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DECIPHERING RET SIGNALLING IN CELL BIOLOGY AND DEVELOPMENT

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DECIPHERING RET SIGNALLING IN CELL BIOLOGY AND DEVELOPMENT

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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"Someone is sitting in the shadow today
because someone planted a tree a long time ago."

Warren Buffet

ABSTRACT

The rearranged during transformation (RET) tyrosine kinase regulates a plethora of biological processes such as cell survival, proliferation and migration and is essential for the normal development of several organs such as the sensory, enteric and sympathetic nervous systems and the kidneys. After RET activation by its ligands several intracellular tyrosine residues are phosphorylated and serve as binding sites for adaptor proteins that activate different downstream signalling pathways. One prominent binding site is tyrosine 1062. This residue is part of a binding motif for the phosphotyrosine binding (PTB) proteins DOK1-6, FRS2 and SHCA,B,C. The binding of PTB adaptors depends on the amino acids N-terminal of the tyrosine, and this feature can be utilised to engineer adaptor-specific receptors.

RET is known to be recruited into cholesterol-rich membrane domains upon activation, but the mechanism and biological importance of this translocation were previously unknown. In **Paper I**, we analyse the influence of the membrane domain localization of RET and its adaptors on their signalling characteristics. We show that the lipid raft-associated FRS2 recruits RET to lipid raft domains, while SHC localizes it to other membrane regions. A lipid raft-bound SHC (SHC^{MLS}) resembles FRS2 both in signalling, translocation of RET and biological functionality, with diminished support of cell survival and increased migration of SHC^{MLS} compared to normal SHC. In contrast to SHC, both FRS2 and SHC^{MLS} functions depend on lipid raft integrity.

RET signalling is important for the development of several organ systems. In particular Y1062 plays a role in both the enteric and sympathetic nervous system and in nephrogenesis, however the specific roles of the different Y1062 binding proteins *in vivo* were unknown. In **Paper II** I investigate the role of RET signalling via DOK, FRS2 or SHC from Y1062 *in vivo*. $Ret^{9Frs/9Frs}$ mice show severe enteric aganglionosis, reduced soma size of dorsal root ganglion (DRG) neurons and mechanical hypersensitivity at early postnatal stages. $Ret^{9Shc/9Shc}$ mice on the other hand show a misregulation of sensory markers together with a hypersensitivity for cold and itch stimuli. In the sympathetic nervous system, $Ret^{9Frs/9Frs}$ animals display a reduced repression of cholinergic markers, with unchanged noradrenergic specification. We conclude that the studied adaptors have tissue- and cell type-specific roles and that they are main regulators of cell type specification both in the sensory and sympathetic nervous system.

One central process during sympathetic nervous system development is the segregation of the noradrenergic and cholinergic lineages. While several regulating factors are known, the knowledge about how they are organized into a regulatory network is incomplete and is still missing several regulatory elements. In **Paper III** we investigate the gene regulatory network that controls this segregation. We show that sympathetic progenitors are a hybrid population expressing markers of both the cholinergic and noradrenergic lineage and that the homeobox transcription factor HMX1 is required both for the repression of the expression of *Ret* and other cholinergic markers and for the maintenance of noradrenergic marker expression. RET on the other hand maintains cholinergic marker expression and supresses HMX1.

LIST OF SCIENTIFIC PAPERS

I. Lundgren TK, Lübke M, Stenqvist A, Ernfors P
Differential membrane compartmentalization of RET by PTB-adaptor engagement
FEBS Journal 2008 May 275 (9), 2055-2066

II. Lübke M, Furlan A, Eleuteri B, Abdo H, Lundgren TK, Li L, Lal M, Oddsson A, Tryggvason K, Ernfors P
Rewiring RET to specific PTB adaptor pathways leads to distinct developmental deficits
Manuscript

III. Furlan A, Lübke M, Adameyko I, Lallemend F, Ernfors P
The transcription factor HMX1 and growth factor receptor activities control sympathetic neurons diversification
EMBO Journal 2013 April 32 (11), 1613-1625

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Adameyko I, Lallemend F, Furlan A, Zinin N, Aranda S, Kitambi SS, Blanchart A, Favaro R, Nicolis S, Lübke M, Müller T, Birchmeier C, Suter U, Zaitoun I, Takahashi Y, Ernfors P
Sox2 and Mitf cross-regulatory interactions consolidate progenitor and melanocyte lineages in the cranial neural crest
Development 2012 January 139 (2), 397-410

CONTENTS

LIST OF ABBREVIATIONS

AKT	Protein kinase B
ARTN	Artemin
DOK	Downstream of kinase
DRG	Dorsal root ganglion
ENS	Enteric nervous system
ERK	Extracellular signal-regulated kinase
Ex (e.g. E10.5)	Embryonic day x
F	Phenylalanine
FRS2	Fibroblast growth factor receptor substrate 2
GDNF	Glial cell-line derived neurotrophic factor
GFL	GDNF family ligand
GFR	GDNF family receptor
GM1, 3	Monosialotetrahexosylganglioside 1, 3
HMX1	Homeobox (H6 family) 1
IR	Insulin receptor
MAPK	Mitogen-activated protein kinase
MEN	Multiple endocrine neoplasia
NRTN	Neurturin
PH	Pleckstrin homology
PI3K	Phosphatidylinositol-3-kinase
PIP2 (3)	Phosphatidylinositol-(3,)4,5-phosphate
PLCγ	Phospholipase C gamma
PSPN	Persephin
PTB	Phosphor tyrosine binding
PTC	Papillary thyroid cancer
Px (e.g. P8)	Postnatal day x
RET	Rearranged during transformation
RTK	Receptor tyrosine kinase
SH2	Src homology 2
SHC	Src homology and collagen homology
SNS	Sympathetic nervous system
TrkA,C	Tropomyosin receptor kinase A, C
Y	Tyrosine

1 INTRODUCTION

1.1 RECEPTOR TYROSINE KINASE SIGNALLING

The adult mammalian body consists of millions of cells of various types and functions. Each of these cells originates from a single oocyte, which, during development, divides and multiplies, generating stem and progenitor cells of progressive specificity, to finally assume a fully differentiated state. This process is tightly controlled by external and internal signals, through direct cell-cell interactions, locally active secreted molecules like growth factors, or systemically acting hormones. All of these signalling molecules are registered and processed by the target cells through receptors that in most cases are localized on the cell surface.

1.1.1 Receptor tyrosine kinases

One class of cell surface receptors are the so-called receptor tyrosine kinases (RTKs). In humans, 58 different RTKs have been described that can be grouped into 20 families (Lemmon and Schlessinger, 2010).

Despite certain functional differences, all RTKs share a common structure and a similar activation mechanism. They all have an extracellular domain that constitutes the ligand binding domain, a single transmembrane domain and an intracellular tyrosine kinase domain. The intracellular domain contains one or more tyrosine residues (Y) that can be phosphorylated upon receptor activation by ligand binding.

With few exceptions, inactive RTKs are monomeric molecules, and ligand binding to the receptor results in receptor dimerization. This dimerization leads to conformational changes that release *cis*-autoinhibitory elements and result in auto-phosphorylation of the receptors (Nolen et al., 2004). The insulin receptor (IR) and insulin growth factor 1 receptor are exceptions, as they are expressed as disulphide dimers and ligand binding merely induces conformational changes and thereby auto-phosphorylation (Ward et al., 2007).

The phosphorylation of tyrosines is the starting point for signalling cascades, resulting in different biological responses. Through the wide variety of signalling pathways initiated by RTKs they control processes such as proliferation, survival, migration, differentiation, cell-cycle control and metabolism (Lemmon and Schlessinger, 2010).

Mutations in RTKs can result in the misregulation of these processes and are therefore the basis of several human pathologies, including cancer. Mutations of an RTK may result in constitutive receptor activation independent of ligand availability, resulting for example in aberrant proliferation and carcinogenesis.

1.1.2 Receptor tyrosine kinase docking proteins

Intense research efforts have delineated signalling pathways underlying the biological effects of RTKs. The phosphorylation of tyrosines by autocatalytic activity of intracellular tyrosine kinase domains results in the generation of binding sites for intracellular docking proteins. Two important classes of such proteins are those that contain phosphotyrosine binding (PTB) or Src homology 2 (SH2) domains (Lemmon and Schlessinger, 2010).

The first PTB domain was reported in SHC, a protein containing both a PTB domain at the amino-terminus and a carboxy-terminal SH2 domain (Blaikie et al., 1994; Gustafson et al., 1995; van der Geer et al., 1995). To date, about 60 different PTB domains have been identified in the human proteome (reviewed in Uhlik et al., 2005).

All PTB domains share a common basic structure: Two orthogonal β-sheets align into a sandwich-like structure, while a carboxy-terminal α-helix caps the positively charged binding pocket. Depending on the mode of phosphotyrosine binding, PTB domains are classified into three groups. The phosphorylation-dependent SHC-like PTB domains, which bind the oxygen of the phosphate group in a triangular fashion between two arginines and one lysine (Zhou et al., 1995), the IRS-like group, which also depends on tyrosine phosphorylation, but binds the phosphoryl oxygen through interaction with two arginine residues (Eck et al., 1996; Zhou et al., 1996) and the phosphorylation-independent group of Dab-like domains (Howell et al., 1999). In addition to the common structure of a β-sheet sandwich with C-terminal cap, the SHC- and DAB-like PTB domains have two additional α-helices, one between two of the β-strands and one at the N-terminus. IRS-like PTB domains in contrast have only very truncated N-terminal α-helices or lack them altogether (Eck et al., 1996; Zhou et al., 1996). The SHC PTB domain contains also a unique elongated loop region between the α2 helix and the β2-strand that seems to partially reach around the bound molecule and that has been reported to be directly involved in the pY interaction, although the biological significance of this interaction is still unknown (Zhou et al., 1995; Deshmukh et al., 2010).

Well-known examples of IRS-like PTB domain proteins are the IRS1, FRS2 and DOK proteins, while the SHC family as well as tensin and Numb are representatives of SHC-like domain containing proteins.

A feature distinguishing PTB proteins from other adaptors like e.g. phospholipase C gamma (PLCγ) is their lack of intrinsic catalytic activity. Instead they usually contain binding sites for other interaction domains, such as the PH, SH2 or SH3 domains of downstream signalling molecules, which link RTK activation to the different intracellular signalling pathways (Uhlik et al., 2005; Pawson, 2007; Lemmon and Schlessinger, 2010).

1.1.3 RTK signalling pathways

The most prominent signalling pathways controlled by RTKs and their PTB adaptors are the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) pathways.

1.1.3.1 MAPK

MAPK signalling can be subdivided into four families of signalling pathways that share the most upstream signal mediators, but differ in their downstream targets ERK, JNK, p38 and BMK-1 (Burotto et al., 2014). Signalling through ERK is the best-described MAPK pathway and is usually seen as the classical MAPK pathway.

