



## Review

# PI3K-AKT-mTOR-Signaling and beyond: the Complex Network in Gastroenteropancreatic Neuroendocrine Neoplasms

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## Abstract

Gastroenteropancreatic neuroendocrine neoplasms are heterogeneous in their clinical behavior and require therapies specially tailored according to staging, grading, origin and expression of peptide receptors. Despite extensive scientific efforts, the therapy options are still not satisfactory. The main reasons are due to the lack of a broad mechanistic knowledge, an insufficient classification of specific diagnostic sub-groups, and predictive markers. GEP-NEN tumors evade early diagnosis because of slow asymptomatic growth behavior and are frequently not detected until metastasized. How signaling networks contribute to tumor progression and how these networks interact remains unclear in large parts. In this review we summarize the knowledge on the growth factor responsive non-angiogenetic pathways in sporadic GEP-NENs, highlight promising mechanistic research approaches, and describe important therapy targets.

Key words: Gastroenteropancreatic neuroendocrine neoplasms, signal transduction, growth factors, kinases, biotherapy, molecular biology, inhibitor.

## Introduction

GEP-NENs (Gastroenteropancreatic neuroendocrine neoplasms) emerge from various neuroendocrine cells of the gastroenteropancreatic system and represent the largest subgroup of neuroendocrine neoplasms. This heterogenic entity of solid tumors, formerly termed GEP-NETs or GEP-NECs (gastroenteropancreatic neuroendocrine tumors and carcinomas) or “carcinoids”, displays a broad spectrum of characteristics concerning behavior during growth and differentiation, functional aspects, localization and prognosis.

Although they are ranked among rare neoplastic diseases in general, their incidence has increased exponentially throughout the last decade. Currently, GEP-NENs state the second most common gastroin-

testinal malignancy after colorectal cancer [1].

The majority of GEP-NENs is characterized by slow proliferating, well differentiated G1 phenotypes (WHO/ENETS classification 2010, refer to table 1), which are often diagnosed late in the developmental course by the occurrence of metastases (NEN G1, previously termed WNET: well differentiated neuroendocrine tumor). In contrast, a small G3 subgroup of rapidly growing and poorly differentiated GEP-NENs display a behavior that is comparable to those of prevalent solid carcinoma entities (NEN G3 or NEC, previously called PDNEC: poorly differentiated neuroendocrine carcinoma). The third group, characterized by an intermediate malignancy or unclear behavior, NEN G2, approximates the former

description of a well differentiated neuroendocrine carcinoma. The tumor grade is dependent on the proliferative behavior marked by mitoses and by the Ki-67 antigen, which is a proliferation-related antigen and is immunohistochemically analyzed during diagnosis by default (refer to table 1). In contrast to previous classifications or the TNM staging, the differentiation of the tumor cells is not involved in grading decisions, which still hampers the evaluation of slowly growing poorly differentiated or highly proliferating well differentiated tumors and does not provide insights regarding invasive behavior. However, the main advantage of the current grading is the possibility to predict proliferation behavior and thus facilitate decisions regarding surgical resection, chemo-, radio- and biotherapy or monitoring.

**Table 1.** WHO 2010 grading for (neuro-)endocrine tumors.

Grade	Mitotic count (10 HPF)	Ki-67 index (%)
G1	<2	≤2
G2	2-20	3-20
G3	>20	>20

Several signaling cascades influence malignant transformation, progression and metastasis in neuroendocrine cancers including RTKs (receptor tyrosine kinases) and GPCRs (G-protein coupled receptors) downstream signaling, which regulate Ras/Raf, MAPK, PI3K-Akt-mTOR and JNK and lead to DNA synthesis and cell proliferation. Pancreatic NENs are highly vascularized and nourished. Accordingly, they exhibit a vast expression of growth factors such as VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor), IGF-1 (insulin-like growth factor 1), bFGF (basic fibroblast growth factor), TGF- $\alpha$  and - $\beta$  (transforming growth factor) and PlGF (placental growth factor). Not surprisingly, aberrant receptor activity, including those of the IGF-1R (IGF-1 receptor) and FGFR3 (FGF receptor 3), and highly activated downstream signaling is frequent in pNENs [2-5].

Analogous to the pancreatic subgroup, gastrointestinal NENs overexpress VEGF, bFGF, TGF  $\alpha$  and - $\beta$ , PDGF, IGF-1 and its corresponding receptors PDGFR (PDGF receptor), IGF-IR, EGFR (epidermal growth factor receptor), VEGFR (VEGF receptor), and c-kit (stem cell factor receptor). They thus exhibit increased growth factor, pro-angiogenic and typically pro-secretory signaling [6-16]. This work will focus on the major growth-factor related signaling networks, namely the PI3 kinase and the MAP kinase cascades and their importance for GEP-NEN therapy and diagnosis today and for future therapy approaches.

## Aberrant receptor activity induces sustained growth factor signaling in GEP-NENs and activates the PI3K and MAPK signaling network.

### IGF receptors

The IGF-1R is one of the crucial RTKs in gastroenteropancreatic neuroendocrine tumor growth factor biology. The intrinsic RTK activity of IGF-1R is activated upon binding of its respective ligand and leading to auto-phosphorylation of intracellular tyrosine residues in the juxtamembrane and C-terminal domains. Those phosphorylated tyrosines serve as docking stations for insulin receptor substrates, such as IRS-1 and Src resulting in PI3K signaling via Grb2/SOS and Ras and MAPK pathways [17].

NEN cells have been shown to secrete high amounts of IGF-1. In gastrinomas, increased levels of both IGF-1 and the corresponding IGF-1R were associated with tumor growth, aggressiveness, and progression [18]. Furthermore, other studies have displayed that the expression of IGF-1R is decreased in functionally inactive neuroendocrine tumors of different offspring in relation to their functional analogues. These findings and further *in vitro* experiments suggest that IGF-1 is not only a major autocrine regulator of neuroendocrine tumor growth but also of neuroendocrine secretion itself. Inhibition of IGF-1R activity, e.g. by direct inhibition or by blocking its regulators, such as HSP90 (heat shock protein 90), resulted in decreased PI3K and ERK1/2 (extracellular signal-regulated kinase) signaling and induction of cell cycle arrest and apoptosis [14, 18-26]. Additionally, an alternatively spliced IGF-1R mRNA transcript could be detected with a higher abundance in neuroendocrine tumors of different offspring, suggesting that post-transcriptional mechanisms may cause regulatory aberrations [19].

In addition to aberrant receptor and ligand abundance, an important regulator of IGF signaling was found to be significantly up-regulated in metastatic NENs in two gene expression studies: IGFBP3 (IGF binding protein 3), which is considered to maintain the serum level of IGF-1 in a tissue specific pro- or antiproliferative manner. IGFBP3 was overexpressed in >80% of lymph node or distant metastases versus <60% in primary pNEN lesions [27-29]. Those data might indicate a stoma or tumor cell-controlled regulation of a distinct IGF-1 homeostasis and allocation even in target tissues with a completely different composition. Adaptive and cooperative behavior of metastasizing NEN cells in the context of circulation and homing should be further explored in the future.

Therefore, IGF-1 and its receptor IGF-R1 are

highly expressed in GEP-NENs with an altered abundance which depends on IGF binding factors and the relative ratio of specific receptor isoforms. IGF-1 has been shown to be a major autocrine regulator of neuroendocrine tumor growth and of neuroendocrine secretion.

### EGF receptors and FGF

The **EGFR** belongs to the HER receptor family that consists of EGFR (HER1 or erbB1), erbB2 (HER2), erbB3 (HER3) and erb4 (HER4). Gastrointestinal and pancreatic NENs express and activate EGFRs. In immunohistochemical analyses of NENs located in different primary locations, 96% of the specimens were positive for EGFR expression and 63% were positive for phosphorylated EGFR [6]. Another study demonstrated a significantly higher expression (> 91%) in metastatic and non-metastatic gastrointestinal NENs in contrast to <25% in primary and metastatic pNEN [30]. A third study retrospectively evaluated the expression of EGFR and one of its ligands, TGF- $\alpha$  (transforming growth factor alpha), in pNENs, demonstrating that 63% of the tumors were positive for TGF-alpha and 65% were positive for the intracellular and/or extracellular domain of EGFR, but failed to prove a correlation with size, functional status, secretory profile, or biologic behavior [31]. These data were confirmed by Nilsson and colleagues, who showed that several human neuroendocrine tumors express both TGF-alpha and EGF receptors *in vivo* and *in vitro*, suggesting an autocrine mechanism [9].

Di Florio and colleagues recently demonstrated that gastrointestinal hormones and neurotransmitters stimulate the growth of the human BON, QGP-1 and the rat Rin-14B-cell lines in an EGFR dependent manner through activation of PKC and Src kinases, matrix metalloproteinase activation and the generation of reactive oxygen species [32].

Although a recent analysis of pancreatic neuroendocrine tumors uncovered frequent single nucleotide polymorphisms (SNPs) in PDGFRA and EGFR, no druggable EGFR, KIT or PDGFRA mutation could be found in these receptors to date. Nevertheless, elevated copy number of the EGFR and HER-2/neu loci could be detected in 38% and 33% of the cases and high expression of PDGFRA in 65%, respectively [21].

The non-secretory protein FGF13 has recently been described as new progression marker in pNENs. Although FGF13 is insufficient to stimulate FGF receptors, it was demonstrated to be an independent predictor of a shorter progression-free survival associated with positive Ki-67 staining. Furthermore FGF overexpression correlates with the occurrence of liver metastases and shortened disease-free survival in patients that underwent complete tumor resection

[33]. FGF13 has recently been identified as microtubule-stabilizing protein in neuronal cells that promotes neuronal migration in the cerebral cortex. These processes might also facilitate dissemination of neuroendocrine tumor cells that share at least some characteristics with neuronal cells, but the underlying mechanisms remain unknown [34].

Taken together, these studies have accounted for high growth factor abundance in GEP-NENs. Although SNPs in several growth factor receptors have been demonstrated, no druggable mutation could be found to date. Therefore growth factor receptors might serve as targets for anti-growth receptor therapy in patients with GEP-NENs, as several IGF-1R and EGFR inhibitors are currently under clinical assessment (refer to Supplementary Material: suppl. 1). Nevertheless further subgroup-specific genetic and post-transcriptional analyses are necessary to clarify the role of the distinct receptor aberrations and improve the therapeutic potential of growth factor receptors as therapeutic targets in GEP-NENs.

### Somatostatin receptors mediate anti-proliferative signals, inhibit PI3K and MAPK signaling and are important therapeutic targets in GEP-NENs

#### Somatostatin signaling

The cyclopeptide family of **SSTs (somatostatins)**, which function as somatotropin-release inhibiting factors, is distributed throughout the central nervous system and peripheral organs and can be found in endocrine, immune and neuronal cells, as well as in certain tumors. Its preserved peptide structure indicates a fundamental regulatory function in vertebrate hormone homeostasis.

