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# Studies of epigenetic deregulation in parathyroid tumors and small intestinal neuroendocrine tumors

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ACTA UNIVERSITATIS UPSALIENSIS UPPSALA 2017

ISSN 1651-6206
ISBN 978-91-513-0097-9
urn:nbn:se:uu:diva-330810

Dissertation presented at Uppsala University to be publicly examined in Fåhraeussalen, Rudbecklaboratoriet Hus 5, Dag Hammarskjölds väg 20, Uppsala, Friday, 24 November 2017 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Associate Professor Christofer Juhlin (Department of Oncology-Pathology, Karolinska Institute).

**Abstract**

Barazeghi, E. 2017. Studies of epigenetic deregulation in parathyroid tumors and small intestinal neuroendocrine tumors. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1377. 53 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0097-9.

Deregulation of the epigenome is associated with the initiation and progression of various types of human cancers. Here we investigated the level of 5-hydroxymethylcytosine (5hmC), expression and function of TET1 and TET2, and DNA methylation in parathyroid tumors and small intestinal neuroendocrine tumors (SI-NETs).

In Paper I, an undetectable/very low level of 5hmC in parathyroid carcinomas (PCs) compared to parathyroid adenomas with positive staining, suggested that 5hmC may represent a novel biomarker for parathyroid malignancy. Immunohistochemistry revealed that increased tumor weight in adenomas was associated with a more aberrant staining pattern of 5hmC and TET1. A growth regulatory role of TET1 was demonstrated in parathyroid tumor cells.

Paper II revealed that the expression of TET2 was also deregulated in PCs, and promoter hypermethylation was detected in PCs when compared to normal parathyroid tissues. 5-aza-2′-deoxycytidine treatment of a primary PC cell culture induced TET2 expression and further supported involvement of promoter hypermethylation in *TET2* gene repression. *TET2* knockout demonstrated a role for TET2 in cell growth and migration, and as a candidate tumor suppressor gene.

In Paper III, variable levels of 5hmC, and aberrant expression of TET1 and TET2 were observed in SI-NETs. We demonstrated a growth regulatory role for TET1, and cytoplasmic expression with absent nuclear localization for TET2 in SI-NETs. *In vitro* experiments supported the involvement of exportin-1 in TET2 mislocalization, and suggested that KPT-330/selinexor, an orally bioavailable selective inhibitor of exportin-1 and nuclear export, with anti-cancer effects, could be further investigated as a therapeutic option in patients with SI-NETs.

In Paper IV, DNA methylation was compared between SI-NET primary tumors and metastases by reduced representation bisulfite sequencing. Three differentially methylated regions (DMR) on chromosome 18 were detected and chosen for further analyses. The *PTPRM* gene, at 18p11, displayed low expression in SI-NETs with high levels of methylation in the presumed CpG island shores, and in the DMR rather than the promoter region or exon 1/intron 1 boundary. PTPRM overexpression resulted in inhibition of cell growth, proliferation, and induction of apoptosis in SI-NET cells, suggesting a role for *PTPRM* as an epigenetically deregulated candidate tumor suppressor gene in SI-NETs.

*Keywords:* Epigenetics, 5hmC, TET1, TET2, parathyroid tumors, SI-NET, RRBS, PTPRM

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ISSN 1651-6206
ISBN 978-91-513-0097-9
urn:nbn:se:uu:diva-330810 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-330810)

*To my beloved family*

# List of Papers

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

I **Barazeghi E**, Gill AJ, Sidhu S, Norlén O, Dina R, Palazzo FF, Hellman P, Stålberg P, Westin G. (2016) 5-hydroxymethylcytosine discriminates between parathyroid adenoma and carcinoma. *Clinical Epigenetics* 8:31.

II **Barazeghi E**, Gill AJ, Sidhu S, Norlén O, Dina R, Palazzo FF, Hellman P, Stålberg P, Westin G. (2017) A role for TET2 in parathyroid carcinoma. *Endocrine-Related Cancer* 24: 329-338.

III **Barazeghi E**, Prabhawa S, Norlén O, Hellman P, Stålberg P, Westin G. Decrease of 5-hydroxymethylcytosine and TET1 with nuclear exclusion of TET2 in small intestinal neuroendocrine tumors. *Submitted*.

IV **Barazeghi E**, Marabita F, Hellman P, Stålberg P, Westin G. Reduced representation bisulfite sequencing of small intestinal neuroendocrine tumors identifies PTPRM as a novel candidate tumor suppressor gene. *Submitted*.

Related Paper

I Svedlund J, **Barazeghi E**, Stålberg P, Hellman P, Åkerström G, Björklund P, Westin G. (2014) The histone methyltransferase EZH2, an oncogene common to benign and malignant parathyroid tumors. *Endocrine-Related Cancer* 21: 231–239.

# Contents

# Abbreviations

| | |
|---|---|
| 5-HIAA | 5-hydroxyindoleacetic acid |
| 5caC | 5-carboxylcytosine |
| 5fC | 5-formylcytosine |
| 5hmC | 5-hydroxymethylcytosine |
| 5mC | 5-methylcytosine |
| AID | Activation-induced cytidine deaminase |
| AKT | RAC-αserine-threonine protein kinase |
| AML | Acute myeloid leukemia |
| ANOVA | Analysis of variance |
| APC | Adenomatous polyposis coli |
| APOBEC | Apolipoprotein B mRNA-editing enzyme complex |
| Aza | 5-aza-2′-deoxycytidine |
| BER | Base excision repair |
| Cadherin | Calcium-dependent adhesion |
| CASR | Calcium-sensing receptor |
| CCDC178 | Coiled-coil domain containing 178 |
| CCND1 | Cyclin D1 |
| CDKN1B, 2A/B | Cyclin-dependent kinase inhibitor 1B, 2A/B |
| cDNA | Complementary DNA |
| CgA | Chromogranin A |
| CGI | CpG island |
| CpG | Cytosine-phosphate-guanine |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CTNNB1 | Catenin beta 1 |
| DAB | 3,3′-diaminobenzidine |
| DKK | Dickkopf |
| DMR | Differentially methylated region |
| DNMT | DNA methyltransferase |
| EC | Enterochromaffin |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| EZH2 | Enhancer of zeste homolog 2 |
| FHH | Familial hypocalciuric hypercalcemia |

| | |
|---|---|
| FIHP | Familial isolated hyperparathyroidism |
| FITC | Fluorescein isothiocyanate |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GATA6 | GATA binding protein 6 |
| GCM2 | Glial cells missing homolog 2 |
| GI | Gastrointestinal |
| H2A/B | Histone 2A/B |
| H3K4me | Histone 3 lysine 4 methylation |
| H3K9me | Histone 3 lysine 9 methylation |
| H3K27ac | Histone 3 lysine 27 acetylation |
| H3K27me | Histone 3 lysine 27 methylation |
| H4 | Histone 4 |
| HIC1 | Hypermethylated in cancer 1 |
| HPT-JT | Hyperparathyroidism-jaw tumor syndrome |
| HRP | Horseradish peroxidase |
| HRPT2 | Hyperparathyroidism 2, CDC73 |
| IDAX | Inhibition of the dvl and axin complex, CXXC4 |
| IDH1/2 | Isocitrate dehydrogenase 1/2 |
| IHC | Immunohistochemical |
| kb | Kilo base pairs |
| kDa | Kilo Dalton |
| Ki-67 | Antigen KI-67 |
| LEF/TCF | Lymphoid enhancer-binding factor/ T-cell factor |
| LRP5 | Low-density lipoprotein receptor-related protein 5 |
| MAPK | Mitogen-activated protein kinases |
| MEN1 | Multiple endocrine neoplasia type 1 |
| MEN2A/B | Multiple endocrine neoplasia type 2A/B |
| mESC | Mouse embryonic stem cell |
| Met | Metastases |
| MLL | Mixed-lineage leukemia |
| mRNA | Messenger RNA |
| MTC | Medullary thyroid cancer |
| mTOR | The mammalian target of rapamycin |
| MUTYH | MutY DNA glycosylase |
| NIH | National Institute of Health |
| NSHP | Neonatal severe primary hyperparathyroidism |
| PA | Parathyroid adenoma |
| PBS | Phosphate buffered saline |
| PC | Parathyroid carcinoma |
| pHPT | Primary hyperparathyroidism |
| PRC2 | Polycomb repressive complex 2 |

| | |
|---|---|
| PT | Primary tumor |
| PTH | Parathyroid hormone |
| PTP | Protein tyrosine phosphatase |
| PTPRM | Protein tyrosine phosphatase receptor type M |
| RASSF1A | Ras association domain-containing protein 1 |
| RB1 | Retinoblastoma gene |
| PI3K | PhosphoInositide-3 Kinase |
| RIZ1/PRDM2 | Retinoblastoma protein-interacting zinc-finger 1/ PR/SET domain 2 |
| RRBS | Reduced representation bisulfite sequencing |
| rRNA | Ribosomal RNA |
| RT-PCR | Real-time polymerase chain reaction |
| SFRP | Secreted frizzled-related proteins |
| sHPT | Secondary hyperparathyroidism |
| SINE | Selective inhibitor of nuclear export |
| SI-NETs | Small intestinal neuroendocrine tumors |
| SSA | Somatostatin analog |
| STAT3 | Signal transducer and activator of transcription 3 |
| TCEB3C | Transcription elongation factor B, polypeptide 3C |
| TDG | Thymine-DNA glycosylase |
| TET | Ten-eleven translocation protein family |
| TSS | Transcription start site |
| UV | Ultraviolet |
| WHO | World Health Organization |
| Wnt | Wingless-type |
| WT1 | Wilms tumor protein 1 |
| XPO1/CRM1 | Exportin-1 |

# Introduction

## Epigenetics

Cancer is a disease of both genetic and epigenetic alterations. The term 'epigenetics' was first introduced by Waddington in 1942, and refers to the study of somatically heritable changes in the gene expression without altering the DNA sequence. Epigenetic modifiers include many genes that modify the epigenome directly through DNA methylations, histone modifications, and nucleosome positioning, to alter chromatin structure and gene expression during differentiation and development, which establishes the tissue-specific gene expression. Additionally, microRNAs are a group of small noncoding RNAs playing important roles in epigenetic regulation. Notably, all epigenetic mechanisms work together to establish the 'epigenetic landscape' of the genome to regulate gene expression (Figure 1) [1].

### DNA methylation

DNA methylation occurs mainly at cytosine residues within CpG dinucleotides, which represent between 1% and 4% of genomic mammalian DNA, and a family of DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group to the 5′ position of cytosine residue. DNMT1 maintains the DNA methylation pattern during replication and prefers hemi-methylated over nonmethylated DNA as substrate, whereas DNMT3A and DNMT3B are responsible for *de novo* methylation, establishing DNA methylation patterns in early development [2].

