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Rewiring Ret:
PTB-adaptor regulated signaling and cell biology

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Abstract

The Ret receptor belongs to a group of transmembrane tyrosine kinase receptors that serve to transduce environmental signals into cellular responses. Upon extracellular ligand activation the receptors dimerize and their structural conformation is altered into one that allows for interaction with proteins in the cell cytosol. Among the most proximal interactors are PTB-adaptor molecules that dock to Ret tyrosine residues. As several PTB-adaptors compete for interaction with the same receptor-sequence the molecule that successfully binds to Ret excludes binding of any other PTB-adaptor at that time. Hence, the resulting signals are dependent on which adaptor that successfully engaged the receptor. In this work, Ret has been rewired to preferentially bind one PTB-adaptor on the expense of others to tyrosine 1062. This makes it possible to experimentally assign biochemical events as well as cell biological outcomes to one specific adaptor and so increasingly reveal how one common receptor may serve a plethora of functions depending on the subcellular milieu in which it operates.

In paper I the important residues for Ret interaction with the Shc and Frs2α adaptors were investigated. Based on data from the effect on adaptor affinity for Ret by specifically substituting amino acids around tyrosine 1062, mutants could be established with selective recruitment of either Shc or Frs2α to this tyrosine. Ret interaction with Shc resulted in capacity for the receptor to mediate ligand dependent survival in the setting of apoptotic stimuli or severe starvation while Frs2α mediated signaling was insufficient to do so.

In paper II the Frs2α adaptor docked to Ret were found to be essential for chemotactic directional migration towards Ret ligands. The molecular basis for Ret promoted migration depended on the membrane associated Src family of kinases that bind to Ret at a different tyrosine than Frs2α such that these two residues are cooperatively involved in assembling the molecular framework required to execute a migratory response downstream of Ret.

In paper III the Dok adaptors, which were recently found to interact with Ret, were investigated. Dok selective Ret mutants could be created and were expressed in neuronally derived cells. Dok binding resulted in prolonged phosphorylation of MAP kinases and allowed for Cdc42 activation. The cellular response was enhanced microspike formation as determined morphologically.

In paper IV local membranous Ret interaction with Shc and Frs2α were investigated. While Frs2α overexpression recruited Ret to distinct lipid raft like partitions of the plasma membrane Shc expression led to Ret being found outside these fractions. A Shc molecule with an appendage forcing its association to lipid rafts resembled Frs2α characteristics in terms of downstream biochemical profile and dependence of ordered cholesterol species in the membrane for signaling, suggesting the importance of adaptors for appropriate relocation of Ret. Moreover, the Frs2α dependent migration was indeed disrupted by cholesterol oxidation while the survival response promoted via Shc showed little dependence on disruption of membrane architecture.

List of publications

I. **Lundgren TK**, Scott RP, Smith M, Pawson T, Ernfors P.
Engineering the recruitment of phosphotyrosine binding domain-containing adaptor proteins reveals distinct roles for RET receptor-mediated cell survival.
J Biol Chem. 2006 Oct 6;281(40):29886-96.

II. **Lundgren TK,** Stenqvist A, Scott RP, Pawson T, Ernfors P.
Cell migration by a FRS2-adaptor dependent membrane relocation of ret receptors.
J Cell Biochem. 2008 Jan 11; [Epub ahead of print]

III. Stenqvist A, **Lundgren TK**, Smith M, Hermansson O, Castelo-Branco G, Pawson T, Ernfors P.
Subcellular receptor redistribution and enhanced microspike formation by a Ret receptor preferentially recruiting Dok.
Neuroscience Letters. 2008 Apr 11;435(1):11-6

IV. **Lundgren TK**, Luebke M, Stenqvist A, Ernfors P.
Differential membrane compartmentalization of Ret by PTB-adaptor engagement,
FEBS J. 2008 May 275 (9), 2055–2066

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Selected abbreviations

ARTN	artemin
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
Dok	downstream of kinase
DRG	dorsal root ganglion
E	embryonic day
Erk	extracellular signal-regulated kinase
FAK	focal adhesion kinase
Frs2α	fibroblast growth factor receptor substrate-2α
Gab	Grb-2 associated protein
GDNF	glial cell line-derived neurotrophic factor
GEF	guanine nucleotide exchange factor
GM1	Ganglioside M1
GFL	GDNF family ligand
GFP	green fluorescent protein
GFRα	GDNF family receptor alpha
Grb	growth factor-bound protein
GPI	glycosyl-phosphatidylinositol
HSCR	Hirschsprung disease
IRS	insulin receptor substrate
KO	knockout
MAPK	mitogen-activated protein kinase
Men2	multiple endocrine neoplasia type 2
mRNA	messenger RNA
MTC	medullary thyroid carcinoma
NCAM	neural cell adhesion molecule
NGF	nerve growth factor
NRTN	neurturin
NT-3	neurotrophin 3
P	postnatal day
PI3-K	phosphatidylinositol 3-kinase
PNS	peripheral nervous system
PSPN	persephin
PTB	phosphotyrosine binding
RET	Ret tyrosine kinase receptor /*re*arranged during *t*ransfection receptor
SCG	superior cervical ganglion
SH	Src homology
Shc	SH2-containing
Src	sarcoma gene product
TGF-β	transforming growth factor beta
TH	tyrosine hydroxylase
Tyr	Tyrosine
WT	wild-type
Y	Tyrosine

1 Introduction

1.1.1 General concepts in cell signaling

After successful fertilization the egg cell divide and the progeny will continue proliferating until millions of cells together make up the complete body structure. The cells in a functioning adult will be of vastly different types and populate different regions of the body, often at a distance from where they first emerged. Therefore, specification of type, location and function needs to be defined for every cell. This is accomplished as the cells keep track of the surroundings by reacting to signals from the extracellular matrix and from neighbouring cells. Signals may consist of secreted soluble molecules, mechanical stress, osmotic differences or local variance in hydroxonium ion concentration (pH). Regardless of the type or origin of the input, in order to affect cell biology, the extracellular signals must translate into an alteration of the intracellular milieu. A large number of receptors that interact with extracellular molecules are expressed on each cells plasma membrane. After ligand engagement the receptors will alter their structural conformation and hence become activated and as receptors are activated further pass-on of the signal occur within the cell interior. Intracellular signaling is executed by the onset of cascades of new molecular interactions and consequent structural modifications of intracellular proteins; broadly referred to as intracellular signal transduction. One and the same receptor commonly has the capacity to conduct several transduction events and the temporal and quantitative mode of various signaling proceedings depend on the subcellular context in which the receptors reside, for example, the expression/abundance of proteins complexing with the receptor and on the presence (or not) of competing receptor-interacting proteins. The focus of the work presented here has been on regulation of intracellular messaging and the effect on cell biology of rewiring the receptor tyrosine kinase Ret away from the normal signal homeostasis. Emphasis has been on the most proximal modifiers of intracellular messaging, namely adaptor proteins, and their importance for biochemical and functional signaling events mediated by the Ret receptor.

1.1.2 Receptor tyrosine kinase signaling

Receptor tyrosine kinases (RTKs) are typically integral transmembrane proteins. Within the RTK group members vary in their extracellular construction as to infer ligand specificity and in

their intracellular part to specify interactions leading to intracellular signal transduction. Ligand engagement to the extracellular part of RTKs result in a structural change of the receptors and homodimerization, which in turn will auto-transphosphorylate intracellular tyrosines on each receptor in the pair. Tyrosine phosphorylation within the kinase domain leads to further conformational alteration that permits autophosphorylation of more C-terminal residues; tyrosines or serines. Importantly, several human cancer forms are the result of deregulated receptor tyrosine kinases, for example, uncontrolled cell proliferation can be the result of constitutive RTK activation unaffected by ligand availability. The phosphorylated RTK residues are contact points for intracellular cytosolic or membrane bound proteins that by binding to the receptor provide a scaffold which specifies further molecular interactions by recruiting the next set of interactors in a cascade-like fashion and thereby regulate downstream signaling events. More often than not, several such scaffolding proteins may be competent for interacting with a particular residue on the RTK and the identity of the ultimate interactor in a given instance depend on the environmental circumstances in the intracellular milieu.

1.1.2.1 Modulatory build-up of signal transduction

Component proteins of intracellular signaling chains are frequently constructed from a set of modular domains. Domains are three-dimensional polypeptides with intrinsic folding properties resulting in a conformation particularly suitable for interaction with specific amino acid sequences on other intracellular proteins. Examples of domains are; the phosphotyrosine recognizing SH2 (Src homology 2), PTB (phospho tyrosine binding) domains, the phosphoinositide recognizing PX (Phx homology), PH (plextrin homology) and FYVE domains or the polyproline recognizing SH3 (Src homology 3) domains (Pawson and Nash 2003). One or several such domains can be incorporated into a larger polypeptide i.e. an adaptor molecule. Proteins equipped with such “cassettes” of modular domains function to mediate a link between the surface bound receptors and intracellular components of signaling complexes. Depending on the characteristics of the receptor-bound adaptor (which domains that are present) one or a number of further downstream interactions are selected for or against. Molecules interacting with the adaptors may have enzymatic activity and catalyze phosphorylation or other post-translational modifications on subsequent proteins. Evolutionary continuous use of modules in different combinations presents a suitable way of creating increasingly complex connectivity of protein segments already encoded by the cell and thus enhance the functionality of a limited set of gene products (Pawson and Nash 2003). Intriguingly, chimeric combination of domains by

in-lab cloning has been shown to completely redirect signaling and consequent cellular biology downstream of transmembrane receptors (Howard et al. 2003).

