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"Role of EPHA2 Serine 897 phosphorylation in thyroid cancer: molecular mechanisms and biological properties of a novel player in thyroid tumorigenesis"

Chiara Allocca

University of Naples Federico II
Dipartimento di Medicina Molecolare e Biotecnologie Mediche

Administrative Location

Dipartimento di Medicina Molecolare e Biotecnologie Mediche
Università degli Studi di Napoli Federico II

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“Role of EPHA2 Serine 897 phosphorylation in thyroid cancer: molecular mechanisms and biological properties of a novel player in thyroid tumorigenesis”

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ABBREVIATIONS

AGC: PK**A**, PK**G**, PK**C** kinases
ALK: **a**naplastic **l**ymphoma **k**inase
ADAMs: **a** **d**isintegrin **a**nd **m**etalloproteinases
ATC: **a**naplastic **t**hyroid **c**arcinoma
CAS: exportin **c**ellular **a**poptosis **s**usceptibility
CCDC6: **c**oiled-**c**oil **d**omain **c**ontaining gene **6**
CCH: **C**-**c**ell **h**yperplasia
CIP: **c**alf **i**ntestinal alkaline **p**hosphatase
CML: **c**hronic **m**yeloid **l**eukemia
CRD: **c**ysteine **r**ich **d**omain
CS: **c**alf **s**erum
CT: **c**alci**t**onin
CTNNB1: **c**adheri**n**-associa**t**ed protei**n** **β1**
CTKD: **C**-**t**erminal **k**inase **d**omain
DMEM: **D**ulbecco's **m**odified **e**agle's **m**edium
DTC: **d**ifferentiated **t**hyroid **c**arcinoma
ECM: **e**xtra**c**ellular **m**atrix
EFN: **E**PH **f**amily receptor i**n**teracting protein
FV-PTC: **f**ollicular **v**ariant **p**apillary **t**hyroid **c**arcinoma
EGFR: **e**pidermal **g**rowth **f**actor **r**eceptor
EPH: **E**rytropoietin-**P**roducing **H**epatocellular carcinoma cell line
FAs: **f**ollicular **a**denomas
FAK: **f**ocal **a**dhesion **k**inase
FBS: **f**oetal **b**ovine **s**erum
EMT: **E**phitelial-**m**esenchimal-**t**ransition
FGFR: **f**ibroblast **g**rowth **f**actor **r**eceptor
FMTC: **f**amilial **m**edullary **t**hyroid **c**arcinoma
FNIII: **f**ibro**n**ectin-type **III** repeats
FNAB: **f**ine **n**eedle-**a**spiration **b**iopsy
FTC: **f**ollicular **t**hyroid **c**arcinoma
Glut-1: **glu**cose **t**ransporter type **1**
GPI: **g**lycosyl**p**hosphat**i**dylinositol
HDAC: **h**istone **d**e**ac**etylase
HGFR: **h**epatocyte **g**rowth **f**actor **r**eceptor (MET)
HIF-1: **h**ypoxia-**i**nducible **f**actor **1**
HUVECs: **h**uman **u**mbilical **v**ein **e**ndothelial **c**ells
IkB: **i**nhibitor **kB**
JAK: **ja**nus **k**inase
JMD: **j**uxta**m**embrane **d**omain
LBD: **l**igand **b**inding **d**omain
LMW-PTP: **l**ow-**m**olecular **w**eight **p**hophotyrosine **p**hosphatase
MAPK: **m**itogen **a**ctivated **p**rotein **k**inase
MDR1: **m**ulti**d**rug-**r**esistance **1**
MEN: **m**ultiple **e**ndocrine **n**eoplasia
MMP: **m**embrane **m**etallo**p**roteinase
MTC: **m**edullary **t**hyroid **c**arcinoma
NIS: **s**odium/**i**odine **s**ymporter

NCOA4: **n**uclear receptor **coa**ctivator gene **4**
NF-kB: **n**uclear **f**actor **kB**
NGF: **n**erve **g**rowth **f**actor
NSCLCs: **n**on-**s**mall **c**ell **l**ung **c**arcinomas
NTKD: **N**-**t**erminal **k**inase **d**omain
NTRK1: **n**eurotrophic **r**eceptor-**t**yrosine **k**inase **1**
OIS: **o**ncogene **i**nduced **s**enescence
PAX8: **pa**ired bo**x8** transcription factor
PDGFR: **p**latelet-**d**erived **g**rowth **f**actor **r**eceptor
PDK-1: **p**hosphoinositide-**d**ependent **k**inase-1
PD-L1: **p**rogrammed **d**eath-**l**igand **1**
PDTC: **p**ooly **d**ifferentiated **t**hyroid **c**arcinoma
PDZ: **p**ostsynaptic **d**ensity-95/discs large/ **z**onula occludens-1 domains
PIF: **P**DK1 **i**nteracting **f**ragment
PI3K: **p**hosphat**i**dylinositol **3**-**k**inase
PKC/A: **p**rotein **k**inase **C**/**A**
PPARγ: **P**eroxisome **P**roliferator **A**ctivated **R**eceptor **γ**
PTC: **p**apillary **t**hyroid **c**arcinoma
PTEN: **p**hosphatase and **ten**sin homolog
PTP1B: **p**rotein **t**yrosine **p**hosphatase **1B**
RBD: **r**eceptor **b**inding **d**omain
RSK: **r**ibosomal **S**6 **k**inase
RET: **RE**arranged during **T**ransfection
RTK: **r**eceptor **t**yrosine **k**inase
SAM: **s**terile **α** **m**otif
SEER: **S**urveillance **E**pidemiology and **E**nd **R**esults
SHIP2: **SH**2-containing 5'-**i**nositol **p**hosphatase **2**
SLAP: **S**rc-**l**ike **a**daptor **p**rotein
SRC: **sa**r**c**oma kinase
STAT: **s**ignal **t**ransducer and **a**ctivator of **t**ranscription
TCGDB: **T**hyroid **C**ancer and Disorder **G**ene **D**ata**b**ase
TERT: **te**lomerase **r**everse **t**ranscriptase
TG: **t**hyro**g**lobulin
TGFβ: **t**ransforming **g**rowth **f**actor**β**
TKD: **t**yrosine **k**inase **d**omain
TKI: **t**yrosine **k**inase **i**nhibitor
TMD: **t**rans**m**embrane **d**omain
TNFα: **t**umor **n**ecrosis **f**actor **α**
TPO: **t**ireo**p**er**o**xidase
TSH: **t**hyroid-**s**timulating **h**ormone
VAB-1: **v**ariable **ab**normal-**1**
VEGFR: **v**ascular **e**ndothelial **g**rowth **f**actor **r**eceptor
WDTC: **w**ell **d**ifferentiated **t**hyroid **c**arcinoma

LIST OF PUBLICATIONS

This Dissertation is based upon the following publications:

1. C. Allocca, et al. – RSK-dependent Serine 897 phosphorylation of EPHA2 mediates oncogenic activity of MAPK drivers in thyroid cancer cells. *Manuscript in preparation (main body of Dissertation)*.

2. MC Cantisani, A Parascandalo, M Perälä, C Allocca, V Fey, N Sahlberg, F Merolla, F Basolo, MO Laukkanen, OP Kallioniemi, M Santoro, MD Castellone – A Loss-of-Function Genetic Screening Identifies Novel Mediators of Thyroid Cancer Cell Viability. Oncotarget, *in press*

ABSTRACT

EPH (Erythropoietin-Producing Hepatocellular carcinoma cell line) receptor tyrosine kinases (RTK) belong to the largest subfamily of RTKs counting 14 genes in humans. Among them, EPHA2 is often overexpressed in a variety of human cancers, including thyroid carcinoma. Thyroid carcinomas are commonly driven by genetic lesions targeting the MAPK signaling cascade including rearrangements of several RTKs, such as RET and NTRK, or point mutations in RAS or BRAF. We have previously demonstrated, through a siRNA-based genetic screen of the human kinome, that EPHA2 expression is essential for viability of thyroid cancer cells in culture. To gain insight into the EPHA2 function in thyroid tumorigenesis, we studied the role of the intracellular domain of EPHA2 and, in particular, of its phosphorylation on Serine 897 (pSer897). Ser897 phosphorylation has been previously reported to mediate EPHA2 oncogenic activity. Ser897 is embedded in the consensus phosphorylation sequence for AGC (PKA, PKG, PKC) family kinases, including p90RSK, a direct MAPK target. Here we show that in thyroid cancer cells bearing oncogenic lesions in the MAPK signaling cascade, EPHA2 is robustly phosphorylated on Ser897. Treatment with chemical inhibitors targeting p90RSK or other MAPK pathway components blunts Ser897 phosphorylation of EPHA2. Recombinant p90RSK phosphorylates *in vitro* EPHA2 Ser897. Finally, RNA interference-mediated knock-down combined with rescue experiments demonstrate that Ser897 phosphorylation of EPHA2 mediates thyroid cancer cell proliferation and motility. Collectively, these findings point to EPHA2 pSer897 as a novel crucial mediator of the oncogenic MAPK signaling cascade, and in particular of p90RSK, in thyroid cancer.

1. INTRODUCTION

1.1 Thyroid cancer

Thyroid carcinoma is a relatively rare neoplasm, representing about 1% of all cancers (Tuttle et al. 2014). Nonetheless, thyroid cancer is the most common malignant endocrine tumor and its incidence has been stably increasing over the past few decades (Fallahi et al. 2014; Frampton 2016). According to the Surveillance Epidemiology and End Results (SEER) database, the rate of new thyroid cancer cases have risen on average 5% per year. The increased thyroid cancer incidence is probably due to the development of high-resolution imaging techniques and early diagnosis upon fine needle-aspiration biopsy (FNAB) (Zevallos et al. 2015). However, other potential causes, such as exposure to ionizing radiation, iodine deficiency, autoimmune thyroiditis and chronic infections (Antonelli et al. 2007; Ferrari et al. 2015; Su et al. 2016), as well as increased life expectancy (Kwong et al. 2015; Su et al. 2016) cannot be excluded.

The vast majority of thyroid cancers derives from follicular cells (thyrocytes) that normally secrete thyroglobulin (TG) and thyroid hormones (thyroxine-T4 and triiodothyronine-T3). Instead, approximately 5% of thyroid carcinomas originate from para-follicular thyroid cells (C-cells, secreting calcitonin); calcitonin-secreting thyroid carcinomas are called medullary thyroid carcinoma (MTC). MTC occurs either sporadically (75%) or as a dominantly inherited disease in the context of multiple endocrine neoplasia (MEN) type 2 (MEN2A or MEN2B) [Ferrari et al. 2015].

Histological examination of the thyroid gland of patients affected by the hereditary form of the disease shows a widespread C-cell hyperplasia (CCH) suggesting that hyperplasia is an early event in C cell transformation (Hinze et al. 1998; Wells et al. 2015).

The follicular cell-derived thyroid carcinomas include several histological histotypes; according to their malignancy and differentiation grade they are classified in well differentiated thyroid carcinoma (WDTC), in turn subdivided in papillary thyroid carcinoma (PTC, 80%) and follicular thyroid carcinoma (FTC, 10%), poorly differentiated thyroid carcinoma (PDTC, 3%) and anaplastic or undifferentiated thyroid carcinoma (ATC, 2%) (Wu et al. 2014; Wells and Santoro 2014; Puxeddu et al. 2009; Shi et al. 2016).

PTC is often diagnosed in the 5th decade of life presenting with a slow-growing thyroid mass. Patients usually have a palpable nodule in absence of any other clinical findings (Puxeddu et al. 2009). PTC is associated to an overall favorable prognosis; tumor recurrence and distant metastases, indeed, are a rare event, found in about 10% of the PTC cases (Romitti et al. 2013; Shi et al. 2016). PTC is characterized by specific nuclear features and typical papillary architecture; several PTC variants are described, including solid-follicular, follicular, and tall-cell (Shi et al. 2016). These variants have different pathological features and clinical outcomes (DeLellis, 2006). Generally, PTCs metastasize to the local lymph nodal stations and feature a survival rate greater than 90% (Schlumberger 1998; Sherman 2003). FTC is defined as a carcinoma with follicular cell differentiation in the absence of the nuclear features typical of PTC (DeLellis, 2006). FTCs are generally unifocal (Passler et al. 2004). Both PTCs and FTCs retain the capacity to accumulate and metabolize iodine (Petrulea et al. 2015).

Poorly differentiated carcinomas are a subset of thyroid tumors considered morphologically and clinically intermediate between WDTC and ATC; PDTC are more aggressive than DTC but less than ATC (Burman 2014; Landa et al. 2016; Volante et al. 2007). Finally, the most aggressive thyroid tumor is represented by ATC. Although ATC is quite rare, occurring in about 2% of thyroid tumors, it represents the first cause of death for thyroid cancer with the median survival time after diagnosis of about one year. ATC can originate *de novo* or represent an advanced stage of WDTC (Romitti et al. 2013). Morphologically, it is defined as a malignant tumor composed by undifferentiated cells with high mitotic rate and massive stroma infiltration (DeLellis 2006; Smallridge et al. 2012) (**Figure 1**).

1.2 Molecular genetics of thyroid cancer

Research conducted in the last 20 years has improved our knowledge of the genetic alterations involved in thyroid tumorigenesis and distinct molecular mechanisms, specific for the vary histopatological subtypes have been identified (Nikiforov et al. 2011; Xing 2013; Wells and Santoro 2014) (**Figure 1**).

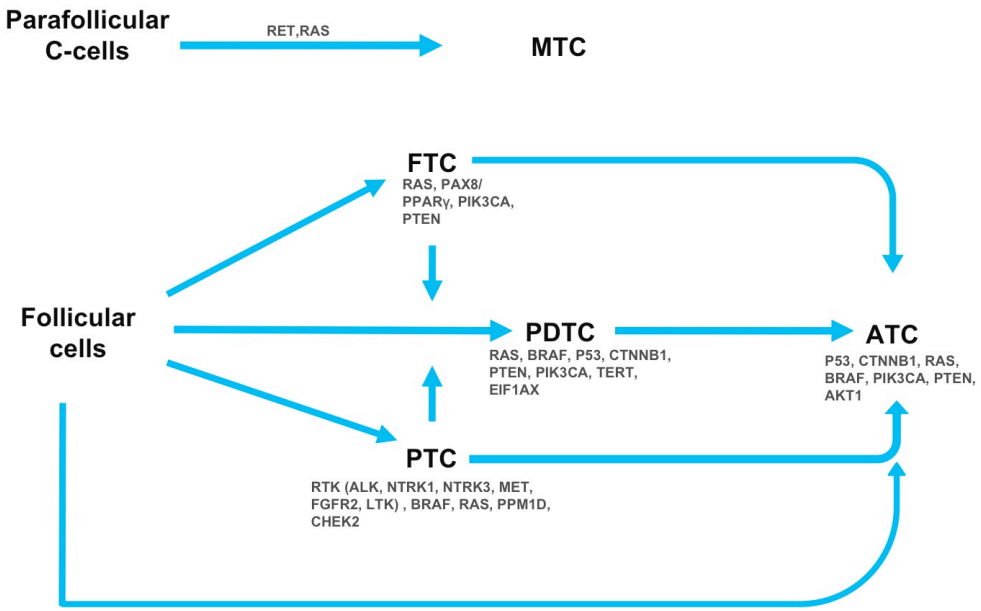

Figure 1. Subtypes of thyroid cancers arising from parafollicular or follicular cells and their related oncogenic lesions.

REarranged during Transfection (RET) gene activating point mutations have been identified in 98% of hereditary MTC and in about 50% of sporadic MTC cases (Wells et al. 2013; Wells et al. 2015). RET encodes a receptor tyrosine kinase for neurotrophic growth factors of the GDNF family (Santoro and Carlomagno 2013). In MEN 2A, the most frequent mutation affect Cysteine 634, located in the extracellular protein domain. In MEN 2B as well as in sporadic MTCs, the predominant RET mutation is the substitution of the Metionine 918 with a Threonine residue, within the tyrosine kinase domain of the receptor (Fallahi et al. 2014; Wells et al. 2015). Recently, RAS somatic mutations have been found in RET-negative sporadic MTCs (Agrawal et al. 2013). Of note (see also below), the aberrant activation of RAS pathway is a common event in thyroid tumorigenesis, being present in different thyroid tumor subtypes such as MTCs, WDTCs, PDTCs and ATCs. RET gene rearrangements, commonly caused by paracentric inversions of the long arm of chromosome 10, are found in PTC, particularly in radiation-associated cases (Nikiforov et al. 2004; Ricarte-Filho et al. 2013; Cancer Genome Atlas, 2014; Giordano, 2016). These rearrangements generate chimeric proteins called "RET/PTC" all resulting in an *in frame* fusion of the 3' portion of RET gene (coding for the tyrosine kinase domain) with the 5'-terminal sequence of unrelated genes. These gene fusions lead to a consititutive RET kinase ligand independent activation which in turn induces cell transformation mainly through the RAS/MAPK and PI3K/AKT pathways, but also through other signaling cascades such as Janus kinase-1 (JAK)/signal transducer and activation factor (STAT), protein kinase C (PKC), sarcoma kinase (SRC), focal adhesion kinase (FAK) or β-catenin pathways (Mulligan, 2014).

Several different RET/PTC rearrangements have been identified, with RET/PTC1 (where the RET fusion partner is coiled-coil domain containing gene 6 [CCDC6], formerly known as H4) and RET/PTC3 (where the RET fusion partner is nuclear receptor coactivator gene 4 [NCOA4], formerly known as RFG/ELE1) as the most common ones (Wells and Santoro 2014; Fallahi et al. 2015; Nikiforov et al. 2011). Moreover, in 2-15% of PTCs another chromosomal rearrangement involves the Nerve Growth Factor (NGF) receptor (Neurotrophic Receptor-Tyrosine Kinase1-NTRK1) gene or other RTKS (Greco et al. 2010, Nikiforov et al. 2011; Cancer Genome Atlas, 2014). Noteworthy, RET gene rearrangements initially thought to be PTC-specific have more recently been described also in other tumor types, including lung adenocarcinomas, colon carcinoma, chronic myelomonocytic leukemias and Spitz nevi (Santoro and Carlomagno 2013).

Gain of function mutations of RAS genes primarily affect codons 12, 13 and 61, with KRAS (24%) being the most common mutated member in follicular-cell derived thyroid carcinomas, followed by NRAS (8%) and HRAS (4%) (Alonso-Gordoa et al. 2015). RAS proteins are small GTP-ases belonging to the mitogen activated protein kinase (MAPK) pathway. RAS mutations are common in FTC, PDTC, ATC as well as in PTC belonging to the follicular variant subtype (Volante et al. 2007; Landa et al. 2016; Cancer Genome Atlas, 2014). RAS mutations have been described to occur also in thyroid benign follicular adenomas (FAs), though their prevalence in benign lesions appears to be lower (Nikiforov et al. 2011).

In FTC cases negative for RAS gene mutations, chimeric proteins have been detected (in about 30-40% of cases) which can be more rarely (2-13%) found also in FAs (Durante et al. 2015).

