannotated[23]. A plausible explanation for the discrepancy is the fact that lncRNAs are often

expressed at rather low levels in a tissue specific manner. Therefore, the number of expressed lncRNAs could increase in the future due to emerging deeper sequencing techniques and by exploring early embryonic stages. Taken together, it has become evident that cells produce large amount of lncRNAs. However, only a tiny fraction of these lncRNAs have been functionally characterized. Therefore, increased functional studies of lncRNAs could aid in understanding the onset and progression of various human diseases.

1.1 CANCER

The World Health Organization estimated 14.1 million new cancer cases worldwide in 2012 and annual rates are expected to rise to 19.3 million before the year 2025[24]. Factors, such as longevity, cancer screening programs and better diagnostics, can greatly contribute to the growing cancer incidence rates. Along with growing incidence, the efforts from academic research and pharmaceutical industries result in the introduction of novel and effective treatment modalities against cancer. In spite of these advances in cancer therapy, drug resistance remains one of the major obstacles in cancer treatment.

Cancer is a heterogeneous disease with common characteristic of abnormal cell growth that goes beyond its boundaries. In 2000 Hanahan and Weinberg described six major hallmarks of cancer: (i) self sufficiency in growth signals, (ii) evading apoptosis, (iii) sustained angiogenesis, (iv) limitless replication potential, (v) insensitivity to anti-growth signals and, (vi) activating invasion and metastasis[25]. Eleven years later the same authors added tumor microenvironment to the hallmarks of cancer[26]. Interestingly, lncRNA have been shown to be involved in all of these fundamental elements that contribute to tumor development[27] (Figure 1).

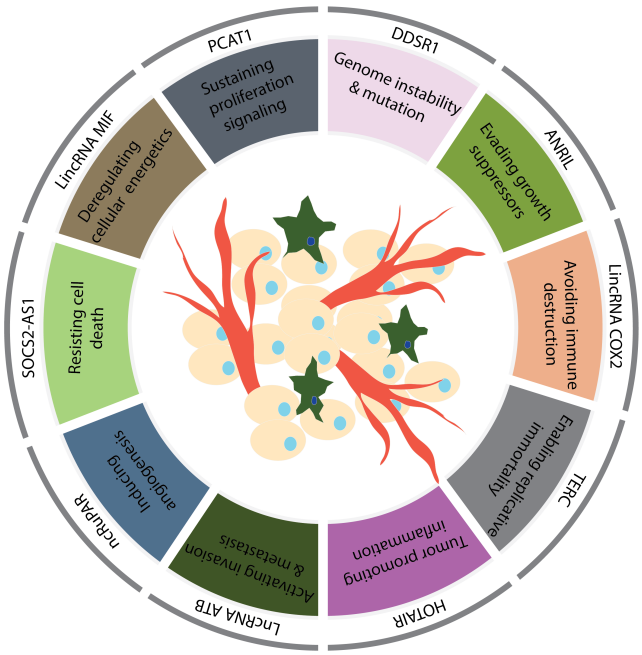

Figure 1 The hallmarks of cancer and an example of a lncRNA involved in each of the hallmarks (modified from[28]).

1.1.1 Cutaneous malignant melanoma

Melanoma is a malignant tumor that originates in melanocytes and commonly arises on the skin surface[29]. Although cutaneous malignant melanoma (CMM) account for only 5% of skin cancer, it causes 75% of all skin cancer-related deaths. Risk factors for melanomas are increased numbers of melanocytic nevi, light skin, hair and eye color and ultraviolet light (UV) exposure[29]. Only about 6-12% of new cases of CMM are family related, often carrying a germline mutations in the *CDKN2A* gene[30]. Other genetic predisposition that increase the lifetime risk of acquiring melanoma are loss of function of the cyclin-dependent kinase 4 (CDK4), mutations in the tumor suppressor *RB1* and missense substitution of the transcription factor *MITF*[30-32]. Somatic mutations contributing to melanomagenesis have been reported. The most common somatic deviations are mutations in the *BRAF* gene (66%) and *NRAS* (20%), which lead to activation of the mitogen-activated protein kinase (MAPK) pathway that stimulates cell growth and survival[33,34]. Although not as common as *BRAF* and *NRAS* mutations, *KIT* mutations are found in a small subset of patients (1%)[35]. Recently, these findings have led to the discovery of small molecule inhibitors that target the mutated forms of *BRAF* and *KIT*. The role of *BRAF* and BRAF inhibitors are described in more details in section 1.2.2.

Tumor progression of melanocytes into advanced metastatic cancer occurs in 5 steps:

Step 1. Common acquired nevus
Step 2. Dysplastic nevi (has architectural and cytological atypia)
Step 3. Radial growth phase (dysplastic lesions start to form)
Step 4. Vertical growth phase (higher risk for metastasis)
Step 5. Metastatic melanoma[36]

Patients who have their tumors detected at early stages of melanoma progression will have increased cure rates and good prognosis. Unfortunately, for those patients diagnosed with advanced metastatic melanoma, the median survival time is only 6-9 months and the cancer has become incurable[37]. For these patients chemotherapy results most often in partial responses with response rates as low as 5-12%[38] and radiotherapy is mainly used as palliative therapy[39]. Thus, detection and prevention of melanomas are a crucial part in fighting this disease. However, recent advances in drug development of small molecule inhibitors have brought some hope for metastatic melanoma patients.

1.1.2 LncRNA in melanoma

At least dozen of lncRNAs have been reported to participate in the pathogenesis of cutaneous melanoma, among those are *HOTAIR*, *MALAT1*, *ANRIL* and *GAS5*[28]. A study reported deregulation of ~100 lncRNAs upon induced expression of $BRAF^{V600E}$. In this screen the lncRNA *BANCR* (BRAF-activated non-coding RNA) was discovered and shown to influence gene expression in *trans* of genes involved in cell migration[40]. Further, knockdown of *BANCR* resulted in disruption of proliferation in melanoma cell lines and high *BANCR* expression levels were correlated with shorter patients survival[41], suggesting its pro-oncogenic functions in melanoma. Another pro-oncogenic lncRNA called *SAMMSON* was

found in 90% of melanomas. *SAMMSON* is frequently co-amplified with the transcription factor MITF but appears to function independent of MITF. *SAMMSON* transcription is driven by the lineage-specific transcription factor SOX10, and was shown to drive mitochondrial function in melanoma. Targeting of *SAMMSON* sensitized melanoma to MAPK-targeting therapeutics[42], indicating *SAMMSON* as a potential therapeutic target. The role of *PTEN* pseudogene acting as a miRNA sponge (see chapter 1.4.1) has also been suggested to contribute to the development and progression of melanoma[43,44]. Interestingly, the *PTEN* pseudogene has been shown to be deleted in ~14-21% of melanoma[43], indicating a possible role in cancer development.

1.2 TUMOR SUPPRESSIOR GENES AND ONCOGENES

Tumor suppressor genes and oncogenes are two classes of genes that can drive tumor development and progression, either through their inactivation (tumor suppressor genes) or an illegitimate activation (oncogenes). Loss of function of tumor suppressor genes can occur through e.g. mutations, chromosomal rearrangements or epigenetic silencing, while an overexpression due to mutations, chromosomal translocations, or an abnormal signaling can lead to activation of oncogenes.

In general, Knudson´s "two hit hypothesis" applies to tumor suppressor genes. Knudson´s hypothesis suggests that tumor suppressor genes loose their function if both alleles become inactive by two mutational events[45]. There are exceptions to this rule where tumor suppressor genes can also loose their function through epigenetic changes or haploinsufficiency[46]. The very well studied *TP53* gene is an example of tumor suppressor gene and has the highest mutation rates in human cancer[47]. P53 has a diverse function but is mostly known for its ability to induce cell cycle arrest and apoptosis[48]. *BRCA1* and *BRCA2* genes are also good examples of tumor suppressor genes. The *BRCA* genes play an important role in error-free DNA damage repair. Inherited mutations in the *BRCA* genes greatly increase the risk of breast, ovarian and prostate cancer[49-52].

The oncogene *Src* was the first oncogene discovered in chicken retrovirus[53]. Since then numerous oncogenes have been discovered. Another example of an oncogene is the transcription factor MYC that is frequently amplified in human cancers. Aberrant expression of the proto-oncogene MYC results in transcriptional deregulation of many genes and is known for its role in genomic instability[54]. In humans three *RAS* genes are found (*NRAS, KRAS* and *HRAS*) and all have been shown to be oncogenic in various types of cancer[55]. RAS proteins are GTPases and oncogenic mutations in *RAS* genes result in a constitutively active protein leading to activation of RAS downstream targets in the PI3K-AKT-mTOR and Raf-MEK pathways[56].

Taken together, tumor suppressors and oncogenes play a major part in tumorigenesis and tumor progression. Here, the tumor suppressor *PTEN* and the oncogene *BRAF* will be discussed in more details below.

1.2.1 PTEN

Early genetic studies revealed a partial or full loss of chromosome 10 in brain, bladder and prostate cancers[57]. Later *PTEN* (Phosphatase and tensin homolog) was mapped to chromosome 10 and germline mutations in *PTEN* were found in patients with cancer predisposition syndromes (e.g. Cowden disease)[58,59]. In addition, PTEN was often found to be dysregulated in various tumor types and *PTEN* knockout mice were shown to be prone to tumor formations[58,60]. These initial studies confirmed the tumor suppressor activity of PTEN. Interestingly, for PTEN, Knudson´t two hit model does not apply because subtle downregulation of PTEN levels can promote cancer susceptibility[61]. Therefore, PTEN needs to be maintained at stable levels in order to avoid tumorigenesis, which implies a necessity for a tight regulation of PTEN expression. PTEN levels can be regulated transcriptionally, post-transcriptionally and post-translationally. The tumor suppressor p53, early growth response protein 1 (EGR-1) and peroxisome proliferator-activated receptor γ (PPAR-γ) have all been found to be positive regulators of PTEN by binding to the *PTEN* promoter to activate *PTEN* transcription[62-64]. PTEN has also been shown to be negatively regulated by the transcription factor c-Jun that can bind to the 5´upstream sequence of *PTEN* and inhibit its transcription[65]. Furthermore, epigenetic remodelers can contribute to transcription of *PTEN* and epigenetic silencing at the *PTEN* promoter is seen in various types of cancer, including melanomas [66-68]. *PTEN* is also regulated post-transcriptionally by plethora of miRNAs that bind to the 3´UTR of *PTEN* mRNA and cause degradation of the mRNA or translational repression[69-72]. Excessive amount of particular miRNAs that target *PTEN* can therefore contribute to tumorigenesis through lowering PTEN levels[73]. Phosphorylation, oxidation, acetylation and ubiquitination can all affect PTEN at the post-translational level. These alterations can change the localization or the stability of PTEN, in addition to interfering with PTEN protein interactions[74-78].

The main catalytic function of this phosphatase is to generate the inactive form PIP_2 (Phosphatidylinositol 4,5-bisphosphate) by dephosphorylation of PIP_3 (also known as PtdIns(3,4,5)P). Typically, the PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) are activated by stimuli (hormones, growth factors, chemokines or cytokines) that bind and signal through receptor kinases or G proteins coupled receptors (GPCRs)[79]. PI3K generates the phospholipid PIP_3 by phosphorylating the 3-postion of the inositol ring of PIP_2[80]. PIP_3 is localized to the cellular inner membrane and recruits and activates PDK (3-phosphoinositide-dependent kinase) and AKT (protein kinase B)[81]. AKT is activated by phosphorylation at two different residues Thr308 and Ser473[82]. Active AKT leads to cascade activation of various proteins that drive cell survival, cell proliferation, angiogenesis and cellular metabolism[82]. Loss of PTEN will therefore lead to constitutively active PI3K-AKT-mTOR pathway and promote cell growth and survival[83] (Figure 2).