CpG sites are not evenly distributed, and there are short regions of 0.5–4kb in length with high CpG densities in the genome known as CpG islands (CGIs). CGIs located proximal to the promoter region of more than half of the genes are mainly unmethylated and are associated with the regulation of gene transcription and DNA accessibility. Regions of up to 2kb from CGIs with comparatively low CpG density, termed 'CpG island shores', are also related to gene expression and, more importantly, exhibit tissue-specific differential methylation [3]. Moreover, the role of DNA methylation in the regulation of gene expression is context-dependent, for instance, when it occurs close to promoters, it is correlated with repression, and when within gene bodies, it activates transcription and may also have an impact on splicing. In repetitive sequences, including centromeres, methylation plays an

important role in maintaining genome stability, and, in distal regulatory elements, it is associated with transcriptional regulation. DNA methylation is necessary for the regulation of imprinted alleles, X chromosome inactivation, and differentiation [4, 5].

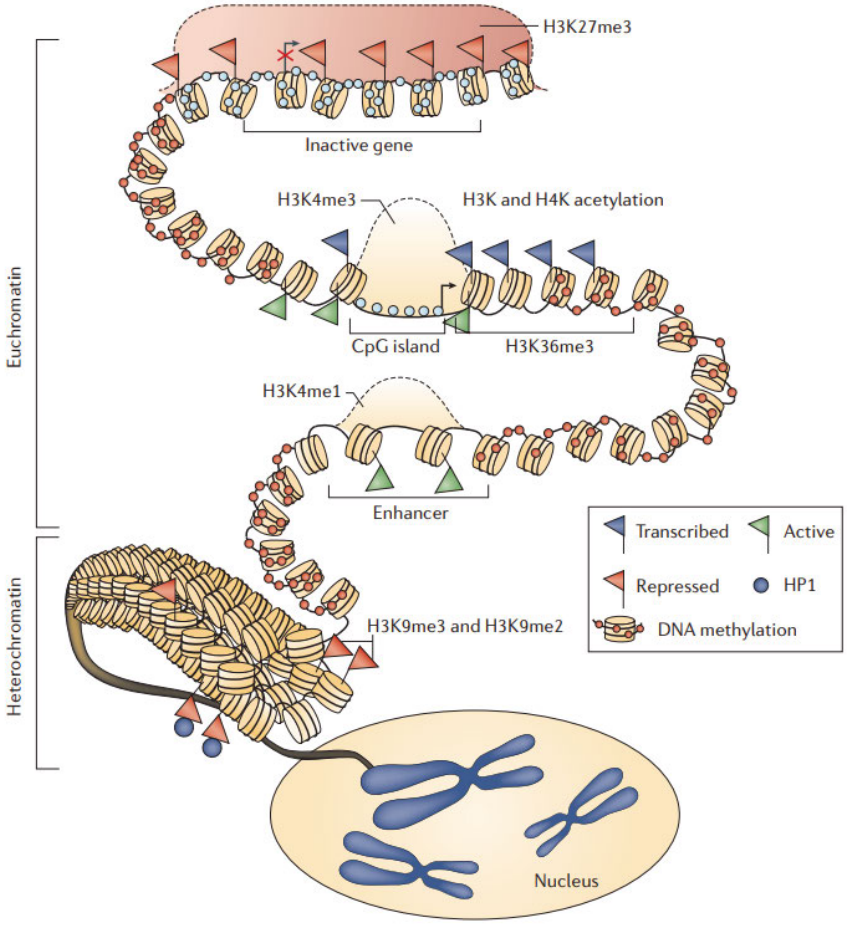


*Figure 1.* Epigenetic mechanisms in normal human cells. This diagram shows DNA methylation, histone modifications, nucleosome positioning, and the balanced state of chromatin. HP1, heterochromatin protein 1. Figure is adopted, with permission, from Baylin and Jones [6].

## 5-hydroxymethylcytosine (5hmC)

5hmC was detected first in mammalian DNA in 1972 [7], and has a distinct distribution from 5mC, with relatively low levels throughout the genome. High levels of 5hmC exist in embryonic stem cells (ESCs) (~2 million 5hmC sites), followed by a significant reduction after differentiation, but levels rise again in some of the differentiated cells, such as specific cell types of the

brain. In ESCs, the enrichment of 5hmC was found in regions with low CpG content, including promoters of genes with low expression. Tissue-specific distribution of 5hmC and accumulation in specific exonic and intragenic regions, as well as in active distal regulatory elements, support the idea that 5hmC is not only an intermediate of DNA demethylation pathway, but also has a role in the regulation of tissue-specific gene expression and may contribute to RNA splicing by recruiting DNA-binding regulatory factors and other unknown mechanisms [8-11].

Additionally, genome-wide analysis of 5hmC revealed that 5hmC is abundant in gene bodies, especially in exons compared to introns, as well as in promoter regions and active enhancers, and its contribution to gene expression regulation is cell-specific [12-14]. Because discriminating between 5hmC and 5mC is impossible when employing bisulfite conversion-based methods, different techniques have been developed to generate maps of 5hmC within the genome, including chromatography, mass spectrometry, antibodies, oxidative bisulfite sequencing (oxBS-seq), and, more recently, TET-assisted bisulfite sequencing (TAB-seq) [15]. In a recent study of the hydroxymethylome in normal and malignant liver and lung tissues by oxBS-seq, 5hmC was found to be highly enriched at active enhancers, CGI shores, along the gene body, and at the transcription termination site of active genes, but was depleted in the transcription start site (TSS) and CGIs, indicating 5hmC as an active epigenetic mark that is associated with active histone modifications and open chromatin [16].

Therefore, the discovery of the TET proteins added another dimension of complexity to the epigenetic regulation of gene expression by introducing 5hmC as an intermediate player during demethylation, and also as a new epigenetic mark leading to the modification of DNA, that may function to recruit specific chromatin remodelers and transcriptional regulatory elements.

## Ten-eleven translocation protein family, TET (TET1, TET2, TET3)

In 2009, the TET proteins were identified as 2-oxoglutarate and Fe (II)-dependent dioxygenases that all have the capacity to oxidize 5mC to 5hmC and, even further, to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which could be followed by active or passive demethylation pathways to restore unmethylated CpGs (Figure 2). Passive demethylation occurs during replication, as DNMT1 is unable to recognize 5hmC in the target hemi-methylated DNA, whereas in the active pathway, TET proteins promote DNA demethylation by triggering specific DNA repair mechanisms, such as thymine-DNA glycosylase (TDG), followed by base excision repair (BER) [17, 18].

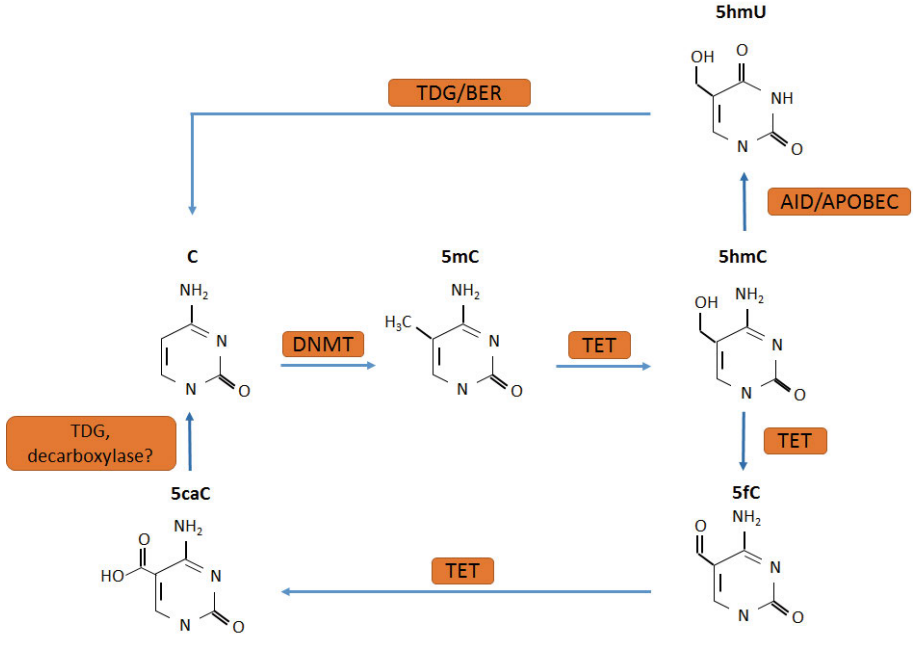


*Figure 2.* Potential pathways for TET-mediated DNA demethylation. 5mC is oxidized by TET proteins to 5hmC and further to 5fC and 5caC. Deamination of 5hmC by activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex (AID/APOBEC) produces mismatches that are recognized and repaired by TDG/BER. 5caC is converted to cytosine through TDG/BER pathway or other unknown decarboxylases.

All TET proteins are large (~180-230kDa) multidomain enzymes with a core catalytic domain consisting of a conserved double-stranded β-helix domain, a cysteine-rich domain, and binding sites for 2-oxoglutarate and Fe (II). In addition to the catalytic domain, TET1 and TET3, but not TET2, have an N-terminal CXXC zinc finger domain that can directly bind DNA. Although TET proteins share catalytic activity, they also have specific functions due to their distinct expression patterns and recruitment mechanisms [19]. TET1 is highly expressed in mouse ESCs (mESCs), and TET2/3 are broadly expressed in different adult mouse tissues [20, 21]. TET2 is also expressed in mESCs and it has been shown that TET2 regulates 5hmC production in gene bodies, enhancers, and transcription factor-binding sites, whereas TET1 tends to regulate TSS, promoter regions, and protects CGIs from hypermethylation [22]. TET3 is mostly expressed in mouse oocyte and preimplantation embryos. *TET* triple-knockout studies in mESCs have revealed that DNA methylation accumulates mostly in DNase I hypersensitivity sites, enhancers, and bivalent promoters, and these findings highlight the essential role of TET proteins in embryonic development [23-25].

**TET1**

TET1, located at 10q21, was first identified in patients with acute myeloid leukemia (AML) as a fusion partner of the histone H3 Lys4 methyltransferase mixed lineage leukemia (*MLL*) gene located at 11q23. In *MLL*-rearranged leukemia, TET1 plays a critical oncogenic role, and its aberrant overexpression leads to a global 5hmC increase [26]. In mESCs, the functional role of TET1 has been studied extensively, where it contributes to the regulation of developmental gene expression and early embryonic differentiation [27, 28]. TET1 is capable of binding to unmodified cytosine, 5mC and 5hmC, and has a preference for CGI and CpG-rich promoters via its DNA binding domain, CXXC-type zinc finger, at the N-terminus part.

In colon cancer, it has been shown that TET1 downregulation and reduced 5hmC levels are correlated with tumor initiation, and TET1 regulates cell proliferation by controlling the Wnt signaling pathway through maintaining the inhibitors of the Wnt pathway, such as DKKs and SFRPs, hypomethylated [29]. Frequent TET1 silencing by promoter methylation has been reported in various tumor cell lines and primary tumors [30].