These rearrangements result in the fusion of the thyroid-specific transcription factor paired box8 (PAX8) with the nuclear-hormone-receptor Peroxisome Proliferator Activated Receptor γ (PPARγ) (Raman et al. 2014; Fallahi et al. 2015; Alonso-Gordoa et al. 2015). The resulting protein acts as a PPARγ dominant negative mutant and displays its oncogenic properties thought the activation of MAPK, transforming growth factor β (TGFβ), Wnt/β-catenin pathways and through the inhibition of phosphatase and tensin homolog (PTEN) [Raman et al. 2014].

The Serine/Threonine kinase BRAF, another member of the MAPK pathway, is also frequently deregulated in thyroid carcinomas particularly in PTC, tall cell variant PTC and in undifferentiated tumors. BRAF mutation is considered a negative prognostic marker (Elisei et al. 2008; Basolo et al. 2010; Xing et al. 2013; Puxeddu et al. 2014; Xing et al. 2015; Collet et al. 2016). Valine to Glutamic acid substitution at residue 600 is the most frequent point mutation of BRAF, occuring in 45% of PTCs, 10-20% of PDTCs and 20% of ATC (Fallahi et al. 2015). Other oncogenic BRAF mutations have been described, including Lysine 601 to Glutamic acid substitution (K601E), commonly found in follicular variant PTCs (FV-PTCs), and several small deletions or insertions (indels) around codon 600 (Moretti et al. 2006; Moretti et al. 2009; Cancer Genome Atlas 2014). Finally, rearrangements such as the AKAP9/BRAF one, can be found in PTC and other thyroid cancers (Ciampi et al. 2005; Landa et al. 2016). BRAF mutations have been associated with tumor recurrence caused by increase in proinflammatory state, with the degradation of nuclear factor kB (NF-kB) inhibitor (IkB) [Zou et al. 2015; Ferrari et al. 2015; Alonso-Gordoa et al. 2015], combined with the downregulation of $CD8^+$ cytotoxic T-cell caused by an increased expression of programmed death ligand 1 (PD-L1) [Brauner et al. 2016].

Moreover, BRAF oncogenic activation induces a radioiodine-refractory state associated to decreased expression of the sodium/iodine symporter (NIS), thyroglobulin, thyroperoxidase (TPO) and glucose transporter type 1 (Glut-1), secondary overexpression of vascular endothelial growth factor receptor (VEGFR) or hepatocyte growth factor receptor (MET) [Durante et al. 2007; Zou et al. 2015; Ferrari et al. 2015; Alonso-Gordoa et al. 2015], and exportin cellular apoptosis susceptibility (CAS) [Holzer et al. 2016]. Consistently, Knauf and colleagues (2005) have demonstrated that transgenic mice carrying BRAF V600E developed a thyroid carcinomas closely related to human PTC, and that these tumors rapidly progressed to PDTCs or ATCs.

Genetic alterations in phosphatidylinositol 3-kinase (PI3K)/AKT pathway are also common in thyroid carcinoma, particularly in less differentiated tumor types and ATC (Xing 2013). Indeed, aberrant activation of PI3K/AKT pathway is related with progressive dedifferentiation and acquisition of new genetic alterations. The PI3K/AKT oncogenic activation may be linked to different genetic alterations, such as mutations of the catalytic subunit of PI3K (PIK3CA) [12-23% ATCs, 2% PTCs and 10% FTCs], as well as PIK3CA amplifications (38-61% ATCs) [Garcia-Rostan et al. 2005; Xing 2013; Romitti et al. 2012], AKT mutations (such as E17K), loss of PTEN through point mutations or gene silencing, and phosphoinositide-dependent kinase 1 (PDK1) gene amplification (Ricarte-Filho et al. 2009; Xing 2013; Alonso-Gordoa et al. 2015; Robbins and Hague 2016). Overexpression of angiopoietin-2, VEGF/VEGFR, HGF/c-MET, EGF/EGFR, PDGF/PDGFR, FGF/FGFR, hypoxia-inducible factor 1α (HIF-1α), among the others, have been correlated with thyroid tumor aggressiveness.

Recently, mutations in anaplastic lymphoma kinase (ALK) (Kelly et al. 2014) and mutations of the telomerase reverse transcriptase promoter (TERT) have been reported in ATCs or other less differentiated thyroid cancers (Wells and Santoro 2014; Melo et al. 2015; Carneiro et al. 2015; Lennon et al. 2016; Tavares et al. 2016). In addition, loss of function of p53 tumor suppressor gene and point mutation in exon 3 of the cadherin-associated protein β1 (CTNNB1) gene encoding β-catenin were related to ATC (Malaguarnera et al. 2007; Garcia-Rostan et al. 2001; Landa et al. 2016).

Most of these evidence point to a crucial role of two major signaling cascades, the MAPK and the PI3K/AKT ones, in thyroid cancer formation and progression (**Figure 2**).

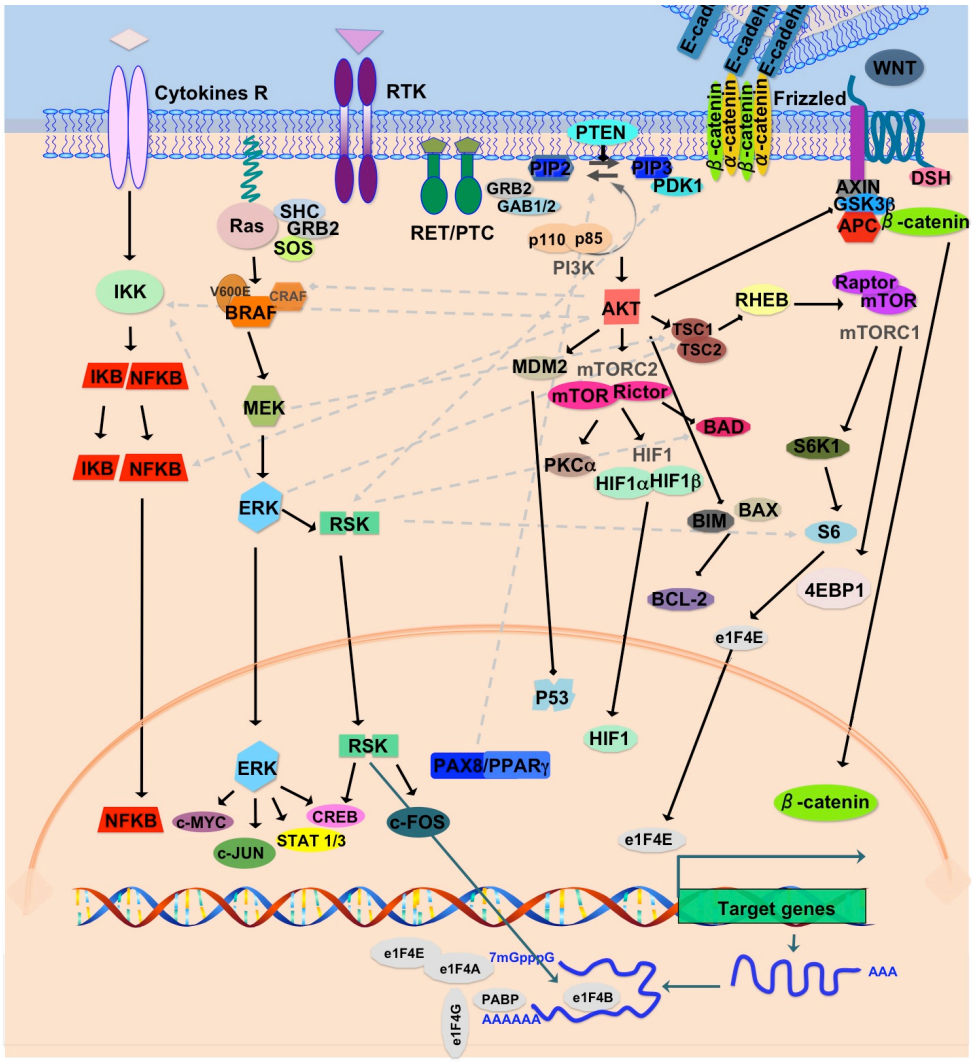

Figure 2. **The main oncogenic pathways in thyroid cancer.** The figure summarizes the most important signaling nodes as well as their cross-talks (dashed lines).

Recently, Wu et al. (2014) through biomedical literature text mining have generated an in silico molecular profiling of thyroid cancers, and found that, in fact, RAS/MAPK and PI3K/AKT pathways, together with WNT/β-catenin and NFkB ones are the best described oncogenic pathways compromised in all thyroid cancers subtypes (**Table 1**).

Table1. Top 4 related pathways and text-mining study for each subtype.

Pathway	Evidence PMIDs			
	MTC	FTC	PTC	ATC
MAPK	23934677	21196179	16896265	16410725
PI3K/AKT	23329180	17426084	18000091	22918703
WNT/β-catenin	N/A	18727708	22204713	17218945
NFkB	N/A	16314832	23528368	19885592

Table 1: The reported numbers are PubMed Identifier (PMID) evidence analyzed by Wu et al. (2014). N/A: Not Applicable: no evidence in literature.

1.3 Targeted therapy for thyroid cancer

Standard treatment for thyroid cancer is based on thyroidectomy and thyroid stimulating hormone (TSH) suppression, followed by radio-iodine ablation to remove residual disease. Despite an overall good prognosis, subsets of patients (about 5%) develop metastatic disease that is refractory to treatment (Puxeddu et al. 2011; Xing et al. 2013; Frampton 2016). For these patients no effective systemic therapies are available and therefore a leading pharmaceutical interest has been to develop specific personalized treatments based on the identification of "druggable targets" (Smith and Nucera 2015). Thus, among the novel therapeutic approaches, targeting oncogenic protein kinases has emerged as promising strategy in different human cancers (Muller et al. 2015).

Effectiveness of kinase inhibitors as cancers targeting agents is linked to the concept of "oncogene addition" (Weinstein and Joe 2008), whereby single constitutive activated protein may maintain cells malignant phenotype causing a selective dependence of cancer cells on the function of that specific protein. The prototypic example of these targeted drugs has been imatinib mesylate (Gleevec), the first kinase inhibitor approved by FDA for chronic myeloid leukemia (CML) in 2001 (Druker et al. 1996; Tsai and Nussinov 2013), followed by gefitinib (Iressa) and erlotinib (Tarceva), targeting the EGFR in non–small cell lung carcinomas (NSCLCs) [Yoishida et al. 2013]. In humans there are 518 different protein kinases, and they still represent the preferred targeted molecules because of their implication in a wide set of crucial cellular events and because of their well characterized (and conserved) activation mechanisms (Manning at al. 2002).

During the last decade, thyroid cancer therapy research has led to development of several drugs against different kinases. Many efforts are still needed to improve selectivity avoiding off-target effects on normal kinases and to enhance their specific antitumor effects, such as in the case of RET TKIs for instance (Newton et al. 2016; Frett et al. 2014; Frett et al. 2015). Among successful examples of the use of TKIs in thyroid cancer, there are multikinase inhibitors (Sorafenib, Cabozantinib, Lenvantinib, Sunitinib), BRAF inhibitors (Vemurafenib), tyrosine kinase inhibitor (TKI) [Vandetanib, Pazopatinib], and PI3K/AKT/mTOR inhibitors (Everolimus, Rapamicin, Tersirolimus); some of these drugs have been registered for the treatment of specific types of thyroid cancer (Frampton 2012; Frampton 2016).

Additional approaches for thyroid cancer may be redifferentiation agents with the aim of increasing expression of thyroid-specific differentiation markers leading to rescue of radioiodine sensititivity.

PPARγ inhibitor Rosiglitazone and HDAC inhibitor Vorinosat, for example, belong to this category of compounds. Importantly, inhibition of protein kinases acting downstream driving oncoproteins, such as with MEK inhibitor Selumetinib, has demonstrated the possibility not only of restraining tumor growth but also of rescuing radioiodine concentration (Ho et al. 2013; Wells and Santoro 2014; Alonso-Gordoa et al. 2015; Ferrari et al. 2015; Ruan et al. 2015). All these drugs proved beneficial outcomes in ongoing phase II trials.

In tumors, it is unlikely that a single targeting drug can elicit a complete remission; this is because the existence of many signaling feedback loops and multiple interconnections of signaling transduction networks that can allow cancer cells to bypass the block determined by a single targeting agent, raising a compensatory activation of a different oncogenic pathway (Mendoza et al. 2011). These evidence have pushed scientists to search for the possibility of targeting different nodes of the same pathway, and crucial core components of signaling transduction networks moving from a "linear signal modular scheme" to a "complex transduction circuit" (Kolch at al. 2015). Consistently, for example cross-influence between oncogenic pathways, in particular the mitogenic RAS/MAPK and the survival PI3K/AKT ones have been described (Zimmermann and Moelling 1999; Rommel et al. 1999; Aksamitiene et al. 2010; Paraiso et al. 2010; Mendoza et al. 2011). Just as examples, c-MET overexpression was found to mediate reactivation of PI3K/AKT signaling in thyroid cancer cell lines harboring BRAF mutation (Byeon et al. 2015), and TSH was described to overcome BRAF V600E-induced senescence [oncogene induced senescence (OIS)] promoting thyroid tumor progression via AKT overactivation and p53 expression loss (Zou et al. 2015).

In this context, this dissertation has focused on the characterization of a novel signaling hub connecting the MAPK signaling cascade and a specific tyrosine kinase receptor of the EPH family.

1.4 EPH receptors and ephrins

Since the cloning of the first EPH gene in 1987 (Hirai et al. 1987) and the subsequent identification of EFN ligands (Bartley et al. 1994; Beckmann et al. 1994; Cheng end Flanagan 1994), EPH/EFN system has captured the attention of many authors for its uniqueness and its involvement in several physiological and pathological phenomena (Arvanitis and Davy 2008). Erythropoietin-producing human hepatocellular carcinoma receptors (EPH) were identified through a screening aimed at identifying novel kinases involved in cancer (Holland et al. 1997). EPH constitutes the largest subfamily of RTKs. Unlike other RTK ligands, EPH ligands, called "ephrins" (EPH family receptor interacting proteins-EFN), are non soluble, attached to the cell membrane and, sometimes, sequestered within extracellular matrix (ECM). Interaction between EPHs and their cognate ligands is mediated by cell-cell contacts and causes bi-directional signaling into the opposing cells (Klein 1999; Noren and Pasquale 2004; Pasquale 2010).

In humans there are 9 EPHs belonging to class A (EPHA1-EPHA8 and EPHA10) and 5 EPHs of class B (EPHB1-EPHB4 and EPHB6) on the basis of their sequence homology and ligands affinity (Pitulescu and Adams 2010). Ligands, EFNs, have also been divided in two groups: the A-subclass (ephrins A1-A5) that contains ligands that are anchored to the cell membrane by a glycosylphosphatidylinositol (GPI) linkage; the B-subclass (ephrins B1-B3) that contains ligands represented by transmembrane proteins with a short cytoplasmatic region (**Figure 3**). EPHA9, EPHB5 and EFNA6 proteins have been identified only in the chicken (Pasquale 2004).

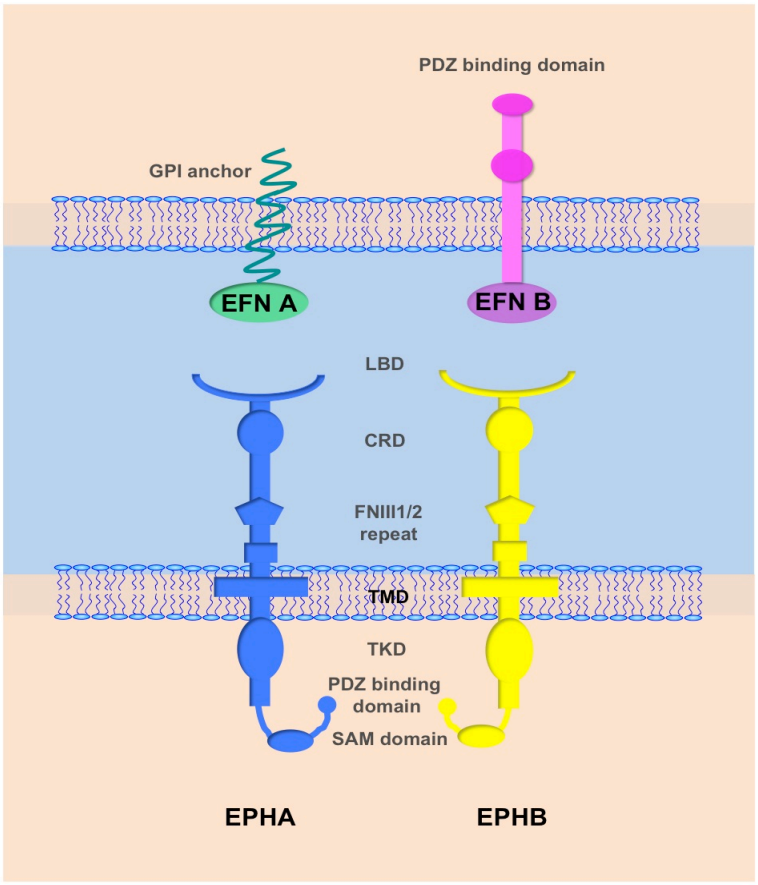

Figure 3. EPHs and EFNs structure. In EPH receptors the N-terminal extracellular region is composed by a ligand binding domain (LBD) followed by a cysteine rich domain (CRD) and two fibronectin-type III repeats (FNIII 1/2). With a single transmembrane domain (TMD), EPHs cross the membrane bi-layer and feature sequentially: a juxtamembrane (JMD), tyrosine kinase (TKD), sterile α motif (SAM) and a postsynaptic density-95/discs large/ zonula occludens-1 (PDZ) binding domains at the C-teminus. On the opposing cells, EFNAs and EFNBs have at the N-terminus an extracellular receptor binding domain (RBD); EFNAs are anchored to the cell membrane with a glycosylphosphatidyl-inositide (GPI) anchor, while EFNBs have a cytoplasmic tail with a C-terminal PDZ binding domain.

Thus, excluding EPHA4 (Takemoto et al. 2002) and EPHB2 (Himanen et al. 2004) that can bind both A-type and B-type ligands with the same binding-affinity, EPHAs bind A-type ligands, and EPHBs bind B-type EFNs (Kullander and Klein 2002; Pasquale 2004). In this complex system, despite each EPH binds with greater affinity its "preferred" ligand, it is described some EPHs/EFNs promiscuity (Pasquale 2004; Haramis and Perrakis 2006; Dai et al. 2014) (**Figure 4**). Capability of EPHs to discriminate among different EFNs was described as being dependent by particular aminoacid interactions, but the precise mechanism is still poorly known (Pasquale 2004). Probably, this cross-binding could be framework-dependent, in response to greater or lesser EFNs availability, and it can modulate different biological responses in different contexts. Ligand genes (such as EFNA1/3/5 genes on the q arm of the chromosome1) may exist as genetic cluster on the same chromosome (Perez White and Getsios 2014), supporting the possibility of a co-regulated expression.