RTK activation and receptor phosphorylation generate binding sites for docking proteins like SHC, GRB2 or GAB1/2 (Schlessinger, 2000; Lemmon and Schlessinger, 2010). Binding and subsequent phosphorylation of these docking proteins lead to the recruitment of further downstream effector molecules such as the Ras-guanine exchange factor son of sevenless (SOS), which in turn can activate RAS. RAS then activates MAPK kinase kinases (MAPKKK) of the RAF family, amongst others. MAPKKK activate MAPKK, most prominently MEK1/2, followed by activation of the MAPK effector ERK1/2. ERK proteins then elicit cell specific responses. It has been shown that effect-determining factors include the localization of ERK, as cytosolic ERK regulates e.g. cytoskeletal proteins (Pullikuth and Catling, 2007), while nuclear ERK regulates gene-expression through phosphorylation of transcription factors (Zassadowski et al., 2012). In addition to subcellular localization, also timing and intensity of the signal seem to be decisive for the outcome. During short-term activation of ERK, c-FOS is rapidly degraded in the nucleus, but following long-term ERK activation it gets

phosphorylated and acts to further strengthen ERK signalling (Murphy et al., 2002; Murphy and Blenis, 2006).

As MAPK signalling is a central factor in the control of cell proliferation, it is not surprising to find deregulated signalling as the underlying cause of several forms of cancer, for example melanoma (Burotto et al., 2014). For this reason, members of this pathway are attractive therapeutic targets and several pharmaceutical drugs have been developed that target MAPKs (Carter et al., 2013; Cossa et al., 2013).

1.1.3.2 PI3K

Another pathway regulated by RTKs are the phosphatidylinositol-3-kinases (PI3Ks).

Their major role is to phosphorylate phosphatidylinositol (PtdIns)-4,5-diphosphate (PIP2) to generate PtdIns-3,4,5-triphosphate (PIP3). This process is reversed by PTEN, which therefore provides a negative regulatory mechanism for PI3K signalling (Maehama and Dixon, 1998). Following PIP2 phosphorylation, AKT is translocated from the cytosol to the membrane-residing PIP3 through its PtdIns-interacting pleckstrin homology (PH) domain (Andjelković et al., 1997). Once at the membrane, AKT is phosphorylated by PDK1 (Wick et al., 2000) and either activates cytosolic target proteins such as the mammalian target of rapamycin (mTOR) complex 1 (Navé et al., 1999; Aoki et al., 2001) or translocates into the nucleus to exert its roles there. AKT controls a variety of cellular processes, for example survival through either inhibition of pro-apoptotic or activation of anti-apoptotic factors, proliferation through control of cell cycle regulators or migration by interaction with cytoskeletal proteins (reviewed in Davis et al., 2015).

Similar to MAPK signalling, also modifications of the PI3K pathway are a common cause of cancer (Marone et al., 2008; Pitt and Chen, 2008). Both inhibitory mutations of PTEN and gain-of-function mutations of AKT can result in aberrant cell growth and tumour formation (Zbuk and Eng, 2007). Interestingly, a profound crosstalk between MAPK and PI3K has been found. In addition to RAF, RAS can also activate PI3Ks, AKT can inhibit RAF, and ERK can assist AKT by activating mTORC1 directly or by inhibition of TSC2 alongside AKT (Mendoza et al., 2011).

1.1.4 Cell membrane organization

Biological membranes are a complex system consisting of a mix of various kinds of proteins and lipids. While the textbook model of a ‘fluid mosaic’ is a great model for illustration and

general understanding of membrane function, evidence has accumulated that it presents a very simplified view of cell membrane biology and function. Due to a much higher protein content of biological membranes than usually visualized, the biophysical implications of membrane composition are very different from a true fluid structure (Engelman, 2005).

The cell membrane is composed of three classes of membrane lipids: Cholesterol, glycerophospholipids and sphingolipids, with glycerophospholipids being the most abundant lipid. Their general structure of a hydrophilic head with a long hydrophobic tail gives rise to the lipid bilayer, with the lipophilic tails facing inwards and the hydrophilic heads facing the aqueous surrounding. This structure enables the cell membrane to integrate proteins that either contain a hydrophobic domain or that are subject to a posttranslational addition of an anchor that recruits and attaches them to the membrane.

Evidence has accumulated that has led to the idea that the membrane is not a uniform system, but that certain lipids form local clusters of somewhat different steric character and density, and that some proteins are preferentially localized in such domains. One such domain are the so-called lipid rafts that represent sterol and sphingolipid-enriched nanodomains and that assemble specific sets of proteins (Simons and Sampaio, 2011).

Disruption of these lipid ordered domains can change or even abolish protein function. In addition to merely serving as an anchoring site, some sphingolipids have also been shown to directly regulate receptor function. For example, GM3 ganglioside can inhibit EGFR autophosphorylation through interaction with a glycan residue on the receptor (Coskun et al., 2011), and GM1 increases RET activity in striatal cells both *in vitro* and *in vivo* and is able to partially recover dopamine defects in a mouse model of Parkinson's disease (Newburn et al., 2014). GM1 was also shown to increase PI3K signalling through Trk receptors (Duchemin et al., 2008).

1.1.5 Establishing signal specificity

In face of the different signalling pathways and potential cell biological outcomes of RTK activation the question arises how signal specificity can be achieved. While some specificity certainly is derived from differential expression patterns of receptors and docking proteins in different cell types, as well as limited ligand availability, some of the docking proteins are shared by several RTKs within the same cell type, and in some cases several adaptor proteins share one and the same phosphotyrosine residue.

PTB proteins share a common NXXpY binding motif (Kavanaugh et al., 1995). Additionally to this core motif, PTB proteins interact with residues amino-terminal of NXXpY. Of particular importance are amino acids at positions -4 to -8 of the pY residue, as they control binding affinity and stabilize an otherwise weak binding. By this mechanism PTB domain proteins can discriminate between different binding sites and generate a receptor-specific PTB protein profile (He et al., 1995; Wolf et al., 1995; Zhou et al., 1995). The PTB motif of the IR for example is readily bound by IRS1/2, but SHC binding to the same motif is of much lower affinity, and while the Tropomyosin receptor kinase A (TRKA) is bound by SHC and FRS2, EGF appears to signal exclusively via SHC family members (Sorkin, 2001; Ceni et al., 2014; Du and Wei, 2014).

Consistent with these studies, the IR, which associates primarily with the PTB domain of IRS1, but not SHC, can be re-engineered to selectively interact with SHCA *in vitro* by amino acid substitution at the -5-position in the sequence N-terminal to the core NXXpY PTB domain binding site (van der Geer et al., 1999).

Interestingly, some PTB domain proteins are able to bind peptides independently of tyrosine phosphorylation. The FRS2 PTB domain binds constitutively to an extended peptide sequence in the juxtamembrane region of the fibroblast growth factor receptor 1 (FGFR1) that is devoid of a NXXpY motif (Xu et al., 1998; Ong et al., 2000). However, although FRS2 is constitutively bound to the FGF receptor, signalling downstream of FRS2 is initiated only upon ligand binding to the receptor.

Another mechanism controlling signalling specificity is the subcellular localization from where the receptor is signalling. As adaptor proteins are built up of different domains and are subject to different posttranslational modifications they are recruited to different subcellular compartments. While some adaptors are primarily cytosolic, such as SHC (Ravichandran et al., 1997), the FRS2 adaptor has a myristoylation sequence that results in its recruitment to lipid raft domains of the cell membrane (Gotoh et al., 2004). As shown in paper I, artificial recruitment of SHC to lipid rafts through a membrane localization sequence (MLS) changes its signalling properties, making it more similar to FRS2 in the context of RET signalling. In addition to these mere biochemical changes this also leads to functional differences in supporting cell survival and migration (Lundgren et al., 2008a).

1.2 THE RET TYROSINE KINASE

1.2.1 RET architecture and interactions

The rearranged during transformation (RET) receptor was discovered in 1985 by Takahashi *et al.* as part of a rearrangement of two genes during transfection of NIH 3T3 cells, resulting in a transforming activity (Takahashi et al., 1985).

Moving from amino- to carboxy-terminus, the extracellular domain of RET (RET^{ECD}) is built up of four cadherin-like repeats and a cysteine-rich domain, with a calcium binding site between cadherine-like domain 2 and 3. The RET^{ECD} is the ligand interaction domain of RET. A single-spanning transmembrane domain connects the extra- and intracellular segments. On the intracellular side, the juxtamembrane segment is followed by the tyrosine kinase domain and the C-terminus (Airaksinen and Saarma, 2002).

In humans, *RET* is expressed in three isoforms with different C-termini that are generated through alternative splicing. The *RET* gene contains 21 exons, of which all isoforms contain the first 19 exons. Splicing of exon 19 into exon 20 generates RET51. RET43 on the other hand skips exon 20, and is spliced into exon 21 instead. Generation of RET9 does not require any splicing, but includes additional codons in exon 19 (Ivanchuk et al., 1997; Fleming et al., 2015). After the last shared amino acid G1063, the isoforms have 9 (RET9), 43 (RET43) or 51 (RET51) additional amino acids (Airaksinen and Saarma, 2002). In contrast to RET9 and RET51 that are found in many vertebrate species, RET43 is only found in primates, and even there only at low expression levels (Carter et al., 2001).

1.2.2 RET signalling complexes

RET is the receptor for the members of the glial cell derived neurotrophic factor (GDNF) family of ligands (GFLs), namely GDNF, Neurturin (NRTN), Artemin (ARTN) and Persephin (PSPN).

Uniquely for an RTK, RET does not interact with its activating ligands directly. Instead, its ligand interaction is dependent on prior GFL binding by a coreceptor of the GFR family, GFRα1-4. GFRs are cell surface molecules and usually anchored in the cell membrane by a glycosylphosphatidylinositol (GPI)-anchor, but do also exist in a soluble form (see below). Each GFL has a specific high-affinity receptor. Generally speaking, GDNF interacts with GFRα1, NRTN with GFRα2, ARTN with GFRα3 and PSPN with GFRα4, although some *in*

vitro studies describe crosstalk and suggest that GFRα1 also can be activated by NRTN and ARTN and GFRα2 by GDNF (Airaksinen et al., 1999; Baloh et al., 2000). Based on their protein structure the GFLs are members of the TGFβ superfamily and have a homology of approximately 40% to each other (Kotzbauer et al., 1996; Baloh et al., 1998; Milbrandt et al., 1998).

The formation of the ternary RET:GFR:GFL complex occurs in a 2:2:2 stoichiometrical fashion. A model of this complex suggests an interaction where a GDNF dimer is bound by two GFRα1 molecules, and this complex is embraced and enveloped by two RET receptors. The RET^{ECD} interacts with the ligand-coreceptor complex at four distinct contact sites, three with GFRα1 and the fourth one as a shared GDNF-GFRα1 binding site. This interaction is calcium-dependent (Goodman et al., 2014).