Moreover, TET1 expression and the level of 5hmC was found to be reversely correlated to the expression of oncogene EZH2 in gastric cancer, and the tumor suppressor activity of TET1 might act through down-regulation of EZH2 [31]. It has also been shown that there is a correlation between TET1 and polycomb repressive complex 2 (PRC2), specific to ESCs, and they share target genes in ESCs [32, 33].

**TET2**

The gene encoding TET2 is positioned at 4q24, and is frequently mutated in different types of hematological malignancies and these mutations are associated with a decrease in 5hmC levels [34]. Disruption of *TET2* in mouse models leads to genome-wide enhancer hypermetylation [35], and increased hematopoietic stem cells proliferation, however, acquisition of other oncogenic events as a ‘second hit’, are required for malignant transformation.

TET2 lacks a DNA binding domain (CXXC) and is recruited to unmethylated DNA by inhibition of the dvl and axin complex (IDAX), which was once a part of an ancestral *TET2* gene encoding the CXXC domain, and during evolution, due to a chromosomal inversion event, became separated from the catalytic domain of this gene. Therefore, the level of TET2 protein can be regulated by IDAX/CXXC4, also known as a negative regulator of the Wnt signaling pathway [36]. Isocitrate dehydrogenase 1 and 2 (*IDH1*/*2*) mutations in AML patients were found to be mutually exclusive with *TET2* mutations and have been associated with DNA hypermethylation in leukemia and brain tumors. *IDH1/2* alterations result in abnormal accumulation of 2-hydroxy-glutarate, which competes with the essential α-ketoglutarate metabolite needed by TET and lysine demethylase enzymes, to regulate chro-

matin and DNA methylation levels. In addition, mutations in the Wilms' tumor 1 (*WT1*) gene are mutually exclusive with *TET2* and *IDH1/2* mutations. *WT1* encodes a transcription factor that regulates expression of genes involved in the Wnt pathway and metabolism, and is a DNA-binding partner of TET2 [37].

### Histone modification

The structure of chromatin consists of nucleosome units, each of which is composed of 147 base pairs of DNA wrapped around two copies of four histone proteins (H2A, H2B, H3, and H4). Nucleosome positions are dynamic and are regulated by chromatin remodeling complexes. The promoters of active genes have nucleosome-depleted regions, which is a key mechanism for transcription activation. The N-terminal tail of histones can undergo various post-translational modifications, including methylation, acetylation etc., and a combination of these modifications in a specific region, leads to a histone code that can determine chromatin structure and consequently regulate gene expression by the recruitment of downstream proteins or complexes, along with other epigenetic marks. H3 tri-methylated on lysine 4 (H3K4me3), for instance, is enriched within active promoter elements, whereas H3K27me3 and H3K9me3 are associated with Polycomb-mediated repressive chromatin states and heterochromatin, respectively. Overlapping presence of H3K4me3 and H3K27me3 establishes a bivalent signature for genes to allow regulatory flexibility during development and differentiation, and epigenetic marks, such as H3K4me1 and H3K27ac, define cell-type specific active enhancers [38-40].

## Epigenetic events in cancer

Aberrant epigenetic regulations, such as hypo- and hyper DNA methylation and altered histone modifications, are commonly observed in the cancer cells, and epigenetic disruption of progenitor cells has been suggested as an early step in tumor progression. More importantly, an intense cooperation between genetic and epigenetic abnormalities leads to the initiation and progression of cancer, and genome sequencing of various types of tumors have revealed frequent mutations in writers, readers and erasers of epigenetic marks, which support an altered epigenome in cancer. However, epigenetic alterations in cancer may be induced by environmental or physiological events, independent of mutations in the epigenetic machinery [41].

The hypermethylation of CGIs is common and renders the silencing of the tumor suppressor genes, whereas global DNA hypomethylation in cancer cells leads to genomic instability and aberrant expression of oncogenes and transposable elements. Moreover, 5-methylcytosine (5mC) increases the rate

of spontaneous mutagenesis due to deamination-induced mismatches in DNA. Hypermethylation of a normally expressed allele through a process termed 'loss of imprinting' can also initiate tumor development. Aberrant expression of histone modifiers, caused by mutations, may also render deregulation of histone marks and result in the disruption of the whole epigenome of cancer cells [6, 42, 43].

The importance of epigenetic alterations in cancer is highlighted by considering that these modifications are reversible, offering potential therapeutic approaches. DNMT inhibitors, mainly azacitidine and its deoxy derivative, decitabine, have been successfully applied for epigenetic therapy in patients affected by hematological malignancies and are approved by the US Food and Drug Administration (FDA) for the treatment of these patients. Recent studies and clinical trials have suggested that epigenetic therapy, in combination with other cancer therapies, such as cytotoxic drugs and chemotherapy, is a promising approach to elevate the efficacy of cancer therapy [44].

## TET1, TET2, and 5hmC in cancer

Global reduction of 5hmC, which is associated with decreased expression of TET proteins, is a general hallmark of many solid tumors, including colorectal, prostate, lung, liver and breast cancer, as well as glioblastoma and melanoma, which suggests that loss of 5hmC could be a cancer-associated feature [45-49]. However, recent studies have revealed that 5hmC content is related to the proliferation rate and at least part of the 5hmC reduction in cancer cells can be attributed to a high growth rate. Although in myeloid malignancies, including AML, 5hmC reduction has been associated with mutations in *TET2* gene, it is unlikely that, in solid tumors, missense mutations in TET genes are also relevant, because TET genes have not been found to be frequently mutated in large-scale sequencing data [45], although somatic mutations have been reported in all three TET proteins in colorectal cancer [50]. Additionally, recent studies have supported the hypothesis that TET proteins and 5hmC have a role in protecting the genome from chromosomal lesions and mutations that may initiate tumor development.

TET1 has been shown to be downregulated in prostate and breast cancers and its depletion as a tumor suppressor gene facilitates tumor growth, cell invasion and cancer metastasis [51, 52]. Moreover, the mRNA level of all three TET family members were downregulated in melanoma, breast and colorectal cancer, which are not known to be associated with mutations in these genes [47, 49, 53]. Mutations in genes encoding IDH1/2, succinate dehydrogenase (*SDH*), and fumarate hydratase (*FH*), result in aberrant accumulation of 2-hydroxyglutarate, succinate, and fumarate, respectively, and the competitive inhibition of the enzymatic activity of TET proteins, which is found in a variety of solid tumors, such as glioma and melanoma [54].

Taken all together, the expression and activity of TET proteins is impaired in different types of cancers and could potentially be considered in the treatment of cancer.

## Protein tyrosine phosphatase receptor type M (PTPRM)

Protein tyrosine phosphatases (PTPs) are the superfamily of enzymes that function as regulators of signaling pathways in cell growth, adhesion, and migration, by opposing the action of protein tyrosine kinases. The receptor-type PTPs include a single transmembrane domain, intracellular segments with tyrosine phosphatase activity, and variable extracellular domains, which are, in most cases, similar to cellular adhesion molecules, and therefore involved in cell–cell and cell–matrix interactions. PTPRM located at chromosome 18p11, is a member of the type IIb subfamily of receptor PTPs and has been shown to mediate homophilic adhesive interactions.

PTPRM has the capacity to dephosphorylate the cadherin-catenin complex to promote cadherin-mediated cell–cell adhesion at epithelial adherens junctions. Cadherins play an essential role in the transition to metastases during tumor progression and interact with actin cytoskeleton through binding to catenins, including α, β, γ, and p120 [55, 56]. Moreover, it has been reported that p120 interaction with E-cadherin is essential to stabilize the E-cadherin complex at the adherens junctions between adjacent cells, and that tyrosine-phosphorylated p120 serves as a substrate for PTPRM [57, 58]. PTPRM re-expression could restore E-cadherin-mediated adhesion in prostate carcinoma cells, regardless of its catalytic activity [59], and low expression of PTPRM was observed in poorly differentiated breast cancer cells associated with poor prognosis, increased proliferation, invasion and migration [60]. In colorectal cancer, the deletion of the chromosomal region, and a hypermethylated promoter, was found in the *PTPRM* gene, supporting the role of *PTPRM* as a tumor suppressor gene [61-63]. Mutations of *PTPRM* in colon and endometrium cancers have been identified, and whole-exome sequencing results revealed frequent somatic mutations (30.7%) of at least one receptor-type PTP family member in head and neck squamous cell carcinoma, which is correlated with elevated expression levels of tyrosine phosphorylated/activated STAT3. STAT3 is a potent oncogenic transcription factor, frequently activated in most cancers, but rarely found mutated [64, 65].

## Parathyroid glands

Usually there are four parathyroid glands located adjacent to the dorsal surface of the thyroid gland. The inferior parathyroid glands arise from the third

pharyngeal pouch, and the superiors from the fourth, during the fifth week of fetal development. The inferior glands have a higher variability of location due to their migrating a longer distance, along with thymic tissue. Normal parathyroid glands are small, pale brown, and usually weigh < 60 mg. The parathyroid glands consist of two main cell types; chief cells, and oxyphil cells. Oxyphil cells are larger than chief cells, with higher mitochondrial content and eosinophilic cytoplasm. Transitional oxyphil cells, representing an intermediate phase from chief cells to oxyphil cells, and chief cells with clear cytoplasm, referred to as 'water-clear cells', are also present within parathyroid glands [66].

The central role of the parathyroid glands is regulation of the serum levels of calcium through the secretion of parathyroid hormone (PTH), an 84-amino-acid peptide with a 3–5 minutes half-life, from chief cells. The serum levels of extracellular ionized calcium is detected by calcium-sensing receptors which are coupled with a G-protein receptor, located on the surface of parathyroid chief cells, and these stimulate the synthesis and release of PTH. Tight regulation of calcium homeostasis is a result of collaboration between the kidneys, small intestine, bone and parathyroid glands. In the kidneys, PTH directly acts on renal tubules and increases calcium resorption and phosphate secretion, and also activates hydroxylation of 25-hydroxyvitamin D to produce the active vitamin D, 1,25-dihydroxyvitamin D. The active vitamin D enhances calcium absorption in the small intestine. In the bone, PTH mobilizes calcium by acting on osteoblasts and osteoclasts and increases bone turnover [67, 68] (Figure 3).