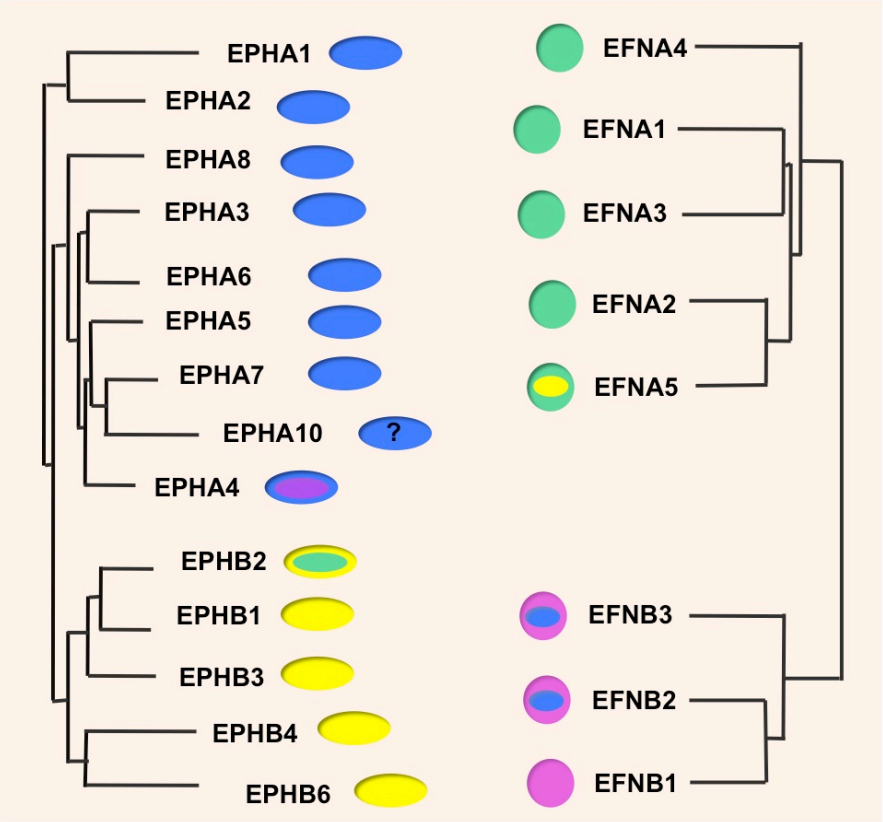

Figure 4. **EPHs/EFNs binding affinity, sequence omology and phylogenetic relationship.** Human receptor/ligand preferential binding is shown with the exception of EPHA10. The dendrogram was obtained by Clustal program using ligands- or receptor-binding domain sequences respectively (The image is a modified version of Pasquale 2004).

Receptor-ligand binding induces a conformational reorganization of EPH LBD that facilitates the interaction with other EPHs, which creates complementary interaction surfaces that causes the laterally joining of different dimer pairs into tetrameric and high-order cluster complexes. This "seeding mechanism" provides a model for how EFNs low level can trigger cluster formation and also result in oligomers but also heterodimers formation (Seiradake et al. 2010). Recent findings demonstrated that heterodimers of different EPHs can form, thus recruiting also EPHs that have not been targeted by the cognate EFN. Examples are EPHB6-mediated suppression of EPHA2 signaling in breast cancer cell (Fox and Kandpal 2011) or EPHA7-mediated inhibition of EPHA2 signaling in lymphoma (Oricchio et al. 2011).

EPH/EFN are among the oldest evolutionarily conserved receptor/ligand pairs (Jones et al 1997). The EPH/EFN system is present also in *Caenorabditis elegans*, where at variance from vertebrates, there is only one EPH gene called variable abnormal-1 (VAB-1), and four EFN genes (Chin-Sang et al. 1999; Wang et al. 1999).

Wykosky and Debinsky (2008) have reported the existence of a functional and soluble unclustered monomeric EFNA1, believed to derive from a proteolytical cleavage. EFNA1 was found to promote or inhibit growth depending on its soluble or membrane-bound state (Alford et al. 2010). Supporting this finding, EFNA1 and its cleaved products was found in serum of tumor patients, as serum biomarkers of tumor burden (Beauchamp and Debinsky 2012).

One of the first EPH/EFN function discovered was in axon guidance; however, this system is nowadays regarded to as an universal cell-to-cell communication pathway that allows cells moving to a specific position and that maintains cellular organization by preventing cell intermingling through chemotatic/chemorepulsive forces (Holmberg et al. 2000; Pasquale 2008; Genander 2012). EPH receptors and EFNs are expressed in all embryonic germ layers in all vertebrates, and mediate cell migration and positioning, boundary formation and segmentation during important developmental processes such as gastrulation (Lisabeth et al. 2013; Park and Lee 2015). Recently, Xavier and colleagues (2016) reported another example of this concept, showing EPHs/EFNs regionally-restricted expression in palate and tongue development, pointing to a fine temporal and spatial-coordinated expression during tissue development. Furthermore, recent insights are focusing on the role of EPH/EFN system in the regulation of adult stem niche in various organs (Genander 2012; Perez White and Getsios 2014) as well as in adult organs specialized functions, such as synaptic plasticity, memory formation, epithelial and vascular homeostasis and integrity, bone remodeling, insulin secretion, inflammatory and immune-response (Kullander and Klein 2002; Gucciardo et al. 2014; Dines and Lamprecht 2015). EPHs/EFNs redundancy and gradient, context-dependent signaling, directionality and intensity of the interactions probably define organs and tissue specific outcomes (Fox et al. 1995; Muñoz et al. 2005).

1.5 EPH/EFN signaling

EPH/EFN signaling system has several important differences with respect to other canonical ligand/RTK signaling pairs. A unique feature of EFN/EPH system relies in the binding step between ligands and receptors. In most of the cases, EPH ligands are bound to surface of neighboring cells rather than freely soluble. Therefore, EPH/EFN interaction elicits "ligand dependent" signal activation in both ephrins- and receptor-expressing cells. In ligand-expressing cells, binding stimulates a "reverse signaling". In this case, EFNAs through their GPI-anchors exert lipid-raft mediated binding with other juxtamembrane proteins (such as Src family kinase), or are able to interacting with membrane-bound receptors such as RET or NTRK1 (Lisabeth et al. 2013). Similarly, EFNBs are phsophorylated by Src family kinases, this in turn mediating association to SH2 containing protein adaptors such as Grb4 (Lisabeth et al. 2013). Binding of PDZ contianing proteins to the PDZ-binding domain of EFNBs can also elicit different intracellular responses, such as angiogenesis, axon guidance and synaptic plasticity.

At the same time EFN binding to cognate EPHs elicits a "forward signaling" in the EPH-expressing cell (Pasquale, 2008; Lisabeth et al. 2013). Like all the other RTKs, EPH dimerization/oligomerization mediated by EFN binding is followed by conformational changes that trigger the autophosphorylation of the receptor on tyrosine residues; in turn, phosphorylation of tyrosines located into the inhibitory juxtamembrane receptor domain facilitate receptor activation and binding and phosphorylation of cytoplasmic downstream signal proteins (Binns et al. 2000; Wybenga-Groot et al. 2001; Davis et al. 2009).

EPHs also contain an autophosphorylated tyrosine residue within their activation loop; however, differently from other RTKs phosphorylation of this residue does not seem essential for kinase activity (Lisabeth et al. 2013).

Several autophosphorylated tyrosine residues in activated EPHs have been identified and involved in forward signaling. Important effectors are represented by Rho-like small GTP-ases as well as signal transducers typically activated by several RTKs such as RAS/MAPK and PI3K/AKT components (**Figure 5**). A striking difference with other RTKs is that typically EPHs use these mechanisms to inhibit rather than promote cell growth and to favor cell repulsion rather than cell attraction, this resulting at the end in tumor suppression rather than promotion (Miao et al. 2000; Barquilla and Pasquale 2015; Lisabeth et al. 2013). This notwithstanding, "ligand-independent" EPH signaling mechanisms exist that confer to these receptors also important tumor promoting effects.

EPH impact on cell cytoskeleton and cell shape is mediated by regulation of Rho-family GTP-ases. The typical cell repulsive effect of several EPHs is, indeed, mediated by an increased balance of RhoA activation *vs* Rac1/Cdc42 activation thereby resulting in increased formation of actin structures named stress fibers, cell process retraction and inhibition of cell movement. In different conditions, however, EPHs can also promote Rac1 activation by activating Rac1 GEF such as Vav and inhibiting RhoA via p190RhoGAP (Wakayama et al. 2011).

At a variance from most common RTKs that signal through RAS/MAPK pathway, EPHs frequently inhibit this signaling system and oppose the activity of other RTKs, such as FGFR, TRKB and IGF1R (Miao et al. 2001; Pasquale 2008).

A mechanism mediating such inhibitory function involves recruitment to EPHs of p120RAS-GAP that promotes GTP hydrolysis and therefore switch-off p21 RAS (Minami et al. 2011; Pasquale 2008; Pasquale 2010). However, it is worth mentioning that in other cases EPHs, similarly to other RTKs, can promote rather than suppress RAS/MAPK signaling. In turn, a feedback loop exists so that MAPK signaling promotes expression of EPHA2 (Macrae et al. 2005).

As in the case of RAS/MAPK cascade, also another signaling cascade that is commonly activated by RTKs, e.g. the PI3K/AKT one, is blunted rather than promoted by EPH forward signaling. For instance, in several cell types EPHA2 stimulation reduces AKT phosphorylation on both T308 and S473 sites (Miao et al. 2009). Also in this case, this peculiar inhibitory activity can be mediated by recruitment to EPH of a negative regulator of the pathway such as a phosphatase. Also in this case, however, evidence exists that EPH may also promote rather than inhibit AKT (**Figure 5**).

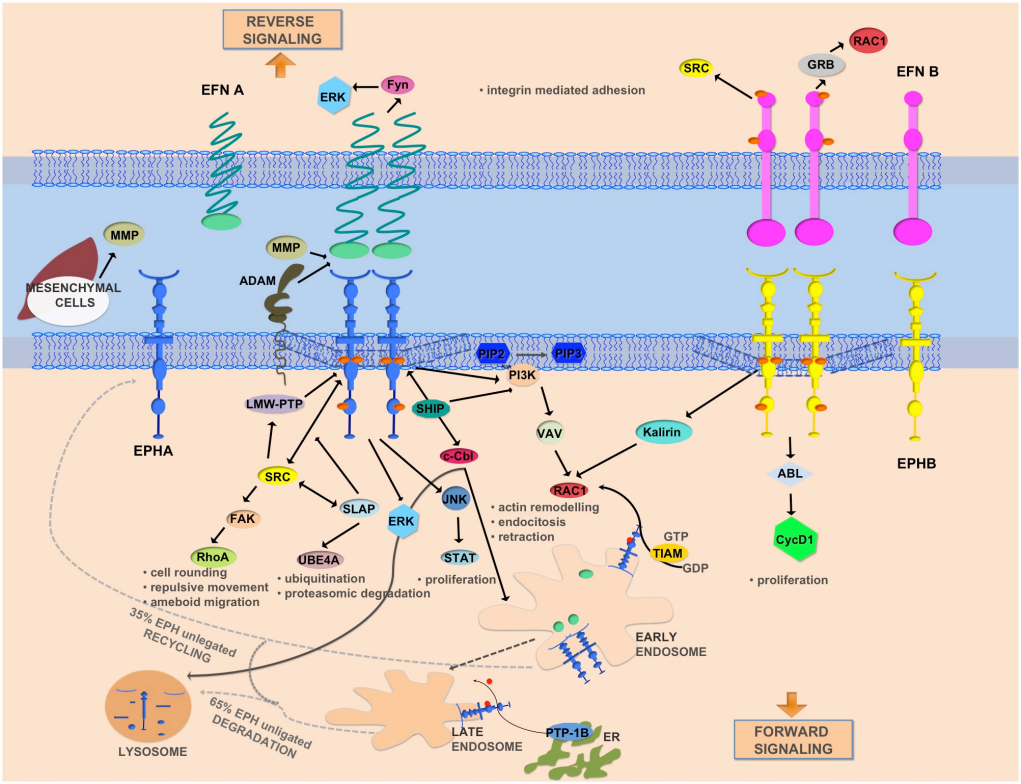

Figure 5. **EPH/EFN signaling.** The image summarizes EFNs-mediated reverse signaling and EPHs-mediated forward signaling events.

Processing of EPH receptors upon EFN binding seems to be an integral part of the signal transduction mechanisms. Protease-cleavage by transmembrane disintegrin [such as A Disintegrin And Metalloproteinases (ADAMs)] or other membrane metalloproteinases (MMP-1, -2, -9, 13) mediates an EPH/EFN irreversible internalization (Atapattu et al. 2014). Receptor/ligand complexes can be internalized in either EFN- or EPH-expressing cells through a clathrin-dependent mechanism of endocytosis termed transendocytosis.

This process is critical for cell separation and repulsive effects (Lisabeth et al. 2013). In endosomes, part of transendocytosed EPHA2 interacts through its juxtamembrane domain with Rho family GEF TIAM1 thus leading to Rac1 (and probably also RhoA/Cdc42) activation. EPHs ubiquitination and degradation were described also to be c-Cbl independent and dependent on the Src-like adaptor protein (SLAP) (Naudin et al. 2014; Boissier et al. 2013; Wybenga-Groot and McGlade 2015). An antagonist of EPH/EFN internalization is the SH2-containing 5'-inositol phosphatase 2 (SHIP2) that is recruited to EPHA2 through SAM domain-mediated interactions. This lipid phosphatase blocks EPH endocytosis via a PI3K-dependent Rac activation (Zhuang at al. 2007). High SHIP2 levels are involved in EPHA2 overexpression in many cancer types. Similarly, low-molecular weight phophotyrosine phosphatase (LMW-PTP) that is activated by Src and commonly upregulated in cancer, mediates EPH receptor dephosphorylation and in turn decreased binding to Cbl and decreased EPH turnover (Kikawa et al. 2002).

1.6 EPHA2 receptor in human cancer

Considering the multiplicity and the versatility played in adult and embryonic cellular activities, it is not surprising that EPHs/EFNs play a key role also in tumors (Pasquale 2008). Next generation sequencing efforts have identified somatic mutations in several EPH members, particularly in melanoma and in lung cancer. Functional implication of these mutations is still

largely unknown, however in most of the cases mutations are scattered throughout the protein sequence making unlikely a gain-of-function effect.
In the case of one particular receptor, EPHA3, mutations identified in cancer have been found to disrupt ligand binding and therefore probably able to interfere with a ligand-dependent tumor suppressive effect of EPHA3 (Lisabeth et al. 2013). Other EPHA family members including EPHA2 do not seem to be frequently mutated in cancer and therefore their deregulation can take place at the transcriptional or post-translational levels.

Controversial tumor-suppressive or tumor-promoting roles have been described for several EPH members. Most of the reports point to a tumor suppressive effect of ligand-dependent EPH stimulation compared to an oncogenic effect of ligand-indipendent stimulation (Lisabeth et al. 2013). Accordingly, in several cancer types it is often documented a reduced expression of the EFN ligands and at the same time an upregulated expression of the EPH receptors. EPHA2 is the EPH family member most commonly upregulated in human cancer. EPHA2 is overexpressed in a variety of human malignancies and it is often associated to increased tumor grade and poor prognosis in diverse cancer types as breast, lung, prostate, skin, esophageal, gastric and renal carcinoma (Amato et al. 2014; Brantley-Sieders et al. 2008; Hafaner et al. 2006; Hatano et al. 2005; Herath et al. 2006; Kinch et al. 2003; Miyazaki et al. 2003; Nakamura et al. 2005; Walker-Daniels et al. 1999; Xu et al. 2014; Zelinski et al. 2001), as well as in thyroid carcinomas.

As far as thyroid carcinomas, Karidis and co-workers have assessed EPHA2 and EPHA4 protein expression by immunohistochemistry in 131 patients with benign and malignant thyroid lesions and found a significant upregulation of EPHA2 in malignant lesions and in particular in papillary carcinomas. In contrast, EPHA4 expression was not changed in malignant versus benign thyroid lesions (Karidis et al. 2011).

O'Malley and colligues reported EPHA2 overexpression in thyroid cancer cell lines and in benign and malignant human thyroid tumors with respect to normal thyroid.

In FTC-238 cell line, EPHA2 knock-down reduced invasion and AKT phosphorylation and the opposite was found upon EPHA2 overexpression in the FTC-133 cell line (O'Malley et al. 2012).

1.7 EPHA2 receptor signaling

Several tyrosine autophosphorylation sites have been identified in EPHA2 and in some cases their role elucidated (**Figure 6**). Phosphorylated Tyr587 and Tyr593 in the EPHA2 juxtamembrane domain bind to Vav2 and Vav3 guanine nucleotide exchange factors, that are able to mediate up-regulation of GTP-bound activated Rac1 GTPase (Fang et al. 2008). Phosphorylated Tyr735 in the N-terminal lobe of the EPHA2 kinase is able to bind to the p85 regulatory subunit of phosphatidylinositol 3-kinase (Fang et al. 2008). Phosphorylated Tyr930 in the SAM domain of EPHA2 is involved in the binding to the Nck adaptor and its phosphorylation is negatively controlled by the LAR phosphatase, thereby attenuating EPHA2-mediated cell migration (Lee et al. 2013). SHIP2 (Src homology 2 domain-containing phosphoinositide 5-phosphatase 2) instead is recruited to activated EPHA2 via a phosphotyrosine-independent mechanism involving a SAM-SAM domain interaction, and this reduces EPHA2 internalization (Zhuang et al. 2007) (**Figure 6**).

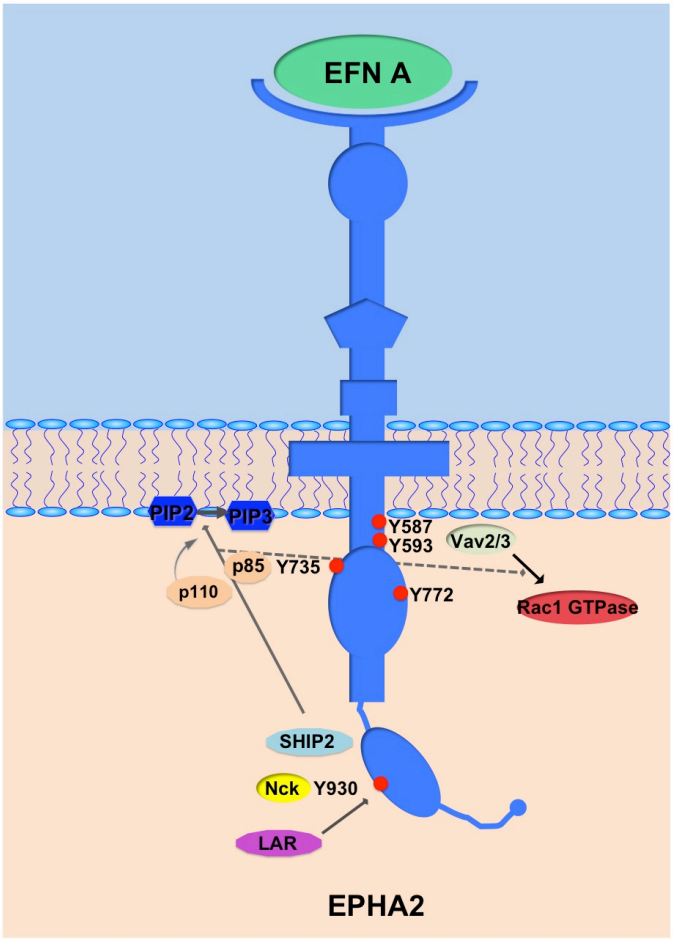

Figure 6. Main EPHA2 tyrosine phosphorylation sites and their role in forward signaling.