Commonly the coreceptors are expressed as membrane proteins in the same cell as RET and associate with and activate it in *cis*, but also soluble forms of GFRs are able to activate RET in *cis* or *trans* and have been described both *in vitro* and *in vivo*. Additionally, also membrane-bound GFRs on neighbouring cells can bind GFLs and activate RET in *trans*. Soluble GFR variants can be generated by cleavage of the GPI anchor by a phospholipase or protease or by alternative splicing (Worley et al., 2000; Lindahl et al., 2001; Paratcha et al., 2001; Arighi et al., 2005; Fleming et al., 2015). In fact, a recent report indicated that the concerted action of *cis*-signalling via GFRα2 and *trans*-signalling of GFRα1 control the survival and the growth of central projections in rapidly adapting mechanoreceptors (Fleming et al., 2015). *Trans* signalling has also been implied in tissue invasion by RET^{+} cancer cells (He et al., 2014).

1.2.3 Expression and functionality of the RET isoforms

As described above, *Ret* is expressed in three different isoforms, generated by alternative splicing. Both RET9 and RET51 are conserved in all vertebrates, but RET43 has to date only been described in primates and only at low levels, and is still uncharacterized (Carter et al., 2001).

Although RET9 and RET51 are coexpressed in most RET^{+} cells and their amino acid sequence is homologous to more than 95%, they have partially different dynamics in maturation and trafficking. Richardson *et al.* found that RET51 is readily matured, while RET9 is found to a higher degree in the immature state. Once matured and localized at the cell surface though, RET51 seems to be internalized quicker. Interestingly, this is partly

counteracted by a recycling of RET51 receptors that is not seen for RET9 (Richardson et al., 2012).

In human kidney development, RET9 and RET51 underlie temporal control and seem to be important at different points during development. RET9 is the major isoform expressed during early stages of embryonic kidney development (week 7.5 of gestation), whereas RET51 is only weakly expressed at this time, but is increased sevenfold until week 9 (Ivanchuk et al., 1998).

In addition to differences in the time of expression and protein dynamics, some functional differences might also arise from the additional tyrosine Y1096 of RET51. This residue constitutes a binding site for the docking protein GRB2 that can serve as an adaptor for both the MAPK and PI3K pathways. Analyses of genetically modified mice carrying either the RET9 or RET51 isoform have shown that RET9 is sufficient for normal development. However, while one study presented evidence that RET51 does not support nephrogenesis and enteric nervous system (ENS) development to the same extent as RET9 (de Graaff et al., 2001), another study showed that both isoforms appear to support development equally well as wild type receptors. Differences between the isoforms became apparent though in the latter study once loss-of-function mutations were induced, and animals on a monoisoformic $Ret^{51/51}$ background seemed to show weaker phenotypes upon tyrosine mutation than on the $Ret^{9/9}$ background (Jain et al., 2006). Although at low penetrance, $Ret^{9/9}$ mice show abnormalities in sphenopalatine ganglia development, and mutations of Y981, Y1015 or Y1062 further increase this phenotype, while RET51 is capable of supporting normal ganglia development even in the presence of mutations (Jain et al., 2010).

These results suggest a compensatory role of Y1096 in the RET51 isoform.

1.2.4 RET phosphotyrosines and associated signalling proteins

The intracellular domain of RET contains 18 tyrosine residues, at least 14 of which can be phosphorylated upon receptor activation. To date, only eight of them have been shown to be functional binding sites though (Liu et al., 1996; Kawamoto et al., 2004).

Y687 is a recently discovered binding site for SHP2 and thereby activates the PI3K pathway. A Y687F mutation diminishes GDNF induced neurite outgrowth and survival in primary superior cervical ganglion (SCG) neurons *in vitro* (Perrinjaquet et al., 2010).

Both Y752 and Y928 are bound by and activate STAT3 (Schuringa et al., 2001), but no cell biological role has been reported *in vitro* or *in vivo* yet.

GRB7 and 10 bind to RET Y905 and stimulate MAPK signalling. An inactivating Y905F mutation greatly reduces the transforming activity of RET-MEN2A *in vitro* in transfected NIH 3T3 cells (Pandey et al., 1995; 1996; Kato et al., 2002).

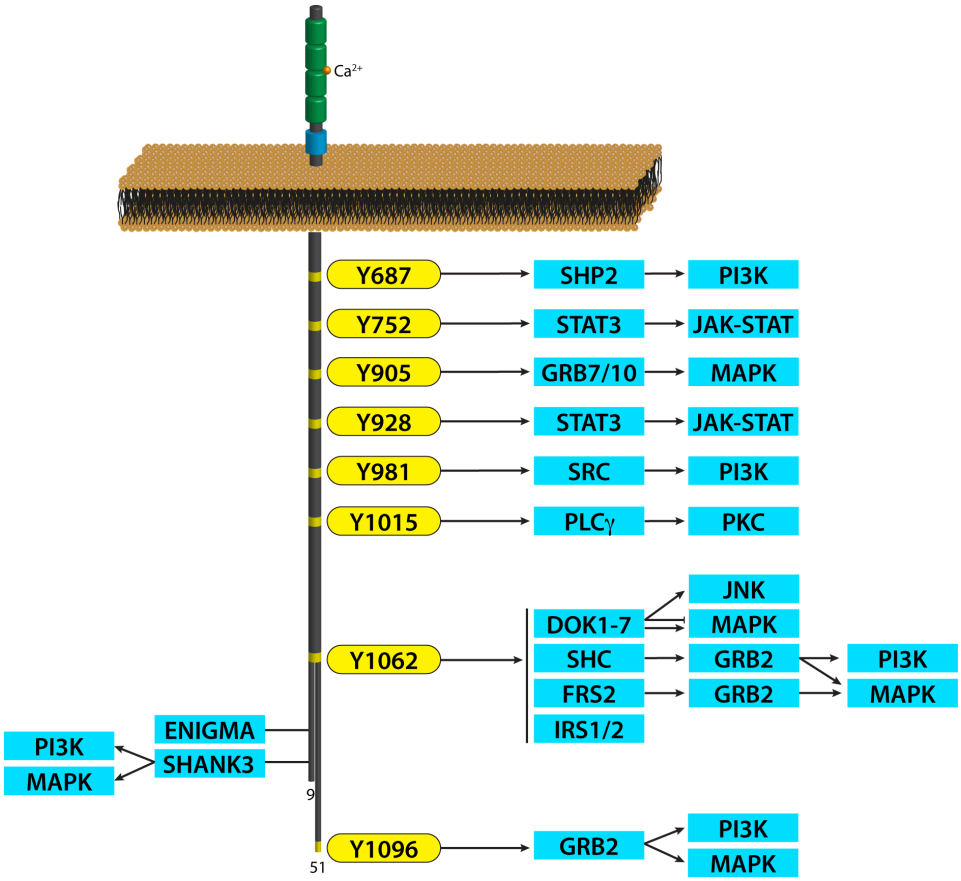

Fig. 1 The RET interaction sites, associated signalling partners and downstream pathways.

Y981 is bound by SRC that links RET to PI3K signalling. A Y981F mutation leads to impaired survival of primary cerebellar granule neurons *in vitro*, and SRC signalling from this residue is required to support full GDNF-mediated migration, while it is not sufficient to support migration on its own in the absence of Y1062 (Encinas et al., 2004; Lundgren et al., 2008b). While animals carrying this mutation on a RET51 background do not show any apparent phenotype, some animals with a RET9(Y981F) variant lack sphenopalatine ganglia, show partial intestinal aganglionosis or colonic hypogonglionosis or faulty ureter development (Encinas et al., 2004; Jain et al., 2010).

Y1015 is a binding site for PLCγ and is crucial for the transforming potential of RET/PTC2 both *in vitro* and *in vivo* (Borrello et al., 1996). It also has been shown to regulate the

migration of neocortical neurons through Ca^{2+} signalling (Lundgren et al., 2012). Both RET51(Y1015F) and RET9(Y1015F) mice show major developmental anomalies, including defects in kidney, ureter and gonad development and partial colonic aganglionosis. Some RET9(Y1015F) mice additionally lack sphenopalatine ganglia (Jain et al., 2010).

The presence of Y1062 is crucial for normal organ development of the ENS and the kidneys. A Y1062F mutation in animals expressing both RET9 and RET51 results in a significant loss of enteric innervation and renal hypoplasia due to reduced ureteric bud (UB) branching, and these mice are not viable (Jijiwa et al., 2004). Mice expressing monoisoformic RET9 with the Y1062F mutation show a phenotype reminiscent of full *Ret* knockout mice, with total enteric aganglionosis and kidney aplasia. The effect in RET51(Y1062F) mice is less pronounced, with colonic aganglionosis but normal kidney development (Jain et al., 2010). Y1062 was also shown to convey neuronal survival signals *in vitro* and *in vivo* (Coulpier et al., 2002; Encinas et al., 2008).

Y1062 is part of an NXXY PTB motif and has been described to be a multidocking site for the PTB adaptor proteins of the DOK, FRS2 and SHC families as well as IRS1/2. It is important to note that binding to multidocking sites creates a competitive situation between potential binding proteins, as only one docking protein can bind at a time (Melillo et al., 2001a; Jain et al., 2006).

The DOK isoforms DOK1-6 all have been reported to bind to and signal from RET Y1062, and regulate MAPK signalling (see 1.2.5.1) (Grimm et al., 2001; Murakami et al., 2002; Crowder et al., 2004; Kurotsuchi et al., 2010).

FRS2 was shown to immunoprecipitate with RET and *vice versa*, and to activate MAPK signalling upon GDNF treatment or in constitutively active variants of RET (see 1.2.5.2) (Kurokawa et al., 2001; Melillo et al., 2001b).

Several proteins of the SHC family have been shown to bind to and get activated by RET. Association with the activated RET receptor activates the MAPK and PI3K pathways, supporting amongst others cell survival and proliferation (see 1.2.5.3) (Asai et al., 1996; Arighi et al., 1997; Pelicci et al., 2002; Gustin et al., 2007).

Two studies suggested activation and phosphorylation of IRS1/2 by RET in response to RET activation. Their results were conflicting though, with one study presenting IRS1 phosphorylation and a weak interaction of IRS1 with constitutively active RET at Y1062,

while the other one explicitly reported IRS1 not to be phosphorylated, and only mentioned phosphorylation, but not direct interaction, between RET and IRS2 (Hennige et al., 2000; Melillo et al., 2001a). As the cells were cultured in serum containing media in both studies and a later study described increased insulin sensitivity in cells stably expressing RET/PTC3 (Miyagi et al., 2004) the increased phosphorylation of IRS1/2 might be a side effect of signalling through the IR and not a direct effect of RET signalling. To date no cell biological process has been coupled to IRS1/2 binding to RET, and both proliferation and neurite outgrowth are independent of IRS1/2 (Gustin et al., 2007).