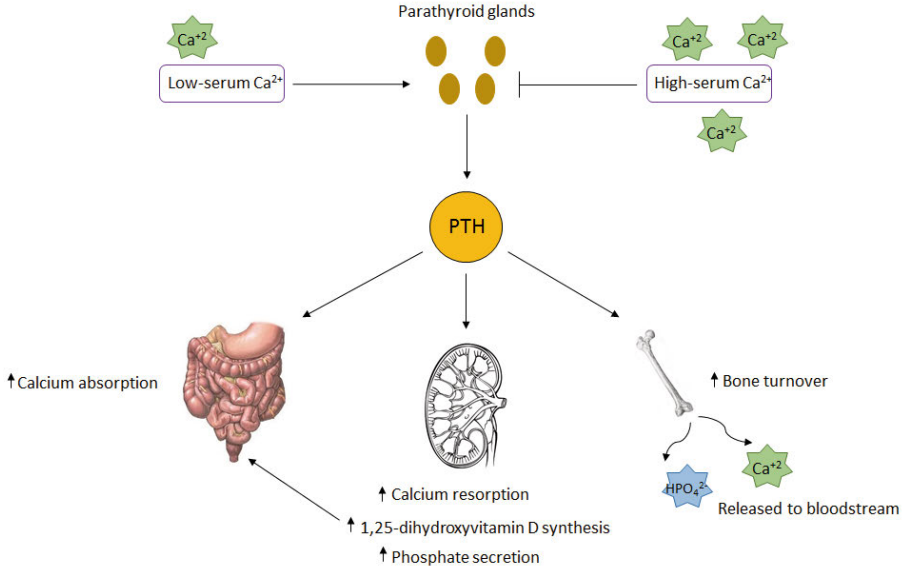


*Figure 3.* Regulation of calcium homeostasis by the parathyroid hormone (PTH).

## Parathyroid tumors

Primary hyperparathyroidism (pHPT) is a common endocrine disease characterized by excessive production of parathyroid hormone from enlarged parathyroid glands, resulting in hypercalcemia. In 80% to 85% of cases, pHPT is caused by an enlarged gland as a single benign adenoma, and in 15% by hyperplasia or multiglandular disease [69, 70]. pHPT progresses slowly and affects ~3 per 1000 of the general population. The incidence of pHPT increases by age and is common among postmenopausal women, with a prevalence of > 1% [71]. Patients affected by pHPT present highly variable symptoms, from being asymptomatic to symptoms of hypercalcemia, including dehydration, kidney stones, fatigue, mental confusion, muscle weakness and osteoporosis. Head and neck exposure to ionizing radiation is a risk factor for developing parathyroid adenomas (PA) [72].

Parathyroid carcinoma (PC) is very rare with an incidence of less than 1–5% of pHPT cases, but is highly aggressive. Progressive transformation of adenoma to carcinoma must be exceedingly rare, as driver mutations in PAs are rarely seen in PCs, although this could be attributed to the removal of adenomas before they progress into carcinoma [73, 74]. Patients affected by PC usually represent higher calcium and PTH levels, and, more frequently, a palpable neck mass, than in benign disease. Patients tend to be younger in age with the same distribution amongst men and women. Metastatic lesions (most common in lung, lymph node, bone, and liver tissues) and local invasion are the only definitive criteria for malignancy, and the rate of recurrence is 66%. Mortality is usually caused by hypercalcemia [75].

Elevated production of PTH in response to hypocalcaemia of another cause, including chronic renal failure, calcium and vitamin D deficiency, renders secondary hyperparathyroidism (sHPT), in which all four parathyroid glands are enlarged, and autonomous areas may develop in the parathyroid glands, leading to persistent hypercalcemia after kidney transplantation (sometimes denoted tertiary HPT). sHPT is a common complication in patients affected by chronic kidney disease and increases the rate of bone resorption and cardiovascular risks [76].

## Diagnosis and treatment

Clinical evaluation of serum calcium and parathyroid hormone (reference upper limits < 2.50 mmol/L and < 6.9 pmol/L, respectively) is a diagnostic approach for pHPT, and is treated by parathyroidectomy in patients with severe symptoms and who are aged less than 50 years. Surgery cures 95–99% of the HPT patients, and, in the case of PC, the main treatment is complete en bloc resection with a 10-year survival rate of 49% [77].

The pathological diagnosis of PC has always been difficult and, based on the World Health Organization (WHO) 2004 criteria, PC is determined ac-

cording to invasive growth patterns and metastases. However, in the absence of malignant features, differentiating PC from benign disease remains difficult. Loss-of-function mutations of the *Hyperparathyroidism 2* (*HRPT2*), encoding parafibromin, are common in sporadic PCs (approximately 70%) and rare in benign disease (< 1%) [78, 79]. Parafibromin nuclear immunohistochemical (IHC) staining has been reported to be useful for the diagnosis of carcinomas in some studies [80-82] and difficult to deploy technically in some other studies, due to variable patterns of staining [83, 84]. In addition, loss of adenomatous polyposis coli (APC) expression and immunoreactivity in PC has been suggested as a potential marker for the detection of PC [85].

sHPT is usually treated by removing the underlying cause of hypocalcaemia. Patients affected by chronic renal disease can be treated by calcium and vitamin D supplementation, chelating excess phosphate in the intestines, and phosphate dietary restrictions, which will lead to a reduction in PTH, and, in case of severe renal hyperparathyroidism, patients require surgery [70]. In hypercalcemic sHPT ("tertiary HPT"), removal of enlarged parathyroid glands or adjusting the sensitivity of the parathyroid calcium-sensing receptor by calcimimetics may be used in addition.

## Hereditary parathyroid tumors

Primary HPT is mostly a sporadic disease, and approximately 10% of pHPT patients have a familial genetic syndrome. Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant familial syndrome characterized by several endocrine lesions, including tumors of the parathyroid glands (> 90%), pituitary (30%), and pancreatic islet cells (40%). MEN1 is associated with heterozygous germline loss of function mutations in the *MEN1* gene located at 11q13, encoding the protein menin, which has a role in transcriptional regulation, DNA repair, and cytoskeletal function. pHPT in MEN1 syndrome develops as multiglandular disease and in the relatively young (< 50 years of age) [71, 86]. MEN2 is also an autosomal dominant tumor syndrome caused by heterozygous germline gain of function mutations in *rearranged during transfection* (*RET*) proto-oncogene, located at 10q11, which encodes a membrane-associated tyrosine kinase. MEN2A patients present medullary thyroid cancer (MTC) (90%), phaeochromocytoma (50%), and PAs (20%), whereas MEN2B comprises MTC, phaeochromocytoma and gastrointestinal ganglioneuromatosis, to name a few [71].

Familial hyperparathyroidism-jaw tumor (HPT-JT) syndrome is an autosomal dominant and hereditary disease caused by germline inactivating mutations in the *HRPT2/CDC73* gene located at 1q31. The *HRPT2* gene encodes the protein parafibromin, which is involved in the regulation of cyclin D1 and the Wnt/β-catenin signaling pathways, and acts as a tumor suppressor gene. HPT-JT is associated with hyperparathyroidism and a high incidence of PCs [87].

The calcium-sensing receptor (*CASR*) gene regulates PTH secretion in parathyroid cells, and germline mutations in this gene at 3q13-q21 cause hypercalcemia in association with heritable syndromes, including Familial hypocalciuric hypercalcemia (FHH) (heterozygous inactivating mutation) and neonatal severe hyperparathyroidism (NSHP) (homozygous inactivating mutation). FHH is characterized by hypercalcemia, a slightly elevated level of PTH, and low urinary calcium excretion. NSHP represents severe hypercalcemia symptoms and is diagnosed in the first week of life. Familial isolated hyperparathyroidism (FIHP) is a rare inheritable disorder representing single adenoma or multiglandular hyperparathyroidism, in the absence of all other endocrine syndromes. A subset of families with FIHP have displayed germline mutations in *MEN1*, *CDC73,* or *CASR* genes, although the genetic etiology of the disease is still unknown [88, 89].

## Genetic and epigenetic alterations of sporadic parathyroid tumors

One of the genetic aberrations in PAs is somatic loss of heterozygosity of the *MEN1* gene in 25%–40%, and, in about 50% of the patients, inactivating mutation of the other allele [73]. Somatic mutations were found in 13% of sporadic PCs [74]. *MEN1*, and two of its target genes, *p18* and *p27*, has been shown to be downregulated in primary and secondary HPTs [90, 91]. Somatic mutations of *CDKN1B* at 12p13, encoding the p27 cyclin-dependent kinase inhibitor, which negatively regulates cell proliferation, have been observed only in a small fraction of adenomas (4.6%) [92] and not in secondary HPTs [93].

The cyclin D1 gene (*CCND1*), initially named parathyroid adenoma 1, at 11q13 is an oncogene, which is overexpressed in some sporadic PAs (20–40%), in 31% of secondary HPTs, and in up to 90% of PCs. However, rearrangement of the *CCND1* as a result of pericentromic inversion, involving the regulatory sequence of the PTH gene, only occurs in 8% of adenomas [94]. As a target gene for the Wnt/β-catenin signaling pathway, cyclin D1 overexpression in the absence of gene translocation could be a result of aberrant accumulation of nonphosphorylated active β-catenin [95, 96], and, in PCs, parafibromin loss of expression could be involved, as parafibromin regulates the expression of cyclin D1 [97].

*HRPT2/CDC73* gene somatic mutations and copy number alterations are rare in PAs, but occur commonly in apparently sporadic PCs (70%), which may represent cases of undiagnosed HPT-JT syndrome and should be considered for germline test, even in the absence of a family history [98, 99].

The Wnt/β-catenin signaling pathway is involved in various cell events and has been shown to be aberrantly activated in parathyroid tumors of pHPT and sHPT patients. *CTNNB1* at 3p22 encodes β-catenin, which regu-

lates the transcription of many downstream genes, including *MYC* and *CCND1*, through binding to the LEF/TCF family of transcription factors. β-catenin accumulation was observed, due to mutation Ser37Ala (TCT > GCT) in β-catenin (7.3%), in adenomas in one study [100], but not in other studies [101, 102]. However, Ser33Cys β-catenin mutation has been reported with a low frequency in PAs [103, 104]. An internally truncated Wnt receptor LRP5 (LRP5Δ) has also been reported as a possible cause of active β-catenin accumulation in adenomas without mutation [105]. In PCs, Wnt/β-catenin signaling pathway activation through aberrant β-catenin accumulation has been shown as a result of the *APC* tumor suppressor gene inactivation, possibly due to promoter DNA methylation [85, 106].

In PC, some studies have shown that abnormal expression of the *RB1* (retinoblastoma) gene at 13q14 could be a potential marker to distinguish PC from benign adenomas [107, 108]. However, it has not been confirmed by other studies whether the allelic loss of *RB1* gene is specific in malignant parathyroid disease [109, 110].

The knowledge of epigenetic aberrations in hyperparathyroidism is sparse. Generally, parathyroid tumors are not hypomethylated in contrast with other types of tumors. Analysis of the differentially methylated CGIs showed that PCs are hypermethylated, while PAs and normal tissues displayed lower methylation, respectively. Hypermethylation of the tumor suppressor genes *RIZ1/PRDM2*, *RASSF1A* and *APC* at promoter CpG sites have been identified in a subset of parathyroid tumors [111-113]. Additionally, a relatively reduced expression of CDKN2A/B, SFRPs and WT1, due to hypermethylation in a number of benign and malignant parathyroid tumors, has been reported [75, 114]. The *HIC1* tumor suppressor gene has been shown to be down-regulated by H3K27me2/3 modification rather than DNA methylation in primary and secondary parathyroid tumors [115]. The *EZH2* gene at 7q36 encodes the catalytic subunit of PRC2 that methylates histone3 lysine27 (H3K27me2/3), and was found to be overexpressed in a large fraction of parathyroid tumors. EZH2 overexpression interfered with the Wnt/β-catenin signaling pathway through epigenetically repressing the Wnt signaling inhibitor genes, contributing to the β-catenin abnormal accumulation and tumor growth in parathyroid tumors [116].