1.1.3 Intracellular signal transduction

Sequential activation of intracellular proteins as a result of receptor activation has often been divided into signaling pathways or cascades. A pathway may be defined as a set of proteins that are activated in chronological order with the most proximal protein interacting with the receptor and the more distal protein(s) executing effects within the cell that determine the cells biological behaviour. More recently, however, it has been made clear that most pathways diverge, converge and interact with each other. Thus, pathways classically assigned to one particular receptor may be absent or altered downstream of that receptor in a different cellular environment. It seems as if the context of activation is essential for regulating the signaling and that conserved "well mapped" pathways are rarely as linear as previously depicted. Some of the most important signal transduction cascades encountered in this work are the phosphatidylinositol 3-kinase pathway (PI3K) and the mitogen activated protein kinase signaling pathway (MAPK) which are briefly delineated below.

1.1.3.1 The phosphatidylinositol 3-kinase pathway

PI3 kinases are lipid kinases that phosphorylate the 3`-OH position of inositol rings on phosphoinositides. The PI3 kinases are divided into class I, II and III and vary some in their competence for phosphorylating different phosphoinositides (Marone et al. 2008). Recruitment of class I PI3Ks, the best characterized class, by adaptors result in the generation of phosphoinositides (3,4,5)P_3 which in turn recruit effector proteins with pleckstrin homology (PH) domains such as Akt (also known as protein kinase-B (PKB) to the

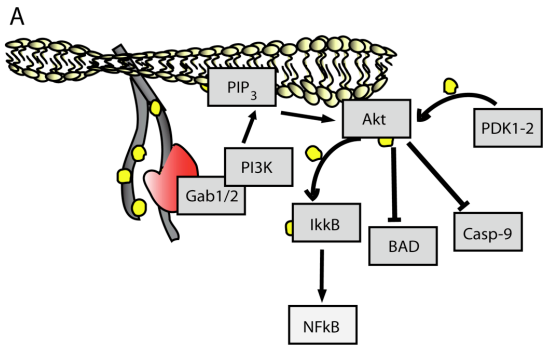

Figure 1. (A) The phosphatidylinositol 3-kinase pathway. An example of the PI3K pathway activated via a receptor bound adaptor protein. Some of the outcomes of active PI3K/Akt such as inhibition of proapoptotic Bad and Caspase-9 or activation of Nuclear factor kappa B are exemplified.

membrane. Akt become phosphorylated by phosphoinositide dependent kinase 1 (PDK-1) and sometimes also by PDK-2 kinases i.e. the mTor complex at the membrane. Akt in turn are capable of regulating a multitude of downstream effectors with effects on cell biology such as survival/anti-apoptosis, motility, process outgrowth and cell cycle progression (Marone et al. 2008). In disease, exaggerated PI3K/Akt positively affect cell growth and prevent apoptosis which may lead to cancerous tumour formation and metastasis (Vivanco and Sawyers 2002) Figure 1A.

1.1.3.2 The mitogen activated protein kinase pathway

The MAPKs are resultant effectors post activation of several RTKs. Upon ligand mediated activation of a transmembrane receptor the resulting intracellular phospho-tyrosines serve as docking sites for proteins including Grb2 and 7 or Gab1/2. These proteins in turn recruit guanine nucleotide exchange factors (GEFs), typically the son of sevenless (SOS) protein. GEFs activate the guanine nucleotide phosphorylase (GTPase); Ras. Ras is membrane bound and become activated by the exchange of bound GDP for GTP. Active Ras binds to one of three (in mammals) Raf proteins that are mitogen activated protein kinase kinase kinases (MAPKKKs). In progressive order the next target in the cascade are the MAPKKs MEK1,2 which become activated by Raf mediated dual phosphorylation. MEK1,2 then activate extracellular regulated kinase1,2 (Erk1,2) also by phosphorylation. Erk1,2 translocate to the nucleus of the cell where several transcription factors are regulated. Depending on which transcriptional systems are activated the MAPK transduction cascade can lead to proliferation, survival, migration and more (Junttila et al. 2007). On multiple levels the MAPK pathway is kept in check by dephosphorylation of incounted proteins by phosphatases such as members of the type-1 (PP1) or type-2 (PP2) phosphatase family. Figure 1B.

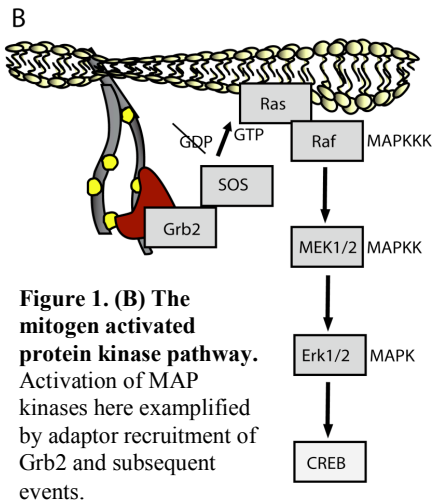

Figure 1. (B) The mitogen activated protein kinase pathway. Activation of MAP kinases here examplified by adaptor recruitment of Grb2 and subsequent events.

1.2 The Ret receptor

1.2.1.1 Ret structure and function

The Ret protooncogene was identified by Takahashi et al. in 1985 by a re-arrangement of two genes during a transfection procedure of NIH 3T3 cells that resulted in transforming activity of the cells (Takahashi et al. 1985). *Ret* as in *re*arranged during *t*ransfection has remained the name of the gene expressing the kinase domain that was fused N-terminally to an unrelated gene product in the Takahashi experiments (Takahashi et al. 1985; Nakamura et al. 1994). The *RET* gene is located on chromosome 10 (10q11.2) and consist of 21 exons (Ishizaka et al. 1989). Wild type *Ret* codes for an approximately 170 kDa, or 150kDa in a less glycosylated form, protein of typical receptor tyrosine kinase type. The expressed receptor is N-terminally made up of an extracellular part with 4 continuous cadherin-like repeats and one calcium ion binding site followed by a region rich in cysteins (Anders et al. 2001; Kjaer and Ibanez 2003). The cadherin-like repeats make Ret a distant member of the cadherin superfamily and it has been speculated that the *Ret* gene is an evolutionary recombination product of a kinase domain with a cadherin gene (Anders et al. 2001). Cadherin domain one is required for ligand interaction whereas the calcium ion binding site is formed by domain two and three (Anders et al. 2001; Kjaer and Ibanez 2003). The Ret molecule continues by a hydrophobic single-pass region transversing the cell membrane. Within the cell the Ret receptors juxtamembrane domain contains two tyrosines with unknown if any signaling properties and one serine (S697) which has recently been found to be a phospho kinase A (PKA) site and to be regulated by cyclic adenosine monophosphate (cAMP) (Fukuda et al. 2002; Asai et al. 2006). In the C-terminal direction the kinase domain follows on; a domain which is divided into two parts by an interfering stretch of 27 amino acids. Activation of the Ret kinase domain most likely depend on transphosphorylation of two neighbouring Ret molecules akin to other receptor tysosine kinases although this has not been specifically proven for Ret (Takahashi and Cooper 1987; Takahashi et al. 1989; Iwamoto et al. 1993). C-terminal to the kinase domain Ret is equipped with several tyrosine residues (14 tyrosines in the Ret9 isoform) that become autophosphorylated upon ligand interaction or by erroneous mutations in the Ret gene (discussed below) (Figure 2A). As for most RTKs when Ret is activated by ligands it will form homeodimers and this will catalyze intracellular tyrosine phosphorylation on several residues in the C-terminal part of the receptor. Intracellular phosphotyrosines activate several signaling cascades some of which are common for more than one tyrosine and others that are unique (residues Y905, Y981, Y1015, Y1062 and in the Ret51 isoform; Y1096 are the best characterized to date). Among the most studied of the transduction

cascades activated by Ret are the mitogen activated protein kinase (MAPK) pathway, the phosphatidylinositol-3 kinase (PI3K) pathway, the phospholipase C gamma (PLCγ) pathway and the activation of c-Src and Jun (reviwed in de Groot et al. 2006). Tyrosine 1062 is an *in vivo* and *in vitro* autophosphorylation residue of Ret that functions as a multidocking site for PTB binding domain containing interaction proteins. Phosphorylation of Y1062 is functionally important since substitution of this residue result in a phenotype by and large resembling *Ret* null mutant mice and also impairs the transforming activity of Ret oncogenic mutants (Asai et al. 1996; Jijiwa et al. 2004).

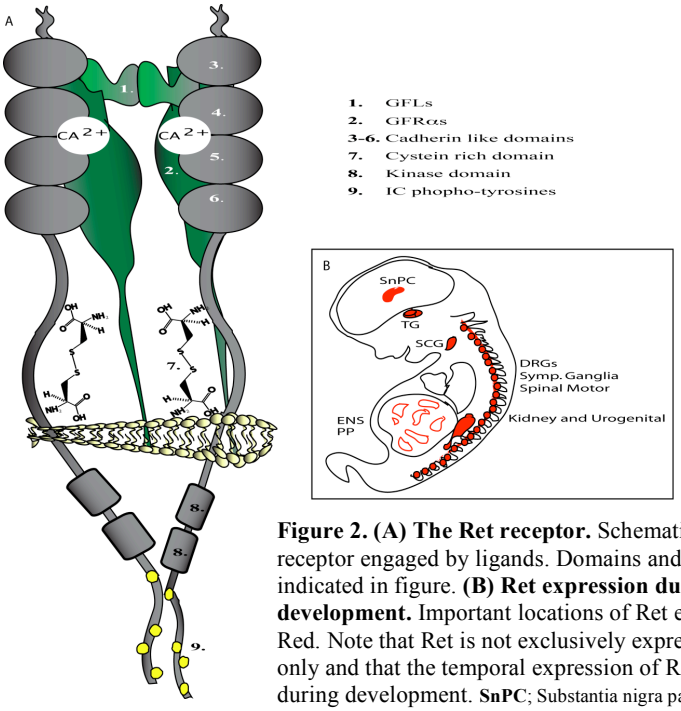

Figure 2. (A) The Ret receptor. Schematic illustration of the Ret receptor engaged by ligands. Domains and important regions are indicated in figure. **(B) Ret expression during different stages of development.** Important locations of Ret expression is indicated in Red. Note that Ret is not exclusively expressed in marked locations only and that the temporal expression of Ret varies between tissues during development. **SnPC**; Substantia nigra pars compacta, **TG**; Trigeminal ganglion, **DRG**; Dorsal root ganglion, **ENS**; enteric nervous system, **PP**; Peyers patches, **SCG;** sympathetic cervical ganglion.

