

The orphan 7TM protein GPR50 as a novel regulator of TGF β signal transduction

Stéfanie Wojciech

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par

Stefanie WOJCIECH

The orphan 7TM protein GPR50 as a novel regulator of transforming growth factor β signal transduction

Directeur de thèse : Dr Ralf JOCKERS, Directeur de Recherche (Institut Cochin, Paris)

JURY

Pr Mohammed TAOUIS Pr Olivier HERMINE Dr Laurent PRÉZEAU Dr Céline PRUNIER Dr Philippe DELAGRANGE Dr Mark SCOTT Président Rapporteur Rapporteur Examinateur Examinateur

Résumé

La protéine à 7TM GPR50 : un nouveau régulateur de la voie de signalisation TGFß

La protéine GPR50, qui fait partie de la famille des récepteurs de la mélatonine, est classée, avec une centaine d'autres protéines à sept domaines transmembranaires (7TM), dans la catégorie des récepteurs couplés aux protéines G hétérotrimériques (RCPG) orphelins, c'est-à-dire pour lesquels aucun ligand n'a pu être identifié. De plus en plus d'études montrent que les 7TM peuvent avoir des fonctions indépendantes d'un ligand. C'est le cas de GPR50 qui inhibe les fonctions du récepteur de la mélatonine MT_1 en interagissant directement avec lui. Nous avons cherché à identifier d'autres partenaires associés à GPR50 en appliquant la technique de purification par affinité en tandem et avons mis en évidence son interaction avec un récepteur du facteur de croissance *Transforming Growth Factor* β (TGF β), le récepteur de type I (T β RI).

Nous décrivons ici la formation d'un complexe entre GPR50 et le récepteur T β RI au niveau de la membrane plasmique, avec pour conséquence l'induction d'une activité constitutive du récepteur et des voies de signalisation en aval en l'absence de TGF β , mais également en l'absence du récepteur T β RI qui est habituellement indispensable pour l'activation de T β RI par phosphorylation. Cette activité constitutive se traduit par la phosphorylation des protéines Smad2 et Smad3, leur intégration dans un complexe avec Smad4, la translocation du complexe dans le noyau et finalement l'activation de la transcription de leurs gènes-cibles. Nous avons décrypté les mécanismes moléculaires de cette activation constitutive en montrant que GPR50 entre en compétition, pour l'interaction avec T β RI, avec le régulateur négatif FKBP12, une protéine inhibitrice de l'activité basale du récepteur en l'absence de ligand. Nous avons identifié dans la queue intracytoplasmique de GPR50 un motif répétitif similaire à la séquence de FKBP12 impliquée dans son interaction avec T β RI, motif qui constitue la base moléculaire de cette compétition.

Nous avons étudié les conséquences fonctionnelles de cette activation en surexprimant GPR50 de manière stable dans la lignée cellulaire MDA-MB-231, dérivée d'un cancer de sein. Nous avons observé dans ces cellules des effets pro-migratoires et anti-prolifératifs similaires à ceux causés par l'administration de TGFβ.

En conclusion, ce travail décrit un nouveau mode d'activation du récepteur T β RI en l'absence de ligand, mais identifie également une nouvelle fonction indépendante d'un ligand pour le RCPG orphelin GPR50. En perspective de ce travail, nous allons essayer d'identifier des conditions biologiques où cette interaction pourrait prendre place afin de confirmer ces résultats dans un contexte plus physiologique.

Abstract

The orphan 7TM protein GPR50 as a novel regulator of TGFβ signalling

During the last years, it became more and more accepted that orphan G Protein coupled receptors (GPCRs) with a transmembrane spanning heptahelical core (7TM) can have ligand-independent functions. One of those 100 orphan GPCRs is GPR50, a 7TM protein with a long cytosolic domain. Recently, studies revealed ligand-independent functions for GPR50, where it has the capacity to modulate the activity of other proteins upon complex formation. By applying a tandem affinity purification approach we sought to identify further putative interacting partners of GPR50. One of the identified binding partners is the transforming growth factor β (TGF β) receptor type I (T β RI).

The TGF β -dependent signal transduction pathway of serine/threonine kinases is a pathway with direct signal flow from ligand over the receptor to its substrates, the Smads which translocate into nucleus where they bind DNA and regulate gene expression. An important question concerns the generation of specificity and fine-tuning of TGF β -dependent signaling. Throughout the years, an important number of proteins which regulate the activity of the TGF β signal transduction pathway in a positive or negative manner have been identified. Most of them act in a cell-context-dependent manner, allowing the regulation of TGF β signaling adapted to the particular circumstances.

We report here the complex formation of GPR50 and T β RI on the plasma membrane. The consequence of this interaction is the GPR50-mediated induction of a constitutive activation of the T β RI and its downstream signaling in a TGF β ligand-independent manner. This has been monitored by Smad2/3 phosphorylation, Smad2/3-Smad4 complex formation and their subsequent translocation into the nucleus, where they activate Smad-dependent gene expression. In order to decipher the molecular mechanism that allows this activation, we showed that GPR50 competes with the negative regulator, that prevents leaky TGF β signaling, the gatekeeping molecule FKBP12, for binding to the T β RI. We identified a motif in FKBP12 involved in the interaction with T β RI with similarities to a motif in GPR50, providing a molecular basis for the replacement of FKBP12 by GPR50 in the T β RI, which normally is required for activating the T β RI by phosphorylation. This reveals a previously unknown mode of activation of the T β RI in absence of the TGF β ligand and T β RII. In order to identify the functional consequences of this crosstalk, we studied migration and growth of MDA-MB-231 breast cancer cells stably overexpressing GPR50. In these cells, TGF β -like pro-migratory and anti-proliferative effects have been observed.

Future research will help to identify tissues and biological circumstances, where this crosstalk could take place for putting this novel mode of regulation of TGF β signaling pathway into a context-dependent-manner. Additionally our work established another ligand-independent task for the orphan 7TM protein GPR50, consolidating its function as binding partner and activity modulator.

« Être parisien, ce n'est pas être né à Paris, c'est y renaître. »

Sacha Guilry

A ma famille

A mes amis

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Un essai en français

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Public presentation of work associated with this thesis

Publications:

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(2) Journée de l'école doctorale, September 2013, Le Kremlin Bicêtre

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2nd annual meeting of the GDR 3545, October 2013, Strasbourg

ABBREVIATIONS

5-HT	serotonin
7TM	seven transmembrane domain
А	alanine
A _{2A}	adenosine receptor subtype 2A
aa	amino acid
AKAP	A-kinase anchor proteins
Ala	alanine
ALK	activin receptor-like kinase
AP2	adaptor protein 2
ARE	activin-response element
ARF	activin-response factor
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATF3	cyclic AMP-dependent transcription factor
Bcl2	B-cell lymphoma 2
BG	betaglycan
bHLH	basic helix-loop-helix
BIM	B-cell lymphoma L11
BMP	bone morphogenic protein
BRET	bioluminescence resonance energy transfer
bZIP	basic leucine zipper
С	cysteine
c/EBP	CCAAT/enhancer-binding-protein
Ca ²⁺	calcium ions
cAMP	cyclic adenosine monophosphat
CCL	CC chemokine ligand
Cdc42	cell division control protein 42
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CNS	central nervous system
COS	CV-1 (simian) in Origin carrying the SV40
CpG	cytosine-guanine
CREB	cAMP response element-binding protein
CSF	cerebrospinal fluid
C-terminal	carboxy-terminal
CTGF	connective tissue growth factor
CXCR	CXC chemokine receptors
Cys	cysteine
D	aspartate
D_2	dopamine receptor subtype 2
DAF	TGFβ receptor homolog in <i>C.elegans</i>
DAG	diacylglycerol
DAPK	death-associated protein kinase

DAT	dopamine active transporter
Dlx	Homeoboxprotein Dlx
DNA	deoxyribonucleic acid
Dnmt	DNA-methyltransferase
DP1	DP1 transcription factor
Dpr2	dapper2
DRAK2	DAP kinase-related apoptosis-inducing protein kinase
E	glutamate
E2F4/5	transcription factor
EBI2	Epstein-Barr virus-induced G protein-coupled receptor 2
EC domains	extracellular domains
ECL	extracellular loop
ECM	extracellular matrix
EEA	early endosome antigen
EGF	epidermal growth factor
ER	endoplasmatic reticulum
ETV	ETS family of transcription factors
ETV6-NTRK3	fusion protein ETV6-NTRK3
F	phenylalanine
FKBP12	FK506-binding protein
FOP	fibrodysplasia ossificans progressiva
FoxH1	FoxH transcription factor
FRAP	fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
FSH	follicle-stimulating hormone
G	glycine
GABA	gamma-aminobutyric acid
GADD34	growth arrest- and DNA damage-inducible protein
GAP	GTPase-activating protein
GATA	GATA transcription factor
GDF	growth differentiation factor
GDP	guanosine diphosphate
GEF	guanine-nucleotide exchange factor
GIP	G-protein-coupled receptor interacting protein
Gln	glutamine
Glu	glutamate
Gly	glycine
GPCR	G protein-coupled receptor
GPS domain	GPCR proteolytic site
GR	glucocorticoid receptor
GRK	G protein-coupled receptor kinase
GS domain	gylcine/serine-rich domain
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
GTPase	GTP hydrolyzing enzyme
Н	histidine

HAT	histone acetylase
HDAC	histone deacetylase
HGF	hepatocyte growth factor
HIF	hypoxia inducible factor-1
His	histidine
Hlx	homeoboxprotein Hlx
HNPCC	hereditary nonpolyposis colorectal cancer
HSC	hematopoietic stem cells
HTRF	homogeneous time resolved fluorescence
Ι	isoleucine
ICL	intracellular loops
ID	inhibitor of DNA binding
IGF	insulin-like growth factor
Ile	isoleucine
IP3	inositol 1,4,5-triphosphate
JAK	Janus protein tyrosine kinase family
JNK	c-Jun N-terminal kinase
Κ	lysine
L	leucine
LAP	latency-associated peptide
LARG	Leukemia-associated RhoGEF
Leu	leucine
LH	luteinizing hormone
LPA	lysophosphatidic acid
LTBP	latent TGFβ binding protein
Μ	methionine
MAPK	mitogen-activated protein kinases
MH	Mad homology
miRNA	micro RNA
MIS	mullerian-inhibiting substance
MMTV	mouse mammary tumor virus
Mrg	Mas related gene
mRNA	messenger RNA
MT_1	melatonin receptor subtype 1
MT_2	melatonin receptor subtype 2
MUPP1	multi-PDZ domain protein 1
Ν	asparagine
NANOG	NANOG homeoboxprotein
NEDD4	neural precursor cell expressed developmentally down-regulated protein 4
ΝΓκΒ	nuclear factor-kappa B
NGF	nerve growth factor
NHERF	Na ⁺ /H ⁺ exchanger regulatory factor
NLS	nuclear localization signal/-sequence
Nogo-A	reticulon4/neurite outgrowth inhibitor
N-terminal	amino-terminal
NTRK	transmembrane surface receptor for neurotrophin-3

Oct4	octamer binding transcription factor 4
ORL1	opioid-receptor-like 1
Р	proline
p300/CBP	transcriptional co-activator of p300 and CREB-binding protein
p53	protein 53
PAC1	procaspase activating compound 1
PAR1	thrombin receptor subtype PAR1
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PH domain	pleckstrin homolgy
Phe	phenylalanine
PI3K	phosphatidylinositide-3 kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
РКА	protein kinase A
РКС	protein kinase C
PLC	phospho lipase C
PP	protein phospatase
Pro	proline
PTEN	phosphatase and tensin homolog
Q	glutamine
R	arginine
RAFT	FKBP12-rapamycin associated protein
RAMP	receptor activity-modifying proteins
RANKL	receptor activator of NF-kB ligand
RGD	Arg-Gly-Asp sequence
RGS4	regulator of G protein signaling 4
RING	Really Interesting New Gene
RLP	Ras-like protein
RNA	ribonucleicacid
ROC1	RING finger protein 1
ROCK	Rho-associated protein kinase
RSTK	receptor serine/threonine kinase
RTK	receptor tyrosine kinase
Runx3	Runx3 transcription factor
S	serine
S1P	sphingosine-1-phosphate
SARA	Smad anchor for receptor activation
SCN	suprachiasmatic nucleus
Ser	serine
SHC-1	Src homology 2 domain-containing-transforming protein C1
SID	Smad-interacting domain
SIM	Smad-interacting motif
Smad	small mothers against decapentaplegic
Smurf	Smad ubiquitin regulatory factor
SOX2	SOX2 transcription factor
SP1	SP1 transcription factor

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SRF	serum-reponse factor
STAT	signal transducer and activator of transcription
STRAP	serine/threonine kinase receptor associated protein
SUMO	small ubiquitin-related modifier
SXS	Ser-x-Ser
Т	threonine
TAB	TGFβ-activated protein kinase 1 (TAK1)-binding protein
TACE/ADAM17	Tumor necrosis factor-alpha converting enzyme/metallopeptidase domain 17
TAK	TGFβ-activated protein kinase
TAS	taste receptors
TFE3	TFE3 transcription factor
TGFβ	transforming growth factor beta
Thr	threonine
TIC	tumor-initiating-cell
TIP60	Tat-interactive protein 60
TNF-α	tumor necrosis factor alpha
TRAF6	TNF receptor associated factor
TrkA/B/C	neurotrophin NT-3 receptors A B and C
TRP	tryptophan
TSH	thyroid-stimulating hormone
Tyr	tyrosine
TβRI	TGFβ receptor type I
TβRII	TGFβ receptor type II
TβRIII	TGFβ receptor type III
Ubc9	SUMO-conjugating enzyme
UCH37	ubiquitin carboxyl-terminal hydrolase 37
UDP glucose	uridine diphosphate glucose
UL	unique long
USP15	ubiquitin specific peptidase 15
V	valine
Val	valine
VEGF	vascular endothelial growth factor
W	tryptophan
WD40	try-asp-rich domain
WWP	Try-Try-Pro proteins
Y	tyrosine
YAP65	Yes-associated protein

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PROLOGUE

Every organism from prokaryotes to high eukaryotes has the capacity to sense and to adapt to the environmental circumstances. This is the result of a multitude of molecular events that are accomplished by the means of cellular communication. Stimuli from outside the cell that range from light, ions and small organic substances over to more complex molecules as peptides and proteins are translated into an intracellular response via transmembrane proteins with a signal-integrating function. This process is termed signal transduction. Advances in research during the last years made it possible to identify the core signaling pathways that are the basis of cellular communication and biological function. These transmembrane proteins, which react upon perception of extracellular signals are grouped into main classes: (I) G protein-coupled receptors (GPCRs), which activate intracellular G proteins that pass the signal to their effectors; (II) receptors with an intrinsic tyrosine kinase (RTKs) or serine/threonine kinase activity (RSTKs) which modulate the activity of intracellular proteins through their covalent modification by phosphorylation; (III) ion channels which modulate the cellular charge through ion in- or outflow; (IV) an exception are nuclear receptors, which are localized in the cytoplasm where they capture the signal from lipophilic molecules that pass through the plasma membrane into the cell. The conversion into an intracellular signal can be accomplished through various ways, like activation of second messenger molecules (cyclic adenosine monophosphate cAMP, calcium ions Ca²⁺, nitrogen monoxide NO), induction of activation cascades (like the RTK - Ras - mitogen-activated-protein MAP kinase cascade) or the change of intracellular charge and pH. They all often converge in transforming the response on the nuclear level into the transcription of genes and their products respond to the extracellular stimulus and allow adaption to the environmental circumstances.

After the core signal transduction pathways and their components had been elucidated during the last century, the focus changed on the identification of pathway-associated proteins which modulate principal signaling. These proteins are necessary to allow the core pathway to adapt a context-specific signaling that fits the requirements of each cell type and the current biological conditions. In the age of "omics", emerging high throughput screening methods facilitated the uncovering of pathway associated- and interacting molecules. Identification of their function often coincides with discovery of novel regulatory mechanisms, which form the basis of common principles in biology, and the revelation of intersection points between different signal transduction pathways. It becomes increasingly appreciated that, instead of functioning independently apart, signal transduction pathways are part of a cellular signaling network. Thus, a cell responds in a certain cellular context or under specific physiological conditions by an adaptation of its signaling upon choosing the appropriate tools out of its repertoire.

The identification of further unexpected protein functions, interplays between the different signal transduction pathways and the elucidation of common principles of biology will help to gain more precise molecular explanations for the functioning of biological systems in health and disease. This knowledge can contribute to the identification of novel drug targets and the development of alternative strategies for disease treatment.

I. INTRODUCTION

1. G protein-coupled receptors (GPCRs)

The family of G protein-coupled receptors (GPCRs) forms the largest entity of receptors mediating signal transduction from outside the cell through the plasma membrane into the cytosol. In humans they are encoded by about 800 genes, accounting for 3 to 4% of the entire genome (Vassilatis et al, 2003).

GPCRs convey the cellular response for an immense variety of ligands, including hormones, neurotransmitters, lipid compounds, chemokines, ions, photons, small organic molecules and nucleotides and also tastes and odorants (Figure 1). This diversity of ligands reflects the important role of GPCRs to manage diverse physiological processes as vision, smell and taste and to regulate neurological, cardiovascular, immune, endocrine and reproductive functions in the organism (Bockaert & Pin, 1999). Consequently, GPCRs are a notable therapeutic target, which is underlined by the fact that about 30% of the pharmaceuticals on the market direct GPCRs (Overington et al, 2006; Tyndall & Sandilya, 2005)

In recent years the research on GPCRs has shown an impressing dynamic due to structural resolutions, which led to an increase in understanding the mechanisms of GPCR signaling and could shed light on the various functional capacities of GPCRs. In 2012, these achievements have been recompensed by the Nobel prize in chemistry for two of the pioneers in GPCR research, Brian Kobilka and Robert Lefkowitz (Benovic, 2012; Bockaert, 2012).



Figure 1 Variety of GPCR ligands (Bockaert & Pin, 1999)

GPCRs are transmembrane proteins that can bind a multitude of different ligands, including large molecules as proteins, different small molecules, as peptides, amino acids, amines, lipids, nucleotides and also percept signals in form of ions, photons from light or odorants and pheromones.

1.1 Structure of GPCRs

Despite the variety of ligands and GPCRs sizes that range from 300 to 1200 amino acids (Baldwin, 1993), all GPCRs share a common structural core. Rhodopsin sequencing in the 70's and cloning of other GPCRs in the late 80's (Dixon et al, 1986) could prove the existence of a shared topology for hormone receptors: all GPCRs are composed of seven transmembrane (7TM) spanning helical domains (see Figure 2 for details). The TM region is preceded by an N-terminus of variable length that can be involved in ligand binding and sometimes presents long sequences that form separate extracellular domains (EC domains) that also contribute to anchoring GPCRs to the extracellular matrix (ECM). TM segments are linked by three extracellular loops (ECLs) that play a role in ligand binding and three intracellular parts (ICLs) that are involved in downstream signaling. Furthermore, a cytosolic part in the C-terminus, including the helix #8 (the only known exception is CXCR4) is responsible establishing downstream signaling and functions as regulatory element in being a target for phosphorylation or a scaffold for binding other proteins. The sequence of the TM domain is quite conserved, while the other parts are more variable and form the basis of specificity concerning ligand binding, downstream signaling and the binding of modulators.



Figure 2 GPCR topology with important structural features of class A GPCRs (Audet & Bouvier, 2012)

The common topological core of all GPCRs are the seven transmembrane helices with the extracellular N-terminus and interhelical ECLs loops. ICLs loops between the helices range into the cytosol, where also the C-terminal part is located. Frequent features of class A GPCRs are the DRY motif in TM3 that forms an ionic lock with TM6, the toggle switch and the NPXXY sequence in the TM7. Ligand binding is often maintained by cooperation of ECL3 and TM6.

After deciphering the principal structure, further insight into GPCR structure was provided by the crystallization of bovine Rhodopsin, the prototypical GPCR, in 2000. (Palczewski et al, 2000). A breakthrough came in 2007 owing to the techniques that had been developed in the previous years that facilitate the obtainment of crystal structures (Cherezov et al, 2010). Application of this methods to the GPCR field resulted in discovery of the structure of the inactive adrenergic receptor β_2 in 2007 (Rasmussen et al, 2007; Rosenbaum et al, 2007), as shown in Figure 3, and β_1 in 2008 (Warne et al, 2008) bound either to an inverse agonist or an antagonist respectively. It was quickly followed by the structure of ligand-free rhodopsin (opsin) (Park et al, 2008; Scheerer et al, 2008). Further advances allowed the visualization of active structures as for the agonist-bound adenosine A_{2A} receptor (Lebon et al, 2011; Xu et al, 2011) and the β_2 adrenergic receptor in complex with the heterotrimeric G proteins (Rasmussen et al, 2011b).

These and other structures in complex with ligand or coupled to G proteins or β -arrestins led the way from a simple observation of the structure to comparative analysis of the structural changes depending on the different conformations of GPCRs.



Figure 3 Crystal structure of β_2 adrenergic receptor (Rasmussen et al, 2007)

The crystal structure of the β_2 adrengic receptor bound to an inverse agonist for stabilization of the inactive conformation.

Currently, more than 20 class A GPCR structures have been obtained (Katritch et al, 2013). The structural identification of other GPCR families has progressed slower, but first results were achieved with the structure of Smoothened (Wang et al, 2013), extracellular parts of the glutamate receptor mGluR₇ (Muto et al, 2007) and class B family members (Hollenstein et al, 2013; Siu et al, 2013). This has helped to identify specific characteristics of each family and to describe the structural diversity in the different GPCR classes (Venkatakrishnan et al, 2013). The beginning of this structure resolution era in GPCRs helped to gain information about the flexibility and functionality of GPCRs and revealed structure-function relations.

1.2 Families of GPCRs

Aside from their common transmembrane core structure, GPCRs vary in their other structural elements. Analysis of shared features led to a first categorization into four different main classes as shown in Table 1 (Kolakowski, 1994).

Table 1 Classification of GPCRs (Lagerstrom & Schioth, 2008)

Current classification of GPCRs (after (Kolakowski, 1994)), with the number of proteins they comprise and the chemical nature of their ligands.

	Class A	Class B		Class C	Others	
Property	Rhodopsin	Secretin	Adhesion	Glutamate	Frizzled	Taste2
Number of full- length receptor proteins*	672 (388 ORs)	15	33	22	11	25
Number of identified major drug targets [‡]	>39	4	0	3	0	0
Number of orphans*	63 (not including ORs)	0	30	7	0	21
Type of ligand	Peptides, proteins (including enzymes), small organic compounds, lipid- like substances, nucleotides	Peptides, proteins	Proteins, glycosaminoglycan	Amino acids, cations, small organic compounds, carbohydrates	Proteins	Small organic compounds

Class A - rhodopsin like GPCRs

The class A is the largest GPCR family with approximately 700 members, which vary a lot in primary structure and ligand specificity. Their common feature is the short extracellular N-terminal part and conserved regions in the 7TM domain. In TM3 is a D/ERY motif, which is also part of the inactivating ionic lock with a Glu-residue in TM6, two cysteines that form a disulfide-bond between ECL2 and TM3 and a NPXXY motif that is located at the end of TM7

attenuating the helical structure and forming a water pocket, as illustrated in Figure 2 (Katritch et al, 2012). Further analysis enabled the formation of subgroups among class A GPCRs: (I) Subclass α regroups receptors that preferentially bind small ligands, which occurs in the 7TM region between TM3 and TM6. (II) Subclass β is responsible for the binding of large peptide ligands. (III) Subclass γ is composed of receptors for peptides and lipidic substances. (IV) Subclass δ GPCRs bind either nucleotides and glycoproteins or some of them are responsible for the perception of odorants and taste. The large amount of nearly 400 olfactory receptors found in humans is also part of this class. In addition to these 4 subclasses, another organization model exists, which divides class A into 19 subfamilies that have common characteristic features and often bind similar ligands, like the of melatonin receptors family (see Chapter 2).

Class B – adhesion and secretin GPCRs

This family comprises two different groups of GPCRs:

(1) Adhesion GPCRs

This family consists of about 30 members. They display unique elements, like their long Ntermini that are often glycosylated. This N-terminal part is often composed of distinct domains, which are also found in other proteins with long extracellular parts like the cadherin-, lectin-, IgG- and EGF domains, which serve for binding ECM components and maintain ligand-receptor-interactions. Some receptors in this family bind proteins and glycosaminoglycans, but most are still orphan receptors (Gupte et al, 2012; Paavola & Hall, 2012).

(2) Secretin family of GPCRs

These 15 family members bind large ligands, such as peptides and proteins, with their extracellular domain. Since these ligands are important players in the maintenance of organism homeostasis, they form an attractive drug target.

Class C – The glutamate family of metabotropic receptors

The glutamate family of GPCRs is comprised of 25 members with amino acid ligands, such as glutamate and GABA, small organic compounds, and the cation Ca^{2+} . Among them are also three taste receptors. Their characteristic feature is their extended N-terminus that is implicated in ligand binding. Crystal structure analyses demonstrate a possible venus flytrap mechanism of this domain (Muto et al, 2007).

Others – frizzled GPCRs and taste receptors (TAS)

This class is composed of 11 frizzled receptors of which are 10 that bind the frizzled ligand, which is the glycoprotein Wnt, and one Smoothened ligand binding receptor.

In this class of receptors are also 25 taste receptors (TAS) that bind small organic compounds.

1.3 Signal transduction of GPCRs

The evidence that GPCRs couple to G proteins was recognized in 1970 as Martin Rodbell discovered that incubation of cells with glucagon in the presence of GTP triggers the activation of the adenylate cyclase. This led to a reformation of the concept of the signal flow of hormone receptors going from ligand via the receptor over to the G protein and its effector. In addition the name "G protein-coupled receptor" for this group of signal transducing membrane proteins became established.

1.3.1 Structural basis of GPCR activation

Ligand binding to a GPCR induces conformational changes which consequently affect its activation state. Experimental findings and structural analysis during the last years gave more insight into the complexity and the different facets of GPCR activation. This led to the shift in seeing GPCR activation as a process implicating a continuum of conformations instead of a simple two-state switch.

The resolution of GPCR structure has aided in revealing the conformational changes that occur upon activation. Surprisingly, it was found that there is not only one active conformation, but several different ones depending on the progress of activation (Figure 4).



Figure 4 Activation mechanism and conformational rearrangements of GPCRs (Katritch et al, 2013)

R is the inactive ground state, small local changes occur in the inactive low-affinity agonist-bound state R'. Achieving R'' is accompanied by substantial conformational changes and rearrangements in the receptor, leading to at least partial exposition of the G protein binding site. R* is the activated state where the structural reorganization allows the interaction with the G α protein. Finally, R*G is the fully active receptor conformation in complex with the heterotrimeric G protein. Additionally R*GRK and R*A are the conformations that receptors have upon the interaction with GRKs or β -arrestin. Noteworthy, most processes are bidirectional (indicated with flashes), while the formation of the R*G complex is unidirectional since it is accompanied by a non-reversible GTP hydrolysis. The TMs that are mainly implicated in the activation process are highlighted in red (5 and 6) and blue (3 and 7).

As a result of the bidirectionality of the different activation conformations shown in Figure 4, there is always equilibrium between receptors in the inactivated and in the activated states.

Structural changes during the activation process

During the activation process, the helices undergo several substantial rearrangements in the helical core in response to ligand binding. Ligand binding induces conformational changes that are propagated from the extracellular portion of the GPCR to the cytoplasmic surface. These conformational changes take place in several steps: ligand binding occurs upon interaction with ECLs and parts of the 7TM domain, the 7TM functions as stabilizing core, and the ICLs are responsible for the intracellular transformation of the response. For rhodopsin-like GPCRs, the movements during activation process have been shown for rhodopsin (Altenbach et al, 2008) and β_2 adrenergic receptors (Yao et al, 2006): in the inactive state, most of the class A members have an ionic lock between TM3 and TM6 that fix

the inactive conformation. Ligand binding then leads to a loss of the ionic lock and an important outward movement of TM5 and TM6, that creates a binding site for the G α protein, allowing the active ternary complex formation and the transformation of ligand binding to an intracellular effect (Rosenbaum et al, 2009).

1.3.2 Downstream signaling of GPCRs

The initiating step in signal transduction of GPCRs is the binding of a ligand. Ligand binding is incredibly variable, occurs in various regions of the GPCR and each receptor has a binding site adapted to the structure of its ligand (reviewed in (Audet & Bouvier, 2012).

1.3.2.1 Ligand efficacy

Beside this specificity in ligand binding, synthetic ligands of similar chemical structure to the natural ligand can differ in their strength to shift the receptor towards the state of full activation or even block its activation. The capacity of a ligand to activate a receptor is termed ligand efficacy. Every natural or synthetic ligand stabilizes an individual set of receptor conformations, which is reflected by its capacities to more or less fully activate or inhibit distinct downstream signaling pathways and biological responses. Different efficacy classes have been defined according to their effects on receptor activation (Figure 5):

(1) An agonist is a ligand that leads to the activation of GPCRs, either partial (= partial agonist) or completely (= full agonists).

(2) An antagonist is a ligand that inhibits receptor activity. Within this classification are inverse agonists, which inhibit spontaneous activity or counteract the basal activation of the receptor. Another type of antagonist are neutral antagonists, which are able to bind the receptor, but lack intrinsic activity, thus blocking the receptor binding site for agonists and inverse agonists (Rosenbaum et al, 2009).



Figure 5 Ligand efficacy and downstream signaling (Rosenbaum et al, 2009)

It can be differentiated between agonists that activate receptors either partially (partial agonist, light blue) or fully (full agonist, dark blue). To counteract GPCR activity, there are neutral antagonists which block receptor access and biological response (black), while inverse agonists reduce basal activity (red).

Constitutive activity of GPCRs

In the case of constitutive receptor activity a significant proportion of the GPCR can shuttle towards active conformations even in the absence of ligand (Tiberi & Caron, 1994). It is described for several GPCRs and goes along with constant coupling to G proteins and downstream signaling. A structural explanation for class A receptors could be the absence of the inhibitory ionic lock, as in the melatonin receptors. Mutations in GPCRs, that shift the balance towards more activated than inactivated receptors are often associated with diseases (Seifert & Wenzel-Seifert, 2002).

1.3.2.2 Signaling via G proteins

After ligand binding has induced conformational changes, the receptor is capable of transducing the signal to different intracellular effectors. Classical downstream signal transduction includes the heterotrimeric G proteins as mediators between receptor and effector. By binding to effectors, like enzymes or channels, they are responsible for intracellular signal transduction.