## Neuroendocrine cells

Neuroendocrine cells are diffusely distributed in different parts of the human body, including the lungs, skin, pancreas and gastrointestinal tract (GI tract). Neuroendocrine cells are also called neurosecretory cells, and they release hormones into the blood in response to neurotransmitters. These cells consist of at least 17 different types of cells, and are able to produce various bioactive peptides and amines. The most abundant neuroendocrine cells in the GI

tract are enterochromaffin (EC) cells, which produce 5-hydroxytryptamine (serotonin) as well as a variety of peptides, such as kinins, and prostaglandins, and are positively stained for chromogranin A [117, 118].

## Small intestinal neuroendocrine tumors (SI-NETs)

SI-NETs arise from EC cells in the second portion of the duodenum, jejunum, ileum, and proximal colon, and account for 25% of small-bowel neoplasms. These are the most frequent GI tract NETs, and are mostly well-differentiated, slow-growing, and, based on embryonic derivation, were previously classified as midgut carcinoid tumors. Foregut carcinoids include endocrine tumors of the respiratory system, stomach, upper duodenum, and pancreas, while tumors of the transverse colon up to the anus arise within the hindgut [119].

According to the WHO classification in 2010, small intestinal neuroendocrine tumors are categorized into three grades based on proliferative activity and mitotic count; well-differentiated NETs with low or intermediate as Grade 1 (G1, Ki67 < 3%) and Grade 2 (G2, Ki67 3-20%), and poorly differentiated NETs of Grade 3 (G3, Ki67 > 20%). Tumor-node-metastasis (TNM) classification guidelines, based on the WHO system, are also used for the staging and grading of NETs [120].

The annual incidence of SI-NETs is 1/100 000 and is on the rise, partly due to advanced diagnostic techniques and increased awareness. More than 25% of these tumors are multifocal, with common symptoms of abdominal pain and intestinal obstruction, and often secrete peptide hormones, such as serotonin and tachykinins, resulting in carcinoid syndrome. Carcinoid syndrome, including diarrhea, flushing, and bronchial construction, occurs in ~ 10% of patients and can lead to carcinoid heart disease, mostly in patients with metastases, due to high levels of serotonin and tachykinin secretion [121, 122]. However, the majority of patients present mild and nonspecific symptoms that can cause diagnosis at an advanced stage with lymph nodes and liver metastases in up to 88% and 61% of patients, respectively, with approximately 67% having a five-year survival rate [123]. Metastases can also be found in distant sites, such as bone, peritoneum, and ovaries, in advanced stages.

### Diagnosis and treatment

NETs are characterized by excess secretion of functional hormones and peptides, such as serotonin and chromogranin A (CgA), which can function as biomarkers for the diagnosis. CgA, a neurosecretory glycoprotein, is stored within chromaffin granules of neuroendocrine cells and is widely used in clinical practice as a biomarker to diagnose both functioning and non-

functioning NETs. Serum levels of CgA are associated with tumor burden and are used to follow disease progression and recurrence, although this is not recommended as a NETs screening test due to low specificity [124]. 5-hydroxyindoleacetic acid (5-HIAA) 24-hour urine measurement is specific for monitoring patients with SI-NETs, and is usually elevated in case of liver metastases. Enzymatic inactivation of serotonin in the liver produces 5-HIAA, which is excreted into the urine where it can be measured. Urine 5-HIAA level is affected by the patient's diet and certain foods and drugs may cause false positive or negative results. In addition to biochemical diagnosis, radiology, nuclear imaging, and histological evaluation of mainly CgA and synaptophysin, can be used to determine the location of tumors and the stage of the disease [125, 126].

Curative surgery is the main therapeutic option in SI-NETs, and may relieve symptoms caused by small intestinal obstruction or ischaemia. However, surgery is not often possible due to metastases in an advanced-stage at diagnosis. Therefore, medical treatment is required for most of the patients to relieve the symptoms caused by hormone overproduction, and to suppress the tumor's growth and progression. Somatostatin analogs are nowadays widely used for carcinoid syndrome therapy. Somatostatin endogenously inhibits secretion of various hormones, such as serotonin, from endocrine cells through binding to the five somatostatin receptor subtypes on secretory endocrine cells. The two most important subtypes, with wider inhibitory effect, are subtypes 2 and 5, which have also been shown to have antiproliferative effects. Somatostatin analogs (SSA), such as octreotide and lanreotide, have been developed for clinical use, as somatostatin has a very short half-life (< 3min). Radiolabeled SSAs have also been provided for both the therapy and diagnosis of SI-NETs. In addition, interferon has been used alone or in combination with SSAs due to its antitumor effects, but has limited use because of its potential side effects [123, 127].

Expression profiling has been performed in SI-NETs in order to identify the relevant genes involved in tumorigenesis as novel targets for treatment, and to obtain tumor classification, which is associated with clinical features and patient outcome. These analyses suggested prognostic biomarkers and novel genes that could be further evaluated for treatment [128, 129]. Epigenetic therapy using histone deacetylase inhibitors, such as valproic acid, has also been suggested in neuroendocrine tumors as a potential therapeutic option in combination with other treatments, although further evaluations are required [130].

The mammalian target of rapamycin (mTOR) is the most important downstream mediator of the PI3K/AKT signaling pathway that regulates cell proliferation, metabolism, and angiogenesis. Inhibitors of the mTOR pathway, such as everolimus, were introduced into clinical trials since mTOR activation has been demonstrated in different types of neuroendocrine neo-

plasms. Everolimus is an oral agent which has demonstrated significant antitumor effect in most NETs [131].

## Genetic and epigenetic landscape of SI-NETs

The most common genetic aberration in SI-NETs is the loss of one copy of chromosome 18, which is known to occur in > 60% of tumors. According to genetic studies, one copy deletion of chromosome 18 is followed by additional losses on chromosomes 3p, 11q, and 13. However, in a smaller group of tumors with intact chromosome 18, gains on chromosomes 4, 5, 7, 14, and 20 have been identified [132-136]. Whole-exome sequencing analysis has revealed a low mutation rate with no highly recurrent mutations in SI-NETs, indicating that these tumors may be genetically stable. In the same study, the amplification of *AKT1* and *AKT2* were detected, which may affect the PI3K/Akt/mTOR signaling pathway [137]. Sequencing analysis of a cohort of 180 tumors identified frameshift mutations (8%) and hemizygous deletions (14%) in the *CDKN1B* gene, located on chromosome 12p, encoding the cyclin-dependent kinase inhibitor, p27 [138]. These findings were corroborated in a study of a larger cohort of 362 tumors [139]. In addition, familial cases of SI-NET have been reported, and a heterozygous mutation was detected as both germline and somatic mutation in the MutY DNA glycosylase gene (*MUTYH*) located at 1p34, involved in oxidative DNA damage repair. However, other mutations in additional genes may also play a role in predisposing to familial SI-NET [140, 141].

SI-NET epigenetics has not been extensively studied. In a study of global DNA methylation analysis of repeat elements, all NET subtypes were found to be hypomethylated compared to normal tissue, which was correlated with the loss of one copy of chromosome 18, metastases, and poor prognosis [142]. *RASSF1A* and *CTNNB1* promoter regions were found to be more methylated in metastases when compared with primary tumors, suggesting that these genes may be associated with tumor progression in SI-NET. *RASSF1A* is a tumor suppressor gene located on chromosome 3p, which has an important role in the RAS signaling pathway, and *CTNNB1*, located in the same region on chromosome 3p, encodes beta-catenin [143]. *TCEB3C*, located at 18q21, encodes elongin A3, and has been shown recently as being epigenetically downregulated in SI-NETs, and may have a growth regulatory role [144]. DNA methylation, gene expression, and copy number variance analysis of 97 SI-NETs [145] identified differentially methylated genes between primary tumors and normal tissue, and the majority of these genes were involved in multiple cancer-related pathways, including the MAPK, Wnt, and PI3K-mTOR signaling pathways.

# Aims of the study

The overall aim of this study was to investigate epigenetic aberrations in parathyroid tumors and SI-NETs in order to identify disease mechanisms and find possible therapeutic targets.

The specific aims of the study were:

**Paper I.** To investigate the 5hmC level and TET1 protein expression and function in parathyroid tumors.

**Paper II.** To clarify the expression, epigenetic regulation, and possible growth regulatory role of TET2 in parathyroid carcinomas.

**Paper III.** To study the level of 5hmC, expression level and function of TET1 and TET2 in SI-NETs.

**Paper IV.** To identify novel candidate tumor suppressor genes using reduced representation bisulfite sequencing (RRBS), with more emphasis on regulatory elements in SI-NETs.

# Materials and methods

The following is a brief description of the materials and methods used in this thesis. All materials and methods are fully described in the papers.

## Tissue specimens

Parathyroid tumors (Papers I and II) were obtained from patients diagnosed and operated upon at Uppsala University Hospital, Uppsala, Sweden, the Royal North Shore Hospital, St Leonards, Australia, and Hammersmith Hospital, London, UK. SI-NETs (Papers III and IV) were collected from patients diagnosed and operated on at Uppsala University Hospital. The tumors were snap frozen or paraffin embedded, and cryo- or paraffin sections were used in the analysis. Informed consent was obtained from all of the patients, and approval to conduct the research was obtained from each of the respective ethics committees.

## DNA/RNA preparations

Genomic DNA was extracted from frozen surgical specimens or cultured cells using the DNeasy Blood and tissue kit (Qiagen GmbH, Hilden, Germany), from paraffin-embedded tissue sections using the QIAamp DNA FFPE tissue kit (Qiagen GmbH), and DNA-free RNA was extracted using the RNeasy Plus Mini kit (Qiagen GmbH) according to the manufacturer's protocol. The TURBO DNA-free™ kit (Life Technologies Corporation) was used to complete DNase I treatment of all RNA preparations, and successful treatment was established by PCR analysis.

## DNA immune-dot blot assay

One microgram of genomic DNA (Papers I and III) was denatured in 0.1 M Sodium hydroxide, then neutralized with 1 M ammonium acetate, and two-fold serial dilutions of the DNA samples were spotted onto Hybond-N+ nylon membrane (GE Healthcare, Piscataway, NJ, USA) using a Bio-Dot apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The spotted mem-

brane was fixed with UV irradiation (GS Gene Linker UV chamber, Bio-Rad), blocked with 5% skimmed milk, and incubated overnight with a rabbit polyclonal anti-5hmC antibody (39791; Active Motif, Carlsbad, CA, USA). After incubation with the appropriate secondary antibody, signals were visualized with the enhanced chemiluminescence system (GE Healthcare). NIH Image-J software was used to quantify signal intensities of the second dot blot signal from the top for each serial dilution according to the program's instructions. Equal spotting of the total DNA was ensured by staining the same membrane with 0.02 % methylene blue in 0.3 M sodium acetate.