Despite this detailed biochemical knowledge, output of EPHA2 signaling in cells and in particular cancer cells is still largely unknown. EPHA2 seems to play an important role in cell-cell contacts (Zantek et al. 1999) and EPHA2-mediated phosphorylation of Claudin-4 at the tight junctions decreases cell-cell contact enhancing paracellular permeability (Tanaka et al. 2005).

In some cases, EPH signaling intersects other RTKs through downstream mediators or through direct interactions. Ligand-activated EPHA2 decreases MAPK signaling (Parri at al. 2005), whereas MAPK activity leads to EPHA2 overexpression (Macrae et al. 2005). There is also evidence of EPHA2-EGFR physical and functional interaction. EPHA2 is an EGF/EGFR transcriptional target gene mediated by MAPK activation, and EPHA2-EGFR colocalize and interact at the plasmamembrane of cancer cells. EPHA2 has ligand-independent effects on EGF-induced cancer cell motility whereas EFNA1 stimulation leads to inhibition of EGF-induced motility, leading to EPHA2 receptor internalization, downregulation and termination of EPHA2 positive effects on cell motility (Larsen et al. 2010). EFNA1 is a tumor necrosis factor α (TNFα) induced gene in human umbilical vein endothelial cells (HUVECs) (Holzman et al. 1990). EFNA1 expression is low in tumor cells but high in endothelial cells (Ogawa et al. 2000), where it acts as chemoattractant for other endothelial cells (Pandey et al. 1995). EFNA1 and EPHA2 expression are upregulated in hypoxic conditions by HIF-1α and VEGFR, leading to EPHA2 phosphorylation (Yamashita et al. 2008; Cheng et al. 2003), that in turn induces VEGFR expression (Beauchamp and Debinsky 2012). Angiostatic Slit2 protein impairs this mechanism (Youngblood et al. 2015).

A wealth of evidence points to a tyrosine phosphorylation- and ligand-independent role of EPHA2 in promoting tumor formation (**Figure 7**). Despite its high expression levels, tyrosine phosphorylation of EPHA2 is often downregulated in malignant cells compared to normal cells (Pasquale et al. 2010). EFNA1 is often lost in tumor cells and this contributes to the loss of cell-cell contact among cancer cells. One phosphotyrosine- and ligand-independent role of EPHA2 has been related to the phosphorylation of a particular serine residue, Serine 897 (Ser897).

Ser897 maps in the EPHA2 SAM domain, and its phosphorylation has been reported to mediate the switch from an anti-oncogenic to a pro-oncogenic role of EPHA2 (**Figure 7**).

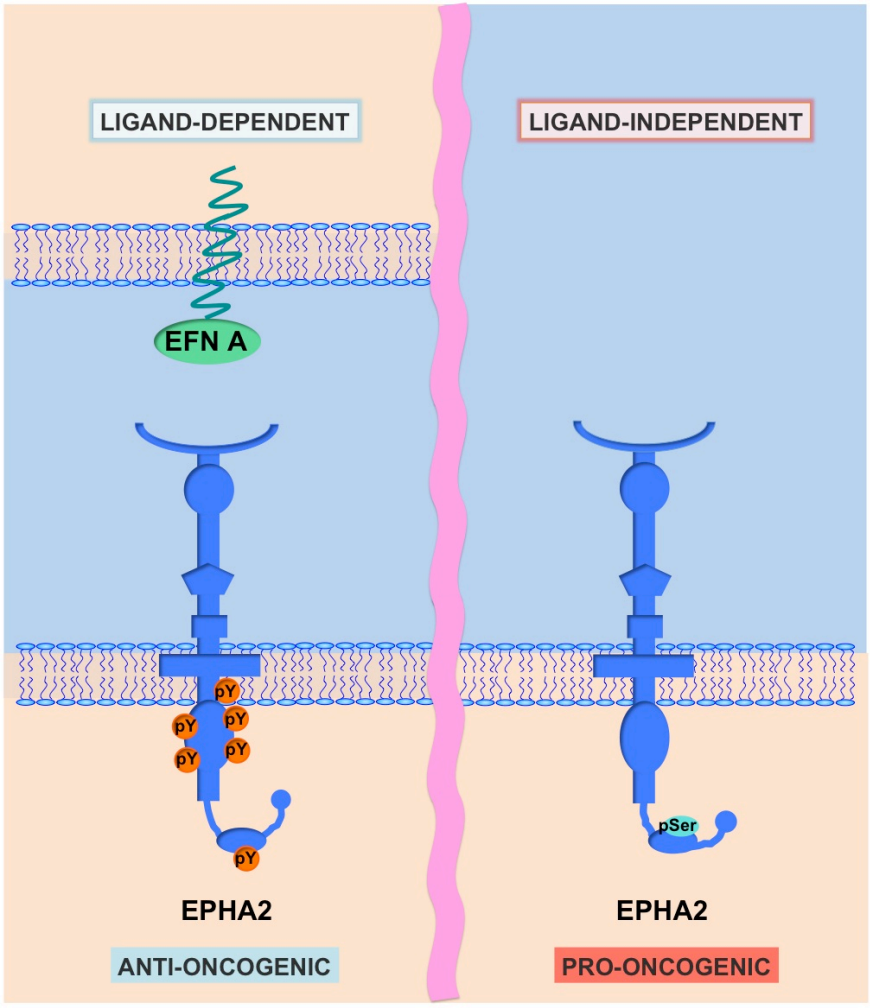

Figure 7. EPHA2 ligand dependent and independent pathway. After cell-cell adhesion, EPHA2/EFNA1 interaction mediates kinase activation and increased phospho-tyrosine content; this results in suppression of tumorigenesis. In contrast, ligand-unbound receptor upon Ser897 phosphorylation is able to emanate pro-oncogenic signals.

Miao and coworkers demonstrated that Ser897 EPHA2 phosphorylation mediated by AKT is crucial for glioma and prostate cancer cell invasive phenotype and stem cell properties (Miao et al. 2009; 2015). S897A mutation abolished such a ligand-independent promotion of cell motility. In contrast, EFNA1 stimulation of EPHA2 blunted AKT and caused EPHA2 dephosphorylation on Ser897 (Miao et al. 2009). Moreover, EPHA2 was highly expressed in glioblastoma cell lines and promoted invasion. These effects required AKT-mediated phosphorylation on Ser897 and did not require EFN binding. Importantly, EFN-null mice featured significantly increased glioblastoma cell invasion. Finally, overexpression of EPHA2 promoted stem cell properties in a kinase-independent manner (Miao et al. 2015).

More recently, Zhou and coworkers reported that inflammatory cytokines are able to promote phosphorylation of EPHA2 at Ser897 mediated by the RSK kinase and this fosters breast cancer cells metastatic properties. Ser897-phosphorylated EPHA2 co-localized with phosphorylated active form of RSK in various human tumour specimens, and this double positivity was related to poor survival in lung cancer patients (Zhou et al. 2015).

Prompted by these new informations, in this Dissertation we have explored role of EPHA2 and of its Ser897 phosphorylation in thyroid cancer.

2. AIM OF THE STUDY

In order to find novel protein kinases that may be involved in thyroid cancer, we have performed a small interfering RNA-based genetic screening in the RET/PTC1-positive TPC1 papillary thyroid cancer cell line using a siRNA library targeting the human kinome. We identified 14 hits whose silencing was able to significantly reduce the viability and the proliferation of TPC1 cells; most of them were active also in BRAF-mutant BCPAP (papillary thyroid cancer) and 8505C (anaplastic thyroid cancer) and in RAS-mutant CAL62 (anaplastic thyroid cancer) cells. Kinases relevant for thyroid cancer cell viability included SRC and MAPK (mitogen activated protein kinases) families and importantly the EPHA2 receptor (Cantisani et al. 2016).

In this framework, Aim of this study has been to understand the role of EPHA2 in thyroid tumorigenesis. In particular, we studied:

1) expression and phosphorylation of EPHA2 in thyroid cell lines harboring different oncogenic lesions;

2) how RET/PTC1 and BRAF V600E thyroid oncogenes influence EPHA2 activation;

3) role and kinases involved in mediating the activatory phsophorylation of EPHA2 on Serine residue 897.

3. MATERIALS AND METHODS

3.1 Cell culture

Thyroid cancer cells featuring different complements of genetic lesions were authenticated by SNP genotyping (**Table 2**). TPC1 and BCPAP cell lines were derived from PTC, while 8505C, SW1736, OCUT-1 and CAL62, were derived from ATC (**Table 3**). Nthy-ori 3-1 (hereafter referred to as NTHY) (ECACC, Wiltshire, UK) is a human follicular epithelial cell line derived from a normal thyroid tissue immortalized by SV40 large T gene. NTHY cells were grown in RPMI-1640 medium supplemented with 10% FBS (Foetal Bovine Serum) (GIBCO, Paisley, USA), while all the other cell lines were grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS, 2 mM L-glutamine and 100 U/mL penicillin-streptomycin (GIBCO). PC Cl 3 (hereafter referred to as PC) is differentiated non tumoral thyroid follicular cell line derived cells from 18-month-old Fischer rat. PC cells were cultured in Coon's modified HAM's F12 medium (LONZA, Walkersville, USA) supplemented with 5% CS (calf serum) [BioWhittaker for LONZA], 2 mM L-glutamine and 100 U/mL penicillin-streptomycin and a mixture of 6 hormones, including thyrotropin (10 mU/mL), hydro-cortisone (10 nM), insulin (10 μg/mL), apo-transferrin (5 μg/mL), somatostatin (10 ng/mL), and glycyl-histidyl-lysine (10 ng/mL) [Sigma-Aldrich Chemie GmbH, Steinheim, DE]. Stably transfected PC-RET/PTC1 and BRAF V600E cells as well as Doxycycline

inducible RET/PTC3 cells have been described previously (Knauf et al. 2003; Castellone et al. 2003; Wang et al. 2003).

Transformed PC cells were grown in the absence of 6H. Phoenix human embryonic kidney cells (hereafter named φχ) were used for transient expression studies. These cells were grown in DMEM contaning 10% FBS, 2 mM L-glutamine and 100 U/mL penicillin-streptomycin.

Table 2: Authentication of the thyroid cell lines used in this study.

MARKERS	NTHY	TPC1	BCPAP	8505C	OCUT-1	SW1736	CAL62
AMEL	X	X	X	X	X	X	X
D3S1358	14-16	16-17	16-17	16-17	15	16-17	16
D1S1656	15.3-17.3	16-17.3	13-17.3	12-18.3	14-15	11	17.3
D2S441	11	11	11	12-12.3	10-13	11-14	11
D10S1248	15-16	13-17	15-16	15	13-15	13-14	14-16
D13S317	11	11-12	12	13	11	11-12	12
Penta E	7-13	18	5-12	12-15	11-16	11-17	5-10
D16S539	12-13	9	11-12	12	9	11-12	12-13
D18S51	14	13-16	13-17-18	16	13	14	16
D2S1338	20-26	16-23	18	17-24	17-24	19-25	19-23
CSF1PO	12	11-12	13	12-13	11	12	9-12
Penta D	8-13	9-13	10-11	9-10	9-12	12	13
TH01	7-(9.3)	9	6-9.3	6-9	6-7	6-(9.3)	7-(9)
vWA	16-18	14-18	14-(17)	17-19	14-18	16-19	16
D21S11	29-30	30-31.2	30-31.2	28-32.2	30-32.2	29-31	32,2
D7S820	7-12	11	10	10	11	8-11	10
D5S818	11	8-10	10-11	10-11	13	12-13	9-12
TPOX	9	11	8-11	10-11	11	11	8-9
DYS391	-	-	-	-	-	-	-
D8S1179	12	11-17	12-13	10-13	13-14	13-(14)	13
D12S391	(17.3)-20	20-26	(18)-(23)	(18)-(22)	20-23	22-(23)	22
D19S433	16.2	13	13.2-15	13-14	13-14	14	14
FGA	21-22	20-21	20-23	23	21-26	22	19
D22S1045	14-16	11-15	16	15	16	15-(16)	15

Table 2: 24 genetic markers (PowerPlex Fusion System kit) were analysed by BMR Genomics s.r.l. in the indicated cell lines.

Table 3: Oncogenic lesions in the thyroid carcinoma cell lines used in this study.

CELL LINE	HISTOTYPE	GENETIC LESIONS
TPC1	PTC	RET/PTC1
BCPAP	PTC	BRAF (V600E), TP53 (D259Y), APC (A927)
8505C	ATC	BRAF (V600E), TP53 (R248G), NF2 (E129STOP), CDKN2A (del150)
SW1736	ATC	BRAF (V600E)
OCUT-1	ATC	BRAF (V600E), PI3KCA (H1047R)
CAL62	ATC	KRAS (G12R), TP53 (A161D), NF2 (E215STOP), CDKN2A (del471 and del522)

Table 3: The indicated mutations were derived from catalogue of somatic mutations in cancer (COSMIC) [www.sanger.ac.uk/genetics/CGP/cosmic/].

3.2 Cell treatments

Drug treatments were performed in low serum conditions (2.5% FBS or CS) for the indicated time points. ZD 6474 (0.25-1 µM), PLX 4032 (0.25-1 µM), U0 126 (5 µM, Cell Signaling, Danvers, USA), BI-D1870 (0.5-1 µM, Selleckchem, Munich, DE), Wortmannin (0.5 µM, Cell Signaling Technology, Lane Danvers, USA), Doxycycline (1µg/mL, Sigma-Aldrich Inc., Saint Louis, USA) were used as specified.

3.3 Plasmids construction and expression

The plasmid encoding human GFP-tagged EPHA2 (RG205725) (GenBank accession number NM_004431) was purchased from Origene Technologies (Rockville, USA) and was used also as template to generate the EPHA2 Ser897Ala (S897A) mutant. Mutagenesis was performed by using the QuickChange Site-Direct mutagenesis kit (Stratagene/Agilent Technologies, Santa Clara, USA). Primers were designed according QuickChange Site-Direct mutagenesis instruction, controlled on Operon tool (http://www.operon.com/tools/oligo-analysis-tool.aspx), and synthesized by the Ceinge Core Service Unit (Naples, IT). The mutations were confirmed by DNA sequencing performed by Ceinge Core Service Unit. Primer sequences were as follows:

Forward: 5'-gct atc cgg ctc ccc gcc acg agc ggc tcg gag-3';

Reverse: 5'-ctc cga gcc gct cgt ggc ggg gag ccg gat agc-3'.

The pRK7-myr-RSK1 (8997) (GenBank accession number NM_001006665.1) plasmid was purchased from Addgene (Addgene, Cambridge, USA). Myr-AKT and HA-MEKEE (activated MEK1 generated by replacing Ser-218 and Ser-222 by Glutamic acid) plasmids were kind gifts of Professor J. Silvio Gutkind (University of California and Moores Cancer Center, San Diego, CA).

For φχ cell transient transfections, cells were plated at 50% confluence in 60-mm poly-D-lysine Hydrobromide (10 μg/mL); 1 μg of each plasmid was mixed with 10 μL of Polyfect transfection Reagent (Qiagen, Hilden, DE) and 100 μL of serum free DMEM, incubated for 48 h in complete medium and starved O/N before collection. 8505C and PC cells were transfected using Fugene reagent (Promega Corporation, USA).

RSK knock-down experiments were performed with ON-TARGETplus SMARTpool RSK1 (L-003025-00) and RSK2 (L-003026-00) human siRNA (Carlo Erba for DharmaconGE, Cornaredo, IT). EPHA2 silencing was performed with ON-TARGETplus SMARTpool EPHA2 (E-003116-00). As suggested by manufacturer instructions, 10 μL of 5 μM siRNA were mixed with 2.5 μL of Dharmafect1 transfection reagent (Carlo Erba) in Optimem medium (GIBCO). The transfection mix was added at 25 nM final concentration and delivered to each plate for 48 h. Instead, in the silencing-rescue experiment, EPHA2 was knocked-down in 8505C cells with a human specific siRNA (SI02223508) from Qiagen and in PC rat cells with a rat ON-TARGETplus SMARTpool (L-099402-02) provided by DharmaconGE (Carlo Erba). In details, in this case, 8505C cells were transfected using 6.6 μL of 20 μM siRNA mixed with 200 μL of Optimem medium and 13.2 μL of HiPerFect Transfection reagent (Qiagen) for 10 min. The transfection reaction was then applied at 22 nM final siRNA concentration to each plate. Rat EPHA2 transient knock-down was performed in the same conditions as described for RSK silencing in medium containing 5% serum. As negative controls for all our silencing experiments, we transfected AllStars Negative Control siRNA (SI03650318, Qiagen) or siGENOME Non-Targeting siRNA (D-001206-13, Carlo Erba), as previously described. Following 36 h of silencing, rescue was obtained by transient transfection; this was performed by using EPHA2 wt (4 μg) or EPHA2 S897A mutant (7 μg) and 12 μL of Fugene (Promega). GFP, EPHA2 wt or EPHA2 S897A plasmids were transfected as control. Trypan Blue exclusion test and dosage of cleaved-PARP in protein lysates were applied to exclude non-specific toxicity of the transfection procedure (data not shown).

3.4 Protein experiments

Cell cultures plates were washed two times with ice-cold PBS 1X solution (GIBCO) and cells were scraped in fresh JS lysis buffer containing 50 mM N-2- hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES pH 7.5), 1% (vol/vol) Triton X-100, 150 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM phenylmethylsulphonyl fluoride (PMSF) and 1 μg/mL aprotinin. Lysates were than clarified by centrifugation at 13,500 rpm for 20-30 min and kept at 4°C during all passages. Protein concentration was measured using a modified Bradford Assay (Bio-Rad Laboratories, Munich, DE). Protein lysates (15-50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on Whatman Protran nitrocellulose membranes (PerkinElmer Health Sciences B.V., Groningen, NE). For EPHA2 pull-down, after precleaning with gamma-bind G sepharose beads (GE Healthcare Bio-Sciences AB, Uppsala, SE), 500 μg of fresh cell lysates in 500 μL volume were incubated overnight at 4°C (with gentle rotation) with 0.1 μg of recombinant Human Ephrin-A1 Fc protein (R&D Systems, Minneapolis, USA) for 1 mg of cell lysate. Ligand-receptor complexes were pulled-down using 50 μL of protein-G sepharose beads for 1 h at 4°C. The samples were centrifuged and washed in JS buffer, eluted in sample buffer loading dye, boiled for 5 min at 99°C and run on 7.5% SDS-polyacrylamide gel. Immunoprecipitations were performed according to standard procedures.

After blotting, membranes were incubated in 5% non-fat dry milk (Bio-Rad Laboratories) or TBS containing 5% BSA (Sigma-Aldrich Inc.) blocking solution for 1 h at room temperature and then with primary antibodies O/N at 4°C. After appropriate washes, the membranes were incubated for 1 h at room temperature with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (dilution 1:3000) from Bio-Rad Laboratories, or with HRP-conjugated donkey anti-goat secondary antibodies (dilution 1:3000) from Santa Cruz Biotechnology (Heidelberg, DE). Immunocomplexes were detected using the enhanced chemiluminescence kit (ECL) from Thermo Fisher Scientific/Life technologies (Rockford, USA); images were scanned with Epson Perfection V750 PRO and signal intensity was acquired by Cawomat 2000 IR. Primary antibodies (**Table 4**) were from Millipore (Merk Millipore Corporation/Life Science, Darmstadt, DE), R&D Systems, Cell Signaling Technology, Santa Cruz Biotechnology, Upstate (Lake Placid, USA), Sigma-Aldrich. Anti-RET is a polyclonal antibody raised against the tyrosine kinase protein fragment of human RET (Santoro et al. 1995).