The Ret mRNA can be alternatively spliced at the 3-prime end which result in different transcripts of Ret with either 9, 43 or 51 unique amino acid stretches downstream the last common residue (Tahira et al. 1990; Lorenzo et al. 1995; Myers et al. 1995). The Ret43 form is least well characterized, Ret51 and Ret9 seem to function somewhat differently and perhaps in different contexts *in vivo* (Tsui-Pierchala et al. 2002; Scott et al. 2005). Ret9 is reportedly the more important isoform for survival and for development of the enteric nervous system as knock-in mice monoisoformic for *Ret9* appear viable and normal in contrast to monoisoformic *Ret51* mice that display kidney hypoplasia and aganglisosis of the colon (de Graaff et al. 2001).

These same authors also showed that *Ret9* but not *Ret51* expressed under the control of the Hoxb7 promotor could rescue kidney agenesis in *Ret* deficient mice. The data on Ret isoforms and the development of the kidney are not clear however, as a later study found kidney development to be sufficiently supported by homozygous *Ret51* mice unless tyrosine 1015 was mutated (Jain et al. 2006). In addition, tyrosine 1096 which is only present in the longer Ret isoform Ret51 could salvage at least some signaling initiated from tyrosine 1062 when the latter was nullified (Jain et al. 2006). For excellent detailed reviews of Ret genomics see also (Arighi et al. 2005; Zbuk and Eng 2007).

1.2.1.2 Ret ligands

The ligands for Ret are four; Glial cell line-Derived Neurotrophic Factor (GDNF), Neurturin (NRTN), Artemin (ARTN) and Persephin (PSPN) (Durbec et al. 1996; Kotzbauer et al. 1996; Trupp et al. 1996; Vega et al. 1996; Worby et al. 1996; Baloh et al. 1998; Milbrandt et al. 1998). Collectively they make up the glial cell line-derived neurotrophic family of ligands (GFLs). GDNF, the first discovered Ret binding partner, was identified in a glial cell line derived from rat and was found to promote embryonic midbrain dopaminergic neuron survival *in vitro* (Lin et al. 1993). GFLs belong to the TGFβ-superfamily of ligands with a defining cystein knot structure. Despite a striking 3-dimensional structural resemblance to the TGFβ-superfamily the GFLs do not have large sequence similarities to other TGFβ members (Daopin et al. 1993; Eigenbrot and Gerber 1997). GFLs function as dimeric molecules but are expressed as prepro-monomers including a signal sequence i.e. prepro-GDNF consist of 211 amino acids and is further processed into the mature 134 amino acid form (Airaksinen et al. 1999).

Interestingly, while other RTK ligands bind directly to their receptor the GFLs form a complex with a co-receptor prior to interaction (Jing et al. 1996; Treanor et al. 1996; Jing et al. 1997; Klein et al. 1997; Baloh et al. 1998) or bind to preassembled Ret with the co-receptor (Eketjall et al. 1999). The co-receptors are referred to as GDNF family receptors alpha1-4 (GFRα1-4) each of which have a favoured ligand partner in the GFL family; GFRα1+GDNF, GFRα2+NRTN. GFRα3+ARTN and GFRα4+PSPN but at least *in vitro* they are somewhat promiscuous in this regard (Airaksinen et al. 1999). The GFRαs have an attached glycosyl-phosphatidylinositol (GPI) moiety that confer membrane anchorage and may also serve to amplify their presence in distinct membrane localities which, as discussed below, can potentially affect signaling. Still, GFRαs are also biologically active in soluble form (Paratcha

et al. 2001). All GFRαs except GFRα4 which lack domain one have a three domain structure; D1,D2 and D3 (Suvanto et al. 1997; Airaksinen et al. 1999). Crystallography combined with modeling studies has revealed at least four important residues in D2 as essential for GDNF binding to GFRα1 and the D2 and the cleft between D2 and D3 to form the Ret binding surface (Airaksinen et al. 1999; Scott and Ibanez 2001; Leppanen et al. 2004). The D1 domain seemingly function to stabilize GDNF binding to GFRα1 (Virtanen et al. 2005).

1.2.2 Ret in development and disease

1.2.2.1 Expression of Ret signaling components

The expression pattern of *Ret* is largely seen in neuronal structures and tissue derived from neural crest cells (NCC) including sensory, sympathetic, central motor, enteric plexa and catecholaminergic neurons which are all dependent on Ret during development (Pachnis et al. 1993; Tsuzuki et al. 1995) (Figure 2B above). Furthermore, Ret is essential for the developing kidney and tubular system (Schuchardt et al. 1994) and recently Ret has been implicated in the formation of immunological aggregates called Peyers patches that surround the intestine (Veiga-Fernandes et al. 2007). Ret functions in the adult by preventing ongoing reduction of dopaminergic substantia nigra pars compacta neurons, the very same neurons that degenerate in Parkinson disease (Kramer et al. 2007). Ret also present occurring implications for the development of early embryos and in spermatogenesis. GDNF and soluble GFRα1 were identified as exogenous factors for the self-renewal of spermatogonial stem cells *in vitro*. The cultured cells could then be transplanted into infertile recipient mice testis, which restored fertility (Kubota et al. 2004). In the female, preovoulatory increases of GDNF in ovarian structures together with Ret and GFRα1 in mouse oocytes enhanced extrusion of the first polar body and moreover GDNF inhibited apoptosis of preimplantation embryos and promoted blastocyst development (Kawamura et al. 2007). Based on these findings GDNF has been suggested as an enhancer/treatment for infertility and conception difficulties in humans.

Physiological functioning or Ret requires the presence of one or more of the GFRαs and GFLs. GFRαs are expressed more widely than Ret (Trupp et al. 1997) and Ret independent signaling via only GFRα1 and GDNF or together with the neuronal cell adhesion molecule (NCAM) has been described (Paratcha et al. 2003; Ledda et al. 2007). The physiological importance of Ret independent signaling remains unclear as mice null for *Gfrα1* in non-Ret expressing cells do not

have an as yet distinguishable phenotype (Enomoto et al. 2004). Furthermore, GFRα can signal both *in cis* and *in trans* via Ret and GFRαs may thus be secreted from surrounding cells to activate the more restrictionally expressed Ret *in trans* (Paratcha et al. 2001). The protease cleaving the glycosyl-phosphatidylinositol anchor of GFRαs remains unknown.

Knock-out mice have provided information on the respective importance of Ret signaling components *in-vivo*. *Ret*$^{-/-}$, *Gdnf*$^{-/-}$, and *Gfrα1*$^{-/-}$ mice develop with rudimentary kidneys, lack enteric neurons in plexa myentericus and die soon after birth (Schuchardt et al. 1994; Enomoto et al. 1998). In several neuronal ganglia there is a gradual deficit of neurons when comparing *Ret*$^{-/-}$, *Gdnf*$^{-/-}$, and *Gfrα1*$^{-/-}$ mice with *Ret*$^{-/-}$ being more severe than *Gdnf*$^{-/-}$ and *Gfrα1*$^{-/-}$ mice displaying the least severe loss of neurons (Moore et al. 1996; Enomoto et al. 1998; Erickson et al. 2001). With regards to kidney development *Gfrα1*$^{-/-}$ mice show more severe disruption than either *Ret*$^{-/-}$ or *Gdnf*$^{-/-}$ counterparts. Additionally, branching of ureteric buds dependent on GDNF also depend on membrane associated heparan sulfate glucosaminoglycans as without those Ret phopshorylation are absent and mice lacking essential enzymes for generating the heparan sulphates resemble *Ret*$^{-/-}$, *Gdnf*$^{-/-}$, and *Gfrα1*$^{-/-}$ mice phenotypically (Bullock et al. 1998). Suggestibly, GDNF binding to heparan sulphates may concentrate the ligand with GFRα1 and/or Ret (Barnett et al. 2002). Mice null for *Gfrα2* (Rossi et al. 1999), *Gfrα3* (Nishino et al. 1999), *Gfrα4* (Lindfors et al. 2006), *Nrtn* (Heuckeroth et al. 1998), *Artn* (Honma et al. 2002) or *Pspn* (Tomac et al. 2002) are all viable and fertile but possesses phenotypes with variable developmental, mostly neuronal and ganglional shortcomings.

1.2.2.2 Ret related disease

The type of dysregulation; either loss or gain of signaling reflects disease manifestations of Ret. As discussed above, mice with homozygous absence of *Ret, Gfrα1* or *Gdnf* but also mice harboring gene mutations in *Ret* that inactivate downstream signaling invariably die soon after birth and present with dysgenesis or absence of the kidneys, absence of superior cervical ganglia, impaired spermatogenesis and loss of enteric innervation (Manie et al. 2001; Arighi et al. 2005). In humans, loss-of-function mutations in *RET* is the most common genetic implication in Hirschprung disease, a condition that presents as early constipation due to loss of enteric neuronal innervation of a variable length of bowel (Manie et al. 2001). In a study examining 33 stillborn human fetuses with renal aplasia or severe renal dysgenesis mutations in

RET was found in approximately one third of subjects (Skinner et al. 2008) indicating the importance of Ret for kidney development also in humans.

