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MOLECULAR ASPECTS OF TUMOR DEVELOPMENT AND TREATMENT FOR SMALL INTESTINAL NEUROENDOCRINE TUMORS

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MOLECULAR ASPECTS OF TUMOR DEVELOPMENT AND TREATMENT FOR SMALL INTESTINAL NEUROENDOCRINE TUMORS
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“I would rather have questions that can't be answered than answers that can't be questioned.”

— Richard Feynman

To Sheler, my loyal fellow-traveler and

Catharina, the polestar for this journey

ABSTRACT

Small intestinal neuroendocrine tumors (SI-NETs) may cause symptoms due to excess secretion of hormones and peptides. The molecular mechanisms behind development of SI-NETs are not well understood. Copy number alterations, especially loss of chromosome 18q, have been reported and recently *p27* mutations were implicated in SI-NET tumorigenesis. Somatostatin analogs (SSAs) have long been used to alleviate the symptoms and have recently been shown to arrest SI-NET growth by unknown molecular mechanisms.

In Study I, copy number alterations were investigated in 30 SI-NETs, using array comparative genomic hybridization. Recurrent alterations and minimal overlapping regions were observed, including losses on chromosomes 18, 16, 11 and 9 and gains on chromosome 20 and 14, 5 and 4. Using qPCR-based TaqMan assays, losses on chromosome 18, 16 and 11 were verified in an extension cohort, comprised of 43 SI-NETs, in total. Using unsupervised hierarchical clustering, a group of tumors was identified that was enriched with gains of chromosomes 20, 14, 7, 5 and 4. Gain in 20pter-p11.21 was associated with shorter survival and loss of 16q and gain of chromosome 7 were associated with metastases.

In Study II, quantitative Pyrosequencing assays were used on 44 SI-NETs for promoter methylation analysis of candidate genes. Promoter hypermethylation was found for *WIF1, RASSF1A, CTNNB1, CXCL14, NKX2–3, p16, LAMA1*, and *CDH1*, but not for *APC, CDH3, HIC1, P14, SMAD2*, and *SMAD4*. Hypermethylation of *WIF1* was concomitant with its mRNA downregulation in SI-NETs vs. normal intestine. Downregulation of *RASSF1A* and *p16* was associated with a worse patient outcome. Global genome hypomethylation was demonstrated in SI-NETs. One group of tumors was identified with hypermethylation of *WIF1*, global hypomethylation and loss of chromosome 18 and another group with hypermethylation of *RESSF1A* and *CTNNB1* and loss of chromosome 16. 5-azacytidine treatment of the SI-NET cell lines HC45 and CNDT2 reduced the methylation of hypermethylated genes and restored their mRNA expression.

In Study III, the molecular mechanisms behind SSA treatment of NETs was examined using HiRIEF LC-MS/MS in HC45 and H727 cells treated with lanreotide at different time points. The results were confirmed for selected candidates using Western blot. The expression of Adenomatous polyposis coli (APC) was increased and survivin was decreased after 2 and 6 hours of treatment. Using shRNA against APC, the expression of survivin was elevated and siRNAs against somatostatin receptor 2 (SSTR2) suppressed APC-survivin regulation. In conclusion, lanreotide induced APC specifically through binding to SSTR2 and APC inhibited survivin. Immunohistochemistry on a tissue microarray comprised 112 NETs showed that survivin expression was associated with worse patient outcome.

In Study IV, HiRIEF LC-MS/MS was used to study the mechanisms behind liver metastasis of SI-NETs. The proteome was compared between SI-NETs with and without liver metastasis at diagnosis. Higher expression of ubiquitin-like NEDD8 was seen in cases that had liver metastasis at the time of diagnosis. The NET cell lines BON-1, CNDT2, HC45 and H727 were treated with MLN4924, an inhibitor of the neddylation activating enzyme, NAE1. The proliferation of all cell lines was inhibited in a dose-dependent way. The proteome of CNDT2 and HC45 after treatment with MLN4924 was investigated using HiRIEF LC-MS/MS. Neddylation seems to play a role in the progression of SI-NET and MLN4924 treatment is a promising strategy in the management of these tumors.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers which are referred to in the text by their Roman numerals (I-IV).

I. Jamileh Hashemi, **Omid Fotouhi**, Luqman Sulaiman, Magnus Kjellman, Anders Höög, Jan Zedenius, Catharina Larsson: Copy number alterations in small intestinal neuroendocrine tumors determined by array comparative genomic hybridization.
BMC Cancer 13:505, 2013.

II. **Omid Fotouhi**, Maral Adel Fahmideh, Magnus Kjellman, Luqman Sulaiman, Anders Höög, Jan Zedenius, Jamileh Hashemi, Catharina Larsson: Global hypomethylation and promoter methylation in small intestinal - neuroendocrine tumors – an in vivo and in vitro study.
Epigenetics 9(7):987-997, 2014.

III. **Omid Fotouhi**, Hanna Kjellin, Catharina Larsson, Jamileh Hashemi, Jorge Barriuso, C Christofer Juhlin, Ming Lu, Anders Höög, Laura G Pastrián, Angela Lamarca, Victoria Heredia Soto, Jan Zedenius, Marta Mendiola, Janne Lehtiö, Magnus Kjellman: Proteomics suggests a role for APC-survivin in response to somatostatin analog treatment of neuroendocrine tumors.
J Clin Endocrinol Metab. 101(10):3616-3627, 2016.

IV. **Omid Fotouhi**, Hanna Kjellin, Catharina Larsson, Mehran Ghaderi, Stefano Caramuta, Magnus Kjellman, C Christor Juhlin, Jan Zedenius, Lukas Orre, Janne Lehtiö: Proteomics identifies neddylation as a potential therapy target in small intestinal neuroendocrine tumors.
In Manuscript

LIST OF RELATED PUBLICATIONS

Publications by the author on related topics outside the thesis.

- Luqman Sulaiman, C Christofer Juhlin, Inga-Lena Nilsson, **Omid Fotouhi**, Catharina Larsson, Jamileh Hashemi: Global and gene-specific promoter methylation analysis in primary hyperparathyroidism. *Epigenetics* 8(6): 646–655, 2013.

- Na Wang, Hanna Kjellin, Anastasios Sofiadis, **Omid Fotouhi**, C Christofer Juhlin, Martin Bäckdahl, Jan Zedenius, Dawei Xu, Janne Lehtiö, Catharina Larsson: Genetic and epigenetic background and protein expression profiles in relation to telomerase activation in medullary thyroid carcinoma. *Oncotarget* 7(16):21332-21346, 2016.

CONTENTS

1 Introduction 1
  1.1 Tumors and the biology of cancer 1
    1.1.1 Abnormal growth and tumor development 1
    1.1.2 The biology of cancer 2
    1.1.3 Tumor classification 3
  1.2 Neuroendocrine Tumors 3
  1.3 Genetic Background 7
  1.4 Epigenetic Background 12
  1.5 Somatostatin Analogs in SI-NET therapy 17
    1.5.1 Somatostatin and its pharmaceutical analogues 17
    1.5.2 Signal transduction 18
    1.5.3 Adenomatous Polyposis Coli and survivin 19
  1.6 Targeting neddylation in SI-NETs 20
    1.6.1 Ubiquitination system and cancer 20
    1.6.2 NEDD8 (Neural Precursor Cell Expressed, Developmentally Down-Regulated 8) 21
    1.6.3 P27 proteolysis inhibition as a strategy in SI-NET management 23
2 Aims 25
3 Materials and Methods 27
  3.1 Tumor samples and cell lines 27
  3.2 Array comparative genomic hybridization 28
  3.3 Quantitative PCR and TaqMan assays 28
  3.4 Pyrosequencing 29
  3.5 HIRIEF Mass Spectrometry proteomics 30
  3.6 Western blotting 30
  3.7 Immunocytochemistry and Immunohistochemistry 31
  3.8 Proliferation assays 31
  3.9 Apoptosis analysis using annexin V marker on a flow cytometer 32
4 Results and discussion 33
  4.1 Genomic studies in SI-NETs 33
    4.1.1 DNA profiling of SI-NETs 33
    4.1.2 Clustering Analysis 34
    4.1.3 qPCR verification of CNAs 34
  4.2 Epigenetic studies in SI-NETs 34
    4.2.1 Promoter hypermethylation and mRNA expression of cancer-related genes 34
    4.2.2 Global hypomethylation in SI-NETs 36
    4.2.3 Clustering and association with DNA copy numbers 36
    4.2.4 Demethylation analyses 36
  4.3 Proteomics of somatostatin treatment in NETs 36
    4.3.1 HiRIEF Mass Spectrometry analysis 36

4.3.2 Network and pathway analyses 37
4.3.3 Western blot verification 37
4.3.4 Cell proliferation analysis for lanreotide and survivin inhibitor YM155 37
4.3.5 Survivin as a prognostic marker in SI-NETs 38
4.3.6 SSTR2-APC-survivin axis 38
4.4 Implication of neddylation in SI-NET 38
4.4.1 NEDD8 is over-expressed in liver metastasis..38
4.4.2 NEDD8 inhibition suppressed proliferation and induced apoptosis 39
4.4.3 The proteomics landscape following neddylation inhbition..................39
4.4.4 UPS-p27 regulatory axis is a target in SI-NET management 39
5 Concluding Remarks 41
6 Acknowledgements..43
7 References 47

LIST OF ABBREVIATIONS

5-aza-CdR	5-Aza-2′-deoxycytidine
5-aza-CR	5´-Azacytidine
5-HIAA	5-Hydroxyindoleacetic acid
ABC-DLBCL	Activated B-cell-like diffuse large B-cell lymphoma
aCGH	Array Comparative Genomic Hybridization
ALL-1	Acute lymphoblastic leukemia 1
Alu	Arthrobacter luteus
APC	Adenomatous polyposis coli
APC/C	Anaphase-promoting complex/cyclosome
BAC	Bacterial artificial chromosome
BCL2	B-cell lymphoma 2
BIRC5	Baculoviral IAP Repeat Containing 5
BMPER	Bone Morphogenetic Protein -binding endothelial regulator protein precursor
BRAF	Serine/threonine-protein kinase B-Raf
BRCA1	Breast cancer 1
BrdU	Bromodeoxyuridine
cAMP	cyclic adenosine monophosphate
CAGE	Cancer-associated gene
CAND1	Cullin-associated and neddylation-dissociated 1
CARD11	Caspase recruitment domain family member 11
CDH	Cadherin
CDH1	Cadherin 1
CDK	Cyclin-dependent kinase
CDKN	Cyclin Dependent Kinase Inhibitor
CDX1	Caudal type homeobox 1
CELSR3	Cadherin EGF LAG seven-pass G-type receptor 3
CgA	Chromogranin-A
CGH	Comparative Genomic Hybridization
CN	Copy number
CNA	Copy number alteration
COBRA	Combined bisulfite restriction analysis
COX2	Cyclooxygenase 2b
CREB	CAMP Response element-binding protein
CRL	Cullin-RING ligase
CSC	Cancer Stem Cell
CT scan	Computed tomography scan
CTGF	Connective tissue growth factor
CTNNB1	β-Catenin
CUL	Cullin
CXCL14	Chemokine C-X-C motif ligand 14
DAB	3,3′-Diaminobenzidine
DAD1	Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit
DAPPLE	Disease Association Protein-Protein Link Evaluator

DCC	Deleted in colorectal cancer
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EMILIN2	Elastin Microfibril Interfacer 2
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal–regulated kinase
EZH2	Enhancer of zeste homolog 2
FACS	Fluorescence-activated cell sorting
FBP1	Fructose-Bisphosphatase 1
FFPE	Formalin-fixed paraffin-embedded
FGFR2	Fibroblast growth factor receptor 2
FIEC	Familial Ileal Endocrine Carcinoma
FYN	Tyrosine-protein kinase Fyn
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI-NET	Gastrointestinal NET
GIPR	Gastric Inhibitory Polypeptide Receptor
GPCR	G-protein–coupled receptor
HECT	Homologous to E6-AP carboxyl terminus
HIC1	Hypermethylated in cancer 1
HiRIEF LC-MS/MS	High-resolution isoelectric focusing liquid chromatography mass spectrometry
HOOK3	Hook microtubule tethering protein 3
HPV	Human Papillomavirus
HRP	Horseradish peroxidase
INSM1	Insulinoma-associated protein 1
IPA	Ingenuity pathway analysis
iTRAQ	Isobaric tags for relative and absolute quantitation
IκBα	Inhibitor of NF-κB
JNK	Jun amino-terminal kinases
KIAA0650	SMCHD1: structural maintenance of chromosomes flexible hinge domain contain
KIP1	Kinesin-like protein 1
KRAS	Kirsten rat sarcoma viral oncogene homolog
LAMA1	Laminin Subunit Alpha 1
LINE-1	Long interspersed nucleotide elements
LOH	Loss of heterozygosity
LPIN2	Phosphatidate phosphatase LPIN2
MAPK	Mitogen-activated protein kinase
MEN	Multiple endocrine neoplasia
MetI	Methylation index
MGMT	O-6-Methylguanine-DNA Methyltransferase

MLF1	Myeloid Leukemia Factor 1
MOR	Minimal overlapping region
MSP	Methylation specific PCR
MST1	Mammalian Sterile Twenty 1
NAE	Neddylation activating enzyme
NEC	Neuroendocrine carcinoma
NEDD8	Neural Precursor Cell Expressed, Developmentally Down-Regulated 8
NET	Neuroendocrine tumor
NF-κB	Nuclear factor-κB
NKX2-3	NK2 Homeobox 3
NONO	Non-POU Domain Containing, Octamer-Binding
OIS	Oncogene-induced senescence
PCR	Polymerase chain reaction
PET	Positron emission tomography scan
PFS	Progression free survival
PI3K/AKT	Phosphatidylinositide 3-kinases / Serine/threonine-specific protein kinase
PKA	Protein kinase A
PPP2R1B	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform
RARb	Retinoic acid receptor beta
RASSF1	Ras Association Domain Family Member 1
RBX	Ring-Box protein
RING	Really Interesting New Gene
RNA	Ribonucleic acid
SCF	SKP1-CUL1-F box protein
SDHD	Succinate dehydrogenase complex subunit D
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHP	Src homology phosphatase
SI-NET	Small intestinal neuroendocrine tumor
SKP	S-phase kinase-associated protein
SMAD1	Sma- And Mad-Related Protein 1
SMIM21	Small Integral Membrane Protein 21
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variation
SRIF	Somatotropin-release inhibiting factor
SSA	Somatostatin analog
SSTR	Somatostatin receptor
TBS	Tris-buffered saline
TGF-beta	Transforming growth factor beta
THBS1	Thrombospondin 1
TMA	Tissue microarray
UBC12	Ubiquitin-Conjugating Enzyme 12
UBE2F	Ubiquitin Conjugating Enzyme E2F
UBL	Ubiquitin like protein

UBL	Ubiquitin like protein
UPS	Ubiquitin-proteasome system
VEGF	Vascular endothelial growth factor
VHL	Von Hippel–Lindau
VIP	Vasoactive intestinal peptide
βTrCP	β-transducin repeat-containing protein

1 INTRODUCTION

1.1 TUMORS AND THE BIOLOGY OF CANCER

“Normal cells are carefully programmed to collaborate with one another in constructing the diverse tissues that make possible organismic survival. Cancer cells have a quite different and more focused agenda. They appear to be motivated by only one consideration: making more copies of themselves.” — Robert A. Weinberg (Weinberg 2014)

The general developmental rule ever since multicellular organisms evolved on earth is that each cell retains all necessary genetic information to build any other differentiated cell types in the body. This versatility is critical for several normal processes of the organism such as wound healing, cell replacing, homeostasis and tissue maintenance. The human body is composed of 3.72×10^{13} cells (Bianconi, et al. 2013) of which as many as 2×10^{11} are replaced in one day (E 1995). The fundamental point for every single cell is to accurately protect the genetic reservoir and to maintain the genetic integrity throughout life.