The human genome includes five non-allelic genes that encode for five or six distinct transmembrane domain G-protein-coupled SSTRs (somatostatin receptors). The gene encoding SSTR2 produces two splice variants (SSTR2A and SSTR2B) in mouse and presumably in humans as well [35-38], whereas the other genes are intronless and generate one receptor in each case [39-42]. The natural ligands of SSTR1-5 (SST-14, SST-28 and cortistatin) are bound with a high affinity. Nevertheless the majority of (longer acting) synthetic peptide analogues, namely MS201-995 (octreotide), RC-160 (vapeotide), BIM 23014 (lanreotide) and MK 678 (Seglitide), only interact with the subtypes SSTR 2, 3 and 5 to a satisfactory extent. Moreover, Pasireotide (SOM 230) shows higher binding capacity towards SSTR1 [43, 44].

Somatostatin receptor signaling is complex. Its activation mediates cell cycle arrest, apoptosis and is involved in the regulation of hormone secretion in

endocrine target cells. Appropriately, binding of somatostatin and its analogues to SSTRs triggers a number of intracellular signaling events, initiated by specific G-Protein activation. These G-proteins function as transducers and in turn modulate the activity of several key enzymes including adenylyl cyclase, phospho-tyrosine phosphatases (PTPs) and MAPKs (mitogen activated kinases) [45-47]. Inhibitory effects of SST on adenylyl cyclase, cAMP production and several distinct ion channels regulate both endocrine and exocrine secretion [39, 48, 49]. Intracellular PTPs exert anti-proliferative activity by deactivation of intracellular PI3K and MAPK signaling. Phosphatases, such as SHP-1 and SHP-2, mediate SST-induced cell cycle arrest in many cell lines *in vitro*. SHP-2 is also considered to inactivate insulin and epidermal growth factor binding RTKs and to repress C-Raf and MAPK signaling, whereas SHP-1 is involved in dephosphorylation of the p85 PI3K regulatory subunit and IRS-1 and thus, decreased PDK-1 (phosphoinositide-dependent kinase 1) and Akt activity. It furthermore inhibits S phase entry through the overexpression of p27kip1 and increase of hypo-phosphorylated retinoblastoma gene product (Rb) [47, 50-61]. A third PTP is involved in the SST-induced proliferation arrest: DEP-1 (density enhanced phosphatase-1, also termed PTP $\eta$  in rats) is a negative regulator of Src-mediated growth factor receptor activity and participates in cellular differentiation [62-66].

The mechanism of SST signaling has been confirmed in different cellular models indicating that this modular multi-effector pathway, which is induced by several SSTRs, transmitted by similar kinases and PTPs and resulting in the activation of final effector PTPs, is a common mechanism in various cells [67, 68]. Furthermore the downstream signaling of the distinct SSTRs differs in certain subtype specific functions: e.g. whereas both SSTR2 and 5 regulate GH secretion, insulin secretion is predominantly controlled by SSTR5. Glucagon secretion and immune response are SSTR2 dependent (reviewed in [39]).

### Somatostatin receptors in GEP-NEN therapy

Gastroenteropancreatic neuroendocrine neoplasms express all five subtypes of SSTRs, although at a variable extent and in various combinations. The most prevalent receptor is SSTR2A, which has been immunohistochemically detected in 84% of the GEP-NEN specimens in a recent study. SSTR3, 4, 5 and 1 have been found expressed in 84, 44, 32 and 32%, respectively [69]. This data agree with previous studies in insulinomas where the SSTR2, 1 and 3 have been found to be predominantly expressed [70]. In midgut NENs SSTR2 is the most prominent as well, but followed by SSTR1 and SSTR5, and, with a less

frequency, by SSTR3 and SSTR4. [70-73]. Comparable findings have been recently published in a cohort of 67 NENs, with an expression of SSTR1, 2a, 3, 4 and 5 in 42, 63, 6, 32 and 65% of the cases, respectively. Interestingly, the SSTR5 immunoreactivity was correlated with the presence of metastases and angioinvasion [74]. Other studies have previously demonstrated a positive correlation of SSTR2 and SSTR5 expression with a better prognosis and higher differentiation [75-80].

Although corresponding data is rare, some studies have demonstrated a frequent co-expression of SSTRs and the D2R (dopamine D2 receptor) in GEP-NENs [38, 77, 81]. Co-expression of SSTR5 and D2R is assumed to contribute to a hetero-oligomerization and creation of a novel receptor with enhanced functional activity [82]. A Co-expression of SSTR2 and SSTR5 with D2R and its correlation with low tumor grade has been described in mixed NENs [77]. The high frequency of SSTR1, SSTR2, and SSTR5 expression and inverse correlation with COX2, a cytochrome C oxidase and component of the respiratory chain, was recently demonstrated. The study also accounted for a better prognosis concomitant with a generally high SSTR expression [76].

Additionally, one group was also able to demonstrate a high expression of the human SSTR2B which raises further questions, whether this splice variant exists in humans [38].

Data concerning the SSTR receptor distribution pattern in different subgroups was published for SSTR2A, which was present in a high frequency in carcinoids and gastrinomas, but detectable in only half of the analyzed insulinomas, suggesting one reason for the high number of octreotide insensitive insulinomas [83, 84]. Another study demonstrated a higher SSTR expression in non-pancreatic NENs versus pancreatic NENs for all receptor isoforms [78].

This high heterogeneity of data, beyond the fact that SSTR2 exerts to be the most prevalent of the generally highly expressed SSTRs in GEP-NENs, is summarized in table 2 which highlights the need for better defined criteria. For example, a discussion about scoring systems as criteria in the use of mono-versus polyclonal antibodies is still ongoing [69, 85-88].

Furthermore the trafficking of SSTRs has become more and more interesting in somatostatin receptor-positive cancer entities and GEP-NENs. Concerning the internalization of SSTRs and its regulation in NENs, mainly controversially discussed immunohistochemical data is available. The involved agonists, phosphorylation sites and phosphatases in NENs are almost unknown, but basic neuroscience research has

increasingly focused on this fundamental aspect of SSTR regulation. [89-95].

Throughout two decades, efforts have already been made to translate the knowledge of neuroendocrine SSTR expression into effective GEP-NEN therapies. During this process a multitude of somatostatin peptide agonists and antagonists and non-peptide agonists and antagonists have been developed, of which the agonists have found their way into clinical applications (refer to Supplementary Material: suppl. 2).

The clinical use of SST analogues is limited in clinical application due to a poor oral bioavailability, short half-life and immunogenicity. Non-peptide analogues are considered to be more advantageous as synthesis can be directed upon a higher specificity, bioavailability and less immunogenicity. Furthermore, somatostatin analogue-conjugated radioligands are applied in PRRT (peptide receptor radionuclide therapy) and scintigraphy-based diagnosis (Reviewed in [96-106]).

The two octapeptide analogues octreotide and lanreotide, which are also available as long-acting repeatable (LAR) depot formulation, bind to SSTR2

with a high and to SSTR5 with a moderate affinity. They inhibit the release of neuroendocrine hormones and have been shown to prolong disease stability in >50% of patients with progressive GEP-NEN disease. Although no objective tumor responses were observed in several studies, including the randomized, prospective Phase III PROMID trial in patients with metastatic neuroendocrine midgut tumors, patients profit from prolonged time to progression [107-117]. The results of the Phase III CLARINET-study using lanreotide in progressive neuroendocrine tumors of various sites are awaited later on this year. Further SST analogues, binding to the other SSTRs, such as Pasireotide, which binds to SSTR1, 2, 3 and 5, have been clinically assessed so far and chimeric molecules directed towards SSTRs and DRs (dopastatins) are under development and preclinical assessment in GEP-NENs [45, 118-123].

In summary, the SSTRs are highly expressed in GEP-NENs. They have evolved into establishing therapy targets, although to date the individual expression patterns of the patients have not been related to therapeutic outcome.

**Table 2.** SSTR expression in GEP-NENs. Several studies have been conducted to date, but with very heterogeneous results. SSTR2 is presumed to be the most prevalently expressed isoform.

Reference	Tissue	Method	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Papotti 2002 [73]	GEP-NENs	RT-PCR IHC pAb	90,1%	84,8%	78,8%	24,2%	42,4%
Reubi 2003 [70]	Midgut NENs	receptor auto-radiography	50-60%	90-100%	10-20%	< 10%	~ 50%
Srirajaskanthan 2009 [77]	Low grade Interm. gr. High grade	IHC pAb		100% 94,4% 66,7%			100% 94,4% 66,7%
Corleto 2009 [75]	functioning NEN	RT-PCR	73%	100%	64%	9%	64%
Zamora 2010 [78]	GEP-NENs	IHC	46%	86%	26%	24%	62%
Kim 2011 [76]	GEP-NENs	IHC m/pAb	84%	72%			55%
Diakatou 2011 [38]	GEP-NENS	IHC pAb	39%	62% (2A) 49% (2B)	38%	15%	38%
Kaemmerer 2012 [69]	GEP-NENs	IHC mAb	32%	84%	84%	44%	32%
Schmid 2012 [74]	GEP-NENs	IHC mAb	42%	63%	6%	32%	65%

## The PI3K-pathway is frequently deregulated in many human cancers entities including neuroendocrine neoplasms of the gastroenteropancreatic system

### PI3 Kinases

The lipid kinase family of **PI3Ks (Phosphatidylinositol 3-kinases)** consists of three classes (I-III) that promote the phosphorylation of 3-hydroxyl-phosphoinositides. Whereas heterodimeric class I PI3Ks influence cellular proliferation, insulin signaling and inflammation, the monomeric

class II PI3Ks determine the regulation of membrane trafficking. The sole class III member is involved in autophagy. The most important subclass in human cancers is formed by class IA PI3Ks [124, 125].

Extracellular growth factor signaling such as by VEGF, PDGF, IGF-1, FGF and TGF-  $\alpha$  and  $\beta$  is transmitted by RTKs to PI3Ks and results in their activation [2, 3]. Activated class I PI3Ks convert their substrate PI(4,5)P<sub>2</sub> (phosphatidylinositol 4,5-biphosphate) into its triple-phosphorylated form PI(3,4,5)P<sub>3</sub> (phosphatidylinositol (3,4,5)-triphosphate). Subsequently, PI(3,4,5)P<sub>3</sub> recruits proteins that contain a PH-domain (Pleckstrin homology domain)

into proximity and thus functions as a docking site for Akt and PDK-1, allowing the latter to phosphorylate Akt at T308 (Threonin-308) [125, 126]. Phosphorylation on both, T308 by PDK-1 and on S473 (Serin-473) by mTORC2 is required for the full activation of Akt kinase activity [127].