Table 4: Antibodies used in this study.

ANTIBODY	BRAND	CODE
Eck/EPHA2 clone D7	Millipore	05-480
phospho-Y1062 RET	R&D Systems	AF5009
phospho-EPHA2 (Tyr772)	Cell Signaling Technology	82244
phospho-Ser897 EPHA2 D9A1	Cell Signaling Technology	6347
phospho-AKT Ser473	Cell Signaling Technology	9271
phospho-MEK 1/2 Ser217/221	Cell Signaling Technology	9121
phospho-p44/42 MAPK Erk1/2 Thr202/Tyr204	Cell Signaling Technology	4370
phospho-p90RSK Ser380	Cell Signaling Technology	9341
phospho-YB1 Ser102 C34A2	Cell Signaling Technology	2900
phospho-AKT Substate RXXS*/T* 110B7E	Cell Signaling Technology	9614
phospho-AKT Substate RXRXXS*/T* 23C8D2	Cell Signaling Technology	10001
phospho-PRAS40 Thr246	Cell Signaling Technology	2640
AKT	Cell Signaling Technology	9272
MEK1/2	Cell Signaling Technology	9122
p44/42 MAP Kinase	Cell Signaling Technology	9102
RSK1 C-21	Santa Cruz Biotecnology	sc-231
RSK2 C-19	Santa Cruz Biotecnology	sc-1430
c-Myc 9E10	Santa Cruz Biotecnology	sc-40
actin	Santa Cruz Biotecnology	sc-1616
BRAF	Upstate	07-4543
tubulin	Sigma-Aldrich	T 9026

3.5 Phosphorylation studies

φχ cells transfected with EPHA2 wt plasmid were lysed without phosphatase inhibitors in EDTA-free JS buffer according to published procedure (www.abcam.com; Feng and Irvine 2009). 30 µg of crude extracts were treated with 1U/µg of CIP (Calf Intestinal alkaline Phosphatase; New England Biolabs, Beverly, USA) for 30 min at 37°C. The reaction was stopped by adding loading dye and heating at 99°C for 5 min before SDS-PAGE. To verify whether RSK kinase was able to phosphorylate *in vitro* EPHA2, 200 ng of recombinant full length active RSK1 (R15-10G) from SignalChem (Richmond, USA) was incubated with 20 ng of EPHA2 GST-tagged cytosolic domain (PV3688) from Thermo Fisher Scientific/Life technologies in kinase buffer [25 mM MOPS (pH 7.2), 12.5 mM β-glycerophosphate, 25 mM MgCl2, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT and 50 µM ATP]. Following 30 °C incubation for 15 min, the reaction was stopped by 99°C heating in loading dye for 5 min. De-phosphorylation was performed directly on the nitrocellulose membrane after *in vitro* phosphorylation reaction. The membrane was blocked with 5% BSA in TBS with 0.1% of Triton X-100 for one hour at room temperature and then incubated with TBS-1% Triton with or without CIP (68 mU/µg) for 30 min in a 37°C water-bath. Membranes were then incubated for 1 h with phospho-S897 EPHA2 antibody. For [^{32}P]-ATP kinase assay, EPHA2 peptides spanning Serine 897 (wt:RVSIRLPSTSGSE, S897A:RVSIRLPATSGSE, R894K:RVSIKLPSTSGSE, 4A: RVAIRLPSAAGAE) were synthesized by PolyPeptide Group Laboratories (Strasbourg, FR). Kinase assay was performed using 10 µM of each peptide, 100 nM of RSK1 recombinant kinase and 50 µM of [^{32}P]-ATP for 15 min at 30 °C.

The reaction was spotted on pre-cut phosphocellulose P81 paper (Merk Millipore Corporation/Life Science). After several washes with 1% orthophosphoric acid (Carlo Erba), filters were air-dried and subjected to Cherenkov counting.

3.6 RNA extraction, cDNA synthesis and RT-PCR

For mRNA expression, each cell line was grown to 70% confluency, total RNA was extracted with RNeasy mini kit (Qiagen) according to the manufacturer's instructions and quantized with NanoDrop 2000c (Thermo Fisher Scientific/Life technologies). RNA (1μg) was reverse transcribed (RT) using a high-capacity reverse transcriptase kit (Quantitect Reverse® Transcription Kit, Qiagen) according to manufacturer's instructions. The mRNA level (40 ng for each cDNA) of RSK family members in thyroid cell lines was measured by semiquantitative RT-PCR assay using JumpStart REDTaq ReadyMix PCR (Sigma-Aldrich) according to manufacturer's instructions for 30 cycles. The levels of the housekeeping GAPDH transcript were used as a control for equal cDNA loading. The thermal protocol was: 94° for 2 min (1 cycle), 94° for 30 sec, 60 ° for 30 sec, 72° for 2 min (30 cycles), 72° for 5 min (1 cycle); reaction was stopped at 4°C. GAPDH was amplified for 25 cycles. PCR products were loaded on 1% agarose gel, stained with ethidium bromide (0.05 μg/mL), and the corresponding image was saved by Bio-Rad Quantity One software 4.5.2 (Bio-Rad Laboratories).

To exclude DNA contamination, each PCR reaction was also performed on untranscribed RNAs (data not shown).

The primers were designed using Primer-BLAST tool (available on www.ncbi.nlm.nih.gov) and synthesized by the Ceinge Core Service Unit. The sequences of used primers were shown in **Table 5**.

Table 5: primers sequence.

GENE	FORWARD	REVERSE
RSK1- RPS6KA1	5'-tgc aca gcc tgg gta tca ttt-3'	5'-ctg tcc cgc aga aag aat agg-3'
RSK2- RPS6KA3	5'-cgc tga gaa tgg aca gca aat-3'	5'- tcc aaa tga tcc ctg cc taa t-3'
RSK3- RPS6KA2	5'- gcc acc cta aaa gtt cgg gac-3'	5'- ttt cct tcc gtc tga aag gca-3'
RSK4- RPS6KA6	5'-cgg cga ggt aaa tgg tct ta-3'	5'-gca act caa act gtg cag ga-3'
GAPDH	5'-cca tca cca tct tcc agg agc g-3'	5'-aga gat gat gac cct ttt ggc-3'

3.7 Cell proliferation assays

8505C cells (4×10^5) or PC BRAF V600E (8×10^5) were plated in 60-mm dishes. Cells were then counted 48 or 72 h after plating. Each count was performed in triplicate and each point was the mean value of 3 dishes with 95% confidence intervals.

3.8 Wound healing assay

8505C cells were transfected as indicated and grown for 60 h to form cell monolayers (80% confluency). A wound of approximately 300μm width was inflicted with a sterile pipette tip. The culture medium was re-freshed to remove non-adherent cells.

Wound closure (healing) was monitored with microphotographs (6X magnification) taken with the Leica DM IL light microscope (Leica Microsystems, Wetzlar, DE) immediately (t0), 24 (t1) and 48 h (t2) after the wound in 5% serum. Experiments were performed in triplicate.

3.9 Statistical analysis

Statistical analyses were performed using a paired, two-tailed Student's t test (GraphPad Prism 3.0, GraphPad Software, San Diego, USA), and differences were considered to be statistically significant at a value of $p \leq 0.05$.

4. RESULTS

4.1 EPHA2 receptor is phosphorylated at Ser897 in thyroid cancer cell lines

Our previous studies, based on a genetic knock-down screening, indicated that EPHA2 was essential for thyroid cancer cell proliferation (Cantisani et al. 2016). In order to investigate further the role of the EPHA2 in thyroid cancer cells, we studied expression and phosphorylation of EPHA2 in a small panel of cell lines either harboring RET/PTC1, BRAF V600E or KRAS G12R point mutations (see **Table 3**). Normal follicular thyroid NTHY cells were used as control. **Figure 8** shows that EPHA2 is robustly expressed in all the cancer cell lines tested. Moreover, while phosphorylation on the autocatalytic Tyrosine (Y772) was variable and not particularly intense, phosphorylation on Ser897 was intense and consistent throughout the cell lines. This was particularly interesting considering previous evidence in the literature pointing to a specific role of Ser897 phosphorylation in transducing trasforming signals in human cancer cell lines (see Introduction).

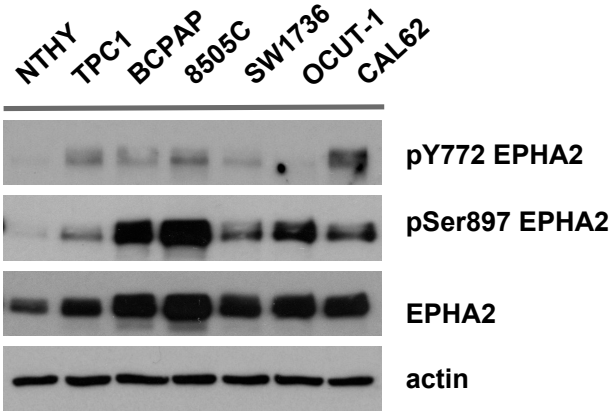

Figure 8. EPHA2 expression and phosphorylation in thyroid cell lines. Serum starved indicated cell lines were studied by Western blot analysis with the indicated antibodies. Actin was used for protein lysates normalization.

Then, to test whether the pSer897 antibody was specific for the detection of the EPHA2 Ser897 only when phosphorylated, we treated the lysates of embryonic kidney φχ cells transiently transfected with EPHA2 receptor with Calf Intestinal Phosphatase (CIP) for 30 min and tested the level of Ser897 phosphorylation by Western blot analysis. A strong reduction of pSer897 reactivity was observed after CIP treatment supporting the specificity of the used antibody (**Figure 9A**). The antibody specificity was further confirmed by transfecting φχ cells with expressing vectors containing the wild-type (wt) or the mutant (S897A) EPHA2. **Figure 9B** shows that the Alanine to Serine replacement impaired the antibody signal, further demonstrating antibody specificity.

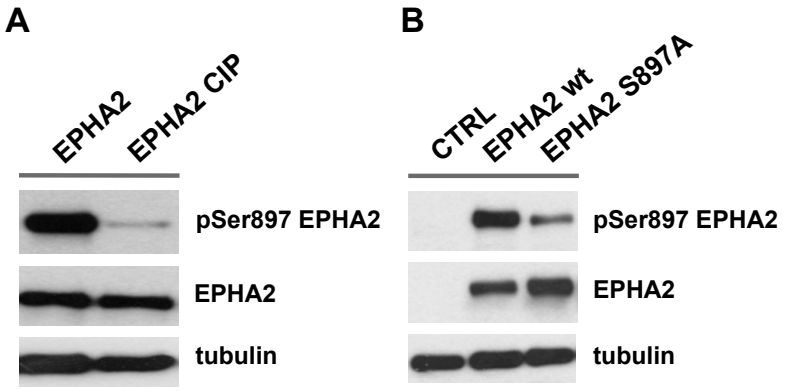

Figure 9. EPHA2 pSer897 antibody test. Reactivity with pSer897 was tested in φχ cells transiently transfected with GFP-tagged full-length EPHA2 and treated or not with Calf Intestinal Phosphatase (CIP) 1U/μg (A) and in φχ cells transfected with GFP-tagged EPHA2 S897A mutant compared to EPHA2 wild-type (wt) (B). CTRL refers to mock transfected cells. Tubulin was used for normalization.

Then, we tested by Western blot the levels of the phoshorylated Ser897 in an expanded panel of human thyroid cancer cell lines (see **Table 2** and **Table 3**): BCPAP, SW1736 and OCUT-1 (harboring BRAF V600E) and CAL62 (harboring KRAS G12R) as well as NTHY as control. Both papillary (TPC1 and BCPAP) and anaplastic (8505C, SW1736, OCUT-1, CAL62) thyroid cell lines featured highly levels of phosphorylated Ser897 compared to control (**Figure 10A**). Noteworthy, some of the cell lines tested featured activating mutation (V600E) in a pure MAPK driver (BRAF) and, accordingly, robust and consistent phosphorylation in MEK and ERK suggesting a role of MAPK pathway in sustaining Ser897 phosphorylation in addition to AKT, a kinase that has been proposed as one of the kinases able to mediate Ser897 phosphorylation in glioma cells (Miao et al. 2009).

To confirm these findings, we used the rat-derived PC thyroid follicular cell line adoptively expressing Myc-tagged versions of RET/PTC1, a MAPK and AKT driver, or BRAF V600E, a pure MAPK driver. According to the capability of MAPK of stimulating EPHA2 expression (Macrae et al. 2005), PC-RET/PTC1 and -BRAF cells expressed high levels of EPHA2 with respect to parental cells. Moreover, phosphorylation at Ser897 residue was strong in both cell lines (**Figure 10B**).

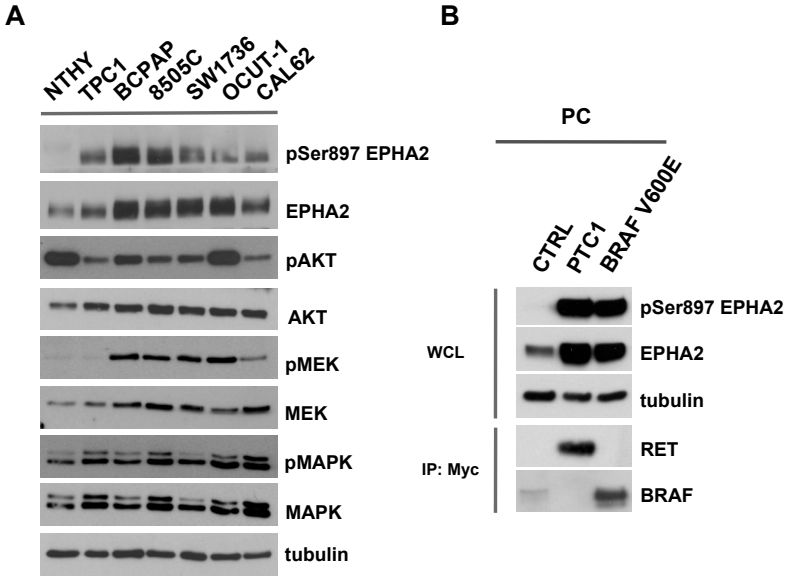

Figure 10. EPHA2 expression and Ser897 phosphorylation in thyroid cancer cells. A) The indicated thyroid cells were serum starved O/N and 15 μg of cellular extracts were immunoblotted with pSer897 and total EPHA2 antibodies. Phosphorylation of MEK (S217/S221), ERK (T202/Y204), AKT (S473) was explored with phosphospecific antibodies; B) Whole cell lysates (WCL) of parental PC cells and PC cells stably transfected with RET/PTC1 or BRAF V600E, were subjected to Western blot analysis for pSer897 and total EPHA2. 500 μg of cell lysates were immunoprecipitated (IP) with anti-tag Myc antibody and immunoblotted with pY1062 RET (RET/PTC1) or BRAF antibodies. Tubulin levels were measured for normalization. These experiments are repeated at least three times.

To dissociate the influence of RET/PTC-mediated EPHA2 upregulation from its increased phosphorylation on Ser897, a short term experiment was performed by using a conditional model represented by PC cells stably expressing another type of RET/PTC fusion (RET/PTC3 variant, e.g. the NCOA4-RET fusion) in a Doxycycline-inducible manner (Knauf et al. 2003). In this experiment, RET/PTC3 up-regulation upon Doxycyline treatment resulted in a gradual and time-dependent increase of Ser897 phosphorylation, while EPHA2 expression levels did not change significantly a these time points. In parallel, RET/PTC3 induced an early (12h-48h) MEK/MAPK and AKT activation, and a delayed (48h) activation of p90RSK, a downstream effector of MAPK (see below); p90RSK activation nicely paralleled S897 phosphorylation (see below) (**Figure 11**). Doxycycline wash-out decreased MEK, MAPK and AKT phosphorylation while Ser897 and p90RSK phosphorylation persisted for at least 48h upon the wash-out.

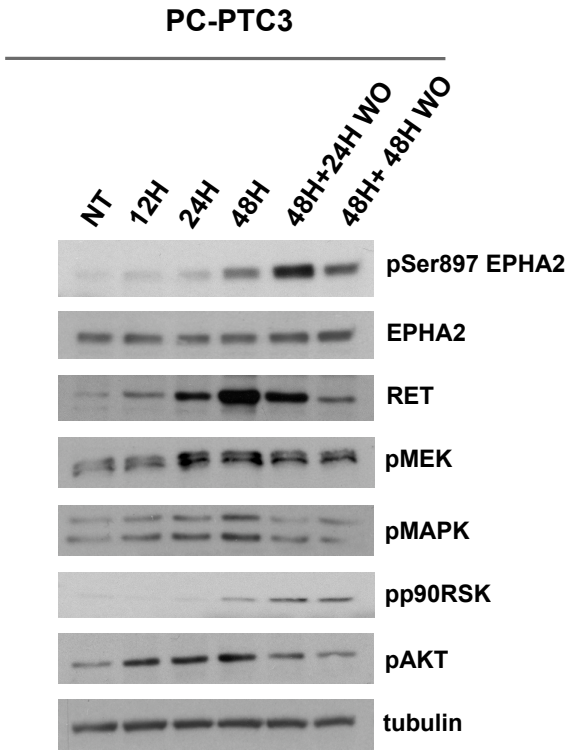

Figure 11. EPHA2 pSer897 induction in RET/PTC3 cells. PC cells stably expressing a conditional RET/PTC3 construct were stimulated with 1 μg/mL Doxycycline in low serum conditions (1%) for the indicated time points; after 48h stimulation, Doxycycline was washed-out (WO) for 24 or 48h and cell lysates were subjected to Western blot analysis with the indicated antibodies; tubulin was used for normalization. These figures are representative of three different experiments.

4.2 EPHA2 Ser897 phosphorylation correlates with oncogene activation in thyroid cancer cell lines

We tested whether the pharmacological inhibition of RET and BRAF in cell lines expressing these activated oncogenes was able to reduce levels of pSer897 EPHA2. Thus, TPC1 and 8505C cells were treated for 1h with the indicated doses of the kinase inhibitors ZD6474 (a RET inhibitor) or PLX4032 (a BRAF inhibitor), respectively. As shown in **Figure 12A**, both inhibitors were able to inhibit their own targets in a dose-dependent manner (as demonstrated by the reduced phosphorylation levels of RET, AKT, MEK and MAPK in the case of ZD6474, or MEK and MAPK in the case of PLX4032). Importantly, in parallel to MAPK pathway inhibition, both the compounds strongly reduced the phosphorylation of EPHA2 at Ser897; this suggested that a kinase in the MAPK cascade could be involved in Ser897 phosphorylation.

Therefore, we examined the aminoacid sequence surrounding Ser897. We found that Ser897 is embedded in the consensus sequence for the phosphorylation mediated by AGC (PKA, PKG, PKC) family kinases (Pearce et al. 2010). EPHA2 in particular bears a RLPS(897) sequence, with an Arginine residue at position -3 relative to Ser897 (**Figure 12B**). Accordingly, in thyroid cancer cells EPHA2 protein, pulled-down with recombinant EFNA1 ligand, was recognized by a phospho-specific antibody targeting the RXXpS/T sequence (containing an Arginine at position -3), but not by an antibody targeting the RXRXXpS/T sequence (requiring also an Arginine at position -5) used as a control (**Figure 12C**). Of note, one member of the AGC family kinases is p90 ribosomal S6 kinase (p90RSK) is a well-known effector of the MAPK signaling cascade (Galan et al. 2014).