The region C-terminal of Y1062 of RET9 has additionally been reported as phosphorylation-independent binding site for the LIM2 domain protein Enigma and the PDZ protein SHANK3 *in vitro*, while RET51 is not bound by either of them (Durick et al., 1996; Borrello et al., 2002; Schuetz et al., 2004).

In addition to these tyrosine residues that are shared by all isoforms, RET51 contains an additional tyrosine at position 1096 that is bound by GRB2 through its SH2 domain (Liu et al., 1996; Lorenzo et al., 1997). Tyrosine replacement by phenylalanine at this position does not result in any apparent phenotype *in vitro* in a neuronal cell scattering assay (Degl'Innocenti et al., 2004).

In summary, based on the currently available data the tyrosines of highest importance in physiological RET signalling seem to be Y1015 and Y1062. Y1015 is the only PLCγ binding site in the RET receptor and is of vital importance not only for the development of the genitourinary system, but also for neocortical neuron migration, and with lower penetrance for gastrointestinal development (Jain et al., 2010; Lundgren et al., 2012). The multidocking site Y1062 controls both the MAPK and PI3K pathways and is of uttermost importance especially, but not only, in the RET9 isoform, as a Y1062F mutation results in a severe developmental failure in multiple organs with loss of enteric neurons and kidney malformation, which is similar to full *Ret*, *Gdnf* or *Gfrα1* knockout animals (Airaksinen and Saarma, 2002; Jain et al., 2006; 2010).

1.2.5 RET PTB adaptor proteins

1.2.5.1 DOK

The downstream of kinase (DOK) family is made up of seven members with different expression patterns and functional characteristics. All family members share an N-terminal

pleckstrin homology (PH) domain, a PTB domain and a C-terminal SH2 domain target motif (Okada et al., 2006).

Sequence analysis suggests a subdivision of the family into subgroups, with DOK1-3 and DOK4-7 forming separate subfamilies. DOK1-3 are mostly expressed in the haematopoietic lineage (Di Cristofano et al., 1998; Cong et al., 1999; Yamanashi et al., 2000). DOK4 is widely expressed in the organism, amongst others in the developing nervous system and endothelial-derived tissues like the intestine, kidney and the lungs. DOK5 is mostly found in the brain, while DOK6 is predominantly localized in the DRG and cortical neurons, but also the UB and the testes (Grimm et al., 2001; Crowder et al., 2004). Despite convincing *in situ* data by Crowder et al., *Dok6* expression could not be detected by single cell RNA sequencing of adult DRG neurons (Usoskin et al., 2014). Interestingly, DOK4 and DOK5 had first been identified as IRS-5 and IRS-6 with a suggested role as substrates of the IR, but were later found to be only weak binders (Cai et al., 2003; Versteyhe et al., 2010).

In situ hybridization showed that DOK4-6 are coexpressed with RET in the ventral spinal cord, the DRG and cells of the UB. Both DOK4, 5 and 6 promote RET-dependent neurite outgrowth in cell lines (Grimm et al., 2001; Crowder et al., 2004), and in primary cortical tissue in case of DOK6 (Li et al., 2010). Considering that RET has been reported to promote and be essential for axonal growth in DRG neurons and in the sympathetic system *in vivo* and that DOK proteins support neurite outgrowth *in vitro* they may likely be involved in this mechanism also *in vivo*.

Dok4 is most abundantly expressed in the kidney and liver and was found to be increased in clear cell renal cell carcinoma (Al-Sarraf et al., 2007), while no physiological role of this protein in the developing or adult kidney has been described yet.

DOK1-3 have been described as negative regulators of MAPK/ERK signalling through binding of RasGDP and inhibition of RAS. Murakami *et al.* showed that while repressing MAPK/ERK, DOK1 activates JNK and c-Jun upon GDNF stimulation of RET (Cong et al., 1999; Suzu et al., 2000; Yamanashi et al., 2000; Murakami et al., 2002). DOK4-6 on the other hand are activators of MAPK/ERK (Grimm et al., 2001; Crowder et al., 2004). Overexpression of a RET variant preferentially binding DOK at Y1062 results in strong MAPK activation and induces microspike formation and receptor redistribution in SK-N-MC cells, which have been shown to express both DOK4 and DOK6 natively (Stenqvist et al., 2008; Kurotsuchi et al., 2010).

1.2.5.2 FRS2

The FRS2 family was first described as signalling factors phosphorylated upon NGF and FGF stimulation and consists of two members, FRS2α (also known as FRS2) and FRS2β (FRS3) (Rabin et al., 1993; Kouhara et al., 1997; Ong et al., 2000). In this thesis, FRS2 is used synonymously with FRS2α.

FRS2 contains an N-terminal myristoylation sequence, resulting in a localization in the plasma membrane (Schlessinger, 2000). Activation of FRS2 depends on RTK binding through its PTB domain. As described above though, FRS2 was also found to bind constitutively to the tyrosine-free juxtamembrane domain of the FGFR, independently of the NXXpY motif usually bound by PTB proteins (Xu et al., 1998; Ong et al., 2000). In addition to the PTB domain, FRS2 contains SH2 and SHP2 binding sites that serve as docking sites for e.g. the downstream adaptor GRB2, linking FRS2 to the MAPK and PI3K pathways (Kouhara et al., 1997; Hadari et al., 1998). In the case of RET, FRS2 seems to activate only the MAPK pathway though (Kurokawa et al., 2001). While FRS2 additionally interacts with GAB1 upon FGFR activation, this interaction seems to be amiss in the RET:FRS2 complex, suggesting a mechanism why FRS2 activation through RET does not result in PI3K activation (Kurokawa et al., 2001; Ong et al., 2001; Melillo et al., 2001b).

FRS2 is expressed from E5.5 and an almost ubiquitous expression is maintained throughout development. Amongst others, *Frs2* expression was detected in the DRG, kidneys and the gut (Gotoh et al., 2005; Gotoh, 2008). *Frs2*$^{-/-}$ animals suffer from defects in anterior-posterior axis formation and die between E7-E8 (Gotoh et al., 2005). Conditional deletion of FRS2 in the UB results in renal hypoplasia, while general branching architecture and mesenchymal stromal development are normal. Interestingly, *Ret* expression in bud cells was reduced in these animals (Sims-Lucas et al., 2009). In addition, FRS2 is crucial for the maintenance of nephron progenitors and conditional knockout animals develop renal cysts (Di Giovanni et al., 2015). Although showing a similar phenotype as *Ret*$^{-/-}$ mice, the role of FRS2 in nephrogenesis might be independent of RET, as FRS2 has also been shown to be an essential part of FGFR signalling in kidneys (Sims-Lucas et al., 2012).

RET is important for cell migration, and FRS2 is a regulator of RET-dependent migration in a neuroblastoma cell line (Lundgren et al., 2008b). Coexpression of FRS2 with RET/PTC3 leads to increased proliferation in NIH 3T3 fibroblasts (Melillo et al., 2001b), while another group

did not find FRS2 to be essential for RET-dependent proliferation in MG87 fibroblasts (Gustin et al., 2007).

In the sympathetic system, a loss-of-function mutation of FRS2 at its SHP2 binding site results in a reduced cranio-ventral migration of the superior cervical ganglion cells and a lack of the carotid body (Kameda et al., 2008). Although suggested as an FGFR-dependent event, a link to RET signalling is also possible, since *Ret*$^{-/-}$ mice display similar deficits in migration and axonal projection of sympathetic neurons (Enomoto et al., 2001). No FRS2 phenotype has been described in the ENS yet.

1.2.5.3 SHC

The SHC family consists of four members, SHCA, B, C and D, named in sequence of their discovery, with different splicing isoforms for each member. While SHCA, B and C are well described, the function of the newest member SHCD is still mostly unknown. SHC proteins are widely expressed throughout most cells of the organism and are recruited to several tyrosine kinases, including, but not restricted to, EGF, FGF, TRKB and RET (Wills and Jones, 2012).

SHC proteins contain both an N-terminal PTB and a C-terminal SH2 domain, connected by a linking CH1 domain, and can interact with growth factor receptors through each of them, given the presence of suitable binding sites (Gustafson et al., 1995; Migliaccio et al., 1997). Upon binding and phosphorylation, SHC serves as a docking protein for GRB2 (van der Geer et al., 1996), linking it to ERK/MAPK through SOS or to PI3K via Gab1/2 (Lowenstein et al., 1992; Pelicci et al., 2002; Nishida and Hirano, 2003). The SHC proteins differ to some extent in their activation kinetics: While SHCA activation results in ERK phosphorylation within five minutes, SHCC activation does not result in phosphorylation before 30min of stimulation (Pelicci et al., 2002).

Two of the three isoforms of SHCA (p52, p46) are based upon different start codons within the same transcript, while the longest isoform (p66) is generated by alternative splicing (Migliaccio et al., 1997). Despite their similar structure, p66 is standing out in terms of function. It is involved in the oxidative stress response, and animals devoid of this isoform show increased ageing and reduced sensitivity to oxidative stress, while knockouts of other SHCA isoforms do not show this phenotype (Migliaccio et al., 1999).

Both SHCB and SHCC are expressed in the DRG of E19 rat embryos, while SHCA is absent (Nakamura et al., 1998). SHCB is expressed in most DRG neurons at E13.5 as well as in adulthood. SHCC on the other hand shows a more restricted expression and is mostly found in large-diameter neurons (Sakai et al., 2000; Usoskin et al., 2014). Consequently, a loss of SHCB results in a loss of more than 50% of $IB4^+$ and $TRKA^+$ neurons that is also reflected in reduced cutaneous innervation, while the neuronal number in *Shcc*$^{-/-}$ mice does not show such an effect. SHCB and SHCC expression are also found in sympathetic cells of the superior cervical ganglia (SCG) and a double-knockout results in a loss of 33% of SCG neurons between E15 and P0, which partially resembles the phenotype of *Trka*$^{-/-}$ or *Ret*$^{-/-}$ animals. In contrast, no phenotype was found in the individual knockouts (Sakai et al., 2000). In the neuronal PC12 cell line, SHCC links RET to the PI3K/AKT pathway and thereby inhibits apoptosis (Pelicci et al., 2002).

SHCC expression is found in enteric glia cells, but not in enteric neurons (Villanacci et al., 2008), and *Shc* knockdown by siRNA does not have an effect on neuronal numbers in the ENS (Jain et al., 2010). In the kidney, the p46 and p52 isoforms of SHCA activate ERK/MAPK signalling and induce pro-survival signals. Under severe oxidative stress though the p66 isoform can disrupt ERK signalling, resulting in a lack of survival signals and in apoptosis of renal proximal tubule cells (Arany et al., 2008).