Ret gain-of-function oncogenic syndromes typically affect neural crest cell derived tissue such as the medullary thyroid, the adrenal glands and ganglionic neuromas. Ret overactivation may result from chromosomal rearrangements where an unrelated gene from chromosome 10 or from another chromosome fuse with Ret N-terminal to the kinase domain resulting in Ret/PTC (papillary thyroid carcinoma) chimeric proteins (Grieco et al. 1990) (Figure 3C). Remarkably, Ret chromosomal gene fusion events occur more often than other gene fusions when cells are exposed to ionizing radiation. It is furthermore interesting to note that the physical proximity of the *RET* locus to other chromosomal loci during interphase folding of chromosomes may govern the specific fusion events. (Bounacer et al. 1997; Lai et al. 2007). In agreement with such a proximity model is that some PTC genes that are normally several mega bases parted from *RET* have been shown to fold into closeness with *RET* in cancerous but not in normal cells during interphase (Nikiforova et al. 2000). At least twelve foreign N-terminal fusion partners of Ret are known all of which have motifs in common that are suitable for dimerization of the expressed protein short of any need for ligand (Asai et al. 2006; Fusco and Santoro 2007; Namba and Yamashita 2007; Wang et al. 2007). Clinically, *RET* involved chromosomal rearrangements lead to sporadic rather than familiar (compare with the MEN2 syndromes discussed below) thyroid tumours. These papillary thyroid carcinomas are in general well differentiated and their cells of origin are the follicular epithelial cells of the thyroid, which are derived form the embryonal endoderm. The Ret/PTC proteins lack the Ret signal peptide and the transmembrane domain of wild type Ret and typically the N-terminal chimerism of Ret/PTC proteins amend the receptor to signal from within the cytosol as dimerization is independent of membrane anchorage. Mechanistically it has been proposed that Ret/PTC may dimerize as a result of coiled-coil structure sequences in the Ret chimeric partner proteins (Rabes 2001). Both the ligand independent signaling and signaling from a different subcellular compartment compared to wild type Ret are likely to contribute to a pathogenic mode of intracellular signal propagation (Arighi et al. 2005; Santoro et al. 2006).

The multiple endocrine neoplasia (MEN2a and 2b) syndromes and familial medullary thyroid carcinoma (FMTC) are commonly caused by, likewise to Ret/PTC, ligand independent Ret activation. In contrast to the fusion events in Ret/PTC the common underlying mechanism in these hereditary diseases are missense point mutations either directly activating the kinase

domain (Santoro et al. 1995; Iwashita et al. 1996) (Figure 3A) or of extracellular cysteins (Figure 3B).

The MEN2a syndrom presents with medullary thyroid carcinoma (MTC) of parafollicular calcitonin producing C-cells, pheochromocytomas developing in adrenal medullary cells and hyperparathyroidism although the latter two are not always present (Chong et al. 1975; Yip et al. 2003). Mechanistically MEN2a Ret result from mutations of cysteins in the extracellular domain that interrupts normally occurring intramolecular disulphide bonds and alter the Ret three-dimensional configuration. Exchange of cysteins for other amino acids lead to that remaining cystein SH-groups loose their intramolecular binding partner and instead become available for bonding to another likewise mutated Ret (intermolecular binding) (Figure 3B). Consequently, these intermolecular disulphide linkages dimerize and constitutively activate the Ret receptors (Borrello et al. 1995; Santoro et al. 1995).

MEN2b mutations in Ret, most often of residue M918, activate the receptors kinase domain and alter the Ret substrate specificity (Satoh and Mori 2006; Fujieda 2007; Raue and Frank-Raue 2007). As the kinase domain is activated without dimerization Ret MEN2b mutations accomplish the onset of signaling also as a monomer. MEN2b patients can be recognized by MTC and pheochromocytomas but also by the prescence of ganglioneuromas of buccal membranes, eyelids and intestinal cells. Additionally, patients may be very tall, have an exaggerated arm span and a high rising palate known as a marfanoid habitus. MEN2b is considered a more aggressive disease than MEN2a and present at an earlier age (You et al. 2006; Lakhani et al. 2007).

Regardless if Ret is activated by mutations or by ligand, propagation of signaling occur by means of the intracellular phosphorylated tyrosines (Xing et al. 1998; Coulpier et al. 2002; Kawamoto et al. 2004) and at least one serine (Ser697) (Fukuda et al. 2002). In both cases phosphorylation of these residues provide sites for interaction with intracellular scaffold/adaptor proteins and further downstream signal transduction eventuate but the specific signaling that occur may be different between ligand activated and oncogenic Ret (Liu et al. 1996). Specific mutations leading to MEN2 syndromes, familiar medullary thyroid carcinoma and Hirschprung are reviewed in (Kato et al. 2000; Iwashita et al. 2001; Yip et al. 2003; Zbuk and Eng 2007).

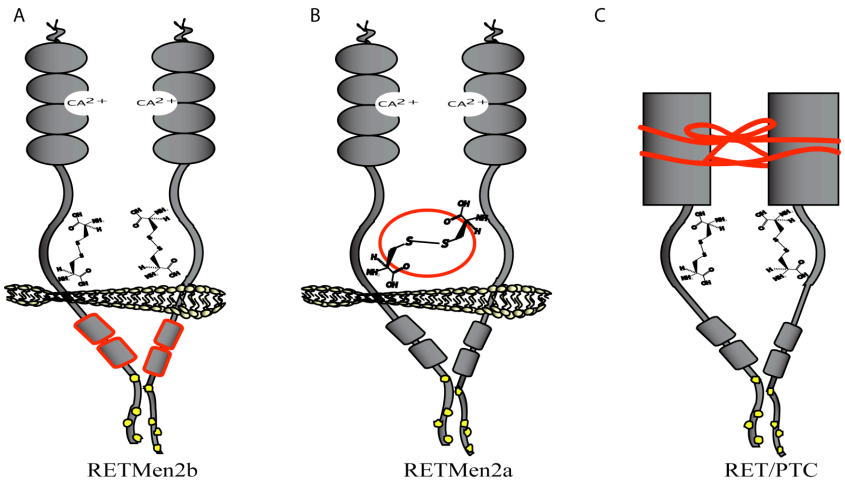

Figure 3. (A) Ret Men2b. Schematic illustration of the Ret receptor affected by Men2b mutations in the kinase domain (demarked in Red) resulting in activation of signaling irrespective of ligand. The Men2b Ret may also signal as a monomer. **(B) Ret Men2a.** Schematic illustration of the Ret receptor affected by Men2a mutations in the extracellular cysteine rich domain. The receptor is activated by formation of covalent intermolecular sulphidebridges (Red) resulting in constitutive dimerization. **(C) Ret/PTC.** A chimeric protein is formed by N-terminal fusion of Ret to another unrelated protein. Signaling is the result of dimerization between the fused proteins (Red) and typically occur in the cytosol rather than as membrane transversing proteins.

1.3 Signal propagation via adaptor molecules

Adaptor proteins are cytoplasmatic molecules equipped with conserved structural domains, the most common being; SH2 domains, SH3 domains (Koch et al. 1991; Pawson 1995) and PTB binding domains (Kavanaugh and Williams 1994) the latter sort will be predominantly focused on in this text. In 1994 a region of the Shc protein distinct from the Shc SH2 domain was found to bind several phosphotyrosines on transmembranous receptors (Blaikie et al. 1994). This was the first described PTB binding domain and set the stage for future discovery of other PTB adaptors. Currently, about 200 PTB binding domain equipped proteins have been noted in eukaryotes (Uhlik et al. 2005).

1.3.1 PTB-binding-domain containing adaptor proteins

PTB binding domains on most adaptors share a similar structural conformation to that of the Shc PTB binding domain; seven antiparallel β-strands together form two β-sheets positioned at right angels (orthogonal) to each other. At the C-terminal end of the two sheets an α-helix caps the sheets and also completes a pocket in which peptides can bind (Zhou et al. 1995; Farooq et al. 2003). Besides this common core different PTB binding domains have additional features that distinguishes them from one another. Depending on the type of additional features and hence on the mode of peptide recognition PTB binding domains are minimally segregated into either "IRS-1 like" or "Shc like" depending on whether they resemble IRS-1 or Shc more. Nevertheless, further sub classifications are often warranted to increase specificity when discussing structural and functional aspects of these molecules (Uhlik et al. 2005). For the following text please note that the Dok and Frs2α adaptors are both more IRS-1 like with a shorter PTB binding domain and they use two arginines for recognizing the phosphorylated tyrosine on Ret (Dhalluin et al. 2000; Shi et al. 2004). This is in contrast to the longer PTB binding domain of the Shc protein and how Shc capture its binding partners phosphotyrosine in a triangulate arginine:arginine:lysine pocket (Zhou et al. 1995).

The common domain architecture on adaptors is such that they sterically fit for binding to short motifs with the consensus sequence NXXY immediate to phosphorylated tyrosines on receptors (Y/pY representing the tyrosine/phospho-tyrosine) yet they each show variable affinity depending on what other amino acid residues surround this consensus stretch (Pawson and Scott 1997). Adaptors with PTB domains discriminate between related interaction sites based on the residues immediately N-terminal to the phospho-tyrosine. Consequently, differences in amino acid residues N-terminal to the phospho-tyrosine promote preferential binding to various PTB-adaptors such as Frs2α, Shc or Dok (Zhou et al. 1995; van der Geer et al. 1999; Uhlik et al. 2005). In rarer cases some PTB-adaptors, as exemplified by the Frs2α adaptors engagement with the juxtamembrane domain of the fibroblast growth factor receptor (FGFR1), can also bind to sequences that does not contain a NXXY stretch (Trub et al. 1995; Wolf et al. 1995; Xu et al. 1998; Ong et al. 2000). Several PTB adaptors may be competent for docking to each receptors NXXpY sequence but importantly there is only room for one adaptor per receptor docking-site at any given time. Hence, in a cellular milieu where many adaptors are present they will have to compete for interacting with the receptor and further downstream signaling ensue according to which particular adaptor that secured the seat (Madhani 2001).