Indeed, p90RSK contains an effector N-terminal (NTKD) kinase domain that belongs to the AGC family. NTKD is linked to a C-terminal (CTKD) regulatory kinase domain, that instead belongs to the CaMK family (Romeo et al. 2012). NTKD phosphorylates substrates containing the RXXS/T consensus (Galan et al. 2014; Moritz et al. 2014). In details, ERKs initiate p90RSK activation via phosphorylation of CTKD on T573; in turn, CTKD phosphorylates S380 within the linker region and this primes NTKD for final activation mediated by PDK1 (Romeo et al. 2012). Noteworthy, p90RSK has been recently demonstrated to be able to phosphorylate EPHA2 on Ser897 (Zhou et al. 2015).

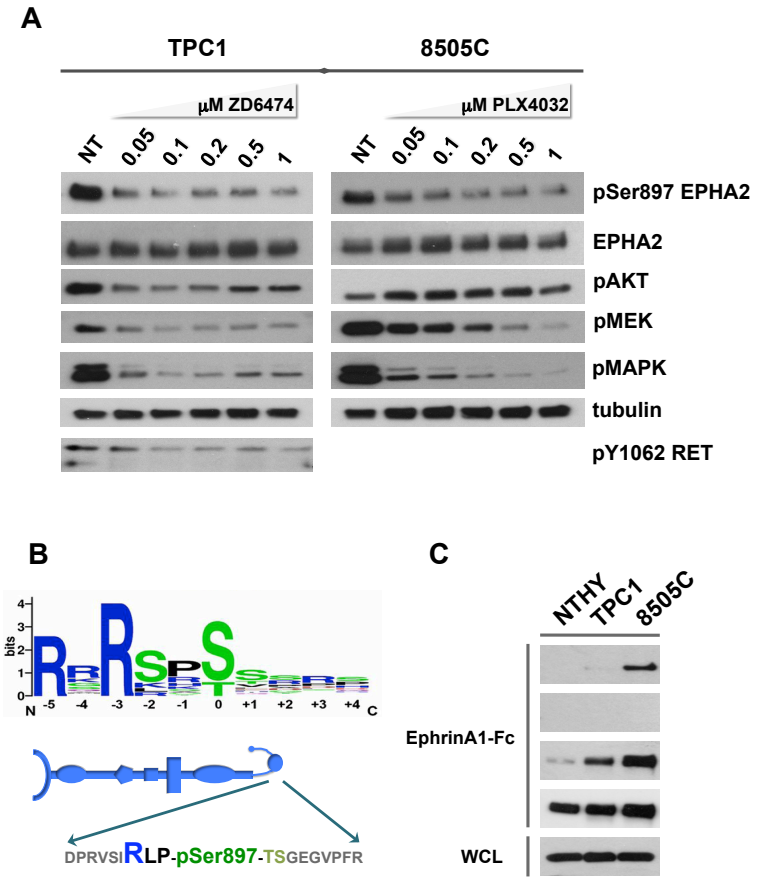

Figure 12. EPHA2 Ser897 as a MAPK pathway target. A) TPC1 and 8505C cells were treated for 1h with ZD6474 or PLX4032 at the indicated doses, and lysates were immunoblotted with the indicated antibodies; B) Ser897 of EPHA2 is embedded in a p90RSK kinase (N-terminal kinase domain: NTKD) consensus sequence; in the reported WebLOGO representation, bits on the ordinate axis show the relative relevance of the conserved aminoacids (Galan et al. 2014); C) NTHY, TPC1 and 8505C cells underwent pull-down assay with EphrinA1-Fc; upon SDS-PAGE, the indicated antibodies recognizing phosphorylated RXXS/T or RXRXXS/T sequences, or pSer897 and EPHA2 were used. Tubulin was used as loading control (WCL). These experiments are repeated at least three times.

4.3 p90RSK inhibition attenuates EPHA2 Ser897 phosphorylation in thyroid cancer cell lines

To dissect the signaling cascade involved in EPHA2 Ser897 phosphorylation, RET/PTC1-positive TPC1 cells and BRAF V600E-positive 8505C cells were treated with the ATP-competitive RET (ZD6474) or BRAF (PLX4032) kinase inhibitors, respectively, as well as with MEK (UO126), NTKD RSK (BI-D1870), or PI3K (Wortmannin) inhibitors. As expected, in TPC1 cells, treatment with ZD6474 abrogated the phosphorylation of the MAPK signaling cascade effectors (RET, MEK, MAPK, p90RSK) (Santoro and Carlomagno 2013), as well as effectors of the PI3K/AKT signaling pathway (AKT). Phosphorylation of YB1 and PRAS40 was also studied since YB1 is a well-known substrate of both AKT and p90RSK and PRAS40 is an AKT substrate (Davies et al. 2015; Wang et al. 2012). In these cells, RET inhibition attenuated Ser897 phosphorylation, suggesting that both MAPK and AKT signaling pathways concurred in the EPHA2 phosphorylation (**Figure 13,** left panel). Accordingly, in these cells both UO126 and Wortmannin treatments caused a significant de-phosphorylation of Ser897 (though it should be noted that Wortmannin also caused a reduced phosphorylation of MEK). Differently, in 8505C cells, PLX4032 treatment abrogated the phosphorylation of MEK, MAPK, p90RSK and YB1, but not AKT or PRAS40, suggesting that, in these cells driven by a BRAF mutation, only the MAPK pathway is involved in EPHA2 phosphorylation on Ser897; accordingly, Wortmannin had virtually no effect on Ser897 phosphorylation (**Figure 13,** right panel). Importantly, in both cell lines treatment with the p90RSK (NTKD) inhibitor (BI-D1870) strongly decreased Ser897 phosphorylation, in parallel to YB1 dephosphorylation (**Figure 13**).

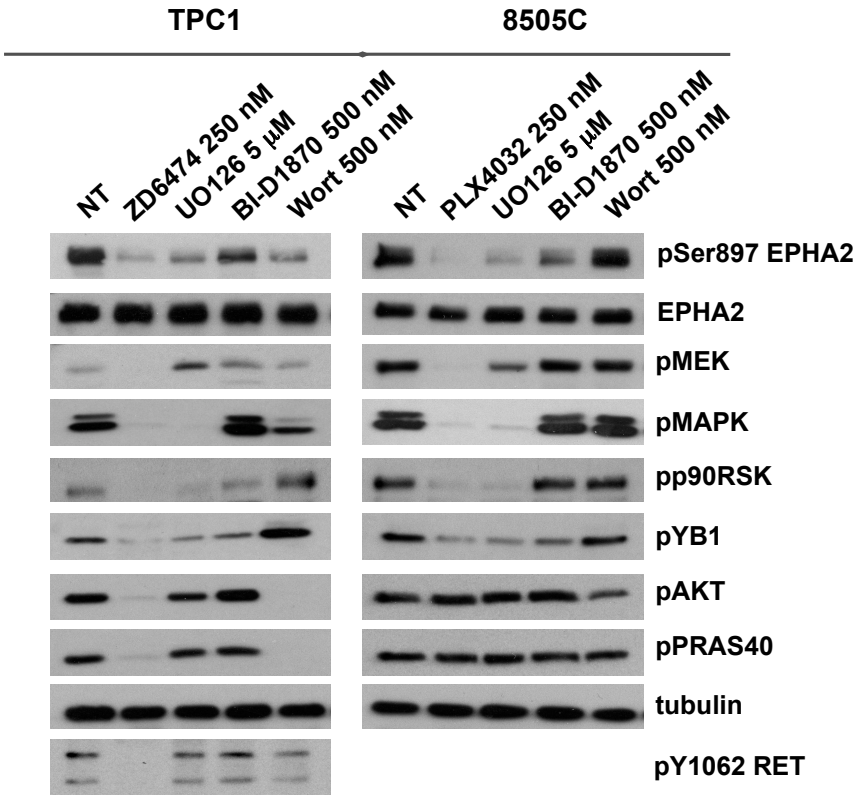

Figure 13. Modulation of Ser897 EPHA2 phosphorylation in thyroid cell lines by different chemical inhibitors: Protein lysates (15 μg) were harvested from TPC1 or 8505C cells treated in low serum condition with indicated doses of ZD6474, PLX4032, UO126, BI-D1870 or Wortmannin (Wort) for 1h. Western blots were probed with the indicated antibodies. Tubulin was used as loading control. These figures are representative of three independent experiments.

Similarly, in PC-RET/PTC1 or PC-BRAF cell lines, the inhibition of RET and BRAF, respectively, induced a reduction of pSer897 levels, whereas the PI3K inhibitor Wortmannin, did not. In particular, despite the efficient AKT inhibition, Wortmannin showed only a modest effect on the phosphorylation of Ser897 in PC-RET/PTC1 and even a more modest effect in PC-BRAF cells (**Figure 14**).

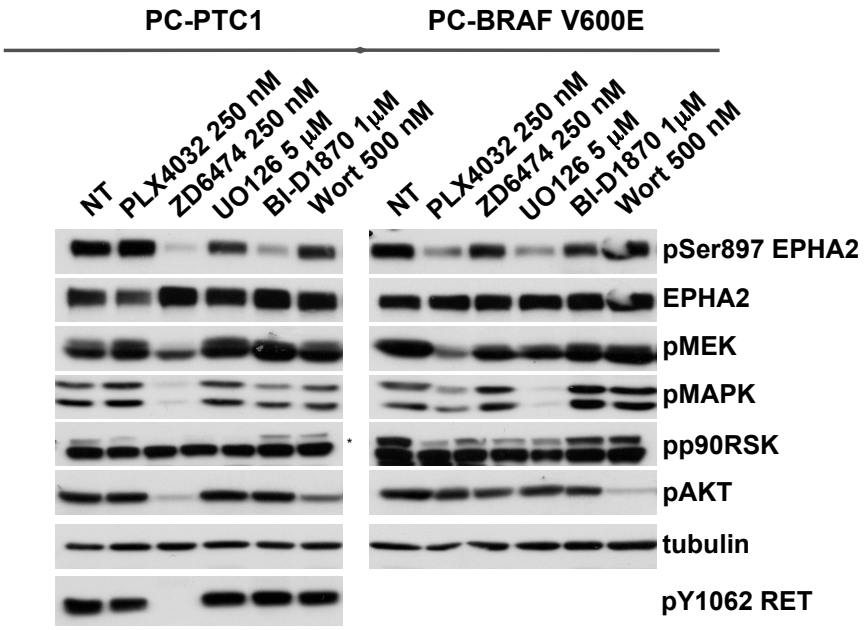

Figure 14. Modulation of Ser897 EPHA2 phosphorylation in thyroid cell lines by different chemical inhibitors: Protein lysates (15 μg) were harvested from RET/PTC1 or BRAF V600E expressing PC cells treated in low serum condition with indicated doses of ZD6474, PLX4032, UO126, BI-D1870 or Wortmannin (Wort) for 1h. Western blots were probed with the indicated antibodies. The asterisk (*) indicates an aspecific faster migrating band identified by the p90RSK antibody. Tubulin was used as loading control. These figures are representative of three independent experiments.

Finally, in agreement with these findings, BRAF, MEK and p90RSK inhibition in additional thyroid carcinoma cell lines (BCPAP and SW1736 carrying BRAF V600E and CAL62 carrying KRAS G12R see **Table 3**) was able to blunt pSer897 levels, whereas Wortmannin had negligible effect (**Figure 15**). Noteworthy, in CAL62 cells, as previously described (Heidorn et al. 2010), BRAF inhibition caused a paradoxical activation of the MAPK signaling cascade rather than inhibition, probably due to the formation of active RAF dimers in a RAS-dependent manner; consistently, this was associated to increased EPHA2 pSer897 levels (**Figure 15**, right-end panel).

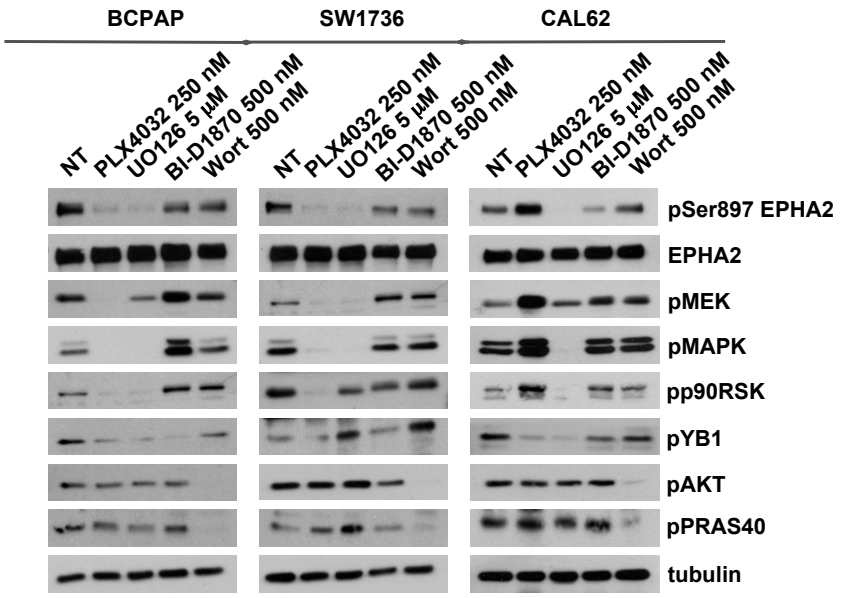

Figure 15. Modulation of Ser897 EPHA2 phosphorylation in a panel of thyroid cancer cell lines. BCPAP, SW1736 and CAL62 cells were treated with indicated doses of PLX4032, UO126, BI-D1870 and Wortmannin (Wort) for 1h. Drug treatments were performed in low serum for 1h; protein lysates were immunoblotted with antibodies recognizing MEK/MAPK/p90RSK pathway components (pMEK, pMAPK, p90RSK, pYB1) as well as AKT pathway (pAKT, pPRAS40). Levels of EPHA2 and pSer897 EPHA2 were tested. Tubulin was used as loading control. These images are representative of three independent experiments.

Altogether, these data showed that, in BRAF transformed cells, EPHA2 phosphorylation at Ser897 is sustained by MAPK signaling and in particular by p90RSK. In RET mutant thyroid cancer cells, PI3K pathway, together with the MAPK pathway, may concur to mediate Ser897 phosphorylation. However, cell treatment with MK2206, an AKT kinase inhibitor, failed to significantly downmodulate Ser897 phosphorylation (data not shown) pointing against a crucial role of AKT in this process.

4.4 p90RSK induces EPHA2 Ser897 phosphorylation

The above reported findings pointed to a role of MAPK system in EPHA2 Ser897 phosphorylation. Thus, in order to formally identify the kinase able to mediate EPHA2 Ser897 phosphorylation, we transfected φχ cells with active forms of AKT (Myr-AKT), MEK (HA-tagged MEKEE) or RSK1 (Myr-RSK1). The full-length GFP-tagged EPHA2 and GFP alone were used respectively as controls, respectively. Transfection with either AKT or MEK and RSK induced a modest increase of endogenous EPHA2 expression. Besides that, it was important to observe that Myr-RSK1 expression was able to induce a strong potentiation of Ser897 phosphorylation. AKT and MEK were clearly weaker that RSK at inducing such a phosphorylation despite enhancing their signaling (pAKT on S473 and pERK on T202/Y204, respectively). It is possible that the weak activity of MEK in this system is due to low levels of endogenous RSK expressed in the used cell type (**Figure 16**).

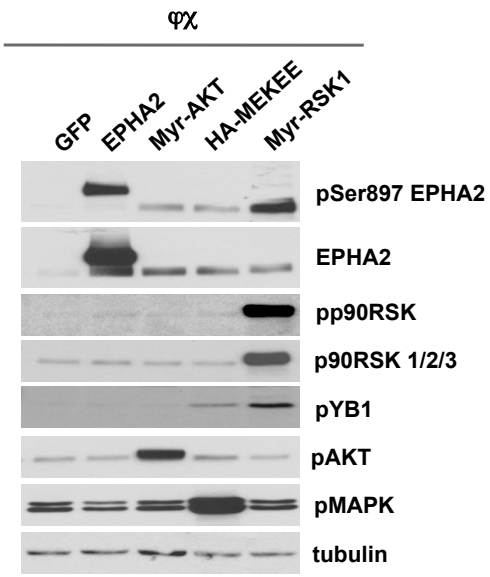

Figure 16. EPHA2 and pSer897 induction. φχ cells were transiently transfected with GFP, GFP-EPHA2, Myr-AKT, HA-MEK-EE or Myr-RSK1 plasmids. Cell lysates (50 μg) were starved O/N after transfection and subjected to Western blotting with the indicated antibodies; tubulin was used for normalization. These figures are representative of three different experiments.

We aimed at obtaining a genetic evidence of the *in vivo* role of p90RSK in mediating Ser897 phsophorylation. p90RSK family contains 4 members, among which p90RSK1 and p90RSK2 are the best characterized and most commonly involved in cancer (Sulzmaier and Ramos 2013). Initially, we analysed by RT-PCR the expression levels of the 4 isoforms in a panel of thyroid cancer cell lines. RSK1 and 2 resulted consistently expressed throughout the cell lines tested (**Figure 17)**. Therefore, we performed a siRNA-mediated knock-down experiment in 8505C cells to blunt expression of p90RSK1, p90RSK2 or both.

Results showed that the simultaneous knock-down of both p90RSK1 and 2 significantly reduced phosphorylation of EPHA2 Ser897 as well as of YB1, a *bona fide* p90RSK substrate, while knock-down of either p90RSK1 or p90RSK2 alone did not significantly affect EPHA2 phosphorylation (**Figure 17**). It has been previously reported that RSK1 and 2 play redundant functions and that therefore it is required to knock-down both to achieve a significant effect (Torchiaro et al. 2015).