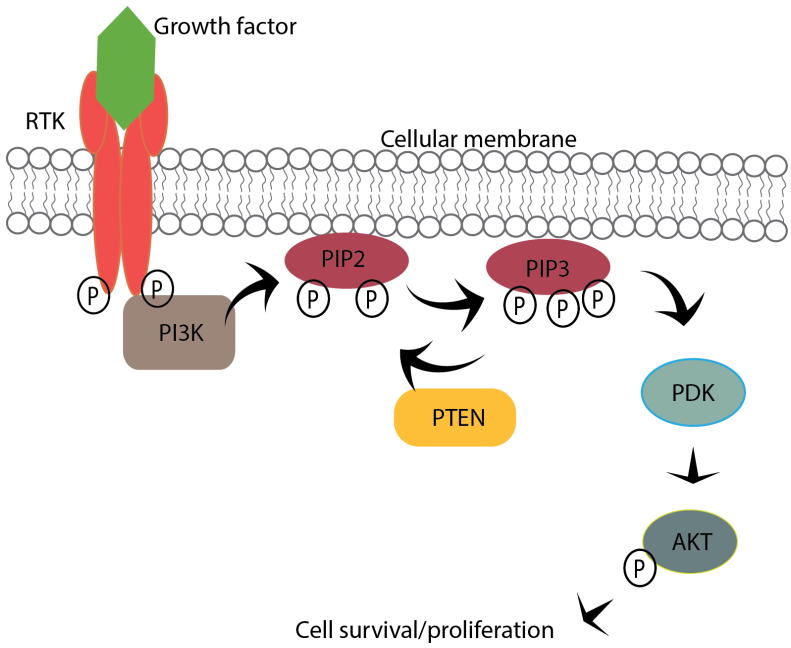

Figure 2 The PI3K/AKT pathway. Upon stimuli, a receptor tyrosine kinase (RTK) activates PI3K kinase, which catalyzes phosphorylation of PIP_2 into PIP_3. PIP_3 then recruits and activates PDK and AKT. PTEN antagonizes the PI3K/AKT pathway by dephosphorylating PIP_3.

1.2.2 BRAF

Activating mutations in the serine/threonine kinase *BRAF* (v-Raf murine sarcoma viral oncogene homolog B) are found in 8% of human tumors and the highest *BRAF* mutation incidence is observed in melanoma (27-67%)[34]. Various *BRAF* mutations have been reported that lead to elevated kinase activity; of which, the most common mutation is the glutamic acid substitution for valine at codon 600 (V600E)[34,84]. Interestingly, $BRAF^{V600E}$ is found in 80% of benign nevi, implying that *BRAF* mutation is not sufficient to induce melanoma and additional molecular event(s) are required for the tumorigenesis[85]. However, $BRAF^{V600E}$ expression in nude-mice leads to an increased ability for non-transformed melanocytes to form tumors[86] and $BRAF^{V600E}$ inhibition in xenograft models induces growth arrest and apoptosis[87,88].

BRAF is a part of the RAS/RAF/MEK/ERK signaling pathway (or MAPK pathway) that controls cell proliferation, survival, senescence and differentiation in melanoma[89]. RAS (HRAS, KRAS and NRAS), RAF (ARAF, BRAF, CRAF), MEK (MEK1, MEK2) and ERK (ERK1, ERK2) proteins are key components of the MAPK pathway. Generally, the upstream signal for activation of RAS proteins are generated by extracellular signals and activation of receptor tyrosine kinases[90]. This leads to the formation of active RAS-GTP complexes from the inactive form RAS-GDP. RAS-GTP mediates phosphorylation and activation of BRAF that promotes its homodimerization[91]. BRAF homodimers phosphorylate MEK, which in return phosphorylate and activate ERK[92]. The active ERK-regulated transcription factors activate transcription of genes leading to cell proliferation. ERK also induces a negative feedback in the MAPK pathway, by inhibiting

phosphorylation of RAS through inhibition of receptor tyrosine kinases and de-phosphorylation of BRAF[93,94].

BRAF^{V600E} inhibitors

Treatment options for melanoma patients largely depend on the stage of the disease at diagnosis. Although surgical excision is sufficient in early stages of melanoma, the therapeutic options for advanced diseases are limited due to the fact that chemotherapy and radiation have not shown to be a great success. Recently, small molecule inhibitors have been developed and shown to be effective in melanoma patients that carry activating mutations in BRAF. Vemurafenib and dabrafenib are examples of BRAF inhibitors, and 80% of patients with advanced melanoma carrying a BRAF^{V600E} mutation initially respond well to vemurafenib treatment and show tumor regression[95]. At the time of the discovery, this drug attracted a broad attention and raised hopes for effective melanoma treatment. However, it also brought a deep disappointment since the vast majority of patients developed resistance to vemurafenib, which typically emerged within 6-7 months of treatment[96]. In recent years, efforts have been made to understand the molecular mechanisms underlying the acquired vemurafenib resistance[97,98] Constitutively active PI3K/AKT pathway, due to loss of PTEN, has been shown to be one of the mechanisms contributing to vemurafenib resistance[99] (Figure 3). Mutational activation in NRAS is also commonly seen in vemurafenib resistance[100]. This activation leads to increased formation of RAF hetero- or homodimers consequently causing activation of MEK. Secondary mutations in MEK leading to activation of the MAPK pathway are also found in vemurafenib-resistant melanomas[101]. Another scenario of vemurafenib resistance is activation of the serine/threonine kinase COT (Cancer Osaka Thyroid oncogene, also known as MAP3K8) that can activate ERK but does not require RAF[102].

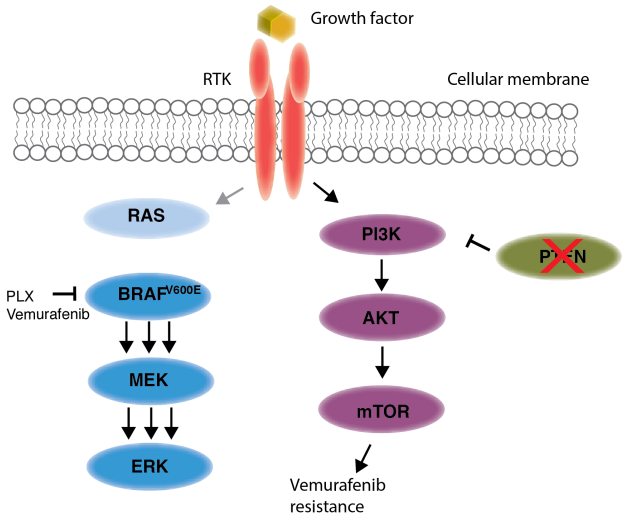

Figure 3 A Schematic depicting how loss of PTEN can contribute to vemurafenib resistance. BRAF^{V600E} leads to hyperactive MEK/ERK signaling pathway without involvement of upstream signaling. Vemurafenib targets BRAF^{V600E} and shuts down the MEK/ERK signaling pathway. Upon vemurafenib resistance the MEK/ERK signaling pathway is restored as well as the PI3K/AKT pathway is activated due to loss of PTEN to promote cell proliferation and survival.

1.2.3 C/EBPβ

Although C/EBPβ (CCAAT/enhancer-binding protein β) has not been titled as an oncogene or a tumor suppressor, it has been shown to be implicated in various types of cancers (reviewed in [103]). Therefore this gene will be included in this chapter.

C/EBPβ is a versatile transcription factor (TF) that can carry out multiple cellular functions, such as cell proliferation, differentiation and senescence. This protein is a part of the basic leucine zipper (bZIP) transcription factor family, which consists of two domains: DNA binding domain and multimerization domain[104]. Commonly, C/EBPβ will homodimerize or heterodimerize to other proteins from the C/EBP family. C/EBPβ binds double-stranded DNA in a sequence specific manner through the consensus sequence, RTTGCGYAAY (R=A/G, Y=C/T)[105], however different binding motifs for C/EBPβ have also been reported[106].

C/EBPβ is translated to three different isoforms, i.e. two long isoforms (LAP1 and LAP2) and one short isoform (LIP)[107]. The proportion between them is important for the cell to maintain normal growth and development[108]. The isoforms have been shown to be capable of carrying out different functions. For example, the LAP1 has been shown to recruit the SWI-SNF nucleosome remodeling complex to silence genes, which leads to increased access of TFs to DNA through chromatin remodeling[109]. Different isoforms of C/EBPβ can also bind to their specific promoters, e.g. only LAP2 can bind to the Cyclin D1 promoter [110].

Although methylation of CpG islands is commonly correlated with repressed transcription, there is recent evidence showing that some DNA methylated sites can have increased TF binding and induced gene activity[111-113]. For example, the promoter region of *FOXA2* gene shows high DNA methylation levels in tissue that express FOXA2[111]. CEBPβ has been reported to show enhanced binding affinity towards methylated promoters and/or CpG islands, in particular the CRE (cAMP response elements) and C/EBP motifs, leading to increased gene activity[114,115].

Dysregulation of C/EBPβ plays a role in tumorigenesis. In primary human and mouse fibroblasts that express oncogenic RAS or BRAF, C/EBPβ has been shown to guard RAS- and BRAF-mutated cells from cellular transformation by inhibiting cell proliferation and inducing senescence[116,117]. Therefore, bypassing the C/EBPβ activity is necessary for continued cell proliferation and several mechanisms of how this occurs have been suggested[118]. On the contrary, CEBPβ expression was found to be essential for formation of skin tumors in the majority of tumors carrying Ras mutations[119]. Additionally, high C/EBPβ expression can also contribute to tumorigenesis. For example, C/EBPβ has been shown to be overexpressed at late stages of breast carcinogenesis[120] and induce epithelial-to-mesenchymal transition in mammary epithelial cells[121]. C/EBPβ can also regulate metastatic genes and survival in prostate cancer cells[122,123] and mesenchymal transformation in human gliomas[124]. A better knowledge of CEBPβ contribution to tumorigenesis is needed to understand if CEBPβ could represent a promising drug target.

1.3 NONCODING RNA

Noncoding RNAs are grouped into small (<200 nucleotides (nt)) or long noncoding RNAs (>200 nt). This arbitrary classification is originally based on the Qiagen RNA isolation kit that will keep long RNAs while short RNAs will filter through[125].

1.3.1 Long noncoding RNA

LncRNAs represent a large proportion of transcripts found in the human genome. To some extent lncRNA resemble messenger RNAs (mRNAs), by often being 5´capped, polyadenylated, spliced and transcribed by RNA pol II[126]. However, unlike protein coding genes many of these lncRNAs lack sequence conservation between species and therefore the hypothesis has been that lncRNAs simply represent transcriptional noise. Indeed only 20% of lncRNA show an inter-species homology between human and mouse and this proportion is even lower, about 5%, for homology between humans and fish[127]. It is, however, important to keep in mind that although sequence conservation usually implies functionality, lack of conservation does not necessarily imply the opposite[128]. Also, the conserved function of RNAs between species could occur through secondary structures, which are not based on exact homology but nevertheless are important for RNA functions; at least this feature has been demonstrated for small non-coding RNA, like tRNA and snoRNAs. Studies on lncRNAs secondary structures are however scarce and difficult to carry out due to a steady uncertainty in the predicting models. Triplex elements are one of the few structures that have been reported to stabilize the 3´ends of the lncRNAs, *MALAT1* (metastasis-associated lung andenocarcinoma) and *MEN β,* that both lack a poly-A tail[129]. Another evolutionarily conserved domain is the tandem stem-loops in *roX1* and *roX2* RNAs in *D. melanogaster* that has been found to bind the MLE RNA helicase and the MSL2 ubiquitin ligase to mediate X-chromosome dosage compensation[130]. Recently the secondary structure of *HOTAIR* was published and notably some secondary structure elements within *HOTAIR* structure are conserved across 33 mammalian sequences[131]. However, a contradicting study claim that lncRNAs, including *HOTAIR,* do not have a conserved structure[132]. In addition to sequence and structure, functional conservation could also be obtained through the position of lncRNAs within a genome: thus the location of antisense transcripts may play an important role in regulating their sense counterpart[133].

Functional characterizations of lncRNAs are still in its infancy. One important aspect to reveal their specialized function is subcellular localization of lncRNAs. LncRNAs localized in the nucleus often act as epigenetic modulators (Figure 4A-B), while those in the cytoplasm often have post-transcriptional functions (reviewed in [134]). For example, the lncRNA *NRON* regulates trafficking of the transcription factor NFAT into the nucleus[135] and the lncRNA *SNHG5* stabilizes the mRNA SPATS2 by forming a RNA:RNA duplex making the mRNA inaccessible to the destabilizing protein, STAU1 lncRNAs (Figure 4F-E)[136,137].