Function and diversity of heterotrimeric G proteins

The heterotrimeric G proteins belong to the family of guanine-nucleotide-binding proteins. They are highly conserved during evolution and composed of three subunits: α (about 40kDa), β (about 35kDa) and γ (about 8-10kDa), which can exist in different isoforms and assemble in various combinations in the heterotrimeric G protein complex (Cabrera-Vera et al, 2003).

In the inactive state, the three G protein subunits are associated with the G α subunit binding GDP. Following its recruitment by an activated GPCR, the G α protein undergoes conformational changes leading to a GDP to GTP exchange. This activation step is accompanied by the dissociation of the G $\beta\gamma$ complex from G α , what allows the subsequent interaction with their corresponding effectors. Termination of G protein signaling occurs through GTP hydrolysis, causing its dissociation from the effector and a reassembly of the three subunits, which are ready for another activation-inactivation cycle.

The capacity of each subunit to interact with different effectors through their protein interacting domains allows a broad spectrum of downstream signaling pathways (Kostenis et al, 2005). The G α proteins can be divided into four subfamilies with specific downstream signal transduction (see Figure 6 for overview):

 $G\alpha_s$ and $G\alpha_i$ proteins either stimulate ($G\alpha_s$) or inhibit ($G\alpha_i$) adenylate cyclaces that convert ATP into cyclic AMP (cAMP). cAMP functions as a second messenger, activates protein kinase A (PKA), which in turn phosphorylates and activates the transcription factor cAMP response element protein (CREB), which regulates the expression of numerous genes.

 $G\alpha_q$ proteins activate the phospho lipase C (PLC) that hydrolyzes phosphatidylinositol 4,5bisphosphate (PIP₂) to diacylglycerol (DAG) and inositoltriphosphate (IP₃). DAG can activate the protein kinase C (PKC) and IP₃ binds to receptors which triggers the release of calcium from internal stores.

 $G\alpha_{12/13}$ proteins can direct downstream signaling towards the regulation of Rho GTPase activity.

The G β and γ subunits remain associated after dissociation from the G α protein, and interact and activate PLC $_{\beta}$ through their pleckstrin-homology (PH) domains, which triggers IP3 and DAG production, like the G α_q proteins. Additionally they can also activate ion channels and phosphatidylinositide 3 (PI3) -kinases.

Other possible actions of G protein subunits have been reported like guanylyl cyclase activation for cGMP generation, regulation of mitogen-activated protein (MAP) kinase activity and the activation of phosphatases, highlighting the diversity of G protein targets inside the cell (Milligan & Kostenis, 2006).



Figure 6 Signaling of GPCRs via heterotrimeric G proteins (Dorsam & Gutkind, 2007)

The heteromeric G proteins can direct GPCR signals depending on their subtype towards different effectors. The different G α proteins are indicated in red and the $\beta\gamma$ subunit in purple.

Structural basis of G protein coupling to the receptor

The activation of G proteins requires their coupling to the receptor, which is also subject to conformational rearrangements in the receptor. Ligand binding induces the loss of the ionic lock between TM3 and TM6 and the outward movement of TM6 creates a binding site for the G protein. Thus, a conformation, that allows the binding of the G protein is stabilized (Audet & Bouvier, 2012). More specific insights for the receptor-G protein interaction were recently gained with β_2 adrenergic crystal structures bound to the G protein (Rasmussen et al, 2011a; Rasmussen et al, 2011b): G α protein interaction sites are found on TM3, TM5, TM6, and the ICL2 of the receptor. After coupling of G proteins to an active receptor, the nucleotide exchange from GDP to GTP for activation is facilitated through structural adjustments and interactions between the receptor and the G protein (Bouvier, 2013).

But the different existing G protein isoforms and the specificity of each ligand and its ability to signal via different G α protein suggest the existence of additional mechanisms to achieve the pairing of a ligand with its specific G protein. It will be a future challenge to describe the mechanisms that allow the recognition of ligand and associated G protein via the 7TM core.

Regulation of G protein activity

Different classes of proteins control G protein activity in order to regulate GPCR-dependent signal transduction. Some proteins can act on the GDP-to-GTP exchange: while guanine-nucleotide-exchange factors (GEFs) are activating, GDP-dissociation inhibitors (GDIs) have an inhibiting function. Activators-of-G protein-signaling (AGS) proteins bind to the $\beta\gamma$ subunit, to form a quaternary complex (Cismowski, 2006). RGS proteins are regulators of G protein activity that mediate their inactivation by promoting GTP hydrolysis, thus functioning like a GTPase-activating protein (GAP). Their expression is often induced via a negative feedback loop mechanism (Ross & Wilkie, 2000).

1.3.2.3 G protein-independent signaling

Different G protein-independent pathways have been discovered in the recent years: GPCRs can function via the β -arrestin coupling that favors the activation of MAP kinases like extracellular-regulated-kinase ERK (see below). GPCRs can also bind to PDZ domain-containing proteins like MUPP1 that modulate their activity and localization (Guillaume et al, 2008). The existence of residues that are phosphorylatable can form a binding motif for SH2 domain proteins like Src and trigger associated downstream signaling events (Marinissen & Gutkind, 2001). Upcoming research will surely uncover more presently unexpected downstream signaling activities in the absence of G proteins.

1.3.3 Regulation of GPCR activity

Beside above described proteins that act on the G proteins, further distinct mechanisms to modulate GPCR activity and signaling:

1.3.3.1 Receptor trafficking and desensitization

The receptor desensitization claims to stop ligand binding or to prevent the receptor from inducing downstream signaling in response to ligand binding. There exists a canonical way to stimulate receptor desensitization.

Receptor phosphorylation

The first event to stop ligand-dependent effects is a phosphorylation on serine/threonine residues in the cytosolic C-terminus of the receptor. Different kinases have been identified to

target GPCRs, like PKA, PKC and GPCR kinases, the GRKs. Seven different GRKs have been identified and all are activated as a downstream signaling event or by the binding of GPCR effectors or second messengers. GRKs also have a GAP domain to inactivate G proteins (Luttrell & Lefkowitz, 2002). Typically, GRK-mediated phosphorylation leads to a decoupling of G proteins, but can also trigger other signaling cascades (Zanello et al, 1997).

β -arrestin recruitment

Phosphorylation is a prerequisite not sufficient for inactivation of GPCRs, but it creates a binding site for β -arrestin. β -arrestins are responsible for receptor internalization by interacting with the adaptor protein AP2 in the endosomes and triggering the formation of receptor-containing vesicles that translocate inside the cell. Afterwards, receptors are either recycled and inactive receptors shuttle back to the plasma membrane, or otherwise, they can also be directed to the lysosomes to be proteolyzed (Luttrell & Lefkowitz, 2002).

β -arrestin-mediated G protein-independent signaling

Besides blocking further G protein docking and activation of GPCRs, β -arrestin recruitment can also have other possible outcomes. They can function as scaffolding proteins for diesterases that degrade cAMP and terminate signaling, ubiquitin ligases for a proteasomal degradation of the receptor complex (Shenoy & Lefkowitz, 2011), or trigger the activation of other pathways by activation of Src (Luttrell et al, 1999) or the PI3K/Akt pathway.

The β -arrestin-mediated internalization into endosomes can also help for the creation of signaling endosomes and prompt ERK activation (DeFea et al, 2000).

In addition to β -arrestin actions on GPCRs, increasing evidence suggests their involvement in other GPCR-independent pathways, as the TGF β signaling pathway (Chen et al, 2003; McLean et al, 2013).


Figure 7 Signal transduction of the β_2 adrenergic receptor (Rosenbaum et al, 2009)

The β_2 adrenergic receptor can mediate different downstream signaling pathways. Dependent on the bound ligand, it can activate $G\alpha_i$ or $G\alpha_s$ proteins that either activate or inhibit cAMP production. The subsequent phosphorylation of PKA triggers the activation of Ca^{2+} channels. The recruitment of β -arrestin to the receptor can induce different scenarios: a bound phosphodiesterase can attenuate adenylate cyclase activity, the MAP Kinases ERK1 and ERK2 can be activated or the receptor can be internalized with subsequent recycling or lysosomal degradation.

1.3.3.2 Regulation of GPCR signaling by interacting proteins

The activity of GPCRs can also be regulated by different classes of interacting proteins:

GPCR-interacting proteins (GIPS) comprise GRKs and β -arrestin, but a lot of other molecules have been identified that can participate in regulation of GPCR localization and activity after complex formation (Magalhaes et al, 2012).

A specific class of proteins are receptor-activity modifying proteins (RAMPs), which were found to interact with the calcitonin receptor and alter its activity and pharmacology (Hay et al, 2006). Other proteins are GPCR-associated sorting proteins (GASPs) that play a role in subcellular localization or influence the trafficking towards the plasma membrane after synthesis.

The PDZ motif in the C-terminal part of the GPCR can mediate the interaction with PDZ proteins like NHERF or MUPP1 that can be required for GPCR activation (Ritter & Hall, 2009).

Additionally, a lot of other proteins exist to regulate activity, pharmacology and localization of GPCRs and are described in more detail in the literature (Maurice et al, 2011a).

1.3.4 Diversity and special features of GPCR signaling

The preceding chapter illustrates that one GPCR can adopt different conformations. In dependence of each natural or synthetic ligand, a distinct conformation with distinct downstream signaling activity is obtained. This diversity of GPCR signaling can be further increased by the following opportunities of GPCR signal transduction:

1.3.4.1 Allosteric regulation of GPCR activity

Allosterism was firstly described for enzymes in 1963 (Monod et al, 1963). This means a compound can bind to an allosteric site in the receptor, which is another than the standard, the orthosteric one. Thus, an allosteric ligand can influence receptor actions or have an effect on the orthosteric ligand activity (Bridges & Lindsley, 2008). The binding of an allosteric molecule leads to conformational changes, compared to the orthosteric-ligand-bound-only-state and is often accompanied by a different signaling outcome. Hence, allosteric ligands also play a role in biased signaling (see below).

Allosteric ligands can be different types of molecules: small compounds but also proteins like interacting and regulating proteins described in the preceding chapter and they function either as positive allosteric modulators (PAMs) or negative allosteric modulators (NAMs). The identification of allosteric modulators and their corresponding binding sites is one of the current major interests in GPCR research (Katritch et al, 2012). They bear a huge therapeutic potential for the development of a new type of synthetic drug for GPCRs and benefit from *in silico* drug design. Furthermore, bitopic ligands, that occupy more than one binding site in a receptor and thus display particular signaling profiles, are another future application of the allosteric mode of action are the benzodiazepines binding to GABA receptors (Sebag & Pantel, 2012).

1.3.4.2 Biased Signaling of GPCRs

The phenomenon of biased signaling is grounded in the ability of one GPCR to induce several downstream signaling pathways. Biased signaling describes the ability of one synthetic ligand to direct signaling into a particular pathway, compared to the natural ligand (Rajagopal et al, 2010).

The structural basis is the existence of several active conformations of one receptor (see 1.3.1.). This is also reflected in the ability of each ligand for one GPCR to stabilize a particular set of conformations implicating a specific rearrangement of certain parts in the receptor in response to ligand, which finds its consequence in a specific ligand-corresponding downstream signaling (Wacker et al, 2013). This capacity of a ligand to preferentially activate only one signal transduction pathway is termed "functional selectivity" (Rosenbaum 2009). Technical developments, like bioluminescence resonance energy transfer (BRET), permit to study conformational changes that underlie biased signaling (Galandrin et al, 2008).

Drug design takes advantage of biased signaling to find suitable ligands with impaired efficacy and reduced side effects (Kenakin & Christopoulos, 2013).

In addition to ligands, receptors can be biased, which is the case in mutated receptors as demonstrated for different variants of the melatonin receptor MT_2 (Bonnefond et al, 2012).

The existence of biased signaling supports the concept of multi-functionality of GPCRs and its action as a center of integration for different ligands by permitting each of them a specific signaling pathway and a different biological response.

1.3.4.3 GPCR variants

With the development of high throughput sequencing to analyze genomic DNA and their application to genetic association studies, it was revealed, that most GPCRs exist in sequence variants or display frequent germline mutations (SNPs, single nucleotide polymorphisms) (Nelson et al, 2012). These variants are sometimes found associated with a higher risk to develop certain diseases. One pioneer study tried to find a connection of variants in melatonin receptor genes and the risk to develop type 2 diabetes, these variants are either located in intronic sequences (Andersson et al, 2010; Bouatia-Naji et al, 2009; Chambers et al, 2009; Sparso et al, 2009) or the coding exon region of the MT₂ receptor gene MTNR1B (Bonnefond et al, 2012). Variants in the coding region might introduce changes in the amino acid sequence that affect the functionality of the protein. The interesting task is now, to

characterize the signaling properties of each of those variants and to identify their potential for biased signaling.

Altogether, these findings gave rise to the supposition that the SNPs we naturally find in everyone of us, might also evoke different signaling properties and functional effects of GPCRs and that this concept could be translated to other receptors and other proteins. In turn, this can explain why certain people have a higher tendency to develop certain disorders and different sensitivities to medication. To generalize, this concept might not only find its application in disease but could also serve as the scientific basis to understand the individuality of everyone.

1.4 GPCR Homo- and heteromerisation

GPCR homo- and heteromerisation means the assembly of two or more monomers into one functional complex, either with the same (di- or oligo-homomer) or with different monomers (di- or oligo- heteromer) of other GPCRs.

1.4.1 Homodi/oligo-merisation of GPCRs

The existence of receptor dimers and oligomers is a quite young concept in GPCR research, while for several other receptor classes dimerisation is indispensable for signaling activity. It is described for different receptor tyrosine kinases (RTKs) like the epidermal-growth-factor receptor (EGFR) (Blakely et al, 2000), serine/threonine receptor kinases as the transforming growth factor β (TGF β) receptor (Franzen et al, 1993), cytokine receptors that are coupled to soluble tyrosine kinases as found in the januskinase (JAK) - signal transducers and activators of transcription (STAT) pathway or even nuclear receptors (Marianayagam et al, 2004). The reason is that their ligands are dimeric and have two symmetric binding surfaces that require binding to two receptors. This often goes along with the fact, that receptor activation requires transphosphorylation from one receptor monomer to the other. For GPCRs, the existence of higher-order structures slowly became accepted with turning of the last century (Bouvier, 2001; Hebert & Bouvier, 1998; Salahpour et al, 2000). A list of GPCRs for whom the existence of multimeric states has been proven can be found at <u>http://data.gpcr-okb.org/gpcr-okb/oligomer/list</u>

1.4.1.1 GPCR homodimers

One receptor monomer is sufficient to perform signaling (Whorton et al, 2007) thus questioning the necessity of dimeric complexes. The development of appropriate biophysical techniques based on energy transfer between donor and acceptor molecules like BRET (Angers et al, 2000; Mercier et al, 2002) and homogeneous time resolved fluorescence (HTRF) (Maurel et al, 2004) have become a versatile tool to study and prove GPCR dimerisation. Further supporting evidence is provided by recent crystal structures, that propose the existence of dimer-interaction surfaces as shown for the opioid receptors (Granier, 2012). Consequently, the concept of receptor dimerisation is now established and it has been shown that it even can be mandatory for correct functioning, for example for class C GABA receptors (Galvez et al, 2001).

Evidence exists that an organization in dimers can be constitutive and that their formation already occurs during protein synthesis (Terrillon et al, 2003). The mechanisms of dimer formation are various and can rise from the formation of disulfide bridges between receptors over transmembrane interactions to coiled-coil interactions of helices in the cytosol (Bouvier, 2001).

1.4.1.2 GPCR homooligomers

The existence of homo-oligomeric assembly of GPCRs has been shown by different techniques. Additionally to BRET and FRET, other light based studies like total internal reflection fluorescence microscope (TIRF) (Boyer & Slesinger, 2010), fluorescence recovery after photobleaching (FRAP) (Dorsch et al, 2009) or single molecule techniques (Calebiro et al, 2013) proposed the existence of higher-order states for GPCRs. The technique of HTRF also allowed their detection in native tissue (Albizu et al, 2010). These techniques brought evidence for GABA receptors being organized as tetramer (Maurel et al, 2008) and M₃ muscarinic receptors as hexamers (Patowary et al, 2013). The crystal structure of the turkey β_1 adrenergic receptors in a lipid membrane displayed oligomeric structures (Huang et al, 2013). The functional effect of oligomerisation remains speculative. It might be a mean to facilitate ligand binding and signaling complex formation (Maurice et al, 2011b), but also could be a way for the receptor to stabilize inactive conformations (Bouvier, 2013).

1.4.2 Heteromerisation of GPCRs

GPCRs form high order structures by the assembly of different GPCR mono- or dimers, what is called heteromerisation.

Examples of heteromer formation

As for homomerisation, heteromers are assembled in the endoplasmatic reticulum (ER) before their insertion in the plasma membrane (Milligan, 2006). It can take place between different types of GPCRs:

(1) Heteromerisation in the same subfamily is often found between closely related GPCRs. The class C GABA receptor GABA_{B1} and GABA_{B2} form obligate heterodimers (Bettler et al, 2004; Kaupmann et al, 1998; White et al, 1998) based on their asymmetric mode of activation, where one monomer is responsible for ligand binding and the other manages the activation of the G protein (Pin et al, 2009). The example of opioid receptors shows a case where both monomers κ and δ are necessary for activation and a strong ligand binding (Jordan & Devi, 1999). In the taste receptor subfamily, T1R₁ and T1R₂ form heteromers with the T1R₃ ligands and their combination defines the taste we experience (Xu et al, 2004; Zhao et al, 2003).

(2) Heteromerisation in different, but related subfamilies of one class often involves receptor transactivation, where ligand binding to one receptor activates the other, despite their different ligand preferences. The heteromeric complex formation between the adenosine receptor A_2 and the dopamine receptor D_2 is an example of allosteric regulation of one receptor by the other (Ferre et al, 1991; Hillion et al, 2002). In contrast, heteromerisation can also have antagonistic effects, as in the case of glutamate binding to the complex of receptor mGluR₂ glutamate receptor and the 5-HT_{2A} serotonin receptor heteromer (Gonzalez-Maeso et al, 2008).

(3) Heteromerisation in different GPCR classes of families occurs less frequently, but has been shown for the adenosine A_1 /glutamate mGluR_{1 α} dimer composed of one class C and class A receptor (Ciruela et al, 2001).

Rare is the case where more than two different receptors assemble into oligomers, but the example of the heteromer of adenosine A_{2A} and dopamine D_2 receptors with the glutamate receptor mGluR₅ (Cabello et al, 2009) proposes the existence of more complex heteromeric structures for GPCRs.

(4) The case of heterodimerisation of with orphan GPCRs will be discussed in more detail separately (see 1.6.2.)

Heteromer specific signal transduction and effects

Heteromer formation increases the spectrum of biological responses and signaling variety of GPCRs. It affects signaling either in an antagonistic, synergistic or receptor-biased fashion and can induce crossactivation or –inhibition (Smith & Milligan, 2010). Heterodimerisation can, for example, change the ligand binding properties. This is accomplished by either obligatory heteromers (GABA_B) or involves conditional allosteric regulation mechanisms where ligand binding to one receptor affects the affinity and efficacy of the other ligand and can enhance or impair signaling (Milligan, 2009). Another facet of heterodimerization is the creation of novel binding sites upon complex formation, which has been found for opioid receptor heteromers (Waldhoer et al, 2005).

Heteromers can induce a switch in G protein activation or coupling to induce distinct downstream signaling pathways. Additionally, they can also be implicated in biasing signaling and preferentially activate a specific downstream pathway (Rozenfeld & Devi, 2011).

Heteromerisation can also modulate surface delivery of receptors (Achour et al, 2008) by inducing a ER retention or inhibiting internalization (Jordan et al, 2001) (Cao et al, 2005).

Physiological and pathophysiological relevance of heteromers

The description of heteromer formation and function was mainly carried out by the use of cellular models, but more and more results prove its *in vivo* existence: A_{2A}/D_2 dimers can be found in the central nervous system (CNS) (Agnati et al, 2005) and a recent example proved evidence for melatonin receptor heteromers and their functional importance in the retina (Baba et al, 2013).

The detection of 5-HT_{2a}/GluR₂ heteromers could provide targets for antipsychotic drugs that diminish hallucination symptoms in psychotic patients (Fribourg et al, 2011).

For the future, the discovery of more heteromer complexes with unique pharmacological properties can reveal novel therapeutic targets (van Rijn et al, 2013). The synthesis of peptides that interfere with heteromer formation (Rozenfeld & Devi, 2010; Rozenfeld & Devi, 2011) and selective or bivalent ligands for heteromers (Milligan, 2006) have enormous potential and might aim to provide new solutions for disorders which lack specific drugs.

1.5 Crosstalk of signal transduction pathways

In addition to their typical signal transduction pathways, GPCR signaling can cooperate with other signaling pathways, which is called signaling crosstalk. A crosstalk is the interplay between independent signal transduction pathways that influence or alter the outcome of one of the involved signaling pathways. A crosstalk can be achieved by (I) direct interaction of members of two different pathways, (II) indirect enzymatic or transcriptional action (III) competition events between signal transduction pathways (illustrated in Figure 8).



Figure 8 Crosstalk in signal transduction (Guo & Wang, 2009)

The figure explains the different possibilities of signal transduction pathways. Compared to the signaling in an independent pathway (left side), this signal can be influenced due to an interplay with other signaling pathways via (a) direct interaction, (b and c) one being an enzymatic or transcriptional target of the other in direct manner or through a mediator M or (d) two members of independent signal transduction pathways can compete on another protein.

1.5.1 Crosstalk between GPCRs

The crosstalk that occurs between GPCRs can have several origins. One source of crosstalk is the above-described formation of heteromers as described above: the physical interaction on the level of the plasma membrane often results in altered downstream signaling. This includes also the phenomenon of receptor transactivation from one to another (Vischer et al, 2011). But the crosstalk between GPCRs can also take place in intracellular downstream signaling. GPCRs can, for example, compete for the same G protein subunits as it has been shown for opioid and cannabinoid receptors, which are not assembled as heteromers but counteract on a later level of signaling (Canals & Milligan, 2008). Another example of crosstalk is the case of heterologous desensitization, which is the downstream activation of protein kinases, like the GRKs, by one receptor, which in turn phosphorylates another GPCR and induces their silencing (Chu et al, 2010).

Especially class C GPCRs serve as a good tool to study and understand the different mechanisms of inter-GPCR crosstalk (Prezeau et al, 2010) and the example of the mGluR_{1a}-GABA_B crosstalk has been physiologically demonstrated in the CNS (Hirono et al, 2001).

1.5.2 Crosstalk with other signal transduction pathways

GPCRs are also able to interfere with and to be regulated by signal transduction pathways implicating other proteins than GPCRs.

Crosstalk with receptor tyrosine kinases

Already in the 90's it has been shown that a GPCRs can transactivate receptor tyrosine kinases (RTKs) in the absence of the RTK ligand. A well-described model is the transactivation of the EGFR by the lysophospholipid receptor LPA (Daub et al, 1997). The dissection of the mechanism showed that non-receptor tyrosine kinases like Src and Pyk function as intermediates through direct interaction with the RTK after their GPCR-mediated activation. Another mechanism could also pass in a ligand-dependent manner by activation of EGF ligand secretion through GPCR stimulation. New models like the triple-membrane-passing model propose the passage of the signal from the active GPCR over to metalloproteases, that act as intermediates for shedding of heparin-binding-EGF which then binds and activates its receptor (Fischer et al, 2006). The result of this transactivation is the formation of MAP kinase activity.

Additionally, other GPCRs, such as LPA-, endothelin- and thrombin receptors can activate the platelet-derived growth factor (PDGF)-, the insulin-like growth-factor (IGF)- or the neurotrophin TrkA and B -receptors (listed in (Wetzker & Bohmer, 2003).

Conversely, RTKs can also transactivate GPCRs in the absence of their corresponding ligand, like the IGF receptor activates CXCR4 and the nerve-growth-factor (NGF) RTK activates the LPA₁ GPCR. This occurs mainly through direct interaction, but other mechanisms like ligand synthesis or transphosphorylation are possible (Delcourt et al, 2007a). A crosstalk between

the fibroblast growth factor (FGF) receptor and GPCRs has been demonstrated in several examples and different physiologic consequences: together with D_2 in schizophrenia, with A_{2A} that has a role in synaptic plasticity (Flajolet et al., 2008) and with 5-HT_{1A} in depression (Borroto-Escuela et al., 2011).

The crosstalk with RTKs explains the implication of GPCRs in growth control and vascular remodeling, which are not part of the variety of biological responses rising from independent GPCR signaling.

Crosstalk with non-receptor tyrosine kinases

Crosstalk can also occur with intracellular proteins. First evidence was provided by the chemical inhibition of soluble tyrosine kinases that led to a decrease in GPCR-mediated activation of MAP kinases, proposing their function as signaling intermediates. An involvement of Src kinase in GPCR-mediated activation of the MAP Kinases ERK, c-Jun N-terminal kinase (JNK) and p38 has been identified (Cao et al, 2000; Ma et al, 2000). Recent findings also propose a strong implication of GPCR bound β -arrestin mediating the activation of MAPKs. This suggests the creation of physical signaling platforms of RTKs and GPCRs and lead to the involvement of GPCRs in growth control (*Pyne & Pyne, 2011*).

Crosstalk with PI3 kinase pathway

GPCRs have also been found to transactivate PI3 kinases and their downstream signaling, either leading to Akt (Murga et al, 2000) or MAP Kinase (Lopez-Ilasaca et al, 1998) activation.

Crosstalk with receptor serine/threonine kinases

Compared to RTKs, only recent findings provide evidence for an extension of the transactivation to the RS/TKs receptors of the TGF β signaling pathway (Burch et al, 2012), further described in chapter 3.4.

Crosstalk with ion channels

An interaction of the opioid-receptor-like GPCR ORL1 receptor can activate ligand gated calcium channels in absence of their ligand (Beedle et al, 2004).

Implication of GPCR signaling elements in other signal transduction pathways

In return, it has been described that RTKs use elements of GPCR signal transduction.

This is the case the IGF receptors that use the constitutive active CXCR12 GPCR, without its CCL12 ligand, to benefit from its $G\alpha_i$ activity in MDA-MD-231 cells (Akekawatchai et al, 2005). In order to activate adenylyl cyclases, the IGF receptor teams up with the pituitary adenylate cyclase-activating polypeptide PAC1 receptor signaling (Delcourt et al, 2007b). The physical interaction of the vascular endothelial growth factor (VEGF) receptor and the sphingosine1-phosphate receptor S1P1 also leads to a specific downstream signal transduction (Bergelin et al, 2010). The transactivation from TrkA to LPA₁ even seems to lead to nuclear translocation of the complex (Moughal et al, 2004). The existence and physiological relevance of the crosstalk has also been demonstrated in the CNS (Shah & Catt, 2004).

These different types of crosstalk add another brick in the multifunctionality concept of GPCRs and make them an important element of the cellular network, instead of functioning individually apart (Marinissen & Gutkind, 2001).

1.6 Orphan GPCRs

All these signal transduction pathways and crosstalks are mainly valid for the GPCRs with a known ligand. But there is still a part of proteins left that display sequence homology with GPCRs and have the same basic 7TM structure, but lack the identification of the corresponding natural ligand. The detection of increasing amounts of sequences of 7TM proteins without known proper ligand caused the definition of them as orphan receptors (Libert et al, 1991).

Today, we count in total more than 100 orphans, meaning 15% of all the GPCRs, and about one third by excluding the ~400 proteins of the olfactory fraction of GPCRs. Most of these orphans are members of the rhodopsin class A family (~ 80), nearly all of the adhesion GPCRs are still orphans (~30), and also 7 class C GPCRs, a secretin family member and some 20 taste receptors have currently no identified ligand (see Figure 8). A database that provides the up to date information about newly identified ligands can be found at <u>http://www.iuphar-db.org</u>.

These orphans build for two reasons an interesting object in research: (I) the identification of the matching ligand, their deorphanization, is still of big interest and (II) increasing evidence



exists for ligand-independent functions of orphan receptors. Therefore, orphan receptors are a source for the identification of further and formerly unappreciated functions of GPCRs.

Figure 8 Orphan GPCRs (Civelli et al, 2013)

The figure shows orphan class A GPCRs according to their homology with existing subfamilies.

1.6.1 Deorphanization of GPCRs

A first aim for all the orphan receptors is to identify their natural ligand, the deorphanization. With the identification of other putative GPCRs due to their sequence homology and following cloning experiments in the early 90's (Marchese et al, 1994), a repertoire of orphan 7TM proteins was available. In order to identify suitable ligands, either *in silico* sequence alignment helped to find similarities to existing GPCRs or neurotransmitter molecule libraries were matched up with orphan GPCRs (Civelli et al, 2006).

Reverse pharmacology

The existence of such big collections of receptors without ligands initiated the development of new strategies in pharmacology. The approach from an available receptor towards ligand identification was termed reverse pharmacology (Mills & Duggan, 1994). It includes the heterologous expression of the orphan protein in cells, the incubation with a ligand from molecular libraries or tissue extracts and a subsequent analysis by functional assays. (Civelli, 2005).

The era of reverse pharmacology led successfully to receptor deorphanization but also the discovery of novel classes of ligand and receptors, like new neuromodulator families with nociceptin, orexins, prolactin-releasing peptide, ghrelin, apelin and kisspeptin (Civelli, 2012). Additionally, deorphanization could identify some lipid-like ligands and surprisingly previously unappreciated substances like uridine diphosphate (UDP)-glucose or citric acid cycle intermediates as GPCR ligands. Some receptors were found to be able to bind several different substances, like for the class C GPRC6A that binds different basic amino acids. This induced a shift away from the "one ligand-one receptor" dogma (Civelli et al, 2013).

The post-reverse pharmacology age – new methods for deorphanization and future challenges Since 2005, we recognize stagnation in ligand identification, marked by a slower deorphanization rate that implemented the development of new approaches that include:

(1) in silico methods

to bioinformatically compare expression profiles of receptors and possible ligands and to use structure predictions of orphan proteins, which allow a forecast for the chemical structure of binding what in turn improves the generation of synthetic ligands.

(2) functional tests and their coupling to new read outs

One way to improve the sensitivity of established assays is the use of light-based methods as fluorescent labeling of receptors to assess their internalization, the fluorescence- (Ferguson & Caron, 2004) or luminescence- (Southern et al, 2013) based detection of β -arrestin recruitment or a combination of the measurement of cAMP production with fluorescence or luminescence. Additionally, the technique of reporter gene assay also made its way into GPCR deorphanization by the utilization of constructs with cAMP- or the PKC-activation-dependent transcription-factor-binding elements in their promoter (Yoshida et al, 2012).

(4) analysis of physiological function of GPCRs

The use of heterologous expression models (Sugita et al, 2013) or transgenic mice models with deletion of the protein of interest (Yang et al, 2013) can give idea about their function and physiological relevance and can in turn narrow down the number of ligands to be tested.