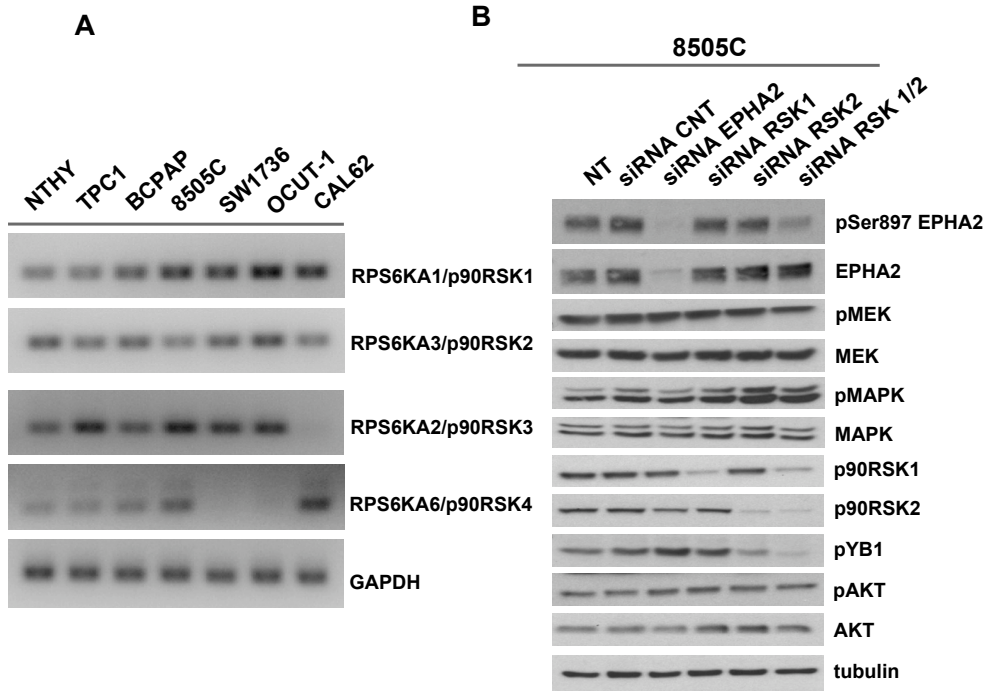

Figure 17. p90RSK as a EPHA2 Ser897 kinase. A) The mRNA levels of p90RSK1-4 were tested by semiquantitative RT-PCR in the indicated cell lines. GAPDH was used for normalization; B) Silencing of endogenous EPHA2, p90RSK1, p90RSK2 or combination thereof was obtained by indicated siRNA (25 nM) transfection in 8505C cells; 48h after transfection, cells were lysed and analyzed by Western blot with indicated antibodies. Tubulin was used as internal control. These figures are representative of three different experiments.

Finally, we sought to obtain the formal proof that RSK was a Ser897 kinase. To this aim, we performed an *in vitro* kinase assay by using full-length GST-tagged recombinant p90RSK1, as a kinase, and the cytoplasmic EPHA2 (cEPHA2) domain, as a substrate. As shown in **Figure 18**, when incubated alone, cEPHA2 showed a detectable level of reactivity with the pSer897 antibody that reflected a baseline level of Ser897 phosphorylation as demonstrated by the knock down of the signal upon incubation with CIP. Importantly, the incubation with p90RSK1 strongly increased Ser897 phosphorylation; again this signal was almost completely abolished upon CIP treatment (**Figure 18**).

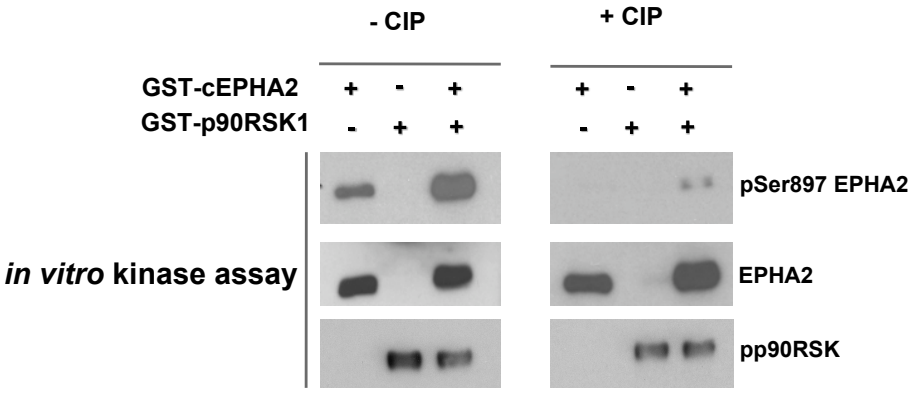

Figure 18. p90RSK phosphorylates EPHA2 on Ser897 *in vitro*. Recombinant GST-tagged EPHA2 cytosolic domain (amino acids 560-976) was incubated alone or with GST-tagged full-length p90RSK for 15 min at 30 °C in kinase buffer. The assay reaction was splitted in two tubes and, after SDS-PAGE, the resulting membranes were blocked in TBS-BSA 0.1% for 1h, and subjected or not to CIP treatment (68 mU/µg, 1h, 37°C) before immunoblotting with pSer897 EPHA2, total EPHA2 or p90RSK antibodies.

To confirm these findings and to finely map the phosphorylation target sequence, we generated minimal EPHA2 peptides either containing the wild type sequence spanning Ser897 (wt), the replacement of S897 with the non-phosphorylatable Alanine residue (S897A), or all the 4 Serine/Threonine residues substituted by Alanine residues (4S/T→A). A peptide where the RXXpS consensus sequence was disrupted by replacing the Arginine at position -3 with a Lysine residue (R894K) was also used. Then, we tested *in vitro* the capability of recombinant p90RSK of inducing detectable phopshorylatio of these peptides. Incubation with p90RSK and γ-^{32}P ATP induced a robust incorporation of ^{32}P in the wt peptide but not in its mutated versions. These data indicated that p90RSK is able to phosphorylate a EPHA2 peptide containing S897, that this phosphorylation depends on the integrity of S897 and finally that it depends on the presence of a consensus sequence (Arginine at position -3) for the phosphorylation mediated by the N-terminal p90RSK kinase domain (NTKD) (**Figure 19**).

Altogether, these findings proved that p90RSK is able to phosphorylate Ser897 both in intact cells as well as *in vitro*.

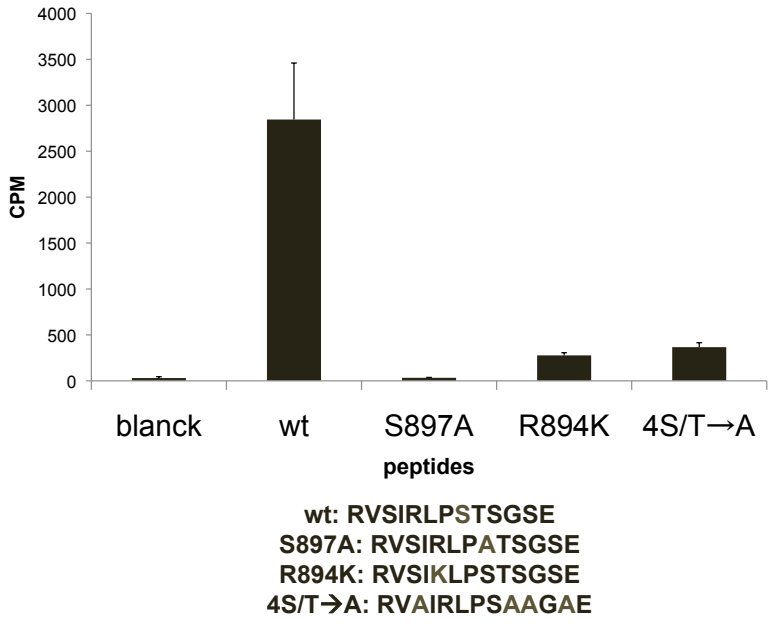

Figure 19. p90RSK phosphorylates EPHA2 peptides on Ser897 *in vitro*. Recombinant p90RSK1 (100 ng) was incubated with the indicated EPHA2 synthetic peptides (wild-type, S897A mutant, R894K mutant, and 4S/T→A mutant) in the presence of γ-^{32}P ATP for 15 min at 30°C. Peptides were spotted onto P81 filters, and the incorporation of ^{32}P was measured by Cherenkov counting and reported as counts per minute (CPM). Data shown are the mean ± SD of three independent experiments.

4.5 Ser897 phosphorylation of EPHA2 regulates growth of thyroid cancer cells

To explore the significance of EPHA2 phosphorylation on Ser897 in thyroid cancer cells, we performed a siRNA-based EPHA2 knock-down combined with EPHA2 rescue in human 8505C and in rat PC-BRAF cells. As shown in **Figures 20** and **21**, EPHA2 silencing reduced number of both cell types compared to control siRNA transfected cells. The co-transfection with a GFP-tagged EPHA2 construct was able to rescue number of cells in both cases; rescued levels of EPHA2 were confirmed by the immunoblot experiment reported in **Figure 22.** More importantly, a non-phosphorylatable S897A mutant version of EPHA2 was virtually inactive in this assay in both cell lines (**Figure 20**-**21**). In the case of 8505C cells both Trypan-blue exclusion assay and dosage of PARP cleavage indicated that effects of EPHA2 silencing were cytostatic neither than apoptotic (data not shown).

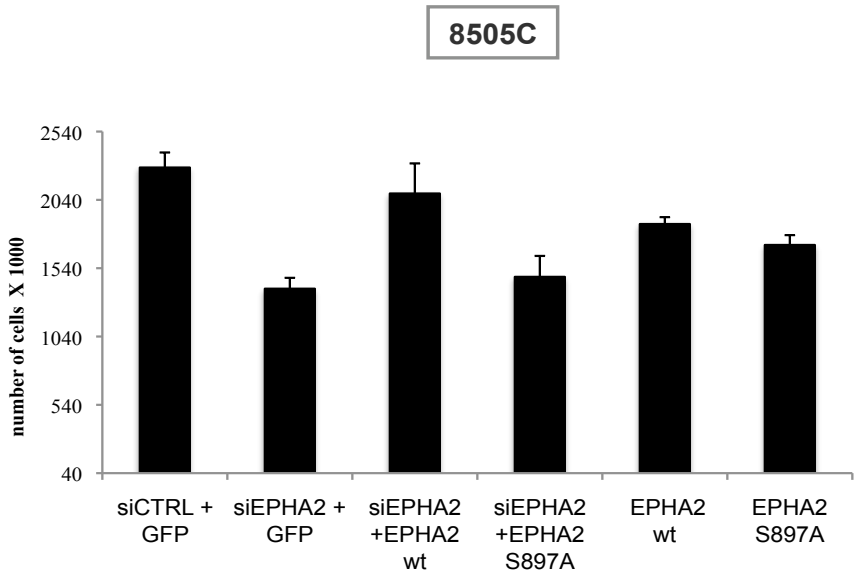

Figure 20. Effects of EPHA2 silencing on growth of 8505C thyroid cells. EPHA2 expression was knocked-down by siRNA transfection in 8505C cells. Control siRNA (siCTRL) was used as negative control. Upon 36h of silencing, rescue of EPHA2 expression was obtained upon transfection with wild-type (wt) or S897A mutant (S897A) EPHA2. 36h after rescue, cells were counted and reported as the average values of triplicate dishes ± SD. These data are representative of three independent experiments.

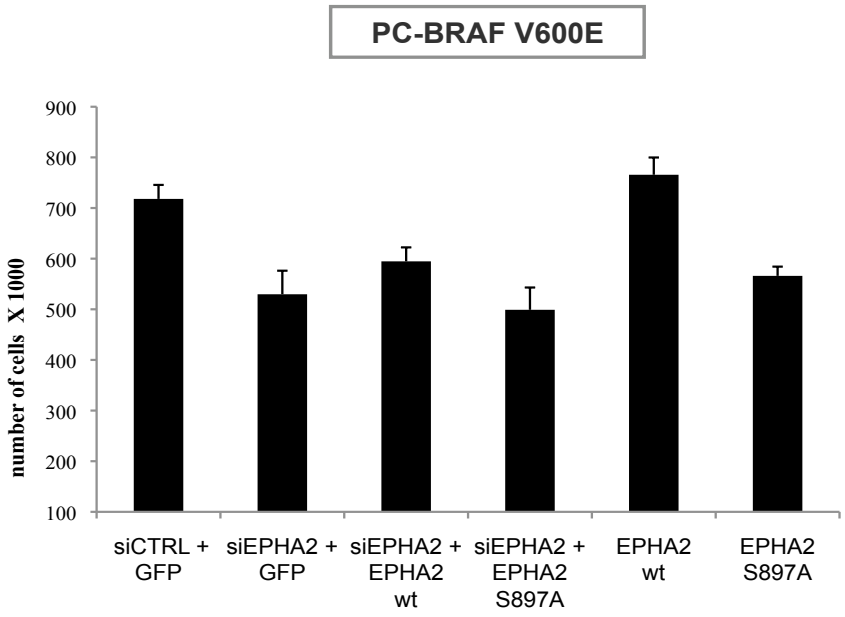

Figure 21. Effects of EPHA2 silencing on growth of BRAF V600E expressing PC thyroid cells. EPHA2 expression was knocked-down by siRNA transfection in PC-BRAF V600E cells. Control siRNA (siCTRL) was used as negative control. Upon 36h of silencing, rescue of EPHA2 expression was obtained upon transfection with wild-type (wt) or S897A mutant (S897A) EPHA2. 36h after rescue, cells were counted and reported as the average values of triplicate dishes ± SD. These data are representative of three independent experiments.

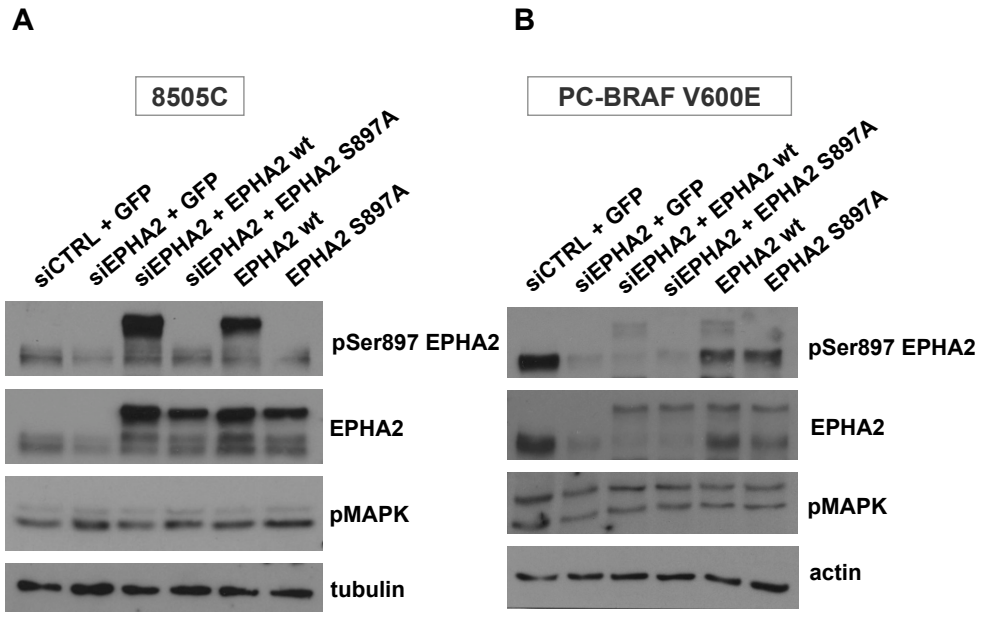

Figure 22. EPHA2 silencing in BRAF mutant thyroid cells. 8505C (A) or PC-BRAF V600E (B) were treated as shown in Figures 20-21. Upon 36h of silencing, rescue of EPHA2 expression was obtained upon transfection with wild-type (wt) or S897A mutant (S897A) EPHA2. 36h after rescue, cells were lysated and immunoblotted with indicated antibodies. Tubulin or actin were used for normalizzation. These experiments were performed at least three times.

4.6 Ser897 phosphorylation of EPHA2 regulates motility of thyroid cancer cells

As previously demonstrated by Miao and coworkers (2009 and 2015) and, more recently, by Zhou and coworkers (2015), Ser897 phosphorylation of EPHA2 regulates cancer cell migration and invasion. We therefore tested whether this applied also to thyroid cancer cells.

siRNA-mediated knock-down of EPHA2 reduced the ability of 8505C cells to close an artificial wound, while transfection with wt EPHA2, but not with the S897A mutant, was able to restore the ability of closing the scratch (**Figure 23**).

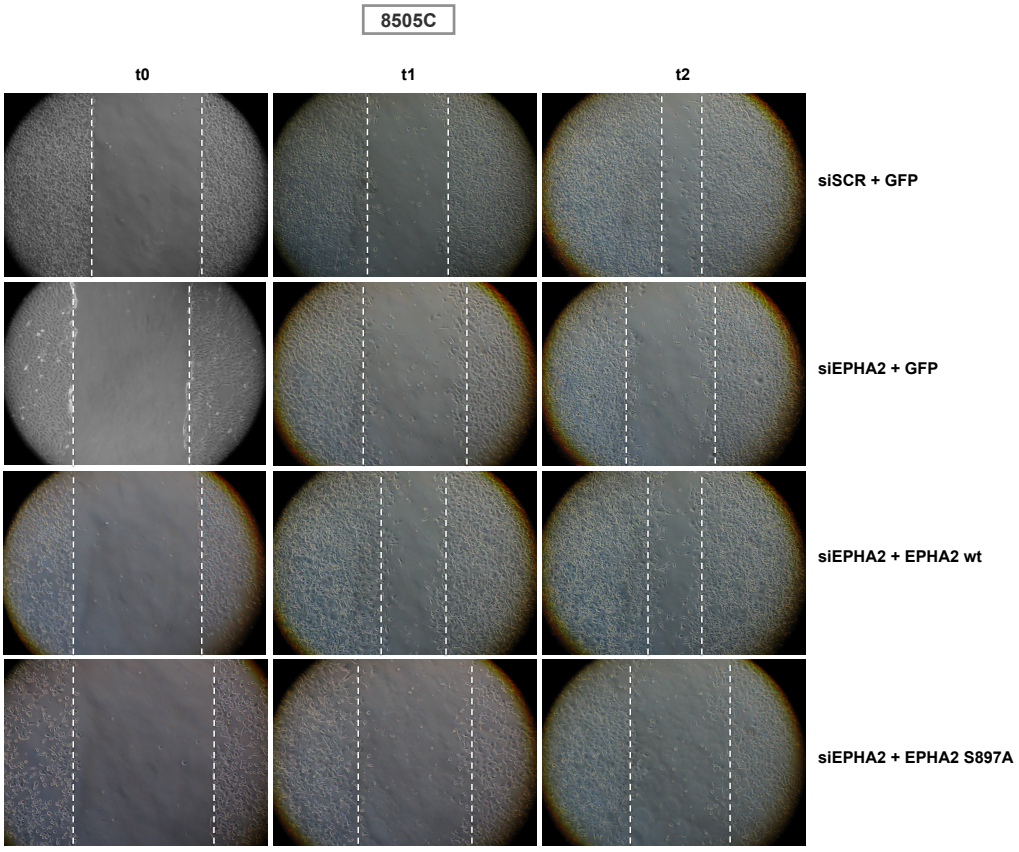

Figure 23. Effects of EPHA2 silencing on *in vitro* migration of 8505C cells. Wounds of about 300 μM width were inflicted to monolayers of 8505C cell cultures treated (EPHA2 knock-down followed by EPHA2 transfections) as in Figures 20 and 22. Wound closure was measured after 24 (t1) and 48 (t2) h. The vertical dotted lines indicate the margins of wound. Representative micrographs are shown: magnification = 6X. These figures are representative of at least three independent assays.