The *PIK3CA* gene, which encodes for p110 $\alpha$  (the catalytic subunit of class I PI3K) and is considered as the only relevant catalytic subunit in the context of cancer associated mutations, was found mutated in only 1.4% and 8% of pNENs, respectively [128, 129]. Data about PI3K-p85 $\alpha$  subunit mutation nor PI3K amplification in NENs have not been published to date.

The regulatory impact of PI3K could be validated by preclinical studies with PI3K inhibitors. LY294002, a quercetin analogue and PI3K inhibitor, decreased cell proliferation in non-gastrointestinal neuroendocrine cell lines when applied as single agent or combined with rapamycin [130, 131]. Studies with LY294002 treatment of rat-derived GEP-NEN cell lines propose an inhibitory effect of LY294002 on the VEGF secretion by neoplastic endocrine cells [132]. The mTORC2-PI3K-mediated activation of the ERK cascade during mTOR inhibition of NENs was demonstrated through stimulation of human neuroendocrine BON (pNEN), GOT-1 (ileal NEN), KRJ-I (ileal NEN), H-STS (hepatic metastasis of ileal NEN) and NCI-H727 (bronchial carcinoid) cell lines with single and dual inhibitors [133-135]. Previous studies on BON cells have demonstrated that LY294002 blocks the constitutive activation of PI3K and ERKs, respectively. PI3K, but not the ERK cascade, regulates expression of cyclin D1 and p27kip1, induced by an autocrine IGF-I loop, in BON cells [136]. Not least, PI3K signaling is negatively involved in NE secretion, as demonstrated by PI3K subunit p110 $\alpha$ -inhibition *in vitro*. Li et al. demonstrated that inhibition of p110 $\alpha$  increases neurotensin granule trafficking by up-regulating  $\alpha$ -tubulin acetylation and regulating Ras-related protein Rab27A in BON and QGP-1 cells [137].

A vast number of agents and inhibitors interfering with PI3 kinases and upstream receptors have been developed so far and are currently in different stages of clinical testing (refer to Supplementary Material: suppl. 3). The majority have not been assessed in GEP-NENs thus far.

The physiological inhibition and termination of PI3K signaling by degradation of PI(3,4,5)P3 is mediated by two major types of phosphatases. The SH2 domain-containing inositol phosphatases SHIP1 and SHIP2 dephosphorylate position 5 of the inositol ring and produce PI (3,4)P2. The loss of SHIP2 results in a significant increase of insulin sensitivity, indicating

that this phosphatase is a crucial regulator of PI3K signaling downstream of insulin [138].

In summary, PI3 Kinases show up to be rarely mutated in GEP-NENs but contribute to several feedback loops as signal transduction mediators. They are therefore prominent targets for dual and combined targeting therapy approaches but their importance for GEP-NEN disease remains to be further explored in clinical and *in vivo* contexts.

### The tumor suppressor PTEN

Another crucial phosphatase and tumor suppressor protein involved in growth factor signaling is **PTEN (phosphatase and tensin homologue)**. After recruitment from cytosol to plasma membrane, PTEN dephosphorylates position 3 of PI(3,4,5)P3 and produces PI(4,5)P2. Loss of PTEN is frequently observed in a wide variety of human cancers, with the highest incidence found in endometrium, central nervous system, skin, and prostate cancers [139, 140]. Beyond its function as upstream regulator of PI3K signaling, loss of PTEN was linked to occurrence of metastases and is regarded as critical marker for therapy resistance and sensitivity towards mTOR inhibition [139, 141-144].

The activity of PTEN is lost through diverse mechanisms in many different entities of cancer, but the majority of *PTEN* mutations induce truncations of the protein. *PTEN* is often mutated in tumor-prone germ line diseases and in cancer-associated somatic mutations [145-147].

The impact of PTEN towards cellular integrity is not limited to its cytoplasm-located lipid phosphatase activity. PTEN is localized in the nucleus under various conditions, such as cell differentiation and cell cycle arrest under stress and apoptotic stimuli, e.g. by regulating the APC/C (anaphase-promoting complex/cyclosome) [148-152].

Nuclear localization has been linked to maintenance of chromosome stability. The absence of nuclear PTEN is thus linked to tumor aggression, which suggests that the nuclear localization of PTEN is tumor-suppressive [148, 153-155]. PTEN also influences cytoskeletal remodeling processes and thereby controls cell size, cell invasion and migration [156, 157].

Interesting work with regard to the sequestration of PTEN was published by Putz and colleagues in 2012. They demonstrated that mono-ubiquitinated PTEN can be exosomally trafficked between cells. In target cells, internalized PTEN had functional activity, which led to a reduction in the abundance of pAkt and a decrease in the extent of target cell proliferation [158]. These findings disclose new insight into tumor-stroma interactions and highlight the unlimited influence of tumor-associated cells on tumor cell sig-

naling.

In analogy to many other cancers, PTEN is frequently affected in pancreatic neuroendocrine neoplasms (pNENs). *PTEN* gene mutations are rare events in 7 and 9% of the cases respectively, but reduced PTEN expression is a frequently observed phenomenon in sporadic pNENs. Allelic loss of heterozygosity of *PTEN*, which is located at 10q23.3, was identified in one-third of sporadic pNENs. In 25% of pNENs a somatic deletion of 10q occurs [33, 128, 154, 159-162].

In nonfunctioning endocrine tumors of the pancreas alterations in microRNA expression have been related to endocrine and acinar neoplastic transformation and progression of malignancy. Assuming that MicroRNA-21 regulates the expression of PTEN in hepatocellular cancer, this scenario is as well discussed as a potential inhibitory mechanism of *PTEN* down-regulation in pNENs [163-165].

In primary pNENs, low expression of PTEN correlates with advanced WHO phenotype and higher proliferation index. Moreover, hyper-phosphorylation of mTOR is associated with significantly lower 5-year overall survival [166].

Several studies have demonstrated that the subcellular localization of PTEN is altered in both sporadic and VHL-associated pNENs, although there are contradictory conclusions whether cytoplasmic or nuclear localization is sufficient to predict therapy outcome or whether an increased or decreased nuclear to cytoplasmic ratio is associated with worse prognosis [33, 154, 167, 168].

*In vitro* data concerning the response of neuroendocrine pancreatic (BON) and insulinoma (CM) cell lines towards triciribine inhibition suggest that PTEN might also influence the sensitivity of pNENs cells to Akt inhibition [169-171].

In general, in GEP-NENs the expression of PTEN was found to correlate with response to streptozotocin-based cytostatic therapy and to systemic therapy with streptozotocin and doxorubicin [168]. As observed in the pNENs subclass study, PTEN expression in overall GEP-NENs was prevalently effected in tumors with lower differentiation in contrast to those with well differentiated phenotype [172].

PTEN might therefore serve as therapy response and malignancy marker, especially in combination with TSC2 expression analyses (see below). The mechanisms that lead to the down regulation of PTEN remain almost unexplored.

### The role of Akt

The **Akt** family of serine/threonine kinases (Akt1/2/3, also known as protein kinase B, PKB $\alpha$ / $\beta$ / $\gamma$ ) is the key mediator of PI3K signaling and

connector to several interrelated pathways. It is involved in the majority of cellular processes, such as protein synthesis and cell growth, survival, proliferation and metabolism. Presuming that these processes constitute the backbone of cancer development and progression [173], Akt isoforms represent a highly prominent target for GEP-NET therapy research and for drug development in general.

The most prevalent member of the Akt family of protein kinases is Akt1, which is implicated in cell growth and survival. Akt2 is predominantly expressed in muscle and adipocytes and is involved in the insulin-mediated regulation of glucose homeostasis. The distribution of Akt3 is almost limited to testes and brain [174-178].

Full Akt activation depends on the concomitant phosphorylation of two distinct sites that can be activated independently: the PDK-1-catalyzed T308 phosphorylation inside of the activation loop serves as readout of PI3K activation. In contrast, the phosphorylation of S473 in the hydrophobic motif of the C-terminal tail indicates a mTORC2 to Akt feedback signaling activity or is induced by PIKK (PI3 kinase-related kinase) superfamily or DNA-PK. Activity of Akt is detected by a fivefold increase upon S473 phosphorylation [179-184]. Furthermore the role of the Akt phosphorylation site is not limited to activation enhancement, but also required for target specification. One of the most prevalent downstream targets of Akt T308 is TSC2 (tuberous sclerosis complex 2). Akt mediated phosphorylation of the TSC2 subunit hinders TSC1/2 complex formation and activates the GAP function of TSC2 toward the small GTPase Rheb. Hereupon Rheb activates mTORC1 at S2448, leading to the phosphorylation of the downstream effectors 4EBP1 (Eukaryotic translation initiation factor 4E-binding protein 1) and p70S6K (70 kDa ribosomal protein S6 kinase 1, also termed S6K) [185]. Phospho-(T308)-Akt mediated PI3K signaling also determines cellular metabolism, cell cycle progression and insulin signaling by affecting the subcellular localization of GSK3 (glycogen synthase kinase 3) [125, 186-189]. Interestingly, *in vitro* and *in vivo* studies on deletion of Rictor, mSIN1 (stress-activated map kinase interacting protein 1) or mLST8 (mammalian lethal with SEC13 protein 8) suggest, that the inhibition of mTORC2 mediated S473 phosphorylation selectively affects Akt substrates FOXO1 and FOXO3a (O-Family Forkheadbox proteins), with little effect on Akt substrates GSK3 and TSC2 [183, 190]. The discrepancy between S473 phosphorylation and resulting Akt activity indicates that S473 phosphorylation might not be the major regulator of Akt activity [191].

Nevertheless, activated Akt promotes survival signaling by inhibition of pro-apoptotic proteins, such

as BIM (Bcl-2 interacting mediator of cell death) and BAD (BCL2-associated agonist of cell death) by phosphorylation, leading to their sequestration and degradation. Akt also inhibits the expression of pro-apoptotic genes and cell cycle inhibitors such as p21cip1 and p27kip1 by targeting their transcription factors and stimulates cyclin D1- and c-Myc-controlled cell cycle progression by inhibiting their negative regulators, e.g. FOXOs and GSK3.

Relatively few studies are available on Akt phosphorylation and expression in GEP-NENs. Weak expression of Akt or presence of the PI3-signaling antagonist PTEN was associated with response to systemic chemotherapy and Akt overexpression has been correlated to shorter median survival rates for patients with well-differentiated and poorly differentiated tumors [168].