Mutations, on the other hand, are inevitable and may occur throughout the genome including non-coding regions as well as the 3% of the human genetic content that is involved in generation of proteins or regulation of expression. Alterations of the integrity of DNA are characteristic of cancer cells and many different types of genetic alterations can cause or contribute to tumor development. In fact, even tiny modifications in genetic information or regulatory epigenetic modulators can lead to aberrant proliferation and spreading of cancer cells. (Weinberg 2014)

1.1.1 Abnormal growth and tumor development

Under normal conditions, cells in a multicellular organism are strictly organized. Tumor development is a multistep process where the cellular organization becomes disturbed. The time frame from tumor initiating event(s) to the development of symptomatic disease is variable and may proceed during years. In addition to established cancers, there are several other types of aberrant growth. (Weinberg 2014)

Exaggerated cell proliferation is the first step. As a result of the abnormal growth and cell division, increasing numbers of that particular cell type accumulate in the tissue. These cells maintain their original cytological characteristics. The histopathological appearance of the tissue architecture seems normal and no threat has been posed to the organism. This kind of abnormal growth is called “hyperplastic”. (Weinberg 2014)

In “metaplasia” cytologically normal cells replace other cell types, which has been associated with increased risk of subsequent cancer development. For example in “Barrett’s Esophagus” the secretory stomach epithelium extends towards the esophagus where it replaces the

squamous epithelium. This phenomenon can increase the risk of esophageal cancer up to 30 times. (Weinberg 2014)

In “dysplasia”, cytological alterations also appear. The dysplastic tissue may exhibit increased mitotic index and over-expression of the proliferation marker Ki-67, altered nuclear shape and increased nucleus to cytoplasm ratio. Dysplasia can be a transient phase between non-spreading and safe “benign” lesions and established cancers. (Weinberg 2014)

A “cancer” represents a new type of tissue, which can invade nearby tissues and spread to the entire body. A cancer in its original location is termed “primary tumor” and when it has spread to a new location it is referred to as “metastasis”. (Weinberg 2014)

1.1.2 The biology of cancer

The two main types of cancer genes are “oncogenes” that after activation promote cell proliferation, escape of cell death and metastasis and “tumor suppressor genes” that normally prevent tumor initiation and progression. The first and best examples are the *RB1* tumor suppressor gene and the *RAS* oncogenes (Cox and Der 2010; DeCaprio 2009; Shih, et al. 1979). “Gain of function” mutations such as point mutations and chromosomal rearrangements activate oncogenes while “loss of function” mutations silence tumor suppressor genes, both of which can drive tumorigenesis. Epigenetic alterations can sometimes play the same role in tumorigenesis (Lee and Muller 2010).

In 1971 Alfred G. Knudson reported that two mutational hits are required for the development of retinoblastoma –later, this was clarified as the two alleles of the tumor suppressor *RB1*. He observed that children with bilateral retinoblastoma tumors had an earlier age of onset as compared to children with unilateral tumors. Through a mathematical calculation he showed that this could correspond to the mutation rate for two alleles of *RB1* in unilateral compared to only one allele in bilateral retinoblastoma (Knudson 1971). Commonly bilateral disease occurs in children with familial disease (inherited or *de novo* mutation), while unilateral disease is seen in sporadic disease. This study funded the important notion of “Knudson’s two-hit” theory. Accordingly, for each loss of function alteration of a tumor suppressor gene there are two alleles that need to be inactivated. The alterations could involve genetic and/or epigenetic events.

A myriad of studies on the biology of cancer has revealed the universal laws to govern cancerous cells that are identified as hallmarks of cancer. There are six core hallmarks: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg 2000). Lately, two non-generalized and non-validated “emerging hallmarks” has been added, i.e. deregulating cellular energetics and avoiding immune distraction. Finally, two hallmarks are introduced as “enabling hallmarks” since they facilitate

acquisition of other hallmarks, including “genome instability and mutations” as well as “tumor-promoting inflammation” (Hanahan and Weinberg 2011).

1.1.3 Tumor classification

Tumors usually arise from “epithelia”, which are layers of cells that cover the surface of other tissues. Malignant tumors of epithelial origin are called carcinomas and are responsible for 80-90% of cancer-related deaths (https://training.seer.cancer.gov/disease). Squamous cell carcinomas originate from protective epithelium that for example covers the skin, mouth and esophagus. Adenocarcinomas arise from epithelia that secrete different substances into the lumen of for example the lung or intestine.

“Sarcomas” are another main group of tumors that originate from mesenchymal tissues. Osteosarcoma, liposarcoma, angiosarcoma are some examples of sarcomas. Sarcomas comprise approximately 1% of all malignancies. (Burningham, et al. 2012)

“Hematopoetic malignancies” are derived from hematopoietic cell lineages and comprise “leukemias” and “lymphomas”.

“Neuroectodermal” tumors are derived from cells within the nervous system. This group includes tumors such as “glioma” and “medulloblastoma”.

Finally, some tumors are derived from neuroendocrine cells. They include tumors with a wide range of aggressiveness from indolent, low or intermediate grade and well or moderately differentiated neuroendocrine tumors (NETs) to aggressive, high grade, moderately or poorly differentiated neuroendocrine carcinomas. (Rindi and Wiedenmann 2011)

1.2 NEUROENDOCRINE TUMORS

Hyperplastic growths can occur anywhere in the body and usually are not life threatening. Benign tumors in the colon for example or papilloma on the cervix of the uterus do not usually pose a direct problem except they can provide the precursor cells of cancers. However, benign tumors may cause symptoms due to hormonal production and secretion, as exemplified by pituitary adenomas with increased growth hormone levels and development of acromegaly.

Many NETs can remain indolent and stay silent for several years. Most NET tumors can secrete various peptides, which depending on the level and type may lead to symptoms. Cases with a “non-functioning” tumor or lack of symptoms might not be identified at all or be found only by chance during a CT (computerized tomography) scan for example. Cases with a “functioning NET” may exhibit symptoms due to excess hormone secretion as seen in SI-

NET patients with "carcinoid syndrome" characterized by diarrhea, flushing and carcinoid heart failure.

Gastrointestinal NETs (GI-NETs) originate from endocrine cells of endodermal origin such as serotonin secreting cells (traditionally known as enterochromaffin cells) in the small intestine. Other types of NETs such as pheochromocytoma (derived from chromaffin cells) and medullary thyroid carcinoma (from calcitonin-producing C cells) originate from cells of the neural crest (Adams and Bronner-Fraser 2009). Neuroendocrine cells and neurons share several features. The two types of storage-release organelles, called "large dense-core vesicles" and "small synaptic-like vesicles", are also observed in neurons (Figure 1). Moreover, development of both neuroendocrine and neuronal cells is controlled by Notch pathway transcription factors such as neurogenin 3 and neuroD/β2 (Schonhoff, et al. 2004).

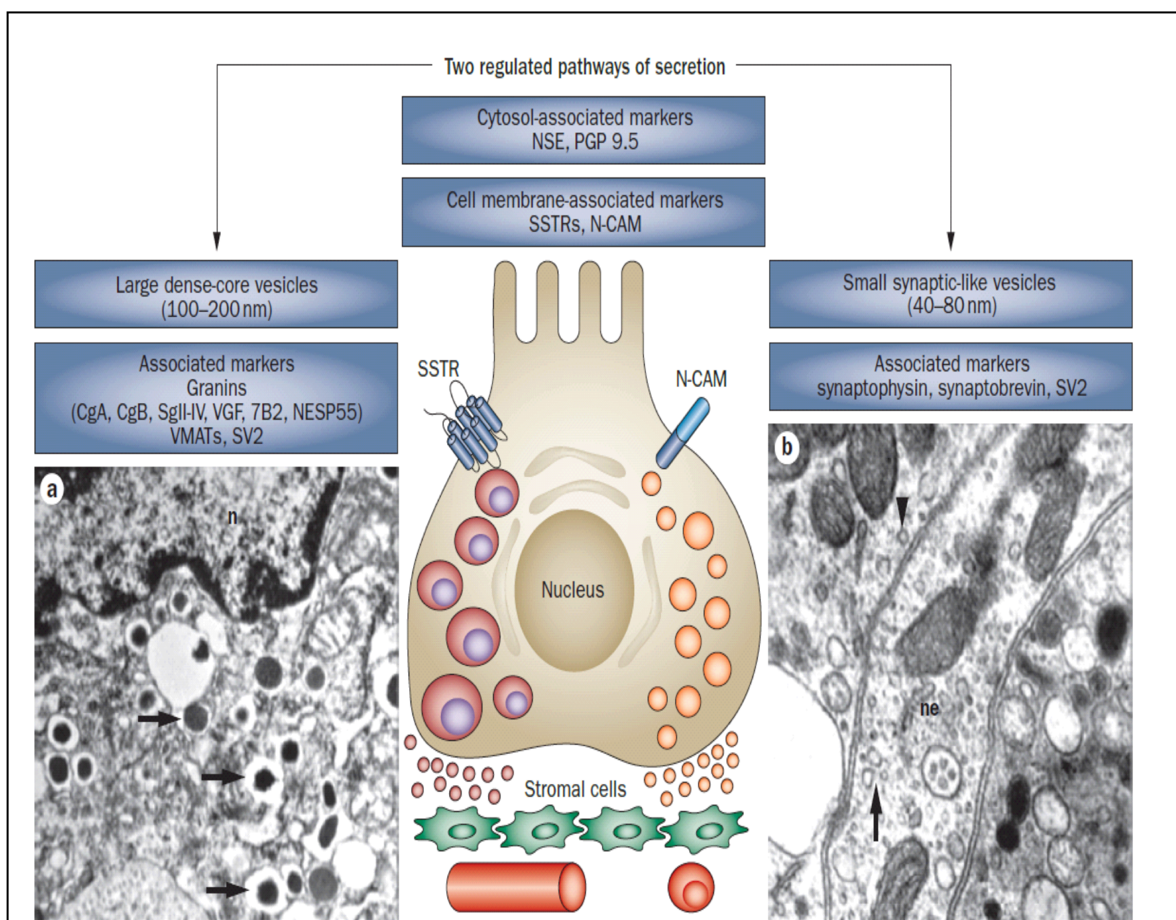

Figure 1. A general demonstration of a neuroendocrine cell, facing the lumen at the top and secreting to stromal cells (paracrine) or to a blood vessel (endocrine) at the bottom. Small synaptic-like vesicles and large dense-core vesicles (shown at left and right electron micrographs, respectively) are the characteristics of neuroendocrine cells. The small synaptic-like vesicles are comparable with their counterparts in neurons (ne in right). n=nucleus. (Courtesy of Rindi and Wiedenmann 2011)

SI-NETs are generally known as sporadic malignancies. However, there are several reports of families with two or more affected members supporting a familial form of the disease in a subset of cases (Eschbach J 1962; Kinova S 2001; Moertel CG. 1973; Pal, et al. 2001; Wale RJ 1983). In one of these reports family members from three generations developed SI-NET (Jarhult, et al. 2010). Additionally, there are epidemiological studies that suggest familial forms of SI-NET (Babovic-Vuksanovic, et al. 1999; Hemminki and Li 2001). Cunningham et al. studied a group of SI-NETs with a family history of the disease and found similar aberrations in DNA dosage and gene expression patterns as in sporadic cases, suggesting common mechanisms of pathogenesis (Cunningham, et al. 2011). Neklason et al. investigated 384 SI-NETs and found a 13.4-fold ($P<0.0001$) and 6.5-fold ($P=0.143$) risk rate for siblings and parents, respectively. Furthermore, for third-degree relatives a 2.3-fold risk rate was revealed ($P=0.008$), suggesting a genetic influence (Neklason, et al. 2016).

In 1907, Siegfried Oberndorfer a German pathologist at the University of Munich suggested the term karzinoide for 6 cases of carcinoma-like indolent tumors in the small intestine, a tumor type that was later on referred to as midgut carcinoids and more recently as SI-NETs (Figure 2). (Soga 2009)

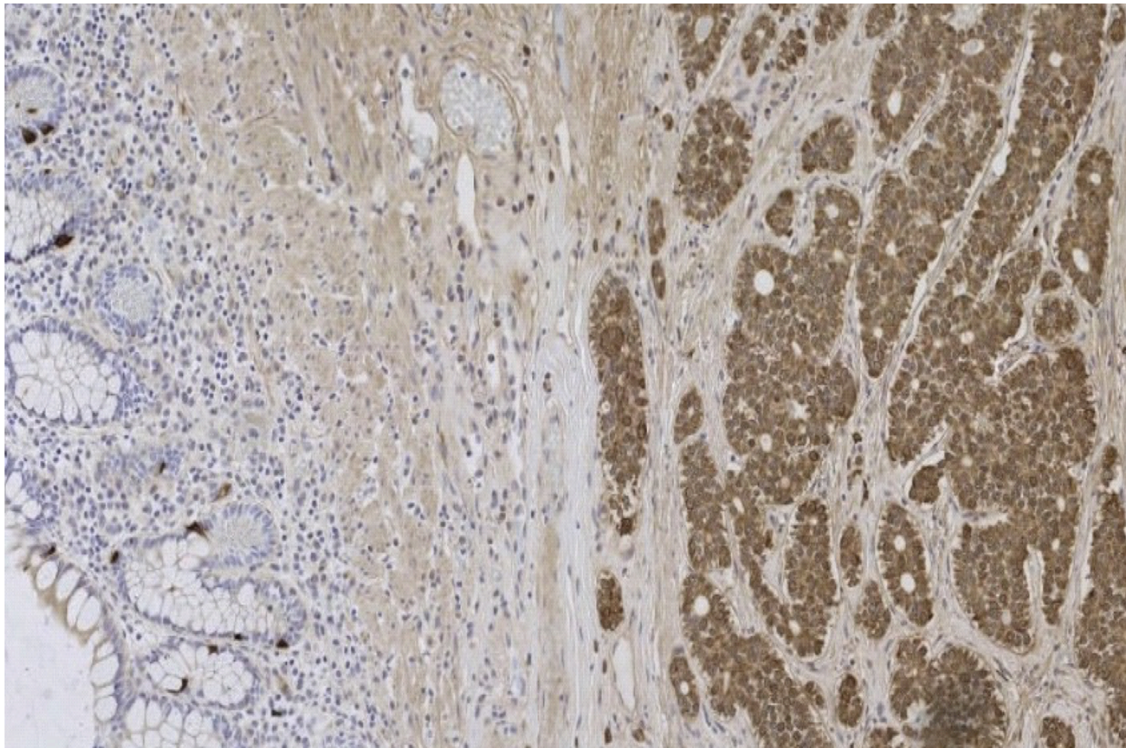

Figure 2. Immunohistochemistry staining for serotonin expressing cells showing SI-NET to the right and normal intestinal epithelium to the left (The immunohistochemistry micrograph courtesy of Anders Höög).

SI-NET is the largest subgroup of NETs. They are often already metastasized at diagnosis. Regional metastases are present in 36% and distant metastases in 48% of SI-NET patients. (Niederle, et al. 2010)

The incidence of SI-NETs in Sweden is 1.12 in 100,000 (Landerholm, et al. 2010). Based on the WHO 2010 guidelines patients with Stage I, II and IIIA have localized disease, with Stage IIIB have regional metastases, and with Stage IV distant metastases. Grading is based on both Ki-67 proliferation index and "mitotic index". Tumors with a Ki-67 of 3-20% and a mitotic index of 2-20 are considered as Grade 2 (G2), tumors with lower values are defined as G1 and those with higher values as G3 (Klimstra DS 2010).

The prognosis has been reported based on both staging and grading. The 5-year tumor-specific survival is 100% for Stage I and II, 97.1% for Stage III and 84.8% for Stage IV. The corresponding 5-year survivals according to grading are 93.8% for G1, 83% for G2 and 50.0% for G3 (Jann, et al. 2011). However, the prognosis is overall better for SI-NET than for other small intestinal malignancies including carcinomas, sarcomas and lymphomas (Lepage, et al. 2006).

The most frequent clinical presentation of asymptomatic SI-NET is vague abdominal pain or weight loss that is seen in 37% of the patients (Niederle et al. 2010). Moreover, in functioning tumors or symptomatic cases, the "carcinoid syndrome" is also present due to excess secretion of serotonin and/or tachikinins, substance-P, TGF-beta (Transforming growth factor beta) and connective tissue growth factor (CTGF). The carcinoid syndrome is characterized by secretory diarrhea, flushing, intermittent wheezing and most dangerously "carcinoid heart disease" (CHD or Heidinger syndrome) in up to 25-50% of the patients with carcinoid syndrome. Carcinoid syndrome is detectable in 95% of patients with liver metastasis, due to bypassing of the serotonin degradation in the portal circulation (Niederle, et al. 2016).

The diagnosis of NET is based on detection of diagnostic markers such as 5-HIAA (5-Hydroxyindoleacetic acid, the degradation product of serotonin) and Chromogranin-A (CgA), together with symptoms. The diagnosis is then verified by CT or MRI imaging followed by histopathological classification, according to strict criteria. The accuracy of imaging has been significantly improved after the introduction of somatostatin receptor (SSTR) imaging. In this method radionuclides are linked to somatostatin analogs (SSAs) that can be detected by PET scan (positron emission tomography) upon binding to SSTR2 on the tumor cell membrane. Other markers have been suggested to have diagnostic values but are not established in clinical practice, such as CDH1, p53, p27 and VEGF (Vascular endothelial growth factor). (Niederle et al. 2016)

Ki-67 index, CgA and synaptophysin determined by immunohistochemistry are applied in the clinical practice as prognostic markers of NETs. SSTR2 has been suggested as a predictive marker in SSA therapy. (Niederle et al. 2016)

1.3 GENETIC BACKGROUND

"Yet to define genes by the diseases they cause is about as absurd as defining organs of the body by the diseases they get…. This is a pitifully small thing to know about a gene, and a terribly misleading one". — Matt Ridley (Ridley 1999)

The term "Genome" was first used by Hans Winkler in 1920. It means the whole information that is carried by the haploid chromosomal set of an organism. "Genomics" refers to the study of the entire information harbored by an organism's genome (Graham Dellaire 2014; SP 2007). Recent studies suggest the existence of approximately 19,000 identified protein-coding genes in the human genome (Ezkurdia, et al. 2014).