1.2.6 Role of RET and signalling partners in development

1.2.6.1 RET in the sensory nervous system

Cell lineages of the sensory nervous system

The dorsal root ganglia (DRG) are positioned bilaterally to the spinal cord and harbour the cell bodies of the sensory nervous system. The sensory neurons present the link for sensory information from the periphery to the central nervous system. The neurons of the mature sensory system can be classified by their respective sensory modality into low-threshold mechanoreceptors (LTMR), proprioceptors and nociceptors. Based on the full molecular profile of sensory neurons established by RNA sequencing of single cells, the sensory neurons can be subdivided into 11 groups: three types of LTMRs, two classes of proprioceptors and six classes of nociceptive, thermosensitive, itch sensitive and mechanosensitive neurons (Usoskin et al., 2014).

Generally speaking, LTMRs and proprioceptors are myelinated, large-diameter cells, innervate subcutaneous end organs and relay information about inoccuous vibration, pressure and light touch, while proprioceptive neurons target muscle spindles and Golgi tendon organs (Marmigère and Ernfors, 2007).

The neurons of the thermo-nociceptive lineage on the other hand constitute small-diameter, unmyelinated neurons. Functionally, nociceptors are the receptors and transmitters of noxious signals of different modi and are stimulated by noxious heat, cold, chemical or mechanical stimuli. While the different modalities are detected by specific receptors, the nociceptors often express several different receptors and are polymodal, i.e. one and the same cell conveys information about more than one modus to the central nervous system (Basbaum et al., 2009).

The thermo-nociceptive lineage can be grouped into six subgroups, namely the non-peptidergic (NP) groups 1-3, peptidergic (PEP) groups 1 and 2, and tyrosine hydroxylase-containing neurons (TH) (Usoskin et al., 2014). While the PEP and NP classes partially overlap in function, one can roughly summarize their main modalities in that NP neurons transmit itch and neuropathic pain sensations, while PEP neurons convey inflammatory pain and thermal information and the TH neurons pleasant touch. TRPM8, a cold receptor, is for example exclusively expressed in PEP1, whereas the itch-receptor MRGA3 is restricted to NP2. Other molecules like NAV1.8, which has been linked to mechanical pain, are found in neurons of all NP, PEP and TH classes (Liu and Ma, 2011; Lallemend and Ernfors, 2012; Usoskin et al., 2014).

Sensory neuron specification

During the time of neural tube closure, neural crest cells (NCC) undergo epithelial to mesenchymal transition and delaminate from the neural tube. NCC migrating along a ventral pathway form the organs of the sensory and autonomic nervous systems. The NCC forming the sensory nervous system commit to their neuronal phenotype around E9.5-E10.5 in mice during or after migration and coalesce into ganglia (Marmigère and Ernfors, 2007).

The first migratory NCC to arrive and exit the cell cycle differentiate into large-diameter myelinated LTMR and proprioceptors of the NF classes, while later differentiating cells form the family of small-diameter unmyelinated nociceptors. Under the control of different neurotrophic factors and transcription factors the sensory neurons undergo lineage

specification in a hierarchical fashion and finally acquire the phenotypes of the 11 different sensory subgroups of the adult DRG (Lallemend and Ernfors, 2012; Usoskin et al., 2014).

Expression of *Ret* can first be seen in the DRG at E10.5-E11.5 (Kramer et al., 2006b; Luo et al., 2009) in a small group of early-born large diameter cells (eRet). They innervate cutaneous sensory structures like longitudinal lanceolate endings, Pacinian corpuscles and Meissner corpuscles. Alongside with RET, these cells are also positive for the transcription factor v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA) and GFRα2 during embryonic and early postnatal stages (Bourane et al., 2009; Luo et al., 2009; Lecoin et al., 2010). Interestingly, *Gfrα*2 expression could not be detected in this population in single-cell RNA sequencing of adult DRG neurons, while GFRα1 was found instead (Usoskin et al., 2014).

At E15, another set of RET^{+} cells emerges (reviewed in Marmigère and Ernfors, 2007; Lallemend and Ernfors, 2012), this time belonging to the population of unmyelinated small- and medium-diameter neurons that constitute the nociceptors (lRet). Highest *Ret* expression is found in cells of the NP and TH classes, alongside with GFRα2 or GFRα3, and in NF classes together with GFRα1. Surprisingly a recent report also described GFRα3 expression in large-diameter neurons, which is in contrast to the above mentioned single cell RNA sequencing data as well as several other reports (Naveilhan et al., 1998; Orozco et al., 2001; Wong et al., 2015).

As $Ret^{-/-}$ mice die within few hours after birth with severe defects in several organs (Schuchardt et al., 1994), functional analysis of RET in the sensory system has long been very limited, but in recent years several groups have published analyses on conditional *Ret* knockouts, where *Ret* was deleted under the control of a cell type specific *Cre* recombinase.

Upon deletion of *Ret* in all neural crest cells, expression of the eRet population marker *Mafa* and the total number of LTMR are greatly reduced, together with a reduced innervation of their target structures like the Paccinian corpuscles. The importance of RET for LTMR is further strengthened by the finding that GDNF and NRTN support the survival of $MAFA^{+}$ DRG neurons *in vitro* (Bourane et al., 2009; Luo et al., 2009).

Deletion of *Ret* under the control of Th^{Cre} or $Nav1.8^{Cre}$ removes RET from the small- and medium-diameter cells of the lRet population. Analyses of these mice found several changed behavioural characteristics, including increased cold and mechanical sensitivity. Molecular

analyses showed that RET plays a critical role in the regulation of the expression of several ion channels and receptors of the NAV, MRG and TRP families (Golden et al., 2010; Franck et al., 2011). While RET does not seem to be required for cell survival in the DRG, several studies found it to be involved in the regulation of the soma size of DRG neurons (Luo et al., 2007; Golden et al., 2010). This phenotype likely involves signalling through NRTN/GFRα2, as it was also found in animals lacking the GFRα2 receptor (Lindfors et al., 2006).

1.2.6.2 Development of the ENS

The ENS is the most complex part of the peripheral and autonomous nervous system, with cell numbers rivalling those in the spinal cord. It is organized into ganglia of the myenteric and submucosal plexi. While it also gets input from the sympathetic and parasympathetic nervous system, it can work completely autonomously and control gut motility, secretion and resorption without external input (Furness, 2006) .

Development of the ENS starts around E8.5 with cells delaminating from the vagal neural tube. These cells reach the foregut at E9-E9.5 and are then called enteric neural crest cells (ENCC) (Durbec et al., 1996; Anderson et al., 2006). During the following days the ENCC undergo a rostro-caudal migration resulting in the colonization of the entire intestine by E15 (Druckenbrod and Epstein, 2005). During this migration along the intestine, GDNF functions as a chemoattractant with highest expression levels in the foregut and caecum region (Young et al., 2001; Natarajan et al., 2002; Mwizerwa et al., 2011; Nishiyama et al., 2012). In addition to the cells originating from the vagal neural tube, another population migrates from the sacral neural tube at E9-E9.5, first forming the extrinsic pelvic ganglia from where they migrate into the hindgut at later stages of intestinal colonization (Serbedzija et al., 1991; Burns and Douarin, 1998).

Migrating neural crest cells upregulate *Ret* expression en route along the dorsal aorta around E8.5-E9 (Durbec et al., 1996; Anderson et al., 2006) and maintain *Ret* expression in the neuronal population until adulthood, while cells differentiating into glia downregulate *Ret* upon completed migration (Pachnis et al., 1993; Young et al., 2003).

ENS development relies on the appropriate establishment of cell number, proliferation, and survival as well as migration of the precursor cells. RET signalling has been linked to all of these processes. In the early phase of ENS development, RET activity is crucial for survival of ENCC, and apoptosis is increased in the absence of RET (Taraviras et al., 1999). GDNF

signalling through RET is a regulatory factor of ENCC proliferation, and *Gdnf*$^{+/-}$ mice have a greatly reduced number of precursor cells (Heuckeroth et al., 1998; Taraviras et al., 1999; Gianino, 2003). Y1062 plays a central role in this process, as mice lacking this residue develop enteric aganglionosis (Jijiwa et al., 2004). When this mutation is introduced on a monoisoformic background, animals expressing only RET9 show a stronger phenotype with total aganglionosis, whereas only the colon is affected in RET51 expressing animals (Jain et al., 2010). Barlow *et al.* showed that a minimum number of cells is required for complete formation of the ENS. While expression of monoisoformic RET9 at 50% of normal RET levels still enabled complete ENS colonization, a reduction to 30% failed to do so (Barlow et al., 2008). These phenotypes resemble human Hirschsprung's disease (HSCR). 50% of familial and 30% of sporadic HSCR cases carry coding sequence mutations in RET, both in the extra- and intracellular domains (Runeberg-Roos and Saarma, 2007; Wallace and Anderson, 2011).

As in the sensory system, NRTN controls cell size of enteric neurons (Gianino, 2003). In addition, NRTN has been suggested to play a role in axon guidance of enteric neurons, as *Nrtn*$^{-/-}$ and *Gfrα2*$^{-/-}$ display a reduced density of cholinergic neurons projecting to the circular muscle layer (Heuckeroth et al., 1999; Rossi et al., 1999).

All in all, GDNF signalling through RET is of great importance for ENS development, and in their absence, no ENS is formed. Loss-of-function mutations that disrupt signalling through Y1062 result in severe enteric aganglionosis.

1.2.6.3 Development of the sympathetic nervous system

The sympathetic nervous system controls homeostasis by regulating internal organ functions, including vasoconstriction, salivation and gut motility. It consists of numerous ganglia throughout the body. Best-described are the superior cervical ganglion (SCG), the stellate ganglion and the paravertebral ganglia, which are organized in two chain-like structures located ventrally and bilaterally to the spinal cord.

Sympathetic neurons either belong to the cholinergic or the predominant noradrenergic lineage. Noradrenergic sympathetic neurons innervate most tissues and organs, while cholinergic neurons represent a small subset of neurons that innervate sweat glands and the periosteum. The innervation of sweat glands by cholinergic neurons has a unique role in the control of thermoregulation (Asmus et al., 2000).

Like the DRG and the ENS, also the SNS is of neural crest origin. SNS development starts with a ventral migration of neural crest cells, which coalesce in the vicinity of the dorsal aorta at E9-E10 and form the primary sympathetic ganglia. BMP factors released by the dorsal aorta have been proposed as regulating factors of sympathetic specification and they upregulate the transcriptional regulators *Mash1*, *Hand2*, *Gata2/3* and *Phox2a/2b* that establish a transcriptional network which defines the sympathetic fate (Schneider et al., 1999; Goridis and Rohrer, 2002; Young et al., 2011). MASH1 and PHOX2B control together the expression of *Phox2a* (Goridis and Rohrer, 2002), which in turn is known to induce expression of neuronal markers such as *Th* and *Dbh*. HAND2 further enhances their expression (Xu et al., 2003).