Shah and colleagues demonstrated a phospho-S473 activation of Akt in 76% of 98 differently graded NEN-tissue samples, indicating that activation of Akt may contribute to GEP-NEN tumorigenesis. Unfortunately the data failed to correlate Akt S473 phosphorylation to tumor grade by multi-variant statistical analysis [6]. These findings were supported by further immunohistochemical analysis of entero-pancreatic NENs respecting Akt S473 phosphorylation. In a subsequent study S473-activated Akt was found in 61 % of the tissues, but also failed to correlate with tumor grade, tumor size or the presence of metastases [192].

Nevertheless, NEN patients treated with everolimus and octreotide in an open-label phase II trial (NCT00113360) showed a significant longer PFS in the case of T308-phosphorylated Akt in pretreatment and on-treatment tumor biopsies. Even partial responders were more likely to have an increased Akt T308 phosphorylation in comparison to non-responders. Therefore, although baseline Akt activation is associated with a more aggressive clinical course, increase of Akt activation has been considered a predictor of rapamycin response. These data have been supported by several preclinical studies on rapamycin treated cell lines of various offspring *in vitro* and xenografted [193-195]. *In vitro* experiments on neuroendocrine cell lines have furthermore evaluated an isoform specific impact of Akt on NEN cancerogenesis that might be important for therapeutic approaches. Only the directed down-regulation of Akt1 and Akt3 decreased the phosphorylation of classical Akt downstream targets such as GSK3 $\alpha/\beta$ , MDM2 and p70S6K and suppressed cell viability. Inhibition and knockdown of Akt1 and Akt3 resulted in decreased ERK1/2 phosphorylation and Akt3 ablation induced apoptosis in BON cells, indicating that this isoform is particularly relevant to neuroendocrine cell survival. Akt1

and Akt3 seem to be important for NEN cell viability while Akt2 may have antitumor activity as demonstrated by pan and isoform-selective knockdown of Akt. The data suggest a particular role for these Akt isoforms in NEN activity [196].

Several specific inhibitors of pan-Akt, Akt isoforms and PDK-1, an Akt regulator, are commercially available. Only a few of them are already under clinical and preclinical investigation in GEP-NENs (refer to Supplementary Material: suppl. 4).

In conclusion, the Akt proteins serve as crucial feedback mediators within the growth factor response network. Nevertheless, the sole focus on pan-Akt S473 phosphorylation might not suffice to understand the complex interplay between various isoforms, phosphorylation sites and scaffold regulators. Akt activation and subtype distribution has a high potential to predict therapy response, but the importance of the specific patterns *in vitro* and in clinical settings remains to be elucidated.

### mTOR complexes and feedback activation

The most important downstream mediator of PI3K-Akt signaling is the serine/threonine kinase **mTOR mammalian target of rapamycin**. It is encoded by the *FRAP 1* gene and contributes to a multitude of cancer associated cellular processes. It forms two distinct protein complexes: mTORC1 and mTORC2. Whereas mTORC1 is sensitive to rapalogues such as rapamycin, everolimus and temsirolimus, mTORC2 is considered resistant to rapamycin and insensitive to nutrient signals [197].

The mTOR complex 1 is formed by the mTOR protein itself, associated with RAPTOR (Regulatory associated protein of mTOR), and two negative regulators, PRAS40 (proline rich Akt substrate 40 kDa) and DEPTOR (DEP domain containing mTOR interacting protein) [198-201]. RAPTOR functions as a scaffold and positively modulates the mTOR kinase reaction towards the mTORC1 key targets 4EBP1 and p70S6K *in vivo* [198]. The second subunit of the mTORC1 complex, mLST8, is considered to bind to the kinase domain of mTOR and to regulate its kinase activity positively. It is also considered to maintain the interaction between mTOR and either RAPTOR or RICTOR (Rapamycin-insensitive companion of mTOR), which is part of the mTORC2 complex, and thus, to be important for shuttling mTOR between the two complexes and for sustaining the intracellular equilibrium of mTORC1 and mTORC2 [183, 202, 203]. Whereas PRAS40 inhibits the mTORC1 activity via raptor, DEPTOR was identified to interact directly with mTOR in both mTORC1 and mTORC2 complexes. However, DEPTOR overexpression leads to decreased p70S6K phosphorylation and to



mTORC2-mediated signaling back to Akt, monitored by S473 phosphorylation [201, 204].

PI3K-mTORC1 signaling controls the transcription of many genes, some of which are involved in metabolic pathways and regulate nutrient-responsive transcription programs [205-208]. Its major downstream effectors, including p70S6K and 4EBP1 are regulated by phosphorylation [209]. Subsequently, activated p70S6K phosphorylates S6 (40S ribosomal protein S6) and other ribosomal proteins and elongation factors and therefore enhances the translation of mRNAs and promotes proteins synthesis [210]. Although p70S6K can also be activated by mTOR independent pathways such as PDK-1, MAPK and SAPK (stress-activated protein kinase), the mTORC1-mediated phosphorylation at T389 is required for its complete activation [211].

Hypo-phosphorylated 4EBP1 inhibits the initiation of protein translation by binding and inactivating eIF4E (eukaryotic translation initiation factor 4E). Phosphorylation of 4EBP1 by mTORC1 promotes dissociation from eIF4E and thereby facilitates eIF4E-dependent translation initiation [212].

Furthermore, mTORC1 controls the activity of many other proteins, such as ODC (ornithine decarboxylase), glycogen synthase, HIF-1 $\alpha$  (hypoxia-inducible factor 1 $\alpha$ ), eEF2 kinase (eukaryotic elongation factor 2 kinase), PKC $\delta$  and PKC $\epsilon$  (protein kinases C delta and epsilon), PP2A (protein phosphatase 2A), p21cip1 and p27Kip1 cyclin-dependent kinase inhibitors and STAT3 (signal transducer and activator of transcription 3) [213-223]. Accordingly, the impact of the mTORC1 complex spans a multitude of cellular processes that modulate cellular behavior in response to local circumstances and links availability of growth factors, nutrients and energy to cell growth, survival, proliferation, angiogenesis and motility.

The rapamycin-insensitive mTOR Complex 2 consists of mTOR mLST8, mSin1, PROTOR1/PRR5 and 5 PROTOR2/PRR5L (proline-rich protein), HSP70 (heat shock 70 kDa protein) and DEPTOR.

The mSin1 protein contains a Ras binding domain and a PH-domain, which allows localizing the protein near the plasma membrane. It is therefore considered to be important for the assembly and dynamic localization of mTORC2 and to the phosphorylation of Akt at S473. HSP70 assures proper assembly of the protein complex under physiological conditions and following heat shock [190, 224-227]. HSP70, which has various cellular functions beyond maintaining proper protein structures, has been found to exist in a neuroendocrine tumor specific truncated isoform in NENs. The authors conclude, that the altered HSP70 isoform equilibrium might contribute to

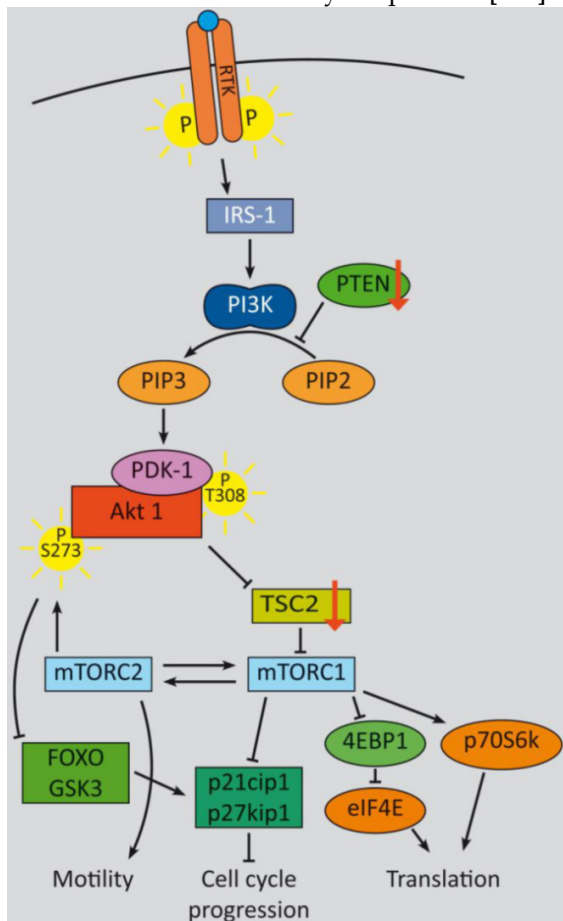
apoptosis inhibition or might be based on similarities with neuronal cells, as protein folding and protection against aggregate formation is of particular importance in the nervous system [228].

The mTOR complex 2 is activated by growth factors, G protein-coupled receptor ligands and cytokines. It phosphorylates PKC- $\alpha$  and paxillin (a focal adhesion-associated adaptor protein) and regulates the activity of the small GTPases Rac and Rho which control motility, invasion and cytoskeletal assembly. Beside motility aspects that play distinct roles in metastasis, the Akt S473 feedback activation, that is mediated by mTORC2 following mTORC1 inhibition, is one of the crucial mechanisms for PI3K cancer signaling [181, 229-231].

In summary, two major feedback loops control PI3K-signaling, which could potentially impact therapy approaches, especially with regard to monotherapy (refer to figure 1): Activation of p70S6K by mTORC1 causes feedback inhibition of IGF-1/insulin signaling by phosphorylating IRS-1 (insulin receptor substrate 1), causing IRS-1 degradation, and leads to decreased PI3K signaling and reduced Akt T308 phosphorylation. Reciprocally, rapalogue-induced inhibition of mTORC1 consequently inhibits p70S6K phosphorylation, but relieves this feedback and induces Akt T308 re-phosphorylation and thus increased mTORC2 activation. Subsequent Akt S473 phosphorylation follows in an mTORC2-dependent manner, which attenuates the therapeutic effects of rapalogues in tumors, in model systems and in patients as well [204, 232, 233]. This feedback activation could be obviated in serum free *in vitro* conditions, due to the known requirement for growth factors for mTORC1 to PI3K feedback loops [204]. For some cancer entities it is evident that even inhibition of both, mTORC1 and mTORC2, e.g. by ATP-competitive mTOR kinase inhibitor AZD8055, did not result in a persistent inhibition of PI3K signaling: although sustained inhibition of mTORC2 activity and AKT S473 phosphorylation was detectable, a distinct activation of RTK signaling occurred, which induced PI3K signaling and reinduction of T308 phosphorylation [234].