Cancer cells are characterized by a background of genetic aberrations such as point mutations, chromosomal rearrangements and abnormal DNA copy numbers. In addition, epigenetic modifications are common. As a result of these abnormalities, cells with selective advantages may proliferate and propagate throughout the body, thus contributing to initiation and progression of cancer.

Targeted and genome-wide sequencing is commonly used in cancer genetic studies. DNA based arrays are also useful methods to compare the DNA copy numbers of the whole genome in cancer and normal tissue specimens. By this method recurrent copy number alterations (CNA) can be identified and evaluated as diagnostic and prognostic markers. Moreover, narrow regions of recurrent copy number aberrations may indicate the location of cancer genes involved in the disease. (Pinkel and Albertson 2005)

DNA microarrays were developed in the mid-1990s. They have played a major role in identifying the genetic background of many diseases including cancers. They have also been used as additional tools to improve diagnostic, predictive and prognostic accuracy for different cancer subgroups. (Laura 2008)

In classical Comparative Genomic Hybridization (CGH) normal and cancer DNA samples are labeled with different fluorescent dyes and co-hybridized to normal metaphase chromosomes (Pinkel and Albertson 2005). The ratio between the two fluorochromes is measured along all chromosomes to create ideogram profiles of regions with loss, gain or normal copy numbers. In array-CGH instead, sheared genomic DNA is introduced to bacterial artificial chromosomes (BACs) generated from *Escherichia coli*, or more lately oligonucleotides or single nucleotide polymorphism (SNP) probes, to identify DNA dosage alterations at an increased resolution of up to 1kb (Martin and Warburton 2015).

The first reports of genetic investigations of SI-NETs were published in the 1990s. Using loss of heterozygosity (LOH) analyses Jakobovitz et al. reported in 1996 that most carcinoids show LOH around the *MEN1* (Multiple endocrine neoplasia type 1) locus in chromosomal region 11q13. Using genome-profiling methods in the beginning of the millennium, recurrent CNAs were identified in a limited number of chromosomal regions. (Jakobovitz, et al. 1996) Recurrent alterations are most frequently observed in the form of losses within chromosomes

18, 16 and 11 and gains within chromosome 14. The results from fine mappings performed in Study I as well as in the literature using different approaches are illustrated in Figures 3, 4, 5 and 6. Overlapping CNAs are considered as important sources of information for the location of important cancer genes (Kim, et al. 2008; Stancu, et al. 2003; Walsh, et al. 2011). In Study I, TaqMan copy number assays were selected among overlapping CNAs.

Kytölä et al. studied CNAs in a subset of SI-NETs (that was extended later in this thesis as Study I), using metaphase CGH. That was one of the first reports that showed a striking proportion of loss of chromosomal region 18q, and less frequently loss of 11q, loss of 16q and gain of chromosome 4. More numerical aberrations were detected in metastases compared with primary tumors. Loss of 18q and 11q were detected in similar frequencies in primary tumors and metastasis, unlike other alterations such as loss of 16q and gain of chromosome 4 that were associated to metastasis. In this study, SI-NET CNAs were compared with available reports on lung NETs, reporting similar loss on chromosome 11q in both diseases. Candidate genes within frequently lost regions were suggested to be *SMAD2* (Sma- And Mad-Related Protein 2) and *SMAD4* (Sma- And Mad-Related Protein 2) and *DCC* (Deleted in colorectal cancer) located in chromosomal region 18q and the *MEN1*, *SDHD* (Succinate dehydrogenase complex subunit D), *ALL-1* (acute lymphoblastic leukemia 1) and *PPP2R1B* (Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform) genes in 11q. (Kytöla, et al. 2001)

Taking a similar approach Zhao et al. had compared gastrointestinal with bronchial NETs and found a significant difference between groups for some of the recurrent alterations, for example loss of chromosome 18 was overrepresented in the first group and loss of chromosome 11 in the second one (Zhao, et al. 2000). Löllgen et al. also found a high proportion of samples with LOH in chromosome 18 and in an attempt to identify the target tumor suppressor gene in this chromosomal region, they sequenced *SMAD4* and *DCC*, however, no mutation was found (Löllgen, et al. 2001).

Wang et al. found frequent LOH on chromosomes 11, 16 and 18 but did not detect any *BRAF* mutation in a series of 47 NET including a subgroup of SI-NETs (Wang, et al. 2005). Using SNP-based array screening, Kulke et al., found recurrent loss of chromosomes 18, 9p and 11q and a minimal region of gain on chromosome 14q encompassing the *DAD1* (Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit) gene. In subsequent immunohistochemical analyses over-expression of DAD1 was demonstrated in SI-NETs compared to normal ileum (Kulke, et al. 2008).

In 2009, Andersson et al. suggested that gain within chromosome 14 may contribute to the morbidity of SI-NETs, based on the association between this abnormality and worse patients' outcome. The study also found frequent loss of chromosome 18 and suggested that there are two distinct patterns of CNAs in these tumors, i.e. loss of chromosome 18 or gain of chromosome 14. Given the high frequency of loss of chromosome 18, this alteration was proposed to have a role in the initiation of SI-NETs (Andersson, et al. 2009). Recently, the same group reported a more detailed study, by which they confirmed the adverse effects of

gain of chromosome 14 on SI-NETs' outcome. Based on gene expression profiling SI-NETs were suggested to fall into clinical subgroups with different molecular characteristics. The study also found a correlation between gain of chromosome 14, higher grades of the disease and over-expression of cell cycle-promoting genes (Andersson, et al. 2016).

Cunningham et al. profiled DNA copy numbers and global mRNA expression in sporadic SI-NETs and a group of SI-NETs with familial background. They detected loss of chromosome 18 in 100% of sporadic SI-NETs compared with 38% in the familial group. They further suggested an autosomal dominant inheritance pattern for the familial form and proposed the term "Familial Ileal Endocrine Carcinoma (FIEC)". A tentative common disease gene for sporadic SI-NET and FIEC was suggested to be located in the distal part of 18q (Figure 3). (Cunningham et al. 2011)

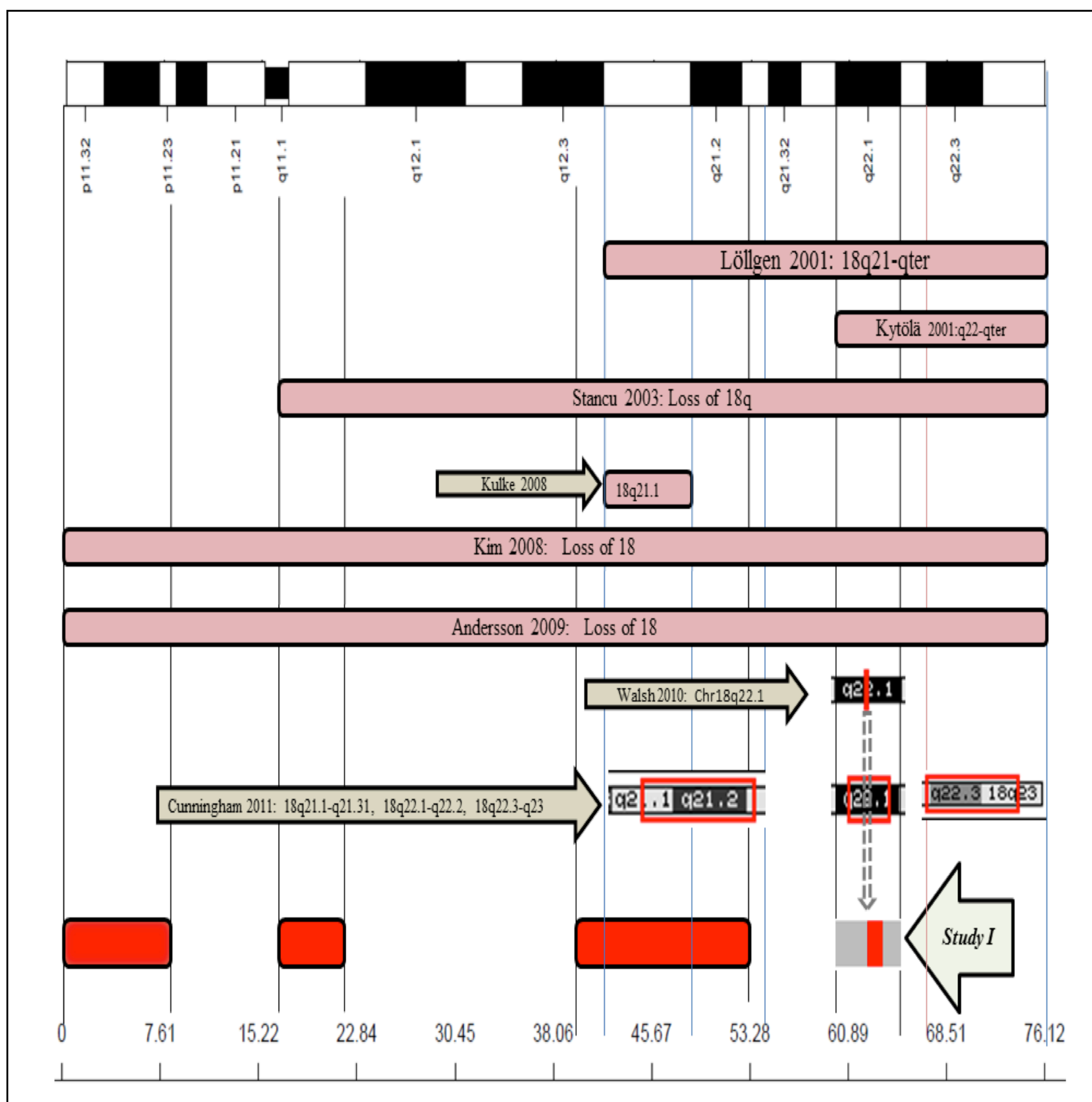

Figure 3. Summary of recurrent regions of loss of chromosome 18 observed in Study I and from the literature.

The application of high throughput sequencing techniques has led to promising results in determining the genetic etiology of SI-NETs and other cancers. Banck et al. used exome sequencing and verified previous reports of recurrent copy number losses in chromosomes 11 and 18 and gains in chromosomes 4, 5, 19, and 20. Furthermore, an overall low mutation frequency was noted. However, single nucleotide variants (SNVs) predicted to induce protein missense alterations were detected in a set of known cancer genes (*FGFR2* (Fibroblast growth factor receptor 2), *MEN1, HOOK3* (Hook microtubule tethering protein 3), *EZH2* (Enhancer of zeste homolog 2), *MLF1* (Myeloid Leukemia Factor 1), *CARD11* (Caspase recruitment domain family member 11), *VHL* (Von Hippel–Lindau), *NONO* (Non-POU Domain Containing, Octamer-Binding), and *SMAD1*). In cases with involvement of *PI3K/AKT* (phosphatidylinositide 3-kinases / Serine/threonine-specific protein kinase) signaling genes, amplification of either *AKT1* or *AKT2* also occurred. (Banck, et al. 2013)

Francis et al. only few months later published the first recurrently mutated gene in SI-NETs ie *CDKN1B* (or *p27*) based on whole-exome and -genome sequencing. Heterozygous frameshift mutations of small deletions or insertions predicted to truncate the p27 protein were identified in 14/180 (8%) of SI-NETs. Copy number losses of *CDKN1B/p27* were also detected. They further hypothesized that p27 can function as a haploinsufficent tumor suppressor in SI-NETs. (Francis, et al. 2013)

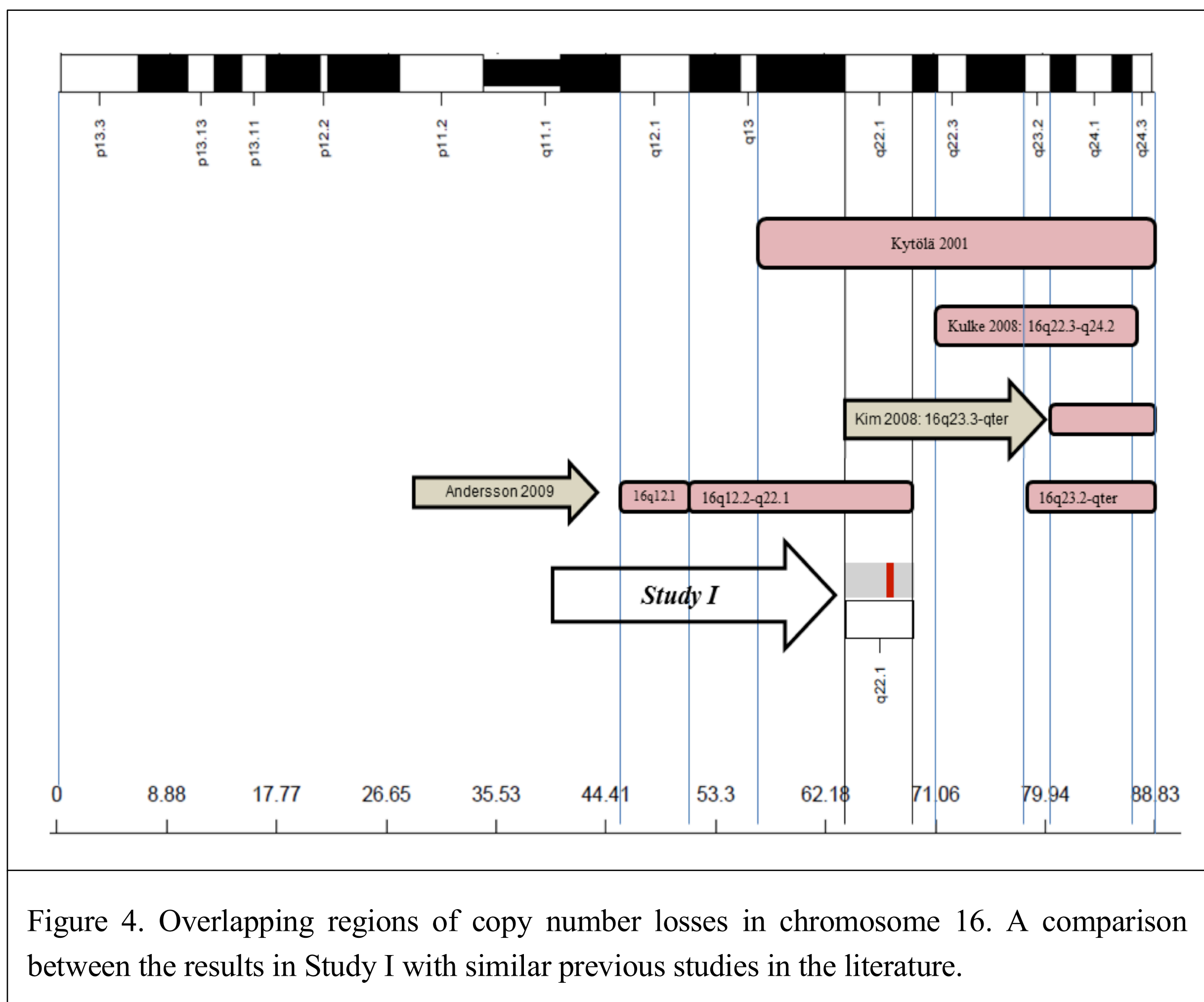

Figure 4. Overlapping regions of copy number losses in chromosome 16. A comparison between the results in Study I with similar previous studies in the literature.