Beside the improvement of deorphanization methods, there are still some points that have to be paid attention to in the future in order to succeed: the discovery of increasing signaling possibilities and unpredictable G protein coupling of GPCRs make it more difficult to choose the appropriate method for deorphanization. It also has to be taken in account that heterologous expression models represent an artificial cellular environment and that either unnatural responses can be detected or additional interacting proteins, co-factors or heterodimerisation, which are required for activation, might be missing. The possibility that one orphan might only be activated by the simultaneous binding of more than one ligand could provide explanation for failure in deorphanization (Levoye & Jockers, 2008).

In contrast, for some GPCRs a ligand once has been identified, but it could never be proven again, as for GPR39 and obestatin binding (Civelli et al, 2013). In the case of GPR37, a first putative ligand, the neuropeptide head activator, has been identified in 2006 (Rezgaoui et al, 2006), what has never been repeated, while recent data provide another ligand possibility with the neuroprotective and glioprotective factors prosaptide and prosaposin (Meyer et al, 2013).

For the future, it will be important to adapt the current state of knowledge about GPCRs and their signaling, including all the new concepts and findings, for the deorphanization of orphan GPCRs and to develop new techniques and strategies for their ligand identification.

1.6.2 Ligand-independent functions of orphan GPCRs

Even though, Civelli hypothesized that the rules of evolution implicate the existence of a ligand for every GPCR (Civelli, 1998), combined with the difficulty to imagine the evolutionary survival of receptor-like proteins without ligand, increasing evidence proposes a ligand-independent function for GPCRs. For these proteins, the term "orphan 7TM protein" seems to be more suitable, because their sequence predicts the existence of the heptahelical core, but their coupling to G proteins is not obligatory.

The characterization of orphan 7TM proteins started with the development of cloning techniques in the beginning of the 90's that enabled overexpression in cells and founds its continuation in methods using genetic ablation in cells or transgenic mice, permitting the identification of functions and modes of action for the 7TM proteins.

Constitutive activity

One possibility for the orphan GPCRs is the case of constitutive activity. This concept has been proven for virus encoded 7TM proteins like UL33 (Waldhoer et al, 2002) and the Epstein-Barr virus-induced G-protein coupled receptor EBI2 (Rosenkilde et al, 2006) and for non-viral 7TM proteins like GPR3 (Ledent et al, 2005; Mehlmann et al, 2004), GPR6 and GPR12 (Tanaka et al, 2007), GPR26 and GPR78 (Jones et al, 2007) that constitutively activate adenylate cyclase. But it remains under speculation whether this could be an artificial effect upon overexpression or the result of permanent occupancy with the endogenous ligand as it is the case for the fatty acid receptor GPR40 (Stoddart et al, 2007). Whether other ligand-independent mechanisms exist to regulate their activity or to inhibit their constitutive activity is unknown, but constitutive activity of orphan GPCRs can be applied for the design of suitable inverse agonists.

Modulation of the function of other proteins upon direct interaction

Another area for the characterization of the function of orphan 7TM proteins lies in their ability to interact with other proteins. The utilization of proteomic approaches can help to identify putative binding partners of 7TM proteins. Recently, a lot of techniques specific for transmembrane proteins have been developed (Daulat et al, 2013), among them the tandem affinity purification (TAP) that allows the isolation under native conditions (Daulat et al, 2007). Their application to orphan GPCRs and other techniques helped to uncover protein complexes.

(1) Obligatory formation of heteromers with other GPCRs

A first role which has been attributed to orphan 7TM proteins, is heteromerisation with other GPCRs, which mainly occurs in members of the same subfamily. In some cases the interaction of an orphan protein can be absolutely required for the activation of the other protein. A well-described system is the one of the GABA_B receptors. Heterodimerisation between GABA_{B1} and GABA_{B2} is mandatory for activation by the GABA ligand. Both proteins must be in complex to be able to transduce a signal, since each one has a specific function: GABA_{B1} binds the ligand and GABA_{B2} is responsible for transducing the signal to the G protein. In this case GABA_{B2}, who has no ligand binding domain, functions as the orphan receptor (Kniazeff et al, 2002).

(2) Activity-modulating, inducible heterodimerisation with other GPCRs

Another possibility is conditional heteromerisation of GPCRs, where the formation of heteromers can alter the function of the homomer. Various examples with different modes of action have been discovered in the last years. The pioneer work in this field demonstrated the heteromer formation of the melatonin receptor MT_1 and its orphan family mate GPR50. In this case, the orphan GPR50 negatively interferes with melatonin-dependent signal transduction (Levoye et al, 2006a) (for more details see Chapter 2).

The β -alanine binding mas related receptor Mrg_D can form a complex with its orphan relate Mrg_E. This interaction is associated with potentiation of signaling and inhibition of internalization of the receptor (Milasta et al, 2006).

But there are also some examples which demonstrate a heteromer formation between nonhomologous proteins. This can even occur between the 7TM proteins of different species. The orphan receptors UL33 and UL78 from the human cytomegalovirus are able to interact with the chemokine receptors CCR5 and CXCR4 of their host cells and modulate their function (Tadagaki et al, 2012). Another example for an interaction of proteins of different species has been shown with EBI that binds to CXCR5 (Barroso et al, 2012).

An interesting example is the one of a conditional orphan receptor. A protein with a known ligand can behave under certain circumstances as orphan receptor. This was recently demonstrated for the ghrelin receptor $GHSR_{1a}$. In hypothalamic neurons, where ghrelin is not present, the receptor forms heteromers with the DRD_2 dopamine receptor and modulates its activity, which induces the anorexigenic properties of dopamine stimulation. The independence of this interaction of the ghrelin ligand was additionally demonstrated with the use of ghrelin KO mice (Kern et al, 2012).

(3) Association with other proteins

So far, only few examples describe the interaction of orphan 7TM proteins with other proteins than GPCRs. The long-time orphan GPR37 has been shown to interact with the dopamine transporter DAT to modulate dopamine uptake (Marazziti et al, 2007). The orphan GPR50 can interact with neurite-outgrowth-inhibitor Nogo-A (Grünewald et al, 2009) and the transcription factor TIP60 (Li et al, 2011), described later in chapter 2.3.3.3. Another example is the inhibition of the constitutive activity of the frizzled class GPCR Smoothened by the hedgehog ligand. Hedgehog binds via the 12TM protein Patched to smoothened, and Patched itself is thereby responsible for inhibiting constitutive activity of Smoothened (Riobo et al, 2006).

Other functions of orphan 7TM proteins

Another axe of research is the identification of functions independent of protein-interaction for orphans. So far, only little data exist giving information about other potential actions of orphan 7TMs proteins. One example is nuclear translocation, either partial or total, which has been shown for GPR50 (Li et al, 2011) and GPR158 (Patel et al, 2013) respectively.

1.7 GPCRs as drug targets

A large part of the available pharmaceuticals target GPCR activity and for about 15 to 30% of each molecular class of ligand, appropriate drugs have been developed, most of them for receptors of biogenic amines.

The emerging knowledge about the functionality and diversity of GPCR signaling will allow the development of more various and specific drugs at once. Promising possibilities arise from biased signaling (Kenakin & Christopoulos, 2013) and allosterism (Valant et al, 2012) to design more specific and selective drugs in order to better target the diseases and avoid undesired side effects. Additionally, the recent concept of GPCR oligo- and heteromerisation can also lead to a development of new classes of curing substances like bivalent ligands or substances that interfere with complex formation (Allen & Roth, 2011). Emerging *in silico* use, can help to improve the generation of synthetic drugs, where a design suitable for orphan receptors can also be imagined.

In combination with modern techniques of industry, drug design for GPCRs will reach another dimension by shifting from general treatment for a GPCR to one that is adapted to target the condition-specific signaling of each receptor.

2. The GPCR subfamily of melatonin receptors and GPR50

 MT_1 and MT_2 receptors for the amine melatonin and the orphan 7TM protein GPR50 form the melatonin receptor subfamily of the class A rhodopsin-like GPCRs.

2.1 Melatonin synthesis and function

The origins of melatonin (5-methoxy-N-acetyltryptamine) go back to 1917, when Allen and McCord described that extracts from bovine pineal glands can bleach tadpole skin. Then, in 1958, Lerner and colleagues successfully isolated melatonin from bovine pineal glands (Lerner et al, 1958).

Melatonin is synthesized during the night primarily by the pineal gland, but also, although to a lesser extent, by tissues like retina, intestinal organs, skin, blood cells and lymphocytes. The amino acid tryptophan is the precursor of melatonin (see Figure 9) (Klein et al, 1997). Pineal melatonin synthesis is regulated by light and the circadian rhythm generated by the hypothalamic suprachiasmatic nucleus (SCN).



Figure 9 Melatonin synthesis

Melatonin is synthesized out of the essential amino acid L-tryptophan. After enzymatic hydroxylation and decarboxylation, serotonin is formed. This intermediate then gets transformed through enzymatic addition of an acetyl group by the AANAT, which is the limiting step during melatonin synthesis. The HIOMT then transfers a methyl residue, which forms melatonin out of N-Acetylserotonin.

Melatonin is a lipophilic molecule, which can easily cross the blood-brain barrier and circulate through the bloodstream to reach peripheral organs. Its half-life is about 10 minutes before it undergoes cytochrome-mediated hepatic transformation into hydroxymelatonin prior to excretion (Vanecek, 1998).

One of the main tasks of melatonin is the synchronization of circadian rhythms and the regulation of reproduction in seasonal animals. The other actions of melatonin in the brain also explain its implication in psychiatric and neurological disorders. It plays a role in depression, sleep and seasonal affective disorders and jetlag-related effects. An association also seems to exist with Alzheimer disease in melatonin decelerating Alzheimer progression (Savaskan et al, 2007; Savaskan et al, 2001). Beside its function in the brain, the melatonin signal reaches tissues either through their neuronal connection to the SCN, or in a humoral manner of melatonin circulating in blood and binding to its corresponding receptors on different organs (Hardeland et al, 2011). In the periphery, melatonin regulates functions of the cardiovascular and the immune system (Calvo et al, 2013; Markus et al, 2007), metabolism and glucose homeostasis (Karamitri et al, 2013).

2.2 The family of melatonin receptors

The effects of melatonin are mediated by its corresponding receptors present at the cellular plasma membrane. The discovery of melatonin receptors became possible with the development of radioactively labeled 2-iodomelatonine in 1984 (Vakkuri et al, 1984a; Vakkuri et al, 1984b). The radioligand enabled the localization and detection of melatonin receptors in different tissues.

First attempts to clone the melatonin receptor occurred in the first half of the 90's and the first receptor to be cloned was Mel_{1c} from Xenopus (Ebisawa et al, 1994). Cloning of the mammalian receptors followed in the next years (Reppert et al, 1995; Reppert et al, 1994). The subfamily of melatonin receptors is composed of three family members, MT_1 and MT_2 and Mel_{1c} the latter only existing in vertebrates (fish, amphibia, birds and reptiles) and having evolved into the orphan 7TM protein GPR50 in mammals. MT_1 is expressed in every species, while MT_2 is not found in hamster. Another melatonin binding protein, called MT_3 , corresponds to the quinone reductase QR_2 (Nosjean et al, 2000; Nosjean et al, 2001). Compared to MT_1 and MT_2 , MT_3 binds melatonin with lower affinity and is structurally very different. MT_3 might be responsible for the implication of melatonin in detoxification and possibly accounts for some of melatonin's antioxidant effects.

Genetics of melatonin receptors

The genes for melatonin receptors are composed of two exons separated by one intron and are located on different chromosomes for MT_1 and MT_2 . Several studies identified different

isoforms or polymorphisms in intronic and exonic regions of the corresponding genes that can slightly affect signaling, as demonstrated for Mel_{1c} isoforms (Jockers et al, 1997). Recent studies have shown that rare exon variants of MT_2 are associated with an increased risk to develop type 2 diabetes (Bonnefond et al, 2012).

Structural features of melatonin receptors

The three melatonin receptors MT_1 , MT_2 and Mel_{1c} share a sequence homology of 60% that increases up to 73% in the TM region (Gubitz & Reppert, 2000). They are about 350 amino acids longs and have a molecular weight of 40 kDa.

Sequence analysis allowed their classification as rhodopsin-like receptors. They share some common features with other class A GPCRs like the existence of the N-terminal glycosylation sites, cysteine residues in ECLs to form disulfide bonds with the 7TM core, phosphorylation sites and palmitoylation sites in the C-terminal part of the receptor. Distinctive structural features only found in the melatonin receptor subfamily are the presence of the NRY motif instead of the E/DRY motif in TM3 and the NAXXY motif instead of the NPXXY motif in TM7.

Tissue distribution and signal transduction of MT_1 and MT_2

Melatonin receptors generally display low expression levels. By lacking a good antibody, information about expression of the receptors was obtained mainly from *in situ* hybridization and radioligand binding studies. MT_1 is more abundantly expressed than MT_2 . The highest amounts of MT_1 are found in the pars tuberalis, SCN and retina, and it is also expressed in hippocampus, cortex and the tanycytes (von Gall et al, 2002). Furthermore, the receptors are found in many of the peripheral tissues. MT_2 displays similar expression patterns with lower levels, but it still remains challenging to distinguish between both receptors.

Signal transduction of melatonin receptors

The studies to decipher melatonin receptor function were carried out in tissue with elevated MT receptor expression or appropriate cellular models. It was proven, that melatonin receptors signal via $G\alpha_i$ proteins by the decrease of cAMP due to inhibition of the adenylate cyclase and its sensitivity to Pertussis toxin (Vanecek & Vollrath, 1989). The $G\alpha_i$ downstream activity has been shown with inhibition of PKA activity and the lack of CREB phosphorylation (Witt-Enderby et al, 2003). Additionally, melatonin can inhibit cGMP

production via MT_2 (Petit et al, 1999). Other signaling pathways that are activated upon Melatonin stimulation implicate $G\alpha_q$ coupling and $G\beta\gamma$ -dependent PLC activation with subsequent IP3 production and calcium release from internal stores. Also the coupling to β -arrestins and subsequent ERK activation has been demonstrated (Kamal et al, 2009).

Receptor heteromerisation

A co-expression of MT_1 and MT_2 in several tissues led to the suggestion, that they might form heterodimers. Utilization of the BRET technique (Ayoub et al, 2002) helped for to the successful detection of homo- and heteromeric structures with a preferential formation of MT_1/MT_2 or MT_1/MT_1 dimeric structures (Ayoub et al, 2004). Recently, the occurrence and relevance of heteromers in retinal physiology was demonstrated *in vivo* (Baba et al, 2013). Another aspect is the heteromerisation with the orphan family member GPR50 that negatively influences melatonin binding and signaling, which will be discussed in detail below.

2.3 The orphan 7TM protein GPR50

The subfamily of melatonin receptors contains another member, which for the first time appeared in rodents during evolution, the orphan 7TM protein GPR50. It was discovered by screening a bank of human pituitary cDNA with degenerated primers in order to amplify proteins with high sequence homology to MT_1 and MT_2 . The protein of 613 amino acids was named melatonin-related-receptor, that later became GPR50 (Reppert et al, 1996).

2.3.1 Origins and structure of GPR50

Phylogenetic origins of GPR50

GPR50 is a protein that is exclusively found in mammals and for long time, its evolutionary origins remained unclear. Sequence analysis revealed, that it developed out of the Mel_{1c} melatonin receptor, which is only found in lower metazoa as reptilia, fish and chicken. The finding that GPR50 is the mammalian ortholog of Mel_{1c} was unexpected because of the low percentage of sequence homology. Further analysis could show that GPR50, compared to Mel_{1c} , underwent translocation from chromosome 4 to chromosome X and fusion with another gene, that is supposed to be an ancestor of the RNA polymerase II (Dufourny et al, 2008). Sequence comparison of Mel_{1c} and GPR50 in the 7TM part revealed, beside the amino acid changes that are the result of evolutionary pressure that a surprisingly high amount of

neutral evolution of 28% occurs in GPR50. One might speculate, that these relaxed substitutions occurred probably because melatonin binding to Mel_{1c} was not required anymore in higher species, diminishing the pressure for amino acid conservation or directed exchange (Tian et al, 2009).

A comparison between the sequences of the different species, which contain the gene for GPR50 shows that its sequence is less conserved among different species compared to the other melatonin receptors. While the 7TM part exhibits about 90% sequence homology between mouse, sheep and humans, the cytosolic region has more evolved. For example, the 591aa long mouse GPR50 (Gubitz & Reppert, 1999a) and the 575aa long sheep GPR50 (Drew et al, 1998) both share only 74% homology in total with the human sequence.

Genetic and protein structure of human GPR50

The GPR50 gene is organized like the other melatonin receptors and composed of two exons separated by one intron. Exceptions are found in horse, where exon 1 is splitted into four exons and in opossum, where it is composed of 7 smaller exons. In the 3' region of the GPR50 gene, an important region for the regulation of gene transcription, a consensus site for a specific micro RNA, the has-mir185 has been recognized (John et al, 2004). This miRNA is implicated in regulation of circadian genes like cryptochrome1 (Cry1) (Lee et al, 2013) and might probably be related to cancer by inhibiting tumor growth (Takahashi et al, 2009; Yoon et al, 2013). Additionally, the identification of CpG islands that are targets of DNA methylation, in the intronic region, propose epigenetic regulation mechanisms for GPR50 gene expression. Indeed transcription of GPR50 is regulated by the DNA methyltransferase Dnmt3a1 (Kotini et al, 2011). The human gene product of GPR50 has a size of 67 kDa and displays in total 45% homology with MT₁ and MT₂. In the hydrophobic transmembrane core part, it rises up to 55% (see Figure 10).



Figure 10 Structure and topology of GPR50

GPR50 is composed of a heptahelical core with extracellular and intracellular loops and a characteristic C-terminus. It shares common features that are unique for the subfamily of melatonin receptors as the NRY motif in the ICL2 and the NAVIY motif. The C-tail is 318 aa long and derived from gene fusion with an ancestor of RNA polymerase II.

The comparison to the melatonin receptor structure shows common characteristics as cysteine residues to form a disulfide bridge between ECL2 and ECL3, the NRY motif in TM3 and the NAVIY sequence in TM7. But some features are also altered or missing. GPR50 has no N-glycosylation site in the N-Terminus and the ECLs (Barrett et al, 2003).

Long C-terminal region

GPR50 has a 318 amino acid long cytoplasmatic C-terminal tail, which is much longer than the typical size of class A GPCRs of 20 to 90aa. This part is the product of genetic fusion with an ancient RNA polymerase II. It contains a highly repetitive sequence of several degenerated heptapeptides (see Figure 11):

GPR50 390	S	R	S	s	S	A	Y
GPR50 397	R	ĸ	S	A	S	т	H
GPR50 404	Н	ĸ	S	V	F	S	H
GPR50 411	S	ĸ	A	A	s	G	H
GPR50 418	\mathbf{L}	ĸ	P	V	s	G	H
GPR50 425	S	ĸ	P	A	s	G	H
GPR50 432	Ρ	ĸ	S	A	т	V	Y
GPR50 439	Ρ	ĸ	P	A	s	V	H
GPR50 446	F	K	A	D	s	V	H
GPR50 453	F	ĸ	G	D	s	V	H
GPR50 460	F	ĸ	P	D	s	V	H
GPR50 467	F	ĸ	P	A	s		
C.parvum RNAPII	Y	s	P	т	s	P	н
RNAPII_CTD_consensus	Y	s	P	т	s	P	s

Figure 11 Heptapeptid repeats in the C-terminus of human GPR50 (Dufourny et al, 2008)

The C-terminal part of human GPR50 presents a repetitive heptapeptid structure: in the first and last position is an aromatic amino acid, the sixth is aliphatic and hydrophobic one, the second is a basic lysine and the fifth one with a hydroxyl-residue like Ser. Below are represented consensus repetitive sequences of the RNA Pol II, the gene of origin of GPR50, displaying similar amino acids.

Furthermore, SH dipeptides in the distal region might be putative phosphorylation sites. Especially this repetitive part is strongly resembling the C-terminal repetitive domain of RNA Pol II, that functions as scaffold for factors that regulate transcription in a phosphorylation-dependent manner (Dufourny et al, 2008). These features lead to the hypothesis that this part of GPR50 might be subject to phosphorylation and form a scaffold for other proteins

Existence of sequence variants

A first targeted gene approach carried out by Thomson in 2005 (Thomson et al, 2005) revealed the existence of sequence polymorphisms in GPR50. Three of them in the coding region of the exons: one insertion/deletion of 12 base pairs that encode for the four amino acids Thr-Thr-Gly-His at position 502 to 505 TTGH, a Thr>Ala exchange at position 532 that is in complete linkage disequilibrium with the deletion. At position 606 we find another substitution of Val>IIe that occurs independently from the other ones (see Figure 12).

These polymorphisms occur with a high frequency of about 40% in the population, consequently one prefers to speak of sequence variants instead of polymorphisms, that usually only account for 1% or less. Another consequence is the denomination of the insertion variant with 617aa as GPR50 wildtype, while the other, originally cloned version of 613aa, is the mutant form of GPR50.



Figure 12 Localization of polymorphisms in the exons of GPR50 (Thomson et al, 2005)

The GPR50 can occur in different sequence variants of the exon 2: (I) A 12 base pair deletion, that leads to deletion of the TTGH amino acids from position 502 to 505 and is always accompanied by (II) an Thr>Ala exchange rising from one A to G base substitution. (III) Another sequence variant is the replacement of a G by an A that changes Val to Ile. All polymorphisms occur with about 40% in the population, classifying them as sequence variants.

2.3.2 Expression pattern and tissue distribution of GPR50

An interesting question is, whether GPR50 displays similar expression pattern as the melatonin receptors or if the evolution also led to an altered tissular distribution. First analysis started with mRNA *in situ* hybridization, northern blot and tissue reverse transcriptase (RT)-PCR based on mRNA levels. Only later, with the development of antibodies against GPR50 (Hamouda et al, 2007a) a characterization of protein localization was performed.

GPR50 expression in the brain

First studies concentrated on the localization of GPR50 mRNA in the brain. It was found in the pituitary and the mediobasal hypothalamus (ventromedial, paraventricular and arcate nucleus) which is overlapping with expression of MT_1 and MT_2 (Reppert et al, 1996). Additionally, a lot of cells around the 3rd ventricle, the tanycytes contain GPR50 mRNA. Sheep studies showed additional expression in retina, pars distalis and pars tuberalis of the pituitary, the region coordinating reproduction (Drew et al, 1998).

Later studies by RT-PCR and *in situ* hybridization in rodents detected GPR50 in additional regions like the amygdale, the chorioid plexus, the subfornical organ, the preoptic area, the

bed nucleus of stria terminalis, the olfactory bulb, the parabrachial nuclei and the vascular organ of lamina terminalis (Drew et al, 2001).

Later studies using the first GPR50 specific antibody (Hamouda et al, 2007b), allowed to detect regions of GPR50 protein expression. Analysis by immunofluorescence could confirm strong expression of GPR50 in the dorsomedial hypothalamus (DMH), tanycytes and the median eminence (Sidibe et al, 2010). A later study showed that GPR50 is among the 6 highest genes expressed in the DMH (Lee et al, 2012), a region with an important role in energy homeostasis under circadian regulation. In the human brain, GPR50 was found also in the CA4 region of the dentate nucleus of the hippocampus (Hamouda et al, 2007b). The use of another antibody provided precise data for mouse, rat and sheep brain, that confirmed existing data obtained from mRNA expression studies (Batailler et al, 2011).

A recent study also investigated the expression of GPR50 in developing and adult mouse brain, that was based on the findings that GPR50 holds a role in neurite-outgrowth regulation (Grünewald et al, 2009). It was found that GPR50 is expressed throughout all developmental stages with peaks at E18, a late stage in embryonic development with axon formation. Furthermore, this study could identify new regions of GPR50 expression, that are involved in neurotransmission, as monoaminergic neurons (Grunewald et al, 2012).

Regulation of GPR50 expression in the brain

In the seasonal Siberian hamster a down-regulation of GPR50 expression was detected in short-day periods in cells of the ependymal layer of the 3rd ventricle, proposing a regulation by photoperiod (Barrett et al, 2006). A later study in a transgenic mouse where the lacZ gene replaced the GPR50 gene, showed that GPR50 expression in the 3rd ventricle is decreased upon high fat diet or fasting (Ivanova et al, 2008). Both studies suggest a regulation of GPR50 expression dependent on environmental and metabolic circumstances.

Expression in peripheral tissues

Expression levels of GPR50 in peripheral tissue were barely investigated. One study could reveal by RT-PCR that eye, testis, kidney, adrenal, intestine, lung, heart, ovary and skin express GPR50 mRNA (Drew et al, 2001). Most of these data could be confirmed by another PCR analysis (Li et al, 2011). For the future, the detection of GPR50 protein level in peripheral tissue will hopefully be achieved to gain information about possible functions outside the brain.

2.3.3 Physiological importance and function of GPR50

Identifying the function of an orphan receptor is always challenging. As a first approach the expression pattern of the protein can already give some hints about its function. These data suggest for GPR50 a strong implication in neuroendocrine functions, like food intake, energy homeostasis, thermoregulation, behavior and reproduction. In addition, different strategies detailed below have profitably helped to find out about different functions of GPR50 during the last years.

2.3.3.1 The molecular basis of GPR50 being an orphan 7TM protein

After the successful cloning of GPR50, studies to test melatonin binding for the melatoninrelated-receptor in COS cells remained negative (Reppert et al, 1996). This result could be confirmed by other groups for the mouse GPR50 (Gubitz & Reppert, 1999a) and the sheep protein (Drew et al, 1998). Also other screening assays by reverse pharmacology did not result in finding a cognate ligand for GPR50, and still nowadays modern techniques failed (Southern et al, 2013). Therefore, GPR50 currently seems to be a genuine orphan 7TM protein.

After having established the absence of melatonin binding in GPR50, further studies tried to identify the structural differences between melatonin receptors and GPR50. The construction of chimeric proteins helped to reveal the regions and amino acids, which are important for high-affinity melatonin binding and which are altered in GPR50. It was shown that especially TM6 of GPR50, notably the Gly257Thr- and some other amino acid substitutions seem critical for losing melatonin high-affinity binding (Conway et al, 2000; Gubitz & Reppert, 2000), as illustrated in Figure 13. Since high-affinity binding depends on the binding of the ligand and the G protein to the receptor, it still remains unclear whether these residues are involved in melatonin binding or in receptor activation leading to G protein engagement.

Later sequence analysis and comparison of amino acids between GPR50 and its Mel1_c ancestor revealed amino acids that have positively evolved (see Figure 13). Their juxtaposition to the extracellular side indicates that they might be involved in a gating function, necessary to permit melatonin access to the ligand binding pocket inside the 7TM region. Probably, the evolution of these gating amino acids in GPR50 explains its loss of melatonin binding. Experimental validation of these predictions and the importance of sites in the ECL loops, especially ECL2 is still in progress (Clément, Guillaume & Jockers, unpublished data).



Figure 13 Structural prediction of GPR50 and sequence alignment with melatonin receptors (Dufourny et al, 2008)

(A) A structure prediction of GPR50 that highlights that the amino acids changes compared to the melatonin receptor. Most of them are located in TM6, as Thr257 (red) and Val258 (light blue). The analysis of amino acids that underwent positive evolutionary pressure (pink) are mainly localized in regions and face the extracellular site.

(B) The sequence alignment reveals amino acids that are important for melatonin binding in MT_1 (dark blue stars), MT_2 (light blue stars) or both (red stars). Comparison with the GPR50 sequence reveals some amino acids, which are changed and might account for the loss of melatonin binding ability in GPR50.

2.3.3.2 Physiological role of GPR50

To determine the physiological impact of GPR50, several research axes and strategies can help to shed light on this question. Transgenic mice with deleted or non-functional GPR50 can serve to reveal an altered phenotype. Furthermore, genetic association studies with GPR50 variants or SNPs can also give us information about its physiological importance.

Expression – function relation of GPR50

A first idea about the function of GPR50 can arise from its expression pattern. The hypothalamic DMH is an important area of the brain to regulate food intake: it has a circadian clock that is food entrainable and additionally, it has an important role in stress responses. Another region with strong GPR50 expression are the tanycytes surrounding the 3rd ventricle. They form the connection between the cerebrospinal fluid (CSF) and the pituitary. Hence, these cells are important in sensing molecule concentration (like glucose) in the CSF, responsible for molecule transport from the CSF into the brain and they govern release of hypothalamic hormones. Additionally, tanycytes are part of the hypothalamus-pituitary-thyroid axe, and express a deiodinase for thyroid hormone activation.

Genetic association studies of GPR50 polymorphisms

The region of GPR50 localization on the X chromosome, Xq28, is associated with some neuroendocrine disorders (Dufourny et al, 2008), which is also supported by GPR50's expression pattern. The findings of the existence of GPR50 sequence variants opened the gate for genetic association studies of different disorders.

(1) Mental disorders

The first study in 2005 established a correlation of GPR50 polymorphisms with different mental disorders, as bipolar affective disorder (BPAD), major depression disorder (MDD) and schizophrenia in a Scottish population (Thomson et al., 2005). The mutant Δ TTGH variant holds a higher risk for BPAD and MDD, especially in females. A later study with further Scottish subjects also demonstrated an association in females with two Δ TTGH alleles with age of onset, increase in episode number and hypomanic periods (Macintyre et al, 2010).

Another intronic polymorphism, rs2072621 is associated with schizophrenia development in females. The strong sex specific component could be underlined by the observation of additive effects of two identical alleles, even though one X chromosome possibly undergoes inactivation. Later studies had difficulties to repeat the association in a Swedish cohort (Alaerts et al, 2006; Thomson et al, 2005), but the findings for association of the rs2072621

intron polymorphism in a French population (Delavest et al, 2012) and another intronic rs1202874 variant in Scottish people (Macintyre et al, 2010) with seasonal affective disorder (SAD) suggest a correlation between GPR50 function and the neuronal activities underlying these disorders.

A tendency for an association of the deletion variant with autism spectrum disorder was also be shown but did not reach statistical significance (Chaste et al, 2010).

With regards to neurological disorders, an increase of GPR50 immunoreactivity was found in the brain of Alzheimer patients (Hamouda et al, 2007a).

(2) Lipid metabolism

The results obtained from KO mice, that GPR50 is an important player in energy homeostasis got further support by genetic association studies. Even though, no differences in body mass index were observed, higher levels of fasting triglycerides in the blood of subjects with homozygous alleles of either intronic, Δ TTGH or V>I GPR50 variants (Bhattacharyya et al, 2006).