## Real-time quantitative PCR

Reverse transcription of RNA preparations was performed with random hexamer primers using the First-strand complementary DNA (cDNA) synthesis kit (GE Healthcare). Real-time quantitative RT-PCR reaction was performed on StepOnePlus RealTime PCR systems (Life Technologies Corporation) using the TaqMan gene expression Master Mix, and assays for GAPDH mRNA and 18S rRNA were used as internal control. *TET1* (Papers I and III), *TET2* (Papers II and III), *PTH, CASR, GCM2* (Paper II), *XPO1* (Paper III), *PTPRM, GATA6,* and *CCDC178* (Paper IV) Taqman assays were used, and all samples were amplified in triplicates.

## Immunostaining

Paraffin-embedded tissue sections were deparaffinized with xylene and rehydrated through descending alcohol concentrations and distilled water. After treatment with 3% hydrogen peroxide, sections were heated in either EDTA pH 8.0 or citrate buffer pH 6.0. The sections then were incubated with normal serum, followed by a primary antibody. The rabbit polyclonal anti-5hmC antibody (Active Motif) (Papers I and III), rabbit polyclonal anti-TET1 (HPA019032; Prestige Antibodies, Sigma-Aldrich Sweden AB, Stockholm, Sweden) (Papers I and III), goat polyclonal anti-PTH antibody (sc-9678; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (Papers I and II), rabbit polyclonal anti-TET2 antibody (21207-1-AP, Proteintech Group, Manchester, UK) (Papers II and III), anti-chromogranin A antibody (LK2H10, Thermo Fisher 13 Scientific) (Papers III and IV), and goat polyclonal anti-PTPRM antibody (ab111207, Abcam) (Paper IV) were used. After incubation with the appropriate biotinylated secondary antibody and ABC complex, DAB (3,3′-diaminobenzidine) was used for visualization. Frozen tissue sections (Papers I and II) were first fixed in formalin and then stained, as described above. Cultured cells (Papers II and III) on chamber

slides were fixed in formalin and incubated with ice-cold 70% alcohol, then stained.

For immunofluorescence staining, the sections were treated and incubated with the rabbit polyclonal anti-5hmC antibody (Active Motif), as mentioned above (Paper I), and cultured cells with the goat polyclonal anti-PTH antibody (Paper II), then incubated with proper fluorescence secondary antibody (Alexa 594 or Alexa 488, Life Technologies). Sections were washed with PBS (0.05% Tween20) and mounted with Vectashield with Dapi (Vector Laboratories, Inc., Burlingame, CA, USA).

## Cell culturing

sHPT-1 parathyroid tumor cells, established from a hyperplastic parathyroid gland removed at operation from a patient with secondary hyperparathyroidism due to renal insufficiency [96] (Papers I and II), and HEK293T cells (Paper I) were cultured in DMEM/10% fetal bovine serum (Sigma Aldrich). CNDT2.5 adhesive cells established from a liver metastases from a patient diagnosed with primary ileal SI-NET [146], and KRJ-I suspension cells from a multifocal metastatic ileal carcinoid tumor [147], were cultured in DMEM-F12 complemented with 10% fetal bovine serum (Sigma Aldrich), 1% non-essential amino acids, and 1% penicillin-streptomycin (PEST). All cells were cultured at 37°C in 5% $CO_2$.

## Transfections and drug treatments

sHPT-1 cells (Papers I and II) and HEK293T cells (Paper I) were distributed onto 35-mm dishes ($2\times10^5$), and transfected in triplicates using FuGENE 6 transfection reagent (Promega Biotech AB). In Paper I, pIRES-hrGFP ll-TET1-FL [148] or empty vector (pIRES-hrGFP ll), and in Paper II, TET2 clustered regularly interspaced short palindromic repeats (CRISPRs) double nickase plasmid (sc-400545-NIC) or control double nickase plasmid (sc-437281) (Santa Cruz Biotechnology), were used.

CNDT2.5 cells ($2\times10^5$) were transfected with TET1 plasmid expression vector (InvivoGen) or empty vector (pUNO1) (Paper III), GATA6, CCDC178, PTPRM expression vectors (ViGene) or empty vector (pEnter), and PTPRM wild type and mutant expression vectors or empty vector (pcDNA3.1) [62] (Paper IV) using Lipofectamin 2000 transfection reagent (Life Technologies). KRJ-I cells were seeded onto twelve-well plates ($1\times10^5$) prior to transfection and transfected with TET1 plasmid expression vector (InvivoGen) or empty vector (pUNO1) (Paper III), and PTPRM expression vector (ViGene) or empty vector (pEnter) (Paper IV) using Attractene transfection reagent (Qiagen).

CNDT2.5 ($2\times10^5$) and KRJ-I ($1\times10^5$) cells were treated with 5-aza-2′-deoxycytidine (Aza) (10 μM and 5 μM, respectively) for 72h (Papers III and IV), leptomycin B (sc-202210, Santa Cruz Biotechnology), the nuclear export inhibitor for 24h, and KPT-330/selinexor (Selleckchem), an orally bioavailable selective inhibitor of nuclear export for 72 hours (Paper III).

## Colony formation assay

sHPT-1 and HEK293T cells (Paper I) were transfected with TET1 expression vector or empty vector, and, after 24h, 2000 sHPT-1 cells and 8000 HEK293T cells were distributed onto six-well plates in triplicates. After 10-day selection in 0.2 mg/ml Neomycin (G418, Sigma Aldrich), the cells were fixed with 10% acetic acid/10% methanol and stained with 0.4% crystal violet, and visible colonies were counted. In Paper II, sHPT-1 cells were transfected in triplicates with TET2 CRISPR double nickase plasmid or control plasmid, and selected with 0.5 μg/ml puromycin for 14 days. Then colonies were fixed, stained, and counted as described above. CNDT2.5 cells were transfected with TET1 (Paper III), GATA6, CCDC178, PTPRM expression vectors or empty vectors, and PTPRM wild type and mutant expression vectors or empty vector (pcDNA3.1) (Paper IV). Then, 2000 cells were seeded onto six-well plates in triplicates and selected in 8 μg/ml blasticidin, 0.5 μg/ml puromycin, and 0.2 mg/ml G418, respectively. After 10 days in selection, colonies were fixed, stained, and counted as mentioned above.

## Cell proliferation and apoptosis assays

In Papers II, III and IV, cell proliferation assay was performed using the CyQUANT cell proliferation assay kit (Invitrogen, Thermo Fisher Scientific). sHPT-1, CNDT2.5 and KRJ-I cells were first frozen in the microplate and then lysed and stained with CyQuant GR dye solution, and fluorescence intensity was measured at 480/520 nm using an Infinite 200 PRO (TECAN) plate reader.

Apoptosis was measured in sHPT-1 cells (Paper I) using the Cell Death Detection ELISA kit (Roche Molecular Biochemicals), and, as a positive control, cells were incubated with 0.1 μg/ml camptothecin (Sigma Aldrich) for 72h. Apoptosis in sHPT-1 transfected cells was also measured by incubation with FITC-labeled annexin V and propidium iodide (Sigma-Aldrich), and was analyzed by flow cytometry on a FACS Canto II (BD Biosciences). In Papers III and IV, the Cell Death Detection ELISA kit (Roche) was used to measure apoptosis. As a positive control, CNDT2.5 and KRJ-I cells were incubated with 0.1 μg/ml camptothecin (Sigma Aldrich) for 48h.

## Cell migration and invasion assays

sHPT-1 cells (Paper II) were transfected with TET2 CRISPR double nickase plasmid or control plasmid in triplicates and selected as described above. After 14 days, migration and invasion capacity was measured *in vitro* using Cytoselect 96-well cell migration assay (CBA-106; Cell Biolabs Inc., San Diego, CA, USA) and Cytoselect 96-well cell invasion assay (basement membrane, CBA-112).

## Bisulfite treatment and pyrosequencing

Genomic DNA (Papers II, III and IV) was bisulfite treated with the EpiTect bisulfite kit (Qiagen) and PCR amplified using MyTaq HS Mix (Bioline USA Inc., Taunton, USA). The pyrosequencing was performed with PyroMark™ Q24 (Qiagen).

## Reduced representation bisulfite sequencing (RRBS) and methylation analysis

Genomic DNA was first digested using 50 U of the restriction enzyme Msp1 (New England Biolabs, #R0106T). Then end repair and A tailing was performed, and NextFlex methylated adapters were ligated. Adapter-ligated constructs were bisulfite-treated using the EZ DNA methylation Gold kit according to the manufacturer's protocol (Zymo Research). Bisulfite-converted libraries were amplified 12 cycles by PCR and were quality controlled and quantified. The RRBS libraries were subjected to cluster generation on a cBot instrument (Illumina) and paired-end sequencing was performed for 100 cycles using a HiSeq instrument (Illumina) with TruSeq SBS Chemistry v3, according to the manufacturer's protocols.

For DNA methylation analysis, first adapter removal and adaptive trimming (quality score < 25) was performed, then trimmed sequences were mapped to the human genome (build hg19) and methylation calls were extracted using Bismark [149]. CpGs with ≥ 5× coverage in all samples were retained, and 278 006 CpGs were included in subsequent analysis. For each CpG site, a *t*-statistic as an indicator of differential methylation was calculated using limma [150]. The moderated *t*-statistics were extracted for the comparison metastasis versus primary tumor and were used to find candidate differentially methylated regions (DMRs) in the genome where its value exceeded an arbitrary threshold. The threshold was computed using 2.5% and 97.5% quantiles on the *t*-statistic, corresponding to 5% of the tested positions. The region was finally summarized using the average difference in methylation between metastases and primary tumors.

## Western blotting

Western blotting analysis (Papers I, II and III) was completed on protein extracts prepared in Cytobuster Protein Extract Reagent (Merck Millipore, Billerica, MA, USA) with a complete protease inhibitor cocktail (Roche Diagnostics Scandinavia AB, Bromma, Sweden). Primary rabbit polyclonal anti-TET1 (GTX124207), goat polyclonal anti-Actin (sc-1616), and rabbit polyclonal anti-TET2 (21207-1-AP) were used. Separate cytoplasmic and nuclear protein extracts (Paper III) were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific) with Halt Protease Inhibitor Single-Use Cocktail EDTA-free (Thermo Scientific). Rabbit polyclonal anti-TET2 (21207-1-AP), mouse monoclonal anti-Lamin A/C (sc-376248), and rabbit polyclonal anti-βtubulin (sc-9104) were used. After incubation with the proper secondary antibody, except for anti-Lamin A/C-HRP conjugated, bands were visualized using the enhanced chemiluminescence system (GE Healthcare).