Methods for investigating adaptor interactions with intracellular receptor-residues are typically techniques such as co-precipitation of the receptor with the adaptor of interest. Silencing of (i.e. render incapable of binding) specific receptor tyrosines can then reveal the specific interaction point as a directed mutation of the correct tyrosine into a phenylalanine or alanine should abolish the co-precipitation of the adaptor when the receptor is pulled down or vice versa. Ret is an excellent general model for adaptor specificity experiments. In particular tyrosine Y1062 of Ret competitively binds to several different adaptors and this tyrosine are *in vivo* and *in vitro* critical for most Ret receptor functions (Jijiwa et al. 2004; Wong et al. 2005) Activated Ret can potentially recruit a mumber of different adaptor proteins to the autophosphorylated tyrosine 1062 site, including Shc (Asai et al. 1996; Arighi et al. 1997; Lorenzo et al. 1997), Frs2α (Kurokawa et al. 2001; Melillo et al. 2001), Dok (Grimm et al. 2001; Murakami et al. 2002), IRS-1 (Melillo et al. 2001), Enigma (Durick et al. 1998) and Shank (Schuetz et al. 2004) (Figure 4).

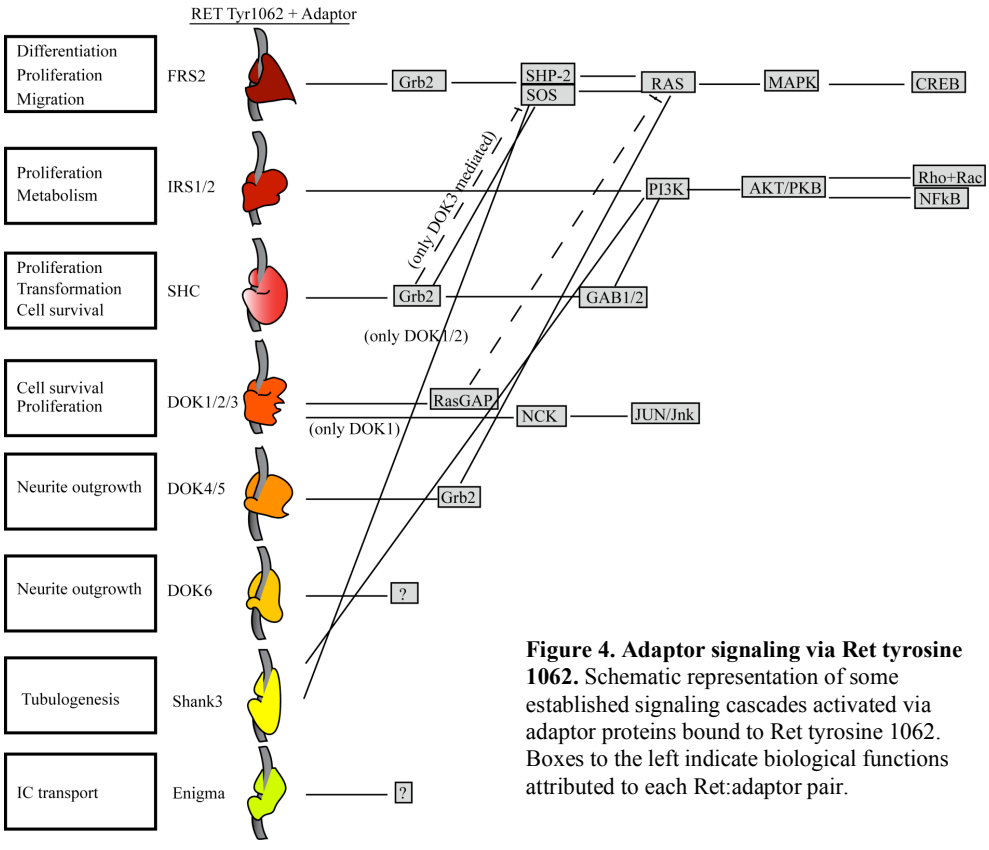

Figure 4. Adaptor signaling via Ret tyrosine 1062. Schematic representation of some established signaling cascades activated via adaptor proteins bound to Ret tyrosine 1062. Boxes to the left indicate biological functions attributed to each Ret:adaptor pair.

By virtue of binding directly to the receptor the adaptor proteins constitute the most proximal part of intracellular effector chains. Once bound, the adaptors form a scaffold for assembly and activation of further downstream proteins (Figure 4). The sterical fit between a particular adaptor and cytosolic signaling proteins create selectivity for what further signaling interactions will occur. Different adaptors that normally compete for phosphotyrosines activate specific intracellular signaling pathways downstream of Ret and other RTKs both qualitatively and quantitatively (Jain et al. 2006). The variety as to which adaptor binds to a receptor site thus provides a way for diversification of signaling from one and the same receptor. For example, Ret tyrosine 1062 binds to at least six PTB-adaptors but the adaptors each propagate signaling differentially. As the adaptors are expressed in different tissues activation of Ret should have very different biochemical outcomes depending on which one is present for Ret engagement in a given cell at a given time (Grimm et al. 2001; Crowder et al. 2004; Uchida et al. 2006). As described onwards, versions of the Ret receptor constructed by mutations around intracellular tyrosine 1062 with resulting specificity for one out of a several potentially binding PTB adaptor molecules could be created. This lends to investigation of specific Ret functions mediated by the allowed adaptor solely.

1.3.1.1 The Shc-PTB adaptors

The Shc adaptor proteins consist of three closely related family members called ShcA, B and C (Pelicci et al. 1996). The ShcA gene encodes three isoforms of 46,52 and 66 kDa size (Pelicci et al. 1992). ShcA is ubiquitously expressed in the organism. ShcB is a 68kDa protein that are expressed predominantly in the nervous system but also in lung, brain and heart (Sakai et al. 2000). ShcC, 53kDa and 67kDa, appear to be almost exclusive to the nervous system.
Shc proteins posses a SH2 domain towards the C-terminus while the PTB binding domain is located N-terminally. Centrally in the protein is a CH1 region. The interaction domains of Shc themselves have distinct functions for controlling signaling in that Shc regulated morphogenesis of specific tissues depend on the SH2, CH1 or PTB domains integrity to different extents (Hardy et al. 2007). When Shc is bound to Ret or other receptors it can mediate further signaling by various means such that Grb2 coupling to the SH2 or PTB domains in turn activates SOS and consequently the MAP kinase and/or the PI3K pathway, alternatively Grb2 when bound to Shc can attract Gab2 and Gab2 directly activates PI3K signaling. Shc also bind SHIP, adaptins and other proteins that recognize the proline rich CH1 region of Shc. (van der Geer et al. 1996; Gu et al. 2000).

1.3.1.2 The Frs2α-PTB adaptor

Frs2α, a 57kDa protein was first described as an adaptor mediating several cellular effects in response to activation of the FGF receptors (Ong et al. 2000) and was later found to bind Ret (Xu et al. 1998; Kurokawa et al. 2001; Melillo et al. 2001). Frs2α interaction with FGFRs, Ret and other RTKs are dependent on the PTB binding domain of Frs2α. However, whereas Frs2α binds to Ret via phospho-tyrosine 1062 it also binds to the juxtamembrane segment of FGFR independent of a phospho-residue (Xu et al. 1998). Frs2α is equipped with a myristoyl anchor near the PTB binding domain at its C-terminus and this lipid moiety locates Frs2α to the plasma membrane and specifically to within membrane lipid raft structures (Ridyard and Robbins 2003). At the C-terminal part of Frs2α are several tyrosine phosphorylation sites to which Grb2 can dock mediating further signaling through in particular the MAP kinase pathway (Kouhara et al. 1997). In addition, Shp2, a tyrosine phosphatase protein, are also able to bind Frs2α whereas Frs2α has not been shown to bind Gab1 or Gab2 when docked to Ret. Altogether, Frs2α docking to tyrosine 1062 enhances Ras-MAPK signaling downstream of Ret (Hadari et al. 1998; Kurokawa et al. 2001).