Transgenic Knock-Out mice

A preferential tool to study the physiological role of a protein consists in the utilization of mice where the gene of interest is deleted. The first KO mice for GPR50 was described in 2006 (Barrett et al, 2006). This mouse has a GPR50 gene that is interrupted through insertion of the LacZ gene, leading to expression of LacZ, which can be visualized, instead of GPR50. This mouse has been studied extensively with regards to metabolism (Ivanova et al, 2008). Mice lacking functional GPR50 show lower body weight, resistance to weight gain after high energy diet and less fat mass development while higher food intake, which makes them have a lower energy efficiency but a higher metabolic rate. Surprisingly, the GPR50 KO mice lose less weight under fasting conditions. In addition they display a tendency for hyperactivity with increase in oxygen consumption and CO₂ production. This is also supported by higher corticosterone concentration in the blood that might reflect higher stress levels. Unfortunately, this study could not clarify whether this hyperactivity is the reason for higher metabolic rate or whether less weight gain enables for higher activity. Additional expression analysis of the GPR50-LacZ protein revealed that GPR50 levels adapt to the energy status: both, high fat diet and fasting conditions lead to decrease in GPR50 expression. Taken together, these results suggest a role of GPR50 in energy homeostasis. Another study with KO mice (Bechtold et al, 2012) revealed the implication of GPR50 in torpor, a possibility for the body to cope with

difficult environmental conditions as reduced food availability and harsh climatic situations upon reduction of physical activity. Mice that lack GPR50 enter faster torpor and generally display lower body temperatures during sleep and fasting, due to less warmth producing uncoupling protein-1 (UCP1) expression and higher active thyroid hormone levels, which lead to a decrease in body temperature. Furthermore, it has been shown that GPR50 expression can be controlled by leptin levels. These findings support the role of GPR50 in energy homeostasis and propose an additional role of GPR50 in adaptive thermogenesis that might prevent the entry into hypometabolic states.

Microarray data

Another way to get information about a protein function are microarray data that try to identify changes in gene expression at the transcriptome level in cells or tissues under specific experimental conditions. A comparative analysis by microarray of mRNA expression in fibroblasts of normal skin and hypertrophic scar could reveal a strong increase of GPR50 expression under hypertrophic conditions in wound healing (Zhang et al, 2010).

A microarray of cells silenced for the regulator of G protein signaling 4 (RGS4) protein, which terminates opioid receptor signaling and is associated with schizophrenia, showed increased expression of GPR50 (Vrajová et al, 2011).

The detection of a highly enriched GPR50 in the trophoblast membrane of placenta proposes a role in pregnancy associated disorders as preeclampsia (Cox et al, 2011).

With regards to cancers, some studies detected an upregulation of GPR50 in early cancer states or tumorigenic tissue in pancreatic neoplasia (Buchholz et al, 2005) and nicotine induced cellular transformation (Bavarva et al, 2013).

2.3.3.3 Molecular function of GPR50

To dissect the molecular role GPR50, proteomic approaches have been used to identify putative binding partners and associated complexes. One example is the yeast two-hybrid (Y2H) screen, which is applicable for the cytosolic part of a protein. In addition, recent techniques were developed that target specifically membrane protein complexes like the TAP Assay (Daulat et al, 2007).

One first hypothesis came up with the emerging evidence that GPCRs form heterodimers (Levoye et al, 2006b). It was discovered, that GPR50 can form heteromers with MT_1 and MT_2 , with a functional influence on MT_1 receptors. GPR50 represses melatonin binding to

MT₁ and inhibits downstream signaling, which is depending on the GPR50 cytosolic Cterminus, which impairs coupling to G proteins and causes constitutive coupling to β -arrestin. A physiological impact of this heteromer might be found in tanycytes, where colocalisation of GPR50 and the MT_1 has been shown. Probably, the seasonally expressed GPR50 (Barrett et al, 2006) can negatively regulate the response to melatonin during the long photoperiod. This prototypic paper gave rise to the concept that the orphan GPR50 could be an interactingand activity modulating protein and was supported by further findings in the following years: With Nogo-A, another interacting protein was revealed in 2009 in a Y2H-screen with the Ctail of GPR50 with both variants in a human adult brain cDNA library (Grünewald et al, 2009). Nogo-A is known to inhibit neurite outgrowth and deviant expression levels were found in schizophrenia, bipolar disorders and Alzheimer disease. Nogo-A and GPR50 are both localized in postsynaptic fractions of neurons and GPR50 overexpression leads to significant increase in neurite outgrowth concerning their number and length and is accompanied by filopodia- and lamellipodia formation. This suggests, that the interaction of GPR50 with Nogo-A might probably block its proper function. Further studies have to establish a connection to brain function and psychiatric disorders.

Another interaction was demonstrated for GPR50 and the transcription factor TIP60 by a Y2H assay of the C-tail with a mouse testis cDNA library (Li et al, 2011). TIP60 functions as a transcriptional co-activator with histonacetylase activity for nuclear receptors as the glucocorticoid receptor. Surprisingly, this interaction occurs in the nucleus, suggesting a nuclear translocation of GPR50. Further studies in this paper proved, that the cytosolic C-terminal part of GPR50 can indeed localize in the nucleus, like its genetic ancestor RNA Pol II. Functionally, it was shown in cellular models and KO mouse, that the presence of GPR50 increases glucocorticoid receptor-dependent gene expression, supporting an involvement of GPR50 in cellular stress response.

The data from the Y2H assay revealed more potential interacting partners implicated in neural developments, stress response, lipid- and steroid metabolism, neurotransmission and signal transduction. Together with other proteomic assays, this can form an ideal basis for future work in order to reveal more molecular functions for GPR50 and to find its place in cellular signaling and protein networks.

3. Transforming growth factor β signal transduction

The transforming growth factor β (TGF β) superfamily of cytokines-induced signal transduction pathway is composed of membrane localized receptors with an intrinsic serine/threonine kinase activity. Activated upon binding of the TGF β ligand, they phosphorylate their substrates, the Smads, transcription factors, which then translocate to and accumulate in the nucleus, where they regulate the expression of target genes. The actions of TGF β signaling are essential for maintenance of cellular- and tissue homeostasis, hence supporting its important role in development, growth and differentiation and its pathophysiological implications, for example in cancer development and progression (Feng & Derynck, 2005; Shi & Massagué, 2003b).



3.1 The TGFβ signal transduction pathway

Figure 14 The TGFB signaling pathway (Massagué & Wotton, 2000)

The TGF β ligand binds to the type II receptor which recruits and transphosphorylates the type I Receptor. Activated type I receptor in turn phosphorylates the Smads which form complexes that translocate into the nucleus and regulate gene transcription. Termination of signaling occurs with proteasomal degradation of the Smads. Proteins that negatively regulate TGF β signaling (FKBP12, BAMBI, Smad6/7, Smurf, MAPK) are indicated with red bars.

3.1.1 TGF_β ligands

In humans, the TGF β family of cytokines counts 33 members divided into two main categories, discriminated by their sequence similarity and the distinct signaling pathways they activate: (I) TGF β /Activin/Nodal- and (II) the bone morphogenetic protein (BMP) / growth differentiation factor (GDF) / muellerian-inhibiting substance (MIS) subfamily. The prototypic ones are TGF β 1, 2 and 3.

Although eliciting various cellular responses, all ligands share common features: they are produced in the cell as dimeric precursors, where they form the C-terminal part of a propeptide. Though getting cleaved during secretion, they get released into the extracellular matrix (ECM) still associated to the N-terminal part (therefore also named the latency-associated polypeptide, LAP) and further proteins like the latent-TGF β -binding-protein (LTBP), retaining the TGF β from binding to their receptors. The activation process requires either acidic conditions, cleavage by extracellular proteases (as metalloproteases) or interaction with the Arg-Gly-Asp (RGD) sequence of integrins (Annes et al, 2003). The active TGF β ligands form dimers and one monomer is composed of several β strands that are associated by disulfide-bonds, a structure known as the cysteine-knot (Sun & Davies, 1995). The presence of the TGF β ligand as a dimer suggests that the ligand is binding to homodimeric structures of its cognate TGF β receptors.

3.1.2 TGFβ receptors

The receptors for the TGF β ligands are membrane-localized kinases with serine/threonine activity, thus forming the only existing family of receptor serine/threonine kinases (RSTK). In humans, 12 genes are encoding for the different receptors, divided into two subfamilies, with seven type I and five type II receptors (Manning et al, 2002). The different type I receptors are further subdivided upon the ligand-specific downstream cascades, they activate. The small number of receptors compared to the high number of ligands is quite surprising, but the existing specificity for each of the 33 ligands is established by the receptor promiscuity, allowing different heteromeric combinations, as illustrated in Figure 15. The best-described and -studied system is the one for the TGF β -1/2/3 ligands that bind T β RII and signal through T β RI (former ALK5).



Figure 15 TGF_β ligands and their receptors (Shi & Massagué, 2003b)

The TGF β superfamily of cytokines is composed of 33 members, divided into two groups, the TGF/Activin/Nodal and the BMP/GDF/MIS group. These ligands are bound by only 12 receptors (seven RI and five RII), which are either activating TGF β /Activin/Nodal- or BMP/GDF/MIS-dependent signaling. Different Type II receptors can combine with different RI receptors, thus forming combinations that are specific for each ligand. Furthermore, accessory receptors like Betaglycan, Cripto and Endoglin are implicated in ligand activation.

Receptor structure

The TGF β receptors T β RI and T β RII receptors are composed of 503 (T β RI) or 567 (T β RII) amino acids, respectively. They are divided into a short, cysteine-rich, extracellular domain responsible for ligand binding, a single-pass transmembrane part and a long dominating cytosolic portion which is enzymatically active (Massague, 1998). Interestingly, sequence analysis and comparison revealed that the receptors have a dual kinase specificity, with a stronger affinity to phosphorylate Ser- and Thr-residues, but a phosphorylation on Tyrresidues has also been demonstrated for the RII. (Hanks & Hunter, 1995; Lawler et al, 1997). Between the T β RI and T β RII exist important structural differences, which are reflected in the mechanism of activation: the T β RI has a characteristic, conserved glycine and serine rich GS region preceding the kinase domain, which is composed of several serines and threonines that form the target of phosphorylation by the T β RII. This GS domain is the activity-regulating unit and the flexible ankle in conformation, explaining the constitutive activity of the T β RII

and the necessity of T β RI being activated. The T β RI also carries an additional L45 loop for Smad recognition (Figure 16).



Figure 16 Schematic overview of the TβRI- and TβRII structure (Runyan et al, 2006)

The TGF β receptors are composed of an extracellular part, a single transmembrane spanning segment and a long cytosolic tail. The cytosolic part bears the kinase domain. In T β RI, additional characteristic parts are the GS domain which is the phosphorylation target for T β RII and the L45 loop for Smad binding.

Mechanism of ligand binding - 2 different models

The different type I- and type II- receptors also diverge in their mode of ligand binding and activation, depending on the ligand. The BMP ligand family has a quite weak affinity for both of their receptors, resulting in a cooperative binding model, where the ligand binds both receptors together to form a stable complex (Rosenzweig et al, 1995). In contrast, the loss of the Phe85 residue, which is creating hydrophobic interactions between ligand and receptor in the BMP receptors (Kirsch et al, 2000), the TGF β receptor T β RI has no ability to bind the TGF β ligand alone. TGF β has a much higher affinity for binding to the T β RII (Attisano et al, 1993). Thus, complex formation occurs in a sequential manner, induced by a strong binding of TGF β ligand to the T β RII, which subsequently enables the recruitment of the T β RI to the complex (Hart et al, 2002).

Mechanism of receptor activation

The heteromeric complex composed of ligand, homomeric T β RII and homomeric T β RI, which is formed after ligand binding, induces a juxtaposition that favors the
transphosphorylation of the inactive T β RI by the constitutive active T β RII (Franzen et al, 1993; ten Dijke et al, 1994). These phosphorylations occur in the characteristic GS domain of T β RI (Wieser et al, 1995), rendering the T β RI in the active conformation and enabling the signal propagation through its kinase function. This ligand binding induced phosphorylation of T β RI by T β RII is absolutely required for signal transduction (Luo & Lodish, 1996; Okadome et al, 1994; Vivien et al, 1995). In contrast to the T β RII, no evidence for an *in vivo* autophosphorylation of T β RI exists (Weis-Garcia & Massague, 1996; Wieser et al, 1995; Wrana et al, 1994). This phosphorylation of T β RI in its GS domain induces a conformational inhibition-to-activation switch, a crucial step in transmission of TGF β signals (Huse et al., 2001a). The phosphorylations lead to a conformational change in the GS domain, accompanied by a disappearence of the binding site of the negative regulator FKBP12 (see Chapter 3.2.3.4.), which causes its dissociation. Consequently, the nine amino acid L45 loop, which forms the binding surface for the TGF β effectors, the Smads (Persson et al, 1998), becomes accessible.

3.1.3 Smad transcription factors

The signal of TGF β is then transmitted via the type I receptors to their substrates, the Smads (short for: small mothers against decapentaplegic homolog), which first have been discovered in 1996 (Derynck et al, 1996). The family of Smad Proteins is composed of eight different members, divided into three different classes: (I) the receptor-regulated R-Smads, Smad1, 2, 3, 5 and 8 which bind to the type I receptor, are a substrate for phosphorylation and form homo- or heteromeric complexes. The R-Smads are furthermore categorized upon their ligand response: Smad2 and Smad3 are phosphorylated after TGF β ligand stimulation, while Smad1, 5 and 8 are BMP-ligand effectors; (II) the common-mediator co-Smad, Smad4, which is indispensable for formation of nuclear translocating complexes with the R-Smads that together bind to DNA and influence gene transcription. (III) The inhibitory I-Smads, Smad6 and 7, compete with R-Smads on receptor binding and target the receptor complex for degradation by recruiting ubiquitin ligases. Smad7 can bind all receptor I subtypes, whereas Smad6 has a specificity for the BMP binding receptor I (Heldin & Moustakas, 2012).

Structure and sequence of the Smads

The R- and Co-Smads comprise about 500 to 600 amino acids and are divided into two major structural entities, the N-terminal MH1 (for Mad-homology) and the C-terminal MH2

domain, connected through a less conserved linker domain (see Figure 17). The MH1 domain of both, R-Smads and Co-Smads, allows nuclear translocation and mediates DNA binding. The MH2 domains of all Smad classes are similar in their sequence and are, in the case of R- and I-Smads, responsible for receptor binding, complex formation with other Smads, nuclear shuttling and transcriptional activation. Also most of the protein interactions are maintained by the MH2 domain. A distinct feature of the R-Smads is their C-terminal SXS motif, which forms the target for phosphorylation by type I receptors. This strong homology of the different MH2 domains also allows the I-Smads to bind the receptor. But lacking the SXS motif explains a part of their inhibiting effect: in the absence of this phosphorylation, no downstream signaling is possible. The domains are connected by the less conserved proline-rich linker region, which is important for crosstalk with other signaling pathways by being a phosphorylation target and implicated in interaction with other proteins (Moustakas & Heldin, 2009).



Figure 17 Smad3 structure (Massague, 2012; Shi & Massagué, 2003b)

The Smads are composed of two main domains, MH1 and MH2 that are connected by a flexible linker region. The MH1 mediates DNA binding and the L3 loop in the MH2 domain is responsible for RI binding in R- and Co-Smads. The C-terminal SXS motif target in R-Smads is the target of phosphorylation by RI (indicated by yellow circle). The linker region is a substrate for phosphorylation by other kinases.

Receptor recognition, activation and nuclear translocation of the Smads

The type I receptor-Smad recognition is obtained between the exposed L45 loop of the type I receptor and the L3 loop in the MH2 domain of the Smads. The interactions between the L45 and the L3 loop and their specific sequences in each type I receptor and Smad-subtype explain the preference of Smad 1,5 and 8 to bind to BMP receptors and of Smad2 and 3 to bind TGF β receptors (Feng & Derynck, 1997; Wu, 2000). After binding to the type I receptor, the Smads get directly phosphorylated by the type I receptor in the serines of the C-terminal SXS motif (Abdollah et al, 1997; Kretzschmar et al, 1997; Macias-Silva et al, 1996), which is enabling the complex formation with Smad4 and leading to the exposure of a domain that is important for nuclear translocation (Wu et al, 2001; Xu et al, 2000).

After phosphorylation has triggered the formation of heteromeric complexes (mainly composed of two R-Smads and one co-Smad4), they translocate to the nucleus. This nuclear import is mediated by a conserved nuclear localization signal (NLS) of the R-Smads (Xiao et al, 2000a), leading to translocation either via the uncommon Importin β pathway for Smad3 (Xiao et al, 2000b), or, in the case of both Smad2/3, by direct binding to the nucleoporin-complex proteins (Xu et al, 2002). For Smad4, the classical importin α pathway is supposed. An important feature is the occurrence of dynamic nuclear-cytosolic shuttling for the co- and R-Smads, permitting repeated cycles of receptor-binding, phosphorylation, complex formation and translocation, explaining the persistence of the TGF β signal for several hours (Inman et al, 2002; Pierreux et al, 2000).

3.1.4 TGFβ-dependent regulation of gene expression

Once located in the nucleus, Smad proteins mediate the TGF β ligand-dependent regulation of gene expression by acting as transcription factors.

DNA binding

Binding of Smads to DNA occurs for all R-Smads (except Smad2) and the co-Smad through a common DNA harbored binding motif: the Smad-binding-element (SBE) that is consisting of only 5 bases, 5'-CAGAC-3' (Yingling et al, 1997). This interaction is carried out by a conserved β -hairpin structure in the MH1 domain. In contrast, Smad2 is bearing an additional 30 amino acid long insert, abolishing its DNA binding ability and explaining the requirement of additional proteins for DNA binding, like the forkhead transcription factor FoxH1. Together they bind DNA at the Smad2-activin-response-element (ARE) together with the

activin-response factor ARF (Chen et al, 1996). With regards to this quite short and unspecific sequence and the weak intrinsic DNA binding affinity of Smads, further mechanisms are essential to achieve specificity in their regulation of transcription.

Regulation of activation and repression

This regulation is made up by the interaction with transcriptional co-factors, who can bind DNA and other transcription factors at once. The interaction with these co-activators or corepressors occurs between the Smad-interacting-domain (SID) in the MH2 domain and a proline-rich Smad interacting motif (SIM), found in some transcription factors, as FoxH1. The interactions with co-factors are responsible for establishing the different Smad responses in a cell type- and context-dependent manner. These interacting proteins can be either (I) general transcription factors, that regulate activation or repression and amplitude, like the coactivators as CBP/p300 and co-repressors like the TGF_β-induced factor TGIF or c-Ski and SnoN that can also modulate chromatin structure through their intrinsic histoneacetylase- or histonedeacetylase-activity respectively, or (II) specific transcription factors, that allow the regulation of specific genes, like proteins of the basic-helix-loop-helix bHLH family like TFE3, the basic leucine zipper domain bZIP family like ATF3, c-Fos, c-Jun and c/EBP, the forkhead family, homeodomain proteins like Dlx and Hox, nuclear receptors like the estrogen receptor, zinc finger proteins like GATA and Sp1, or signal integrators, which are responsible for mediating crosstalk with other signaling pathways as β -catenin, HIF, NF κ B, p53 and SRF (Feng & Derynck, 2005).

Regulated genes

These various interactions and the sequence alterations in the different R-Smads enable for each of it a regulation of an own subset of genes, which underlines that the Smads, dependent on the cellular context, can control the expression of a myriad of genes. Some of the abundant events are activation of the cell cycle inhibitors p21kip and p15Ink4b or the extracellular matrix protein plasminogen activator inhibitor PAI-1 and the repression of c-myc. Furthermore, some of the regulated genes are "selfenabling genes" which later get involved in regulating or participating in the TGF β signaling pathway, like Smad6/7 or ATF3. An overview is given in Table 2.

Table 2 TGF β regulated genes in epithelial cells (Siegel & Massague, 2003)

Overview of common regulated genes in epithelial cells of breast, skin and lung

Functional group	TGF-β response	Genes
Cytostatic programme	Upregulated Repressed	CDKN2B, CDKN2A c-MYC, ID1, ID2, ID3
Extracellular matrix	Upregulated	ΡΑΙ1, uPA, Col VI-A1, ADAM19, ITGα5, ITGβ6
Paracrine network	Upregulated Repressed	IL11, VEGF, CTGF, JAG1, FSTL3, ANGPT4 IL1β, BMP4
Signalling network	Upregulated Repressed	BMPRII, VDR, EPHB2, RHOGEF114, MEKK4 LDLR, PGE-R4, βAR-2
Transcriptional network	Upregulated Repressed	ETS2, c-JUN, JUNB, ATF3, GADD45B, PIM1, MAD2, MAD4 C/EBPδ, MRG1, TRIP-Br2
Other responses	Upregulated Repressed	TBX3, MN1, IGI, SIAT4A SPRY2, IAP3
Negative feedback	Upregulated	SMURF1, SMURF2, SMAD7, SNON

*Table adapted from REF. 11. The gene responses are subdivided into functional groups and those that are upregulated or downregulated by TGF-β are indicated. Only a few gene responses are specifically devoted to mediating the cytostatic response of these cells. Other gene responses regulate the interaction of the target cell with the environment, either by controlling the production of cytokines and extracellularmatrix components, or by modifying the signalling networks and transcriptional regulators that sense and process external signals including the TGF-β signal itself. *ADAM19*, a disintegrin and metalloproteinase domain 19; *ANGP74*, angiopoietin-like 4; *ATF3*, activating transcription factor 3; β*AR*-2, adrenergic β2 receptor; *BMP4*, bone morphogenetic protein 4; *BMPRI*, bone morphogenetic protein receptor type II; *C/EBP6*, CCAAT/enhancer binding protein (C/EBP) δ; *CDKN24*, cyclin-dependent kinase inhibitor 2A; *CDKN2B*, cyclin-dependent kinase inhibitor 2B; *Cd*/*V*-A1, collagen, type VI, α1; *CTGF*, connective-tissue growth factor; *FSTL3*, follistatin-like 3; *GADD45B*, growth arrest and DNA-damage-inducible β; *IAP3*, apoptosis inhibitor 3; */D*, inhibitor of DNA binding; *IGA*, immunoglobulin λ; */L*, interleukin; *ITGα5*, integrin α5; *ITGGP*, integrin β5; *JAG1*, jagged 1; *JUNB*, JUN B proto-oncogene; *LDLR*, low-density-lipoprotein receptor; *MAD*, MAX dimerization protein; *MEKK4*, mitogen-activated protein kinase kinase kinase 4; *MN1*, meningioma (disrupted in balanced translocation) 1; *MRG1*, myeloid ecotropic viral-integration-site-related gene 1; *PAI1*, plasminogen activator inhibitor type 1; *PGE-R4*, prostaglandin E receptor 4; *RHOGEF114*, RHO-specific guanine nucleotide exchange factor p114; *SIAT4A*, sialyltransferase A4; *SMURF*, SMAD ubiquitylation regulatory factor 1; *SPRY2*, sprouty homolog 2; *TRIP-Br2*, transcriptional regulator interacting with the PHS-bromodomain 2; *TBX3*, T-box 3; *uPA*, urokinase-type plasminogen activator; *VDF*, vitamin D receptor; *VEGF*, vasc

Besides the regulation of genes that encode for proteins, Smads were also reported, to be involved in the regulation of the expression of certain microRNAs or other regulatory nucleic acids (Blahna & Hata, 2012).

End of signaling

Once the Smads exerted their role as signal transducers by regulating gene expression, the question arises, how signaling gets terminated. With regards to Smads, either dephosphorylation that induces nuclear export or their degradation after ubiquitination leads to abolishment of signaling. Other mechanisms involve the action of interacting proteins or negative regulators are discussed in chapter 3.2.4

3.2 Regulation of TGF β signaling - the establishment of signaling specificity and diversity

The TGF β signaling pathway is, in addition to being the only described receptor serine/threonine kinase pathway, also marked by other characteristic features, discriminating

it from other classical signal transduction pathways. In the TGF β pathway, we observe a straight forward linear signaling flow after ligand binding from type II receptor to type I

straight forward linear signaling flow after ligand binding from type II receptor to type I receptor via the Smads into the nucleus, while other pathways like the RTK EGF-Ras-ERK pathway, display more complex cascades with a multistep transmission including several enzymes and scaffolding proteins. This case is termed "wiring" with a non-linear signal amplification as a consequence (Schmierer & Hill, 2007). However, in contrast to RTK pathways, the TGFβ signals are converted into a cellular effect in a slow manner, grounded in the need to establish a Smad nuclear accumulation for a response to the ligand. But signals are highly sustained, because signal termination necessitates not only a switch-off mechanism, but a longtime degradation process. Comparing these distinct modes of signal transduction pathways gives rise to the question, how specificity and fine-tuning of signaling are obtained in the case of TGF β signaling. Furthermore, the contrast of the large number of ligands, their cognate receptor combinations and the small number of signal transducing Smads stirs up the issue how they can be capable of reflecting this ligand diversity. The solution lies in three characteristic features of the TGFB signaling pathway: (I) the pleiotropy of interaction partners and regulating proteins (II) their coordination and (III) the context dependency based on the cell type specific distribution of signaling components, which place the TGFβ signaling from a linear pathway into a network (Massague, 2012). In the following, these different components of the network and their TGFβ-signaling specific effects will be explained:

3.2.1 Mechanisms of regulation

The regulation of the TGF β signaling can be obtained through a multitude of regulatory mechanisms:

(I) Regulation of expression of pathway components

The amount of available signaling components is a critical part of signaling, enforcing a regulation on the genetic level. This regulation of expression of their genes is often induced as an effect of other signaling pathways. For example, the expression of inhibitory Smads is regulated through cytokines via the JAK/STAT (Ulloa et al, 1999) or the tumor necrosis factor (TNF)- α / nuclear factor (NF) κ B pathway (Bitzer et al, 2000). Moreover, epigenetic regulatory mechanisms gain more attention in influencing the amount of translated protein for example through miRNA regulated gene expression.

(II) Enzymatic covalent modification of pathway components

Enzymatic actions are a much quicker possibility to regulate the activity of a signal transduction pathway than genetic events. Numerous possibilities of modifications like phosphorylation/dephosphorylation, ubiquitylation/deubiquitylation/sumoylation, acetylation, methylation, glycosylation and ADP-ribosylation of the TGF β signaling pathway components have been reported (Xu et al, 2012). Especially the dynamics and the interplay of these posttranslational modifications add a strong touch of flexibility to TGF β signaling.

(III) Protein interactions

In addition to these protein-mediated modifications, direct interactions of the TGF β signaling core proteins with other proteins can be another possibility for affecting their activity or subcellular localization.

(IV) Crosstalk

A further step is the integration of the TGF β signaling pathway into the signaling network of a cell, meaning the crosstalk with other signaling pathways, which will be discussed in chapter 3.3.

These modes of regulation can occur on every level of TGF β signaling, which is going to be dissected in the following:

3.2.2 Ligand activity and availability

The TGF β ligand, existing as latent complex associated with the LAP and anchored by LTBPs in the extracellular matrix (see 3.1.1), requires further processing for activation. This occurs either by cleavage through extracellular proteases like metalloproteases or upon integrin interaction - steps which can be subject of regulations (*ten Dijke & Arthur, 2007*). Furthermore the interaction of the ligand with other extracellular or transmembrane proteins can interfere with ligand activation: (I) Soluble proteins like noggin (acting on BMP7) or inhibin (acting on activin) function as ligand traps, that sequester the ligand and thus impede receptor binding (Groppe et al, 2002), (II) membrane bound proteins that often operate as correceptors (see also Figure 15): one group of them is often referred as type III receptor, receptors with ligand binding ability but without intrinsic signaling activity, like betaglycan and endoglin. Betaglycan, a membrane anchored proteoglycan (Brown et al, 1999a) can either participate in presenting the TGF β ligand for the receptor (Lopez-Casillas et al, 1993) or otherwise, after its shedding into the extracellular matrix, apply antagonistic affects as a

ligand scavenger (Lopez-Casillas et al, 1994). The homologic endoglin is mainly expressed in endothelial cells (Cheifetz et al, 1992), Cripto proteins binds the Nodal and Activin ligands (Gray et al, 2003). Connective-tissue growth factor (CTGF) is a selfenabling gene induced by TGF β , that enhances its receptor binding, with an important role in ECM formation (Abreu et al, 2002). Another ligand availability regulating protein is the pseudoreceptor Bambi (Onichtchouk et al, 1999), which acts as a decoy receptor for BMP and competes with the BMP-RI upon incorporation into the heteromeric receptor complex.

3.2.3 Regulation of TGF^β receptor activity

A huge number of proteins is implicated into the modulation of receptor activity, either by exerting enzymatic modifications or regulation of activity and downstream signaling due to a direct interaction.

3.2.3.1 Enzymatic receptor modifications

Regulation by ectodomain shedding

Enzymatic shedding can have, dependent on the involved enzymes, different possible outcomes. Shedding of the T β RI via the metalloproteinase TACE/ADAM17 leads to a decrease of cell surface receptor levels and thus negatively influences the sensitivity of cells for the ligand (Liu et al, 2009a).

Regulation by phosphorylation and dephosphorylation

In addition to the transphosphorylation of type I receptors by type II receptors, which is crucial for signal transduction, further regulatory phosphorylations on both receptors have been described. The T β RII can autophosphorylate itself not only in an activity-promoting- (on Ser213 and 409), but also in an inhibiting manner (Ser416). Also tyrosine phosphorylation can be found, either as a result of the dual specificity by an autophosphorylation that is supposed to be necessary for activation (Lawler et al, 1997), or it can be carried out by other proteins like Src what leads so subsequent activation of the p38 protein (Galliher & Schiemann, 2007). For T β RI a phosphorylation on Ser165 was reported, that is associated with modulation of the cellular response (Souchelnytskyi et al, 1996). These regulatory phosphorylations in both receptors are often involved in the creation of binding sites for other interacting partners.

The other way round, desphosphorylation is a mean to inactivate the receptor and to arrest signaling. Concerning this, evidence exists that proteinphosphatases PP1c, together with Smad7 binding (Shi et al, 2004), and PP2a are involved enzymes (Griswold-Prenner et al, 1998).

Regulation by ubiquitylation and sumoylation

Typically, polyubiquitylation targets proteins for proteasomal degradation by the sequential action of the enzymes E1 and E2 and the ubiquitin ligase E3. For proteasomal degradation of the TGF β receptor complex, the inhibitory Smad7 functions as an adaptor by recruiting Smurf1/2 (Kavsak et al, 2000) and WWP1 (Komuro et al, 2004) ubiquitin ligases. Reversion of this effect can be achieved by deubiquitinating enzymes, as the deubiquitinases USP15 (Eichhorn et al, 2012) and UCH37 (Wicks et al, 2005). In addition to the polyubiquitylation, evidence exist for a regulatory receptor monoubiquitylation: it was shown that the combination of metalloproteinase ADAM12/TACE-dependent T β RI shedding and a TRAF6 E3-ligase-mediated monoubiquitylation leads to a nuclear translocation of the T β RI, where it regulates gene transcription (Mu et al, 2011). Another covalent protein modification is sumoylation that influences subcellular localization. Sumoylation by Ubc9 was found on phosphorylated T β RI that further enhanced its activity (Kang et al, 2008).