Expression and activation level of mTOR in GEP-NENs has been analyzed in a vast number of studies and experiments but mainly failed to correlate to clinical outcome in a clear statistically significant matter. Nevertheless, a distinct tendency of high malignant NEN to show highly activated mTOR expression was obvious. Catena and colleagues demonstrated that mTOR was expressed in the majority (80%) of poorly differentiated neuroendocrine carcinoma patients (WHO 2000 classification) however with no relationship to tumor origin or proliferation

rate determined by MIB-1 (Ki-67 antibody for paraffin-embedded tissue specimens) [235]. Another immunohistochemical analysis detected high levels of S2448 phosphorylated mTOR in 67% of poorly differentiated neuroendocrine carcinomas (including positive staining of all large cell neuroendocrine carcinomas) in contrast to 27% mTOR activation in well-differentiated tumors and carcinomas (WHO 2000 classification). Further statistical validation failed due to the low number of analyzed patients [236].



**Figure 1:** PI3K signaling feedbacks: PI3K signaling is highly activated and de-regulated in GEP-NENs. Several feedback loops contribute to this effect [330, 331]. RTK activation by ligand binding triggers a phosphorylation cascade onto IRS-1 and PI3K. The latter phosphorylates PIP2 and thereby activates PDK-1 and Akt at T308. Akt in turn inhibits TSC2 by phosphorylation, which leads to mTOR activation. The mTOR complex I activates translation via eIF4A and p70S6K and cell cycle progression, whereas mTORC2 relieves a feedback loop by fully-activating Akt at S273. S6K promotes a negative feedback onto IRS-1 and thereby counteracts mTORC1 inhibition. The two major negative regulators, PTEN and TSC2 are frequently down regulated in GEP-NENs which lead to a highly de-regulated Akt activation [33, 128].

Significant correlation of mTOR expression with primary tumor location and metastatic status in GEP-NENs could be demonstrated by Kasajima and colleagues. Expression of mTOR, 4EBP4 and phosphorylated p70S6K was found to be higher in foregut than in midgut tumors. Furthermore, higher prolifer-

ation indices (indicated by Ki-67) were associated with significantly higher mTOR and 4EBP1 expression, as well as with higher activation of the mTOR targets 4EBP1, p70S6K and eIF4E (indicated by phosphorylation) [237].

One of the major regulatory proteins of mTOR activation, TSC2, is frequently down-regulated in pNENs and correlates with worse prognosis concerning overall survival, time to progression and disease-free survival. Low levels of TSC2 significantly correlate with functional tumor status and aggressiveness [33]. Although neither TSC2 nor PTEN were significant independent prognostic indicators regarding occurrence of metastases in a multivariate analysis by Missiaglia and colleagues, conjoint low levels of PTEN and TSC2 are assumed to be related to liver metastasis and might predict response to PI3K-pathway inhibitors. While somatic mutation of TSC2 and PTEN was found in only less than 10% of pNENs, 85% of primary pNENs showed altered protein levels of TSC2, PTEN or both. [33, 128].

Germline mutations in TSC1 and TSC2 tumor suppressor genes, resulting in the activation of the mTORC1 pathway, have been described in patients with tuberous sclerosis complex. TSC is an autosomal dominant genetic disorder and most of the occurring neoplasms are benign with an early onset [238]. A study on 219 TSC patients revealed that the incidence of pNENs was 1.8% as compared to the rate of 0,002% in the general population. Therefore it seems likely that pNENs are a dominant pancreatic pathology in the setting of TSC and aberrant TSC1 and TSC2 proteins may play a crucial role in the development of pancreatic neuroendocrine lesions [238, 239, 114, 240-242].

In summary, several studies have highlighted the importance of high mTOR expression and activation, activation of its targets as well as the functional impact of the low expression of its direct negative regulator, TSC2. Relevant somatic mutations that influence the expression of either TSC2 or mTOR have not been detected thus far. Additional regulatory mechanisms such as transcriptional or post-transcriptional gene silencing might be involved in their regulation and require further investigation.

### Inhibition of mTOR as therapeutic strategy

Analyses of mTOR aberrations have furnished the rationale for use of inhibitors of mTOR and its downstream targets (refer to Supplementary Material: suppl. 5) [33, 165]: The mTORC1 inhibitor and rapalogue everolimus (RAD001, Afinitor®) is the most clinically advanced and the furthest developed target directed therapy in GEP-NENs. It was approved by the FDA for advanced well-differentiated pNENs in

2011.

Studies on genetic determinants of rapalogue response have demonstrated that drug-resistant cells (such as HT-29, HCT116, and DLD-1) carried mutations in both *PIK3CA* and *KRAS/BRAF*. Everolimus-sensitive cells displayed PI3K pathway alterations but no mutations in the *KRAS/BRAF* genes, rendering neuroendocrine tumors a favorable target for everolimus treatment [239]. This effect is assumed to be dependent on mTOR-Ras/Raf crosstalk and its outcome depends on Raf mutational status (paradoxical activation, see below).

Nevertheless in preclinical studies of everolimus, inhibition of mTORC1 in neuroendocrine cell lines was demonstrated to induce growth inhibition and induction of apoptosis [240]. However it also led to a global upregulation of upstream PI3K signaling and to cross-activation of Ras/Raf/Erk signaling via mTORC1-p70S6K-IRS-1 mediated negative feedback loops. This cross-activation resulted in upregulation of VEGF secretion, through a raise of NF- $\kappa$ B (nuclear factor- $\kappa$ B)-mediated VEGF expression and HIF-1 $\alpha$  induction [134, 135, 195]. *In vivo*, Rapamycin monotherapy was notably efficacious in pNEN bearing transgenic mice and prolonged survival concomitant with stable disease. Nevertheless, the tumors developed resistance [241]. Preclinical xenograft studies on mouse cells that mimic PDNEC revealed a significant deduction of tumor mass and Ki-67 in response to everolimus treatment, suggesting further exploration in poorly differentiated neuroendocrine cancers, that particularly lack therapy options to date [242].

Three human phase II or III studies have been conducted, including 600 patients with advanced pNENs [114, 243, 244]. The response rate as assessed by conventional Response Evaluation Criteria in Solid Tumors (RECIST) criteria has been shown to be very low; however, everolimus significantly affected progression-free survival. The RADIANT-3 clinical study, which led to FDA approval, resulted in a stable disease of 73% and 51% in the everolimus and placebo arms respectively, rather than a partial response with 5% and 2%.

Concluding, everolimus delayed tumor progression without changing the pattern of progression among patients with advanced pNENs [244, 245]. Recent therapeutic approaches have therefore focused on inhibitors of alternative components of the PI3K pathway, dual target inhibitors and effective combinatory bio/chemotherapies.

### Outlook: PI3 signaling in diagnosis and therapy

The multiple results of PI3K signaling analyzes in GEP-NENs are summarized in table 3. Noticeably, with few exceptions, no study detected serious acti-

vating mutations of PI3K-pathway mediators. In contrast to other cancer entities, where activating mutations in receptors or the PI3 kinase itself are frequent, GEP-NENs thus lack appropriate druggable targets. Nevertheless, a high level of deregulation (as indicated by extensive activation of kinases) triggers proliferation, neoangiogenesis and a secretory phenotype. The complex interactions within the autoregulatory network bypass the current therapeutic approaches. It is therefore all the more important to identify bottle neck factors that might not have been recognized as key players to date. Evaluating the role of regulator non-coding RNAs might also answer some of the questions, why the neuroendocrine growth factors pathways are deregulated to that high extent although almost no appreciable mutations could be detected responsible to date. Furthermore, the tumor stroma and tumor-associated cells also contribute to a growth factor-saturated microenvironment in general. Studying their importance for GEP-NEN cancerogenesis and metastasis might also reveal a high potential of prognostic factors and therapeutic interventions.

**Table 3.** Summary of studies that analyzed the role of PI3K signaling in GEP-NENs.

Study results	Reference
Expression of multiple growth factors is very high in GEP-NENs	[2-16].
Expression of IGF-1 and IGF-1R is elevated in GEP-NENs	[14, 18-26]
Elevated copy number of the EGFR and HER-2/neu loci might contribute to high receptor expression	[21, 246]
EGFR expression is high in GEP-NENs and correlates with metastasis in pNENs	[6, 9, 30, 31]
<i>PIK3CA</i> mutations are rare events	[128, 129].
PI3K signaling stimulates the ERK pathway of NE cells <i>in vitro</i>	[133-135]
PI3K signaling triggers secretion <i>in vitro</i>	[132, 136, 137]
PTEN mutations are rare events, but LOH may contribute to malignant transformation in (pancreatic) NENs	[33, 128, 154, 161, 162]
Low PTEN expression correlates with prognosis and response to therapy	[166, 168, 169, 172]
Subcellular localization of PTEN might serve as prognosis marker	[33, 154, 167, 168]
Akt overexpression associated with shorter median survival in with well- and poorly differentiated tumors	[168]
Phospho-T308 Akt might serve as marker of rapamycin response	[193-195]
Phospho-S473 Akt might not serve as a valuable marker	[6, 192]
Subtype specific activity: Akt1 and Akt3 pro-survival; Akt2 pro-apoptotic	[196]
Expression and activation of mTOR, 4EBP4 and p70S6K is associated with higher proliferation index and might correlate with low differentiation	[235-237]
Low level of TSC2 is correlated with tumor status and aggressiveness	[33, 128]

## The MAPK pathways promotes growth and cancer progression as well as feedback bypasses to PI3K signaling

### The MAPK cascades

Alteration of the Ras-MAPK cascades has frequently been described in human cancer. These pathways comprise several kinases that transmit extracellular signals from growth factors, chemokines and ECM signals and regulate cell growth, differentiation, proliferation, apoptosis and migration. They are classified in several interconnected branches constructed of functional analogues of MAPKs (mitogen-activated kinases), their MAPK kinases and the latter's MAPKK kinases. The four most important branches are (1) the ERK/MAPK, (2) the JNK (c-Jun amino-terminal kinase)/SAPK pathway, (3) the p38 pathway and (4) the BMK (big mitogen-activated protein kinase)/ERK 5 pathway [247]. We will therefore focus on the classical ERK/MAPK cascade and glance to other important members.

The classical cascade is induced by ligand binding to RTKs, such as VEGFR or EGFR, leading to its dimerization and auto-phosphorylation of the intracellular c-terminal region. Thereby binding sites for adaptor proteins are generated that in turn recruit GEFs (guanine nucleotide exchange factors) to the plasma membrane. GEFs facilitate the binding of GTP to Ras proteins. GTP-bound Ras recruits Raf kinases and activates the latter's serine/threonine kinase function to phosphorylate MEK and induce the phosphorylation of ERK effector kinases [248].

Abnormal activation of receptor tyrosine kinases or gain-of-function mutations in the *RAS* and *BRAF* genes have been frequently identified in a vast number of cancers. Multiple cross links and feedback loops to other mitogen pathways induce therapy resistance and bypass inhibitory approaches. The MAPK pathway contributes to neuroendocrine cancerogenesis, although many aspects in GEP-NENs remain to be explored.