1.3.1.3 The Dok-PTB adaptors

The Dok family of adaptor proteins consists of Dok1-6 (62, 48, 48, 37, 52, 38kDa respectively), an additional splice form of Dok4 called Dok4b and a recently discovered Dok7 (55kDa) (Carpino et al. 1997; Cong et al. 1999; Crowder et al. 2004; Okada et al. 2006; Baldwin et al. 2007). Dok4, 5, 6 have only remote sequence similarity to Dok1,2,3 and may be regarded as a Dok subfamily (Grimm et al. 2001; Crowder et al. 2004). Dok 4,5,6 is highly expressed in the devloping nervous system, specifically in DRG´s, SCG´s and Dok4 are expressed also in endothelial tissue which results in a more broad Dok4 expression in the adult mice. Dok5 is mostly expressed in the brain in adult mice (Carpino et al. 1997; Di Cristofano et al. 1998; Cong et al. 1999; Grimm et al. 2001). Dok6 is besides neuronal tissues also expressed in the ureteric buds and testis (Crowder et al. 2004). Dok 1,2,3 is mainly expressed in hematopoetic tissue (Grimm et al. 2001; Crowder et al. 2004). All Dok1-6 have been suggested or shown to bind to Ret in a phosphotyrosine dependent manner (the kinase dead Ret K758M does not interact) as seen by yeast two hybrid assays (Grimm et al. 2001). The interaction with Ret is dependent on the Dok PTB binding domains and most likely through a common mechanism for all isoforms (Grimm et al. 2001; Murakami et al. 2002). Common among the full length Dok proteins are one pleckstrin-homology and one PTB binding domain in the N-terminal region and a C-terminal portion with SH2 domain target motifs. The downstream signaling mediated by Dok

isoforms are complex and variable; Dok1,2,3 at least in some systems inhibit the MAP kinase pathway by binding to rasGAP with subsequent inhibition of Ras (Grimm et al. 2001) whereas Dok4 and 5 signaling after Ret engagement promote activation of the MAP kinases and does not bind rasGAP (Grimm et al. 2001). Dok3 also attenuates v-Abl and several aspects of immunoreceptor signaling. To further complicate matters the splice variant of Dok4, Dok-4b inhibited the tyrosine kinase-induced activation of both MAPK proteins and Elk-1 more strongly than Dok4 and in experiments where the C-terminal portion of Dok-4 was truncated the inhibitory activity of Dok-4 was enhanced. Expression of only the C-terminal domain heightened Elk-1 activation. (Cong et al. 1999; Lemay et al. 2000; Bedirian et al. 2004; Baldwin et al. 2007). It thus appears as if the various parts of Dok proteins possess inhibitory and stimulatory properties respectively and local molecular circumstances of Dok activation strongly impact the outcome.

1.4 Receptor/adaptor localization as a distinguishing facet for downstream signaling

1.4.1.1 Subcellular compartmentalization

Rapid advances in cell signaling research currently happen within the area of subcellular localization of proteins within the lipid membrane (Simons and Toomre 2000). The cell membrane consists of mainly phospholipids, sterol species and proteins. The protein content in human cell membranes are uncertain but data from hamster cells reported an approximate number of 30000 per micrometer, much more than classical textbook drawings may suggest. Membrane associated proteins are not uniformly distributed; rather many proteins tend to cluster in groups within their lipid surrounding. Recent evidence prompts that the cellular lipid membrane is far from a uniform layer but instead constitutes a constantly changing environment with ordered and disordered regions (Brown and London 2000; Jacobson et al. 2007). The precise identity of ordered intramembrane domains is debated as their nanometer size scale make them technically difficult to describe in detail (Brown and London 2000).

1.4.1.2 Lipid ordered domains

Lipid ordered micro-/nano- domains are of transient nature complicating their study by classical means and domains may even form artificially by extraction or detergent dependent solubilization methods which are employed to study them in cells (Hanzal-Bayer and Hancock

2007). In the literature lipid rafts and caveolae are probably the most frequently used denotations of such type domains. Caveolae are morphologically distinguishable by their "flask shaped" microscopical features whereas lipid rafts have no such visually observable characteristic, perhaps one of the reasons the mere existence of the latter is less established as yet. To distinguish lipid rafts from the surrounding membrane in intact cells the experimenter typically uses indirect methods such as tagging or staining of domain-enriched molecules or probe acyl chain order. Measurements on the relative proximity of molecules and their assembly into clusters can then be performed or anomalous diffusion measured (Simons and Vaz 2004). It is beyond this text to in detail discuss the current ideas on the exact molecular composition that defines lipid ordered domains but an comprehensive review was recently published (Jacobson et al. 2007). Despite arguments on raft identity their mere presence and functionality is suggested by the fact that no alternative explanation exist for i) differential distribution of lipid species and lipid anchored proteins in ongoing membrane fluxes ii) formation of endocytotic/exocytotic vesicles without (known) coat proteins iii) functional differences between protein complexes within or outside structures marked as rafts (Hanzal-Bayer and Hancock 2007).

1.4.1.3 Membrane domain regulation of intracellular signaling

Intriguingly, it appears that membrane domains have profound functions on RTK receptor signaling (Simons and Toomre 2000). The lipid molecule species amplified in lipid rafts are most notably gangliosides, sphingolipids and sterols (Fiedler et al. 1993; Brown and London 1997; Ayuyan and Cohen 2008). These dense raft compartments attract certain proteins that are equipped with satured acyl lipid modifications ie. myristolylation, palmitoylation, dual-acylation (McConville and Ferguson 1993; Resh 1999) which are attached to distinct amino acids on proteins. Raft associated proteins may be either cytosolic or membranous. Assembly of lipid domains thus makes for a platform in support of clustering signaling proteins into complexes and lipid raft fractions are indeed highly enriched in some signaling proteins compared to the uniform plasma membrane (Foster et al. 2003). Ret (Tansey et al. 2000) as well as the Fibroblast growth factor (FGF) receptor (Ridyard and Robbins 2003) and immune receptors (Langlet et al. 2000) cluster in lipid rafts once activated and their downstream signaling propagation is strongly affected by this (Tansey et al. 2000; Paratcha et al. 2001).

Ret present in lipid rafts are resistant to ubiquitin degradation which prolong the receptors temporal signaling competence post activation (Pierchala et al. 2007) and GDNF ligand activation translocates Ret to detergent resistant regions of the cell (Pierchala et al. 2006).

Several RTK binding proteins are enriched in rafts. It has been shown that Frs2α and Ret associate in distinct locations corresponding to lipid rafts since markers of rafts was found in the same density fractions as this complex (Paratcha et al. 2001). Frs2α has a myristoylation tail and because of this is exclusively localized within lipid rafts (Ridyard and Robbins 2003). SRC, another lipid raft protein (Liang et al. 2001), is dependent on lipid raft association of Ret for Ret to be able to activate it (Tansey et al. 2000). Furthermore, pharmacological disruption of sterols abolishes some but not all Ret mediated signaling events and functions (Pierchala et al. 2006), which is broadly consistent with what Liu et.al. found earlier when platelet derived growth factor (PDGF) receptor signaling were investigated in the presence or absence of disrupted membrane sterols (Liu et al. 2000). The fact that activation of Ret triggers different events in different cells has been suggested to be a result of Ret localization (Kodama et al. 2005) and recruitment of different adaptors depending on whether Ret is in rafts or not (Paratcha et al. 2001). It thus seem plausible that some adaptors are not only passively located in lipid rafts but also can steer receptors into rafts and that such compartmentalization of Ret and other receptors profoundly regulate signaling and cell biology (Figure 5).

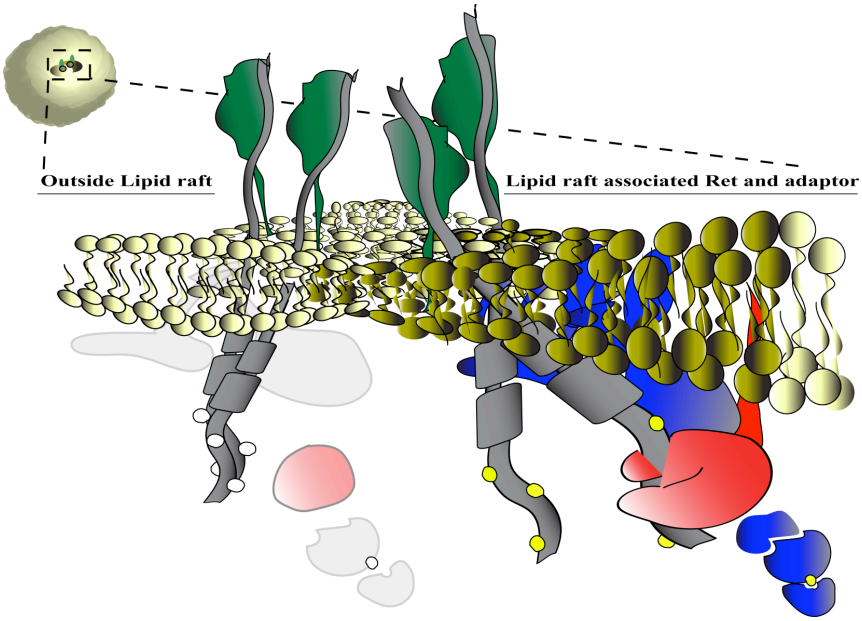

Figure 5. Ret within or outside lipid rafts. Illustration of Ret within Lipid rafts (right half of figure) or outside raft regions (left side of figure). When present in rafts Ret may complex with particular molecules which lead to specific signaling events. Outside rafts the same molecules may not be in proximity to the receptor or may not be sterically fit for interaction (represented as shaded proteins) such that these particular molecules will not propagate signaling and other biochemical cascades are instead initiated.

1.5 Complementary means of Ret signal regulation

Ret activity can be regulated also by slow kinetic mechanisms. The nerve growth factor (NGF) and its receptor tropomyosin receptor kinase A (TrkA) has recently been shown to alter Ret signaling. The presence of NGF augment Ret induced cellular growth independent of Ret ligands (Pierchala et al. 2007). Both protein expression of Ret receptors and the phosphorylation status of Ret tyrosines 905 and 1062 were augmented by NGF treatment of sympathetic neurons. The mechanism for increased Ret phosphorylation were the NGF mediated inhibition of Ret degradation by the cellular proteasome and lyzosome (Pierchala et al. 2007). Specifically, the ubiqutination and internalization of Ret that occur by the Cbl ubiquitin ligase after GDNF ligand activation (Scott et al. 2005) are reduced by NGF supplementation to cells. NGF treatment resulted in mono- rather than poly-ubiqutinylation of Ret and hence Ret protein catabolism is inhibited presenting a system of means of local cross-talk between the TrkA and Ret receptors.