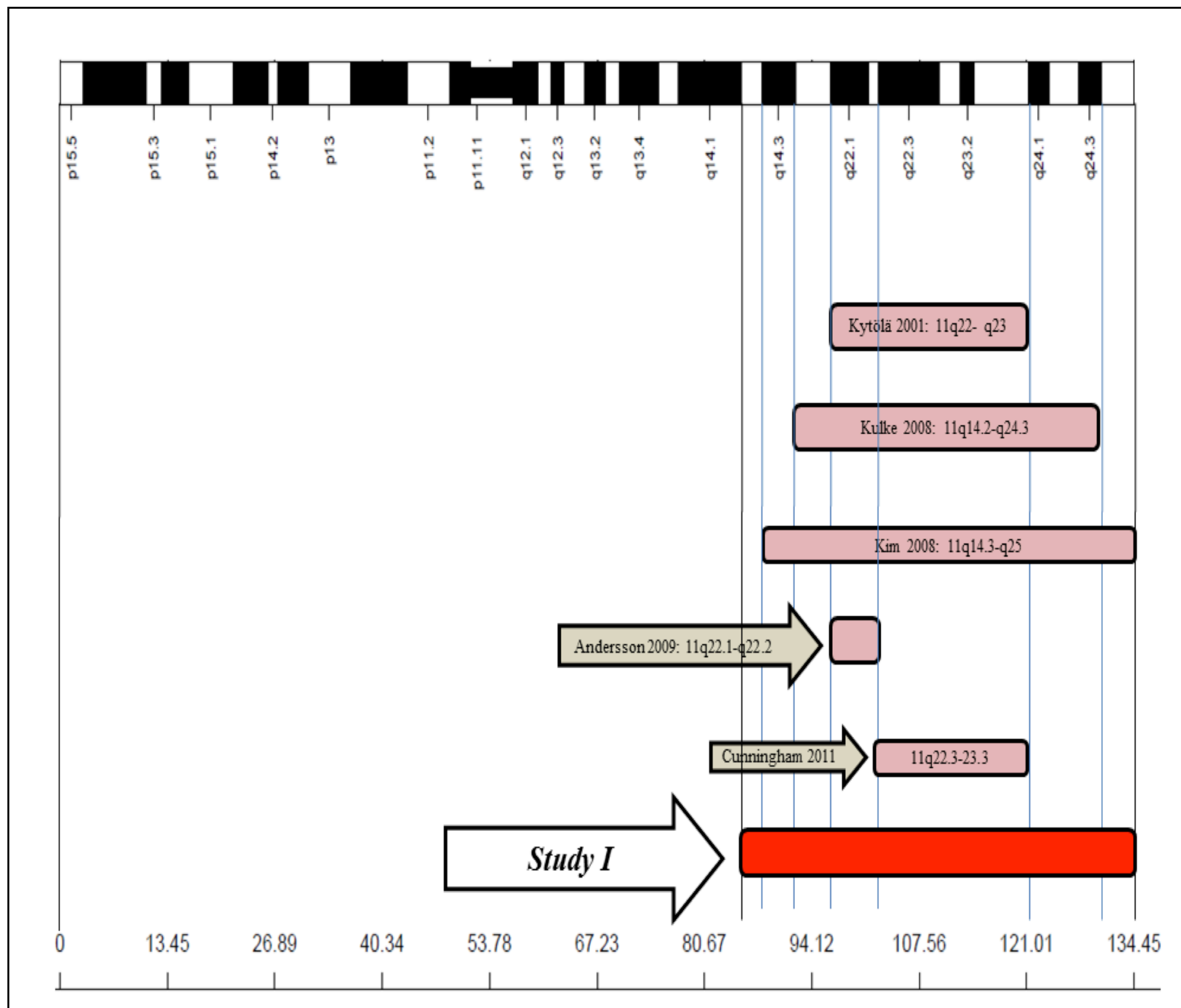

Figure 5.Regions with recurrent copy number losses in chromosome 11 revealed in Study I and the literature.

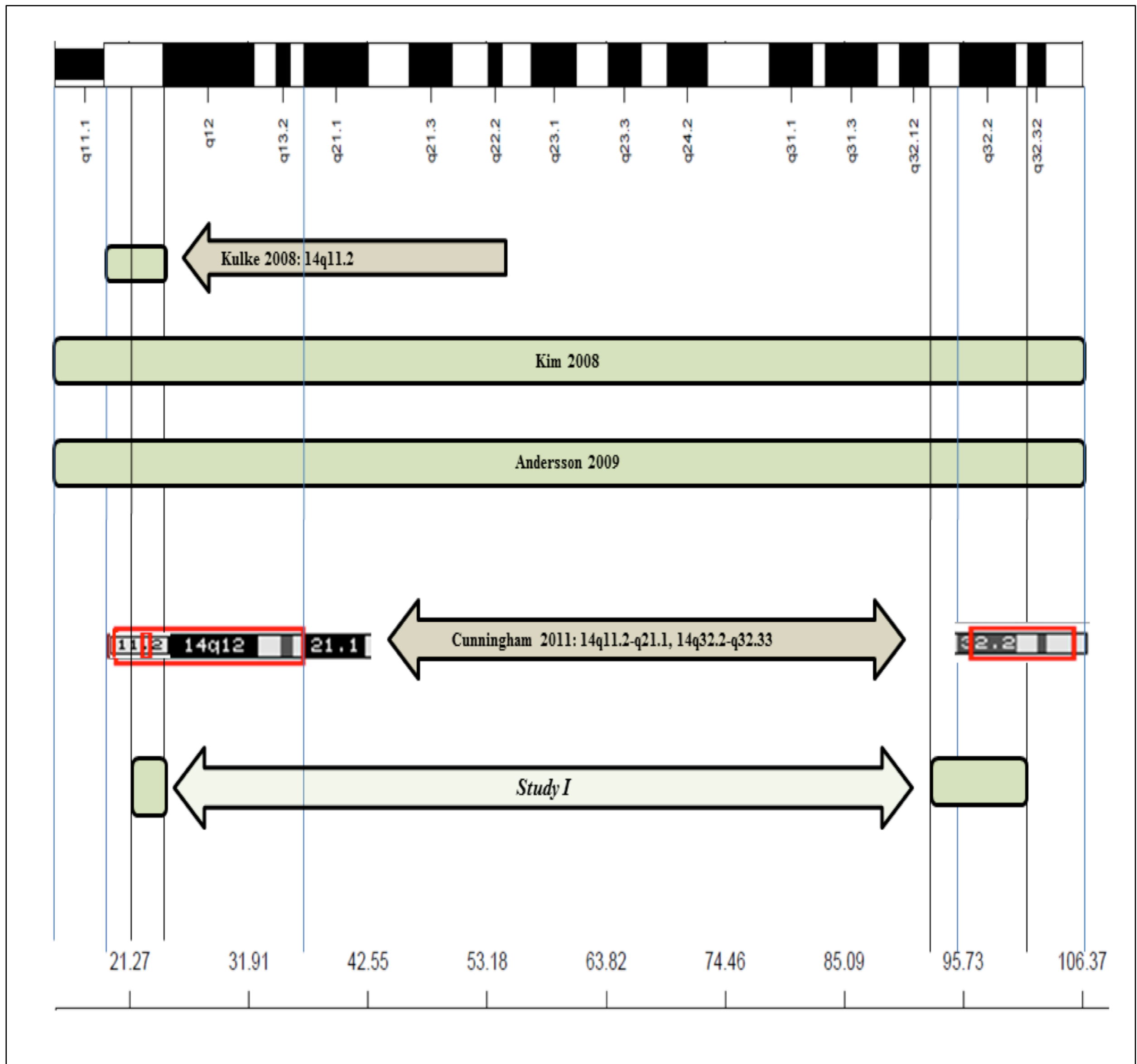

Figure 6. Summary of frequently observed regions with copy number gains in chromosome 14. There were 2 different regions of gains in Study I that also overlapped with previously reported CNAs of SI-NETs.

1.4 EPIGENETIC BACKGROUND

"There's more to heredity than genes ... You are what your grandmother ate" — Lisa Melton (Melton 2004)

The term epigenetics is used to describe heritable cellular information other than the DNA sequence itself. One example of epigenetic modification frequently studied in cancer is DNA cytosine methylation that may lead to gene expression dysregulation (Feinberg and Tycko

2004). Initially DNA methylation studies based on methylation-sensitive restriction enzymes and Southern blotting demonstrated hypomethylation of cancer cell DNA in comparison with DNA from normal counterpart tissues (Feinberg and Vogelstein 1983). Other researchers used high-performance liquid chromatography to compare DNA methylation levels in cancer and normal DNA, and obtained similar results with hypomethylation in tumor DNA that was particularly pronounced in metastases (Gama-Sosa, et al. 1983). To search for functional effects of the aberrant DNA methylation, researchers focused on oncogenes known to be over-expressed in cancer, and could thus show recurrent hypomethylation in promoter regions of e.g. *KRAS (Kirsten rat sarcoma viral oncogene homolog)*, *CAGE* (Cancer-associated gene) and *Cyclin D2* (Feinberg 1983; Oshimo, et al. 2003).

The first proof for the significance of promoter hypermethylation in cancer gene down-regulation (Figure 7) came from studies of the tumor suppressor gene *RB1* (Greger, et al. 1989). Studies showed that *RB1* promoter activity is reduced to only 8% in methylated samples (Ohtani-Fujita, et al. 1993). In subsequent studies, down-regulation of multiple tumor suppressor genes has been associated with increased promoter methylation, for example concerning *VHL*, *BRCA, RASSF1A (Ras Association Domain Family Member 1A)* and *p16 (CDKN2A)* (Catteau, et al. 1999; Dammann, et al. 2003; Herman, et al. 1996; Merlo, et al. 1995). The involvement of promoter methylation in human cancer has since been increasingly recognized, and is today implicated for diagnostic, prognostic and therapeutic purposes.

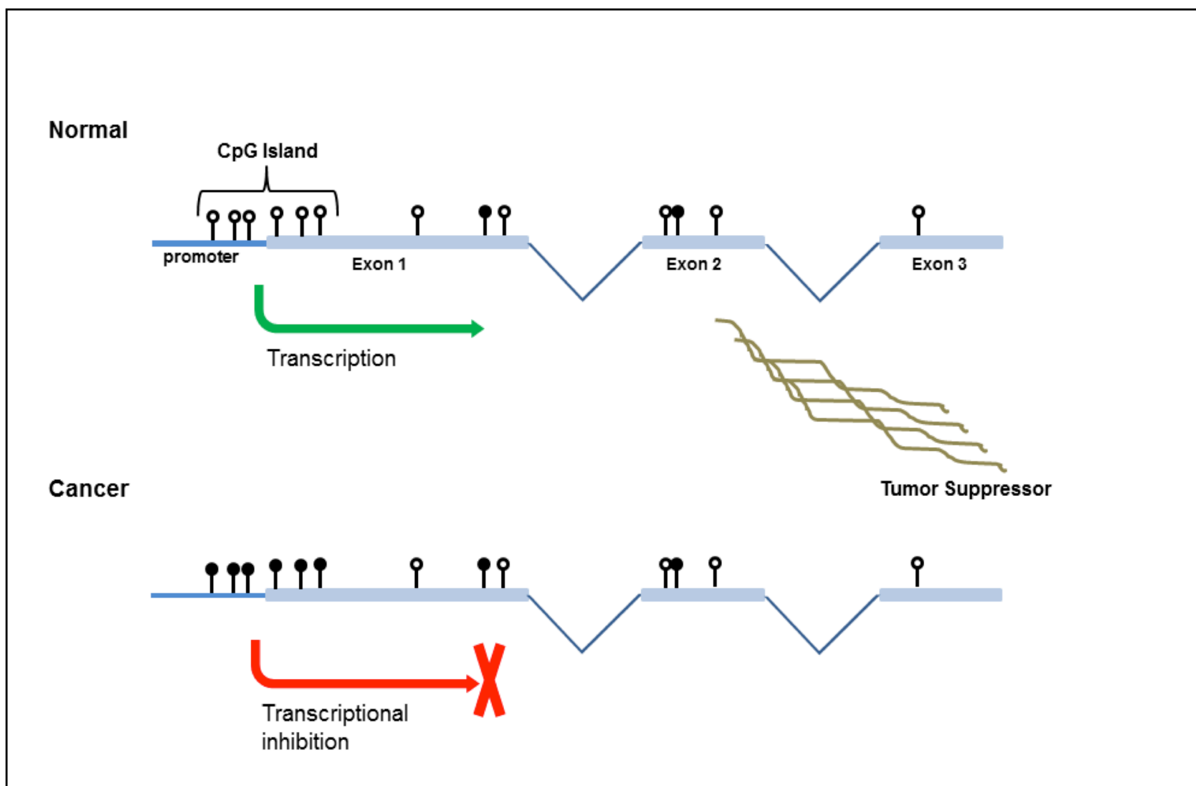

Figure 7. Inactivation of a tumor suppressor gene in cancer by hypermethylation of a CpG island in the promoter region leading to inhibition of the transcription machinery, inhibition of expression and promotion of cancer development.

Both promoter DNA methylation and global genome hypomethylation has been described in SI-NETs. Methylation of repeat elements that are frequent in the human genome such as long interspersed nucleotide elements (LINE-1) and Alu have been employed as an indicator of the global DNA methylation level. Since methylation of LINE-1 is associated with retrotransposon suppression, hypomethylation can also be attributed to transposon over-expression and hyperactivation and genomic instability. (Daskalos, et al. 2009; Lander, et al. 2001)

In a study of 35 NETs (15 ileal, 11 pancreatic and 9 others) all tumor samples were found to be hypomethylated compared to normal tissue at LINE-1 and Alu repeats. In the SI-NET subgroup, LINE-1 hypomethylation was associated with shorter survival, regional metastasis and loss of chromosome 18. (Choi, et al. 2007)

Previous studies have investigated promoter methylation status of selected genes in NETs. The results indicate that aberrant DNA methylation can play a role in NET tumor development; however, non-quantitative methods were commonly used. Pizzi et al. in 2005 reported that *RASSF1A* promoter hypermethylation occur together with LOH in 3p and over-expression of Cyclin D1 in foregut carcinoids (duodenal, pancreatic and gastric endocrine tumors) -but not in midgut (ileal NET) and hindgut carcinoids (colorectal NET) (Pizzi, et al. 2005). In another study, *RASSF1A* and *CTNNB1/β-Catenin* promoter methylation were more frequently observed in metastasis compared to primary SI-NETs (Zhang, et al. 2006). DNA methylation has also been reported for *RARb, MGMT* (O-6-Methylguanine-DNA Methyltransferase), *p16, COX2* (Cyclooxygenase 2b), *p14, THBS1* (Thrombospondin 1), and *ER* (Estrogen receptor) based on methylation specific PCR (MSP) or combined bisulfite restriction analysis (COBRA) in varying frequencies of SI-NETs (25-63%) (Chan, et al. 2003). Recent studies have employed global genome analyses of DNA methylation using array technology, which have identified new candidates for further epigenetic analysis (How-Kit, et al. 2015; Verdugo, et al. 2014). In a very recent study whole genome sequencing and DNA methylation array analyses were combined. This Study Identified a set of 21 epigenetically dysregulated genes, including among others *CDX1* (Caudal type homeobox 1) (86%), *CELSR3* (Cadherin EGF LAG seven-pass G-type receptor 3) (84%), *FBP1* (Fructose-Bisphosphatase 1) (84%), and *GIPR* (Gastric Inhibitory Polypeptide Receptor) (74%) (Karpathakis, et al. 2016). Moreover, bioinformatic analyses with integration of data for *CDKN1B/P27* mutation status and copy number alterations identified three subgroups of SI-NETs with different patient outcome (Karpathakis et al. 2016).

In Study II, global genome methylation and candidate gene promoter methylation was compared to normal ileum, using quantitative Pyrosequencing in SI-NETs. The candidate genes were chosen based on the following criteria:

<u>*WIF1 (WNT Inhibitory Factor 1)* and β-catenin</u>*:* The Wnt/beta-Catenin axis has been implicated in SI-NETs and other tumors of neuroendocrine origin (Sulaiman, et al. 2013; Zhang et al. 2006). *WIF1* is an antagonist of Wnt ligands such as WNT1, and may thereby inhibit Wnt signaling with effects on cell proliferation and survival, as well as on

differentiation and cell migration in tumor development. In cancer, the Wnt pathway plays a role in Cancer Stem Cell (CSC) homeostasis. A complex network of agonists and antagonists are involved in the regulation of the Wnt pathway (Hsieh, et al. 1999; Ramachandran, et al. 2014).

WIF1 promoter methylation, which can be one of the mechanisms involved in down-regulation of WIF1 and activation of Wnt signaling, is found in several different cancer types including cervical, breast, bladder, colorectal, nasopharyngeal, esophageal and non-small cell lung carcinoma (Ai, et al. 2006; Chan, et al. 2007; Delmas, et al. 2011; Kim, et al. 2013; Roperch, et al. 2013; Urakami, et al. 2006).

Kim et al. studied epigenetic modifications of Wnt pathway components in the NET cell line BON-1 (Evers, et al. 1991) based on their previous observations of nuclear accumulation of β-catenin in 25% of NETs (81). They also identified down-regulation of Wnt signaling inhibitors that could be restored by treatment of BON-1 with the demethylating agent 5-aza-CdR. However, using MSP, they did not detect *WIF1* promoter methylation (Kim et al. 2013). In Study II, the *WIF1* promoter was highly methylated in SI-NET tumors and the cell lines CNDT2 and HC45. This inconsistency between the two studies can be due to differences between the cell lines studied and the methodology.