One might speculate that the nuclear lncRNAs are kept in the nucleus by interacting with proteins that facilitate their subcellular localization (Figure A-C). For example, the matrix

protein hnRNP U regulates localization of the lncRNAs *XIST* and *FIRRE* to specific chromosomal loci and knockdown of hnRNP U leads to their mislocalization[138,139]. In addition, lncRNAs could be kept in the nucleus by binding to protein complexes, such as PRC2, preventing them from exiting to the cytoplasm (reviewed in [140]). Generally, it is assumed that processed lncRNAs will be exported to the cytoplasm in a similar manner as mRNAs, however this remains to be experimentally tested (reviewed in[140]).

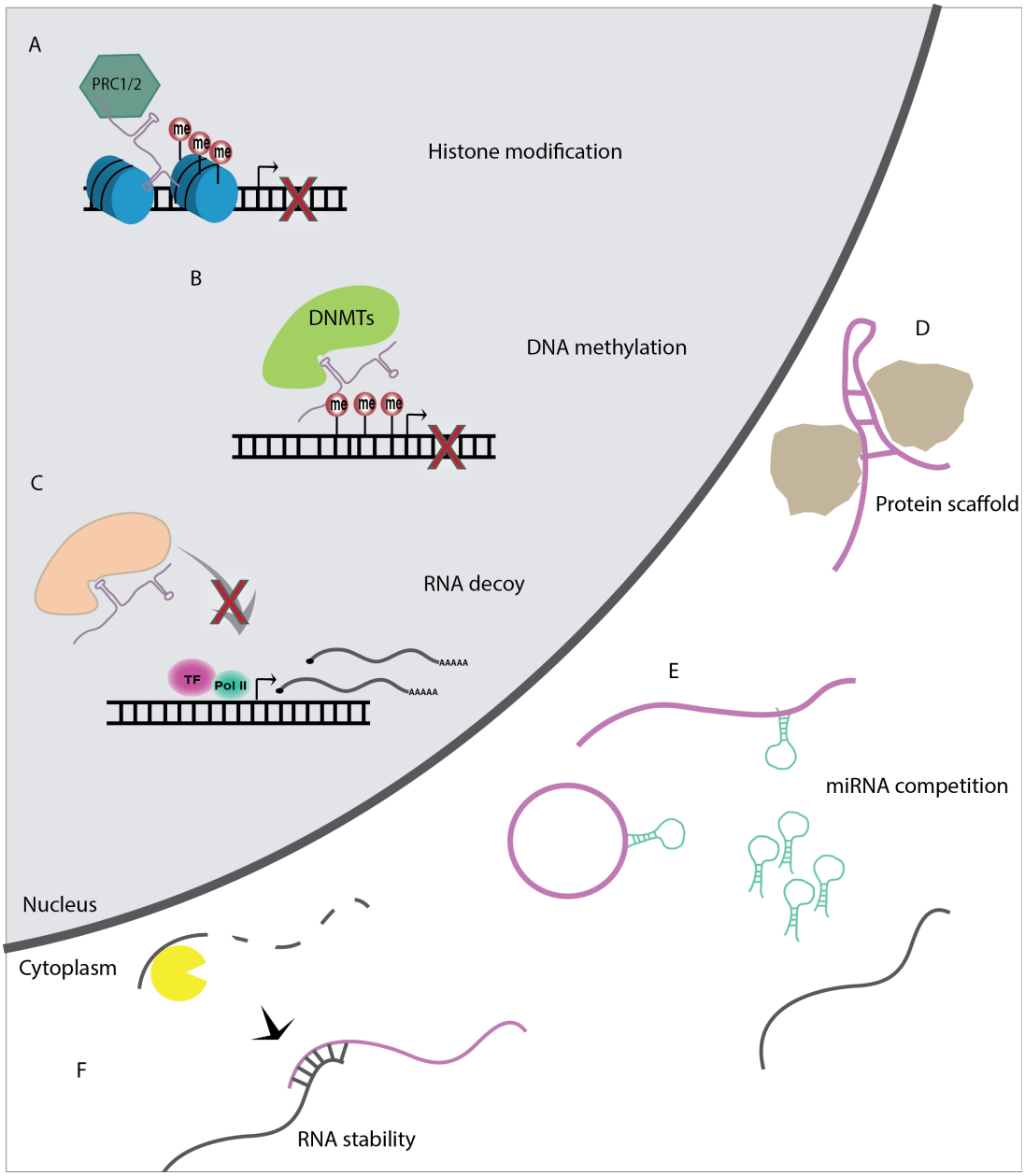

Figure 4 Functions of lncRNAs. (A-B) LncRNA recruit epigenetic modifying proteins to chromatin. (C) LncRNA can act as decoy keeping proteins away from the DNA. (D) LncRNA can act as scaffolds pulling proteins together. (E) LncRNA (and ciRNA) can act as miRNA sponges. (F) LncRNA can block accessibility of RNA degradation proteins by forming RNA:mRNA duplexes.

Although the subcellular localization of a lncRNA can help predict its function, lncRNAs are currently classified according to their genomic location and relation to protein coding genes. The main subclasses of lncRNA (Figure 5) are[22]:

- Antisense RNA (asRNA)
- Large intergenic RNA (lincRNA)
- Sense overlapping RNA
- Sense intronic RNA
- Processed transcripts (without an open reading frame (ORF) and does not fit in of the other categories)
- Trancribed psuedogenes[141]

There are other subgroups of RNA that are emerging, for example enhancer RNA (eRNA) and circular RNA (ciRNA) but they coincide in part with other subgroups of ncRNAs. LincRNAs is the most abundant class of lncRNAs and their expression is highly tissue specific[142]. Although not as common as lincRNAs, antisense RNAs that overlap their sense counterparts will be discussed here in greater details.

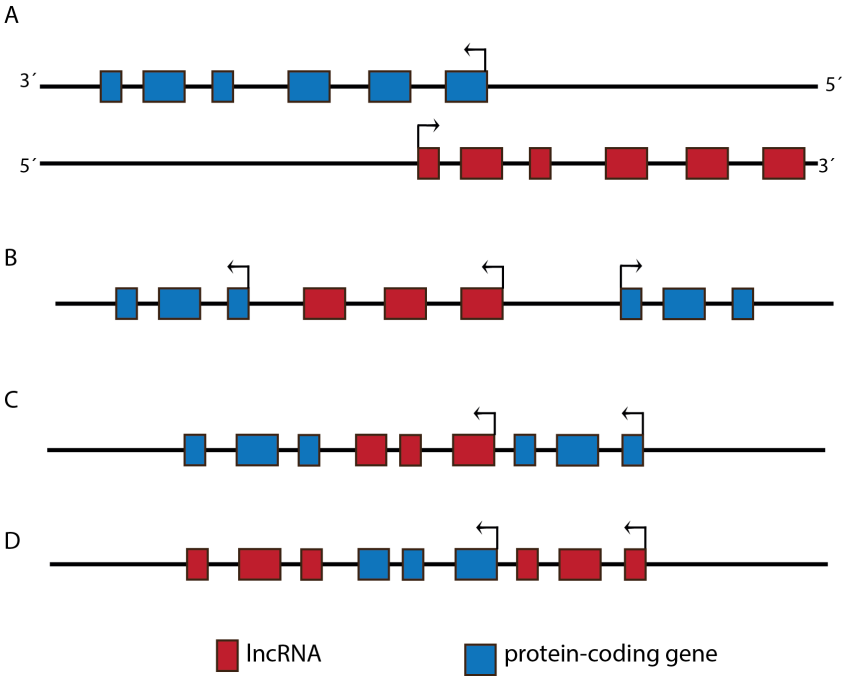

Figure 5: A schematic depicting the main subclasses of lncRNAs. (A) Antisense RNA (B) LincRNA (C) Sense-intronic RNA (D) Sense-overlapping RNA

1.3.2 *Cis*-acting lncRNA with focus on antisense RNA

First reports from the FANTOM consortium suggested that about 20% of transcribed genes have an antisense transcription[143]. Later, the FANTOM3 project and other groups reported that antisense transcripts are emitting from about 2/3 of protein-coding genes in the mammalian genome[3,144]. Antisense RNAs arise from the same genomic loci as their sense counterpart but are transcribed from the opposite DNA strand. They may be coding or noncoding RNA complements. The orientation of antisense RNA towards its sense counterpart is described as being “head to head” or “tail to tail”. There are also transcripts that

have full overlaps and antisense transcripts that are only nearby the head or the tail and therefore literally do not overlap. Antisense transcripts can act in *cis* or in *trans*. *Cis*-antisense RNAs interact with genes at the same genome loci while *trans*-antisense RNAs interact with distant genes on the same chromosome or genes on different chromosomes [145]. *Cis*- acting lncRNA can influence gene expression of overlapping gene counterpart or a gene that has a proximal location towards the lncRNA. The following are a few examples of *cis* regulatory lncRNAs.

X-chromosomal inactivation

In spite of low gene density on the X-chromosome, inactivation of one X-chromosome is vital in eutherian females to compensate for gene dosage imbalance between sexes. LncRNAs are key players in X-chromosomal inactivation (XCI) and the X inactivate specific transcript (*Xist*) is one of the most extensively studied lncRNA. *Xist* is a nucleus-localized lncRNA transcribed from the X-inactivation center (Xic) and physically coats the inactive X-chromosome[16,17,146]. *Xist* is 17 kilobases (kb) with several tandem repeats, however only one of the repeats located in the 5´end is relatively well conserved and thought to be critical for *Xist* function[146,147]. *Xist* orchestrates along with other lncRNAs a cascade of chromatin changes that lead to transcriptional silencing of the X-chromosome that is destined to be inactive, by modification of core histones through acetylation or methylation and exclusion of RNA polymerase II [148].

Xist has a 40 kb antisense transcript called *Tsix* that prevents *Xist* to be transcribed from the active X-chromosome[149]. Before the onset of X-chromosomal inactivation, *Tsix* is expressed from both X chromosomes, however, after the process has been initiated *Tsix* is only transcribed from the active chromosome[149]. Thus, on the inactive chromosome where *Tsix* is not expressed *Xist* will accumulate. It is believed that *Tsix* blocks the XCI by interacting with the polycomb repressor complex 2 (PRC2) but instead of recruiting it to the XCI it sequesters PRC2 away from *Xist* and inhibits their interactions[150]. Interestingly, *Tsix* has also been shown to interact with DNA methyltransferase 3a (DNMT3a), which are known to methylate gene promoters leading to epigenetic silencing. When this interaction was abolished DNMT3a was not recruited to the promoter of *Xist*[151] and thus could not DNA methylate the promoter leading to transcriptional activation of *Xist.*

JPX is another lncRNA involved in X chromosomal inactivation. The transcription factor CTCF (CCCTC-binding factor) represses *Xist* transcription prior to the XCI; however, after initiation of XCI, *JPX* becomes upregulated and binds to CTCF and titrates it away from the *Xist* promoter leading to *Xist* expression[152].

The complexity of the involvement of lncRNAs in XCI is a good example of the many facades of the functional role lncRNAs and the importance of the tight transcriptional regulation executed by lncRNAs.

Airn (Insulin-like growth factor 2 receptor (Igf2r) lncRNA)

The imprinted *Igf2r* gene is important in fetal and placenta development[153,154]. The IGF2 receptor has a role in binding to the major fetal growth factor IGF2 and target it for lysosomal degradation[155]. The *Igf2r* gene has two differentially methylated regions: one is located at the promoter and the other in intron 2 of *Igf2r* gene. This latter region is a promoter for an asRNA named *Airn* (antisense Insulin–like growth factor receptor). In mice, *Airn* is paternally expressed lncRNA that acts in *cis* by mediating transcriptional silencing of *Igf2r*[156,157]. Previous speculation of the mechanism of *Airn* suggested that *Airn* mediates silencing of *Igf2r* through recruitment of epigenetic proteins to *Igf2r* promoter[158]. However, more recent studies claim that *Airn* transcription will interfere with RNA pol II and thereby block the capacity to transcribe *Igf2*[159]. Interestingly, a human homolog for *Airn* is transcribed from the same genomic loci as in mice but has not been shown to be involved in imprinting of *IGF2R* and therefore has an undescribed role in humans[159].