On another note, Ret may also be negatively regulated as shown recently by the leucine-rich repeat protein Lrg1 that physically interact with Ret and as a result prevents GDNF binding to the receptor (Ledda et al. 2008). This in turn limited Ret recruitment into lipid rafts, autophosphorylation of Ret and downstream signaling at least for MAPKs. Similarily, the Sprouty family of proteins that represent proteins regulating downstream signaling from several receptor tyrosine kinases and are expressed in neuroblastoma cells in response to GDNF negatively affect Ret signaling. Sprouty2 expression markedly reduced Ret induced sustained MAPK/Erk phosphorylation and neurite outgrowth whereas cells expressing a phospho-mutated (inactive) form of Sprouty2 led to further differentiated neurons (Ishida et al. 2007). Importantly, Sprouty proteins are downregulated in among others breast-, colon- and prostate cancers (Lo et al. 2006)

2 Results and discussion

2.1 PAPER I

2.1.1.1 Distinguishing important amino acids around tyrosine 1062 in Ret for Shc versus Frs2α interaction.

The prominent importance of tyrosine 1062 in Ret signaling and the competence of this tyrosine for mediating execution of such a multitude of downstream biology made us want to contrast and compare the signaling cascades downstream this residue. To render tyrosine 1062 selective for either Shc or Frs2α PTB adaptor interaction we hypothesized that specific mutagenesis of key amino acids would impose a sterical preference for either the Shc or Frs2α PTB binding domain. First we aligned the corresponding sequences of other known RTK:PTB-adaptor pairs. By comparison, a number of residues surrounding the PTB domains seemed more or less conserved among several RTKs favouring Shc vs. Frs2α PTB-adaptors and thus were suggested important for adaptor discrimination. Next, a peptide binding screen were set up whereby sequential mutations of all amino acids surrounding Ret tyrosine 1062 were swapped into all other amino acids. The strength of interaction between Ret mutants with Shc or Frs2α fused to a GST tag was compared on peptide array spots. The peptide arrays confirmed the adherence to the concensous sequence NXXpY that are the canonical motif for PTB-adaptor interaction with RTKs. Beyond the shared canonical sequence residues that were deemed promising for enhancing or reducing adaptor binding based on the peptide arrays were taken forward for testing in cellular systems.

2.1.1.2 Rewiring Ret.

Based on the above, constructs expressing Ret9 equipped with single, double or triple amino acid mutations were created and expressed in Cos7 cells. By co-precipitation of mutated Ret versions together with either of the two tagged adaptors several Shc favouring mutants could be found that were also week in binding Frs2α. The opposite result; a Ret mutant favouring Frs2α interaction on the expense of Shc was not obtained by single or even up to quadruple residue mutagenesis in cellular systems were endogenous Shc compete with the introduced Frs2α for binding. Hence, a chimeric receptor in which the juxtamembrane segment from the fibroblast growth factor receptor, that interacts with Frs2α only, replaced an equivalent number of residues around Ret tyrosine 1062 were created. As expected this mutant were selective for Frs2α and

together with the best Shc selective mutant these constructs were assayed in further experiments.

2.1.1.3 Downstream biochemical and functional effects of selective adaptor engagement

Biochemically the Shc and Frs2α signaling Ret versions displayed distinct manners of downstream effector protein activation with regards to both quantity of downstream protein phosphorylation and the temporal pattern of phosphorylation. The phosphatidylinositol 3-kinase pathway as determined by Akt phosphorylation was strongly activated via Shc but not Frs2α. Likewise, the Shc selective mutant of Ret displayed enhanced phosphorylation of the NFκB molecule which is a downstream target of Akt. The p42,44 MAPK Erk pathway and the downstream effector CREB, on the other hand, were more strongly phosphorylated by activation downstream of Frs2α although Shc also activated these effectors to some degree. Intriguingly, Frs2α mediated phosphorylation of p42,44 MAPK Erk equated to a distinct temporal alteration seen as high sustained P-p42,44 MAPK Erk levels compared to Ret wild type or Ret mutants selective for Shc.

To investigate whether the altered signaling had functional consequences on cell biology we examined Ret mediated proliferation and survival in the mutants. While both Shc and Frs2α binding Ret mutants showed levels of trophic support comparable to Ret WT (and somewhat higher than Ret Y1062F) after ligand stimuli of neuroectodermal cells, Shc interaction with tyrosine 1062 was necessary for furnishing the antiapoptotic effects of GDNF when cells were challenged by severe starvation or the apoptosis inducing agent Anisomycin.

2.1.2 Conclusions

In conclusion we show in paper I that targeted selective mutagenesis of the sequence N-terminal to the Ret PTB domain can be employed to rewire the receptor:adaptor interaction from a competitive multicompetent order to a selective one. The selective Ret receptors show distinct biochemical characteristics and are also functionally different which provide a means for distinguishing the Shc and Frs2α adaptors respective functionality. In this case suggesting that Ret:Shc interaction is important in Ret promoted cell survival via activation of PI3K/Akt and NFκB.

2.2 PAPER II

Ret is instrumental for generating of the adult nervous system body plan. In essence all cells destined to become functional neurons derive from the neural crest and migrate from there to reach their position in the full grown organism. Cells expressing Ret migrate directionally towards gradients of Ret ligands and Ret is one of few receptors that have a defined migratory function on neuronal cells that is conserved from animal models to clinical disease in humans.

2.2.1.1 Ret induced cellular motility and migration requires Frs2α interaction

To dissect the Ret tyrosine 1062 signaling important for migration to occur we examined Shc and Frs2α selective Ret mutants for motility and directional migration towards a contained source of Ret ligands. Motility was examined by measuring cell movement into an empty field created in confluent cell layers. The results showed that Ret wild type as well as Ret selectively binding Frs2α moved into the field in ligand supplemented cultures while Ret binding Shc or Ret Y1062F mutants displayed little motility. By supplying cells to a compartment separated from the ligand source by a semipermeable membrane the directionality of movement towards Ret ligands were examined. Ret was competent for executing a migrational response in neuronal and non-neuronal cells expressing Ret wild type, as was cells expressing the Frs2α selective mutant. On the other hand, cells expressing Ret mutants selective for signaling via Shc were poorly migrating at levels comparable to the tyrosine 1062 null mutant.

2.2.1.2 Ret mediated migration depend on the Src family of kinases

Src proteins are required for migratory execution downstream several RTKs and Src binds to Ret but its importance for Ret induced migration has not been examined. Activation of the Src family of kinases after Ret ligand induction was investigated by *in vitro* kinase assays. Ret^{WT} and Ret^{Frs+} induced Src phosphorylation at 1.5 to 2 times higher levels than Ret^{Shc+} or Ret^{Y1062F}. Using both pharmacological inhibition of Src and a genomic approach employing mouse embryonal fibroblasts null for Src/Yes/Fyn Src family activition was found to be necessary for cellular migration towards Ret ligands in neuroectodermal cells. Interestingly, SRC interact with Ret at a tyrosine in the kinase region of Ret, namely tyrosine 981, a fair distance from tyrosine 1062. Our results indicate that both tyrosine 981 to which Src binds and tyrosine 1062 that is utilized for Frs2α engagement is needed for migration to occur and hence they seem to work in concert to this end. The proximal molecular machinery necessary for Ret induced migration was

investigated further. The Src activated focal adhesion kinase protein (FAK) that is known to bridge RTKs with the substratum to which cells adhere was examined. Src inhibition was reflected in a slight reduction in the levels of phosphorylated FAK and FAK knock down using siRNA led to marked abolition of cell migration. This suggests a complex of Ret and Src that also regulate FAK in response to migrational cues. Downstream of these molecules the p42,44 MAPK Erk pathway was induced as determined by Erk phosphorylation. Frs2α activates this pathway as known from our and others previous results but also Src is known to mediate Erk phosphorylation and inhibition of Src activation resulted in abrogated levels of phospho-Erk.

2.2.1.3 Migratory competent mutants show distinct membrane localizations of FAK and Ret. We found that the localization and not merely activation per se of FAK was a distinguishing feature in cells expressing Ret migratory competent and incompetent mutants. FAK was found to cluster at sights of focal adhesions/actin bundling in cells expressing Ret^{Frs+} or Ret^{WT} in contrast to Ret^{Shc+} or Ret^{Y1062F} mutants. Src as well as Ret assemble in lipid ordered regions of the membrane. When exposing cells to the cholesterol oxidizing agent Cholesterol Oxidaze levels of phosphorylated FAK were reduced indicating a possible lipid ordered platform where molecular interactions required for migration take place. Morphologically, the migratory competent versions of the Ret protein itself were distinguishable from cells expressing Ret mutants with little ability to mediate migration as the receptors tagged to fluorescent GFP clustered at the membrane by focal adhesions/actin bundles in the former.

2.2.2 Conclusions

In Paper II we conclude that Frs2α interaction with tyrosine 1062 in Ret is necessary for migration but that SRC interaction with an upstream tyrosine is also needed. SRC activation, localization of active FAK to focal adhesions and phosphorylation of the p42,44 MAPK Erk pathway directly downstream Frs2α as well as via SRC/FAK mediation revealed molecular insight towards the mechanics of Ret induced migration. Further investigation on the role of PTB-adaptors in Ret translocation is presented in Paper IV.

2.3 PAPER III

The most recently described PTB-adaptors to interact with Ret Y1062F are the Dok proteins. At least Dok 1,4,5 and 6 binds Ret (Grimm et al. 2001; Murakami et al. 2002; Crowder et al. 2004). In paper III Ret was rewired with the attempt of studying Dok adaptor contribution to cellular responses downstream of Ret.