In another report based on MSP analyses, Zhang et al. studied promoter methylation of β-catenin and other candidate genes. The authors reported increased gene expression and promoter methylation of *CTNNB1* in metastases as compared with primary tumors (Zhang et al. 2006). Similar studies also indicate a possibly non-canonical role for β-catenin as a tumor suppressor protein (Ebert, et al. 2003). This can be explained by the function of this protein in cell-cell binding and the effect that its down-regulation can play in dissociation and metastasis. It has also been demonstrated in Epithelial-mesenchymal transition (EMT), in which E-cadherin (CDH1) becomes down-regulated and its association with β-catenin in membranous adherence junctions is disrupted. β-catenin becomes degraded in the proteasomal system unless it is protected for instance by Wnt signaling (Lamouille, et al. 2014).

<u>*RASSF1A:*</u> Independent studies by Liu et al. and Zhang et al. have shown that in metastases compared to primary SI-NETs, the *RASSF1A* promoter is hypermethylated and RASSF1A expression is concomitantly down-regulated (Liu, et al. 2005; Zhang et al. 2006). Indeed, *RASSF1A* is a typical tumor suppressor for which down-regulation is caused by promoter hypermethylatyion in cancer. *RASSF1A* was shown in lung carcinoma to suppress cell cycle at G1. This is achieved by suppressing cyclin D1, which is mediated by down-regulation of JNK (Jun amino-terminal kinases) pathway (Whang, et al. 2005). Moreover, RASSF1A plays an important pro-apoptotic role binding to and activating MST1 (Mammalian Sterile Twenty 1) (Oh, et al. 2006). Another tumor suppressor function of RASSF1A includes its affinity to microtubules with inhibition of cell migration and tumor progression (Dallol, et al. 2005).

CXCL14 (Chemokine (C-X-C motif) ligand 14) and *NKX2-3(NK2 Homeobox 3):* These genes are among the top down-regulated genes in a microarray comparing metastases with primary SI-NETs (Leja, et al. 2009). CXCL14 is a is epigenetically down-regulated in prostate cancer cell lines where its expression can be restored by 5-aza-CdR in those cells and leading to an increased chemo-attraction for dendritic immune cells (Song, et al. 2010). In lung cancer cell lines *CXCL14* is down-regulated by DNA methylation. Ectopic expression of the gene in these cells induces necrosis and tumor shrinkage in xenograft models (Tessema, et al. 2010).

NKX2-3 is a homeodomain-containing transcription factor. In melanoma cell lines, the *NKX2-3* promoter is hypermethylated (Tellez, et al. 2009). In colorectal cancer cell lines siRNA suppression of the gene alters proliferation, growth and tumorigenesis, characteristics of the cells that are predicted to be regulated by Wnt pathway (Yu, et al. 2010).

P16/CDKN1A is a putative tumor suppressor located on chromosome 9p that was found recurrently lost in Study I. p16 is a CDK4 inhibitor and arrests the cell cycle at G1 through regulation of the retinoblastoma protein (Nobori, et al. 1994; Serrano, et al. 1993).

P16 promoter methylation and gene expression suppression has been widely detected in many cancers (Shima, et al. 2011; Yu, et al. 2014). Epigenetic regulation of this gene in SI-NETs, however, is disputed (Arnold, et al. 2007; Chan et al. 2003; Liu et al. 2005). This raises the possibility of false positive results produced by low-tech PCR-based DNA methylation assays.

P16 can unexpectedly be over-expressed in more advanced cancer stages. This phenomenon is attributed to either onco-viral infection such as Human Papillomavirus (HPV), age associated (replicative) senescence or oncogene-induced senescence (OIS) (Romagosa, et al. 2011). OIS can be a factor to suppress an otherwise rapid tumor progression in often non-invasive, indolent SI-NETs.

CDH1 (cadherin 1) and *CDH3* were among candidate genes due to their location on chromosome 16q, a recurrently observed loss in SI-NETs based on Study I. Aberrant promoter methylation of these genes has been reported in other cancers (Esteller, et al. 2001; Milicic, et al. 2008).

LAMA1 (Laminin Subunit Alpha 1), *SMAD2* and *SMAD4* are located on chromosome 18, which recurrent loss is a hallmark of SI-NET. Along with *CDH1* and *CDH3*, the candidate selection approach was based on Knudson's two-hit hypothesis, where the first hit was already detected in CNA analysis in Study I (Knudson 1971).

APC (Adenomatous polyposis coli), *HIC1 (Hypermethylated In Cancer 1)*, *p14* and all above mentioned genes have been reported as hypermethylated in SI-NETs or other tumors, often using low-resolution PCR-based methods (Arnold et al. 2007; Chan et al. 2003).

LINE-1 constitutes 17% of the human genome (Lander et al. 2001). Only a proportion of LINE-1 has maintained its original retrotransposon potential and the rest is not functional and

is considered as molecular fossil. LINE-1 hypomethylation and over-expression are common phenomena in cancer. Hence, analyzing methylation of LINE-1 gives an estimation of the global genome methylation and integrity as well as retrotransposon activity that is an important cause of many mutations (Beck, et al. 2010). Hypomethylation activates expression of LINE-1 that is a driver of genome instability and may lead to tumorigenesis. In many cancers including breast, colon, lung, head and neck, bladder, esophagus, liver, prostate, and stomach cancers, LINE-1 is hypomethylated.

1.4.3. Demethylation analyses

5-aza-CR (Azacytidine) and 5-aza-CdR (5-Aza-2′-deoxycytidine) are therapeutic agents targeting epigenetic abnormalities in cancer. These agents are derivatives of cytidine, to which DNMT (DNA methyltransferase) can bind and get trapped. Thus, they prevent DNA methylation in the newly synthesized DNA strand. This phenomenon is being used in the management of myelodysplastic syndrome. 5-aza-CR and 5-aza-CdR, also known as Vidaza and Dacogen were approved by FDA in 2004 and 2006, respectively (Matoušová , et al. 2011).

In epigenetic studies, these agents are often used to examine the implication and causality of promoter hypermethylation in transcription regulation. This also provides hope in therapeutic epigenetic agents targeting reversible alterations in comparison with permanent genetic alterations.

1.5 SOMATOSTATIN ANALOGS IN SI-NET THERAPY

1.5.1 Somatostatin and its pharmaceutical analogues

In 1972, researchers at Salk institute, while searching for growth hormone releasing factor in sheep hypothalamus, incidentally found a growth hormone inhibitory hormone that was named somatostatin (Brazeau, et al. 1973). *In vitro* addition of somatostatin to pituitary isolated cells inhibited growth hormone secretion. Further investigation revealed the peptide sequence of the molecule. It was composed of 14 amino acids (Burgus, et al. 1973).

Somatostatin is also called somatotropin-release inhibiting factor (SRIF). It is now known that there are two different types of somatostatin with 14 and 28 amino acids, both C-terminal products of proto-somatostatin. Either of these somatostatin isoforms can potentially agonize 5 different somatostatin receptors (SSTRs) -1 through -5. *SSTRs* are intronless except *SSTR2* that encodes 2 splice variants SSTR2-A and SSTR2-B with different C-terminals. Five distinct SSTRs are classified in 2 groups, based on their structural and pharmacological features: SRIF1 is comprised of SSTR2, SSTR3 and SSTR5 that show a nano-molar sensitivity to somatostatin and its analogs, while the range of somatostatin sensitivity for SRIF2 including SSTR1 and SSTR4 is micro-molar. (Weckbecker, et al. 2003)

Somatostatin is expressed by normal endocrine, gastrointestinal, immune and neuronal cells and also some tumors such as SI-NETs. Somatostatin is a neurotransmitter in the neural system and it inhibits a broad range of hormones such as growth hormone, insulin, glucagon, gastrin, cholecystokinin, vasoactive intestinal peptide (VIP) and secretin as well as exocrine secretion of gastric acid, intestinal fluid and pancreatic enzymes. (Weckbecker et al. 2003)

Its pan-antisecretory features have made somatostatin an attractive candidate in a variety of disorders including NETs and acromegaly. Plasma half-life of natural somatostatin does not exceed 3 minutes; hence attempts have been made to generate clinical somatostatin analogues that sustain its pharmacophore (crucial amino acids tryptophan and lysine), whilst modified to resist degradation. (Harris 1994)

1.5.2 Signal transduction

"The Royal Swedish Academy of Sciences has decided to award the Nobel Prize in Chemistry for 2012 to Robert J. Lefkowitz and Brian K. Kobilka, for groundbreaking discoveries of G-protein–coupled receptors (GPCRs)". — Nobelprize.org

GPCRs constitute a large group of cell membrane receptors. G proteins have three subunits, Gα, Gβ and Gγ. The classification of GPCRs is based on 4 different types of G-alpha namely $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$.

SSTRs are GPCRs. Signaling through SSTRs is cell context dependent and complex. Besides, SSTRs vary in terms of the downstream activators and pathways that they govern. SSTR2 and SSTR5 are the main targets of somatostatin analog therapy. Receptor stimulation occurs in nano-molar blood concentrations of the ligand. (IPSEN 2011; Weckbecker et al. 2003)

Upon somatostatin or SSA – SSTR binding, both pertussis-toxin-sensitive G proteins such as Gαi and Gαo and pertussis-toxin-insensitive Gαq, Gα14 and Gα16 proteins become activated. The most famous pertussis-toxin-sensitive downstream enzyme for all SSTRs is adenylyl cyclase. SSTR activation inhibits this enzyme and reduces signal transduction through cAMP. The best-documented downstream effector of cAMP is protein kinase A (PKA). PKA can phosphorylate and activate transcription factors such as cAMP response element-binding protein (CREB), thereby render therapeutic potential of somatostatin analogs (Tentler, et al. 1997). Marked up-regulation of both cAMP and CREB in SI-NETs suggests the possible importance of this pathway (Drozdov, et al. 2011).

Another secondary messenger that is also down-regulated upon SSTR activation is Ca^{2+}. This can be a direct inhibition of Ca^{2+} channel and reduced Ca^{2+} influx and intracellular release or may proceed indirectly, through K^+ channel activation and cell membrane hyperpolarization. This pathway is associated with Phospholipase C inhibition and Inositol 3 Phosphate down-regulation. (Weckbecker et al. 2003)

Another proposed mechanism for anti-tumor activity of somatostatin and SSAs is activation of protein tyrosine phosphatases, such as the Src homology phosphatases, SHP-1 and SHP-2, which can counteract tyrosine kinases by dephosphorylation of target proteins. (Reubi and Schonbrunn 2013)

For unknown reasons, SSAs play an anti-proliferative role in contexts such as pituitary adenoma, but not on many NETs. It seems that different SSTR expression signatures or other unknown factors in the downstream signaling machinery can cause distinct effects. Cytosolic Ca^{2+} is generally reduced in somatostatin treated cells such as pituitary adenoma cells, whereas it is elevated in pancreatic tumor cells. SSAs inhibit ERK (Extracellular signal–regulated kinase) phosphorylation in pituitary adenoma and medullary thyroid carcinoma cells, but stimulate it in many of examined NET cells (Figure 8). (Reubi and Schonbrunn 2013)

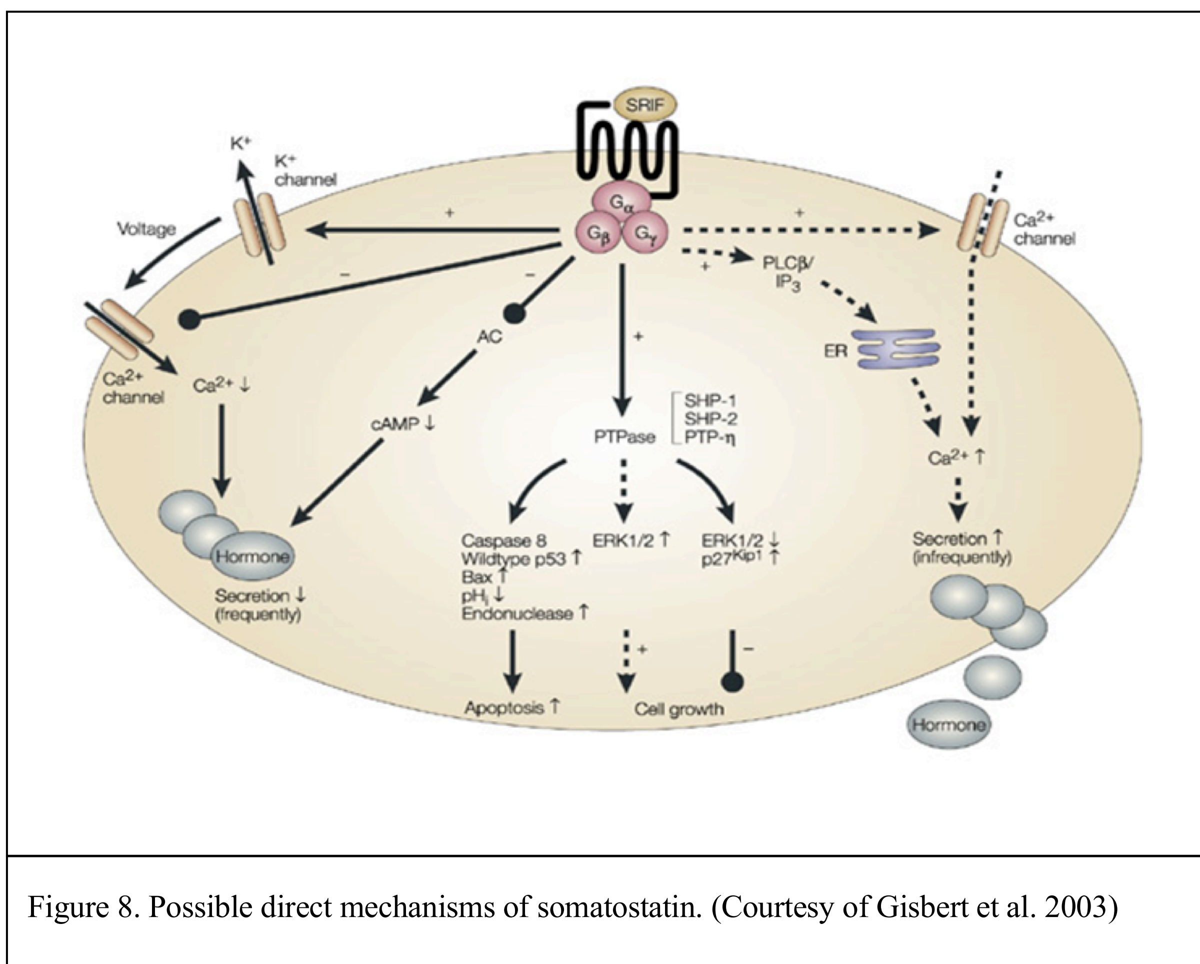

Figure 8. Possible direct mechanisms of somatostatin. (Courtesy of Gisbert et al. 2003)

1.5.3 Adenomatous polyposis coli and survivin

APC plays a critical role in the maintenance of the intestine crypts and their homeostasis. Mutations in this gene are accompanied by a high ratio of colorectal cancer that occurs via pathways for β-catenin phosphorylation and stabilization. (Morin, et al. 1997)

BIRC5 (Baculoviral IAP Repeat Containing 5)/survivin is an inhibitor of caspase 9, thereby playing an important role in inhibition of apoptosis. Survivin also plays an important role in Anaphase through cytokinesis by attaching to mitotic spindles and kinetochores, contributing to chromosomal dissociation. Therefore, over-expression of survivin promotes tumorigenesis in many cancer types. (Mita, et al. 2008)

An association between loss of APC and survivin expression has long been known and is attributed to cancer stem cell characteristics. (Zhang, et al. 2001)

1.6 TARGETING NEDDYLATION IN SI-NETS

1.6.1 Ubiquitination system and cancer

Ubiquitin is a 76 amino acid protein that binds to target proteins in a complex post-translational regulatory process known as ubiquitination and plays an important role in cell homeostasis. In some lethal disorders, such as cancer, it is often dysregulated. One or several ubiquitin molecules bind to a target protein and direct diverse downstream pathways. Mono-ubiquitination occurs when only one molecule of ubiquitin binds to a lysine residue of a substrate. This process regulates endocytosis, DNA repair, protein transfer, and histone regulation. (Woelk, et al. 2007)

Multiple ubiquitin molecules may each bind to a distinct lysine residue in the target protein in a process called multi-ubiquitination, which is involved in endocytosis. A ubiquitin itself has seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33 Lys48 and Lys63) that can function as substrates of other ubiquitin molecules. In a process known as poly-ubiquitination a ubiquitin chain binds to a target protein. Depending on which lysine in the ubiquitin structure is ubiquitinated, the fate of the target will be different. Poly-ubiquitination on lysine 63 directs endocytosis of the target protein, DNA repair and protein assembly in the signaling pathways such as NF-κB (Nuclear factor-κB). Poly-ubiquitination on lysine 48 (and to some extent on lysine 11) is best characterized and involved in proteolysis through the ubiquitin-proteasome system (UBS). (Groettrup 2010; Ikeda and Dikic 2008; Skaar, et al. 2014)

Ubiquitination starts with a ubiquitin activating enzyme (E1). Two ubiquitin molecules bind to an E1 and activate it to transfer one molecule of ubiquitin to a ubiquitin conjugating enzyme (E2) in a transthiolation reaction. E2 transfers ubiquitin to a target protein when in a complex with one of several hundred ubiquitin ligases (E3s), which in turn catalyze the ubiquitination of the substrate on one of its lysine residues. (Frescas and Pagano 2008)

E3 ligases are classified in 3 groups, each containing a distinct domain in their core protein: 1- HECT (homologous to E6-AP carboxy terminus); 2- U-box E3s; 3- RING (Really interesting New Protein) finger. Classic RING-finger ligases contain domains to bind to both substrate and E2-ubiquitin and to directly proceed with substrate ubiquitination. The main subgroup of RING finger ligases contains a scaffold protein, cullin, that binds to a substrate at its N-terminus. At the C- terminus, cullin binds to a RING finger domain containing protein,

RBX1 or RBX2 (RING-box protein) that binds to an ubiquitin loaded E2. This class of E3s is called cullin-RING ligase (CRL) (Petroski and Deshaies 2005; Soucy, et al. 2010) and is the largest family among more than 600 putative E3 ligases in humans (Wang, et al. 2014). Ring-finger proteins including CRLs are the most studied ubiquitin ligases. CRLs are responsible for 20% of cellular proteasome-dependent degradation (Soucy, et al. 2009). Moreover, there is another CRL-like ligase containing cullin- homology domain, named APC2 subunit of the anaphase-promoting complex/cyclosome (APC/C) that has ubiquitin ligase activity (Frescas and Pagano 2008; Petroski and Deshaies 2005).