3.2.3.2 Regulation of subcellular localization of the receptors

Another important step in signaling is the subcellular localization of receptors. Compared to RTK signaling, an increase of internalization and the constitutive formation of signaling endosomes as a consequence of ligand binding is not described for the TGF β signaling pathway. Nevertheless, receptor endocytosis exists to modify activity for which two distinct mechanism were shown (Di Guglielmo et al., 2003): (I) clathrin-dependent internalization, involving a dileucin motif which locates the T β RI to early-endosome-antigen1 (EEA) positive endosomes that can cycle back to the membrane via Rab11 endosomes. This mode of internalization is a way of receptor recycling and can favor enhancement or endurance of signaling. In contrast, (II) clathrin-independent internalization via calveolar-positive vesicles in lipid rafts is favoring proteasomal or lysosomal degradation of the receptor complex. An additional mechanism for degradation of the receptor complex is the possibility of a β -arrestin 2-mediated internalization, a protein responsible for GPCR trafficking (see Chapter 1.3.), carried out by interaction of the short cytoplasmic tail of the T β RIII betaglycan with β -arrestin 2 (Chen et al, 2003).

3.2.3.3 Regulation by interacting proteins

During the last years a huge number of proteins that interfere with TGF β signaling by interaction with receptors have been identified. Their effects can be enhancing and repressing and their modes of action and interaction are various: they can act as scaffolds, influence subcellular localization, others direct signaling towards non-canonical pathways or mediate the crosstalk with other pathways (Runyan et al, 2006).

(I) NEGATIVE REGULATION

Interactions with proteins exerting a negative regulation can either act to prevent the occurrence of signaling or to restrict its duration.

Bambi, is the BMP decoy receptor, which inhibits downstream signaling by competing with BMP-RI for binding to BMP-RII as a result of their sequence homology. This leads to the formation of inactive Bambi/RII complexes (Onichtchouk et al, 1999). Later findings suggest cooperative mechanism of Smad7 and Bambi in inhibiting signaling what might extend the role of Bambi towards TGF β -ligand-induced signaling (Yan et al., 2009).

Smad 6 and 7, the inhibitory Smads are signaling antagonists which are expressed under the control of R-Smads, thus being part of a negative feedback loop of TGF β signaling (Hayashi et al, 1997; Nakao et al, 1997a). Like the R-Smads, they bind to the receptor (Hanyu et al, 2001), but lacking the SXS motif, they cannot be phosphorylated, what is resulting in a competition with R-Smads, that blocks downstream signaling (Hayashi et al., 1997). Furthermore, they can serve as scaffold for the recruitment of other proteins: GADD34 which binds the phosphatase PP1c and causes receptor dephosphorylation (Shi et al, 2004), the E3 ligases Smurf1 and 2 (Kavsak et al, 2000; Suzuki et al, 2002) and WWP1 (Komuro et al, 2004), that are polyubiquitylating the receptors, which triggers the proteasomal degradation of the complex. Other synergizing proteins are STRAP, which binds Smad7 and can also bind T β RI and T β RII with its WD40-domain repeats and negatively regulates Smad2/3 (Datta et al, 1998) and YAP65, a member of the Hippo pathway, that increases the T β RI-Smad7 association (Ferrigno et al, 2002). Further roles are also described for inhibiting complex formation of R-Smads and DNA (Zhang et al, 2007).

DRAK2 (DAP kinase-related apoptosis-inducing protein kinase) is a recently identified TGF β -inducible protein kinase, which constitutively interacts with T β RI and gets increased

upon ligand stimulation. DRAK2 seems to be an important player in attenuation of TGF β signaling (Yang et al, 2012).

Further proteins that were described to negatively regulate signaling upon receptor interaction are the chimeric oncoprotein ETV6-NTRK3 (Jin et al, 2005) and the RTK TrkC (Jin et al, 2007) that both interact with T β RII, thus preventing interaction with T β RI. Dpr2 binds to the receptor I and targets it for lysosomal degradation (Su et al, 2007b), and c-Ski, that beside its nuclear function (see below) can also negatively regulate the receptors (Ferrand et al, 2010). Further examples are described in Table 2.

3.2.3.4 FKBP12 as a signaling-preventing negative regulator

A regulator, which holds an important role as inhibitor of basal TGF β signaling activity is the immunophilin FKBP12. The immunophilins are a group of proteins that can bind immunosuppressive substances and function as peptidyl-prolyl-cis-trans-isomerases (PPI). Their main role is associated with protein folding and -refolding and stabilization of distinct protein conformations. FKBP12 (FK506 binding protein 12) is able to bind the macrolides FK506 (tacrolimus) and rapamycin and has a size of 12kDa. The structure of FKBP12 is highly conserved among the different species and is composed of five β turns and two extremities, the 40s and 80s loop, flexible regions and essential parts for binding to other proteins. By being a PPI, the recognition sequence in other proteins is composed of at least one proline, but preferentially composed of a Pro-Leu dipeptide. Most functions of FKBP12 arise from its complex forming properties together with FK506 and calcineurin, a calcium activated phosphatase, or together with rapamycin FKBP12 can bind PI3K like kinases RAFT. Another function is the regulation of Ca²⁺ channels by binding to ryanodine- and IP3-receptor-calcium channels and influencing their closure kinetics (Ivery, 2000).

The interaction of FKBP12 with the $T\beta RI$

An interaction that is maintained independently from macrolides is the one with the T β RI. By performing yeast two-hybrid assays all five type I receptor family members were found to interact with FKBP12, but none of the type II receptors (Wang et al, 1994). The interaction has been further studied especially for the T β RI. FK506 and rapamycin inhibit this interaction, suggesting a competition for the same binding site in FKBP12. Mutation studies have shown, that FKBP12 binds to the ¹⁹³Leu-Pro-Leu-Leu¹⁹⁶ motif in the GS domain of the

TβRI, what is coherent with its PPIase-activity, requiring a Leu-Pro motif for interaction (Charng et al, 1996; Chen et al, 1997). This got support by the publication of the crystal structure (see Figure 18), revealing more details about the interaction (Huse et al, 1999): The extreme 40s and 80s loop in FKBP12 recognize a distinct structure, the VI β turn of the TβRI, lying the GS domain and bearing the ¹⁹³LPLL¹⁹⁶ motif as recognition site for FKBP12. FKBP12 is directly interacting with two Leu residues (195 and 196) and Pro194 is assisting in stabilization. Additionally, a contact is made between the exposed His87 and Pro88 of the 80s loop in FKBP12, which directly contact the β 3 and β 4 sheet in the L45 loop, the surface for R-Smad binding. Upon this interaction, TβRI gets stabilized in an inactive conformation by the formation of an inhibitory wedge in the GS domain, providing a structural explanation for FKBP12 locking the inactive conformation and constraining the TβRII-mediated transphosphorylation in the GS domain and R-Smad binding to the L45 loop.



Figure 18 Crystal structure of FKBP12 and TßRI (Huse et al, 1999)

The crystal structure of FKBP12 and the cytoplasmic domain of the T β RI provides important information about the interacting domains. FKBP12 (in red) contacts the T β RI with its 40s and 80s loop in the GS domain of the T β RI at the ¹⁹³LPLL¹⁹⁶ sequence (light green). Another side of interaction is made between the H87 and P88 of the 80s loop in FKBP12 and the L45 loop of the β turns 3 and 4 (in blue).

Functional effect of the $T\beta RI$ -FKBP12 interaction

If the binding site for FKBP12 in the T β RI is mutated, a basal, ligand-independent signaling can be observed. That is suggesting a role of FKBP12 in preventing signaling instead of actively participating in the ligand-induced response (Charng et al., 1996). Treatment with FK506 also leads to tonic TGF β signal transduction and an increased response to low doses of

ligand, whereas overexpression of FKBP12 can block or diminish the TGF β signaling response, an effect also described for BMP receptors (Gruendler et al, 2001; Spiekerkoetter et al, 2013). It was show that depletion of FKBP12 from the T β RI induces its hypersensitivity for T β RII-mediated transphosphorylation in the GS domain (Chen et al, 1997). These findings suggest that FKBP12 is important for blocking basal TGF β signaling in the absence of ligand. FKBP12 KO mice further support this function of FKBP12: These mice display defects in heart function and neural tube closure (Shou et al., 1998) and mouse-derived cells display a higher amount of TGF β downstream signaling effects such as increased p38 phosphorylation (see Chapter 3.3.1) and p21 expression, leading to increased cell cycle arrest (Aghdasi et al, 2001; Shou et al, 1998).

Additional functions of the T β RI-FKBP12 interaction are the blocking of T β RI internalization (Yao et al, 2000), probably through an orchestration of T β RI degradation in complex with Smad7 (Yamaguchi et al, 2006) or still unsolved issues as the influence of its PPI-activity or a role in the recruitment of other proteins.

Mechanism of FKBP12 release

A still unsolved part of the puzzle is the exact mechanism that triggers FKBP12 release. Studies have shown that the release of FKBP12 occurs (I) upon ligand stimulation and (II) by transphosphorylation of the GS domain in T β RI by T β RII, but the exact point of FKBP12 release could not be determined. This release should be preceding any GS domain phosphorylation event, because mutating all GS domain phosphorylation sites does not prevent the release of FKBP12. But a deletion of the T β RII-kinase domain locks FKBP12 bound to the T β RI, evoking the possibility of other T β RII-mediated events like phosphorylation of another residue outside the GS domain or the binding of other proteins, that trigger the release of FKBP12 (Wang & Donahoe, 2004; Wang et al, 1996).

(II) POSITIVE REGULATION

Another group of interacting proteins are positive regulators that are either essential for TGF β signaling or that can potentiate and prolong signaling.

TRAP1 (TGF β receptor associated protein) and TLP (TRAP1 like protein) are two proteins that are similar in their sequence (25%) and that can bind to T β RI and T β RII. TRAP1 is supposed to be implicated into the recruitment of Smad4 upon ligand binding via direct

interaction, thus facilitating formation of heteromeric Smad complexes (Wurthner et al, 2001). TLP, in contrast, is a protein that seems to have important functions in specifying TGF β signaling responses by its ability to preferentially activate Smad3- and inhibit Smad2 dependent signaling (Felici et al, 2003).

Dab2, is an adaptor molecule that constitutively interacts with T β RI, which is indispensable for active signaling by functioning as Smad2/3 adaptor (Hocevar et al, 2001). Together in complex with AP2, it can also favor receptor endocytosis to clathrin-coated vesicles (Penheiter et al, 2010).

The molecular chaperon **heat shock protein HSP90** is binding to the T β RI/II complex, to protect it from Smad7/Smurf binding and the following receptor degradation (Wrighton et al, 2008).

The metalloprotease **ADAM12** facilitates signaling by inhibiting Smad7 association with the receptor (Atfi et al, 2007).

Other proteins are important for the mediation of crosstalk with other signal transduction pathways (see Chapter 3.3.) or to induce non-canonical signal transduction pathways, like TGF β -activated protein kinase TAK1 (Yamaguchi et al, 1995) that mediates TGF β -induced MAPK activation of p38 and JNK, or Daxx, that induces apoptosis by favoring JNK activation (Perlman et al, 2001). The cell polarity protein Par6 can be directly phosphorylated by T β RII to promote RhoA degradation by the Smurfs (Ozdamar et al, 2005), and the scaffolding protein SHC1 that binds to T β RII and gets phosphorylated by RI leads to MAPK activation (Lee et al, 2007). Further positive regulating proteins are explained in Table 2.

3.2.4 Regulation of Smad activity

The Smads are also an important object of positive and negative regulation of TGF β signaling either through modifications or complex formation with proteins.

Regulation by covalent modifications

Despite the classical Smad phosphorylation by the type I receptors, phosphorylation on the SXS motif can be carried out by other kinases like RTKs (de Caestecker et al, 1998) or the

kinase Mps1 (Zhu et al, 2007), which were reported to induce a ligand-independent activation of the Smads. The Smad linker region is another part of the molecule that can be subject to activity modulating phosphorylations. ERK (Kretzschmar et al, 1999) and the Ca²⁺ /calmodulin-dependent protein (CaM) kinase II (Wicks et al, 2000) -mediated phosphorylations negatively regulate the Smads, whilst via JNK-mediated phosphorylation contributes to Smad activity (Brown et al, 1999b). The cyclin-dependent kinase CDK 2/4- (Matsuura et al, 2004) or CDK 8/9- (Alarcon et al, 2009) dependent phosphorylation in the linker region can either inhibit (CDK 2/4) or enhance Smad transcription activity (CDK 8/9). Also dephosphorylation is an important step for modifying Smad activity: The phosphatases PPM1A (Lin et al, 2006) or PP2a dephosphorylate serines in the C-terminal SXS motif

(Heikkinen et al, 2010), while small C-terminal domain phosphatases (SCPs) act on linker phosphorylations (Sapkota et al, 2006; Wrighton et al, 2006).

Similar to the receptors, polyubiquitylation of the R-Smads by different E3 ligases like Smurf2 (Zhang et al, 2001), ROC1 (Fukuchi et al, 2001), WWP1 (Komuro et al, 2004) and NEDD4 (Kuratomi et al, 2005) can trigger proteasomal degradation. A monoubiquitination of R-Smads by Itch/AIP4 that interacts with the receptor T β RI increases Smad activity (Bai et al, 2004). In contrast, monoubiquitination of Smad4 is supposed to promote their nuclear export (Moren et al, 2003). Smad3-Sumoylation by PIAS is another possibility for negative regulation by targeting Smads for nuclear export (Lin et al, 2006). 4

Regulation by interacting proteins

(I) NEGATIVE REGULATORS

TMEPAI was found to be a TGF β -induced negative regulator of Smads by sequestering them in the cytoplasm through direct binding and inhibition of complex formation with SARA, an endosomal protein (Watanabe et al, 2010).

c-Ski and SnoN, part of the family of the proto-oncogene of the Ski family, function as inhibitors of Smad-dependent gene transcription by impairing the interaction of Smad4 and the R-Smads in the cytoplasm (Ferrand et al, 2010; Krakowski et al, 2005; Prunier et al, 2003) or upon the recruitment of co-repressors in the nucleus (Sun et al, 1999). During signaling, this negative regulation is abandoned by either Smurf2- or APC-mediated degradation of Ski and SnoN (Bonni et al, 2001; Stroschein et al, 2001).

TGIF, a homeobox transcription factor competes with p300/CBP to R-Smad binding and also recruits repressors and HDACs (Wotton et al, 1999a; Wotton et al, 1999b). TGIF is additionally implicated in a negative crosstalk regulation: an ERK-triggered activating phosphorylation of TGIF can negatively regulate Smad-dependent transcription (Lo et al, 2001).

(II) POSITIVE REGULATORS

SARA is an endosomally (EEA1 positive endosomes) located FYVE domain protein, which functions as anchor between Smads and the receptor, by having both, a Smad-binding-domain (SBD) and an receptor-interacting motif. It is described to induce proximity of R-Smads and the receptor and to be necessary for their phosphorylation by the T β RI (Tsukazaki et al, 1998). Further studies showed, that SARA might be dispensable for Smad3 signaling and only essential for Smad2-dependent signaling (Bakkebo et al, 2012; Goto et al, 2001; Lu et al, 2002).

Other proteins, such as **Hrs** (Miura et al, 2000) and *cPML*, the cytoplasmic form of the promyelocyticleukemia tumor suppressor, (Lin et al, 2004) act as enhancer of SARA activity.

Axin1, an RGS/DIX domain protein, interacts with the MH2 domain of Smad3, possibly also Smad2 to facilitate signaling (Furuhashi et al, 2001). Further evidence exists that it also promotes TGF signaling by negatively regulating Smad7 through inducing its degradation via the E3 ligase Arkadia (Liu et al, 2006) (Koinuma et al, 2003).

Other interacting proteins are summarized in the following tables 2 and 3 and for interaction with transcription factors on the nuclear levels, further information can be found in (Feng & Derynck, 2005)

Proteinname	Target	Proteintype	Mode Of Action	Reference
Receptor inter	racting			
Bambi	RI	Decoy receptor	Competes for BMP binding and formation of nonfunctional RII complexes	Onichtchouk et al., 1999

Table 3 Proteins negatively regulating TGF^β signaling by receptor- or Smad interaction

c-Ski	RI	Adaptor protein	Promotes formation of nonfunctional receptor/R-Smad complexes	Ferrand et al. 2010
Dpr2	RI	Adaptor protein	Triggers lysosomal degradation of receptor	Su et al., 2007b
DRAK2	RI	Kinase	Attenuation of TBRI activity	Yang et al., 2012
ETV6-	RII	chimeric	Prevents interaction with of RII with RI	lin et al 2005
NTRK3	MI	oncoprotein		5m et al., 2005
FKBP12	RI	Peptidyl-prolyl- isomerase	Prevents ligand-independent RI activation	Wang & Donahoe, 2004
PP1c	RI	Phosphatase	RI dephosphorylation	Shi et al., 2004
PP2a	RI	Phosphatase	RI dephosphorylation	Griswold-Prenner et al., 1998
Smad 6/7	RI	I-Smad	Competes with R-Smads, mediates receptor ubiquitylation and dephosphorylation	Nakao et al., 1997, Kavsak et al. 2001
Smurf1/2	RI/RII	Ubiquitinligase	Ubiquitin-mediated proteasomal degradation	Kavsak et al. 2001
STRAP	RI/RII	WD 40 adaptor protein	Potentiates Smad7 effects and inhibits R-Smads	Datta et al. 1998
TACE/ ADAM17	RI	Metalloprotease	Shedding of T β RI extracellular domain	Liu et al., 2009a
TrkC	RII	RTK	Prevents interaction of RII with RI	Jin et al., 2007
WWP1	RI/Smads	Ubiquitinligase	Ubiquitin-mediated proteasomal degradation	Komura et al.,
				2004
YAP65	RI/RII	SH3 adaptor protein	Increase of Smad7 association	Ferrigno et al. 2002
YAP65 Smad interac	RI/RII ting	SH3 adaptor protein	Increase of Smad7 association	Ferrigno et al. 2002
YAP65 Smad interac c-Ski	RI/RII ting S2/3/4	SH3 adaptor protein Adaptor protein	Increase of Smad7 association Impairment of heteromeric Smad complex formation	Ferrigno et al. 2002 Prunier et al.,2003
YAP65 Smad interac c-Ski Erbin	RI/RII ting S2/3/4 S2/3	SH3 adaptor protein Adaptor protein PDZ-domain protein	Increase of Smad7 association Impairment of heteromeric Smad complex formation Sequesters Smads in the nucleus	Ferrigno et al. 2002 Prunier et al.,2003 Dai et al.,2007
YAP65 Smad interac c-Ski Erbin NEDD4	RI/RII ting S2/3/4 S2/3 S2/3	SH3 adaptor protein Adaptor protein PDZ-domain protein Ubiquitinligase	Increase of Smad7 association Impairment of heteromeric Smad complex formation Sequesters Smads in the nucleus Ubiquitin-mediated proteasomal degradation	Ferrigno et al. 2002 Prunier et al.,2003 Dai et al.,2007 Kuratomi et al.,2005
YAP65 Smad interac c-Ski Erbin NEDD4 PIAS	RI/RII ting S2/3/4 S2/3 S2/3 S2/3 S3	SH3 adaptor protein Adaptor protein PDZ-domain protein Ubiquitinligase	Increase of Smad7 association Impairment of heteromeric Smad complex formation Sequesters Smads in the nucleus Ubiquitin-mediated proteasomal degradation Nuclear export	Ferrigno et al. 2002 Prunier et al.,2003 Dai et al.,2007 Kuratomi et al.,2005 Lin et al., 2006
YAP65 Smad interac c-Ski Erbin NEDD4 PIAS PP2a	RI/RII ting S2/3/4 S2/3 S2/3 S2/3 S3 S3	SH3 adaptor protein Adaptor protein PDZ-domain protein Ubiquitinligase Sumoligase Phosphatase	Increase of Smad7 association Impairment of heteromeric Smad complex formation Sequesters Smads in the nucleus Ubiquitin-mediated proteasomal degradation Nuclear export SXS dephosphorylation	Ferrigno et al. 2002 Prunier et al.,2003 Dai et al.,2007 Kuratomi et al.,2005 Lin et al., 2006 Heikkinen et al. 2010
YAP65 Smad interac c-Ski Erbin NEDD4 PIAS PP2a PPM1A	RI/RII ting S2/3/4 S2/3 S2/3 S2/3 S3 S2/3	SH3 adaptor protein Adaptor protein PDZ-domain protein Ubiquitinligase Sumoligase Phosphatase Phosphatase	Increase of Smad7 association Impairment of heteromeric Smad complex formation Sequesters Smads in the nucleus Ubiquitin-mediated proteasomal degradation Nuclear export SXS dephosphorylation SXS dephosphorylation	Ferrigno et al. 2002 Prunier et al.,2003 Dai et al.,2007 Kuratomi et al.,2005 Lin et al., 2006 Heikkinen et al. 2010 Lin et al., 2006
YAP65 Smad interac c-Ski Erbin NEDD4 PIAS PP2a PPM1A ROC1	RI/RII ting S2/3/4 S2/3 S2/3 S3 S3 S2/3 S2/3	SH3 adaptor protein Adaptor protein PDZ-domain protein Ubiquitinligase Sumoligase Phosphatase Phosphatase Ubiquitinligase	Increase of Smad7 association Impairment of heteromeric Smad complex formation Sequesters Smads in the nucleus Ubiquitin-mediated proteasomal degradation Nuclear export SXS dephosphorylation SXS dephosphorylation Ubiquitin-mediated proteasomal degradation	Ferrigno et al. 2002 Prunier et al.,2003 Dai et al.,2007 Kuratomi et al.,2005 Lin et al., 2006 Heikkinen et al. 2010 Lin et al., 2006 Fukuchi et al.,2001
YAP65 Smad interac c-Ski Erbin NEDD4 PIAS PP2a PPM1A ROC1 SCP	RI/RII ting S2/3/4 S2/3 S2/3 S2/3 S2/3 S2/3 S2/3	SH3 adaptor protein Adaptor protein PDZ-domain protein Ubiquitinligase Sumoligase Phosphatase Phosphatase Ubiquitinligase Phosphatase	Increase of Smad7 association Impairment of heteromeric Smad complex formation Sequesters Smads in the nucleus Ubiquitin-mediated proteasomal degradation Nuclear export SXS dephosphorylation SXS dephosphorylation Ubiquitin-mediated proteasomal degradation Smad linker region dephosphorylation	Ferrigno et al. 2002 Prunier et al.,2003 Dai et al.,2007 Kuratomi et al.,2005 Lin et al., 2006 Heikkinen et al. 2010 Lin et al., 2006 Fukuchi et al.,2001 Sapkota et al., 2006, Wrighton et al. 2006

SnoN	S2/3/4	Adaptor protein	Impairment of heteromeric Smad complex formation	Krakowski et al.,2005
TGIF	S2/3/4	Homeobox protein	Competes with Smads for transcriptional co- factor binding	Wotton et al.,1999
TMEPAI	S2/3	Adaptor protein	Competes with RI for R-Smad binding	Watanabe et al.,2010

Table 4 Proteins positively regulating TGF β signaling by receptor- or Smad interaction

Proteinname	Target	Proteintype	Mode Of Action	Reference	
Receptor interacting					
ADAM12	RI	Metalloprotease	Protects from Smad7 binding	Atfi et al., 2007	
Dab2	RI/Smads	Adaptor protein	Required as Smad adaptor and facilitates endosomal trafficking	Hocevar et al., 2001 Penheiter et al., 2010	
Daxx	RI	Death domain protein	Favors apoptosis induction	Perlman et al., 2001	
HSP90	RI	Chaperone	Protects from Smad7 binding	Wrighton et al., 2008	
Itch-AP4 E3	RI/Smads	E3 ligase	Associates with RI and monoubiquitinates Smads for signaling potentiation	Bai et al., 2004	
Par6	RII	Adaptor protein	RhoA degradation	Ozdamar et al., 2005	
Shc	RII	SH2 docking protein	MAPK pathway activation (ERK)	Lee et al. 2007	
Src	RII	Kinase	Tyrosine phosphorylation inducing p38 activation	Galliher and Schiemann, 2007	
TAK1	RI	Kinase	MAPK pathway activation (p38, JNK)	Yamaguchi et	
TLP	RI/RII	Adaptor protein	Promotes Smad3 but impairs Smad2 phosphorylation	Felici et al., 2003	
TRAF6	RII	Monoubiquitinase	Targets intercellular domain of RI	Mu et al. 2011	
TRAP1	RI/RII	Adaptor protein	Facilitated Smad4 recruitment	Wurthner et al., 2001	
Ubc9	RI	Sumoylation	Sumoylated RI and enhances Smad recruitment	Kang et al. 2008	
UCH37	RI	Deubiquitinase	Reverses Smurf-mediated ubiquitination	Wicks et al., 2005	
USP15	RI	Deubiquitinase	Reverses Smurf-mediated ubiquitination	Eichhorn et al., 2012	
XIAP	RI	Multidomain protein	Cooperates with TAK1	Neil et al., 2009	
Smad interacting					
Arkadia	Smad7	Ubiquitinligase	Targets Smad7 for degradation	Koinuma et al., 2003, Liu et al. 2006	
Axin	Smad2/3	RGS/Dix domain protein	Associates with Smads to facilitate signaling	Furuhashi et al., 2001	

cPML	Smad2/3	Adaptor protein	Enhances SARA actions on Smads, favors endosomal localisation of receptor complexes	Lin et al., 2004
Elf	Smad2/3/4	Beta spectrin	Required for Smad activation and nuclear localisation	Tang et al., 2003
Hrs	Smad2/3	FYVE domain protein	Enhances SARA actions on Smads	Miura et al., 2000
JNK	Smad2/3	Kinase	Linker region phosphorylation	Brown et al., 1999b
Mps1	Smad2/3	Kinase	SXS motif phosphorylation	Zhu et al., 2007
SARA	Smad2/3	FYVE domain protein	Favors Smad association with RI	Tsukazaki et al., 1998

3.3 Crosstalk of TGFβ signaling

Another possibility for the creation of diversity in TGF β signaling relies on the phenomenon of crosstalk with other signal transduction pathways (for definition see Chapter 1.4.).

3.3.1 Non-canonical signaling of TGF^β receptors

Non-canonical signaling of the TGF β signal transduction pathway is a Smad-independent signaling, that implies all the events, where a TGF β ligand does not signal via the traditional pathway, but other molecules being part of another signal transduction pathway. The activation of these pathways is also often correlated with a distinct functional outcome of the ligand binding. The molecules that are interacting with the TGF β core components have also an important impact on directing signaling into a specific direction (Zhang, 2009)

Activation of MAP kinases

The possibility of a signaling through MAP kinases came up with the findings that Smad KO mice or cells with Smad-activation-deficient receptors could yet evoke a cellular response to ligand binding. As signaling mediators could be identified the MAPK members JNK, which mediates the TGF β -dependent synthesis of extracellular matrix (Atfi et al, 1997; Hocevar et al, 1999) and p38 (Hanafusa et al, 1999; Yu et al, 2002) that is responsible for inducing TGF β -mediated apoptosis. The T β RI interacting proteins TAK1 and TAB, a MAP3K and its activator respectively, have been found to be responsible for converting the ligand binding into the activation of the MAP kinases JNK and p38 (Hanafusa et al, 1999; Shibuya et al, 1996) independent of receptor kinase activity (Sorrentino et al, 2008). Additional regulators

that are essential for the induction of apoptosis of the complex are the T β RI-interacting XIAP (Yamaguchi et al, 1999) and the T β RII-interacting Daxx (Perlman et al, 2001).

It has also been shown, that TGF β signals can lead to activation of the MAP kinase ERK by two different mechanisms: One mechanism seems to involve a transcriptional regulation occurring only late after stimulation by regulating the expression of Ras-like protein RLP, a receptor binding protein that favors ERK activation (Piek et al, 2004). Another mechanism is a short-term response, which is accomplished by the adaptor protein SHC1 that binds through its SH2 domain to a p-Tyr site in T β RII that, after being phosphorylated by the T β RII, can recruit other proteins that activate ERK by phosphorylation. This direct activation seems to be important in the process of epithelial-to-mesenchymal-transition.

Activation of Rho GTPases

An effect of the Rho family of small GTPases RhoA, Cdc42 or Rac1 by TGF β has also been reported (Bhowmick et al, 2001; Edlund et al, 2002). A mechanism, implicating Par6 binding to the T β RII and subsequent Par6 phosphorylation seems to be important for the induction of RhoA degradation, allowing membrane ruffling and lamellipodia formation through Rac1 and Cdc42 during epithelial-to-mesenchymal transition (see Chapter 3.5.1).

Activation of others pathways

Further reports could show, that TGF β signaling can also imply activation of NF κ B that is phosphorylated by TAK1. PI3K activation occurs via direct association with the receptor complex and a following Akt phosphorylation (Bakin et al, 2000).

3.3.2 Crosstalk of TGFβ signaling with other signal transduction pathways

The TGF β signaling pathway can be the object of activity modulation in a synergistic or antagonist fashion through the crosstalk with other signal transduction pathways (Guo & Wang, 2009).

Crosstalk with MAPK pathways

One of the most important crosstalk occurs upon interplay with the MAP kinase ERK. The basis is, that both evoke opposed responses in epithelial cells (TGF β : cytostatis vs. Erk: proliferation), leading to antagonizing effects of the EGF-Ras-Erk axe on TGF β signaling. Activated Erk can phosphorylate Smads in their linker region, attenuating nuclear import, thus

inhibiting Smad-dependent transcription (Kretzschmar et al, 1999). Ras was also described to decrease Smad4 protein stability (Saha et al, 2001), which can also be maintained by JNK/p38 in cancer cells (Liang et al, 2004). In contrast, during processes like epithelial-to-mesenchymal transition, synergistic effects with Ras-Erk signaling have been observed. TGF β can elicit an increase in cytokine secretion like PDGF. A phosphorylation of R-Smads in their SxS motif induced by RTKs has been be detected (de Caestecker et al, 1998).

Other MAP kinases, with a role in growth inhibition as apoptosis have also been described to affect Smad activity. On one hand, JNK-mediated phosphorylation of Smad3 in the linker region can have enhancing effects (Brown et al, 1999b), while the JNK effector c-Jun blocks Smad activity (Dennler et al, 2000; Pessah et al, 2001).