### The role of Ras

The Ras superfamily is divided into five main families which in total comprise more than 150 members in humans: Ras, Rho, Rab, Arf, and Ran. The Ras family members are the most intensively studied. Three human *RAS* genes encode four Ras protein isoforms, designated as *H-Ras*, *N-Ras*, *K-Ras4A* and *K-Ras4B*. Ras proteins exhibit GTPase function and a structure that is related to the  $G_{\alpha}$  subunit of heterotrimeric G proteins. G proteins are molecular switches that oscillate from inactive GDP-bound to active GTP-bound states [249]. The cyclic process of GDP/GTP is facilitated by GEF and GAP (GTPase

activating proteins) classes of regulatory proteins that build a three-protein-complex with Ras.

The Ras proteins are anchored in close proximity to adaptor proteins such as Grb2 (growth factor receptor bound protein 2) and GEFs in the plasma membrane by farnesylation. The SOS family (son of sevenless) of RasGEFs, facilitates the exchange of Ras bound GDP with GTP and thereby activates Ras by conformational change [250].

Activating point mutations of the three Ras family genes are common in human cancers (30%) with a very high incidence in pancreatic adenocarcinoma, colorectal and lung cancers. Mutations in other Ras superfamily GTPases are rare and thus, their hyper-activation requires alternative induction mechanisms [251-255]. Deregulation of their regulators is a common event.

Aberrant signaling from growth factor receptors, in particular, RTKs and GPCRs (G protein-coupled receptors), or up-regulated gene expression can lead to aberrant GEF regulation and thus to enhanced activity of small GTPase proteins of the Ras superfamily.

GAPs, which return the GTPase to its GDP-bound inactive state, have been shown to exert crucial roles in curtailing GTPase activity in cancer. Since activation of Ras superfamily GEFs has been frequently described in human cancers, loss of GAP activity permits uncontrolled GTPase activity and can promote cancer [256-262].

In contrast to other entities such as pulmonary neuroendocrine carcinomas, mutations in the Ras genes are uncommon and rarely documented in GEP-NENs. Early studies could demonstrate that Ras mutations are virtually absent in gastroenteropancreatic neuroendocrine neoplasms [21, 263-265]. H-Ras and K-Ras expression could be detected in 65% and 10%, respectively. However, further information regarding its activity has not been generated to date [266], but much more comprehensive data is available for the Ras downstream targets, especially for B-Raf.

### Wild type Raf and the importance of paradoxical activation

Activation of Raf family members (A-Raf, B-Raf and C-Raf or Raf-1) is initiated by binding of their Ras binding domain to Ras-GTP and release from a 14-3-3 dimer bound to the N-terminal phosphorylation site. Concomitant conformational changes stimulate their serine/threonine kinase activity, dimerization and trigger sequential phosphorylation and activation of their targets MEK and ERK [267, 268].

B-Raf is the family member most easily activated by Ras, since both A-Raf and C-Raf need additional steps, such as phosphorylation of activating residues

and dephosphorylation of negative regulatory residues, to reach maximal activation [269, 270]. Furthermore the kinase activity of B-Raf is higher than those of the other family members [271].

Recent studies discovered that besides its involvement in oncogenic Ras signaling, *BRAF* itself is also mutated at a high frequency in human cancers, especially in melanoma (30-60%), thyroid cancer (30-50%) and ovarian cancer (~30%) [272]. A very common *BRAF* mutation, *B-RAFV600E*, is involved in the expression of hypoxia-inducible factor-1 $\alpha$  and VEGF and thus contributes to neoangiogenesis in those entities [273-275].

Overexpression or gain of function mutation of full-length Raf (or the truncated catalytic domain) leads to the activation of the ERK pathway and increases proliferation and tumor growth. Although there is no evidence that Raf activation participates in senescence evasion to date, it has been demonstrated to retrain apoptosis by regulating the expression and/or the activity of Bcl-2 family members [276].

Activated Raf is also involved in EMT (epithelial to mesenchymal transition), invasion and metastasis by promoting the production of TGF $\beta$ . Additionally, both B-Raf and C-Raf antagonistically control cell contractility and migration: B-Raf increases Rho-dependent contractility and opposes migration in an ERK-dependent manner, whereas C-Raf reduces contractility and increases migration by interfering with the activity of the cytoskeleton-based Rho effector ROCK2 (Rok- $\alpha$ ) [277-284].

The C-Raf protein appears as part of a multi-protein complex composed of HSP90, p50, and several scaffold proteins, such as 14-3-3. This complex is required for controlling its stability and activation status as well as its activity [285-287]. The phosphorylation state of C-Raf is influenced by multiple further protein kinases, including Src, PKC (protein kinase C) family members, the p21cip1-activated protein kinase PAK, and Akt [288-291].

In the case of the ubiquitous C-Raf, other targets potentially contributing to tumor progression have been identified such as the NF- $\kappa$ B, Rb and BAD [292-294]. C-Raf also contributes to genomic instability. Loss of RKIP (Raf kinase inhibitor protein) or C-Raf overexpression lowers the activity of the Aurora-B kinase, allowing cells to bypass the spindle assembly checkpoint [295].

The serine/threonine phosphorylation of MEK proteins as an intermediate step of the MAPK cascade features two special objectives: to enhance the cooperativity of activation of the MAPK and to allow modulation by other signaling events. In the case of the ERK1/2 pathway, amplification occurs at the Raf-MEK step, because MEK1 is much more abundant

than Raf. Another controlling feature of this step in the MAPK cascade depends on the dual phosphorylation of the MAPK by MEK. The tyrosine residues of ERK1/2 are phosphorylated with a higher affinity than threonine, leading to a nonprocessive phosphorylation and to the establishment of a threshold. The tyrosine phosphorylated proteins remain in an inactive state and accumulate until the threshold is reached. Subsequently the kinases are rapidly converted to the active state by threonine phosphorylation [296-303].

MEK mutations are rare events in human cancers with an incidence of 3% in melanomas and 2% in colon carcinomas [304].

The data concerning Raf and MEK downstream signaling and the discussion whether raf inhibition is reasonable or not is very contradictory and is still in the focus of preclinical investigation. Several further inhibitors of Raf and MEK are under preclinical assessment in GEP-NENs however with not very promising results to date (refer to Supplementary Material: suppl. 6).

Genetic analyses demonstrated that B-Raf mutations are rare in GEP-NENs [263, 305-307], with the exception of colorectal NENs, which have been demonstrated to harbor ~60% *KRAS* and *BRAF* mutations, but presumably due to its high content of adenoma and/or an adenocarcinoma cells [308].

Nevertheless the small GTPase Rap1 and B-Raf are highly expressed in GEP-NEN specimens, and both contribute to ERK1/2 and E26-like kinase (Elk-1) activation in NE cell lines [309]. Disrupting Raf-MEK-Erk signaling by the B-Raf inhibitor Raf265 significantly decreased Bcl-2 level and sensitized to TRAIL signaling in neuroendocrine cell lines. Raf265 inhibited Erk1/2 phosphorylation but in turn induced Akt phosphorylation and VEGF secretion, suggesting the existence of a compensatory feedback loop onto PI3K-Akt signaling [135, 310, 311].

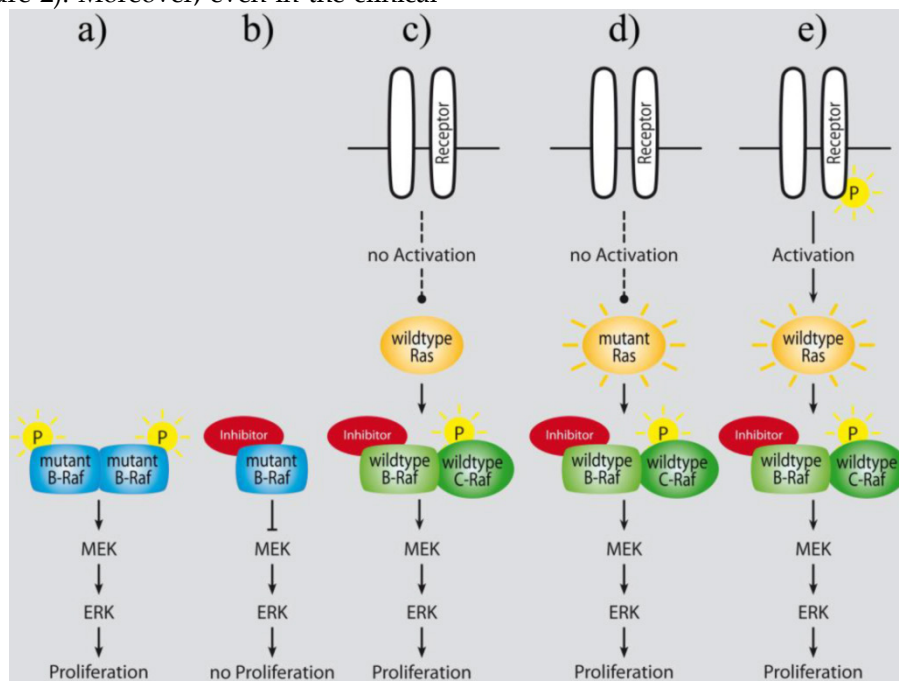
*In vitro* experiments demonstrated the crucial functional role of another Raf isoform: C-Raf activating retroviral transduction of BON cells resulted in high levels of phosphorylated ERK1/2 as well and caused remarkable morphologic and functional changes, such as reductions in 5-HT (5-hydroxytryptamine, the neurotransmitter serotonin), chromogranin A (marker of large dense core vesicles), and synaptophysin (marker of small synaptic vesicles) levels, which are strong neuroendocrine-related secretion markers. Accordingly, treatment of C-Raf-transduced BON cells with MEK inhibitors blocked morphological changes and hormone suppression but not ERK1/2 phosphorylation, indicating a dependency of NE dedifferentiation on MEK-mediated Raf to ERK signaling. Furthermore,

activation of the C-Raf signaling cascade in BON cells resulted in significant decrease in cellular adhesion and migration in a FAK (focal adhesion kinase) dependent manner [312-314]. Although a reduction of NE marker production, especially those of chromogranin A and synaptophysin, might be generally referred to worse differentiation in GEP-NENs *in vitro* and *in vivo* [315-317], cellular adhesion and migration are a prerequisite for invasion and metastatic dissemination and thus, markers of malignant phenotypes. Paradoxically, studies with several ATP-competitive C-Raf activators could demonstrate, that wild type Raf activation can not only suppress the expression of chromogranin A, but also the proliferation via p21cip1 upregulation in p21 wild type NEN cells [318-321]. These data conform with several publications that have proved an insensitivity of wild type B-Raf cell lines from several cancer entities to ATP-competitive Raf inhibitors. On the other hand, the exposure to Raf inhibitors resulted in a dose-dependent and sustained paradox activation of mitogen-activated protein kinase signaling in cells and tumors with wild type B-Raf. These paradoxical effects of Raf inhibition were seen in various malignant and normal cells *in vitro*, *xenografted* and *in vivo*, leading to entry into the cell cycle, enhanced proliferation, and significantly stimulated tumor growth *in vivo* (refer to figure 2). Moreover, even in the clinical

setting this paradoxical ERK-activation by the B-raf-Inhibitor Vemurafenib could be observed and led to a restriction on use of the drug [322]. The mechanism for this paradox activation is assumed to depend on an inhibitor-induced C-Raf/C-Raf or B-Raf/C-Raf homo- and heterodimerization, respectively, following sustained upstream signaling via Ras or enhanced RTK activation such as via IGF-1R or HER2 [20, 323-329].