ANRIL (antisense noncoding RNA in the INK4 locus)

In the INK4b/ARF/INK4a locus resides three possible tumor suppressor genes *p15/CDKN2B, p16/CDKN2A* and *p14/ARF*[160], and they are under tight regulation of the polycomb group complex[161]. *ANRIL* is a large antisense RNA overlapping the INK4b/ARF/INK4a locus and was first discovered in a melanoma-neural system tumor family[162]. *ANRIL* is expressed from the proximal promoter of INK4a and encodes for a 125 kb long transcript[162]. *ANRIL* binds to chromobox protein homolog 7 (CBX7), a subunit of the polycomb repressive complex 1 (PRC1), and together they mediate silencing at the INK4/ARF locus[163]. *ANRIL* has shown to have multiple isoforms and that some of them can form circular RNAs[164]. Interestingly, one *circANRIL* was shown to modulate ribosomal RNA maturation by binding to a 60S pre ribosomal assembly factor and inhibiting exonucleases mediated processing[165,166]. Furthermore, *ANRIL* can also function in *trans* by modulating various gene expression networks[167]. *ANRIL´*s different isoforms and functions is a good example of how transcriptional complexity can contribute to different functions.

WRAP53

Interestingly, p53 itself is regulated by an antisense transcript, WRAP53α, encoded by the *WRAP53* gene that overlaps the *p53* gene in a "head-to head" orientation. In this case, WRAP53α RNA interacts with the complementary region of p53 RNA and thereby stabilizes p53 RNA. Disruption of this interaction by knocking down WRAP53α or blocking the WRAP53α/p53 hybrids leads to decreased p53 RNA levels[168]. Moreover, WRAP53α RNA was found to interact with the CTCF important for the transcriptional regulation of p53 RNA expression [169].

1.3.3 *Trans*-acting lncRNA

LncRNA that directly influence gene expression on different chromosomes are *trans*-acting lncRNAs. *HOTAIR* was the first lncRNA that was reported to function in *trans*. Since then a number of publications have observed this phenomenon as a mechanism of action of a variety of lncRNAs.

HOTAIR (Hox transcript antisense intergenic RNA)

In the human genome there are 39 genes that encode for HOX transcription factors. They all reside within four different loci, HOXA-HOXD. One of the best studied lncRNA, HOX transcript antisense intergenic RNA (*HOTAIR*), resides within the HOXC locus and functions in *trans* by mediating gene silencing covering 40 kb of the HOXD locus. HOTAIR is a 2.2 kilobase (kb) long ncRNA that has two protein binding domains at the 5´ and 3´ end. Initial studies showed reduced levels of SUZ12 (a subunit of the epigenetic silencing complex PRC2) and H3K27me3 at the HOXD locus after siRNA-mediated knockdown of *HOTAIR*[170]. Interestingly, no effect on gene expression on the HOXC locus (where the *HOTAIR* gene is located) was observed. Pulldowns using biotinylated *HOTAIR* show a direct interaction with the PRC2 components EZH2 and SUZ12. *HOTAIR* is therefore thought to guide the PRC2 complex to the HOXD locus[171]. While the PRC2 complex binds to the 5´domain of *HOTAIR*, the 3´domain is thought to interact with the histone demethylases, LSD1 that is a part of a histone modification complex called CoREST/REST (Figure 4D). *HOTAIR* can therefore acts as a modular scaffold between these two complexes leading to transcriptional repression at the *HOXD* locus[172,173]. Overexpression of *HOTAIR* has been reported in breast cancer metastasis; an ectopically induced *HOTAIR* expression in breast cancer cell lines lead to cancer cell invasion and promotes colony growth [174]. Since then numerous publications have linked *HOTAIR* expression to cancer-associated processes, such as metastasis and tumor invasion as well as to prognosis [175-178].

MALAT-1 (metastasis associated lung adenocarcinoma transcript 1)

The ~8 kb long *MALAT-1* was one of the first lncRNA that was linked to human cancer and metastasis and has been shown to be overexpressed in numerous cancers[179,180]. Later the function of the *MALAT-1* was uncovered. *MALAT-1* localizes to the nuclear speckles and interacts with serine/arginine splicing factors that regulate alternative splicing. The interaction between *MALAT-1* and these splicing factors influences the distribution of these splicing factors in a nuclear speckle domain. Therefore, repressed expression of *MALAT-1* leads to alterations in alternative splicing of endogenous mRNAs[181]. *MALAT-1* has also an alternative function where it can interact with a component of the PRC1 complex (CBX4 (Chromobox homolog 4)) and thereby actively regulate gene expression, including genes associated with metastasis[182,183].

LincRNA-p21

The lncRNA *LincRNA-p21* is regulated by p53 and in return inhibits the expression of hundreds of genes that are repressed by p53. *LincRNA-p21* physically interacts with the RNA binding protein, hnRNP-K and mediates the localization of this protein to gene loci, which consequently leads to transcriptional repression[184] . In addition, *LincRNA-p21* has an alternative function as a modulator of translation. The HuR protein binds to *LincRNA-p21* leading to recruitment of let7/Ago2 and causes instability of *LincRNA-p21.* In the absence of HuR, *LincRNA-p21* levels increase, an interaction of *LincRNA-p21* with mRNAs is induced, in return leading to a lower translation of the mRNA products[184]. Since the discovery of *LincRNA-21* numerous publications have reported a link between *LincRNA-21* deregulation and cancer[185-187], yet again stressing the importance of lncRNAs in cancer development.

1.3.4 Short ncRNA

Short ncRNAs have been more extensively studied than lncRNAs. They are classified into the following subgroups: microRNAs (miRNAs), small-interfering RNAs (siRNAs), piwi-interacting RNA (piRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), extracellular RNA (exRNAs) and Small Cajal body-specific RNAs (scaRNAs). Short ncRNAs are typically between 20-30 nt and are associated with Argonaute (AGO) family proteins. MicroRNAs is the group of short ncRNAs has been most extensively studied and over 2.000 miRNAs genes have been annotated in humans [188,189].

1.3.4.1 MicroRNA

MicroRNAs are generally 20-24 base pairs (bp) in length and bind to mRNAs in a sequence specific manner, usually in the 3´ untranslated region (UTR) of mRNAs. Generally, miRNA originates from a long primary transcripts transcribed by RNA pol II called primary miRNA (pri-miRNA)[190]. The pri-miRNA contains a stem loop structure that is recognized by the nuclear RNase III Drosha that is a part of the microprocessor complex that crops the pri-miRNA down to a small hairpin structure called pre-miRNA, typically around 70 nt in length[191]. Following Drosha-mediated processing, pre-miRNAs are exported to the cytoplasm where another RNase III enzyme called Dicer cleaves of the loop leaving a miRNA duplex[192,193]. Finally, the mature miRNA duplex is loaded onto the AGO protein, which will form the RNA induced silencing complex (RISC)[194]. The miRNA will then serve as a guide for the RISC complex to the target mRNA. MiRNA targeting often leads to post-transcriptional gene silencing, and it has been suggested that about 30-60% of all human mRNA are under regulatory roles of miRNAs [195,196].

1.3.5 Promoter associated RNA

Promoter associated RNAs (RNAs) are also known as promoter-proximal transcription start site RNAs, promoter upstream transcripts or transcriptional start site-associated RNAs[197]. Deep RNA sequencing and Global Run-on-Sequencing (GRO-seq) lead to the discovery of paRNAs, which are most often lowly expressed and unstable transcripts[198,199]. There are three main subgroups to be found, again categorized according to size, tiny, short and long paRNAs. Typically, paRNAs are being transcribed in the same direction as their downstream genes[198,199] but antisense transcription can also occur. They are often associated with highly expressed mRNA transcripts and RNA Pol II binding sites[200] and thought to modify the chromatin state of their corresponding genes' promoters.

Tiny paRNAs

Tiny paRNAs or transcription initiation RNAs (tiRNAs) are typically 18 nt in length and map within -60 to +120 nt of transcription start sites (TSSs)[200]. They are not Dicer-2-, Ago1- or Ago2-dependent in contrast to miRNAs[200]. Transcription of tiRNAs is often found downstream of TSSs and therefore it is thought unlikely that they are products of RNA pol II run offs or truncated 5´capped ends[200]. A possible hypothesis for how tiRNA are generated is that RNA pol II binds to the DNA strand to initiate transcription but does not manage to push through the 1st nucleosome and therefore causes pausing or backtracking (reviewed in [201]). The functionality of tiRNAs is not fully known but they are thought to play a role in transcription. For example tiRNAs are frequently found in CTCF binding sites and inhibition of tiny paRNAs transcription leads to increased binding of CTCF to the DNA[202].

Short paRNAs

Short paRNAs are generally between 20-90 nt and originate within -50 bp to +250 bp of the TSS[198]. They have been described to be polyadenylated with a high turnover rate[203]. In embryonic stem (ES) cells short paRNAs have been shown to be lowly expressed transcripts found to be bound to chromatin loci that are enriched with RNA Pol II and histone H3 lysine 4 trimethylated (H3K4me3). Interestingly, short paRNA appear to be fairly common phenomenon and are transcribed in more than half of mouse genes. Like tiRNAs, short paRNA were found to be Dicer independent in Dicer deficient ES cells[198]. In humans, the expression levels of short paRNAs correlate to mRNA expression levels and promoter activity of the corresponding gene[198]. Short paRNAs are thought to influence gene transcription by maintaining an accessible chromatin for TFs, either by releasing negative supercoils or keeping a nucleosome-free region at TSSs[197]. Short paRNA have also been reported to act as transcriptional repressors. For example, cells transfected with short paRNA that correspond to the c-MYC TSS showed reduced MYC mRNA abundance[204]. In addition, similar to the long paRNAs, short paRNA may also give rise to endo-siRNA [197].

Long paRNAs

Accumulation of unstable long paRNAs, spanning around 250-500 nt, were first noticed in yeast that had defects in an exosome component RRP6, which degrades RNA[205]. Although long paRNAs have not been thoroughly studied they are thought to influence DNA methylation. For an example, the *Sphk1* gene has an antisense transcript that emits from the CpG island at its promoter and is capable of influencing DNA methylation status at this region[206]. Another example is a cryptic unstable antisense paRNA of TY1 in *Saccharomyces cerevisiae* that mediates silencing of TY1 expression [207]. It has also been speculated that long paRNAs could be a source for the formation of double-stranded RNAs with their overlapping counterparts giving rise to endo-siRNAs [208].

1.4 PSEUDOGENES

The term pseudogene was created on their discoveries in 1977[209]. To date, there are thought to be around 11.000 pseudogenes in the human genome thereof 876 that are transcribed[210]. In general, pseudogenes are described as duplicated genes that have lost their capacity to code for protein. It should however be stated that a recent proteomics study observed peptides being encoded by 107 pseudogenes[211]. It is, however, unknown if these peptides are being accidentally translated or if they have a physiological function. Due to the nature of pseudogenes they often retain high sequence homology to their coding parental gene[210]. Over time pseudogenes have accumulated mutations that cause premature stop codons or frame shift mutations that prevent them from being expressed. Although before thought of as "junk" DNA emerging evidence exists for their functional properties [141,209]. Pseudogenes are classified according to how they are generated from their ancestral functional genes, and the main subgroups are:

- Processed pseudogenes
- Unprocessed pseudogenes
- Unitary pseudogenes

Processed pseudogenes are mRNAs that have been spontaneously reversed transcribed and randomly inserted back into the genome, often far from the parental gene (Figure 6). Processed pseudogenes lack introns, have poly-adenine features at the 3´end and are flanked by target site duplication. Processed pseudogenes lack a promoter and are therefore dependent upon the location of its insertion for becoming transcriptionally active. Vast majority of pseudogenes are processed pseudogenes[212] and are thought to be a result of burst in retrotransposone activity in ancestral primates about 40 million years ago.

Unprocessed pseudogenes are duplicated gene loci that often are inserted near the parental gene. This process occurs when cells in DNA replication make an extra copy of a gene locus (Figure 6). Duplicated pseudogenes can therefore bare parts or full replicas of parental genes including promoters, introns and exons[213].

Unlike unprocessed and processed pseudogenes that arise from genomic insertion, unitary pseudogenes are genes that have lost their function and therefore do not have a functional parental gene in the genome. There could also be an argument for these genes gaining a new function[210].