Crosstalk with the PI3K/Akt pathway

Different activators of the PI3 Kinase like insulin and IGF can decrease TGF β -dependent cytostatic effects by mainly targeting Smad3 (Chen et al, 1998). The discovery of an inhibitory interaction of Akt with Smad3 further supports this negative interplay of both pathways (Conery et al, 2004; Remy et al, 2004). PTEN, a lipid phosphatase that is a negative regulator of the PI3K pathway, was described to be repressed by TGF β signaling (Chow et al, 2007).

Crosstalk in embryonal development: Wnt, hedgehog and notch

Due to the effects of TGF β on embryonal development, crosstalk occurs with other pathways having an important role in development. The most important is the Wnt pathway, composed of Wnt, a lipid ligand, with its GPCR frizzled, that regulates β -catenin activity and nuclear translocation. During development a reciprocal regulation of ligand concentration of Wnt and TGF β has been observed, and synergistic effects can be obtained through gene transcriptionmediated by complexes of R-Smad/ β -catenin/Lef. Furthermore, interactions between TGF β and the hedgehog- and notch pathways seem to be critical for accurate embryonic development (Wu & Hill, 2009).

Crosstalk with interleukins (IL), TNF and interferon (IFN)-y pathways

Acting also on the immune systems, crosstalk with pathways responsible for the immune reaction of a cell, like IL-pathways, the $TNF\alpha/\beta$ or IFN γ pathway are also occurring (Li et al, 2006).

Crosstalk on a nuclear level

In the nucleus, crosstalk has been observed with the AP-1 transcription factor complex, required for apoptosis (Schuster & Krieglstein, 2002). Another important crosstalk that synergizes the cytostatic effects of TGF β signaling occurs due to the interplay with tumor suppressor p53 related signaling (Atfi & Baron, 2008).

Pathways involving Smad phosphorylation

As for example the crosstalk with MAP kinases has shown before, the Smads and their multiple phosphorylation sites are a key target for integrating signals of other pathways. Further kinases were identified to modulate Smad activity by their phosphorylation: a negatively regulating phosphorylation can be exerted by the calcium-calmodulin dependent-kinase. (Wicks et al, 2000), and a PKC phosphorylation on the MH1 domain has a negative effect by abolishing DNA binding (Yakymovych et al, 2001). Also the cyclin-dependent-kinases CDK2 and CDK4 phosphorylate Smad and inhibit Smad-dependent gene transcription and thereby their cytostatic effects (Matsuura et al, 2004). In addition, also the casein kinase I, glycogen synthase kinase GSK3 or GRK2 (see below) can modify Smad activity through phosphorylation (Wrighton et al, 2009).

3.3.2.1 Crosstalk with GPCR signaling and transactivation by GPCRs

So far, only little information exists concerning the phenomenon of crosstalk and transactivation of TGF β signaling pathways and GPCR-mediated signaling.

Crosstalk between GPCRs and TGF β signaling

Some work could provide evidence for the existence of crosstalk between GPCRs and TGF β signaling. The GPCR regulating protein β -arrestin 2 has also been linked to the modulation of TGF β signaling: the association of β -arrestin 2 with the T β RIII betaglycan can induce internalization of the T β RII/T β RIII complex and subsequently downregulate TGF β signaling (Chen et al, 2003). Another work reported also its direct association with the T β RII, even in the absence of the RIII, that might have promoting effects in directing signaling towards non-canonical pathways, like p38-induced apoptosis (McLean et al, 2013). This goes along with the role of β -arrestins in GPCR signaling by favoring MAP kinase activation (see Chapter 1.3.). Furthermore, evidence is provided for TGF β signaling regulating GPCR signaling proteins, such as GRK2 for being a TGF β response gene. As a result of its TGF β -stimulated

expression, GRK2 associates with and phosphorylates the linker region of Smads, hence inhibiting their nuclear translocation (Ho et al, 2005). So far, there has been no proof, whether GRK2 might also have an effect on receptor phosphorylation, what might trigger the described β -arrestin 2 association with the receptors.

Besides, studies have shown, that the Serotonin receptors $5\text{-HT}_{1B/D}$ can induce, after stimulation with serotonin, a phosphorylation of Smad1, 5 and 8, which normally signal through BMP ligand (Liu et al, 2009b), in a RhoA Kinase (ROCK) -dependent manner. An example for TGF β -induced regulation of GPCRs was shown for the TGF β -induced delay of neurokinin receptor internalization in T-cells leading to prolonged signaling (Beinborn et al, 2010).

Receptor transactivation

Compared to crosstalk, transactivation is a more limited term, to describe the interplay of signaling pathways. While a crosstalk also implies *de novo* gene transcription or signalmediator-dependent responses, a transactivation is "where one receptor activates a heterologous receptor" (Wetzker & Bohmer, 2003), meaning an immediate effect on the receptor level. Compared to the big amount of sources describing an interplay between GPCR signaling and RTK-triggered pathways (see Chapter 1.3.), just recently the group of Peter Little demonstrated an association of TGF β - and GPCR-mediated signaling pathways on the receptor level, what led to the expansion of the transactivation concept of GPCR towards serine/threonine kinases (Burch et al, 2012). They have shown, both for endothelin and thrombin GPCRs, that ligand binding induced a transactivation of the TGF β signaling pathway via Smad phosphorylation. The transmission of the signal from the GPCR to the TGF β receptor was proven by inhibition of the T β RI kinase activity that abolished these events (Burch et al, 2010; Little et al, 2010). But the exact mechanisms underlying this transactivation remain unknown and so far, their studies form the only example for GPCR-ligand-induced transactivation of the TGF β receptor.

3.4 TGFβ signaling in physiology and pathophysiology

TGF β ligands are multifunctional molecules that are known to regulate many cellular processes like proliferation, cell fate determination, differentiation, motility, adhesion, that are essential for the development and maintenance of tissue and organism homeostasis. TGF β cytokines act mainly in an autocrine or paracrine manner and virtually all cells can respond to

ligand stimulation. In contrast, the physiological effects strongly dependent on the cellular context and the expression pattern of different molecules implicated in TGF β signaling, leading to the variety of possible TGF β signaling outcomes that can be completely opposite dependent on the physiologic environment. These pleiotropic actions of TGF β also explain its large implication in human disease (Massague, 2012).

3.4.1 Biological actions of TGFβ signaling

TGF β ligands have been found to be implicated in a huge variety of processes in organisms, the most important ones will be highlighted in the following and are summarized in Figure 20:

3.4.1.1 Cytostasis and apoptosis

In most of the cell types, the cellular response to $TGF\beta$ is an inhibition of cell proliferation. This can observed in epithelial, endothelial, hematopoietic and immune cells, while in mesenchymal cells as fibroblasts, the opposite effect can take place. On the molecular level, the TGF β -induced cytostatis is the result of activation and repression of gene expression of important players of cellular proliferation in the G1 phase of the cell cycle (see Figure 19). An early TGFβ response effect is the repression of the expression of the proto-oncogene c-myc (Alexandrow et al, 1995). This is accompanied by a loss of the repressive actions of myc on cell cycle inhibitors p15INk4b and p21kip. TGF_β promotes additionally the expression of p15Ink4b that inhibits cyclin D/CDK4 and CDK6 in early G1 stage and p21Kip that acts on cyclin E/A-CDK2 activity. This repression-activation cascade is the result of concerting actions: in order to repress myc transcription, a preformed cytoplasmic complex composed of the transcription factors E2F4/5, DP1 and p107 exists, which translocates with activated Smads to the nucleus, binds to the myc promoter and represses it expression. Myc itself is a repressor of p15Ink4b. By inhibiting myc transcription, the formation of the repressive complex with the miz protein is abolished, conducting to the feedback activation of p15Ink4b (Seoane et al, 2001; Staller et al, 2001).



Figure 19 Mechanism of TGFβ-induced cell cycle arrest (Massague et al, 2000)

TGF β induces a repression of the growth promoting protein c-myc. This in turn leads to an arrest of the repressive actions of myc on the cell cycle inhibitors p15, p21 and cdc25A. Their expression is also stimulated by TGF β signaling. Their activity leads the repression of the cell cycle promoting cycline dependent kinases CDK2 and CDK4, thus explaining the cytostatic effects of TGF β .

Other events that play a role in exerting cytostatic effects are the repression of the ID proteins (ID1, ID2, ID3), performed by a complex of Smads with the selfenabling ATF3 protein (Kang et al, 2003a). Also the interaction with the transcription factor Runx3 and the crosstalk with p53 participate in growth control by TGF β (Pardali & Moustakas, 2007).

Under certain conditions, TGF β can not only induce a block of the cell cycle, but can also directly induce apoptosis. So far, several mechanisms have been discovered that propose an involvement of caspases, Bcl-2 family members like the protein BIM, and death-associated protein kinase (DAPK) triggering the mitochondria associated pathway of apoptosis (Chen & Chang, 1997; Jang et al, 2002; Ohgushi et al, 2005). The T β RII-interacting protein Daxx seems to be important to direct signals towards apoptosis via JNK- and p38 activation (Perlman et al, 2001). Physiologically this induction of apoptosis occurs for example in the mammary gland size reduction after pregnancy.

3.4.1.2 Development and embryonal stem-cell differentiation

TGF β signaling is strongly implicated in the orchestration of embryogenesis and development. The occurrence of ligand gradients is fundamental for correct developments at specific stages. Different ligands are required for establishment of the left-right (nodal), correct vascular, cardiac, lung and craniofacial development (TGF β) and the Smads have been shown to be required for the anterior-posterior axe establishment and endoderm formation (Wu & Hill, 2009).

With regards to embryonic stem cells, BMP-dependent actions promote self-renewal of stem cells by inducing a feed-forward cycle with the differentiation repressing triad of the transcription factors OCT4/SOX2/NANOG (Chen et al, 2008). In contrast, Smad2 can drive me endodermal differentiation by activating transcription of genes with an ARE motif, recognized by a Tripartite motif-containing 33 (TRIM33)-Smad2 complex (Xi et al, 2011). In progenitor cells, the synergistic action of identity factors and Smads on gene expression implements differentiation programs like for myoblast (MYOD1), mesenchymal and lymphoid (PU.1), myeloid (C/EBPα) and erythroid (GATA) progenitors (Watabe & Miyazono, 2009).

3.4.1.3 Epithelial-to-mesenchymal transition (EMT)

EMT is a process mainly driven by TGF β , meaning the switch from epithelial cells into a migratory mesenchymal phenotype like in (myo)fibroblasts. During development, it is necessary for gastrulation, embryonic tissue formation and in adult tissues required for regenerative processes. The molecular events underlying EMT are on the one hand a loss of cell polarity, which is mediated by Par6 that interacts with and gets phosphorylated by T β RII and then induces RhoA degradation (Ozdamar et al, 2005). On the other hand, the Smads also induce the expression of the selfenabling transcriptional repressors Snail and Slug that together repress the expression of E-cadherin, a marker of epithelial cells and favor the expression of N-cadherin, marker of invading and migrating cells. Additionally, a preceding crosstalk with Wnt signaling activity creates an environment favoring TGF β to induce EMT instead of growth arrest. Also other crosstalks are implicated to realize a switch from the proapoptotic Smad-pathway to pathways that favor migration (Heldin et al, 2009). Besides the beneficial effects of EMT in tissue repair and wound healing, EMT is a process crucial for cancer progression and occurs pathologically in fibrosis (see below).

3.4.1.4 Regulation of extracellular matrix (ECM)

The ECM is surrounding the cells, forms the major component of the connective tissue and is composed of characteristic proteins as collagen, elastin, fibrillin, fibronectin, lamin and proteoglycans. A lot of genes encoding for ECM components are found to be regulated by Smads, as collagens (Chen et al, 1999), making TGF β a critical regulator of ECM synthesis (Verrecchia & Mauviel, 2007). The plasminogen-activator-inhibitor 1 (PAI-1), is a TGF β response gene important in maintenance of ECM and inhibition of fibrinolysis (Laschinger et al, 1991). This TGF β -stimulated ECM formation is of big importance in wound healing (O'Kane & Ferguson, 1997), but can turn out negatively by excessive signaling and consequently lead to fibrosis.

3.4.1.5 Angiogenesis

Though having a repressive effect on proliferation in endothelial cells, TGF β acts as potential angiogenic factor by favoring endothelial cell migration (Roberts et al, 1986). But things are much more complex, because TGF β effects seem to depend on ligand concentration and the receptor they choose for signaling: in endothelial cells, TGF β can signal either via the classic T β RI with its anti-proliferative actions, while signaling through ALK1 is associated with proproliferative actions. The endothelial cells are an example where the balance of receptor utilization is important for the functional outcome (Pardali et al, 2010). In order to promote angiogenesis, TGF β has been found to induce transcription of angiogenesis stimulation vascular endothelial growth factor VEGF (Ferrari et al, 2009).

3.4.1.6 Hematopoiesis

TGF β acts as potent inhibitor of hematopoietic stem cell proliferation. The TGF β -1 ligand inhibits hematopoietic stem cell (HSC) proliferation, leaves them in a quiescent state and downregulates receptors for hematopoietic cytokines that would promote their differentiation (Yamazaki et al, 2009). This in turn also affects immune cell development that arise from HSCs.

3.4.1.7 Bone formation

Bone is the tissue that gives us mechanical support and organ protection, regulates calcium levels and hematopoiesis. The bone is composed of osteoblasts and osteoclasts. The role of TGF β 1 as predominantly expressed isoform lies in osteoblast progenitor recruitment, stimulation of proliferation and differentiation and inhibition of apoptosis, as revealed with TGF β KO mice that lead to reduced bone growth and mineralization (Janssens et al, 2005). Also the BMP ligand has important functions in bone homeostasis and its deletion leads to impairment in bone formation.

3.4.1.8 Reproduction

TGF β signaling also has critical functions in the female reproductive system: it regulates ovarian follicle development, gonadotropin receptor expression, oocyte maturation, ovulation and luteinization (Abassi & Vuori, 2002). These actions are mainly mediated by the activininhibin-follistatin system: activin is the ligand, while inhibin functions as a decoy ligand and follistatin directly sequesters activin through direct binding. An important role in controlling reproduction role might also be assigned to TGF β signaling in the tanycytes, the brain cells surrounding the 3rd ventricle that are implicated in the regulating of reproduction (Bouret et al, 2004; Bouret et al, 2002; Prevot et al, 2000).

3.4.1.9 Immune system

The importance of TGF β signaling in the immune system becomes obvious with regards to TGF β ligand KO mice, displaying a strong immune deregulation with hyperactivity of the immune system and infiltration of organs with immune cells, showing that TGF β is an important immunosuppressant agent. It is acting negatively on the proliferation, differentiation and activation of T cells, B cells, natural killer (NK) cells, monocytes, macrophages, neutrophils and eosinophils. In contrast, it can exert pro-inflammatory effects, by directing immune cells into the development of a secretory phenotype, leading to the secretion of IL-6, IL-11 (Li et al, 2006).

3.4.1.10 Nervous system

TGF β ligands are implicated in the establishment of synaptic plasticity and play a role in cognition and behavior. They are supposed to have neurotrophic and neuroprotective roles and are involved in excitatory and inhibitory neurotransmission. Furthermore, TGF β influences neurite outgrowth and synaptogenesis (Krieglstein et al, 2011). TGF β mediates axon specification during axon development in a Par6-dependent manner (Yi et al, 2010).

3.4.1.11 Energy homeostasis

Recently actions of TGF β in energy homeostasis were proposed, by showing a role for Smad3 in inhibiting the transformation of white adipose into adult brown adipose tissue through negative regulation of expression of markers of the brown adipose tissue (Yadav et al, 2011).



Figure 20 Effects of TGFβ on different cell types (Siegel & Massague, 2003)

The effects of TGF β are cell type specific. In epithelial cells (yellow), mainly cytostatic and apoptotic actions are observed and adhesion and ECM production are supported. In endothelial cells (brown) their migration is promoted. Immune cells (blue) mainly receive proliferation- and differentiation-inhibitory signals. In fibroblasts (red), TGF β can stimulate proliferation and ECM production.

3.4.2 Pathophysiological implication of TGF^β signaling

Being such a pleiotropic molecule, malfunctioning of the TGF β pathway can result in numerous diseases. Germline or somatic mutations and alterations in expression of signaling components can result in abnormal physiological function. Dissecting the effects of TGF β signaling in disease helps to reveal and understand the physiological functions and their analysis can lead to identification of underlying mechanisms and the development of appropriate therapeutic strategies (Gordon & Blobe, 2008).

Cardiovascular processes and cardiovascular disease

TGF β ligands are implicated in cardiac development and angiogenesis, as supported by KO mice of the TGF β ligand (1,2,3) that show ventricular septum defects, myocardial thinning, double outlet right ventricles, failed coronary vessel and epicardial development. Smad4 KO in the heart leads to a hypocellular myocardial wall defect (Bobik, 2006). Disorders that were

found to be associated with TGF β are *hereditary hemorrhagic teleangiectasia*, with a vascular dysplasia that results in teleangiecstasia and arteriovenous malformation, based on inactivating mutations in the type I receptor ALK1 (Abdalla & Letarte, 2006). Impairment of receptor activity was also found to take place in aorta diseases or pulmonary hypertension. Loss of TGF β signaling activity is associated with a higher risk to develop pre-eclampsia during pregnancy and atherosclerosis. In contrast, hypertension seems to be correlated to an increase of TGF β levels, which might also rely on the effects by the blood pressure regulating actions of the renin-angiotensin-system to upregulate TGF β expression (Wolf, 2006).

Connective tissue diseases

By positively regulating the production of ECM molecules and being dependent on ECM molecules for its activation, TGF β signaling malfunction can lead to different disease phenotypes.

The Marfan Syndrome is a hereditary disorder, displaying a mutation of the ECM protein fibrillin-1, that leads to altered anchoring of inactive TGF β ligand and reduction in TGF β release, resulting phenotypically in aneurysms, aortic dissections and skeletal manifestations (Lindsay & Dietz, 2011)

Another disorder that is associated with TGF β actions on ECM is fibrosis. Fibrosis occurs when the positive effects of TGF β -mediated disposition of ECM molecules by myofibroblasts, for example during wound healing, lose their balance and become excessive. In combination with the pro-proliferative effect of TGF β on myofibroblasts, an accumulation of fibroblast that produce too many ECM molecules is the consequence. A specific type of fibrosis is restenosis, a fibrotic response to revascularization therapies, where TGF β can have a contributing effect, but the increased ECM production is often leading to complications (Verrecchia & Mauviel, 2007).

Other diseases

The other important functions of TGF β in different organs listed in 3.4.1. can give rise to other diseases associated with TGF β signaling.

TGF β and BMP ligands are indispensable for a proper bone homeostasis. Therefore, defective TGF β signaling can lead to diseases as osteoporosis (Su et al, 2007a). In contrast, hyperactivity of the signaling pathway due to an amino acid substitution mutation in the GS domain of the ACVR1 type I receptor, which leads to its constitutive activation causes

increased bone mass and ossification as in *fibrodysplasia ossificans progressiva* (Groppe et al, 2007; Groppe et al, 2011)

A dysfunction of TGF β can also lead to altered development of reproductive organs, as shown for impaired testis or female maturation. A non-functional MIS ligand or its corresponding receptor causes a loss of regression of the Mullerian duct, leading to male persons having both male and female reproductive system, resulting in complete infertility, the persistent Mullerian duct syndrome (Josso et al, 2005).

A failure of appropriate signaling during embryogenesis at the stages of blastula formation, gastrulation or organ development can either have lethal consequences, lead to mild disorders as the cleft palate, where palate fails to undergo EMT, or cause severe deformations, as the inversion of human organ asymmetry (*situs inversus* and *situs ambiguus*), caused by mutations of the ligands important for their establishment as the TGF superfamily members Nodal, GDF and Lefty (Lowe et al, 1996).

Due to its huge immunosuppressant actions, defective TGF β signaling can be the reason for diverse autoimmune diseases (Li et al, 2006).

Alterations of TGF β signaling have also been associated with neurological and psychiatric disorders as parkinson, multiple sclerosis, schizophrenia and Alzheimer disease (Vivien & Ali, 2006).

3.4.2.1 TGFβ signaling in cancer

One of the cruelest diseases and one of the main challenges of modern civilization is cancer. Cancer is the occurrence of malignant neoplastic transformations in one organ that can lose growth control and invade throughout the whole body. This is affecting proper function of the organism and often leads to death of the concerned individuals. Cancer development, progression and therapy are among the most researched fields and the findings helped to reveal the unique properties of cancer cells ("hallmarks of cancer" (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011)).

With its growth inhibitory function, the TGF β signaling pathway is supposed to be an important actor in cancer development and progression. The huge amount of data that correlate malfunction or inactivation of TGF β signaling components as a result of mutations found in tumor cells, is coherent with its important tumorsuppressing role. But the actions of TGF β are paradox: while in early stages, TGF β functions as a tumorsuppressor, in late stages, TGF β signaling can enhance cancer progression and metastasis via its ability to support

cellular migration and EMT. A cancer must overcome the barrier established by TGF β signaling in order to develop, but then it can benefit of TGF β signals in progressing and spreading. Thus, maintenance of homeostasis in TGF β signaling is vital for inhibition of cancer development. Consequently, the understanding of TGF β signaling is a notable research objective to help to increase the knowledge about cancer and to identify possible therapeutic targets, either to prevent cancer onset in early stages or to limit its progression.



Figure 21 Pleiotropic actions of TGFβ in cancer (Massague, 2008)

During cancer development, TGF β has multiple roles. While acting as a tumorsuppressor in early stages, through its cytostatic actions, malignant tumors can profit from TGF β signaling with its abilities to promote migration, suppress immune response and to induce EMT to favor invasion and metatasis.

A - Tumor suppressing actions of $TGF\beta$ signaling

In early stage tumors, TGF β exhibits growth inhibitory function and protects against tumor progression through autocrine and paracrine mechanisms. In autocrine, cell autonomous mechanisms of suppression, TGF β signaling induces its cytostatic program by myc-repression, p15 and p21 activation and ID1 downregulation. Furthermore, the induction of cellular differentiation for certain cell lines can have, dependent on cancer type and cell,

cancer-limiting effects. In conditions of excessive proliferation, as we find them in cancer with strong proliferating phenotypes e.g. as a result of Ras mutation, TGF β can even induce apoptosis of cells in order to eliminate these deviant cells (Massague et al, 2000).

In a paracrine manner, the interplay of tumor and stroma can also generate suppressing effects, which emphasizes the influence of cellular communication of the tumor environment on cancer development. The tumor adjacent cells secret TGF β in order to protect the tumor development by inducing cytostasis and repression of the expression of fibroblast derived mitogens (Massague, 2008).

Loss of TGF β dependent tumor suppression

Cancer cells are genetically unstable and selective pressure gives advantage to those cells, who gained a surviving function due to the occurred modifications in their DNA. TGF β signaling pathway related genes display an important target for a cancer to gain forces, explaining the high amount of genetically based dysfunction of the TGF β signaling in cancer samples. Growing significance concerns the incidence of epigenetic changes that affect expression levels in cancer (Inman, 2011). The loss of TGF β tumorsuppressing effects can have two distinct reasons:

(1) Loss or inactivation of core components and signaling

This type of defective TGF β signaling is mainly found in colorectal, pancreatic, ovarian, gastric, head & neck cancer and elicits the complete loss of tumorsuppressing effects. Evidence was obtained from transgenic KO mouse models that display an increase of tumor progression (Pardali & Moustakas, 2007). Also human studies revealing inactivating germline and somatic mutations in TGF β signaling core components show the connection of TGF β and its tumorsuppressing role in cancer. Concerning the receptors, a frequent T β RII microsatellite instability in a polyadenine region, that is leading to expression of a non-functional receptor, can, as germline mutation lead to the hereditary non-polyposis colorectal cancer (HNPCC), or as somatic mutation to gastric tumors (Chang et al, 1997). Also other inactivating mutations in T β RII and T β RII or a decrease in their expression was found to be associated with lung, ovarian or pancreatic cancer. Additionally, Smad mutations are often referred to be tumor promoting. A Smad4 deletion, loss of heterozygocity or inactivating mutations are found in 50% of pancreatic carcinomas (Hahn et al, 1996; Schneider & Schmid, 2003) and other

intestinal forms, as colon carcinoma. In contrast, the R-Smad mutations are less frequent in cancer (Massague et al, 2000).

(2) Loss or disabling of the tumor suppressor arm

Another possibility is only the loss of the tumor suppressing arm of TGF β signaling. This form is mainly observed in breast and prostate cancer, glioma, melanoma and hematopoietic neoplasias. A partial loss of growth inhibiting actions of TGF β signaling often occurs beyond the core signaling pathway of ligand, receptor and Smads, and enables a cell to still benefit from the intact core parts of TGF β signaling. This gain of function transforms TGF β to a tumor promoting factor. Thus, a tumor can take advantage of pro-tumorigenic actions of TGF β and incorporate them to increase its proliferative, metastatic and invasive capacities.

One reason for this transformation lies in the affection of TGF β signaling regulating proteins (see 1.3.3.) concerning their expression levels or activity. Negative regulators of TGF β signaling are often proto-oncogenes and an increase in activity or expression can lead to a loss of effectiveness of TGF β signaling, as show for DRAK2 or the Ski/SnoN complex. The other way round, TGF β signaling enhancing molecules can function as tumorsuppressors and a decrease in their activity or expression, can lead to a loss of growth inhibition, as known for example for Runx3.

A loss of the cytostatic response can also be completely independent from TGF β signaling molecules, but due to a deregulation of non-signaling parts (Gomis et al, 2006). Reasons can be an increased myc-expression that is minimalizing the effect of TGF β on myc repression and consequently blocks the upregulation of cell cycle inhibitors. These cell cycle inhibitors, being TGF β effector genes, can also be the object of genetic loss or disabling mutations. Also signaling crosstalk plays another role: an increasing amount of proteins that accomplish inhibitory phosphorylations on Smads can diminish their activity, as for example an often described Ras or CDK overexpression (Matsuura et al, 2004). Generally, an imbalance of tumorpromoting and tumorsuppressing events in tumorous tissue can lead to a weakening of the tumorsuppressing effects of TGF β .

B- Tumor promoting actions of TGFβ signaling

The paradoxon: a switch from tumor suppression to tumor promotion

The described loss of tumor suppression is the first step to accomplish the vicious change of TGF β from being a tumor suppressor to becoming a tumor promoter. Having lost the growth

inhibitory branch of signaling, but remaining intact in its components, a cancer cell can benefit from the TGF β core pathway to pursue its aggressive and invasive actions. There is also correlation of TGF β expression and severeness of tumor. And tumors, often displaying an overproduction of TGF β for growth inhibitory purposes, can utilize this amount for their own advantage.

TGF β -induced secretion of mitogens

Under certain conditions, TGF β can also become a pro-proliferative agent, as it does in mesencyhmal and vascular smooth muscle cells. By stimulating the autocrine or paracrine production of mitogenic substances as PDGF or HGF in gliomas or liver, TGF β can stimulate cell growth (Ikushima & Miyazono, 2010).

Immune-suppressive actions of $TGF\beta$

The paracrine TGF β actions on tumor stroma can also be advantageous for tumor progression via suppression of the immune system. This is harbored in the suppressing effect of TGF β on hematopoietic stem cell differentiation and with regards to the tumor environment in a suppression of immune cell maturation (CD4+ and CD8+ cells) and immune cells secreting pro-apoptotic factors like dendritic cells, cytotoxic T lymphocytes and natural killer cells. This renders a tumor being partially protected from immune surveillance (Torre-Amione et al, 1990).

$TGF\beta$ -induced renewal of tumor-initiating cells (TICs)

Another important tumorpromoting effect of TGF β lies in the occurrence of the cells that gain pluripotent stem-cell-like capacities during tumor progression, the tumor-initiating cells (TIC) (Iwasaki & Suda, 2009). TGF β can promote their self-renewal and inhibit their proliferation and differentiation, what promotes the persistence of this cancer-promoting cell type. This was reported to be especially of relevance in glioma-initiating and leukemia-initiating cells (Yamazaki et al, 2009). Another contributing TGF β -mediated action is the epithelial-tomesenchymal-transition that breeds cells with a similar phenotype as the TICs.

Role of epithelial-to-mesenchymal transition on tumor progression and metastasis

A process that resembles TIC formation is the transformation of epithelial cells into mesenchymal-like cells (Mani et al, 2008). The EMT implies the creation of a motile cell

type, the myofibroblast (or "tumor associated fibroblast") with migratory and invasive capacities that can contribute to the metastatic capacities of tumors.

Metastasis is a succession of migrations in different compartments: first local invasion from epithelial cells into lymph- or blood vessels, then circulation and invasion into the metastatic site and finally adaption and growth in a new environment. For each tumor subtype, depending on their tissue of origin, exist distinct patterns and organ preferences in metastasis, as breast cancers preferentially spread into bone or lung. The survival rate of a tumor patient decreases with its associated metastasis rate. The function of TGF β in the onset of metastasis is backed by the existing correlation of increasing TGF β concentrations in invading tumors compared to primary ones and the association of metastasis with higher levels of TGF β . Also mouse models with TGF β overexpression tend to have more invasive and aggressive cancer forms (Heldin et al, 2012).

Generally, EMT is a crucial step for metastasis: the cells escape the contact with the environment by disassembly of cell-cell junctions and develop migratory abilities what allows the transformation into invasive carcinomas (Thiery, 2002; Thiery et al, 2009).

In addition to the described mechanisms, especially the p53 crosstalk (Adorno et al, 2009), epigenetic alterations, and the involvement of TACE-induced cleavage of T β RI and its translocation to the nucleus can support the TGF β -dependent expression of genes for EMT induction (Mu et al, 2011).

Once, the motile phenotype is generated, the cells require further priming for their dispersion in the organism. A molecular target, that has been identified to be relevant for breast cancer cell priming for distant metastasis into lung is effected by Angiopoietin-4 induction (Padua & Massague, 2009). These spreading cells then need to use their invasive capacities in order to enter and colonize in the new tissue. This has been well studied for the metastasis of MDA-MB-231 breast cancer cells as well as the mechanism they use to invade into bone tissue. The cells induce the production of substances that promote osteolysis and enable their nesting in the bone (Yin et al, 1999).

Tumor angiogenesis

Another hallmark of cancer and necessity for survival is neoangiogenesis, providing a tumor with new blood vessel for ensuring metabolism and oxygen provide. A lot of evidence exists for correlation of angiogenesis and TGF β levels (de Jong et al, 2001). TGF β can also promote the expression of VEGF, an important angiogenic driving force. However, dependent on the cancer subtype, also inhibiting effects have been described.
With all these described actions of TGF β , we can conclude that it is a main actor in cancer, with its protecting functions in early stages and its supporting actions on late stage tumors to generate an aggressive and invasive phenotype. This underlines the importance to study the signaling of TGF β in suitable cellular models.