There are 8 different cullins that build up 8 cullin-RING ligase families: CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CRL7 and CUL9 (Wang et al. 2014). For a cullin to bind to a ubiquitination substrate, it must bind to an adapter and through this to a receptor. The receptor confers substrate-targeting specificity to a CRL. The typical CRL in mammals is constituted of a CUL1 scaffold that recruits a ubiquitin loaded E2 through a RBX1. At its other end, CUL1 binds to the N-terminal of the adaptor protein SKP1 (S-phase kinase-associated protein 1) that is bound through its C-terminal to the substrate receptor protein, which is an F-box protein. This complex is called SCF (SKP1-CUL1-F box protein) (Petroski and Deshaies 2005).

There are 69 F-box proteins in humans that based on their targets are divided into 4 groups: tumor-suppressors, oncogenes, context-dependent or undetermined functions. SKP2 (S-phase kinase-associated protein 2) is the only well-established oncogenic F-box among all. This F-box protein is also the only one that targets p27. (Wang et al. 2014)

1.6.2 NEDD8 (Neural Precursor Cell Expressed, Developmentally Down-Regulated 8)

NEDD8 is an 81-amino acid protein and the best-characterized ubiquitin-like protein. It shares 60% of its amino acid sequence with ubiquitin, the highest similarity with ubiquitin among 16 ubiquitin like proteins (UBLs). It binds to target proteins in a cascade similar to ubiquitination. First, it binds to its specific E1, neddylation-activating enzyme (NAE). One of its two E2 enzymes transfers NEDD8 to the target: UBC12 (Ubiquitin-Conjugating Enzyme 12) or UBE2F (Ubiquitin Conjugating Enzyme E2F). (Enchev, et al. 2015; Soucy et al. 2010)

The best-established targets of neddylation are cullins. Neddylation is necessary for normal cellular function of CRLs. Neddylation of CUL1 does not require an E3 ligase but is performed by RBX1 (Petroski and Deshaies 2005). For cullin neddylation, UBC12 only pairs with RBX1 and neddylates CUL1-4 and UBE2F pairs to RBX2 and neddylates CUL5. (Huang, et al. 2009)

Upon neddylation, cullins undergo configurational modification and become activated. This active configuration enhances their binding capacity to E2 that subsequently facilitates ubiquitination and proteasomal degradation of target proteins. (Petroski and Deshaies 2005)

Neddylation has a critical role by activation of CRL, for example in cell cycle regulation, and by degradation of tumor suppressors such as p27 and p21. Another example is CRL1-mediated degradation of IκBα (inhibitor of NF-κB) that activates and promotes cell proliferation for instance in ABC-DLBCL (activated B-cell-like diffuse large B-cell lymphoma). βTrCP (β-transducin repeat-containing protein) is known as the F-box protein in the CRL1 that conducts such a process (Staudt 2010). In addition, NEDD8 has other roles beyond CRL, by directly attachment and inactivation of tumor suppressors such as p53 and p73 (Enchev et al. 2015).

Neddylation regulates activation of CRLs in coordination with CAND1 (cullin-associated and neddylation-dissociated). CAND1 binds selectively to unnedylated CUL1 and makes a ternary complex with CUL1-RBX1. It competes with SKP1 in binding to CUL1, so that SKP1 only binds to CUL1 and makes an active SCF complex when CUL1 is already neddylated. CAND1 dissociates CUL1-SKP1 complex and it can inhibit SCF activity (Figure 9). (Liu, et al. 2002)

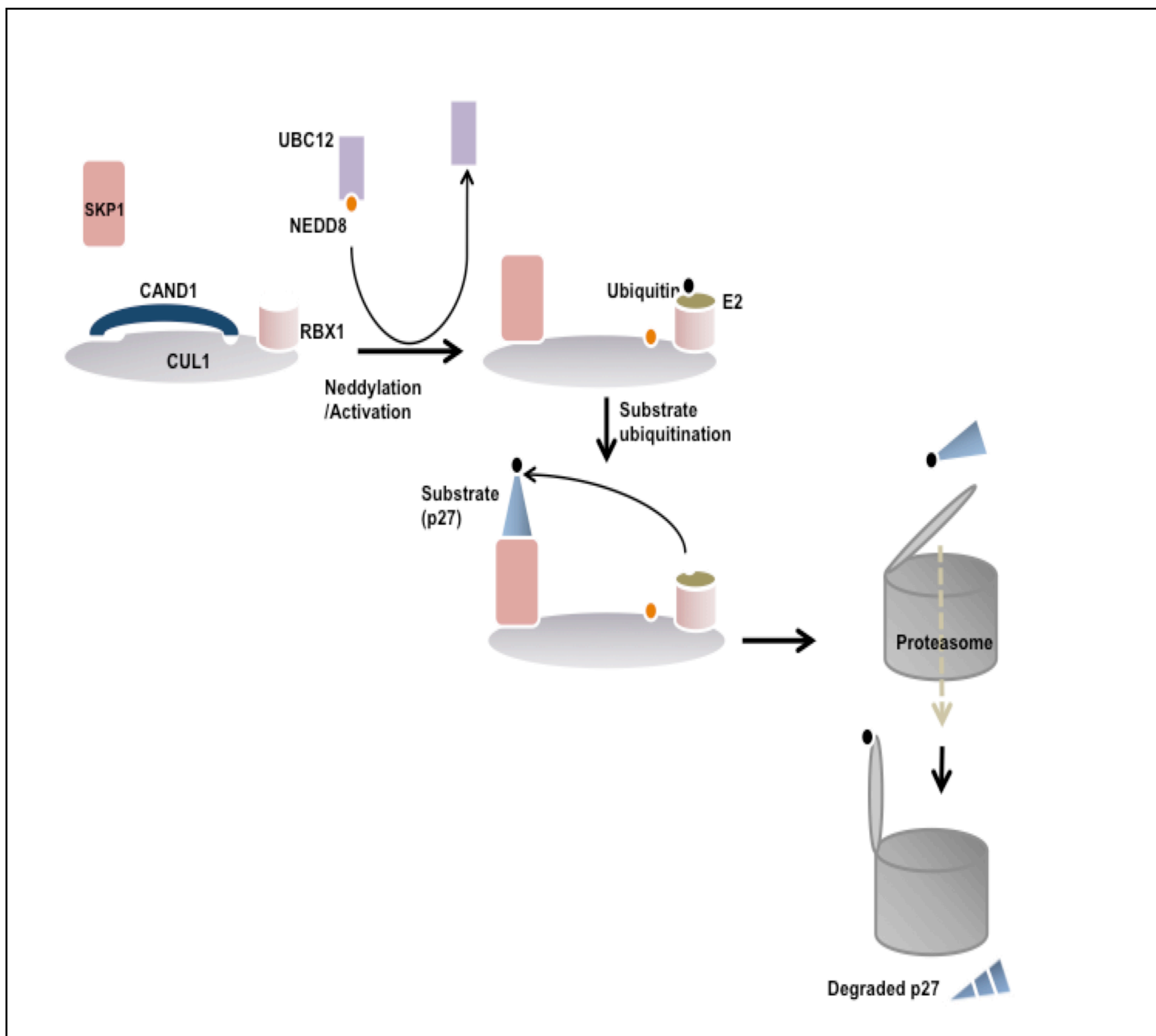

Figure 9. UPS control of p27 levels in the cells. Neddylation activates UPS, rendering the degradation of p27 and hindering cell cycle progression.

MLN4924 is an adenosine sulfamate analog and a selective and first-in-class inhibitor of NAE. MLN4924 competes with ATP and is applied by NAE to form MLN4924-NEDD8 adduct. The adduct binds tightly to the adenylation site and inactivates NAE (Soucy et al. 2009) (Soucy et al 2011). MLN4924 may be effective in cancers with up-regulation of SKP2 (consequently down-regulation of tumor-suppressors such as p27 and p21) or in NF-κB – based cancers (Soucy et al. 2010). The compound is currently administered in several clinical cancer trials (Nawrocki, et al. 2012).

1.6.3 P27 proteolysis inhibition as a strategy in SI-NET management

Genetic information regarding SI-NET initiation and progression has been limited to recurrent CNAs, especially loss of chromosome 18q for many years, until a high throughput genome sequencing approach was exploited in the studies of these tumors. An outstanding study based on exome and genome sequencing of SI-NETs suggested - although in a small proportion of ~10% - that *CDKN1B* mutation or CNA probably being in charge of tumorigenesis (Francis et al. 2013). *CDKN1B* encodes p27/KIP1 (Kinesin-like protein 1) that is a CDK (cyclin dependent kinase) inhibitor and controls G1 to S phase transition in cell cycle. In G0 and early G1, p27 is translated and remains stable to bind to and inhibit cyclin A-CDK2 and cyclin E-CDK2. During G1, p27 is gradually degraded and allows the aforementioned complexes to transcribe necessary factors for G1-S transition and initiation of DNA replication (Chu, et al. 2008).

The finding of *CDKN1B* alterations in SI-NETs was a breakthrough, since it provided a logical clue for further investigations. Losses on one chromosome or heterozygous mutations were consistent with the notion of haploinsufficiency of p27. Germline mutations in p27/MEN4 have been found in families with MEN1 syndrome, without any genetic alteration of *MEN1 per se* (Pellegata, et al. 2006). Furthermore, menin, a protein encoded by *MEN1*, regulates the expression of *CDKN1B* epigenetically (Karnik, et al. 2005). Reduced p27 expression is implicated in many cancers. Reduced expression of P27 is an independent prognostic marker in non-small cell lung cancer (Esposito, et al. 1997; Hommura, et al. 2000). Different studies in ovarian cancer (Schmider-Ross, et al. 2006), breast cancer (Catzavelos, et al. 1997), prostate cancer (Tsihlias, et al. 1998; Yang, et al. 1998), colorectal cancer (Loda, et al. 1997), mantle cell lymphoma (Chiarle, et al. 2000) and head and neck cancer (Pruneri, et al. 1999) show that different P27 expression levels have prognostic value (Chu et al. 2008).

On the other hand, p27 is not a classic tumor suppressor like p53. $P27^{-/-}$ mice were shown to be larger in size and developed thymic and pituitary hyperplasia that progressed to pituitary adenocarcinoma over a longer period (Fero, et al. 1996). Mutations in *CDKN1B* are rare. Loss of heterozygosity and total protein loss has not been observed in cancer (Frescas and Pagano 2008). Therefore, it seems that down-regulation of p27 and subsequent

tumorigeneisis originates from upstream regulatory mechanisms. Lysate from tumors show proteolytic activity against recombinant p27 *in vitro* (Loda et al. 1997).

SKP2 is associated with worse patient outcome in all studied cancers. CRL1^{skp2} is known as the main ligase regulating p27 levels. It degrades p27 and promotes cell cycle and tumorigenesis (Frescas and Pagano 2008). CRL1^{skp2} degrades p21, p27, p57, RB1 and other tumor suppressors; however, it seems that p27 is the key substrate of CRL1^{skp2}. In mouse, deletion of *Cdkn1b* is sufficient to rescue the *Skp2*$^{-/-}$ phenotype (Catzavelos et al. 1997; Frescas and Pagano 2008; Kossatz, et al. 2004). While SKP2 expression always leads to p27 down-regulation, reduced levels of p27 have not always been associated with SKP2 over-expression; therefore other mechanisms should be implicated in the regulation of p27 (Frescas and Pagano 2008).

2 AIMS

This thesis aimed at characterizing the genetic, epigenetic and proteomic mechanisms behind tumor development of SI-NET and its treatment with somatostatin analogs. The particular aims for each study were:

Study I:

To define and refine regions of recurrent DNA copy number alterations in SI-NETs

Study II:

To identify epigenetic modifications including aberrant promoter CpG methylation and global genome methylation levels behind SI-NET tumor development *in vivo* and *in vitro*

Study III:

To characterize the molecular effects of somatostatin analog therapy of NETs, particularly their effects through the APC-survivin axis

Study IV:

To characterize the proteomic signature of SI-NETs and the role of neddylation as a potential target for SI-NET therapy

3 MATERIALS AND METHODS

3.1 TUMOR SAMPLES AND CELL LINES

3.1.1 Tumor samples

For the array-CGH analyses in Study I, 19 primary tumors and 11 metastases, 30 samples in total, from 29 patients were used. For TaqMan copy number experiments, the sample cohort was extended to 43 samples from 32 patients. In total 24 primary tumors and 19 metastases were used in Study I. Seven of the metastases were distant (five liver and two ovarian) and twelve were regional metastases (mesenterial, omental or regional lymph nodes).

The same samples were used in Study II, but in addition a lymph node metastasis from a patient with a familial background of SI-NET was also used.

In Study III, 20 formalin-fixed paraffin-embedded (FFPE) tissue samples with tumor tissue and adjacent normal epithelial tissue from 13 SI-NET patients were used for a comparison between somatostatin treated / untreated samples and tumors/normal adjacent tissue with respect to expression of survivin and APC. In addition a tissue microarray (TMA) was then used which included 112 NET tumors/patients obtained from the Department of Pathology, La Paz University Hospital, Madrid, Spain.

For Study IV, 70 SI-NETs were included of which 37 were primary tumors, 23 were regional metastases and 10 were distant metastases.

Except for the TMA in Study III, all clinical samples were obtained from the biobank at Karolinska University Hospital, Sweden.

3.1.2. NET cell lines

NET cell lines were used for studies II, III and IV. BON-1 is a serotonin positive pancreatic NET cell line. H727 is a pulmonary NET cell line, and CNDT2 and HC45 are both derived from SI-NET liver metastases. H727 was purchased from ATCC, and the other cell lines were kindly provided by other research groups. The non-commercial cell lines were genotyped for a standardized set of SNPs at Biosynthesis Inc.

A primary cell culture was developed from an SI-NET lymph node metastasis. The tumor was chopped in MEM, incubated in type II collagenase overnight, and treated with hyaluronidase. The cells were cultured in DMEM with 10% FBS at 37°C, 5% CO_2 in a humidified incubator for up to 10 passages.

3.2 ARRAY COMPARATIVE GENOMIC HYBRIDIZATION

Array-CGH is used to examine DNA copy number losses and gains corresponding to deletions or gains/amplifications on the chromosomal level. The method has an improved resolution compared to its preceding chromosome based form so called metaphase- CGH. In array-CGH short single strand genomic DNA probes are arrayed on micro slides (chips). The resolution depends on the length of the probes and on the genomic distance between probes.

Test genomic DNA and normal reference DNA are denatured, labeled by different fluorescent dyes, and hybridized together to the array. The ratio between fluorescent emission from the two fluorochromes is read in a digital scanner and analyzed to identify gain or loss of the corresponding DNA sequence (Theisen 2008). Study I reflects the development in the field concerning array platforms and array resolution. One Mb arrays were applied (from Spectral Genomics, currently Perkin Elmer) on a subset of the 30 SI-NETs from 29 patients in the study. Data was analysed with the Spectralware 2 software applying cut-offs of 1.2 and 0.8 to detect gains and losses, respectively. Then, human tiling 33 K and 38 K BAC arrays were applied (produced at the SCIBLU Genomics Centre at Lund University, Sweden). These arrays contained 33,370 and 38,000 BAC clones, respectively (CHORI BACPAC resources) giving an improved resolution of one clone per 50–100 kb.