Expression of the transcription factor *Tlx3* has been reported as early as E10.5, with 82% of PHOX2B$^+$ neurons also being positive for TLX3 and it further increases until E12.5, when 95% of PHOX2B$^+$ cells are TLX3$^+$ (Huang et al., 2013). At this point, most, if not all, sympathetic cells express both noradrenergic and cholinergic lineage markers, such as tyrosine hydroxylase (*Th),* vesicular monoamine transporter 2 (*Vmat2*) and dopamine β-hydroxylase (*Dbh*) or choline acetyltransferase (*Chat*), vesicular acetylcholine transporter (*Vacht*) and *Ret*, respectively (Apostolova and Dechant, 2009; Furlan et al., 2013).

From E13.5 onwards the transcription factor *Hmx1* is gradually upregulated and in parallel *Trkc* is extinguished in TLX3$^+$ cells. A concerted action of the pro-noradrenergic HMX1 and the pro-cholinergic RET leads to a gradual segregation of the noradrenergic and cholinergic lineages, until only a small population of 10% of the paravertebral ganglion neurons maintains a cholinergic identity with expression of *Chat* and *Vacht* while the remaining sympathetic neurons obtain a noradrenergic phenotype (Furlan et al., 2013).

RET activity is crucial for cholinergic specification of sympathetic neurons. *Ret*$^{-/-}$ animals show a drastic reduction of cholinergic marker expression at E15.5, while they upregulate *Hmx1* and *Trka* instead (Burau et al., 2004; Furlan et al., 2013). Throughout sympathetic development, *Tlx3* is almost always coexpressed with *Ret*, however, TLX3 and RET have partially different functions during development of the cholinergic sympathetic neurons. While the number of VIP$^+$ and SOM$^+$ cells is greatly reduced in *Tlx3*$^{-/-}$ mice at both E12.5 and E18.5, *Ret*$^{-/-}$ have normal levels of VIP and SOM at E12.5, but show a reduction at E15.5 and E18.5 (Furlan et al., 2013; Huang et al., 2013). Thus, TLX3 seems to initiate marker expression while RET is critical for its maintenance. Both TLX3 and RET are dispensable for

normal expression of *Vacht* at E12.5 and E13, but are required for its expression at E15.5 and E18.5, indicating that neither of them is required for the induction of functional cholinergic markers, but for their maintenance in face of the repressive activity of HMX1 (Burau et al., 2004; Furlan et al., 2013; Huang et al., 2013).

Cholinergic sympathetic neurons of neonatal mice express *Gfrα2* and expression of its ligand *Nrtn* can be detected in footpad sweat glands and the periosteum . Interestingly, *Gfrα2*$^{-/-}$ mice have a normal density of sympathetic axons innervating sweat glands at P4, but have lost 50-70% of this innervation after three weeks and at adult stages, and innervation of the periosteum is completely absent. This supports the hypothesis of a target-dependent role of RET signalling for the maintenance of cholinergic target tissue innervation. Additionally, GFRα2 is a regulator of soma size in sympathetic neurons (Hiltunen and Airaksinen, 2004). The origin of at least part of RET-mediated survival signalling could be narrowed down to Y1062, which has been shown to be important for sympathetic neuron survival *in vitro*, through activation of IKK via B-Raf (Encinas et al., 2008).

To summarize, RET is a central regulator of cholinergic lineage specification and is required for the maintenance of the expression of different cholinergic markers as well as the suppression of a noradrenergic phenotype.

1.2.6.4 RET in nephrogenesis

Ret and *Gdnf* are expressed in the kidney anlagen as early as E8.5 (Jain, 2009; Coskun et al., 2011). By E10.5, RET is found in distal Wolffian duct cells, while *Gdnf* is expressed in the metanephric mesenchyme (MM) to stimulate outgrowth of the UB into the MM through activation of the RET-GFRα1 axis (Murakami et al., 2002; Chi et al., 2009). During ongoing branching, RET and GFRα1 get downregulated at the UB stalk and become limited to the branching bud tip cells, while GDNF is mostly found in the undifferentiated peripheral mesenchyme. At later stages of kidney development, RET and GFRα1 are mostly found in the nephrogenic zone. In adult mice, both RET and GFRα1 are almost undetectable in the kidney, except for in some collecting ducts and proximal tubules (Kouhara et al., 1997; Ong et al., 2000, reviewed in Jain, 2009; Davis et al., 2013).

Knock-out animals for *Ret*, *Gdnf* or *Gfrα1* die early perinatally with bilateral renal agenesis or aplasia (Schuchardt et al., 1994; Pichel et al., 1996; Enomoto et al., 1998). Interestingly, while NRTN is expressed in UB cells at E14 and into adulthood, mice lacking NRTN have normal

renal morphology and function (Heuckeroth et al., 1999). While there is consensus that RET9 alone supports normal kidney development, results on RET51 are inconclusive and system-dependent. While one study showed renal agenesis in monoisoformic RET51 mice, another one described full competence of RET51 in kidney development. The latter one also found that Y1062 of RET51 is not essential for nephrogenesis, but a Y1015F mutation results in uni- or bilateral dysgenesis in both isoforms (de Graaff et al., 2001; Jain et al., 2006).

Summarizing, RET activity controls and is essential for UB outgrowth and branching and is required for normal kidney development. The central residues involved in this process are Y1015 and Y1062.

1.2.6.5 RET in the central nervous system

The RET ligand GDNF was originally found as a factor supporting the survival of midbrain dopaminergic neurons *in vitro* (Lin et al., 1993). *Ret* is expressed together with *Gfrα1* and *Gdnf* in the substantia nigra in embryonic and adult mice, with highest expression levels being detected at postnatal stages (Trupp et al., 1996). Surprisingly, mice lacking one of these factors do not show any phenotype in the nigrostriatal system at birth. However, it was shown that while the development and early postnatal maintenance of the nigrostriatal system are not affected, conditional $Ret^{-/-}$ mice show deficits in long-term neuronal survival, with a loss or degeneration of dopaminergic neurons of midbrain and striatum (Kramer et al., 2007). Not only loss of RET, but also of GDNF, results in impaired survival of catecholaminergic neurons (Pascual et al., 2008). Given these survival effects of GDNF signalling it is an attractive target for the development of treatments for Parkinson's disease (PD), and clinical trials are ongoing (Patel and Gill, 2007; Patel et al., 2013).

Also cells of the medial ganglionic eminence and the cortex express *Gdnf* and *Gfrα1* and promote differentiation and migration of GABAergic neurons *in vitro* in dissociated primary cultures and slice cultures (Pozas and Ibáñez, 2005). Recently, Lundgren and coworkers presented that PLCγ signalling from RET via Y1015 regulates neuronal migration in the murine neocortex *in vivo* after *in utero* electroporation (Lundgren et al., 2012).

Kramer *et al.* also showed that RET/GDNF signalling provides guidance cues for migrating motor neurons *in vivo* (Kramer et al., 2006a), and in cranial motor neurons the absence of RET results in maturation deficits (Baudet et al., 2008).

1.2.6.6 RET in the parasympathetic nervous system

RET is also involved in the development of the parasympathetic nervous system. Most parasympathetic ganglia are derivatives of cranial and sacral neural crest cells (Le Douarin and Kalcheim, 1999). These *Ret* expressing parasympathetic precursor cells migrate first along a ventrolateral pathway and follow then a chemoattractive GDNF gradient to their final position. Mice lacking either RET, GFRα1 or GDNF have strongly reduced otic and sphenopalatine ganglia, while submandibular ganglia are less affected (Rossi et al., 2000). Once at their target destination, a switch of ligand dependency occurs and mature parasympathetic cells require NRTN/GFRα2 for survival and maintenance (Enomoto et al., 2000).

Both the otic, ciliary, sphenopalatine and submandibular ganglia express *Ret* and the coreceptors *Gfrα1* and *Gfrα2* (Enomoto et al., 2000; Rossi et al., 2000). Their target tissues on the other hand express GDNF and NRTN, which serve as chemoattractors for axonal growth and provide trophic support. While animals lacking GFRα2 or NRTN have normal cell numbers in the otic and sphenopalatine ganglia, the target innervation is reduced, along with a smaller neuron size (Heuckeroth et al., 1999; Rossi et al., 2000). In contrast to the otic and sphenopalatine ganglia, the submandibular and pancreatic neurons have fewer ganglion neurons in the absence of GFRα2 or NRTN, and this cell loss appears to be due to neuronal apoptosis (Heuckeroth et al., 1999; Lähteenmäki et al., 2007).

1.2.6.7 RET signalling in spermatogonia

After birth, Sertoli cells express GDNF, which controls the spermatogonial stem cell (SSC) fate through RET (Meng et al., 2000; Naughton, 2006). GDNF expression depends on follicle stimulating hormone (FSH), tumour necrosis factor alpha (TNFα), fibroblast growth factor (FGF) 2 as well as interleukine (IL)-1β (Tadokoro et al., 2002; Simon et al., 2007). Loss of RET seems to be without consequences for spermatogonia at prenatal stages and the testes of *Ret*$^{-/-}$, *Gfrα1*$^{-/-}$ and *Gdnf*$^{/-}$ mice appear normal at birth, but show greatly reduced SSC numbers by P7 (Naughton, 2006). This phenotype is recapitulated by mice with a Y1062F mutation in RET that show a strong atrophy of their testes. By P7, their RET^{+} spermatogonia in seminiferous tubes are decreased and practically absent at P21, indicating a central role of this tyrosine also in spermatogonia (Jijiwa et al., 2008).

1.2.6.8 RET in the immune and haematopoietic system

Vargas-Leal *et al.* found RET to be expressed in circulating immune cells, such as B cells, T cells and monocytes, alongside with *Nrtn* and *Gfrα2*. While also *Gfrα1* expression was found, it was more restricted than *Gfrα2* (Vargas-Leal et al., 2005). RET signalling can stimulate monocytes to secrete pro-inflammatory cytokines and chemokines (Rusmini et al., 2013). Additionally, RET is important for the formation of Peyer's patches (PP), which constitute the gut associated lymphatic tissue. Animals lacking RET or GFRα3 show severe defects in PP organogenesis, suggesting a central role of the RET/GFRα3/ARTN axis (Veiga-Fernandes et al., 2007). Also survival and proliferation of haematopoietic stem cells (HSC) is controlled by RET through stimulation of expression of the anti-apoptotic factors *Bcl2* and *Bcl2l1*. HSC of *Ret*$^{-/-}$ embryos have a normal differentiation potential, but are more susceptible to apoptosis and therefore present with lower HSC numbers. They are also unable to reconstitute a functional hematopoietic system in fully irradiated mice (Fonseca-Pereira et al., 2014).

1.2.7 *RET* in human pathologies

Both loss- and gain-of-function mutations of RET have been associated with human pathologies.

1.2.7.1 Loss-of-function RET mutations

The probably best-known loss-of-function pathology of RET is Hirschsprung's disease (HSCR), which is characterized by colonic aganglionosis of variable severity. This disease has a prevalence of 1:5000 and 50% of familial cases as well as 15-35% of sporadic cases have been shown to carry mutations in *RET* (Theocharatos and Kenny, 2008).