3.4.3 TGFβ signaling as therapeutic target

This multitude of implications of TGF β signaling in physiology and pathophysiology makes clear that TGF β is an interesting therapeutic target. It might also function as biomarker, to identify cancer stages and their malignancy, and TGF β serum levels can serve as marker in cardiovascular diseases or preeclampsia.

The huge beneficial actions of TGF β can claim for a curative enhancement of signaling, for example in osteoporosis, diseases with a hyperactive immune system or in early cancer stages. This can be obtained by administration of ectopic agonist or by the means of gene therapy via an increase of the expression of signaling components.

But with regards to disorders where TGF β signaling is not desired, strategies for its inhibition are required. A lot of effort has been put into the development of antagonizing methods and they include neutralizing antibodies, ligand traps, soluble receptors that function as scavenger, receptor antagonists, antisense oligonucleotides or small molecule receptor inhibitors. Their application can be beneficial to cancer treatment, especially in the late state, the Marfan syndrome or fibrosis. Antibodies against the T β RII to treat glioblastoma are already in the clinical trial phase (Akhurst & Hata, 2012). But still being in its infancy, a further description of the TGF β signaling pathway and the identification of more context-specific molecular targets that are part of the signaling network can improve the development of further therapeutic strategies.

OBJECTIVES OF THIS WORK

After having gained evidence for ligand-independent functions of GPR50 within the heterodimer with the melatonin receptor MT₁ and additional reports on Nogo-A and TIP60, proposing that GPR50 behaves as an interacting and activity-modulating protein, our laboratory tempted to identify further putative binding partners of GPR50. Upon application of the tandem affinity purification (TAP) technique, native GPR50 complexes were purified and subsequent mass spectrometric analysis revealed several potential interaction partners. Among these proteins was the transforming growth factor β receptor type I (T β RI). After a first *in vitro* verification of this interaction, the objectives of my work where the following: a further characterization of the interaction, including revelation of the interacting domains and the establishment of an endogenous complex formation. Another important part of my work was to characterize the impact of GPR50 on TBRI-dependent signal transduction on different levels of the pathway by the use of different methods. After having deciphered GPR50's role in signal transduction, the unraveling of the underlying mechanism displayed another objective of the work. Finally, after the accomplishment of the mechanistic part, the last aim was, to establish a functional role for GPR50 in relation to TGF\beta-dependent (patho)physiological effects. A first model concerned the TGFβ-signaling-deficient gastric cancer cell line SNU638 to gain further insight in signaling mechanisms and the effect of a presence of GPR50. Furthermore, we chose the MDA-MD-231 breast cancer cells as a model to study differences in the absence and presence of GPR50. The idea behind was, to determine the impact of GPR50 on TGF^β signal transduction and to correlate this to functional effects for this novel regulator of TGF β -dependent signal transduction. Research based upon the described axis during this thesis project should enable us to gain information about how and when the complex of GPR50 and T β RI is formed and which are its consequences on signal transduction and (patho)physiological processes.

II. RESULTS

Article

Ligand-independent activation of type I TGF β receptor (T β RI) by the orphan 7-transmembrane protein GPR50 in the absence of the T β RII

1. Introduction

Emerging evidence exists that the orphan 7TM protein GPR50, a member of the melatonin receptor GPCR subfamily that lost its melatonin binding ability during evolution, has ligand-independent functions. This consists in the binding of other proteins and the modulation of their activity, as it has been shown on the membrane level for heteromerisation with the MT₁ (Levoye et al, 2006a) and in the cytosol for Nogo-A (Grünewald et al, 2009) and TIP60 (Li et al, 2011). In order to further establish this role, we sought to identify further putative binding partners for GPR50. By the application of a method specifically established for GPCRs in our laboratory, the tandem affinity purification (TAP) assay (Daulat et al, 2007), we tempted to isolate GPR50-associated complexes spontaneously forming in HEK293 cells. One of the identified proteins is the transforming growth factor β (TGF β) receptor type I (T β RI) serine/threonine kinase, which is part of the TGF β signal transduction pathway.

The TGF β superfamily of cytokines contains molecules, which are important for the maintenance of organism homeostasis as they regulate processes like cytostasis, apoptosis and cellular migration (Massagué, 2000). The TGF β signal transduction pathway is a simple and direct pathway: the ligand binds to one membrane type II receptor, the T β RII, which recruits the T β RI into the complex and propagates the signal through an activating phosphorylation of the T β RI. The activated T β RI then transduces the signal to the R-Smads 2 and 3, which subsequently, after their phosphorylation, form a complex with Smad4, translocate to the nucleus and regulate the transcription of target genes that execute TGF β -mediated biological responses (Shi & Massagué, 2003a).

The ubiquitous TGF β signaling pathway is composed of a various number of ligands and different receptors that can evoke a multitude of cellular responses. This stands in contrast to the simple way of signaling flow that is mediated by a small number of different Smad proteins. Therefore, a lot of regulatory proteins acting on different levels of the pathway have been discovered in the last years. They are implicated in specifying the TGF β signaling pathway in a context-dependent manner upon a multitude of different modes of action (Kang et al, 2009; Xu et al, 2012). In the following work, we wanted to identify the mechanism and mode of action of GPR50 on T β RI dependent signaling. Additionally, we wanted to investigate whether the two frequent variants of GPR50, the GPR50wt and the GPR50 Δ 4 comprising the ⁵⁰²Thr Thr Gly His⁵⁰⁵ deletion in the C-terminus display any functional differences.

The first part of the work focused on the description of the interaction and its character. We used different techniques, in order to validate the interaction, as co-immunoprecipitation and the BRET technique. A study in different cell types upon overexpression and endogenous levels, helped to gain further information about the circumstances of interaction. In order to study the impact of GPR50 in T β RI-dependent signal transduction, the aim was to study the activation status of different levels from receptor phosphorylation onto gene transcription of the TGF β signal transduction pathway in absence and presence of GPR50 Δ 4 and GPR50wt by the use of different techniques, as western blotting, Co-IP, nuclear extraction and reporter gene assay. Furthermore, we studied the molecular mechanisms responsible for signaling activation in the presence of GPR50.

With the purpose of putting our findings into another context, we used the gastric cancer cell line SNU638 which is deficient of an intact T β RII receptor and thus devoid of TGF β signaling. We addressed the question, whether GPR50 has an the capacity to restore T β RI signaling activity.

For the functional assays, we decided for the MDA-MB-231 cells as a model for cancer and TGF β signaling. MDA-MB-231 cells are epithelial mammary gland derived metastatic cells from an invasive breast cancer with highly metastatic potential. Being responsive to TGF β findings based on utilization of this model helped to obtain knowledge about the effects of TGF β signaling in cancer and during cancer progression. They have a hyperactivity of the Ras pathway leading to a less strong TGF β -mediated growth inhibition as a result of the loss of myc repression (Chen et al, 2001). But still, TGF β -dependent anti-proliferative effects are remarkable (Yang et al, 2012), thus MDA-MB-231 cells are forming an appropriate model for

studying the effects of TGF β -mediated cytostasis. In addition, they served as a model to study the effects of metastasis formation due to their intact TGF β signaling and it could be demonstrated, that TGF β promotes their invasion and angiogenesis (Safina et al, 2007). These MDA-MB-231 cells also provided insight into the gene repertoire important for migration of those cells (Kang et al, 2003b) and the molecular basis for metastasis of cells had been deciphered (Padua et al, 2008). In addition, the mechanisms of bone metastasis have been dissected: homing of MDA-MB-231 cells to the bone marrow, its invasion, angiogenesis and osteolysis underlie a specific mechanism: TGF β increases parathyroid hormone-related protein (PTHrP) secretion that leads to production of the osteolytic factor RANKL that promotes osteolysis and enables nesting of MBA-MB 231 cells in bone (Yin et al, 1999).

Though our results indicated a constitutive activation of the T β RI in the presence of GPR50, we decided to use a model with overexpression of GPR50 to induce and study the effects of a constitutive TGF β signaling. The functional effects of GPR50 were addressed in migration studies in a wound-healing assay and proliferation effects were tested *in vitro* in an anchorage-independent-growth-assay and *in vivo* in a xenograft study.

All these different aspects should enable us, to confirm GPR50 as a new interacting partner of the T β RI, to describe its mode of action on activating T β RI in ligand- and T β RII-independent manner and to examine its functional impact on breast cancer development. The details of our project conception and results are topic of the following publication, which implicates all the principal research tasks for this thesis.

2. Article

Ligand-independent activation of type I TGF β receptor (T β RI) by the orphan 7transmembrane protein GPR50 in the absence of the T β RII

Stefanie Wojciech ^{1,2,3}, Zakia Belaid-Choucair ^{3,4}, Raise Ahmad ^{1,2,3}, Anne-Sophie Journé ^{1,2,3}, Avais Daulat ^{1,2,3}, Jean-Luc Guillaume ^{1,2,3}, Anastasia Saade ^{1,2,3}, Nathalie Clément ^{1,2,3}, Thomas Courivaud ⁵, Kenjiro Tadagaki ^{1,2,3}, Philippe Delagrange ⁶, Céline Prunier ⁵ and Ralf Jockers^{1,2,3*}

¹ Inserm, U1016, Institut Cochin, Paris, 75014, France

² CNRS UMR 8104, Paris, France, 75014, France

³ Univ. Paris Descartes, Paris, 75014, France

⁴ Hôpital Necker, CNRS UMR 8147 Paris, 75015, France

⁵ INSERM U938, Centre de Recherche Saint-Antoine, Paris, 75012, France

⁶ Institut de Recherches SERVIER, Suresnes, 92150, France

* Correspondence should be addressed to:

Ralf Jockers, Institut Cochin, 22 rue Méchain, 75014 Paris.

Phone: +331 40 51 64 34; Fax: +331 40 51 64 30; e-mail: ralf.jockers@inserm.fr

Short title: Constitutive activation of TBRI by GPR50

ABSTRACT

The current dogma predicts that transforming growth factor-ß (TGFß) signaling is induced by TGFß binding to the type II TGFß receptor (TßRII), recruitment of TßRI into the complex and trans-phosphorylation of the GS domain of TßRI by TßRII. Here we report the formation of a molecular complex between TßRI and the orphan GPR50 receptor. Binding occurs through the respective transmembrane domains and the ATSHP motif in the cytoplasmic domain of GPR50 that competes with the ATGHP motif of FKBP12, a negative regulator of TGFß signaling, for binding to TßRI. This new complex leads to the spontaneous, ligand-independent activation of TßRI that does not require TßRII. Overexpression of GPR50 in MDA-MB-231 cells promotes *in vitro* cell migration and inhibits tumor formation in a xenograft model. Our results describe a previously unappreciated spontaneous activation mode of TßRI and identify GPR50 as a TßRI co-receptor with potential impact on breast cancer development.

HIGHLIGHTS

- 1. The interaction of GPR50 and T β RI induces constitutive T β RI-dependent signaling
- 2. GPR50 competes with FKBP12 for binding to the T β RI
- 3. GPR50 activates the T β RI in a ligand- and T β RII-independent manner
- 4. Overexpression of GPR50 induces TGFβ-like responses in breast cancer cells

INTRODUCTION

Transforming growth factor β (TGF β) is a cytokine, which regulates many cellular processes and plays an important role during normal embryogenesis due to its multiple effects on proliferation, differentiation, apoptosis and migration (Massague, 2012; Shi & Massagué, 2003b). The deregulation of components of the TGFB pathway is at the basis of many diseases including cancer (Ikushima & Miyazono, 2010; Massague, 2008). TGFB elicits its effects through two single-transmembrane spanning serine/threonine (Ser/Thr) kinases called type I and type II TGFB receptors (TBRI and TBRII, respectively) (Franzen et al, 1993). Binding of TGFB to TBRII triggers the recruitment of TBRI (Wrana et al, 1994). The constitutively active TBRII kinase activates TBRI by phosphorylating several Ser/Thr residues in the highly conserved GS region (¹⁸⁵TTSGSGSG¹⁹²) located N-terminal to the kinase domain of TBRI (Wieser et al, 1995). This induces the so-called "inhibitor-to-substrate" activatory switch, which consists in the dissociation of the FKBP12 inhibitor and the subsequent binding of SMAD2/3 proteins (Huse et al, 2001). Phosphorylation of SMAD2/3 by the TBRI kinase (Zhang et al, 1996) induces their dissociation from the receptor, which then dimerize, form a complex with the Co-SMAD, SMAD4, translocate to the nucleus, and regulate gene transcription upon DNA binding (Heldin & Moustakas, 2012). Alternatively, TGF β can also signal through Smad-independent pathways like the activation of MAP kinases p38 and JNK (Zhang, 2009). The framework of this relatively simple pathway, which is based on one single signaling mediator that shuttles from the receptor to the nucleus to regulate gene expression, was revealed more than a decade ago (Massagué, 2000). Over the last couple of years multiple regulators have been identified that allow a context-dependent integration of the core signaling pathway (Massague, 2012). Most of them are facilitating the onset of signaling after stimulation or are regulating signaling sustainability, which underlines the primary importance of precisely controlling the TGFB/SMAD pathway (Kang et al, 2009; Xu et al, 2012) Among these are positive regulators, such as SARA, that assists in SMAD recruitment to the T β RI (Tsukazaki et al, 1998), the inhibitory SMAD7 (Nakao et al, 1997a), which recruits E3 ligases that induce proteasomal degradation of the receptor complex (Kavsak et al, 2000) and TMEPAI, which interferes with SMAD2/3 phosphorylation (Watanabe et al, 2010).

Only little information exists about the regulation of TGF β signaling in the absence of ligand. As eluded above, FKBP12 has been described as a gatekeeper to prevent ligand-independent signaling by locking T β RI in its inactive conformation (Chen et al, 1997; Wang et al, 1996) (Wang & Donahoe, 2004). Knowing the far-reaching consequences of TGF β signaling, tight regulation of its ligand-independent activity appears to be a crucial issue.

G protein-coupled receptors (GPCRs), also called 7-transmembrane (7TM) spanning proteins, represent the most abundant class of cell surface receptors with approximately 800 members. GPCRs are major drug targets accounting for up to 30% of currently marketed drugs (Rask-Andersen et al, 2011). Many reports indicate that GPCRs have the potential to interact with themselves (homomers) and with other GPCRs or receptors from other families (heteromers) (Maurice et al, 2011a). Within these heteromeric complexes, allosteric regulation of one protomer by the other is often observed. Among the different GPCR members, approximately 100 are considered as orphans for which no endogenous ligand has been identified so far. Apart from the ongoing deorphanization of these receptors, there is increasing evidence for ligand-independent functions of orphan GPCRs (Levoye et al, 2006c). Indeed, by physically interacting with other membrane receptors with know ligand and function, these orphan TBR have not been described yet.

Among these orphan GPCRs figures GPR50, which shares highest sequence homology with melatonin receptors, but which lost its ability to bind melatonin during evolution (Dufourny et

al, 2008; Jockers et al, 2008). The carboxyl terminal tail (C-tail) of GPR50 of approximately 320 amino acids is one of the characteristic features of GPR50. This C-tail has been shown to scaffold several interacting partners (Grünewald et al, 2009; Li et al, 2011) and to modulate in a ligand-independent manner the activity of other membrane receptors such as the melatonin MT_1 receptor within heteromeric complexes (Levoye et al, 2006b). In addition, a frequent sequence variant (MAF=0.4) lacking 4 amino acids (⁵⁰²TTGH⁵⁰⁵) of the C-tail (GPR50 Δ 4) exists in humans and has been associated with mental disorders (Thomson et al, 2005) and altered lipid metabolism (Bhattacharyya et al, 2006).

We report here for the first time the complex formation between TBRI and GPR50, an orphan 7-transmembrane spanning GPCR. Within this complex composed of TBRI and GPR50, but not TBRII, GPR50 enhances the basal, TGFB-independent, capacity of TBRI to activate SMAD2/3, most likely by excluding the inhibitory FKBP12 from binding to TBRI and by stabilizing activatory conformations in TBRI.

RESULTS

GPR50 interacts with TBRI

To identify novel interacting proteins of GPR50, we applied the tandem affinity purification TAP) protocol recently optimized for GPCRs (Daulat et al, 2007) by fusing the TAP-tag to the carboxyl terminal tail of the full-length human GPR50 Δ 4 variant and by stably expressing the GPR50 Δ 4-TAP construct in HEK293T cells. Mass spectrometric analysis of purified proteins identified 5 unique peptides corresponding to the TBRI in 3 independent purifications but not in control purifications with naïve HEK293T cells (Figure 1A). Co-immunoprecipitation experiments in HEK293T cells confirmed that the human TBRI constitutively interacts with the human GPR50 Δ 4 variant and the GPR50wt isoform (Figure 1B). Similar results were observed in breast cancer MDA-MB-231 cells stably expressing

either GPR50 Δ 4 or GPR50wt (Figure 1C). To evaluate the role of TGF β activation on this interaction, HEK293T cells were stimulated for 2 hours with TGFB or transfected with the constitutively active TBRI T204D mutant (TBRI ca) (Figure 1B). The amount of coprecipitated GPR50 was not altered indicating that the TBRI/GPR50 interaction occurs independently of the activation state of T β RI. To further validate these results in intact cells, we performed bioluminescence resonance energy transfer (BRET) donor saturation experiments. The *Renilla* luciferase 8 (Rluc8) energy donor and the yellow fluorescent protein (YFP) energy acceptor were fused to the carboxyl terminus of TBRI. Co-transfection of a fixed amount of TBRI-Rluc8 expression plasmid and increasing amounts of TBRI-YFP, GPR50wt-YFP or GPR50∆4-YFP (Figure 1D) resulted in a hyperbolic saturation curve with increasing YFP/Rluc ratios for all receptor combinations, reflecting a specific interaction between BRET donor and acceptor pairs (BRET₅₀=1.342±0.185, 0.025±0.006 and $0.031\pm0.0.010$ (n=3-4)) for TBRI homomers and TBRI/GPR50wt and TBRI/GPR50\Delta4 heteromers, respectively. Incubation with TGFB had no apparent effect on the BRET signal of TBRI/GPR50wt and TBRI/GPR50A4 heteromers within the first 30 minutes after addition (Supplementary Figure 1A,B). Expression of TBRI-Rluc8 donor with the insulin receptor (IR)-YFP or leptin receptor (OBRa)-YFP negative controls resulted in a linear, non-saturable BRET increase, characteristic of random interactions. Overall, co-immunoprecipitation and BRET experiments confirm the formation of a constitutive TBRI/GPR50 complex in HEK293T and MDA-MB-231 cells.

Formation of a TBRI/TBRII complex is an obligatory step of the current TGFB receptor activation model. To verify whether TBRII is necessary for the formation of the TBRI/GPR50



complex, we used gastric carcinoma SNU 638 cells, which are devoid of T β RII.

Figure 1. GPR50 interacts with the T β RI

(A) Tandem affinity purification was performed with naïve HEK293T cells or HEK293T cells stably expressing GPR50Δ4-TAP. After purification, mass spectrometry was employed for protein identification.

(B) HEK293T cells were co-transfected with GPR50 Δ 4 or GPR50wt and HA-T β RI-wt or HA-T β RI-ca. Stimulation with 2 ng/ml TGF β was done for 1 h. Cell lysates were subjected to co-immunoprecipiation with anti-HA antibody and blotted against GPR50. Expression was checked in total lysates using anti-GPR50 and anti-HA antibodies.

(C) MDA-MB-231 stably expressing GPR50 Δ 4 or GPR50wt cell lysates were precipitated with an anti-GPR50 antibody, the binding of T β RI was checked with an anti-T β RI antibody. Lysates were used to reveal the total expression level.

(F) HEK293T cells were transfected with HA-TβRI and the indicated GPR50 constructs. Lysates were precipitated with an anti-HA antibody and blotted against GPR50 and GFP. Total lysates were analyzed for expression with anti-HA-, anti-GPR50- or anti-GFP antibody.

TBRI was readily co-immunoprecipitated with GPR50 Δ 4 in these cells indicating that TBRII is not necessary for the formation of the TBRI/GPR50 complex (Figure 1E).

To characterize the molecular determinants involved in the interaction, we expressed the TM

domain (GPR50ACter) and the carboxyl terminus of GPR50 (GPR50Cter) separately (Figure

F). Only the GPR50 ACter construct co-immunoprecipitated with TBRI indicating that the TM

region of GPR50 contains the major molecular determinants necessary for the TBRI/GPR50

interaction. Taken together, the TBRI/GPR50 complex is insensitive to TGFB stimulation,

occurs in the absence of TBRII and involves predominantly the TM domain of GPR50.

Expression of GPR50 induces basal T β RI-dependent signaling

Our next aim was to study the effects of GPR50 expression on TGFβ signaling. We first evaluated the basal SMAD2/3 phosphorylation state, which was dramatically increased in the presence of GPR50 (Figure 2A,B). The effect was prevented by pre-incubating cells with the TβRI kinase specific SB 431542 inhibitor (Figure 2C). Increased basal activation of the SMAD pathway in the presence of GPR50 was also observed at further downstream steps like complex formation between SMAD2/3 and SMAD4 (Figure 2D,E), nuclear translocation of SMAD2/3 (Figure 2F) and ARE- and CAGA-dependent reporter gene assays (Figure 2G,H). Collectively, the GPR50Δ4 variant showed more pronounced effects on basal activity of the SMAD pathway than the GPR50wt variant. Depending on the specific signaling event

⁽E) SNU638 cells were co-transfected with HA-T β RI and GPR50 Δ 4 plasmids, GPR50 was precipitated with an anti-GPR50 antibody and blotting against T β RI was performed with an anti-T β RI antibody. Total lysates were used as expression control.

monitored, TGFB stimulation had only marginal to significant effects on the increased basal activation of the SMAD pathway in the presence of GPR50 than the GPR50wt variant.



Figure 2. Expression of GPR50 induces ligand-independent activation of T βRI -dependent signaling

(A and B) HEK293T cells were transfected with myc-SMAD2 (A) or myc-SMAD3 (B), and GPR50Δ4 or GPR50wt. Cells were starved overnight and stimulated with 2 ng/ml TGFB for one hour. To test SMAD2 phosphorylation, myc-SMAD2 was enriched with preceding precipitation with an anti-myc antibody. SMAD3 lysats were directly separated by SDS-PAGE. Immunoblots were revealed with p-SMAD2- or p-SMAD3 antibodies respectively, anti-SMAD2/3 and anti-GPR50 antibodies were used to check expression.

(C) HEK293T cells were transfected and prepared as described in (A and B) but additionally treated overnight with 10 μ M of SB 431542 T β RI-kinase activity inhibitor before stimulation.

(D and E) To check SMAD2- or SMAD3 complex formation with SMAD4, cells were transfected with corresponding myc- or FLAG-tagged constructs, respectively, in the absence or presence of GPR50Δ4 and GPR50wt. Additional transfection of a constitutive active HA-TβRI-ca served as positive control. SMAD4 was precipitated with an anti-FLAG antibody and bound SMAD2 or SMAD3 was revealed upon blotting against SMAD2 or SMAD3 via the myc-tag. Total lysates were used to determine total expression.

(F) Cells were transfected as in (A and B) and nuclear extracts were prepared. The presence of SMAD3 in the nucleus was detected by the use of an anti SMAD2/3 antibody. Lamin- and Tubulinexpression were checked to ensure purity of the nuclear extracts and GPR50 expression was controlled in the cytosolic fraction.

(G and H) To test TGFβ-dependent transcription of genes, HeLa cells were transfected with a Firefly-Luciferase coupled ARE- or CAGA- promoter construct and a Renilla Luciferase for normalisation. Increasing doses of GPR50Δ4 and GPR50wt were added to the cells. Cells were stimulated overnight with 2 ng/ml of TGF β and luciferase activity was measured. Figures show a representative experiment performed in triplicates as mean \pm SEM (* = p<0,05). (I) Cell were transfected as in (A and B), cell lysates were checked for phosphorylation of p38 with a

phospho-p38 antibody. Total p38 and GPR50 plasmid expression were blotted as control.

(J) HEK293T cells were transfected with GPR50∆4 and GPR50∆Cter and treated as described in (A and B). GPR50 expression was revealed with an anti-GPR50 or -GFP antibody.

(K) For reporter gene assay, HeLa cells were transfected with the CAGA-Firefly Luciferase construct, the Renilla Luciferase and constant doses of GPR50∆4 and GPR50∆Cter. Cells were further treated and analyzed in reporter gene assay as in (G and H).

Depending on the specific signaling event monitored, TGFB stimulation had only marginal to significant effects on the increased basal activation of the SMAD pathway in the presence of

GPR50. Significantly increased basal activation was also observed for the non-canonical p38

signaling pathway (Hanafusa et al, 1999; Yu et al, 2002) in the presence of GPR50 Δ 4 with no

further increase upon TGF^B treatment and to a much smaller extend for GPR50wt suggesting

that TGFB-independent activation of TBRI by GPR50 is not restricted to the SMAD2/3

pathway but can also be extended towards the p38 pathway (Figure 2I).

We next wanted to define the molecular determinants of GPR50 involved in increased TGFB

signal transduction. Surprisingly, the GPR50 \Delta Cter construct was unable to increase the basal

SMAD phosphorylation suggesting that despite the fact that the TM domain of GPR50 is involved in the interaction with TBRI, it is not sufficient for the functional effect on the

SMAD pathway (Figure 2J). The absence of effect of the GPR50 Δ Cter construct was further

confirmed in the CAGA reporter gene assay (Figure 2K). These observations suggest that the cytosolic C-tail of GPR50 is somehow involved in the functional effect of GPR50 on the SMAD pathway, a hypothesis that is also consistent with the differential effects observed for the two GPR50 variants that differ by a 4 amino acid deletion/insertion in the C-tail.

Collectively, these data show that GPR50 promotes T β RI-dependent signaling in the absence of TGF β ligand through the SMAD2/3 and p38 pathways. This effect tends to be more pronounced for the GPR50 Δ 4 variant and relies on the C-tail of GPR50 and the T β RI kinase activity.

GPR50 interferes with FKBP12 binding to TBRI

We then set out to identify the molecular mechanism by which GPR50 potentiates the basal activation of the TBR/SMAD pathway. Initial radioligand binding competition experiments with ¹²⁵I-TGFB could rule out the potential modification of TGFB receptor cell surface expression or improved affinity of TGFB for its receptor (Supplementary Figure 2A). Furthermore, induction of TGFB production or secretion of cells by GPR50 expression is unlikely to occur as supernatants of cells expressing GPR50 were unable to promote SMAD phosphorylation in naïve cells (Supplementary Figure 2B).

The absence of effect of the GPR50 Δ Cter construct on the basal activation of the SMAD pathway hints to the potential implication of the C-tail of GPR50 in this effect. Along this line, we decided to investigate the potential effect of GPR50 on proteins interacting with the TBR. We first verified the possibility of a direct interaction of GPR50 with SMAD2 or SMAD4 that might facilitate the recruitment of these proteins to the TBR and thus potentiate the response of the TBR/SMAD pathway. Co-immunoprecipitation experiments between GPR50 and SMAD2 or SMAD4 did not provide any support for this hypothesis (Supplementary Figure 3). We then focused our attention on FKBP12, which binds to TBRI in

its inactive conformation thus stabilizing TBRI in its inactive state and preventing SMAD2/3 binding (Chen et al, 1997; Huse et al, 2001). FKBP12 has been classified as TGF β signaling gatekeeper as its depletion is causing hypersensitivity towards T β RII, leading to transphosphorylation of T β RI and subsequent induction of downstream signaling. We therefore tested the influence of GPR50 on the interaction of T β RI and FKBP12 by coimmunoprecipitation. Coexpression of GPR50 Δ 4 and GPR50wt variants diminished the amount of FKBP12 interacting with T β RI by 70% and 40%, respectively (Figure 3A). This effect could not be explained by the scavenging of FKBP12 by GPR50, as GPR50 did not interact directly with FKBP12 (Figure 3B). Collectively, these results suggest that GPR50

competes with FKBP12 for binding to TBRI.

To further characterize the relationship between FKBP12 and GPR50, we used the FK506 macrolide compound, which is known to bind to FKBP12 at a site that overlaps with binding to TBRI (Wang et al, 1994). Pre-incubation of cells with FK506 in the absence of GPR50 triggered indeed the expected increase in basal SMAD3 phosphorylation, an effect that was not further increased in the presence of GPR50 (Figure 3C). The overexpression of FKBP12 completely abolished basal SMAD3 phosphorylation irrespective of the absence of presence of GPR50. The fact that the effects of GPR50 on basal SMAD3 phosphorylation can be blocked by FKBP12 overexpression and that the effects of GPR50 and FK506 are not additive are compatible with a common action mechanism, the competition of GPR50 and FK506 with FKBP12 for TBRI binding. To further verify this hypothesis, we aimed to identify the molecular basis of this competition. Sequence analysis of the C-tail of GPR50 and FKBP12 revealed the existence of a repetitive 5 amino acid motif (**AXZHP**) (X=A, T, S; Z=G, S) in GPR50 that is similar to the ⁸⁴ATGHP⁸⁸ motif of FKBP12 (Figure 3D, left part). Interestingly, the ⁸⁴ATGHP⁸⁸ motif of

FKBP12 corresponds to a loop that is part of the binding pocket in the co-crystal structure of



Figure 3. GPR50 is competing with FKBP12 for the binding to TβRI due to a similarity motif

(A) (Left) HEK293T cells were transfected with HA-T β RI and myc-FKBP12 and either GPR50 Δ 4 or GPR50wt. Lysats were precipitated for FKBP12 using an anti-myc antibody and blotted with an anti-T β RI to reveal complex formation. Total lysats were addressed for expression of myc-FKBP12, HA-T β RI and GPR50 with corresponding antibodies. (Right) Three independent experiments were densitometrically analyzed and data represent the mean ± SEM (* = p<0,05).

(B) Co-immunoprecipation was performed according (A) with cells co-transfected with myc-FKBP12 and either GPR50 Δ 4 or HA-T β RI. Precipitates were blotted against GPR50 and T β RI.

(C) HEK293T cells were transfected with myc-SMAD3, myc-FKBP12 and GPR50 Δ 4 as indicated. Cells were starved and stimulated for 1h with 2 ng/ml of TGF β or of 100 ng/ml FK506. Total lysates were immunoblotted for SMAD3-phosphorylation and total expression of myc-SMAD3, GPR50 and myc-FKBP12 with suitable antibodies.