5. DISCUSSION

5.1 Role of EPHA2 phosphorylation on Serine 897 in thyroid neoplastic transformation

Here we have explored role of EPHA2 in thyroid carcinogenesis. Our interest in studying EPHA2 was prompted by a previous screening conducted in our laboratory to identify protein kinases involved in thyroid cancer cell viability (Cantisani et al. 2016). In this screening, we used a library of synthetic siRNAs targeting the 518 human protein kinases and we tested the capability of the individual siRNAs of blunting viability of the RET/PTC1-positive human papillary thyroid carcinoma TPC1 cell line (**Table 3**). Silencing of about 50 genes reduced TPC1 cell viability. Then, the number of positive hits was reduced to 14 based on subsequent validation experiments involving the identification of the most potent siRNAs (able to reduce cell viability by at least 30%), through the use of control siRNAs, and the use of a control cell line (NTHY). Proliferation of additional thyroid cancer cell lines (BCPAP, 8505C and CAL62 cell lines) with different histotypes and different complements of genetic lesions with respect to TPC1 (BCPAP and 8505C cells are positive for BRAF V600E, CAL62 are positive for KRAS G12R) (**Table 3**) was also inhibited by virtually all 14 antiproliferative hits. The 14 hits included EPH receptors (EPHA2, EPHA7, EPHB2, EPHB6), SRC family kinases (FYN, HCK), kinases of the p38 and JNK signaling cascades (MAP3K7IP1, MAPKAPK2 and PKN1) or of the PI3K/mTOR (AKT2) or the PKA/cyclic AMP pathway (PRKACB) [Cantisani et al. 2016].

Among these hits, we foused in particular on EPHA2 based on previous evidence supporting the role of this receptor in different human cancer types, including thyroid carcinoma (see below).

Data discussed in this Dissertation show that EPHA2 is strongly expressed in a panel of human thyroid carcinoma cells lines deriving from both RET/PTC1- or BRAF V600E-positive papillary thyroid carcinomas or BRAF- or KRAS G12R-positive anaplastic thyroid carcinomas. In these cell lines, EPHA2 is robustly phosphorylated on Ser897. RNAi-based silencing experiments confirmed the role of EPHA2 in thyroid cancer cells proliferation and motility and rescue experiments with wild-type and S897A mutant EPHA2 showed that Ser897 is essential for these oncogenic effects. Transfection of rat thyroid PC cells with BRAF V600E and RET/PTC variants confirmed the capability of the two oncogenes of inducing expression and Ser897 phosphorylation of EPHA2. Use of a Doxycycline-inducible PC-RET/PTC3 model showed, in a time-course experiment, that oncogene expression was directly linked to Ser897 phosphorylation. Phosphorylation on Ser897 was impaired by chemical inhibitors of the driving oncoprotein (RET/PTC1 in the TPC1 and BRAF V600E in the 8505C cell lines, respectively) and by inhibitors of the MAPK (MEK) pathway. Since Ser897 is embedded in a consensus sequence (Arginine at position -3 with respect to the phosphorylatable residue) for the phosphorylation mediated by AGC family Serine/Threonine kinases, we hypothesized that a member of this family that was involved in the MAPK cascade could be directly or indirectly involved in EPHA2 Ser897 phosphorylation and pointed to ribosomal S6 kinase p90 (p90RSK) as a reasonable candidate.

Accordingly, both p90RSK1 and p90RSK2 were consistently expressed in thyroid carcinoma cells and a chemical inhibitor of p90RSKs (BI-D1870) was able to reduce phospho-Ser897 content of EPHA2. Similarly, p90RSK1 overexpression in φχ cells increased Ser897 phosphorylation, confirming again that, directly or indirectly, p90RSK was able to phosphorylate EPHA2 on Ser897. Finally, an *in vitro* kinase assay using the cytosolic EPHA2 domain as a substrate showed that recombinant p90RSK1 was able to increase reactivity with a specific phospho-Ser897 antibody and another *in vitro* kinase assay using as substrate peptides spanning the EPHA2 sequence containing S897 proved that p90RSK1 was able to directly phosphorylate Ser897; such a phosphorylation required the Arginine residue at position -3 with respect to Ser897, consistent with the substrate selectivity of p90RSK. However, while in BRAF-mutant thyroid cancer cells p90RSK is the dominant Ser897 EPHA2 kinase, a role of AKT in RET/PTC-positive cells can also be envisaged.

Our findings are consistent with previously published observations. In particular, Miao and coworkers showed that while EFNA1-mediated RTK activation of EPHA2 blunted migration of glioma and prostate cancer cells. Ser897 phosphorylation mediated by AKT promoted in an EFNA1-independent and RTK-independent manner cell migration, multiple growth factors (EGF, FGF, PDGF, HGF) stimulated Ser897 phosphorylation and this event in human glioma samples correlated with tumor grade (Miao et al. 2009). Mechanistically, Ser897-mediated and EFN- and RTK-independent EPHA2 activity promoted invasiveness and stem cell properties of multiple glioma cell lines (Miao et al. 2015). Of note AKT is part of the AGC family kinases as well as p90RSK and the two kinases share a common consensus sequence for phosphorylation (see below) [Mendoza et al. 2011]. More recently, Zhou and coworkers reported that TNF-α induced Ser897 phosphorylation of EPHA2, while EFNA1 induced its phosophorylation on Y588 in HeLa cells.

Moreover, similar to our findings p90RSK was able to directly phosphorylate Ser897 and this mediated motility of breast cancer cells as demonstrated by the use of p90RSK siRNAs as well as chemical inhibitor BI-D1870. Finally, the p90RSK-EPHA2 Ser897 pathway was found constitutively active also in BRAF or NRAS mutant melanoma cell lines (Zhou et al. 2015).

5.2 Role of EPHA2 in cancer

EPHA2 is a member of the A family of EPH receptors and it is the EPHA subtype most commonly involved in human cancer.

Physiological role of EPHA2 seems to be mainly related to regulation of cell-to-cell interactions, based on its capability of regulating components of tight and adherens junctions (Miao et al. 2009). In mice, the only two phenotypes related to EPHA2 ablation are the presence of a kinked tail and cataract. Interestingly, EPHA2 (at chromosome 1p36) rare genetic variants have been found to exert a strong linkage with cataract occurrence in Caucasians. One of these variants, Arg721Gln increases basal kinase activity of EPHA2 (Jun et al. 2009).

While its role in development is still poorly understood, EPHA2 has a strong association with cancer. EPHA2 is rarely mutated but it is frequently overexpressed in carcinomas of the breast, lung, and several other cancer types (Amato et al. 2014; Brantley-Sieders et al. 2008; Hafaner et al. 2006; Hatano et al. 2005; Herath et al. 2006; Kinch et al. 2003; Miyazaki et al. 2003; Nakamura et al. 2005; Walker-Daniels et al. 1999; Xu et al. 2014; Zelinski et al. 2001).

In particular, EPHA2 has been involved in thyroid carcinoma (Lisabeth et al. 2013). In an immunohistochemistry-based screening, EPHA2 was demonstrated to be upregulated in papillary carcinomas (Karidis et al. 2011) and it was essential for invasion of the FTC-238 and FTC-133 thyroid cancer cell lines (O'Malley et al. 2012).

In non-small cell lung carcinomas (NSCLC), EPHA2 was associated to poor outcome and its genetic disruption in model of KRAS-induced NSCLC impaired tumor growth. *In vitro* EPHA2 knock-down reduced growth and survival of NSCLC cell lines and the EPHA2 ATP-competitive kinase inhibitor ALW-II-41-27 reduced tumorigenicity in a NSCLC xenograft model (Amato et al. 2014). More recently, EPHA2 inhibition by ALW-II-41-27 was also demonstrated to be able to overcome resistance of NSCLC cell lines to the EGFR TKIs erlotininb or AZD9291 (Amato et al. 2016).

In mammary carcinomas, EPHA2 has been reported to be ovrexpressed in aggressive subtypes featuring a poor prognosis. Moreover, EPHA2 ablation reduced initiation and metastatic progression of mammary carcinomas in MMTV-ERBB2 transgenic mice. Accordingly, EPHA2 formed a protein complex ERBB2 and enhanced activation of RAS/MAPK and Rho pathways (Brantley-Sieders et al. 2008).

In melanomas, EPHA2 was recently reported to be involved in resistance and adaptation to BRAF/MEK kinase inhibitors. Chronic exposure to BRAF inhibitors caused upregulation of EPHA2 and of its phosphorylation on Ser897 accompanied by increased cell invasion and reduced expression of EFNA1 ligand (Paraiso et al. 2010).

Similarly, in melanoma cell lines that developed resistance to BRAF chemical inhibition as well as in tumor specimens from patients who relapsed upon BRAF inhibitor treatment, EPHA2 was found overexpressed and hyper-phosphorylated on Ser897 and treatment with ALW-II-41-27 EPHA2 TKI was able to suppress BRAF inhibitor-resistant melanoma cells (Miao et al. 2014)

Finally, knock-out mice showed that EPHA2 was dispensable for normal hematopoiesis as well as for acute myleoid leukemias initiated by the MLL-AF9 oncogene, however radiolabeled anti-EPHA2 monoclonal antibodies blocked leukemogenesis in experimental mice (Charmsaz et al. 2015)

Accordingly, EPHA2 has been considered as a promising molecular target for cancer treatment and various tools are being explored to hit EPHA2 in different cancer types, including EPHA2 monoclonal antibody, polyspecific antibodies designed to target different EPH simultaneously, soluble or endotoxin-conjugated EFNA1, small molecule tyrosine kinase inhibitors (such as ALW-II-41-27), gold- or polyethylene glycol (PEG)-coated EFNA1 nanoschells, human adenoviruses engineering with EFA1 extracellular domain or with EFNA1 mimetic homing peptide (YSAYPDSVPMMSK named YSA), functionalized YSA-nanocarriers for drug delivery (www.clinicaltrials.gov; Guo et al. 2015; Tandon et al. 2011; Ozcan et al. 2015). Moreover, some studies have tested the combination of EPHA2-targeting therapies with chemotherapeutics or other targeted therapies, such as combination of EPHA2-antibody and paclitaxel in ovarian tumors, EPHA2 siRNA with FAK or Src siRNAs, EPHA2 antibody with tamoxifen, EPHA2 inhibitors and gemcitabine in pancreatic cancer (Koch et al. 2015; Amato et al. 2016; Quinn et al. 2016).

5.3 Role of MAPK signaling cascade in thyroid cancer

Thyroid cancer genome is dominated by lesions targeting the MAPK signaling cascade (Nikiforov et al. 2011; Xing, 2013; Xing et al. 2013; Wells and Santoro 2014) (**Figure 24**). This pathway is initiated by the activation of a transmembrane receptor, typically a growth factor tyrosine kinase receptor (RTK), at the plasmamembrane level. This is followed by SOS exchange factor recruitment to the inner side of the membrane and by GTP-for GDP exchange on membrane-bound RAS family p21 small GTPases. GTP-p21RAS in turn mediates dimerization and activation of RAF family Serine/Threonine kinases (ARAF, BRAF, CRAF) that in turn phosphorylate and activate MEK1/2 at positions 217 and 221 in the activation loop. Activated MEKs dually phosphorylate p44 and p42 ERKs (MAPK) at Threonine 202 and Tyrosine 204, which results in ERK activation. Once active, ERKs can either directly or indirectly through additional kinases mediate the phosphorylation and activation of transcription factors, including ELK1, MYC, STAT1, thereby promoting multiple cell responses (Mendoza et al. 2011; Pearce et al. 2010).

p90RSK family kinases (p90RSK1-4) are prototypic effectors of the MAPK signaling cascade. p90RSK is composed of a regulatory C-terminal kinase domain (CTKD) that belongs to the calmodulin dependent kinase family and an effector N-terminal kinase domain (NTKD) that belongs to the AGC kinase family separated by a linker region. ERKs mediate phosphorylation of the p90RSK C-terminal kinase domain (CTKD) on Thr573, and of linker region on Thr359 and Ser363. This initiates a sequence of events leading to phosphorylation of the linker on Ser380 by the CTKD and of the NTKD on Ser221 by PDK1 finally resulting in the activation of the NTKD (Moritz et al. 2010; Romeo et al. 2012; Galan et al. 2014).

MAPK is a prototypical oncogenic signaling pathway; several evidence points to a role of p90RSKs, in particular, in cancer. p90RSK is overexpressed in lung cancer where it has been considered a suitable therapeutic target (Poomakkoth et al. 2016). p90RSK1 is involved in prostate cancer cell growth and metastatization (Yu et al. 2015). p90RSK inhibition was found to impair selectively anchorage-independent growth of several tumor cell types (Aronchik et al. 2014). p90RSK was involved in squamous cell carcinomas by increasing MYC expression and cell invasion (Degen et al. 2013). Finally, p90RSK was involved in BRAF-mediated melanoma cell growth (Romeo et al. 2013) and in breast cancer stem cell maintenance and trastuzumab resistance via the phosphorylation of YB1 (Stratford et al. 2012; Astanehe et al. 2012)

The various subtypes of thyroid cancer are typically associated to oncogenic conversion of MAPK, with MTC frequently harboring RET or RAS mutations, PTC associated to RAS (FV-PTC) or BRAF V600E (or other less common BRAF lesions) and RET or other RTKs lesions (CV-PTC), FTC harboring in about half of the cases RAS mutations, and finally ATC commonly harboring either RAS or BRAF lesions (Nikiforov et al. 2011; Xing et al. 2013; Wells and Santoro 2014). Importantly, p90RSK has been found consistently phosphorylated in human papillary thyroid cancer samples, in particular in RAS-mutant PTCs (Cancer Genome Atlas, 2014).

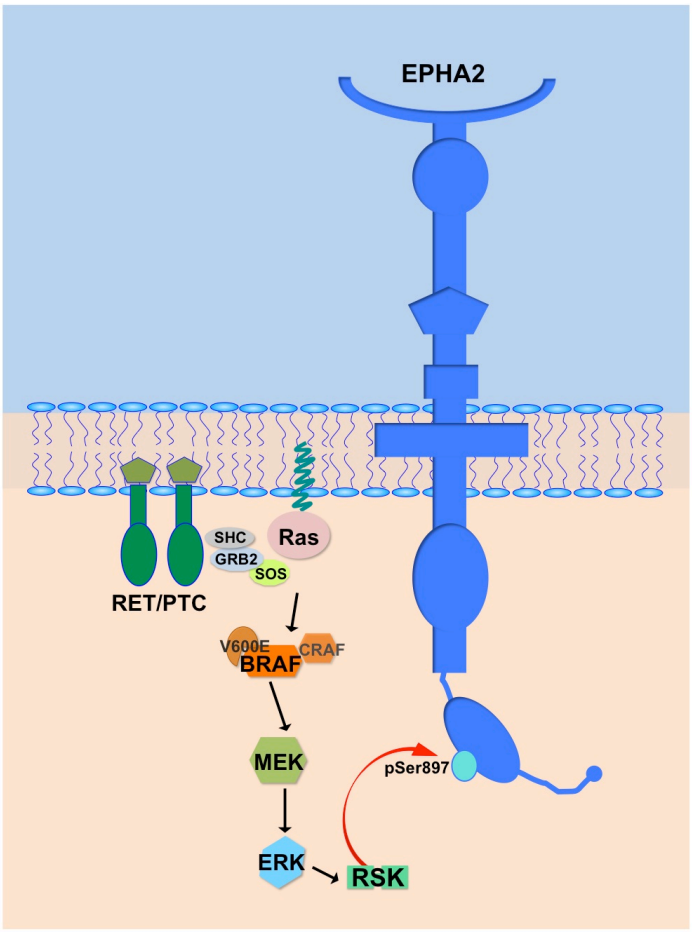

Figure 24. MAPK pathway activation mediates EPHA2 Ser897 phosphorylation through p90RSK in thyroid cancer cells.

6. CONCLUSIONS

Mechanisms through which p90RSKs can contribute to neoplastic transformation are diverse. In some cases, oncogenic activity of p90RSK has been demonstrated to be mediated by activation of specific transcription factors, such as YB1 (Shiota et al. 2014). In other cases, p90RSK has been involved in overcoming DNA damage checkpoint arrest through phosphorylation of MRE11 and CHEK1 (Chen et al. 2013; Ray-David et al. 2013). p90RSKs are able to blunt apoptosis via phosphorylation of APAF1 (Kim et al. 2012). Finally, p90RSKs are able to promote cell growth via phosphorylation of CDC25 isoforms (Wu et al. 2014). Moreover, the notion that p90RSK is able through phosphorylation of modulating the activity of membrane proteins is not unprecedented. For instance, p90RSK is able to phosphorylate β4-integrin on T1736 thus reducing adhesion through hemidesmosomes (Te Molder et al. 2015)

In this context, the mechanism through which p90RSK-mediated Ser897 phosphorylation of EPHA2 functions in thyroid cancer is still unclear. Two possible mechanisms can be conceived.

One possibility is that phospho-Ser897 acts by recruiting intracellular signaling transducers to EPHA2. Accordingly, Ephexin 4 interaction with EPHA2 is regulated by Ser897 phosphorylation. Ephexin 4 is a member of Dbl-type guanine nucleotide exchange factors (GEFs) family. It is able to promote cell proliferation and survival by activating RhoG and Rac small GTPases through the ELMO–Dock180 or ELMO–Dock4 complexes as well as the PI3K/Akt signaling pathway (Hiramoto-Yamaki et al. 2010; Kawai et al. 2013).

We are currently exploring whether Ephexin 4 is expressed in thyroid cancer cells and plays any role in EPHA2 signaling.

Another possibility is that Ser897 phosphorylation modulates in some way EPHA2 signal trasduction. In this frame, while ligand-mediated EPHA2 oligomerization leads to increased tyrosine phosphorylation, cell contraction and reduced tumor growth and motility, EPHA2 mutants that are unable to dimerize feature increased motility promoting effects, Ser897 phosphorylation, and reduced tyrosine-phosphorylation, thus pointing again to opposite effects mediated by ligand mediated oligomerization/EPHA2 tyrosine phosphorylation and AGC kinases-mediated Ser897 phosphorylation (Singh et al. 2015). We are currently exploring whether tyrosine kinase activity of EPHA2 influences or is influenced by Ser897 phosphorylation and whether this has any impact on the ability of EPHA2 of driving intracellular pathways such as the MAPK and the AKT one.

Elucidating this pathway will be crucial to devise strategies to intecept it to blunt thyroid tumorigenesis.

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These pages are mine...but also yours.

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APPENDIX

Publications summary

MC Cantisani, A Parascandalo, M Perälä, C Allocca, V Fey, N Sahlberg, F Merolla, F Basolo, MO Laukkanen, OP Kallioniemi, M Santoro, MD Castellone – A Loss-of-Function Genetic Screening Identifies Novel Mediators of Thyroid Cancer Cell Viability, Oncotarget, ***in press***

In this study we have performed a loss of function screening by transfecting thyroid cancer cells (TPC1 cells) with a library of siRNA targeting the whole human kinome in order to discover kinases involved in thyroid cancer cell viability. Our data identify a novel set of thyroid cancer regulators, including several members of EPH receptor tyrosine kinase family as well as SRC and MAPK (mitogen activated protein kinases) families.

G Vecchio, A Parascandolo, C Allocca, A Strazzulli, M Moracci, C Ugolini, F Basolo, MD Castellone, M Santoro, N Tsuchida – Downregulation of FUCA-1 in human thyroid anaplastic carcinomas - ***manuscript in preparation***

In this work we have studied the role of the alpha-L-Fucosidase-1 (FUCA-1) in thyroid cancer. This enzyme is involved in the removal of fucose from glycans and has been shown to be downregulated in highly aggressive and metastatic histotypes of human cancers. Our data demonstrate that the expression is significantly reduced in anaplastic thyroid cancer (ATC) as well as thyroid cancer cell lines. Moreover, the transfection of FUCA-1 in ATC cells is able to impair their tumorigenic properties.