Most mutations underlying HSCR seem to affect the extracellular domain, with some of them having been shown to result in misfolded proteins that are degraded prematurely. Interestingly, two mutations have been identified that are adjacent to Y1062 and lead to an L1061P amino acid replacement or a deletion of N1059. Both of them result in a modification of RET's NXXpY PTB motif and impaired binding of SHC (Geneste et al., 1999). L1061P was also shown to be crucial for RET adaptor binding in PTB domain binding assays, where the mutation resulted in the loss of binding of DOK, FRS2 and SHC (Lundgren et al., 2006; Stenqvist et al., 2008). Hence, RET receptors that only display a loss of PTB binding, because Y1062 phosphorylation and the SH2 binding motif C-terminal of Y1062 are unaltered, result

in intestinal aganglionosis, illustrating the critical role of PTB domain proteins in development of the ENS also in humans.

Unexpectedly, only very few polymorphisms in GFLs are associated with HSCR (Fernández et al., 2008; Ruiz-Ferrer et al., 2011; Wallace and Anderson, 2011).

There is currently no consensus on the extent of the role of *RET* mutations as an underlying cause of congenital anomalies of the kidneys or lower urinary tract (CAKUT) associated disorders such as renal agenesis or hypodysplasia in humans. While a study by Skinner *et al.* described *RET* and/or *GDNF* mutations in 30% of foetuses that presented with renal aplasia or dysgenesis, other studies by Jeanpierre *et al.* and Chatterjeh *et al.* only found a relation of 5-6% (Skinner et al., 2008; Jeanpierre et al., 2011; Chatterjee et al., 2012).

1.2.7.2 Gain-of-function RET mutations

Pathologies based on increased RET activity are caused by chromosomal rearrangements leading to RET fusion proteins, by increased *RET* expression or by activating point mutations in *RET*, rendering the protein constitutively active or independent of ligand binding.

20-40% of patients that are suffering from papillary thyroid cancer (PTC) are carriers of chromosomal aberrations of the *RET* gene. In most cases, these *RET/PTC* rearrangements fuse the intracellular part of RET, without the trans- or juxtamembrane domain, with the N-terminus of another protein, resulting in a constitutively active cytosolic RET variant. The most common rearrangements are a fusion of RET with either the cell-cycle regulator cytoskeleton protein coiled-coil containing domain 6 (CCDC6) or the androgen-responsive transcription regulator nuclear receptor co-activator 4 (NCOA4), both of which are co-localized with *RET* on chromosome 10 in humans. Together they account for >90% of all PTC-associated rearrangements (Nikiforov, 2002).

Oncogenic point mutations in RET are associated with multiple endocrine neoplasia type 2 (MEN2). All types of MEN2 are associated with medullary thyroid carcinoma (MTC), but they differ in the presence and nature of additional symptoms. Patients with familial medullary thyroid carcinoma (FMTC) only present with MTC. MEN2A patients suffer additionally from phaeochromocytoma and hyperparathyroidism, and MEN2B patients present with phaeochromocytoma as well as other developmental defects like skeletal malformations, marfanoid habitus, ganglioneuromas and myelinated corneal nerves. The best-described oncogenic point mutations of RET are C634R in MEN2A and M918T in

MEN2B, but many additional mutations have been described (Asai et al., 1995; Runeberg-Roos and Saarma, 2007).

MEN2A associated mutations such as C634R are mostly located in the cysteine-rich domain of the RET^{ECD}. These mutations lead to the breach of an intramolecular disulphide bond and result in misfolded proteins with an unpaired cysteine. This residue can then engage with neighbouring RET monomers to establish a covalent disulfide bond, leading to receptor auto-phosphorylation even in absence of ligands (Asai et al., 1995; Santoro et al., 1995). FMTC patients have been found to carry mutations in the same sites as MEN2A patients, plus an additional set in the juxtamembrane domain. Due to the similarity between MEN2A and FMTC with respect to shared mutations it is speculated that they might represent different severities of the same disease, instead of two different disease classes (Kloos et al., 2009). Interestingly, some MEN2A/FMTC patients also show symptoms of HSCR, possibly due to premature degradation of misfolded proteins in some cell types, causing a lack of RET signalling (Ito et al., 1997; Chappuis-Flament et al., 1998; Kjaer et al., 2006).

MEN2B associated mutations are mostly found in the kinase domain and result in overactivation of this domain. The MEN2B mutation M918T has been shown to increase ATP-binding and RET with this mutation is already active when still localized in the endoplasmatic reticulum (Gujral et al., 2006; Runeberg-Roos et al., 2007). Salvatore *et al.* found an increased phosphorylation of Y1062 and enhanced SHC recruitment of RET-MEN2B *in vitro* compared to the MEN2A mutation C634Y and linked it to a constitutive activation of RAS/MAPK and AKT/PI3K signalling (Salvatore et al., 2001).

2 RESULTS AND DISCUSSION

2.1 PAPER I

Lipid rafts are lipid-ordered membrane domains that have been shown to harbour amongst others myristoylated proteins, as well as some transmembrane proteins like tyrosine kinases. One such tyrosine kinase is RET, which can be recruited to lipid rafts upon activation both through *cis* and *trans* GFRα1 association, and interacts with different adaptors inside and outside of lipid rafts (Paratcha et al., 2001). After stimulation with GDNF, RET quickly becomes ubiquitinated outside of lipid rafts, which initiates RET degradation. RET ubiquitination and degradation within rafts is considerably lower, suggesting a mechanism for sustained RET signalling via the raft-bound adaptor FRS2 (Pierchala et al., 2006).

Taking advantage of the different affinities of PTB proteins for different sequences -4 to -8 N-terminal of the PTB motif NXXpY, RET receptors were genetically engineered to preferentially bind either SHC or FRS2 at Y1062 (Lundgren et al., 2006). In functional *in vitro* studies we found that overexpression of these RET mutants resulted in different membrane localization patterns and that RET was detected in membrane foci upon FRS2 recruitment (Lundgren et al., 2008b).

Both SHC and FRS2 binding resulted in pY1062-dependent localization of RET into detergent-resistant membrane (DRM) domains, and this relocalization was abolished upon treatment with methyl-β-cyclodextrin, which extracts cholesterol from membranes and thereby disrupts lipid-ordered regions including lipid rafts. As DRM fractions consist of different lipid-ordered membrane domains and not exclusively of lipid rafts (Brown, 2006), we investigated the precise membrane localization of eGFP-tagged RET in combination with SHC or FRS2 overexpression. Co-staining for the lipid raft-specific ganglioside GM1 showed that RET^{eGFP} is efficiently recruited into lipid raft domains in the presence of FRS2, but not SHC.

In contrast to FRS2, SHC lacks a myristoylation tail anchoring it in lipid rafts. To analyse the importance of adaptor localization for its signalling outcome, we modified SHC and attached a raft targeting sequence (MLS). SHC^{MLS} was able to recruit RET to lipid rafts in a fashion similar to FRS2 as shown by cytochemistry of transfected SK-N-MC cells and density fractionation of the membrane of electroporated chicken embryo spinal cord cells. Regarding

intracellular signalling, we found that ERK activation by SHC^{MLS} resembled FRS2 in that it was stronger and more sustained than that of regular SHC, while AKT phosphorylation of SHC^{MLS} was at an intermediate level between those of FRS2 and SHC. The activation of ERK was attenuated in FRS2 and SHC^{MLS} upon lipid raft disruption, while ERK activation by SHC was not affected. These results could be recapitulated in chicken embryo spinal cords that were electroporated with overexpression constructs for RET in combination with SHC, SHC^{MLS} or FRS2. Interestingly, not only the PTB adaptors themselves, but also phospho-ERK was found in the DRM fraction when overexpressing the lipid raft bound adaptors.

This intermediate position of SHC^{MLS} in terms of signalling led to consequences in functional *in vitro* assays. Both FRS2 and SHC^{MLS} supported cell migration of SK-N-MC cells in a lipid raft-dependent fashion while SHC failed to do so, and neither FRS2 nor SHC^{MLS} promoted cell survival to the same extent as SHC. Thus, SHC^{MLS} resembled FRS2 more than SHC in functional assays.

In summary, this publication shows that specific compartmentalization of RET adaptors is a mechanism that controls which and how signalling pathways are activated, as well as that subcellular localization is a regulatory factor for the desired functional outcome of cell signalling. This is probably not specific to RET signalling, but may be a common mechanism also for other RTKs, explaining how one and the same signalling receptor can have different outcomes, depending on which intracellular adaptor it recruits. In case of polarized cell biological events such as migration, in contrast to more systemic situations like survival signals, recruitment of activated receptors to defined membrane domains like lipid rafts can also aid in the polarization of not only cytoskeletal rearrangements, but also the signalling machinery in general.

2.2 PAPER II

In vivo studies of RET signalling have so far only been performed in knockout animals for different signalling components like RET itself, one of the coreceptors GFRα1-4 or their ligands, or in receptor variants that were based on tyrosine replacements that affect all phosphotyrosine-dependent docking proteins that normally bind the mutated amino acid. Taking advantage of the different affinities of PTB domain proteins for different sequences N-terminal of the PTB motif, we previously generated RET receptor variants that preferentially bind the Y1062 adaptors DOK, FRS2 or SHC (Lundgren et al., 2006; Stenqvist et al., 2008). To analyse the role of these adaptors *in vivo* we generated mice expressing the adaptor-

specific RET receptors and studied their phenotypes. *Ret*$^{9/9}$ mice express only the RET9 isoform, while *Ret*$^{9Dok/9Dok}$, *Ret*$^{9Frs/9Frs}$ and *Ret*$^{9Shc/9Shc}$ mice express the RET9 variants signalling preferentially via DOK, FRS2 or SHC, respectively. The RET9 variants will henceforth be referred to as RET9-DOK, RET9-FRS2 or RET9-SHC.

Kidney development depends to a large extent on the availability of RET, GDNF and GFRα1. Both RET and GFRα1 are found in the tip cells of the branching UB, while GDNF is found in the surrounding tissue, acting as a chemoattractant (Murakami et al., 2002; Chi et al., 2009). We failed to find any phenotype in P8 kidneys in our mouse lines. This is an interesting finding, as *Ret*$^{9/9}$ mice that have a Y1062F amino acid replacement fail to develop normal kidneys, indicating a central role of this residue in the RET9 isoform (Jain et al., 2006). Thus, activation of signalling through any of the adaptors recruited to Y1062 is sufficient for sustained kidney development, probably via the RAS/MAPK pathway, as this is the only signalling pathway that is activated by all three adaptors. The importance of this pathway is also supported by the finding that a loss of the MAPK/ERK inhibitors Sprouty 1 and 2 can rescue the RET knockout phenotype in nephrogenesis (Basson et al., 2005; Miyamoto et al., 2010).