2.3.1.1 Construction of a Dok selective Ret receptor mutant

Peptide spot arrays were performed to guide towards potential Dok binding enhancing mutations around Y1062 in Ret. Dok binding characteristics were similar to what had previously been found for the Frs2α adaptor which is in accordance with the related interaction mode of these two proteins to phosphopeptides (Uhlik et al. 2005). A single site mutation (Glycine to Proline) introduced at position +1 with respect to the Ret phospho-tyrosine proved to bind Frs2α and Dok with high affinity but was a poor binder for Shc. The G1063P mutation (Ret^{Dok+}) was however highly capable of sole Dok binding once Dok was overexpressed in cells. Compared to Ret^{WT} only 20% of transfected Dok was needed to completely outcompete Frs2α and therby create a system of high Ret:Dok exclusiveness.

2.3.1.2 Dok docked to Ret; biochemical and functional consequences

Ligand initiated Ret signaling via Dok led to that the p42, 44 MAPK, Erk pathway reached a phosphorylation status similar to Ret wildtype at short to intermediate lengths of stimuli. In contrast to wildtype however, the p42, 44 MAPK Erk phosphorylation was sustained in the Dok enhanced Ret mutant with maintenance of signal as long as 12 hours and beyond. The Dok mutant did not markedly alter the levels of phosphorylated Akt or other key signaling pathway proteins examined at any timepoint compared to wild type Ret.

Dok has been implicated in neurite outgrowth dependent on Ret activation but this effect is mechanistically poorly understood. To investigate this relationship fluorescently tagged Ret mutants were expressed in neuronally derived SK-N-MC cells. Cells were stimulated with Ret ligands and examined by confocal microscopy of stained actin filaments and protrusions. At short periods of ligand stimuli massive formation of microspikes extending from the cell soma were detected in Ret^{Dok+} and to a lesser extend in Ret^{WT} but rarely in Ret^{Y1062F}. The GFP tagged Ret localized to the very edges and along the length of these spikes in Ret^{Dok+}. To investigate if

the microspikes could be a priming structure for neurite outgrowth the Rho GTPase Cdc42 which has been implicated in formation of similar structures (Ikeda et al. 2001; Aoki et al. 2004) were studied. Using a purified peptide only binding to activated Cdc42 we found that Cdc42 activation was augmented by enhanced Dok binding to tyrosine 1062 in Ret. The kinase dead Ret^{Y1062F} displayed very low levels of Cdc42 activation illustrating the importance of this tyrosine for Cdc42 dynamics downstream of Ret.

2.3.2 Conclusions

A Dok favouring Ret mutant could be constructed and was found to result in augmented and sustained p42,44 MAPK Erk signaling. By high-resolution microscopy we could detect delicate protrusions from the cell somas. These microspikes were 5 times more numerous in the preferential Dok binder Ret G1063P compared to wildtype or the kinase dead Ret^{Y1062F}. In the latter two the few spikes that could be detected were also shorter than in Ret G1063P. Interestingly Ret was seen to cluster at microspikes, either at the very tip of spikes only or at the tip and also further proximal along the spikes. We suggest that the activation of Cdc42 is a downstream mediator of Dok for the formation of such structures.

2.4 PAPER IV

In paper IV wild type Ret was initially used rather than the adaptor selective mutants to acquire knowledge on whether high levels of the respective adaptors are sufficient to promote subcellular translocation of the receptor even when the receptor is less discriminate for adaptor interaction, as should be the case *in vivo* in health and disease. Overexpression of either Shc or Frs2α adaptor proteins was sufficient to precipitate nearly all of the Ret^{WT} present in cells.

2.4.1.1 Fractionation of Ret bound to Shc or Frs2α into detergent resistant partitions

By crude fractionation of cell membranes into detergent insoluble and detergent soluble fractions respectively we found that Ret partitions largely into the former regardless of Shc or Frs2α interaction. As we had found that cholesterol oxidation (Paper II) disrupted signaling mediated via Frs2α but not via Shc more detailed analysis of signaling characteristics were done. To this end a Shc construct with an addition of the RAS protein membrane localization signal (Shc^{MLS}) which result in Shc localizing to lipid rafts and hence mimicking Frs2α in terms of subcellular distribution was used. Employment of two different drugs that disrupt lipid ordered domains both severly inhibited Ret:Frs2α and interestingly Ret: Shc^{MLS} signaling but little effect was seen on Ret:Shc. Furthermore, the Shc^{MLS} pattern of temporal and quantitative signaling resembled that of Frs2α in the absence of disrupting drugs as phosphorylation of Erk in Shc^{MLS} approached levels found when overexpressing Frs2α. When pharmacological disruption of lipid ordered domains were used on cells phospho-Erk was markedly decreased.

2.4.1.2 Adaptor effects on Ret distribution to lipid rafts

We performed immunocytochemical experiments and found that Ret co-localizes with lipid raft markers when co-expressed with Frs2α and Shc^{MLS} but not Shc. This prompted us to undertake more detailed analysis of the distribution of the adaptors. Density fractionations of lysates from transfected embryonal chicken tissue revealed that Frs2α does indeed partition into lower density fractions than Shc and that Ret distribution was contingent on the adaptors.

In functional terms, led by previous findings, we investigated the importance of intact raft structures for Ret mediated migration or cell survival. In the prescence of lipid domain disrupting pharmaceuticals migration was much repressed indicating the need for intact lipid rafts in order for Ret to execute such a migrational function in cells. On the other hand, cell

survival was strictly dependent on Shc and Shc^{MLS} could partly but not fully substitute for wildtype Shc. Disruption of rafts had little effect on cell survival promoted via the Ret:Shc axis.

2.4.2 Conclusions

We conclude in total that Shc and Frs2α does distribute differentially in neuronal cells with the latter locating to regions corresponding to lipid rafts. Forcing Shc to a Frs2α like localization by a membrane locating appendage promote Ret mediated signaling to occur in a Frs2α-like biochemical pattern and subcellular distribution and the functional outcome at least in part is corresponding to adaptor subcellular location besides/rather than adaptor identity in cells.

3 Future prospects

In order to investigate the *in-vivo* phenotypical result of rewiring Ret the production of transgenic mice has been undertaken. The mutated Ret versions favouring interaction with a specific PTB-adaptor to tyrosine 1062 has been incorporated by in-frame cloning into exon 7 of the *Ret* gene. The targeting construct consisting of Ret cDNA from exon 7 to the 3-prime end, a neomycin resistance gene and a polyA-tail have been electroporated into embryonal stem (ES) cells, which were screened for homologous recombination based on neomycin resistance. ES cells were then injected into blastocysts of foster mothers. At this time two constructs; Ret^{Frs+} and Ret^{WT} have given chimeras that have produced germline transmission. The heterozygots are currently breeding to expand the colonies before crossing to receive homozygous mice (Figure 6).

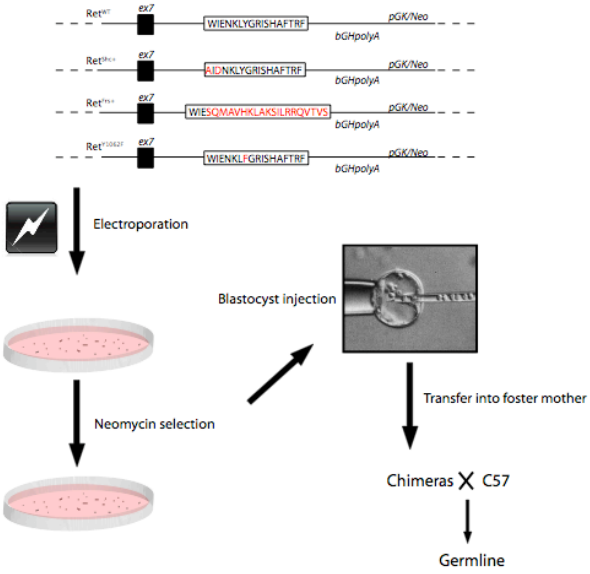

Figure 6. Schematic representation of the production of the transgenic mice. The targeting constructs were electroporated int ES cells. Cells were selected based on antibiotic resistance and injected into the blastocyst of pseudopregant foster mothers. Chimeras were crossed with wild type mice to give rise to germline offspring carrying the mutated Ret allele.

4 Epilogue

In physiological circumstances and in disease alike each cell receives continuous external inputs. It is well established that the receptors that transmit these inputs must act in accordance with their respective physiological role or else disease such as cancer occur. Intracellular signaling pathways are in general not exclusive to any one receptor but rather several signaling cascades with common intermediates integrate and converge in the cytoplasm. It has recently been shown that intracellular signaling can be readout as molecular networks in different states of activation determined by quantitative phosphorylation of several key components, and further, that altered networks predict tumour behaviour perhaps more accurate than gene expression levels (Reinhardt et al. 2007). Moreover, relating signaling-network-states to cell behaviour can be used to track-back altered geneproducts in polygenic disease (Rual et al. 2005; Huang et al. 2007; Huang et al. 2007; Pujana et al. 2007). The domain-like architecture of adaptors as well as of intracellular effector proteins make possible to search for small molecule inhibitors that by binding to conserved domains re-direct or inhibit signaling. Directing drugs towards this level of the signaling cascades could be more specific to the disease at hand compared to receptor blockade because only the disease-causing receptor functions are targeted while the remaining receptor biology may be left untampered. It also presents a possibility for intervening at proteins acting as “hubs” by being signaling intermediates downstream more than one geneproduct (i.e. polygenic disease) (Linding et al. 2008; Pawson and Linding 2008) and may therefore be more efficacious.

In order to reveal on a greater scale how signaling works global investigation by modeling of signaling networks, definement of structural aspects that govern molecular interactions and results that assign biological effects to specific molecules such as adaptors are needed. By rewiring proteins to selective binding partners and monitoring cell behavior it can be defined how specific biological outcomes are generated and networks altered. From such knowledge the hope would be to develop drugs for exogenous administration that rewire erroneous signaling into that of physiology. If it would be possible to define unhealth at that kind of a proteomics level it should lead to a major advance in designing therapy.

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7 Appendices I-IV