After slide scanning, data were processed with GenePix Pro 6.0 package analysis software and BioArray Software Environment. Log2 ratio cut-offs at +0.25 and −0.25 were applied to identify gains and losses and +1 and -1 for amplification and homozygous loss, respectively. Alterations detected using the software were also inspected manually, and particular concern was taken for alterations in telomeric and centromeric regions. To distinguish CNAs fromnormal genomic copy number variants the Database of Genomic Variants (http://dgvbeta.tcag.ca/dgv/app/home?ref=NCBI36/hg18) was used.

3.3 QUANTITATIVE PCR AND TAQMAN ASSAYS

QPCR experiments were performed to quantify mRNA expression levels and DNA copy numbers. Two different platforms were used based on either TaqMan or SYBR Green. SYBR Green binds to the minor groove of DNA and when more double stranded DNA is produced in the PCR reaction, the fluorescent emission will increase.

In TaqMan assays, a fluorescent probe with a reporter and quencher dye binds to the template DNA. Upon primer extension, the two dyes will separate allowing the reporter dye to emit fluorescencein proportion to the number of DNA templates. In mRNA expression assays, equal amounts of RNA from the samples are reverse-transcribed to cDNA and compared. In DNA copy number assays, the result from genomic DNA of test samples is normalized to a reference locus (usually *RNase P*, located in chromosomal region 14q) expected to be present in two copies. In Study I the test samples were then normalized to a normal DNA sample

(pooled leukocyte DNA from 10 healthy individuals) to detect the copy number of the locus under study. (Heping Liu 2006; Livak and Schmittgen 2001)

3.4 PYROSEQUENCING

Pyrosequencing is a quantitative assay for targeted DNA analyses of for example DNA methylation density. The DNA sample studied is first subjected to bisulfite conversion, whereby unmethylated cytosines are converted to uracil and methylated cytosines remain unchanged. In this method, nucleotides are added to an extending strand of DNA, complementary to the bisulfite-converted template. This is followed by release of a pyrophosphate group that reacts with adenosine 5' phosphosulfate (APS) to produce ATP. ATP is consumed by luciferase to convert luciferin to oxyluciferin that produces light. The light is detected by a CCD camera and read as reporter of the nucleotide that has just been sequenced (Figure 10).

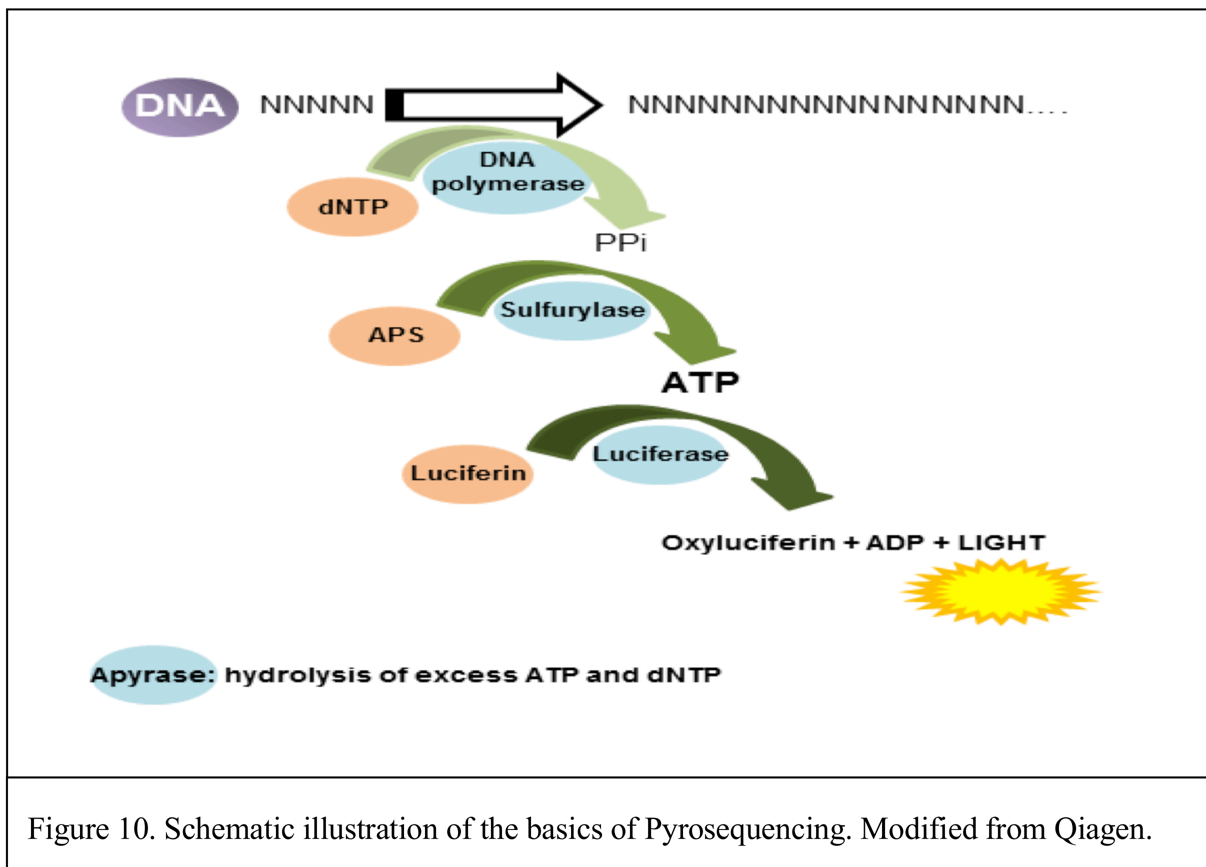

Figure 10. Schematic illustration of the basics of Pyrosequencing. Modified from Qiagen.

For the Pyrosequencing experiments, genomic DNA was bisulfite converted using EpiTect Bisulfite conversion kit (Qiagen). 17 ug of bisulfite converted DNA was amplified using biotinylated primers and applied to the Pyrosequencing workflow. Biotinylated DNA was denatured in NaOH and captured by streptavidin beads. The non-biotinylated strand was washed off in Tris buffer. The sequencing process was performed in the Pyrosequencer, using a sequencing primer that targeted the region of interest.

Pyrosequencing primers were either commercially available as used in Study II and IV or may be designed in-house using a designated software as was done in the related papers. The mean methylation density of the CpGs analysed for a particular gene were used to calculate a methylation index (MetI) for comparisons between samples. (Ronaghi, et al. 1998)

3.5 HIRIEF MASS SPECTROMETRY PROTEOMICS

Proteomics was used to establish protein expression profiles for SI-NET tumors and NET cell lines after modulations with SSA treatment or inhibition of neddylation with MLN4924. The methodology applied is based on isoelectric focusing and liquid chromatography mass spectrometry so called high resolution LC-MS/MS (HiRIEF LC-MS/MS) (Branca, et al. 2014). In Study III, one million cells of HC45, SI-NET and H727, pulmonary NET cell lines without or with lanreotide treatment at 2, 6 and 48 hours were lysed in duplicates in SDS and labeled with 8-plex iTRAQ (Isobaric tags for relative and absolute quantitation) kit (Applied Biosystems). All samples from each cell line were pooled and excess reagent was filtered using an SCX-cartridge (StrataSCX, Phenomenex). iTRAQ- labeled peptides were trypsinized and dissolved in Urea 8M and subjected to narrow range IPG-strips for peptide focusing and peptide separation at pH 3.7 - 4.9 together with dry sample application gels (GE Healthcare Bio-Sciences AB). Samples were then freeze-dried in a SpeedVac and kept at -20 ºC. HiRIEF LC-MS/MS analysis was carried out later on each fraction following re-suspenssion in 3% acetonitrile and 0.1% formic acid.

Each fraction was then injected into a C18 guard-desalting column (Zorbax 300SB-C18, 5 x 0.3 mm, 5 μm bead size, Agilent) in a LC-MS/MS experiment. Tryptic peptide readout was then analyzed using Sequest under the software platform Proteome Discoverer (v1.3.0.339, Thermo Scientific). A threshold of ≥ 1 high-confident unique peptide and a false discovery rate of <1 % was taken for quantification and comparison of different condition.

The same procedure was followed for mass spectrometry in Study IV for tumors and the HC45 and CNDT2 SI-NET cell lines, except for the 10-plex iTRAQ kit (Applied Biosystems) and 3, 6 and 12 and 24 hour-time points of treatment with 400 nM MLN4924.

A threshold of confidence interval was calculated as mean ± 2× (standard deviation) of the non-treated samples to eliminate exceeding noisy values in non-treated controls. The same values were then applied to each treatment condition to define up- or down-regulated proteins.

3.6 WESTERN BLOTTING

Proteins were extracted using NP40, separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred to a nitrocellulose membrane that was blocked with milk or bovine serum albumin. After overnight incubation with primary

antibodies (APC, BMPER (BMPER Bone Morphogenetic Protein binding endothelial regulator protein precursor), SMIM21 (Small Integral Membrane Protein 21), SPAG16 (Sperm Associated Antigen 16), C14orf14, FYN (Tyrosine-protein kinase Fyn), survivin, Chromogranin A and INSM1 (insulinoma-associated protein 1)), the blots were incubated with mouse or rabbit secondary antibodies conjugated with a fluorophore or Horseradish peroxidase (HRP). When HRP antibodies were used, membranes were developed with HRP substrate. Either fluorescent or emitted light was detected by a LI-COR ODYSSEY system. Similar procedure was carried out for an endogenous protein (GAPDH (Glyceraldehyde 3-phosphate dehydrogenase GAPDH) or Vinculin) to which target intensity could be normalized. Expected mass sizes of proteins of interest are according to information at www.uniprot.org.

3.7 IMMUNOCYTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

For immunohistochemistry, tissue sections were deparaffinized in xylene and rehydrated in a series of decreasing concentration of ethanol in water. Antigen retrieval was then carried out in citrate buffer (pH 6). Endogenous peroxidase reactivity was blocked by Hydrogen peroxidas. The sections were then incubated for 60 min with a rabbit polyclonal survivin antibody diluted at 1:400 and a rabbit monoclonal APC antibody diluted at 1:100. Detection was performed with EnvisionPlus Detection System (Dako) using an Olympus BX43 microscope with DP72 Olympus camera and LabSense software.

Nuclear and cytoplasmic staining intensity was conducted and defined as absent (0), weak (1), moderate (2) or high (3). The overall staining intensity was calculated for survivin by summing up both nuclear and cytoplasmic intensities (0-6). For APC only cytoplasmic staining was detected and classified as absent, moderate or high expression.

For immunocytochemistry, 0.1 million cells were spun with a cytospin at 1000 rpm for 5 minutes on a slide. The slides were air-dried for 30 minutes at room temperature and fixed in paraformaldehyde 4% for 8 minutes. After washing in TBS (Tris-buffered saline), the cells were permabilized in 0.5% Triton for 10 minutes. Endonuclease blocking was performed in 1% H2O2, followed by washing and primary antibody incubation overnight at 4 ˚C. The next day, cells were washed and incubated with secondary antibody for 30 minutes and DAB (3,3′-Diaminobenzidine) for few seconds until the cell color changed (both provided in EnvisionPlus Detection System -Dako kit). The slides were then washed in running water for 5 minutes, incubated in Hematoxyline for 5 minutes, washed in running water for 10 minutes and dehydrated in an increasing series of ethanol solution in water. After 2 changes in xylene the slides were mounted with coverslips using Pretex glue.

3.8 PROLIFERATION ASSAYS

Cell proliferation assays were performed using different methods:

BrdU (Bromodeoxyuridine) proliferation assay:

In the 96-well plates, 4000 cells/well for SI-NET and HC45 or 8000 cells/well for BON-1 or H727 were incubated and after corresponding treatment, BrdU labeling agent was added for a continued incubation period of 4-24 hours at 37 ˚C in a humidified incubator. BrdU incorporates to the newly synthesized DNA in place of thymidine. The amount of incorporated BrdU is proportional to the ratio of DNA synthesis in the S-phase.

Cells were fixed and DNA was denatured by fixing buffer (Roche cell proliferation kit). The cells were incubated with BrdU antibody conjugated with peroxidase at room temperature for 90 minutes. The color of the solution turns blue in proportion to the amount of incorporated BrdU, following the addition of the substrate, tetramethyl-benzidine.

XCELLigence real time:

The xCELLigence real time proliferation assay uses gold covered electrodes located at the bottom of E-plates. Upon adhesion of the cells to the plates the impedance of the electrical circuit is affected and a higher density of the cells is read as a higher cell index. Cell indices were measured automatically in 1 or 4 hours intervals under different types of treatment. The first acquired cell index value was assigned the arbitrary value of 1 to which further receiving data were normalized.

Immunocytochemistry using the MIB1 antibody:

In a long-term design of proliferation experiments cell proliferation ratio was assessed by immunocytochemistry using the MIB1 antibody. This measures the expression of Ki-67 as a marker of proliferation.

3.9 APOPTOSIS ANALYSIS USING ANNEXIN V MARKER ON A FLOW CYTOMETER

Flow cytometry (FACS) is a quantitative method to visualize the characteristics of the cells based on the fluorophores that they carry. Fluorescent antibodies are dyes frequently used to specifically stain cells for FACS. Annexin V binds to cell membrane phospholipids with higher affinity for phosphatidylserine. Phosphatidylserine is located at the inner surface of a normal cell membrane. Once the cell is going through apoptosis phosphatidylserine goes to the outer surface of the cell membrane and is recognized by Annexin V. Applying propidium iodide in this experiment serves to exclude false positive necrotic cells.

4 RESULTS AND DISCUSSION

4.1 GENOMIC STUDIES IN SI-NETS

4.1.1 DNA profiling of SI-NETs

We profiled the genome of 30 tumors, including 19 primary tumors and 11 metastases from 29 SI-NETs. We analyzed the samples with array-CGH and found CNAs in all samples. Generally more gains were detected than losses. Recurrent gains were found on chromosomes 20, 14, 4, 5, and 7 and recurrent losses on chromosomes 18, 16, 11, 9 and 13. These data confirms a previous study by metaphase CGH on a subset of this cohort (Kytöla et al. 2001), besides it reveals unknown details of smaller aberrations, owing to its higher resolution. The most frequent CNA was loss of chromosome 18 (70%) followed by gain of chromosome 20 (37%) and gain of chromosome 4 (27%). In Study I, for the first time we reported recurrent gain on chromosome 20 (20pter-p11.21) in SI-NET that is associated with worse patient outcome. Furthermore a minimal overlapping region (MOR) in this chromosome (q13.33) was detected in 9/30 cases (30%).

Smaller common regions of alterations can provide information about the location of candidate genes that may play a driver role in tumor development. Therefore, we focused on MORs as the smallest regions of recurrent loss or gain. A MOR in 18p was detected (18p11.32-p11.31) that comprises only 3 genes, *KIAA0650* (or *SMCHD1*: structural maintenance of chromosomes flexible hinge domain containing 1), *LPIN2* (Phosphatidate phosphatase LPIN2) and *EMILIN2* (Elastin Microfibril Interfacer 2). An additional MOR of only 2 Mb size was implicated at 18q22.1, which encompasses only 2 protein coding genes, *CDH7* and *CDH19*. The high incidence of losses of chromosome 18 has been reported by every SI-NET DNA profiling study, an interesting observation of a very high frequency of recurrent losses especially on 18q (Andersson et al. 2009; Löllgen et al. 2001; Walenkamp, et al. 2014). Nevertheless, no widely appreciated candidate has been found on this chromosome yet, despite implementing next generation sequencing in more recent studies (Francis et al. 2013; Verdugo et al. 2014).

An explanation for this discrepancy could be that chromosome 18 is the most gene poor chromosome. Indeed chromosome 18 along with chromosome 13 and 21 are the only chromosomes that could be found in surviving trisomic patients (Nusbaum, et al. 2005). Therefore, its loss may not only be tolerated by cancer cells, but might also allow a neoplasia to obtain more chromosomal aberrations, leading to tumor invasion and metastasis.

Another explanation for many unsuccessful studies to find the driver gene on chromosome 18 could be the old-fashion strategies applied focusing only on protein coding sequences. Research shows that chromosome 18, despite having the smallest number of genes; harbors a genome-wide average proportion of evolutionary conserved mammalian non-protein-coding sequences (Nusbaum et al. 2005). An alternative approach can be to search for small or large

non-coding genes and/or a structural role for this chromosome in the nuclear architecture instead of a solemn genetic one. It has been shown that chromosome 18 possesses a unique spatial configuration in peripheral regions of the nucleus. This finding suggests an exclusive structural role for chromosome 18, in addition to its conventional genetic function, as opposed to for example the gene rich and similarly sized chromosome 19 with a nuclear centric position (Cremer and Cremer 2001; Croft, et al. 1999). Proofs for this hypothesis came from observations that deletions as long as 1 Mb of non-coding DNA in mice does not compromise its survival (Nobrega, et al. 2004).