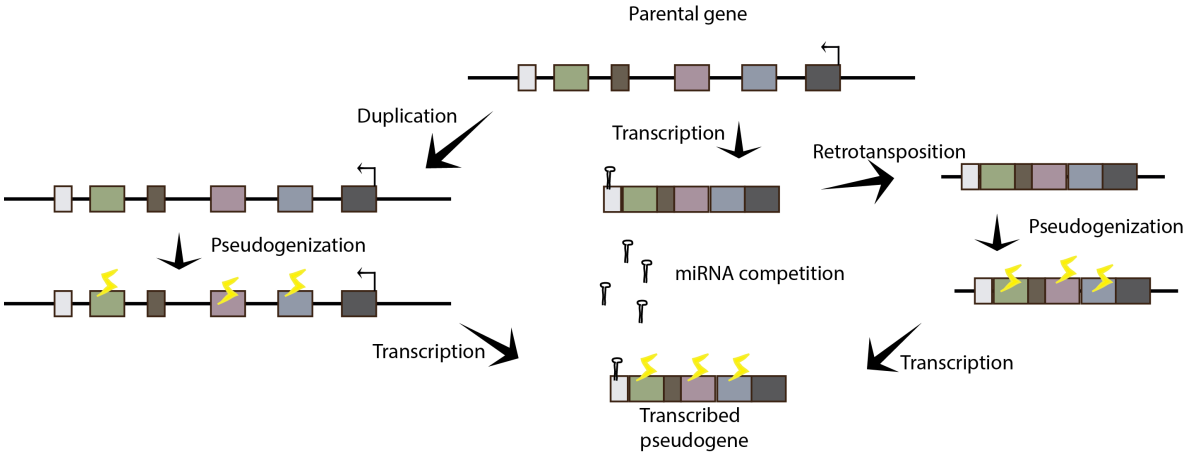

Figure 6 Pseudogenization occurs through duplication or retrotransposition from mRNA consequently followed by mutations. Transcribed psedudogenes can act a competitive endogenous RNAs (modified from[214]).

1.4.1 Pseudogenes as competitive endogenous RNA

Recent data shows that pseudogenes can regulate their parent counterpart on the transcriptional level. The tumor suppressor gene *PTEN* has a pseudogene (*PTENpg1, PTENp1, PTENψ*) located on chromosome 9 that has high sequence homology with only 18 nt mismatches throughout the sequence. The 3´UTR of *PTENpg1* is about 1 kb shorter than that of PTEN. It possesses two regions, one with very high homology and another one with low homology to PTEN. Therefore, within the high homology region of *PTENpg1* the *PTEN*-targeting *miR-17*, *miR-21*, *miR-214, miR-19* and *miR-26* families have perfect matches in seed sequences, and some miRNAs are capable of repressing both *PTEN* and *PTENpg1* transcripts. Upregulation of the 3´UTR of *PTENpg1* showed a de-repression of PTEN transcripts and protein levels, indicating that *PTENpg1* can act as a decoy of competing miRNAs[141](Figure 6). Interestingly, in the same study a similar observation was seen between the proto-oncogene *KRAS* and its pseudogene *KRASP1*[141]. Further, *PTEN* deletions are a common event in human melanomas[215] and partial or full deletions of *PTENpg1* are found in 21% of melanoma tissues. Nine out of 33 samples with *PTENpg1* deletions showed reduced PTEN expression suggesting a competitive endogenous RNA (ceRNA) network between *PTEN* and *PTENpg1*[216]. Another study showed the *PTENpg1* to be downregulated due to methylation in clear-cell renal cell carcinoma and overexpression of *PTENpg1* reduces cell proliferation, invasion, tumor growth and metastasis[217]. In this study the pseudogene *PTENpg1* served as a ceRNA to modulate PTEN expression by miR21 regulation.

The proto-oncogene, *BRAF* has a pseudogene that is frequently overexpressed in various tumor types[218,219] and ceRNA network between *BRAF* and its pseudogene in humans and in mice has been reported. Interestingly, overexpression of the *BRAF* pseudogene in mice,

Braf-rs1, causes aggressive malignancy resembling B-cell lymphoma while silencing of its human ortholog, *BRAFP1* effected MAPK signaling and proliferation in human cancer cells[220].

The ceRNA network has also been proposed for lncRNA and mRNA that are thought to regulate each other[221-223], whereas the lncRNAs can sequester miRNA away from mRNAs. But the ceRNA network has also received some critics. A recent study tried to address this by expressing varying amounts of a competitor RNA to the very abundant miR-122 (120.000 molecules per hepatocyte). The results showed that about 150.000 miR-122 binding sites were needed in order to affect the expression of an endogenous mRNA, a target of miR-122 [224]. However, the RNA expression levels are not even nearly close to generate this amount of binding sites. Another study showed that ceRNA network was not effective for highly expressed miRNAs; however, the ceRNA network could be fully functional in a physiological setting for the low expressed miRNA[225].

1.4.2 Antisense transcription from pseudogenes

About 20% of pseudogenes have shown to have antisense transcript and vast majority of them are coming from duplicated pseudogenes. This phenomenon gives rise to the possibility for antisense transcripts to influence its parental gene in *trans* [226,227]. In 1992, the first antisense transcript from a eukaryotic topoisomerase I (*TOPI*) pseudogene was discovered in humans. Although at that time the function of this antisense transcript was not described, it was speculated that antisense transcripts to a pseudogene would function through RNA:RNA duplex formation[228] . Few years later another antisense transcript from the fibroblast growth factor receptor (*FGFR*) pseudogene in mouse was reported. The study confirmed the transcript to be tissue specific although they did not address its function[229]. Still to date, very few studies managed to reveal how these antisense transcripts function. Studies on antisense transcripts from the *NOS* and *Oct4* pseudogenes are examples of these attempts. A pseudogene to the nitric oxide synthase (*NOS*) gene is found in the central nervous system of snails. This pseudogene has an antisense homology region to the parental gene and functions in *trans* by forming a RNA:RNA duplex, which leads to decreased translation of the *NOS* gene[230]. The pluripotency-associated factor *Oct4* has six related pseudogenes (Oct4-pg1-6)[231]. One of those pseudogenes (*Oct4-pg5*) has an antisense transcript that negatively regulates *Oct4-pg4* and *Oct4-pg5*. This antisense is thought to function by mediating epigenetic silencing at the *Oct4* promoter[209].

1.4.3 Pseudogenes: source for endo-siRNA

SiRNA can arise between two transcripts that share complementary regions. Pseudogenes share high homology with their parental genes providing a possible source for endo-siRNAs. Endo-siRNAs are common small RNA in plants and are known to be produced by RNA-dependent RNA polymerase (RdRP) that catalyzes the replication of RNA from a RNA template. In mammals the existence and biogenesis of endo-siRNAs are obscure, however, there is evidence for their existence as mentioned here. Endo-siRNAs can be formed when a

spliced transcript from a protein coding gene forms a RNA duplex with an antisense transcript emitting from a pseudogenes or when pseudogenes have inverted repeats that bind to a transcript from the parental ancestral gene[232]. Another *in vivo* study on growing mouse oocytes found an endo-siRNA being regulated by the RNAi pathway, a unique way for pseudogenes to regulate mRNAs[233].

1.5 EPIGENETICS- A LNCRNA PERSPECTIVE

Epigenetic modifications at gene promoters play a major role in creating discrepancy between tissues and individual cells. LncRNAs have been recognized as effectors of epigenetic changes at specific gene promoters. For epigenetic changes to take place at a gene promoter, an orchestrated effort of different components is vital. Here, the epigenetic modifications such as DNA methylation and histone modification will be discussed.

DNA methylation

CpG islands span a tiny fraction of the whole genome covering around 0.7% and are usually located in gene promoters[234,235]. DNA methylation is a biochemical modification of the DNA, where DNA methyltransferases catalyze the transfer of a methyl group (CH_3) onto a carbon group located at the C5 position of the pyrimidine ring of cytosines and this occurs predominantly at CpG islands[236,237]. This reaction is catalyzed by a family of DNA methyltransferases identified as DNMT1, DNMT3a, DNMT3b and DNMT3L[238].

Unlike other family members, DNMT1 can identify hemi-methylated portion of newly replicated DNA and maintain the methylation status at specific gene loci. A study on methylation investigated RNA transcripts associated with DNMT1 and found frequent interaction between RNAs and DNMT1 at numerous gene loci. These DNMT1 bound RNAs are thought to evict DNMT1 from their site of expression. One example showed DNMT1 binding to a lncRNA called extra coding CEBPα (*ecCEBPα*) arising in the sense direction from the methylation-sensitive gene CEBPα, which prevented methylation of the *CEBPα* gene locus[239]. Interestingly, the DNA methylation levels were inversely correlated with *ecCEBPα* expression levels. Although there is uncertainty regarding the mechanism of RNA binding to proteins, the study suggests a stem-loop structure that could possibly facilitate the interaction between *ecCEBPα* and DNMT1[239]. In contrast to previous study lncRNAs can also keep DNMT1 at site. For example, the lncRNA *Kcnq1ot1* interacts with DNMT1 in mice and mediates silencing of ubiquitously imprinted genes by binding to DNMT1 and keeping it in place for maintaining allele-specific methylation[240]. *Dum* is another lncRNA that interacts with DNMT1 along with other DNMT family members. In skeletal myoblasts, *Dum* is upregulated in the differentiation process and effects in a *cis* action the expression of nearby genes by interacting with DNMT family members[241]. In order to address this more globally Merry *et. al.* did RNA immunoprecipitation (RIP) of DNMT1 and sequencing of associated RNAs revealed 148 lncRNA bound to DNMT1 in

HCT116 cells. Interestingly, only 31 mRNAs were pulled down indicating that DNMT1 has increased affinity for lncRNA[242].

DNMT3a and DNMT3b are *de novo* methyltransferases[243] that catalyze the transfer of a methyl group onto cytosine. There is also some new evidence that they can maintain methylation[244]. In spite of the importance of this enzyme there are still facades of DNMT3a that remains to be elucidated. In order to investigate the role of DNMT3a on transcriptional repression one study fused DNMT3a to Cas9 enzyme and targeted it to specific gene loci. Interestingly, this resulted in only moderate transcriptional repression[245] and not strong gene silencing that normally is obtained upon DNMT3 binding to promoters. Possibly, DNMT3a needs to form a complex with other proteins in order to maximize its function. DNMT3a has been shown to bind to the DNA methyltransferase 3- like protein (DNMT3L) and the chromatin remodeling ATPase, LSH, that assist DNMT3a in enhancing its DNA affinity or activity[246,247]. In addition, PIWI proteins can recognize transposons that have escaped *de novo* methylation and this leads to recruitment of DNMT3a to transposons. Until recently it has been poorly understood how DNMT3a finds its way to promoters of differentially expressed genes. LncRNAs have been suggested to bind to and recruit DNMT3a to gene promoters and influence gene expression. For example; in the very intrinsic process of X chromosomal activation, the lncRNA *Tsix* recruits DNMT3a to the promoter of *Xist*, which leads to epigenetic silencing [151,248]. In addition, the aforementioned lncRNA *Dum* has also been shown to interact with *DNMT3a*[241].

Histone modification

Polycomb group proteins (PcG) function in two complexes, PRC1 and PRC2, which trigger histone modifications that subsequently lead to transcription silencing.

Polycomb repressive complex 2 (PRC2)

The PRC2 complex is conserved from *Drosophila* and causes mono-, di- or tri- methylation of histone 3 at lysine 27 (H3K27me3)[249-251]. The main players of the PRC2 complex are the following PcG components; enhancer of zeste 2 (EZH2) (or its homolog EZH1), suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED). EZH2 or EZH1 act as the catalytic subunit and SUZ12 - as the regulatory subunit of PRC2. In *Drosophila,* the PRC2 is recruited to the DNA by sequences called polycomb response elements (PRE)[252] and this process is thought to occur independent of other proteins. The recruitment of PRC2 complex in humans however has been a matter of debate. Indeed, PRC2 complex binds predominantly to CpG islands but the DNA sequence itself can not directly recruit the complex and therefore the recruitment has to be mediated through other components. Transcription factors[253], methyltransferases[254] and lncRNAs[150,170] have all been suggested as major players in this process.

The lncRNA *HOTAIR* and the *RepA* element of Xist were the first lncRNA that were suggested to bind to and recruit PRC2 to the specific gene loci[150,170]. In addition, a genome wide study showed that about 20% of all lincRNAs are bound to PRC2 complex[171]. Since

then there have been several reports showing interaction between lncRNA and the PRC2 complex[10,255]. These studies have prompted further investigation on the connection between PRC2 and lncRNA.