The intestine is colonized by ENCC in a rostro-caudal direction, and reduced RET signalling can result in incomplete colonization (Uesaka et al., 2008). We found that signalling of RET Y1062 via FRS2 alone results in intestinal aganglionosis, while both RET9-DOK and RET9-SHC are fully capable of supporting normal ENS development. This phenotype is already detectable as early as E10.5, when ENCCs in *Ret*$^{9Frs/9Frs}$ mice had barely left the foregut, while they had almost reached the caecum in control animals. RET signalling is important in the developing ENS for different cell biological processes such as proliferation, migration and survival (Heuckeroth et al., 1999; Taraviras et al., 1999; Barlow et al., 2003), and critical numbers of enteric precursor cells are required for complete colonization and ENS formation (Barlow et al., 2008). Hence, each of these functions of RET might be the underlying cause of the phenotype observed in *Ret*$^{9Frs/9Frs}$ mice. As described above, a knockout of the MAPK signalling regulators Sprouty1 and 2 can rescue the *Ret* knockout phenotype in the kidneys, but it can only partially do so in the stomach and not at all in the intestine. This suggests that PI3K signalling might be of higher importance in the ENS, while MAPK signalling controls nephrogenesis (Basson et al., 2005; Miyamoto et al., 2010).

RET is a known regulator of sensory neuron development. Mice lacking RET in sensory neurons present with changes in the expression of several ion channels and receptors, accompanied by behavioural deficits (Golden et al., 2010; Franck et al., 2011). NRTN and GFRα2 on the other hand control soma size of DRG neurons (Luo et al., 2007; Golden et al., 2010). We found that RET9-FRS2 does not provide sufficient trophic support for DRG neurons, resulting in reduced cell soma size, while normal cell numbers are maintained. This suggests a NRTN-GFRα2-DOK/SHC axis of signalling that is essential for soma size development. RET9-SHC expression results in aberrant subtype specification and behaviour. We found a downregulation of *Nav1.8* and *P2x3* and a markedly increased expression of *Trpm8*, similar to *Ret* sensory neuron conditional mutant mice (Franck et al., 2011). $Ret^{9Shc/9Shc}$ mice have higher cold sensitivity and show stronger itch behaviour upon stimulation with chloroquine and 5-HT. Interestingly, $Ret^{9Frs/9Frs}$ mice showed increased mechanosensitivity at an early postnatal age, similar to *Ret* conditional mutant mice, but this phenotype was observed without any detected difference in expression levels of sensory markers. This indicates either that RET controls expression of a gene product which is critical for mechanical sensation but was not analysed in our study, or that a change of soma size alone results in a shift in sensitivity.

All observed phenotypes in the $Ret^{9Frs/9Frs}$ and $Ret^{9Shc/9Shc}$ mice are also found in conditional *Ret* knockout mice. However, not all molecular changes observed in the knockouts were found in the rewired RET receptor bearing mice. This suggests that other tyrosine residues than Y1062 might affect sensory specification.

In the sympathetic nervous system, RET plays a central role in the regulation of lineage specification. *Ret* knockout mice lack cholinergic neurons at E15.5, with a corresponding increase in the noradrenergic population (Burau et al., 2004). During lineage specification, a former hybrid population expressing markers of both the cholinergic and noradrenergic lineage downregulates cholinergic markers in most sympathetic neurons, while only a small population of 10% maintains cholinergic and turns off noradrenergic markers. Interestingly, we found an increase in the cholinergic population in $Ret^{9Frs/9Frs}$ animals, consistent for all analysed factors of the cholinergic lineage such as VAChT, VIP, TLX3 and RET. Surprisingly, we did not detect any difference in the number of cells expressing noradrenergic markers. Further analysis showed a striking nearly 4-fold increase of sympathetic neurons expressing both *Th* and *Vacht*.

Since the total number of noradrenergic neurons was unchanged, we concluded that *Ret*$^{9Frs/9Frs}$ mice partially fail to diversify sympathetic neurons into the distinct noradrenergic and cholinergic phenotypes and that cholinergic markers fail to be downregulated in a group of noradrenergic cells.

In summary, we showed that RET adaptor proteins at Y1062 have tissue-specific roles, with binding of DOK or SHC being crucial in the ENS, for trophic support of sensory neurons and for lineage segregation in the SNS. Signalling via DOK or FRS2 on the other hand is essential for subtype specification of sensory neurons.

It would be interesting to extend our analysis to other organ systems, for example the nigrostriatal and parasympathetic systems, where GDNF signalling has been reported to be essential for neuronal survival (Pascual et al., 2008). The pro-survival effect of GDNF on nigrostriatal neurons has been suggested to rely on MAPK/ERK and does not depend on PI3K/AKT (Peterziel et al., 2002). Although also Y905 activates MAPK/ERK through GRB7/10, there is reason to expect an involvement also from Y1062 and/or Y1096, both of which represent binding sites for other MAPK activators, such as SHC, FRS2, DOK and GRB2.

2.3 PAPER III

Lineage specification in the SNS is controlled through a network of growth and transcription factors. Already at E10.5, the transcription factors PHOX2B and HAND2, in concert with GATA2/3, are required for the establishment of the noradrenergic lineage and promote expression of *Th* and *Dbh* (Apostolova and Dechant, 2009). For later stages of SNS development, it has been suggested that lineage specification is under the control of extrinsic factors (Habecker and Landis, 1994; Asmus et al., 2001).

Although previous reports described the expression and function of key regulators in the early transcriptional network, a detailed analysis of the mechanisms of the noradrenergic and cholinergic lineage segregation during embryonic development has not been presented.

Our aim was to elucidate the molecular network of transcriptional regulation and growth factor signalling underlying the lineage segregation of sympathetic neurons.

First, we investigated the expression of several marker molecules throughout embryonic development and found that sympathetic progenitors express both noradrenergic and

cholinergic features at E12.5. This suggests that the first sympathetic neurons are formed as hybrid cells with a mixed phenotype. The transcription factor HMX1 was expressed in hybrid cells from E13.5 and already at E18.5 the noradrenergic and cholinergic populations were largely segregated. Using genetic tracing we could show that both neuronal populations are derived from a common RET^+ pool of progenitors, suggesting that emergence of the distinct neuronal populations is by large a process of gene repression.

The upregulation of HMX1 was accompanied by a downregulation of TRKC and RET. At E18.5, virtually all $HMX1^+$ neurons were $TRKA^+$ and noradrenergic, whereas the remaining RET^+ cells were $ChAT^+$ and cholinergic.

Knockout of *Trkc* resulted in increased *Hmx1* expression, paralleled by a gain of TRKA and a loss of RET. Knockout of *Hmx1* on the other hand resulted in a marked loss of TRKA at E15.5 and P0 and TH expression at P0. The few remaining $TRKA^+$ cells at P0 coexpressed RET. This indicates an antagonistic effect of HMX1 and TRKC in the control of lineage specification. Interestingly, TRKA was already lost from E15.5, while TH was still found at normal levels at this stage and was lost only later on. Vice versa, the cholinergic markers *Ret*, *Vip* and *Sst* were all found at highly increased levels at E15.5 and P0 due to a de-repression, while *Chat* and *Vacht* were unchanged. HMX1 therefore seems to supress both *Ret*, *Vip* and *Sst* expression, while *Chat* and *Vacht* regulation is independent of HMX1. RET is required for the maintenance of cholinergic markers, but increased RET levels as a consequence of a de-repression are not sufficient to maintain *Chat* and *Vacht* expression, suggesting additional repressive factors besides HMX1.

While the loss of HMX1 did not change the expression of *Chat* and *Vacht*, $Trkc^{-/-}$ mice showed both a decrease of ChAT and an increase of TH and therefore present an actual switch of cell fate.

We found that $Ret^{-/-}$ mice showed ectopic expression of *Hmx1* in cells that usually would be expressing *Ret* at this stage, together with a gain of TRKA and a loss of the cholinergic markers VAChT, SST and VIP, which is in line with previously published results (Burau et al., 2004).

In summary our results uncover a gene regulatory network that controls the specification and diversification of sympathetic neurons. *Hmx1* expression is initiated in early sympathetic hybrid neurons and turns on TRKA expression. HMX1 is crucial for the maintenance of the

noradrenergic population and for the suppression of a cholinergic fate and therefore represents a critical part of the gene regulatory network during noradrenergic sympathetic neuron development. TRKC, expressed in early progenitors, is an upstream regulator supporting *Ret* expression and inhibiting *Hmx1* expression and is therefore participating in the establishment of the cholinergic lineage. Maintenance of *Ret* expression suppresses *Hmx1* and thereby drives cholinergic neuron specification. Possible candidate ligands that may initiate the above gene regulatory networks include WNT or BMP factors, both of which are expressed at early stages of neural crest development, and BMPs released by the dorsal aorta have been shown to be essential for the establishment of a sympathetic phenotype (Schneider et al., 1999; Goridis and Rohrer, 2002).

A remaining question is which of these early factors are controlling the onset of expression of *Hmx1* and repress *Trkc*. A recent study suggested a role for PROX1 in developing sympathetic ganglia in chicken, with *Prox1* being expressed in proliferating cells (Holzmann et al., 2015). While it is unknown which sympathetic factors are coexpressed in these cells, it opens up for speculation if *Prox1* might be expressed in the TRKC^{+} precursor population that shows a similar decrease during development as PROX1.

3 CONCLUSIONS

In the presented thesis I investigated the role of RET signalling in cell biology and during different processes in murine development.

We show that RET signalling via the membrane anchored FRS2 adaptor depends on the integrity of lipid rafts. A membrane-localized variant of the otherwise lipid raft independent SHC adaptor differs in its signalling outcomes from normal SHC and resembles that of FRS2 instead. This indicates that the specificity of downstream pathways is not only controlled by *which* adaptor is signalling, but also from *where* within the cell it does so.

I describe organ and cell type specific roles of different adaptors at RET Y1062 *in vivo*. While some organ systems develop normally irrespective of which adaptor is signalling, others show erroneous development upon adaptor restriction, indicating non-redundant adaptor functions and a dependence on defined pathways. I also show that the approach of amino acid replacement for engineering of adaptor-specific RTKs is not only feasible for *in vitro* studies, but can also be employed for *in vivo* analysis of receptor signalling.

In the sympathetic nervous system, the specification of the cholinergic and noradrenergic lineages is tightly controlled by transcription factors and cell surface receptors. We show that early markers of both lineages regulate and supress each other, establishing a complex network of cross-regulatory effects. RET is required for the maintenance of cholinergic markers, while HMX1 favours the noradrenergic lineage and supresses some cholinergic markers including RET.

To conclude, I described that the subcellular receptor localization influences biochemical and functional properties of RTKs *in vitro*. In addition, I found that the different RET Y1062 adaptors have both overlapping and tissue-specific roles during development. Last but not least, I presented a gene regulatory network that governs the lineage segregation in the sympathetic nervous system.

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