Assuming that ATP-competitive wild type Raf inhibition indeed enhances paradoxical mitogen ERK downstream signaling and C-Raf activation in GEP-NENs (under the prerequisite of wild type Ras) and thus inhibits the neuroendocrine phenotype [318], the p70S6K/PI3K/RAS crosstalk under rapamycin treatment [330, 331] might emerge as an desirable side effect. This might e.g. comprise the reduced secretion of the bioactive hormones and thus alleviate the symptoms of functional active neuroendocrine tumors, but better understanding requires further mechanistic exploration.

Cross-regulation of Raf by inhibition of the PI3K-Akt axis might lead to unexpected and (under certain circumstances) harmful therapeutic outcome. Although Raf mutations are rare events in GEP-NENs tumors, subgroup-specific assessment of Raf and Akt inhibitors might improve therapeutic precision.



**Figure 2:** Paradoxical activation of Raf downstream signaling following treatment with ATP-competitive Raf inhibitors lead to an enhanced proliferation of B-Raf wild type cells *in vitro* and *in vivo* (adapted from Cichowski et al. [327]): a) mutant B-Raf constitutively activates MAPK signaling in cancer cells; b) ATP-competitive inhibition of mutant B-Raf counteracts ERK activation and leads to tumor growth reduction; c) ATP-competitive wild type Raf inhibition in normal cells results in increased ERK activation and proliferation *in vitro*, and in hyperplasia in normal tissues of mice *in vivo* [323]; d) and e) sustained RTK or Ras dependent upstream signaling activates MAPK signaling under ATP-competitive wild type Raf inhibition [323, 324, 330].

## The MAP Kinases ERK1/2 and their downstream effectors

ERK1/2 and other MAP kinases target a vast number of effector proteins, including membrane proteins, such as phospholipase A2, cytoplasmic proteins, such as downstream kinases and cytoskeletal proteins, and nuclear proteins, such as transcription factors. In response to cytokines, stress and chemotactic factors, the MAPK p38 and ERK2 regulate transcription by phosphorylation of MAPKAP kinase-2, which induces phosphorylation of the transcription factors CREB (c-AMP response element binding Protein) and ATF-1 (activating transcription factor) in cells and thus regulate the transcription of a large variety of genes.

Furthermore a recent publication demonstrated that p38-ATF/CREB signal transduction pathway can coordinately induce (promote transcription and RNA stability) and repress (promote RNA decay) transcript levels for distinct sets of genes, without shutting off transcription itself. This Stress-Activated RNA decay provides a mechanism to reduce the expression of target genes as it is required for cellular decisions in response to stress and other stimuli [332].

Ras responsive genes can be transcriptionally activated by ETS/AP-1 transcription factors. Hereby AP-1 comprises c-Jun, c-Fos and ATF-2 and controls the early transcriptional response to extracellular signals [333-340]. The TCFs (ternary complex factors) are substrates of the MAPKs ERK1/2, JNK/SAPK and p38. These targets, such as Elk-1, mediate transcription of genes containing SREs (serum response elements) in their promoters [341-343].

ERK2 has been shown to phosphorylate SRC-1 (steroid receptor coactivator-1), which shows histone acetyltransferase activity and is a coactivator of steroid nuclear receptors. SRC-1 also interacts with CREB to enhance estrogen and progesterone receptor-mediated gene activation [344-346].

Not least, MAP kinase pathways are considered to phosphorylate STAT3 (signal transducers and activators of transcription) and thus stimulate cytokine production, induction of pro-angiogenic factors and invasion in tumor cells [347-353].

MAPKs also contribute to chromatin remodeling, for instance by phosphorylating Rsk2 (ribosomal protein S6 kinase), which can subsequently phosphorylate histone H3, or by interacting with topoisomerase II. Further targets, Msk1 and 2 (mitogen and stress-activated protein kinase), are able to phosphorylate CREB and its co-factors, but also are very potent histone H3 and HMG-14 kinases [334, 354-360].

These mitogen effector functions of MAPK has put forward the development of several MAPK in-

hibitors in recent years (refer to Supplementary Material: suppl. 7). Few of those are under preclinical assessment for GEP-NEN therapy.

The activation of MAP Kinases in GEP-NENs is complex and due to multifunctional effects (such as differentiation and proliferation) not yet fully understood. Phosphorylated and thus activated ERK could be detected very frequently in an early immunohistochemically analysis of the MAPK pathway in GEP-NENs [263]. These data were supported by a second study where 96% of the analyzed specimens were positive for phospho-ERK1/2 and ERK activation could be related to EGFR and Akt phosphorylation, suggesting a simultaneous induction of Akt and ERK mediated pathways in NENs under EGFR kinase activity [6]. Consistently, *in vitro* treatment of neuroendocrine cell lines with EGFR or Raf inhibitors resulted in a time and dose-dependent dephosphorylation of ERK1/2 [309, 361].

In summary, analogous to PI3K signaling, the MAPK pathway is highly activated but the triggering mechanisms remain unclear. It is highly involved in the generation of the NE phenotype as inhibition of MAPK signaling results in impaired secretion and migration (summarized in table 4).

**Table 4.** Summary of study results concerning MAPK signaling in GEP-NENs.

Study results	Reference
Ras is expressed in GEP-NENs but mutations are rare	[21, 263-266]
Raf mutations are rare events	[263, 305-307]
Rap1 and B-Raf are highly expressed in GEP-NEN	[309]
Raf inhibition triggers feedback activation of Akt	[135, 310, 311]
MAPK signaling triggers NE secretion and migration <i>in vitro</i>	[312-314]
Raf activation induces a paradox inhibition of proliferation and NE secretion	[318-321]
ERK activation is frequent in GEP-NENs	[6, 263, 309, 361]

## PI3K and MAPK signaling are highly interwoven pathways and cooperate in therapy resistance in GEP-NENs

Although both, the PI3K and the MAPK pathway can be activated by the same RTKs, the agonists only partially overlap, since e.g. insulin, and IGF-1 are weak Ras-ERK activators, but strong PI3K-mTORC1 activators. Nevertheless the response of a certain pathway to specific growth factors depends on the factor's abundance, the receptor status in relation to expression and localization and the expression level of pathway mediators as well as of required docking and scaffold proteins [362-364].

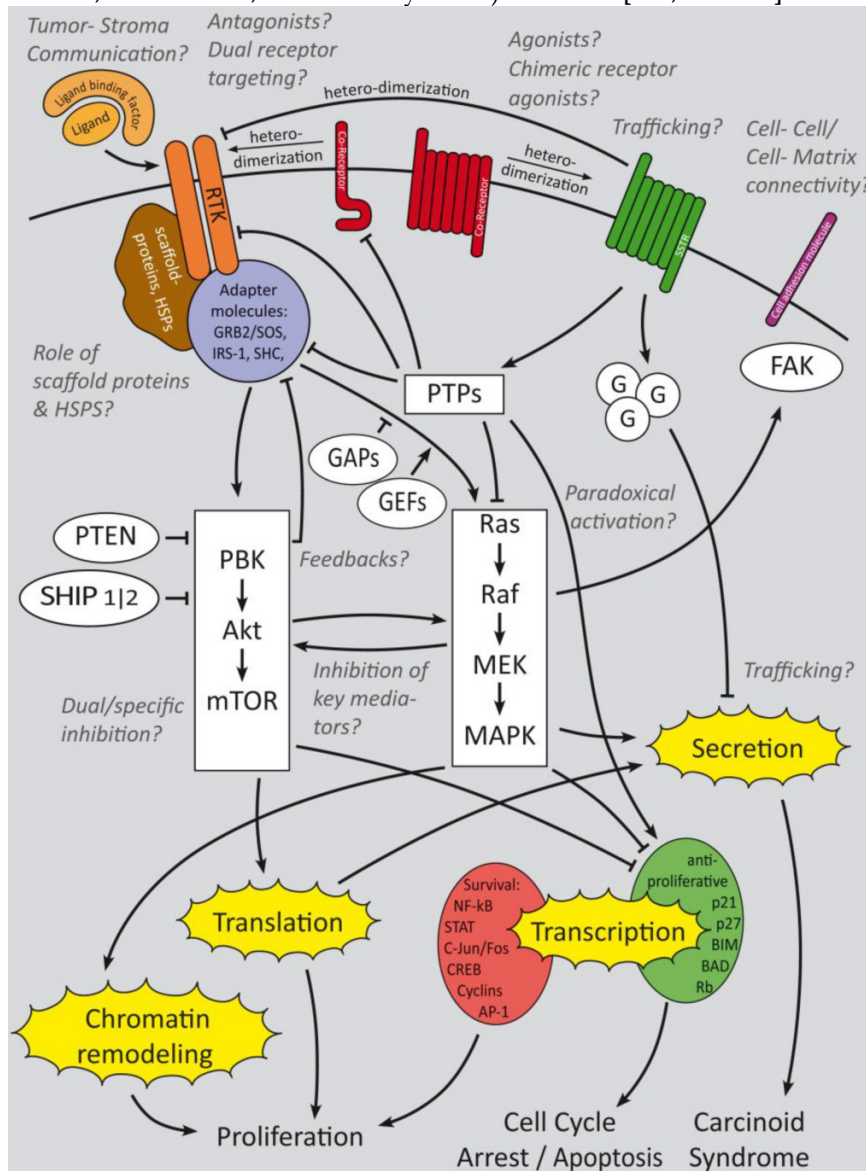
The PI3K pathway can be cross-activated upstream by direct Ras-GTP interaction with PI3 Kinases

or downstream by ERK and Rsk mediated phosphorylation of TSC2 and RAPTOR. In the latter case, phosphorylation inhibits the GAP function of TSC and promotes mTORC1 activity [365-369].