4.1.2 Clustering Analysis

Using hierarchical clustering, we found 2 groups of tumors that were enriched for different recurrent CNAs. A group of tumors named group II was linked to extra hepatic metastasis. This group was enriched with cluster d of CNAs comprising gains of chromosome 4, chromosome 5, 7p22.3, 7p22.2-22.1, 7q22.1, 7q22.3-qter, 14q11.2 and 14q32.2-32.31 (paper I, Fig. 3A). Group II included more metastasis (57%) compared to group I (32%) and was enriched for gain on 20pter-p11.21 which was associated with a worse patient survival. These results suggest a connection between these chromosomal alterations and tumor progression and metastasis.

With regard to CNAs potentially linked to metastatic disease, loss on 16q12.2-qter and gain on 7q22.3-qter were more frequent in metastasis vs. primary tumors.

4.1.3 qPCR verification of CNAs

Using copy number TaqMan assays, we confirmed the CNs and CNAs detected in 61% and 78% of SI-NETs, respectively. The assays targeted gene loci on chromosome 18 including *EMILIN2* on 18p; *DCC* (deleted in colorectal cancer), *BCL2* (B-cell lymphoma 2) and *CDH19* on18q; *CDH1* on chromosome 16, and *SDHD* on chromosome 11, all located on the recurrent regions of CNA.

We also conducted the same qPCR experiments on an extended cohort of 13 paired SI-NET samples of primary/metastasis or two metastases and a high ratio of losses was observed for all assays (paper I, table 3).

4.2 EPIGENETIC STUDIES IN SI-NETS

4.2.1 Promoter hypermethylation and mRNA expression of cancer-related genes

In Study II, promoter methylation was analyzed in a panel of 44 SI-NETs, including 43 samples from Study I, using Pyrosequencing assays. The candidate genes were chosen based

on their location on chromosomal regions with frequent copy number loss (Paper I) in agreement with Knudson's two hit hypothesis, or they were reported as hypermethylated in SI-NET with traditional non-quantitative methods (Arnold et al. 2007; Chan et al. 2003; Liu et al. 2005), or they were reported as down-regulated in SI-NETs (Leja et al. 2009).

Promoter methylation was investigated for *WIF1, RASSF1A, CTNNB1, CXCL14, NKX2–3, p16, LAMA1, CDH1, CDH3, p14, SMAD2, SMAD4, HIC1,* and *APC* out of which increased MetI (10% at least) in tumor samples compared to normal ileum was detected in the first eight genes (Paper II, Fig.1A). This study showed for the first time the implication of *WIF1* hypermethylation in SI-NETs. We also confirmed hypermethylation for *RASSF1A* and *CTNNB1* that had been reported before in SI-NETs (Zhang et al. 2006). For *RASSF1A*, hypermethylation was not only detected in tumors vs. normal ileum, but also in distant metastasis vs. either primary tumors or regional metastasis. These findings suggest a role for *RASSF1A* in initiation and progression of SI-NETs. In distant metastasis at the same time, *RASSF1A* was found down-regulated compared with regional metastasis. These results confirmed a previous study of these genes based on MSP (Zhang et al. 2006). Study II also showed an association between *RASSF1A* expression and better patient outcome in SI-NETs.

P16 promoter methylation has been implicated in cancer (Liu et al. 2005), however with the exception of 3 cases, we did not find a hypermethylation status in the 44 SI-NETs analysed. Nevertheless, the expression of the *p16* gene was associated with better patient outcome. We also found over-expression of this tumor suppressor gene in distant metastasis vs. the rest of tumors; an unexpected phenomenon that could be attributed to "oncogene-induced senescence" in some tumors and could explain their low proliferative status (Romagosa et al. 2011; Serrano, et al. 1997).

Down-regulation of *CXCL14* and *NKX2-3* (Leja et al. 2009) in SI-NET was confirmed in Study II and was also shown to be affected at least partially by promoter hypermethylation. Promoter hypermethylation of *CXCL14* has been reported before in prostate cancer (Song et al. 2010) and of *NKX2-3* in lung cancer and melanoma (Tellez et al. 2009; Tessema et al. 2010).

The most outstanding result was observed for *WIF1*. WIF1 is a Wnt inhibitory factor and inhibits accumulation of CTNNB1 and its oncogenic role of transcriptional activation (Wu, et al. 1999). A high MetI was found in many SI-NETs and the expression of the gene was dramatically lower in metastasis than in primary tumors or normal ileum. However, *WIF1* promoter hypermethylation has not been detected in the BON-1 pancreatic NET cell line (Kim et al. 2013). Such a marked difference between *in vivo* and *in vitro* experiments is observed and expected in DNA methylation studies (Smiraglia, et al. 2001). *WIF1* promoter hypermethylation and down-regulation, however, is also observed in squamous cell carcinoma of the cervix (Delmas et al. 2011), breast cancer (Ai et al. 2006), bladder cancer (Urakami et al. 2006), colorectal cancer (Roperch et al. 2013), nasopharyngeal and esophageal carcinoma (Chan et al. 2007), and non-small-cell lung cancer (Mazieres, et al. 2004).

4.2.2 Global hypomethylation in SI-NETs

Using the LINE1 Pyrosequencing methylation assay, we detected hypomethylation in SI-NETs vs. normal ileum. In distant metastasis, MetI was lower than in primary tumors and regional metastasis (Paper II, Fig. 1C). We also used an ELISA (Enzyme-linked immunosorbent assay) based global methylation assay and confirmed the hypomethylated status observed in many tumors vs. normal samples. LINE1 MetI was inversely correlated with the highest methylated genes *WIF1* and *RASSF1A*, however, inversely correlated with *CDH1* and *LAMA1*. The methylation levels obtained by ELISA were inversely correlated with *WIF1* MetI and were positively correlated with *LAMA1* MetI.

4.2.3 Clustering and association with DNA copy numbers

Three clusters of samples were identified in an unsupervised hierarchical clustering for MetI of the eight methylated genes. Cluster I was associated with hypermethylation of *WIF1* and ELISA-based global hypomethylation; cluster II with hypermethylation of *RASSF1A* and *CTNNB1* and cluster III with *NKX2-3*. These clusters were interestingly characterized by different CNAs detected in paper I for example Cluster II included more samples with chromosome 16q loss than the other two clusters (Paper II, Fig. 2).

4.2.4 Demethylation analyses

DNA methylation density above the arbitrary level of 20% was detected for *CDH1* and *WIF1* in HC45 cells and for *WIF1, CTNNB1, CXCL14, NKX2–3, p16, LAMA1, CDH1* in CNDT2 cells. As mentioned above another study did not detect *WIF1* promoter methylation in BON-1 cells. This discrepancy could be due to local effects on the DNA methylation and/or the non-quantitative method used (Kim et al. 2013). The cells were treated with the demethylating agent 5-Azacytidine for 4 days. All methylated genes showed different levels of demethylation and in return mRNA expression was significantly increased for *WIF1, RASSF1A, CTNNB1, CXCL14, p16, LAMA1* and *CDH1* in HC45 and for *WIF1, p16, CDH1, LAMA1,* and *CTNNB1* in CNDT2 (Paper II, Fig. 5).

4.3 PROTEOMICS OF SOMATOSTATIN TREATMENT IN NETS

4.3.1 HiRIEF Mass Spectrometry analysis

Somatostatin analogs (SSAs) have long been approved as the first line therapy against hormonal symptoms caused by SI-NETs and in the past few years also against their tumor progression (Caplin, et al. 2014; Rinke, et al. 2009). However, their molecular mechanisms are not well understood. The expression of SSTRs was examined in NET cell lines, BON-1,

CNDT2, HC45 and H727, and a primary cell culture. Except CNDT2, other models express SSTR2 and SSTR5. Conducting a Mass Spectrometry-based proteomics analysis on HC45 and H727 cell lines treated with lanreotide at pharmacological concentration of 10 nM, 6,451 and 7,801 proteins were quantified, respectively of which 5,264 were common between the two cell lines. When compared with non-treated cells, 747 proteins in HC45 and 656 proteins in H727 were statistically different in their ratio of expression at one of the three time points, 2 hours, 6 hours or 48 hours.

4.3.2 Network and pathway analyses

DAPPLE (Disease Association Protein-Protein Link Evaluator) was used for prediction of physical interaction networks of the altered proteins in each cell line (http://www.broadinstitute.org/mpg/dapple/dapple.php). APC and survivin were present in the core networks after 2 hours and 48 hours. The same biological significance for these two proteins was observed when analyzed with Ingenuity Pathway Analysis (IPA). IPA also revealed the involvement of the PI3K/Akt and p38 MAPK (mitogen-activated protein kinase) signaling pathways in HC45. Cell cycle, cell growth, proliferation and interactions were among the top five altered pathways regardless of which cell line or time point was analyzed by IPA.

4.3.3 Western blot verification

A panel of the most clinically and biologically relevant proteins or of the highest fold change alteration was verified using Western blot. These included survivin, APC, SMIM21, BMPER, FYN and C14orf42 for HC45 and APC, SPAG16 and INSM1 for H727, which were all detected using specific antibodies and the general direction of alterations were shown for them.

4.3.4 Cell proliferation analysis for lanreotide and survivin inhibitor YM155

Cell proliferation of BON-1, HC45, H727 and a primary cell culture was examined, after treatment with lanreotide or when we combined this agent with YM155, a small molecule inhibitor of survivin.

End point cell proliferation assays BrdU ELISA and Ki-67 immunocytochemistry and real time assay xCELLignence, all failed to detect a significant cell proliferation inhibition after treatment with lanreotide. In BON-1 only a small decrease in proliferation rate could be observed with as much as 10 uM or more lanreotide. These findings are in accordance with previous SSA proliferation studies on NETs (Moreno, et al. 2008; Ono, et al. 2007).

On the other hand, the survivin inhibitor YM155 was able to dramatically inhibit cell proliferation, from 5 nM for HC45 and the SI-NET primary cell culture and from 100 nM for BON-1 and H727. This finding seems promising, considering the favorable safety profile of the drug in many clinical trials for different cancers (Clemens, et al. 2015; Kelly, et al. 2013) and the fact that survivin is expressed in SI-NETs (Vikman, et al. 2005).

4.3.5 Survivin as a prognostic marker in SI-NETs

In the immunohistochemistry panel of Study III, over-expression of survivin was confirmed in tumor cells. Also a worse progression free survival (PFS) was found for over-expressing cases in TMA experiments with 112 NET samples. This association was independent of disease stage, morphological Grade, Ki-67 proliferation index and tumor localization. Moreover, in patients with low proliferation index <=2% or morphological Grade 1 NETs, the link still holds true, a situation that can be implicated in clinical prognosis, where Ki-67 is not of a strong prognostic significance. This finding has potentially high clinical significance, since majorities of SI-NETs are in low proliferation index level (G1) and clinical routine lacks an additional marker to Ki-67 to predict the outcome of the patients.

4.3.6 SSTR2-APC-survivin axis

A hypothesis was examined in Study III, i.e. whether the concomitant over-expression of APC and down-regulation of survivin upon short time treatment of the cell lines with lanreotide could reflect inhibition via the APC-survivin axis. It is already known that APC can inhibit survivin expression in colorectal cancer cell lines (Zhang et al. 2001). We inhibited the APC expression in H727 cells with 2 different shRNAs and observed augmentation of survivin levels. On the other hand, when we over-expressed APC in this cell line, we detected a lower expression of survivin.

To investigate whether this regulatory effect is controlled specifically by lanreotide stimulation of SSTR, an siRNA against SSTR2 was used to knock down the somatostatin receptor SSTR2. In knock down cells, APC expression was decreased and survivin expression was no longer regulated with lanreotide treatment.

4.4 IMPLICATION OF NEDDYLATION IN SI-NET

4.4.1 NEDD8 is over-expressed in liver metastasis

SI-NET patients commonly exhibit metastases and the liver is a common site for distant metastases associated with the carcinoid syndrome. In an attempt to investigate the molecular mechanisms behind SI-NET metastasis, we compared 7 primary SI-NETs in patients without

detectable liver metastases at diagnosis with 7 other primary tumors from patients that had already developed metastasis at the time of diagnosis.

HiRIEF LC MS/MS was performed and 6,775 proteins in total were quantified. Comparing the protein expression of the two groups, only a handful of proteins showed a significantly different expression (2-fold or higher). NEDD8, one of these proteins demonstrated a distinct pattern of expression, both in unsupervised clustering and in a supervised clustering when the 2 groups of tumors were compared. NEDD8 over-expression has also been observed in other cancers. Neddylation of cullins are necessary for their critical function on protein turnover in CRL1 and CRL4 that regulate p27 proteolysis (Chairatvit and Ngamkitidechakul 2007; Salon, et al. 2007).

4.4.2 NEDD8 inhibition suppressed proliferation and induced apoptosis

To investigate the significance of NEDD8 expression in the context of NET and its pharmaceutical potential, the anti-proliferative effects of MLN4924 the first-in-class NAE inhibitor was assayed in 4 NET cell lines BON-1, CNDT2, HC45 and H727 in Study IV. BrdU incorporation as an indicator of DNA synthesis and cell proliferation was suppressed at 3 days of treatment in a dose dependent manner in all cell lines.

Neddylation of cullins was reduced in a time and dose dependent way and p27 and cleaved PARP expression was elevated, the latter used as an indicator of apoptosis. P27 induction has been observed with MLN4924 treatment in other cancer cell lines and is expected, due to inhibition of cullin neddylation and proteolysis function. (Soucy et al. 2009)

4.4.3 The proteomics landscape following neddylation inhbition

The SI-NET cells, CNDT2 and HC45 that showed robust dose-dependent proliferation inhibition were treated with MLN4924 for subsequent proteomics analysis. An increased expression was observed for a group of CRL substrates in a time-dependent way. P27 is among the targets and was chosen for further analysis.

4.4.4 UPS-p27 regulatory axis is a target in SI-NET management

The expression of p27 in a group of SI-NETs was investigated and an association between p27 and patient survival was observed. The same results have been reported for many other cancers. (Chu et al. 2008)

To exclude the role of genetic and epigenetic alterations of *CDKN1B*/p27 on its mRNA altered expression pattern, copy number loss or promoter methylation of the gene was examined. Copy number loss was observed in a subset of samples as it has been reported before (Francis et al. 2013) and promoter methylation was not detected except in one sample.

None of those genetic aberrations or epigenetic characteristics of the SI-NETs was correlated with expression status of *p27* or associated with patients' outcome. In conclusion upstream regulatory mechanisms including neddylation of CRLs can be implicated in *p27* down-regulation and SI-NET tumorigenesis.

5 CONCLUDING REMARKS

Genomic copy number alterations and aberrant DNA methylation of tumor-suppressor gene promoters and genome-wide repeats are implicated in initiation and progression of SI-NETs. Somatostatin analogs may exert their direct anti-tumor effects on NETs through the APC-survivin axis and the ubiquitin-proteasome system (UPS) plays a role in tumorigenesis of SI-NETs and neddylation is a candidate for targeting in the management of this disease.

Study I:

Copy number alterations were revealed in SI-NETs most frequently involving loss on chromosomes 18, 16, 11 and 9 and gains on chromosomes 20, 14, 5 and 4, of which gains of chromosome 20 was associated with a shorter SI-NET survival.

Study II:

Hypermethylation was detected in the promoter region of *WIF1, RASSF1A, CTNNB1, CXCL14, NKX2–3, p16, LAMA1*, and *CDH1* but not of *APC, CDH3, HIC1, P14, SMAD2*, and *SMAD4.* LINE-1 is hypomethylated in tumors compared to normal samples and in metastases compared with primary tumors. Treatment with 5-aza-CR reduced promoter methylation and restored the expression of methylated genes in SI-NET cell lines CNDT2 and HC45.

Study III:

The proteomics signature of NET cell lines HC45 and H727 was changed after treatment with the somatostatin analog lanreotide. Expression of APC was induced upon interaction between lanreotide and somatostatin receptor 2. APC suppressed the expression of survivin. Survivin over-expression was associated with a worse survival of NET patients and targeting survivin with the small molecule inhibitor YM155 had anti-proliferative effects on NET cell lines and primary cell culture.

Study IV:

Comparing the proteomic signature of primary SI-NETs in patients who had developed liver metastasis at the time of diagnosis to the ones without liver metastasis indicated that NEDD8 over-expression may be implicated in the development of the disease. MLN4924 a first-in-class small molecule inhibitor of the neddylation-activating enzyme inhibited proliferation of NET cells and stabilized the UPS targets including p27. Increased expression of p27 was concomitant with induction of apoptosis.

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