## Statistical analysis

An analysis of variance (ANOVA) test (Paper I) was used to calculate differences in relative 5hmC level between the three biological groups and to compare clinical data between the four groups of adenomas. A Bonferroni correction was performed to adjust the $p$ values. Unpaired (all papers) and paired $t$-tests (Papers II and III) were used for statistical analysis. All data were presented as mean ± S.E.M, and, in Paper III, mean ± S.D. with $p < 0.05$ was considered significant.

# Results and discussion

## Paper I. 5-Hydroxymethylcytosine discriminates between parathyroid adenoma and carcinoma

*Clinical Epigenetics 8:31 (2016)*

PC is rare and accounts for less than 1–5% of pHPT cases. However, pathological diagnosis of PCs remains a challenge due to the lack of a completely sensitive, specific and widely applicable IHC marker.

In this study, 43 PAs and 17 PCs were included, and DNA immune-dot blot assay, immunohistochemistry, Western blotting, quantitative RT-PCR, and a colony forming assay were performed. The global level of 5hmC was determined in PCs ($n$ = 9), PAs ($n$ = 15), and apparent normal parathyroid tissue specimens ($n$ = 4) using the DNA immune-dot blot assay. Our results revealed a reduced level of 5hmC in PAs and PCs compared to normal parathyroid samples, and most severe reduction in PCs. In line with these results, IHC analysis of additional PCs showed negative staining for 5hmC ($n$ = 17), whereas normal parathyroid cells stained positively, and the staining pattern of adenomas ($n$ = 43) were generally heterogeneous. Seven adenomas showed positive staining regardless of strength (+), while 36 of them stained positively with aberrant appearances, including mosaic staining (M), a mixture of positive and negative cells, and variably heterogeneous (VH) areas of positive staining together with areas of negative staining. These results suggest that 5hmC may be a marker to distinguish between benign and malignant parathyroid tumors.

Next, we performed IHC analysis to investigate expression of TET1 in parathyroid tumors because TET protein family members oxidize 5mC to 5hmC, and reduced 5hmC levels may be caused by deregulated expression of TET proteins. TET1 staining patterns resembled those of 5hmC in PAs and PCs. TET1 staining in PCs showed undetectable staining (7/17), VH pattern of staining (6/17), and positive staining in 4 tumors. Western blotting analysis of two available PCs showed very low expression level of TET1. PAs were grouped according to 5hmC and TET1 staining results, and those with a severe aberrant pattern for both 5hmC and TET1 were found to be

associated with higher tumor mass, and western blotting analysis displayed low to variable expression levels of TET1 in analyzed PAs.

To further investigate the possible role of TET1 in parathyroid cell growth, parathyroid tumor cell line sHPT-1, and also HEK293T cells, were transfected with TET1 expression plasmid or empty vector followed by colony forming assay. TET1 overexpression increased the level of 5hmC and resulted in a significantly reduced number and size of the sHPT-1 cells, but not of the HEK293T control cells, caused by reduced proliferation of sHPT-1 cells, as no effect on apoptosis was detected after transfection.

In conclusion, 5hmC can discriminate between parathyroid adenomas and carcinomas and could be a potential biomarker in parathyroid tumors. An association of a more aberrant staining pattern of 5hmC and TET1 to tumor weight was also found for PAs, and a growth regulatory role of TET1 *in vitro* was demonstrated in parathyroid tumor cells.

## Paper II. A role for TET2 in parathyroid carcinoma

*Endocrine-Related Cancer 24:309-318 (2017)*

In Paper I, all PCs stained negatively for 5hmC, indicating very low or undetectable levels of 5hmC in PCs, with undetectable or aberrant expression patterns for TET1 in 41% of the tumors. Because TET2 is also capable of oxidizing 5mC to 5hmC, deregulated expression of TET2 could contribute to decreased levels of 5hmC, as observed in the PCs.

IHC analysis of PCs ($n$ = 15) from 13 patients revealed very low or undetectable TET2 staining for all tumors compared to normal parathyroid tissue. Western blotting analysis of 2 available PCs further supported TET2 downregulation in PCs.

In order to investigate a possible growth regulatory role of TET2 in parathyroid tumor cells, TET2 CRISPR double nickase plasmids were used to efficiently knockout TET2 mRNA and protein expression in sHPT-1 cells. The knockout of TET2 expression, in a colony forming assay, significantly increased number and size of sHPT-1 cell colonies when compared to empty vector transfected control cells, which was further supported by the increased cell viability observed after transfection. Additionally, the migration velocity of the sHPT-1 cells *in vitro* increased under *TET2* knockout conditions, even in the presence of the cell proliferation inhibitor mitomycin C, although no effect on invasion capability of the cells was observed. These results strongly support a role of TET2 as a tumor suppressor gene with cell growth regulatory and migratory function in parathyroid tissue.

To further identify a possible explanation for reduced expression of TET2 in the PCs, *TET2* coding exons in nine PCs, *TET2* exon 11 in additional five PCs, and one blood sample, were DNA sequenced. Two common variants were found in exon 11 located in the catalytic region of TET2 protein, a result which has been reported previously in acute myeloid leukemia [151] and also in a protein-coding genetic variation dataset [152]. The potential effect of these two variants on TET2 protein structure and function is unknown.

Next, we measured DNA methylation levels for 29 CpGs located at the promoter region of transcript TET2-201 (encoding 2002 amino acid long polypeptide), which are situated in a CGI, by quantitative bisulfite pyrosequencing. Significantly higher methylation levels (%) were observed for the PCs ($n$ = 11) when the methylation level at each CpG site (CpGs number 4-16) was compared to those of four normal tissues. Two PCs from the same patient were also analyzed, and the methylation levels (%) were significantly higher in comparison to four parathyroid normal tissues, and the PC representing a metastasis after re-operation showed higher methylation level. sHPT-1 parathyroid cells and normal kidney tissue displayed low methylation levels for the 29 CpG sites, in line with high TET2 expression levels observed by western blotting analysis. An *in vitro* primary PC cell culture treated with the DNA methylation inhibitor Aza showed induction of TET2 mRNA expression, whereas no induction was observed in sHPT-1 cells treated with Aza. Taken together, these results suggest that increased methylation level of the *TET2* promoter region caused deregulated TET2 expression, observed in the PCs.

In conclusion, deregulated expression of TET2 by the promoter DNA hypermethylation may contribute to low/undetectable levels of 5hmC in PCs, and our results further supported a growth regulatory and migratory role of TET2 in parathyroid tissue.

## Paper III. Decrease of 5-hydroxymethylcytosine and TET1 with nuclear exclusion of TET2 in small intestinal neuroendocrine tumors

*Submitted*

Deregulated expression of TET1 and TET2 proteins associated with loss of 5hmC have been demonstrated in various types of human cancers. In this study, we investigated the level of 5hmC, and TET1 and TET2 expression level and function in SI-NETs.

The relative level of 5hmC was determined in 40 primary tumors (PT) and the 47 corresponding metastases (Met) from 40 patients by applying the semi-quantitative DNA immune-dot blot assay. Our data separated the 40 patients into 3 groups, although statistical analysis showed no correlation with clinical data. IHC analysis in 32 paired PTs and Mets displayed variable levels of 5hmC and a mosaic pattern of staining with a mixture of positive and negative cells. 9/32 tumors also showed mosaic pattern together with negative areas. IHC staining of TET1 and TET2 in the same tumors resembled the mosaic pattern observed for 5hmC, with a mixture of positive and negative cells, and mosaic regions together with negative staining was observed in a number of tumors for both TET1 and TET2. TET2 staining also displayed cytoplasmic staining in addition to the mosaic nuclear pattern.

Aza treatment of SI-NET cell lines CNDT2.5 and KRJ-I induced TET1 mRNA expression in the CNDT2.5 cells, without any effect on TET2 expression in both cell lines. Consistent with this result, quantitative bisulfite pyrosequencing analysis of TET1 promoter and exon 1 CGI showed high levels of methylation (90%) in CNDT2.5 cells, and KRJ-I cells displayed low levels of methylation (20%). Very low levels of methylation (10%) were observed in 20 PTs and Mets, suggesting that hypermethylation of the *TET1* promoter region could not explain aberrant expression of TET1 in SI-NETs.

To investigate a possible growth regulatory role of TET1 in SI-NET cells, the gene was overexpressed in CNDT2.5 cells and colony forming assay was performed, which resulted in a significantly reduced number of colonies. Moreover, overexpression of TET1 induced apoptosis in CNDT2.5 and KRJ-I cells, as measured by quantifying cytoplasmic histone-associated-DNA-fragments. These results support the role of TET1 as a candidate tumor suppressor gene in SI-NET cells.

Next, we investigated the possible involvement of the nuclear export machinery and the nuclear export protein exportin-1 (XPO1/CRM1), as lack of TET2 nuclear localization but presence in the cytoplasm was observed in 17/32 of the tumors analyzed by IHC staining. Treatment of CNDT2.5 and KRJ-I cells with the nuclear export inhibitor leptomycin B showed nuclear accumulation of TET2 in treated cells by IHC and western blotting, supporting a role of the XPO1 nuclear export machinery in TET2 mislocalization to the cytoplasm. Furthermore, treatment of both SI-NET cell lines with leptomycin B resulted in cell proliferation inhibition and induction of apoptosis. KPT-330/selinexor is a selective oral reversible inhibitor of XPO1 and nuclear export with less toxicity than leptomycin B, which has shown promising results in clinical trials in patients affected by other cancer types. KPT-330/selinexor displayed the same anti-cancer effects as leptomycin B in SI-NET cells. These novel findings suggested that KPT-330/selinexor or future developments of selective inhibitor of nuclear export (SINE) compounds should be considered and further evaluated for potential treatments in patients with SI-NETs.

# Paper IV. Reduced representation bisulfite sequencing of small intestinal neuroendocrine tumors identifies PTPRM as a novel candidate tumor suppressor gene

*Submitted*

In this study, RRBS was performed in 8 SI-NETs to identify differentially methylated regions (DMR) and candidate driver genes contributing to tumorigenesis. A total of 94 DMRs were identified when comparing PTs ($n$ = 3) to Mets ($n$ = 5), and three of them were located upstream of genes or intragenically on chromosome 18, including *GATA6*, *CCDC178*, and *PTPRM*. These were selected for further investigations because the loss of one copy of chromosome 18 is a very common event in SI-NETs.

To explore whether any of the 3 candidate tumor genes on chromosome 18 have a potential growth regulatory role in SI-NETs, colony formation assay was performed by transfecting expression vectors encoding GATA6, CCDC178, or PTPRM to the SI-NET cell line CNDT2.5. A reduced number of colonies was observed for all three candidate tumor genes compared to control transfected cells, and PTPRM, which is involved in cell-cell adhesion, was chosen for further analysis.