Furthermore, studies have demonstrated that also the KSR (kinase suppressor of Ras) scaffold, which maintains the co-localization of RAF, MEK, and ERK during ERK activation, interacts with mTOR, RAPTOR, RICTOR, and the TSC2-activating kinases AMPK and GSK3 [366, 370-372]. Consequently both, the PI3K and the MAPK pathways frequently converge on the same downstream targets and promote cell survival, proliferation, metabolism, and motility

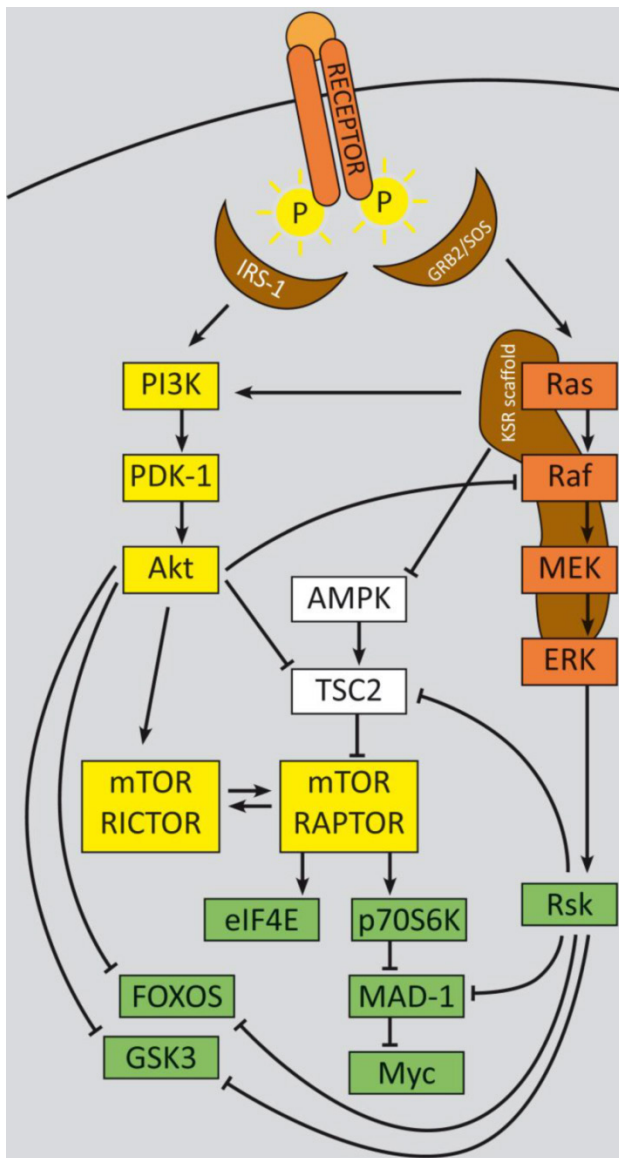
(refer to figure 3). Prominent proteins that are regulated by both pathways are the FOXOs and the c-Myc early transcription factors, as well as BAD and GSK3. FOXOs suppress cell survival and proliferation and once phosphorylated they undergo nuclear export and ubiquitin-proteasome-mediated degradation, whereas c-Myc induces pro-survival genes [373-378].

Furthermore, Rsk, Akt and p70S6K share the ability to induce protein translation by regulating initiation and elongation factors, such as eIF4B (eukaryotic initiation factor 4B), eEF2K (eukaryotic elongation factor 2 kinase), RPS6 (ribosomal protein S6) and eEF2 [365, 379-381].



**Figure 3:** Summary of growth factor-induced cellular mechanisms and inhibitory effects of somatostatin signaling in GEP-NENs. The major receptors of interest are RTKs, SSTRs and their Co-receptors. Cell adhesion molecules are of minor research interest but might have an interesting potential. Whereas RTK activate downstream PI3K and mitogen activated signaling that triggers gene regulation and secretion, SSTRs activate G-protein coupled signaling which has a predominant regulatory effect onto growth factor signaling cascades in GEP-NENs. Beside the main questions, which crosstalks are relevant for GEP-NEN cancerogenesis and how to inhibit their key mediators, the effect of paradoxical activation has not been clinically evaluated. Trafficking, intercellular and cell-matrix interactions have been analyzed only in a small number of publications [90, 92, 94] but might have a high potential for therapeutic and prognostic issues. Ligand binding factors and receptor agonists and antagonists are of high research interest and the focus is on dual receptor targeting to develop a more subgroup specific therapy approach [27-29]. *Italic: major fields of interest in current research and remaining open questions.*





**Figure 4:** Major crosstalks between the PI3K and the MAPK cascades. The PI3K and the MAPK pathway are highly interconnected and can be activated by the same RTKs, dependent on the triggering ligand. Direct Ras-GTP interaction with PI3 Kinases can trigger Ras-activated PI3K signaling [365-369]. The KSR scaffold is also a potent regulator of key PI3K signaling mediators. Several cross-activations and -inhibitions have been assessed to date. For instance, Akt can inhibit Ras function [289, 331, 382-386] and Rsk can interfere with TSC2 [365-369]. Furthermore, both pathways converge on the same downstream targets, such as the FOXO proteins, GSK3, Myc or p70S6K [373-378], and thus promote cancer associated-phenotypes.

A very important cross-inhibitory mechanism in the context of therapy resistance is the crosstalk of C-Raf and Akt. Highly activated Akt suppresses Raf kinase activity by phosphorylation of S259. Phospho-S259 results in binding of the 14-3-3 protein and in Raf inactivation [289, 331, 382-386]. Consequently, inhibition of PI3K signaling can activate Raf as demonstrated for everolimus in GEP-NETs [204]. This mechanism is discussed to be one reason why mTOR inhibition has limited impact in GEP-NET therapy.

Nevertheless the effect of paradoxical MAPK pathway activation has not been integrated in this debate to date and requires further investigation.

In summary, the PI3K and the MAPK pathways share overlapping activation patterns and converge on a number of common targets (refer to figure 4), although with varying affinities. The role of distinct posttranscriptional modification sites and multi-protein-complex assembly is analyzed in a large number of more detailed publications. Inhibiting one of those non-specific pathway mediators thus harbors the danger of activating other cascades and states the rationale for many dual inhibition approaches, unless with little success in GEP-NENs so far.

### Conclusion

In sporadic GEP-NENs, growth factor receptor downstream signaling is ensured by large networks of triggering kinases, stabilizing scaffold proteins and regulating phosphatases, as well as by various transcription factors and co-factors. This network is highly upregulated and triggers cell growth, proliferation and secretion. Although basic cell-biological research has elucidated a vast number of mechanisms and interrelations that might explain observations that have been made in GEP-NENs, the majority of molecular information still remains to be elucidated. In contrast to cancer entities, where the oncogenic input can be “translated” in a manageable number of mutated proteins which facilitates the development of “target-directed” therapies, main sources of growth factor deregulation in sporadic GEP-NENs have not been identified to date. Genomic studies have revealed a number of chromosomal alterations but their relation to specific genes remains unclear. Subgroup specific data is rare due to the already limited number of cases, but might be important to understand differences between distinct groups of patients. Moreover, the “crosstalk” of different signaling pathways in a NEN cell may be much more complex and interactive as it is already known and the inhibition of one pathway can easily activate others by feedback loops, as described for mTOR inhibition.

In this review we have compiled several studies on the molecular basis for growth factor induced mitogen downstream-signaling in GEP-NENs. The two most promising and approved therapies in GEP-NENs are target-directed approaches, namely targeting the mTOR protein and somatostatin receptors. Recommendations for the diagnostics and therapy of gastroenteropancreatic neuroendocrine neoplasms are given by experts of the European Neuroendocrine Tumor Society in consensus conferences and are updated regularly [387]. Certainly, somatostatin analogues have been introduced as an-

ti-proliferative agents after the positive results of the PROMID study [116] and are used in midgut tumors, whereas Everolimus and Sunitinib have their indication in metastatic pNENs [244, 388]. Everolimus might possibly also evolve as therapeutic option in midgut NENs [389] as results of RADIANT-4 study are awaited.

Nevertheless the therapy outcome depends on the abundance and activation pattern of these targets. Subgrouping data by therapy predictive biological markers, such as the phosphorylation status of Akt or the SSTR expression pattern, might improve the therapeutic precision for choosing the right therapy. Here we provide some evidence that even the most advanced therapeutic agent, everolimus, which is widely used in GEP-NENs therapy and was approved by the FDA two years ago, may still hold some drawbacks which may diminish its success.

In preclinical models Everolimus leads to a global upregulation of upstream PI3K signaling and cross-activation of Ras/Raf/Erk signaling via negative feedback loops. Therefore, recent therapeutic approaches focus on inhibitors of alternative components of the PI3K pathway, dual target inhibitors and effective combinatory bio/chemotherapies. Another example is the crosstalk between B-Raf/C-Raf and Ras/MEK/ERK-pathway. In the clinical setting, the use of a B-Raf-inhibitor in a melanoma patient led to the (paradoxical) activation and progression of a second malignancy (n-Ras mutated AML) in the same patient [322]. This effect was reversed by withdrawing the inhibitor – but it impressively demonstrated the need for further investigations on the crosstalks and the complexity of different pathways.

There is also a need for entity-specific research data concerning processes that trigger GEP-NEN-specific cellular behavior, such as intra- and intercellular trafficking, tumor-stroma interactions or the regulatory role of scaffold proteins. Although a large variety of inhibitors and molecular pathway regulator are available to date, their application is almost limited to their assessment for clinical purposes rather than to identify their mechanisms of actions. For instance, the observations that mutations in key mediators of growth factor activated pathways are rare in GEP-NENs, is an important fact for novel therapeutic strategies and, especially in the case of Raf, fundamental to define a certain outcome. Without understanding the underlying mechanism, interpretation of unexpected study results will remain unsatisfying. The importance of molecular markers and genetic data to predict therapy outcome before starting cost-intensive clinical trials has been increased dramatically due to the enormous rise of available substances in the context of growth factor

signaling. Significant mechanistic knowledge, deducted from generally valid cancerogenetic contexts, needs to be analyzed for its validity in GEP-NENs and thus to furnish stronger rationales for distinct target directed and subtype specific therapeutic trials. Therefore the identification and assessment of new markers and their evolution throughout neuroendocrine cancerogenesis is a promising field of research. Detailed pathway analyses are essential to understand the limitations of the current therapies and to elucidate new possibilities of molecular imaging and targeting in this heterogeneous cancer entity.

## Supplementary Material

Suppl. 1-7. <http://www.thno.org/v04p0336s1.pdf>

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## Competing Interests

The group of Dr. Patricia Grabowski cooperates with Ipsen Pharma, Novartis and Pfizer Inc. in the context of preclinical trials and receives financial support from Ipsen Pharma and Novartis.

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