Although RNAs can physically bind to the PRC2 complex, this RNA:protein interaction is poorly understood. Recent studies have shown promiscuous binding of the PRC2 complex to RNAs. For example, EZH2 has been found to promiscuously bind to RNAs with submicromolar affinity *in vitro* [256] and the PRC2 complex can bind to nascent RNA transcript at nearly all active genes, possibly protecting the DNA from inappropriate PRC2 binding[257]. SUZ12 has also been shown to be capable of interacting with RNAs[258,259]. Although the binding affinity between EZH2 and PRC2 is higher than of SUZ12, one study suggests that SUZ12 is the leading protein in establishing contact between PRC2 and RNAs[257].

Some studies have tried to establish in greater depth the physical interaction between PRC2 and RNAs. One such study showed that the PRC2 complex is more likely to bind to exon-intron boundaries and to 3′UTRs. It has also been suggested that the PRC2 complex has a higher affinity for RNA motifs that include short repeats of consecutive guanines[260] as well as to short hairpins structures within RNAs [150].

The interaction between *HOTAIR* and PRC2 complex has been fairly well studied however there still remains some discrepancy in their relationship. A study showed that in spite of strong binding between *HOTAIR* and PRC2, that the transcriptional mediated silencing by *HOTAIR* could still be carried out without the PRC2 complex[261]. This study used artificial tethering of *HOTAIR* to chromatin and suggested the recruitment of PRC2 to be a consequence of gene silencing rather than a cause. *HOTAIR* can also bind to another repressive chromatin modifying complex (LSD1/coREST/REST) that catalysis demethylation of H3K4me2 [173]. Therefore, a better fundamental understanding of the relationship of PRC2 and lncRNAs is needed.

Polycomb repressive complex 1 (PRC1)

The PRC1 complex is typically composed of Bmi1/Mel8, mPh1/2, Pc/Chromobox and RingA/B proteins. PRC1 is rather thought to maintain repressed chromatin but can also monoubiquitinate histone H2AK119 causing transcriptional silencing by inhibiting the elongation of RNA pol II[262]. The PRC1 complex can recognize trimethylation of H3K27, which surfs as a docking site for the PRC1 subunit, chromobox-domain. In addition, the PRC1 complex is also thought to interact with RNAs and the aforementioned antisense transcript *ANRIL* binds to CBX7 of the PRC1 complex and represses transcription[163].

Associations between DNA methylation and histone modification

DNA methylation and histone modifications both contribute to gene silencing; however DNA methylation establishes more of a long term silencing while modification of histones can more easily be converted. In spite of fundamental differences in how these two pathways induce silencing they exhibit cross-talk where they can guide each other to

chromatin. It is unclear if this cross-talk happens through the modifications themselves or through the protein-protein interaction.

EZH2 can facilitate DNMTs binding to promoters that are repressed by EZH2 and the presence of EZH2 is essential for DNMT methylation at these promoters[254]. In addition, H3K27me3 enriched promoters in embryonic stem cells are more likely to be DNA methylated during differentiation and carcinogenesis than those that lack H3K27me3[263-265]. Also, other epigenetic complexes can influence and support each other's functions. G9 and GLP are Histone H3 Lysine 9 methyltransferases that can mono- or di-methylate's H3K9. G9a and GLP can physically interact and are thought to functionally interact with each other through a subunit of EZH2[266]. Taken together, histone modifications and DNA methylations seem to facilitate each other however the exact underlying mechanism needs to be elucidated.

AIMS OF THIS THESIS

The overall aim of this thesis is to expand and deepen our understanding of the functional role of lncRNAs in molecular processes and to investigate their involvement in cancer, particularly in cutaneous malignant melanoma.

Paper I:

The aim of **paper I** was to untangle the regulation of expression of the tumor suppressor PTEN and the functional role of the PTEN pseudogene antisense transcript (*PTENpg1 asRNA*) in this process.

Paper II:

The aim of **paper II** was to unravel the molecular mechanism of the recruitment of *PTENpg1 asRNA* to the PTEN promoter by investigating the role of paRNA emitting from the 5´UTR of *PTEN*.

Paper III:

The aim of **paper III** was to explore the down regulation of PTEN expression and the involvement of *PTENpg1 asRNA* in the drug resistance in melanoma using vemurafenib-resistant cell lines as well as investigation of *PTENpg1 asRNA* expression levels in melanoma patient samples. We also aimed to reveal the regulation of *PTENpg1 asRNA* by the transcription factor *CEBPβ*.

Paper IV:

The aim of **paper IV** was to investigate the role of *CEBPβ-AS* in the regulation of *CEBPβ* expression and consequently in drug sensitivity in vemurafenib-resistant melanoma cell lines.

RESULTS AND DISCUSSIONS

1.6 PAPER I

A pseudogene long-noncoding-RNA network regulates PTEN transcription and translation in human cells

1.6.1 Rational

Pseudogenes were once thought as genomic fossil or "junk" DNA; however in recent years they are gaining attention for being functional[141,220]. For example, the *PTEN* pseudogene (*PTENpg1, PTENp1, PTENψ*) has been shown to function as a mircoRNA sponge that soaks up miRNAs and sequesters them away from their targets[141]. Pseudogenes can also be a source for antisense transcription and these overlapping transcripts often share sequence complementarity to their protein-coding counterparts. Although it has been clear that antisense transcription was emitting from pseudogenes[226], functional studies of this phenomenon are rare but it has been reported that an asRNA emitting from the Oct4-pseudogene 5 affects the expression levels of its protein coding counterpart, Oct4[209]. Here we aimed to investigate an antisense transcript emitting from the *PTENpg1* and its effect on the tumor suppressor gene *PTEN*. In addition, we set out to untangle the distinctive roles of various isoforms of *PTENpg1 asRNA*.

1.6.2 Main findings and discussions

The PTENpg1 encodes an antisense RNA

Initially, we observed one aligned EST (Expressed sequence tags) designated as BX374997 in the UCSC (University of California, Santa Cruz) genome browser. This EST was located upstream of the *PTENpg1* transcription start site (TSS), which encouraged us to look further into the possibility of an antisense transcription emitting from the *PTENpg1* locus. Additionally, the ENCODE ChIP–sequencing data showed enrichment of the active transcriptional histone mark H3K4me3 and the RNA pol II at two different locations within the *PTENpg1* locus, indicating two different TSS that overlap with the *PTENpg1* sense promoter. To experimentally validate these indications from the UCSC genome browser we performed 5´RACE and designed primer walks. This analysis indicated two TSS giving rise to at least two different isoforms that we designated as α and β. Fractionation analysis showed both of the spliced *PTENpg1 asRNA α* and *β* isoforms to be found predominantly in the cytoplasm while an unspliced *PTENpg1 asRNA* was expressed solely in the nucleus. We attempted to further untangle the diversity of isoforms of *PTENpg1 asRNA* using Northern blotting, however, without any success. This was possibly due to the fact that the antisense transcript is composed of two segments: one with high sequence complementarity to the *PTEN* gene and the other - with highly repetitive elements (mostly LINE repeats) making it difficult to design specific probes.

PTENpg1 asRNA α recruits epigenetic modifiers to silence PTEN transcription

We investigated the expression levels of the discovered transcripts in a panel of human cell lines and, interestingly, their expression inversely correlated to the *PTEN* mRNA expression levels. This prompted us to explore if knockdown of *PTENpg1* asRNA α with siRNA or shRNA would affect the expression of *PTEN*. Indeed, *PTEN* levels were elevated after *PTENpg1 asRNA* knockdowns, and this induction was seen already at the pre-mRNA levels indicating an effect on transcription. This was further supported by nuclear run-on assay and ChIP for RNA pol II binding at the *PTEN* promoter. Next, we fluorinated and biotin-linked the *PTENpg1 asRNA* in order to investigate if the *PTENpg1 asRNA* binds to the *PTEN* promoter. Pull-down of the biotin-linked transcript showed that *PTENpg1 asRNA* localizes to the *PTEN* promoter. Further, we knocked down *EZH2* and *DNMT3a* and observed induced expression of PTEN, while simultaneous knockdown of *EZH2* and *PTENpg1 asRNA α* showed no additive effect, indicating that these factors work in the same pathway. RIP showed an existing interaction between the *PTENpg1 asRNA* and DNMT3a supporting the hypothesis that they form a RNA-protein complex together. In order to investigate if the *PTENpg1 asRNA α* recruits EZH2 and DNMT3a to the *PTEN* promoter, ChIP analysis was carried out, and we observed a decreased enrichment of EZH2 and H3K27me3 at the *PTEN* promoter after depletion of *PTENpg1* a*sRNA α*. Further, siRNA depletion of *PTENpg1 asRNA* α resulted in induced protein levels of PTEN and concomitant downregulation of the pAKT, and therefore inhibition of the PI3K/AKT pathway. This also resulted in reduced levels of the cell cycle regulator p21, and FACS analysis showed a G0-G1 arrest after *PTENpg1 asRNA* α depletion.

Although studies have reported lncRNAs to guide DNMT3a to gene loci, our study was the first to our knowledge that demonstrated an interaction between lncRNAs and DNMT3a. Since our study was published, there have been at least two other publication showing an interaction between lncRNAs and DNMT3a[241,267].

Taken together, *PTENpg1 asRNA* α recruits the epigenetic modifying proteins, EZH2 and DNMT3a to the promoter of *PTEN,* which consequently leads to epigenetic silencing of the tumor suppressor. This is in agreement with accumulating data on the interaction of ncRNAs with epigenetic modifiers [163,209,254,268]. In addition, these results add to the understanding of possible functional roles of pseudogene transcripts in transcriptional silencing.

PTENpg1 asRNA β stabilizes its sense transcript through RNA:RNA interaction

Sense-antisense transcripts have shown to form a partial duplex that stabilizes the transcripts[168,269] and therefore we sought out to explore if the *PTENpg1 asRNA* could stabilize its sense transcript through such interactions. We observed that the *PTENpg1* sense transcript lacks a poly-A tail; poly-A tailing of RNA transcripts usually leads to an increased RNA stability and assists in shuttling of RNAs from the nucleus to the cytoplasm [270-272]. By blocking transcription with Actinomycin D, however, similar stability was found for the polyA-tail lacking *PTENpg1* sense, as well as for *PTEN* and the *PTENpg1 asRNA*. When we

used endoribonuclease RNase-A to digest single stranded RNA, we observed an overlapping region of the transcripts that was not degraded clearly pointing at the formation of duplexes. We then used antisense phosphorothioate oligodeoxynucleotides (ODNs) and shRNA to interfere with the interaction between the *PTENpg1* sense and *asRNA β* that both resulted in a decreased expression of the sense transcript. Interestingly, this also led to decreased expression of PTEN at the RNA as well as at the protein level, an observation that is in line with the previously published ceRNA network[141]. Although the *PTENpg1 asRNA α* transcript shares a longer sequence overlap than *PTENpg1 asRNA β* with PTENpg1 sense we did not observe any binding between *PTENpg1 sense* and *asRNA α*. Interestingly, the *WRAP53* asRNA (see section 1.3.2) has been reported to have at least three different isoforms, among those only one forming an RNA:RNA interaction with its sense counterpart, *p53*[168]. A possible explanation is that some isoforms might form specific secondary structures or bind to proteins that block potential interactions. Further studies are needed in order to understand this phenomenon in more details.

Taken together, *PTENpg1 asRNA β* stabilizes the *PTENpg1* sense transcript through an RNA:RNA interaction and our findings also suggest that *PTENpg1 asRNA β* is involved in export of the PTENpg1 sense transcript where it serves as miRNA sponge. Unlike its *α* transcript, *PTENpg1 asRNA β* functions at the post-transcriptional level.

1.7 PAPER II

The molecular dynamics of long noncoding RNA control of transcription in PTEN and its pseudogene

1.7.1 Rational

Subtle changes of *PTEN* alter cancer susceptibility[61] and it is therefore vital for the gene to be under tight regulation in order to keep adequate expression levels. The aforementioned *PTENpg1 asRNA* network fine-tunes *PTEN* expression by recruitment of chromatin remodeling proteins to the *PTEN* promoter. However, it is poorly understood how lncRNAs that are functioning in *trans* can find their way to gene promoters. Previously, small transiently transfected RNAs directed towards promoter associated RNAs have been shown to mediate transcriptional regulation of protein coding genes[208,273]. Here, we set out to investigate if the *PTEN* locus encodes for such promoter associated RNAs and whether such transcripts are involved in recruitment of *PTENpg1 asRNA α* to the promoter of *PTEN,* possibly through RNA:RNA interactions.