Quantitative RT-PCR analysis revealed significantly reduced PTPRM mRNA expression in metastatic tumors ($n$ = 27) when compared to the paired PTs ($n$ = 23), in line with a lower level of the DMR methylation, located far upstream (140kb) of the *PTPRM* gene, in PTs ($n$ = 3) compared to Mets ($n$ = 5) observed in RRBS analysis. IHC analysis of 19 PTs and 21 paired Mets revealed an undetectable/very low level or variable heterogeneous pattern of staining in the majority of tumors. These results demonstrated that PTPRM expression is frequently down-regulated in SI-NETs.

Quantitative bisulfite pyrosequencing displayed overall high levels of methylation of the DMR in 23 PTs, 27 paired Mets, 3 small intestinal tissue specimens, and the SI-NET cell lines CNDT2.5 and KRJ-I. Treatment of both cell lines with the DNA methylation inhibitor Aza induced PTPRM mRNA expression. Previously, it has been shown that the *PTPRM* upstream regulatory region, containing a CGI spanning the promoter, exon 1, and part of intron 1, is hypermethylated in colon cancer [62]. Therefore, we measured the level of methylation at 13 CpG sites in the promoter region and 25 CpG sites in the exon 1/intron 1 region in PTs, corresponding Mets, and both SI-NET cell lines. High levels of methylation (90%) and moderate levels (30-40%) were observed in CNDT2.5 and KRJ-I cells, respectively. However, very low levels of methylation (10%) were detected in all tumors. Next, we determined the methylation level of CpG sites in the presumed CGI shores, located approximately 700–1600 base pairs upstream the TSS of the *PTPRM*

gene, in 20 PTs and Mets, and 3 small intestinal tissue specimens. All tissue specimens that were analyzed and the two SI-NET cell lines were methylated in these CpG sites. In line with the results of a previous study [145], the CpG site # 3 in shore 2 showed significantly more methylation in the SI-NETs compared to small intestinal tissues. Taken together, these results suggested that methylation in the DMR located far upstream of the *PTPRM* gene and in the presumed CGI shores, rather than at the promoter or the exon 1/intron 1 region, could be involved in inactivation of *PTPRM* in SI-NETs.

Overexpression of PTPRM in both CNDT2.5 and KRJ-I cells resulted in inhibition of cell proliferation and induction of apoptosis. Furthermore, transfection of CNDT2.5 cells with an expression vector encoding a double mutant of PTPRM, with disrupted phosphatase activity, showed the same growth inhibiting activity as wild type PTPRM, suggesting that the phosphatase activity was not involved in cell growth inhibition in SI-NET cells.

In conclusion, our results supported a role for PTPRM as an epigenetically dysregulated candidate tumor suppressor gene in SI-NETs.

# Concluding remarks

Epigenetic dysregulation is the hallmark of cancer and associated with the initiation and progression of various types of human tumors. The discovery of 5hmC and TET protein enzymes provided a greater understanding of the epigenetic regulation of gene expression and the importance of the epigenetic abnormalities to tumorigenesis. Therefore, we identified the level of 5hmC, TET1 and TET2 expression level and function in parathyroid tumors and small intestinal neuroendocrine tumors.

In parathyroid tumors, the undetectable/very low level of 5hmC distinguished PC from benign tumors of adenoma with positive staining. Thus, as a major finding of Paper I, 5hmC may present a novel potential marker for parathyroid malignancy because diagnosis of PC is difficult due to lack of a highly specific and sensitive IHC marker. We also identified that a more aberrant IHC staining pattern of 5hmC and TET1 was associated with increased tumor weight in PAs, strongly suggesting a growth regulatory role of 5hmC and TET1. *In vitro* experiments further demonstrated a role of TET1 as a tumor suppressor gene in parathyroid tumor cells.

Moreover, in order to cast light on a potential reason behind the undetectable/very low level of 5hmC in PCs, we analyzed TET2 expression level as aberrant expression of TET1 was observed in 41% of tumors, and TET2 is also capable of oxidizing 5mC to 5hmC. The very low expression of TET2 observed in all analyzed PCs indicated that both TET1 and TET2 are aberrantly expressed in PCs, and we could also demonstrate a growth regulatory role for TET2 in parathyroid tumor cells. Interestingly, *TET2* knockout resulted in increased migration of the parathyroid tumor cells, supporting a role for TET2 in cell migration. In the absence of inactivating mutations, we moved our interest to epigenetic regulation of TET2 expression. We detected promoter hypermethylation in PCs when compared to normal parathyroid tissues. Induction of TET2 by Aza in a primary PC cell culture further supported the involvement of DNA hypermethylation in *TET2* gene repression.

Variable levels of 5hmC and aberrant expression of TET1 and TET2 were observed in SI-NETs. Although the *TET1* promoter hypermethylation has been previously reported in multiple cancers [30], very low levels of methylation were observed in the PTs and Mets analyzed in Paper III, suggesting that other mechanisms rather than promoter methylation could be involved in the down regulation of TET1 in SI-NETs. Moreover, the overexpression of TET1 *in vitro* suppressed colony formation and induced apoptosis, indi-

cating a growth regulatory role for TET1 in SI-NET cells. For TET2, IHC analysis displayed cytoplasmic expression with absent nuclear localization in 17/32 tumors. Treatment of the SI-NET cells with the nuclear export inhibitor leptomycin B revealed the involvement of nuclear export protein exportin-1 (XPO1/CRM1) in the aberrant transport of TET2 from nucleus to the cytoplasm, and also resulted in reduced cell proliferation and the induction of apoptosis. Due to leptomycin B toxicity, other small molecules have been developed as SINE compounds. In SI-NET cells, the SINE compound, KPT-330/selinexor, showed the same anti-cancer effects as leptomycin B. Therefore, selinexor could be further investigated as a potential treatment in patients with SI-NETs, as, in phase I clinical trials in patients with other types of cancers, it has shown promising results [153-156].

DNA methylation profiling has been used to unravel driver genes in cancer cells as tumor suppressors and oncogenes and therefore we analyzed DNA methylation in a small cohort of SI-NETs by RRBS. SI-NETs are tumors with no highly recurrent mutations, as detected so far, but they are epigenetically disrupted, and the loss of one copy of chromosome 18 is the most common aberration in these tumors. We detected a DMR upstream of the *PTPRM* gene located on chromosome 18, and further demonstrated a role for the gene as a tumor suppressor in SI-NET cells. PTPRM is involved in cell–cell adhesion, cell proliferation and migration, which is associated with tumorigenesis in various types of cancers [56]. PTPRM showed aberrant expression profile in SI-NETs, although no DNA methylation was detected in the promoter or in the exon 1/intron 1 region in the tumors analyzed here. High and variable levels of methylation were detected in the presumed CGI shores upstream of the *PTPRM* gene in all of the investigated tissue specimens and in the two SI-NET cell lines. However, the CpG site # 3 in shore 2 was found to be significantly more methylated in the SI-NETs compared to small intestine tissues, suggesting that *PTPRM* is an epigenetically deregulated candidate tumor suppressor gene in SI-NETs. Inactivated PTPRM associated with loss of PTPRM- or E-cadherin-dependent cell adhesion has been described previously [59] and could be further investigated in SI-NETs. Moreover, *GATA6* and *CCDC178*, located on chromosome 18, were also detected by RRBS analysis, and showed a potential growth regulatory role in SI-NET cells. *GATA6* is a transcription factor gene that has been reported as an oncogene that is frequently amplified or overexpressed in different types of cancers, including gastric, breast, and pancreatic adenocarcinomas [157, 158]. CCDC178 has also been reported as being upregulated and associated with cell metastases in hepatocellular carcinoma [159]. These genes should be considered for future investigations with the aim of discovering novel driver genes that are important for small intestinal neuroendocrine tumorigenesis. It may be that several tumor genes on chromosome 18 cooperate during tumor progression.

# Acknowledgements

This thesis work was performed in the Endocrine Surgery group at the Department of Surgical Sciences, Uppsala University. I wish to express my sincere gratitude to all who have helped and supported me along this journey.

First and foremost, I would like to express my appreciation to all of the patients who have contributed to this study. This research would not have been possible without your contributions.

My main supervisor, Professor *Gunnar Westin*; I would like to thank you for introducing me to the epigenetic world. Your great knowledge, enthusiasm, and dedication to science have always inspired me, and I am grateful to you for sending me all those interesting papers to read and for encouraging me to think independently. Our every meeting was like a lecture to me and I have learned so much!

My supervisor Professor *Peter Stålberg*; I am very grateful for all the encouragement, your support, and your scientific expertise. Thank you for the guidance whenever it was needed with your profound knowledge in the field, and for so patiently clarifying clinical aspects of diseases for me!

My supervisor Professor *Per Hellman*; I appreciate your invaluable input and sharing your vast scientific knowledge.

*Birgitta Bondeson*. Thank you so much for all your contributions and technical support. You are a wonderful person and I have always enjoyed your company and our Swedish talks in the lab. *Cihan Cetinkaya* and *Peter Lillhager*. Thank you for teaching me different techniques when I started in the lab, and for the great company. You have been helpful and friendly. *Monica Pettersson*. Thanks for all the help and for showing me how a proper pyrosequencing is actually done!

I would also like to thank all my former and present colleagues, and co-authors. *Katarina Edfeldt*, *Rajani Maharjan*, *Johan Kugelberg*, and *Samuel Backman*, for great company, and *everyone at the former CKMF*, especially *Malin Grönberg*, for all the fun after work, at lunch times and fikas. *Nooshin*

*Talebizadeh*, for becoming a dear friend, and for all the fun times, parties, and travels we had together. My former student and co-author, *Surendra Prabhawa*, for all your hard work and contribution.

I am fortunate and very grateful to have wonderful friends in Uppsala, and would like to thank you *all* for making my PhD life an amazing experience (survivor!). *Shirin*, I am so blessed to know you, and thanks for always nourishing me with your kindness and delicious foods! *Homa*, what can I say, your incredible sense of humor as well as our scientific discussions about evolution (oh no! ... sex chromosomes) reminds me of a nerdy scientific life but filled with joy and hardy laughs. *Mersedes*, thanks for being fabulous. I will never forget how we became real friends☺! To *All* my friends in Iran, *Mehri*, *Mehrnaz*, *Narges*, *Sharieh*, *Neda*, and *Sara*; your friendship means a lot to me. You made my visits to Iran so much fun!

Special thanks to *Dr. Mahnaz Mazaheri* for accepting me in your lab. Working with you was an inspiration to me, both scientifically and personally.

To my dear *Mom*, this wouldn't be possible without you. Thank you for believing in me and supporting me no matter what. Your endless love and affection is the warmth in my heart. My dearest *Iman*, thanks for being the best big brother ever! My sweet nephew, *Iraj*, you bring beauty and joy to my life. To *Madarjoon*, *Grandpa*, aunt *Maryam* and *Fahimeh*, thank you for all the love and support.

And above all, I would like to dedicate this work to my beloved *Dad*.

Thank you all! ♥

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Editor: The Dean of the Faculty of Medicine

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