1.7.2 Main findings and discussions

Detection of PTEN promoter-associated RNA/5´UTR transcript

In the UCSC genome database, ESTs were found to span the *PTEN* 5´UTR. We used strand-specific directional reverse transcription (RT) PCR to detect if these transcripts exist in cellular settings. Indeed, a 5´UTR transcript spanning this region was detected; it was, however, difficult to further characterize it due to its extensive overlap with other *PTEN* associated transcripts. In order to explore the functional role of this transcript we designed a single-stranded antisense ODNs targeting the *PTEN* paRNA/5´UTR transcript. ODNs are single-stranded DNA oligos with the capacity to hybridize to the targeted RNA in a sequence-specific manner and sterically interfere with the targeted transcript. The ODNs used in this study are phosphorothioate ODNs, which are resistant to nucleases. We designed multiple phosphorothioate ODNs and picked one, ODN2, with the capacity to interfere with paRNA/5´UTR. Interestingly, a biotin-labeled ODN2 also managed to pull-down PTEN-5'UTR associated transcripts, including the full length of *PTEN* mRNA.

The PTEN paRNA/5´UTR transcript interacts with the PTENpg1 as RNA network

ODN2 inhibition of the *PTEN paRNA/5´UTR* transcript resulted in less binding of DNMT3a at the *PTEN* promoter and consequently less CpG methylation, indicating a role for the *PTEN paRNA/5´UTR* transcript in the recruitment of DNMT3a to the *PTEN* promoter. These observations prompted us to further investigate if the *PTEN paRNA/5´UTR* transcript interacts with the *PTENpg1 asRNA* network, previously shown to mediate recruitment of DNMT3a to the *PTEN* promoter (see paper I). We initially immunoprecipitated DNMT3a and discovered that both the *PTENpg1 asRNA* α exon 1 and the *PTEN* paRNA/5´UTR transcript could bind to DNMT3a, suggesting that these RNA transcripts could interact by forming RNA:RNA duplexes. To address this more specifically, HEK293 cells were co-

transfected with biotin-labeled *PTENpg1 asRNA* α transcript and ODN2. Pull-down of the biotinylated RNA showed less binding to *PTEN* paRNA/5´UTR transcript after ODN2 transfection, supporting that ODN2 indeed interfered with an interaction between paRNA/5´UTR and *PTENpg1 asRNA*. In addition, the interaction was stable upon RNase A treatment, which predominantly targets single-stranded RNA structures, further suggesting the presence of RNA:RNA interactions.

Next, we set out to investigate the binding between *PTENpg1 asRNA α* and paRNA/5´UTR in more detail. To this end, various truncations of *PTENpg1 asRNA* α exon 1 were labeled with biotin and transfected into HEK293 cells. Again the biotin-labeled transcripts were pulled down and we observed binding to the *PTEN* promoter by two variants, F4R1 and F5R2, and the full-length transcript. Strikingly, only the F4R1 variant and the full-length *PTENpg1 asRNA α* exon 1 transcripts were capable of directing DNMT3a to the *PTEN* promoter while this capacity was lost for the F5R2 transcript.

Analyses of secondary RNA structures suggested that both the F4R1 variant and *PTENpg1 asRNA* α exon 1 have a major loop structure. We hypothesized that this loop structure could be important for the recruitment of DNMT3a to the *PTEN* promoter and therefore generated multiple truncations of this loop. Indeed, the truncations lead to the inability to bind to *PTEN* promoter indicating that the loop structure is necessary for the *PTEN* promoter targeting. Interestingly, the sequence of the loop structure maps to a locus where we observed high levels of DNMT3a and *PTENpg1* asRNA α.

Taken together, the *PTEN* paRNA/5´UTR transcript binds and assists the *PTENpg1 asRNA* α exon 1 transcript in recruiting DNMT3a to the promoter of *PTEN,* which causes epigenetic silencing of *PTEN.* The function of *PTEN* paRNA/5´UTR transcript in epigenetic silencing of the *PTEN* promoter adds to the regulatory layers of the tumor suppressor. The *PTEN* paRNA/5´UTR transcript is an example of the variety of transcripts that can originate from the same promoter and one of the diverse functions they are capable to carry out. Most importantly, our studies on *PTEN* illustrate the presence of multiple functional non-coding RNAs, long as well as short, with the capacity to regulate the same gene.

Interestingly, this study suggests the existence of a major loop structure of the *PTENpg1 asRNA* transcript important for its functional role; however, more studies will be needed in order to fully understand the structural interaction between the *PTEN* promoter and *PTEN* associated transcripts, as well as *PTENpg1 asRNA* and DNMT3a. For example, are there specific nucleotide sequences within the loop structure that are important for the recruitment of DNMT3a or is it just the loop structure itself? Further, is it the stem and/or the loop that maintains the function and how is the localization of *PTENpg1 asRNA* coordinated with the recruitment of DNMT3a?

1.8 PAPER III

PTENpg1 antisense RNA mediates PTEN suppression in vemurafenib resistance and predicts clinical outcome in melanoma patients

1.8.1 Rational

Although $BRAF^{V600E}$ inhibitors (e.g. vemurafenib) improve survival rates for cutaneous melanoma patients, almost all patients will eventually develop drug resistance. Reduced PTEN expression, e.g. through epigenetic inactivation, has been suggested to be involved in the development of melanoma and also to contribute to vemurafenib resistance[68,99,274]. We therefore speculated that the *PTENpg1 asRNA* network could mediate suppression of *PTEN* upon the development of resistance to vemurafenib and possibly also be involved in the development of melanoma.

1.8.2 Main findings and discussions

PTENpg1 asRNA expression is induced in vemurafenib resistant A375 melanoma cell lines

In order to investigate the *PTENpg1 asRNA* network we produced vemurafenib-resistant melanoma cell lines by culturing the melanoma cell line A375 at increasing doses of vemurafenib/PLX47020 for extended period of time. Next, we measured *PTEN* and *PTENpg1 asRNA* expression to see if the drug-resistant melanoma cell lines had acquired changes in their expression levels. All resistant cell lines (A375 PR1, A375 PR3 and A375 PR4) showed upregulation of the *PTENpg1 asRNA* and sense transcripts, and downregulation of *PTEN* levels. PTENpg1 sense has previously been shown to sequester miRNA away from *PTEN* mRNAs[141], and we therefore measured the *PTEN* mRNA and protein level expression. Strikingly, we did not observe the expected effect from the miRNA sponging activity, which would be manifested by an increased expression of *PTEN*, but instead - suppression of *PTEN* in all resistant cell lines. These data therefore suggest that *PTENpg1 asRNA* is the dominant regulator of *PTEN* in this model system.

Next, we more specifically investigated which *PTENpg1 asRNA* isoform was being induced in the resistant cell lines. We predominantly observed induced expression of the *PTENpg1 asRNA* α isoform in the A375 PR1 cell line and the transcript was also specifically enriched in the nuclear fraction. We next investigated if suppression of *PTEN* is a reversible process and if knockdown of *PTENpg1 asRNA* α could reactivate *PTEN* expression. We designed gapmer antisense oligonucleotides (ASO) that specifically target the nuclear pool of *PTENpg1 asRNA* α transcripts and indeed observed that knockdown of *PTENpg1 asRNA* α induced *PTEN* expression in the resistant cell lines; strikingly, this was not the case in the sensitive cells.

EZH2 and DNMT3a mediate epigenetic silencing of the PTEN promoter in A375 vemurafenib-resistant cell lines

These observations motivated us to further investigate if epigenetic silencing caused low PTEN mRNA and protein levels in A375 PR1. We used the McrBc enzyme that cleaves methylated DNA at Pu^{m}CG sequence elements to assess DNA methylation at the *PTEN* promoter in the A375 and A375 PR1 cell lines. We observed increased enzymatic digestion at the *PTEN* promoter in the A375 PR1 cell line compared to the A375 cell line thus indicating enrichment of methylated DNA. Further, knockdown of EZH2 and DNMT3a caused upregulation of *PTEN* in the A375 PR1 resistant cells, while not affecting *PTEN* in the sensitive cells. These results indicate that epigenetic silencing of the *PTEN* promoter in A375 PR1 cell line is mediated through EZH2 and DNMT3a. The histone methyltransferase EZH2 is a subunit of the PRC2 complex that mediates tri-methylation on histone 3 lysine 27 and has also been reported to interact with DNMT3a, thus bridging chromatin remodeling and DNA methylation together[254]. Therefore, we pulled down EZH2 and its downstream target H3K27me3 and found both to be enriched at *PTEN* promoter in A375 PR1 resistant cell lines. In addition, we pulled down EZH2 and DNMT3a and observed binding to *PTENpg1* asRNA.

This allowed us to propose a model for epigenetic silencing of *PTEN* in the vemurafenib-resistant A375 melanoma cell line caused by *PTENpg1 asRNA* α that recruits epigenetic modifying proteins, EZH2 and DNMT3a, to silence *PTEN* transcription.

Interestingly, the expression of *PTENpg1 asRNA* and *PTENpg1 sense* was concordantly upregulated in the resistant A375 cell lines. However, we observed a negative correlation between the *PTENpg1 asRNA* and *PTEN*, which indicates an epigenetic regulation of *PTEN* instead of a positive correlation that would be expected from a ceRNA network. One could speculate that equimolar concentrations of all components of the *PTENpg1* ceRNA network are necessary to observe the effect; alternatively, very low expression of *PTENpg1 asRNA* are sufficient to carry out epigenetic silencing at the *PTEN* promoter while higher expressions of *PTENp1* sense, than seen in the A375 resistant cell lines, is needed to sponge away miRNAs.

The transcription factor C/EBPβ induces transcription of PTENpg1 asRNA α in vemurafenib-resistant A375 cell line.

In order to investigate the underlying mechanisms to induction of *PTENpg1 asRNA* α we set out to identify transcription factors potentially regulating expression of this locus. ChIP data from the ENCODE project showed strong binding of the transcription factor C/EBPβ upstream from the *PTENpg1 asRNA* α transcriptional start site. C/EBPβ was confirmed to be upregulated in the A375 PR1 cell line compared to A375 cell line, and knockdown of C/EBPβ caused downregulation of *PTENpg1 asRNA* α and, consequently, induced expression of *PTEN*. Interestingly, knockdown of C/EBPβ did not appear to reduce the expression of *PTENpg1* sense. In addition, C/EBPβ was enriched at the *PTENpg1 asRNA* promoter in the resistant A375 PR1 compared to the sensitive A375 cell lines. Taken together, C/EBPβ is

upregulated in the A375 PR1 cell line and appears to be an important regulator of *PTENpg1 asRNA*. Thus, this is the first TF identified regulating this locus, and it appears to act on a strand-specific level (on the antisense strand). We speculate that C/EBPβ is binding to the consensus sequence GTTGCGCAAT, this sequence is not found within the matching region of *PTEN* (~70 kb downstream from the *PTEN* TSS). In line with this, there is no indication from the ENCODE ChIP sequencing data that C/EBPβ could have binding sites at this position on *PTEN* or of a transcript emitting from this locus. Further, the nucleotides that make up the consensus sequence for this TF in the *PTEN* pseudogene are located on separate exons in *PTEN,* suggesting that during pseudogenization the *PTEN* pseudogene has acquired a regulatory sequence that is not found in the DNA of its parental gene.

Increased cell death upon targeting of the PTENpg1 asRNA α network in vemurefenib-resistant melanoma cell lines and PTENpg1 asRNA α as a potential biomarker

Next, we set out to investigate if disruption of the *PTENpg1 asRNA* network could re-sensitize vemurafenib-resistant A375 melanoma cell lines to vemurafenib through reactivation of *PTEN,* and a consequently suppressed PI3K/AKT survival pathway. Simultaneous knockdown of EZH2 and DNMT3a induced cell death in the resistant A375 cell lines when combined with vemurafenib treatment. In line with this, knockdown of *C/EBPβ* as well as *PTENpg1 asRNA* also resulted in increased cell death upon vemurafenib treatment in A375 resistant cell lines. These results encouraged us to further investigate the clinical impact of *PTENpg1 asRNA* in melanoma patients. To this end, we used two different cohorts of stage III melanoma patients, and in both cohorts, we determined *PTENpg1 asRNA* expression in lymph node metastases by RT-qPCR. The first cohort patients were further divided into groups with long or short overall survival. Interestingly, patients with high *PTENpg1 asRNA* expression showed shorter survival. In the second cohort we divided the groups according to high or low expression of *PTENpg1* asRNA. The survival plot showed a significant difference in the overall survival of melanoma patients between groups with high or low *PTENpg1 asRNA* expression.

Here we described a novel role for the *PTENpg1 asRNA* in vemurafenib resistance in melanoma cell lines. DNMT3a and EZH2 were enriched at the *PTEN* promoter in the vemurafenib-resistant A375 cell lines. We observed interaction of these proteins with *PTENpg1 asRNA*, and propose that the *PTENpg1 asRNA* is mediating the recruitment of EZH2 and DNMT3a causing tri-methylation on lysine K27 on Histone 3 and DNA methylation of the *PTEN* promoter. Taken together, experimental manipulation of the *PTENpg1 asRNA* network can re-sensitize resistant A375 melanoma cell lines to vemurafenib treatment and manipulation of the *PTENpg1 asRNA* network may provide us with a therapeutic tool to de-repress *PTEN* and consequently inhibit the PI3K/AKT pathway. Moreover, the levels of *PTENpg1 asRNA* can represent a potential predictive biomarker for cutaneous malignant melanoma patients.

1.9 PAPER IV

C/EBPβ-AS inhibits transcription of C/EBPβ through a negative feedback loop

1.9.1 Rational

Transcription factors have been extensively studied as important regulators of genes' activity and the dynamic mechanism of their transcriptional regulation is of a great interest. C/EBPβ is a versatile transcription factor that regulates multicellular functions, and deregulation of this transcription factor has been implicated in various types of cancer[103, 124, 121, 119]. In **paper III** we found *C/EBPβ* to be a transcriptional regulator of *PTENpg1* asRNA. This prompted us to investigate the involvement of *C/EBPβ* in cancer development by using the Cancer Genome Atlas (TCGA) dataset and further, what dictates the transcriptional expression of *C/EBPβ*. Previously, antisense transcripts have been shown to transcriptionally regulate their sense counter parts[149,156,157] and therefore we sought out to investigate if the annotated antisense RNA transcript of *C/EBPβ* could regulate its transcription.

1.9.2 Main findings and discussions

Expression patterns of C/EBPβ and C/EBPβ-AS in human tumors and relation to overall survival

In order to investigate the association between *C/EBPβ* and *C/EBPβ-AS* we took advantage of TCGA database. We found that the expression of the two transcripts showed moderate or strong positive correlation to each other in 32/33 cancer types including melanoma. Furthermore, *C/EBPβ* and *C/EBPβ-AS* expression showed some correlations to patient´s survival in various types of cancer. This correlation was, however, not consistent. In some cases it was the high expression while in other cancer types - it was the low expression that correlated to worse prognosis. In addition, we found that high *C/EBPβ-AS* expression correlated to a better prognosis in melanoma patients.

The yin and yang of C/EBPβ regulation

We noticed in the ChIP-seq data from ENCODE that C/EBPβ is enriched at both *C/EBPβ-AS* and *C/EBPβ* promoters suggesting that it should regulate both transcripts. Therefore, we explored if knockdown of *C/EBPβ* would affect transcription of *C/EBPβ-AS.* Indeed, knockdown of *C/EBPβ* showed downregulation of *C/EBPβ-AS,* in line with the observed positive correlation between *C/EBPβ-AS* and *C/EBPβ* expression in human tumors.

We further set out to characterize *C/EBPβ-AS* and showed the transcript to be a polyadenylated RNA that localized both in the cytoplasm and in the nucleus. We decided to investigate its nuclear function in more detail, such as whether *C/EBPβ-AS* could be a transcriptional regulator of C/EBPβ. We designed siRNAs to knockdown *C/EBPβ-AS* expression. Upon knockdown of *C/EBPβ-AS,* we observed upregulation of *C/EBPβ* mRNA

and protein expression in the majority of cell lines. This was in contrast to our expectations, since we had observed concordant and positive correlation between *C/EBPβ-AS* and *C/EBPβ* in human tumors.

In order to understand the negative regulation of *C/EBPβ* by *C/EBPβ-AS,* we investigated if *C/EBPβ-AS* expression could induce epigenetic changes at the *C/EBPβ* promoter. We knocked down *C/EBPβ-AS* and, to our surprise, observed an enrichment of EZH2 and H3K27me3 at the *C/EBPβ* promoter. Further, we used the McrBc enzyme that specifically cuts methylated DNA and observed more DNA methylation at the *C/EBPβ* promoter upon silencing of *C/EBPβ-AS*. Attraction of methyltransferases to the promoters, histone methylation and the methylated DNA are usually associated with gene silencing. However emerging recent data provide evidence that some specific gene loci can be activated upon DNA methylation[111-113]. Interestingly and in line with latter observation, *C/EBPβ* has been shown to bind more strongly to methylated sequences leading to increased gene activity[114,115]. Therefore, this TF may actually be attracted to methylated regions to activate gene transcription (its own, in this case). *C/EBPβ-AS,* on the other hand, may fine-tune the activity of *C/EBPβ* promoter in that low levels of *C/EBPβ-AS* allow for the activation of *C/EBPβ* promoter while its increased expression may attract and sequester epigenetic factors away from the promoter.

Resensitization of vemurafenib-resistant melanoma cell lines

In paper III, we reported that suppression of C/EBPβ could re-sensitize the A375 PR1 cell line to vemurafenib treatment, and therefore we set out to investigate if manipulating *C/EBPβ-AS* levels would affect sensitivity towards vemurafenib treatment. We knocked down *C/EBPβ-AS* in vemurafenib-sensitive and -resistant A375 as well as in dabrafenib-sensitive and -resistant MNT1 cell lines, and treated the cells with two different concentrations of vemurafenib. All cell lines showed increased sensitivity towards vemurafenib treatment after *C/EBPβ-AS* knockdown. This was an unexpected finding since a decrease in *C/EBPβ-AS* would lead to the activation of C/EBPβ. However, there are examples in the literature when overexpression of C/EBPβ has been shown to cause reduced cell growth and increased cell death. For example the overexpression of C/EBPβ *LIP* isoform has shown to induce cell death in mouse melanoma cells[275], and C/EBPβ overexpression in prostate cancer cells has been shown to reduce cell growth after doxycycline treatment[276].

The observed sensitization to vemurafenib treatment after *C/EBPβ-AS* knockdown promoted us to further look into how *C/EBPβ-AS* affects the two major pathways that contribute to vemurefenib resistance: the PI3K/AKT and the MAPK pathways. Interestingly, our results showed that *C/EBPβ-AS* knockdown induced p-AKT (T308 and S473). This is in line with our data on C/EBPβ being a transcriptional activator of *PTENps1 asRNA*, a negative regulator of PTEN. This is also in agreement with a study suggesting C/EBPβ to be an activator of the PI3K/AKT pathway through the IL-8 cytokine[276]. It is, however, well established that activation of the PI3K/AKT and MAPK signaling pathways would promote

cell proliferation and survival. It remains therefore to further reveal the molecular mechanisms of sensitization to vemurafenib by *C/EBPβ-AS* knockdown. Activation of some pathways, e.g. EGFR that also activate the PI3K/AKT pathway, can under some conditions cause cell death independent of the PI3K/AKT pathway[277]. Therefore, the hypothesis is that activation of other proteins through C/EBPβ is important to trigger increased vemurafenib sensitivity upon *C/EBPβ-AS* knockdown. Alternatively, manipulation of *C/EBPβ-AS* levels affects other pathways by acting in *trans* on other genes' promoters.

Taken together, we characterized a novel transcript, *C/EBPβ-AS* that can be induced by and fine-tune the expression of C/EBPβ. Manipulation of C/EBPβ levels can clearly reverse the resistance towards vemurafenib in melanoma cell lines, and so does knockdown of *C/EBPβ-AS.* However, the exact mechanism behind this remains unclear. We have also revealed an unusual affinity of C/EBPβ to its own methylated promoter in that levels of *C/EBPβ-AS* play a crucial role in mediating these epigenetic changes.

CONCLUDING REMARKS

The results of the work included in this thesis revealed novel regulatory mechanisms and the role of ncRNAs in cancer. The main findings are:

- An antisense RNA emitting from the *PTEN* pseudogene, *PTENpg1 asRNA*, can transcriptionally and post-transcriptionally regulate the tumor suppressor gene *PTEN*.
- *PTENpg1 asRNA α* recruits epigenetic modifying proteins to the *PTEN* promoter.
- *PTENpg1 asRNA β* binds to and stabilizes the *PTENpg1 sense.*
- A *PTEN* 5´UTR/promoter associated transcripts assist the *PTENpg1 asRNA α* transcript to the *PTEN* promoter mediating recruitment of DNMT3a and consequently leads to epigenetic silencing of the promoter.
- The *PTENpg1 asRNA* contributes to vemurafenib resistance by mediating epigenetic silencing of *PTEN* in melanoma cells.
- *PTENpg1 asRNA* is a potential biomarker in melanoma patients.
- The transcription factor C/EBPβ the regulates *PTENpg1 asRNA.*
- C/EBPβ is a major regulator of its own and its antisense transcript, *C/EBPβ-AS*. *C/EBPβ-AS* inhibits *C/EBPβs* positive feedback-loop by mediating epigenetic changes at the *C/EBPβ* promoter.

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In memory of my principal supervisor, professor Dan Grandér:

This is not how our story was supposed to end. I was suppose to defend my thesis with you at me side. Then, at the party you would have started dancing at a too timely hour for my taste. I can just imagine it, your tiny dance moves and you with the biggest smile on your face. After we had gone our separated ways, we would have remained friends and as we always talked about, one day you would visit me in Iceland, although I had warned you that we might have to leave the city and experience "nature".

Before my Phd studies I had decided that I would aim to find a kind supervisor otherwise it would not be worth it. With Danne that was exactly what I got. I was never questioned about my working hours or that I was somehow not performing at my job. If my experiments would fail, he would simply say "sometimes the magic works and sometimes it doesn´t". It was no one to blame and not my fault. Many of his master and bachelor students describe Danne as one of their best teachers – his lectures were supposedly something to witness. However, for us, his phd students, this is not how Danne taught us. To us he would not lecture or tell us what to do (maybe we should already know). Of course he would guide us in the right direction but first of all he would sit back, listen, and observe how we would tackle projects. Easy as is sounds this can be very difficult. You will have to come in terms with your own abilities and limitations and at times, that can be challenging and disappointing. I think this is what Danne thought of as the essence of the highest education possible - YOU should be able to ask and answer questions that have not been answered before.

Coming to your apartment that day, it was nice to see that you were surrounded by so many loved ones. I had been told I should not expect you to reach out to me. As soon as I walked into your room, you knew I was there. I told you my PhD application had been accepted and you said that you thought that you would not be able to be there for me. I know how difficult it was for you to tell me but at that point I already knew. You also asked about the manuscript and how it was progressing at the lab. At your last fighting moments, you were still concerned about the lab and still wanting for me to exceed. I held your hand and we said our last good-bye. The next day you were released from your pain.

Everyday Danne would come into the lab after a long day of meetings, sometimes, I think, just to hang out. It was his favorite part of the working day. This is, of course, my interpretation. Maybe he really was enjoying those thousands of meetings. He would kick off his shoes and place his feet on Iryna´s chair. I would ask how the day was and I always got the same sarcastic answer; "I barely survived". Ironically through out the years the question and the answer would remain the same but with very different meanings. We, the students, and Danne would talk about science or more importantly, life - like the time Danne won a brand new SAAB in a slogan competition or his dream to move to Tel Aviv. We would also complain about tiny things; like the Swedish weather (except me, I moved south), why Danne would stare at us for such a long time or why he would place his feet on our chair. Those

were the days and little did we know. In the next few years, us sitting in this tiny office were about to experience some of life's "unfairs". It was my turn. My saddest day came and it broke my heart. Later, my colleges also had their moments, some overcame them and some are still overcoming them. Danne´s "unfair" was however too great of a challenge and it truly saddens me that he was not given more time.

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