(D) Sequence alignment of FKBP12 and GPR50 revealed sequence similarities between a C-terminal motif in FKBP12 and a repetitive sequence in GPR50 (upper left panel). Analysis of localization of the repetitive motifs in GPR50 Δ 4 and GPR50wt shows the proximity of one to the Δ 4 deletion (lower left panel). Existing structural data from Huse et al. (Huse et al, 1999) highlight the implication of the HP loop in binding to the T β RI (right).

(E) HEK293T cells were co-transfected with HA-TβRI or the HA-TβRI-P194K mutant and the indicated FKBP12 constructs. Co-immunoprecipitation was performed as in (A).

(F) HEK293T cells were transfected with indicated plasmids and precipitated as in (A)

(G) Reportergene assay was performed as in (2 G and H) with cells transfected with constant doses of the GPR50 constructs. Figure shows one representative experiment performed in triplicates as mean \pm SEM (* = p<0,05).

FKBP12 and the unphosphorylated GS region and kinase domain of TBRI (Huse et al, 1999)

(Figure 3D, right part). To directly demonstrate the importance of the ATGHP loop of

FKBP12 in TßRI binding, we designed two mutants predicted to abolish FKBP12 binding to

TBR1 (FKBP12-H87L, FKBP12ΔHP). Figure 3E shows that both mutants are unable to bind

TBRI similar to the previously reported TBRI-P194K mutant that served as a positive control

of the loss of interaction (Chen et al, 1997).

To address the importance of the **AXZHP** motifs in GPR50, we concentrated on the ${}^{495}\text{ATSHP}{}^{499}$ motif located next to the ${}^{502}\text{TTGH}{}^{505}$ deletion in the GPR50 Δ 4 variant. Disruption of the ${}^{495}\text{ATSHP}{}^{499}$ motif (H498L, Δ HP) in the GPR50 Δ 4 variant fully restored FKBP12 binding to levels seen in the absence of GPR50 (Figure 3F). Similar observations were made at the reporter gene level using a CAGA promoter construct underlining the importance of the ${}^{495}\text{ATSHP}{}^{499}$ motif of GPR50 (Figure 3G).

Taken together, whereas GPR50 and TBRI interact mainly through their respective transmembrane domains, the functional effect of GPR50 on TBRI signaling relies on the ⁴⁹⁵ATSHP⁴⁹⁹ motif in the C-tail of GPR50 that competes with the ATGHP loop of FKBP12 for binding to TBRI. Displacement of FKBP12 contributes at least partially to the potentiation of the basal activity of the TBR/SMAD pathway in the presence of GPR50. The proximity of the ⁴⁹⁵ATSHP⁴⁹⁹ motif in GPR50 to the ⁵⁰²TTGH⁵⁰⁵ deletion might be at the origin of the

more pronounced effect of the GPR50 Δ 4 variant as compared to GPR50wt to modulate the TBR/SMAD pathway.

GPR50 activates TBRI-dependent signaling in the absence of TBRII

According to the current dogma, TBRII fulfils two essential functions in the TBR activation process, namely binding of TGFB and transphosphorylation of TBRI in the GS region (Wieser et al, 1995). As our results presented in figure 1E show that formation of the GPR50/TBRI complex does not require TBRII, we asked the question whether TBRI with GPR50 could activate SMAD3 in the absence of TBRII. Native SNU638 are indeed devoid of any TGFB response as monitored at the level of SMAD3 phosphorylation and expression of TBRII in these cells restores the TGF^B responsiveness (Figure 4A). Signaling through this pathway was similarly restored by exogenous TBRII expression at the level of CAGA-driven reporter gene activation (Figure 4B). A potentiating effect of GPR50 on the basal activation of the TBR/SMAD pathway in the absence of TBRII was observed on the level of SMAD3 phosphorylation in SNU638 cells (Figure 4C). This effect was entirely dependent on the TBRI kinase activity, as sensitive to the SB 431542 inhibitor (Figure 4D). A similar increase in basal activity in the presence of GPR50 was observed at the level of the CAGA reporter gene assay (Figure 4B). No further increase was observed upon TGF^β addition, as expected, in the absence of TBRII. To explore the possibility that the effect of GPR50 is dependent on FKBP12 release from TBRI as observed in the presence of TBRII, we overexpressed FKBP12 in SNU638 cells and monitored SMAD3 phosphorylation. Overexpression of FKBP12 clearly reduced the effects evoked by GPR50 further supporting the hypothesis that GPR50 competes with FKBP12 for binding to $T\beta RI$ (Figure 4E).

Taken together, we established that GPR50 expression in SNU638 cells can induce ligandindependent activation of T β RI/SMAD signaling, which is sensitive to FKBP12 expression levels and dependent on TBRI kinase activity. Activation of TBRI kinase activity in the absence of TBRII establishes a new operation mode of TBRI in the complex with GPR50.



Figure 4. SNU638 cells show the capacity of GPR50 to activate T_βRI independently of T_βRII.

(A) SNU638 cells were transfected either with Mock or HA-T β RII plasmid and stimulated for one hour with 2 ng/ml of TGF β . Lysats were immunoblotted for phospho-SMAD3 and total SMAD3 and expression of T β RII plasmid was revealed with an anti-HA-antibody.

(B and D) SNU638 cells were transfected with the indicated plasmids, stimulated during one hour with 2 ng/ml TGF β and in (D) additionally pretreated over night with SB 431542 at 10 μ M. Cells were lysed and blotted as described before in (2A and B).

(C) For the reporter gene assay HA-T β RII-, GPR50 Δ 4- and GPR50wt transfected SNU638 cells were treated as in (2H). Data represent the mean ± SEM (* = p<0,05) of one representative experiment performed in triplicates.

(E) SNU638 cells were transfected with the indicated plasmid and lysats were blotted with an pSMAD3 antibody. Additionally, HA-T β RII, GPR50 and FKBP12 expression were verified with corresponding antibodies.

GPR50 expression in MDA-MB-231 cells is sufficient to promote TGFβ resembling effects

To further establish the functional relevance of GPR50 expression on the TBR/SMAD2/3 pathway, we choose the well-described and widely used TGFβ responsive MDA-MB-231 breast cancer cell line We first recapitulated the ligand-independent basal signaling in the presence of GPR50 and the presence of the GPR50/TBRI complex in this cell model (Figure 5A) and then the interaction between GPR50 and TBRI (Figure 5B). We then established stable cell lines expressing similar levels of GPR50wt or GPR50ACter (Figure 5C) and performed several functional tests. The effect of GPR50 expression on the migratory ability of MDA-MB-231 cells was assessed in the wound healing assays (Figure 5D). Analysis of wound closure after 30 hours showed, that the presence of GPR50 in MDA-MB-231 cells increased the migratory capacities of these cells as seen by the higher number of cells in the wound area (Figure 5D). The same effect was observed when cells were treated during the wound healing process with TGF β , suggesting a common mechanism (Figure 5D). In the soft agar anchorage-independent growth assay, cells stably overexpressing the GPR50 Δ 4 variant showed an inhibition of 60% in the number of colonies formed during nearly 3 weeks (Figure 5F). Apart from the number of colonies, their size was also reduced in cells expressing the GPR50Δ4 variant as compared to mock and GPR50wt transfected cells. Similar results were obtained in xenograft experiments. Monitoring tumor growth over 34 days in nude mice injected with MDA-MB-231 cells in the flanks revealed that the presence of GPR50wt or GPR50 Δ 4 slows down tumor growth compared to mock-injected mice starting from day 23 until the end (day 34) (Figure 5E).



Figure 5. Overexpression of GPR50 in MDA-MB-231 cells induces promigratory and antiproliferative effects.

(A) MDA-MB-231 cells were transiently transfected with GPR50Δ4 or GPR50wt and lysats were tested for SMAD 2 and SMAD 3 Phosphorylation and SMAD 2/3 and GPR50 total expression.

(B) For the Co-IP, MDA-MB-231 cells were transiently transfected as indicated and IP was performed as indicated in (1E).

(C) Analysis of GPR50 expression in lysates of stably overexpressing MDA-MB-231 cells

(D)MDA-MB-231 cells were seeded into IBIDI μ plates, after removal of the insert, cells were stimulated with 2 ng/ml of TGF β and migration of the cells and surface closure was monitored every 6 hrs. Images show representative migration after 30 hrs of one experiment out of three.

(E) Anchorage-independent growth assay of MDA-MB-231 cells stably overexpressing GPR50 monitored for 18 days. Images show an example of colony size and distribution. Diagram shows the mean value \pm SEM of the colony number of 3 dishes for each condition in one representative experiment.

(F) Xenograft experiments after injection of MDA-MB-231 cells into the flanks of nude mice. Images show 5 representative out of 10 (8 for the GPR50 Δ 4 condition) tumors. The graph shows the development of the tumor growth during 34 days.

DISCUSSION

We describe here a previously unappreciated activation mode of TBRI when engaged into a molecular complex with the orphan GPR50 receptor. Whereas in the classical mode of action binding of TGFB to TBRII promotes the association and phosphorylation of TBRI by TBRII, complex formation of TBRI with GPR50 leads to a spontaneous and ligand-independent activation of TBRI and induction of downstream signaling through TBRI regulated pathways. Spontaneous activation of TBRI involves reduced binding of FKBP12 to TBRI and was sufficient to promote several TGFB-like physiological responses like promotion of migration, reduction of anchorage-independent growth *in vitro* and reduction in tumor growth in a xenograft mouse model. Complex formation between GPR50 and TBRI is likely to fine-tune the TBRI signaling capacity in a cell context-dependent manner, defines a new ligand-independent function for the orphan GPR50 receptor and represents the first example of direct crosstalk between a member of the GPCR super-family and TBRs.

Activation mechanism of TBRI in the GPR50/TBRI complex

Our results indicate that TBRI activation in the GPR50/TBRI complex is different from the activation mode in the TBRII/TBRI complex. Whereas TBRI activation in the TBRII/TBRI complex is dependent on TGFB binding to TBRII, complex formation with TBRII and phosphorylation of TBRI by TBRII (Wrana et al, 1994), the GPR50/TBRI complex appears to be independent of TBRII interaction and phosphorylation. In contrast, both activation modes



Figure 6. Proposed Model of GPR50 action on T_βRI and T_βRI-dependent signaling

In the basal state (upper part), the T β RI and the T β RII form homodimers, that are apart from each other. The T β RI is stabilized in its inhibitory conformation by FKBP12 where H87 and P88 contact the region around the GS domain. The R-SMADs are non-phosphorylated in the cytosol and no transcription of target genes occurs. In the classical activation mode (lower left side), the TGF β ligand binds to the T β RII, which enables recruitment of T β RI into the complex. The T β RI gets phosphorylated in the GS domain by the T β RII, what is accompanied by a dissociation of FKBP12 and the stabilization of the active conformation. Hence, the T β RI exerts its kinase activity on the R-SMADS 2/3, which in turn form a complex with Smad4, translocate into the nucleus, bind to DNA and regulate gene transcription. If the T β RI forms a complex with GPR50 (lower right side), GPR50 induces the dissociation of FKBP12 from the T β RI due to a similarity motif of the amino acids H87 and P88 in its C-tail on position 498 and 499. Furthermore, GPR50 stabilizes the active conformation, in a classical manner via SMAD2/3 and SMAD 4 to the nucleus where they regulate gene expression.

converge further downstream on the level of TBRI kinase activation, which is necessary for SMAD2/3 signaling. These results raise the question how the TBRI kinase is activated in the TBRI/GPR50 complex in the absence of TBRII? The simplest explanation would be that GPR50 behaves as a TBRII-like receptor that contains a kinase that phosphorylates TBRI. Since the cytoplasmatic domain of GPR50 shows no homology to any know kinase, this scenario appears very unlikely. A further option might be that GPR50 recruits a kinase into the TBRI/GPR50 complex, which then phosphorylates TBRI. Although we are unable to rule this possibility completely out at the current stage, no Ser/Thr kinase associated with GPR50 has been identified in our TAP assay and there are no such candidates reported in the literature. To better understand the potential influence of GPR50 on TBRI, a closer look on the functional consequences of TBRI phosphorylation might be insightful. Phosphorylation of TBRI by TBRII on a yet to be identified site is believed to dissociate FKBP12 from TBRI thus liberating the two contact points of FKBP12 on TBRI, the L45 loop and the GS region, the latter, which can then be phosphorylated by the TßRII kinase at multiple Ser/Thr residues in the GS sequence (TTSGSGSG). A further, more indirect, consequence of FKBP12 dissociation is the destruction of the inhibitory wedge that inhibits the TBRI kinase in the presence of FKBP12. In the next step, SMAD2/3 proteins are recruited to TBRI through the phosphorylated GS sequence and the L45 loop of TBRI completing the so-called "inhibitor-to substrate-binding switch" (Huse et al, 2001). SMAD2/3 signaling is then initiated by phosphorylating SMAD2/3 by TBRI and dissociation of P-SMAD2/3.

What happens in the GPR50/TBRI complex? We have shown that the ⁴⁹⁵ATSHP⁴⁹⁹ motif of GPR50 competes with the ⁸⁴ATGHP⁸⁸ motif of FKBP12 for binding to the L45 loop of TBRI. Apart from this similarity, the binding mode of FKBP12 and GPR50 to TBRI appears to be different. Formation of the inhibitory wedge is unlikely to occur in the GPR50/TBRI complex as TBRI is constitutively active in this complex. Binding of GPR50 to the GS region, if it

occurs at all, will be different from binding of FKBP12 as the latter is highly dependent on the activation state of TBRI and the phosphorylation status of the GS sequence. Indeed, GPR50 binding is independent of the activation state of TBRI. Altogether, this shows that GPR50 competes with FKBP12 for binding to TBRI but the binding modes are likely to be fundamentally different providing a plausible molecular bases for the striking functional differences of FKBP12 binding (inhibition of TBRI) and GPR50 binding (activation of TBRI). Several questions remain still open concerning the precise impact of GPR50 on TBRI activity. For example, is the absence of FKBP12 in the GPR50/TBRI complex sufficient to explain constitutive phenotype? The amplitude of the SMAD2/3 signaling pathway activation of the GPR50/TBRI complex and independence of TBRII, together with the likely differences in the interaction modes of FKBP12 and GPR50 with TBRI argue for a possible additional effect on the stabilization of active TBRI conformation by GPR50. A second open question concerns the phosphorylation status of the GS sequence in the GPR50/TBRI complex. Based on the absence of the TBRII kinase, the GS sequence is predicted to be unphosphorylated. Furthermore, according to our current knowledge, TBRI is unable to phosphorylate itself yet its kinase activity is required for signaling (Bassing et al, 1994; Carcamo et al, 1995). Whether this is also true in the GPR50/TBRI complex remains to be established. Lastly, if the GS sequence turns out to be unphosphorylated, the binding mode of SMAD2/3 to the GPR50/TBRI complex is likely to be different. An intrinsic affinity of SMAD2/3 for GPR50, suggesting the stabilization of a common complex, can be ruled out according to our data.

Other constitutively active forms of TBRI:

Constitutive TBRI activity has been previously observed for receptors of the TGFB family. The TBRI-T204D mutant, which does not interact with TBRII anymore, constitutively activates the SMAD2/3 signaling pathway. Position 204 is part of the RTI sequence adjacent to the kinase domain, which is not phosphorylated itself but has a positive allosteric effect on the phosphorylation of the GS sequence. This mutant receptor does not interact anymore with FKBP12 and shows increased TBRI kinase activity *in vitro* (Wieser et al, 1995). This mutant shows that, similar to GRP50, activation of TBRI is possible in the absence of TBRII, most likely by stabilizing an active conformation of TBRI. However, in contrast to the GPR50/TBRI complex, the TBRI-T204D mutant is still sensitive to TBRII as TGFB stimulation generates a further increase of SMAD2/3 signaling.

Another reported case of a constitutively active TBRI is the naturally occurring R206H mutant of the activin A receptor type I (ACVRI). This mutant is associated with fibrodysplasia ossificans progressiva, a rare genetic and catastrophic disorder characterized by progressive heterotopic ossification (Song et al, 2010). Similar to the constitutively active T204D mutation of TBRI, the R206H mutation is located in the part of the GS region that is close to the kinase domain and that allosterically regulates phosphorylation of the GS sequence. Molecular analysis revealed indeed a modest constitutive activity and impaired FKBP12 binding of the R206H mutant (Groppe et al, 2011). Recent studies indicate that the simple presence of TBRII, but not its kinase activity nor TGFB binding capacity, is necessary for the constitutive activity of the R206H mutant. This suggests that in the context of an activating TBRI mutant, the scaffolding function of a co-receptor like TBRII is sufficient for TBRI signaling (Bagarova et al, 2013).

More evidence for constitutive TBRII-independent activation of TBRI comes from DAF-1, the TBRI of *C. elegans* (Gunther et al, 2000). Interestingly, signaling of DAF-1 can occur in the absence of TBRII (DAF-4) kinase activity and promote larval development. Differences in the structure of the GS region of DAF-1 in comparison to other TBRI isoforms are possibly at the origin of this autonomous signaling capacity of TBRI. In addition, DAF-1 can also signal through the more classical DAF-1/DAF-4 complex. This example suggests that the TBRII-

independent signaling mode might have occurred early in evolution providing different options to fine-tune the TBRI signaling capacity.

Furthermore, the recently reported activation of TBRI by exposing glomerular mesangial cells to stretch in the absence of any TGFB provides further supports for the existence of alternative activation modes of TBRI (Chen et al, 2013)

Taken together these examples support the notion that TBRI has an intrinsic capacity to be constitutively active and that this activity can be assisted/amplified by the presence of other receptors like TBRII or GPR50 that solely function as scaffolding proteins.

Formation and regulation of the complex

Formation of the GPR50/TBRI complex adds a further dimension of the regulation of TBRI signaling, which is likely to happen in a cell context-dependent manner. Whereas expression of TBRI is widespread, the expression pattern of GPR50 is more restricted. Expression of GPR50 has been mainly studies in the brain and identified in the pituitary, the dorsomedial hypothalamus, tanycytes, the median eminence and the CA4 region of the dentate nucleus of the hippocampus (Batailler et al, 2011; Gubitz & Reppert, 1999b; Hamouda et al, 2007a; Sidibe et al, 2010). Expression of GPR50 in peripheral tissue is less well documented. The GPR50 mRNA has been observed in eye, testis, kidney, adrenal, intestine, lung, heart, ovary and skin (Drew et al, 2001). In addition, GPR50 expression has been shown to be highly regulated during different developmental stages with highest expression at E18 (Grunewald et al, 2012).

With regards to known expression of GPR50 and putative *in vivo* occurrence of the crosstalk, TGF β signaling and T β RI expression has been observed in the *median eminence* (Prevot et al, 2010; Prevot et al, 2000), a region with high GPR50 levels, and might have an impact on the regulation of hormones implicated in reproduction. TGF β signaling has also been reported to

specifies axons during brain development (Yi et al, 2010). As GPR50 is highly expressed in late embryonal stages (Grunewald et al, 2012) and is implicated in neurite outgrowth (Grunewald et al, 2009), this crosstalk might be of relevance during brain development or in synaptic plasticity of the adult brain.

Expression of GPR50 seems to be highly regulated. Significant variation of GPR50 expression has been observed depending on the photoperiod (Barrett et al, 2006), the energy content of the diet and the nutritional status (fed/fasted) of the animal (Ivanova et al, 2008). Proteolytic cleavage of the C-tail of GPR50, as reported recently, might be another way to regulate constitutive activation of TBRI (Grünewald et al, 2009; Li et al, 2011) as the truncated GPR50 Δ Cter construct was devoid of any effect on TBRI function.

Differential action of regulators such as GPR50 in time and space is likely to add to our understanding of how the cellular context determines the response to TGF β .

Little is known about modified expression levels in cancer tissues. With regards to cancers, some studies detected an upregulation of GPR50 in early cancer states or tumorigenic tissue in pancreatic neoplasia (Buchholz et al, 2005) and nicotine-induced cellular transformation (Bavarva et al, 2013). Our results show that GPR50 might have a beneficial effect on tumor size and growth as shown in the xenograft experiments. Specific assays should help in the future to decipher the precise effect of GPR50 on tumor growth. Furthermore, the study of breast cancer microarrays/expression data will help to gain information about expression levels of GPR50 in tumor tissue.

Revelation of first functional differences of the two frequent GPR50 variants

Genetic association studies identified two common GPR50 variants, GPR50wt and GPR50 Δ 4, in the general population (Thomson et al, 2005). The GPR50 gene is located on the X

chromosome, in the Xq28 region. The GPR50 Δ 4 variant is associated with a higher risk for bipolar affective disorder (BPAD), major depression disorder (MDD) especially in females (Thomson et al, 2005). In another study, the GPR50 Δ 4 variant was associated with higher blood levels of fasting triglycerides and lower HDL levels (Bhattacharyya et al, 2006). Up to date no functional differences have been reported between the GPR50 Δ 4 variants. Our study provide a first hint for the existence of such functional differences, which seem to be directly related to structural elements such as the ⁴⁹⁵ATSHP⁴⁹⁹ motif located in close proximity to the ⁵⁰²TTGH⁵⁰⁵ insertion/deletion. The ATSHP motif corresponds actually to one out of seven similar repetitive motifs within the C-terminal domain of GPR50 (Dufourny et al, 2008). Similar motifs are found in the C-terminal repeat domain of the RNA polymerase II, that functions as scaffold for auxiliary transcription factors in a phosphorylation-dependent manner. A similar function can be postulated for the GPR50 repeats, which is possibly modulated depending on the presence of the ⁵⁰²TTGH⁵⁰⁵ insertion/deletion motif.

Crosstalk between GPCRs and TBR - modulatory function of orphan GPCRs

Previous studies showed that stimulation of proteoglycan synthesis in vascular smooth muscle cells by the thrombin receptor (PAR-1) requires TBRI kinase activity and Smad2 phosphorylation suggesting a possible transactivation mechanism between these two receptors. The authors did exclude an effect of PAR-1 on TGFB release but apart from that were unable to define the precise level of crosstalk at the origin of this effect. The GPR50/TBRI complex is the first example of a previously unrecognized crosstalk between the TGFB receptors and GPCRs at the receptor level. The capacity of GPCRs to engage into molecular complexes with other receptors, either of the same family (GPCR heteromers) or with proteins of other receptor families or transporters is increasingly recognized. Indeed, such complexes significantly diversify the repertoire of pharmacological targets with a limited number of proteins. Such complexes might be of particular importance for orphan GPCRs. There exists indeed more than 100 orphan GPCRs for which no ligand has been identified yet. Apart from the ligand-dependent function that still have to be elucidated, an alternative hypotheses based on the existence of ligand-independent functions of orphan GPCRs is emerging (Levoye et al, 2006c). This also applies to GPR50, which has been shown to heteromerize with the melatonin MT₁ receptor and to inhibit ligand binding, G protein coupling and β-arrestin recruitment to MT₁ in the common GPR50/MT₁ complex (Levoye et al, 2006a). As in the GPR50/TBRI complex, the C-terminal domain of GPR50 appears to play an important role in the modulation of the function of the interacting partner. Complex formation with TBRI clearly expands the idea that GPR50 is a co-receptor fine-tuning the function of other receptors with known function. These functions might be of particular importance for the evolutionary conservation of GPR50, which is likely to be a true orphan without ligand, as GPR50 orthologs in non-mammalian species bind melatonin, a property that has been lost in the mammalian GPR50 (Dufourny et al, 2008).

6) Potential relevance for other members of the TBRI family

In our study we used the prototypic type I receptor T β RI, which is one of seven type I receptor family members. Though the T β RI was the only member of the T β RI family identified in the TAP assay, we cannot exclude that GPR50 interacts with other type I receptors as our results might be biased by the expression levels of other T β RI members in HEK293T cells.

The other six members, four activin-like-receptors and two BMP-receptors, display strong sequence homology, underlie the same activation modus and were also found to bind FKBP12 (Wang et al, 1994). Alignment of the expression patterns of GPR50 and the other members of

the TßRI family, which are more restricted as TßRI might help to identify tissues of interest in the organism where a crosstalk might be of physiological relevance.

In conclusion, we describe here a new molecular complex composed of an orphan GPCR and TBRI that renders TBRI constitutively active towards the SMAD2/3 and the p38 pathways by dissociating the negative regulator FKBP12 from TBRI and most likely stabilizing an activated state of TBRI in the absence of TBRII. Such a constitutively active complex might be of interest in the context of the cell migration promoting and tumor inhibiting effect of TGFB.

EXPERIMENTAL PROCEDURES

Cell culture

HEK 293T, HeLa and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle medium (GIBCO) containing 8% fetal bovine serum (GIBCO) and 2% penicillin/streptomycin (GIBCO). Selective Medium for maintenance of MDA-MB-231 clones contained 250 µg/ml Geniticin (G418) (Sigma Aldrich). SNU638 cells were cultured in RPMI-1640 medium (GIBCO) with 10% FCS and 2% pen/strep.

Reagents and antibodies

Reagents: FK506 and SB431542 were purchased from Sigma Aldrich and recombinant TGFβ-1 was used from Peprotech.

Antibodies: Phospho-SMAD2, phospho-SMAD3, p38 and phospho-p38 came from Cell Signaling, SMAD2/3 was used from BD Biosciences, anti-myc (A14 and 9E10), -TβRI V22, - Lamin (M20), and -GPR50 came from Santa Cruz Biotechnology. Mono- and polyclonal Flag-Antibodies were used from Sigma, HA- and GFP-antibodies were used from Roche. Anti-Tubulin was purchased from AbD Serotec. GPR50 antibody7 was produced by Kernov Antibody Services (Hamouda et al, 2007a). All antibodies were employed according to recommended dilutions for either immunoprecipitation or western blotting.

Cell transfection and generation of stably overexpressing cells

Transient transfection was performed by using Lipofectamine® LTX reagent (Life Technologies) in HEK 293T cells, Xtremegene® 9 (Roche) for reporter gene assay in HeLa cells and JetPRIME (Polyplus) reagent for MDA-MB-231 and SNU638 cells, each employed according to the manufacturer's instruction. Cells were incubated for 48 hrs before experimental use. Stably GPR50 overexpressing cells were generated by JETprime

transfection of G418 resistant GPR50 plasmid, selective pressure was established by using conditioned DMEM medium with 1 mg/ml G418 (Sigma Aldrich)

Plasmid mutagenesis

Primers for point mutations were designed with the help of the Agilent QuikChange Primer Design program. Mutagenesis was performed by PCR with the Phusion High Fidelity Polymerase (Finnzymes, Thermo Scientific).

Tandem affinity purification

All purification steps were conducted at 4 °C in the presence of a protease inhibitor mixture (Roche Applied Science), 1 mm orthovanadate, and 2 mM NaF. Crude membranes were prepared from ~ 2×10^8 HEK 293 cells and solubilized overnight in solubilization buffer (75 mM Tris, 2 mM EDTA, 5 mM MgCl₂, pH 8.0) with 0.25% Brij96V at a concentration of 2 mg of protein/ml. The supernatant was recovered after centrifugation at 40,000 × g for 30 min and incubated for 4 h with 400 µl of rabbit IgG-Agarose (Sigma). The resin was washed three times with 1 ml of solubilization buffer, resuspended in 500 µl of the same buffer, and incubated overnight with 100 units of TEV protease (Invitrogen). The supernatant was collected, mixed with 500 µl of calmodulin buffer (75 mM Tris, 5 mM MgCl2, 50 mM CaCl₂, and 0.25% Brij96V, pH 8.0) and incubated for 2 h with 100 µl of calmodulin beads (Stratagene, La Jolla, CA). Beads were washed three times with 1 ml of calmodulin buffer and two times with 1 mL calmodulin rinsing buffer without detergent (50 mM ammonium bicarbonate, 2 mM CaCl₂) and resuspended into 100 µL of 50 mM ammonium bicarbonate, pH8.0. Perform trypsin digestion directly on beads by adding 1 mg of trypsin overnight at 37°C.
Cellular lysis, co-immunoprecipitation and western blotting

For preparation of cellular lysates, cells were harvested after transfection and stimulation according to protocol, in TNMG - Buffer with 0,5% NP-40 and a cocktail of protease inhibitorsd (Prunier et al, 2001) for 15 min, centrifuged at maximum speed for 20 min and supernatants were kept. Samples containing 500 µg to 1 mg protein were subjected to immunoprecipitation by incubating for 3 hrs with 2 to 5 µg of antibody. Protein G beads (Sigma), to enrich precipitates, were added for additional 2 hrs, prior to three washing steps in a Tris-EDTA-Magnesium buffer with 0,05% NP-40 buffer. Samples were diltuted in 2x Laemmli with 4% SDS and heated for 5 min at 95°C preceding SDS-PAGE. Cell Lysates for protein analysis were obtained after lysis with TNMG buffer, protein estimation was performed with BCA Assay (Thermo Scientific), 20 to 100 µg of sample were prepared and 4x SDS-Laemmli was added. Samples were heated at 95°C and separated on a 12% SDS Gel. Proteins were blotted on a PVDF membrane (Dutscher), blocked and incubated over night with in 3% milk or BSA-solution antibody. Incubation with fluorescence coupled secondary antibodies enables readout on an Odyssee reader.

BRET analysis

HEK 293T cells were transiently transfected in 6 well plates with 100 ng or 100 to 2000 ng respectively of the corresponding Luciferase- and YFP-coupled plasmids, grown over night and transferred in to 96-well-Optiplates (PerkinElmer Life Sciences), pre-coated with 10 µg/ml poly-L-lysine (Sigma), where they were grown for additional 24 hrs, washed with PBS, coelenterazine (Molecular Probes) for Luciferase stimulation was added and cells were subjected to measurement of emission at Luc and YFP wavelength on a Berthold Mithras[™] as previously described (Maurice et al, 2010).

Nuclear Extracts

HEK 293T cells were seeded in 100 mm culture plates and transfected with mock, SMAD3 alone or co-transfected with GPR50wt and GPR50ATTGH variant. The cells were starved for 16 hrs in DMEM media without FBS and stimulated with 2 ng/mL of TGF- β for 2 hrs. The culture plates were rinsed twice with ice-cold phosphate-buffered saline (PBS). 500 µL of hypotonic buffer containing 1% NP-40 was added to each culture plate, and allowed to swell on ice for 15 min. The cells were scraped and taken into fresh eppendorf tube. The lysate was vortexed for 10 seconds, and the nuclei were pelleted (14000 rpm for 1 min). Supernatant was collected which is a cytoplasmic fraction. The nuclear pellets were resuspended in 100 to 200 µL of hypertonic buffer and rotated for 30 min. at 4°C. This extract was then centrifuged (13000 rpm for 20 min), and supernatant was collected which is nuclear fraction. The amount of protein was estimated with a BCA estimation kit. The buffer compositions were as follows. (i) Hypotonic buffer contained 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 25mM β -glycerophosphate, 1 mM Na₃VO₄, 1 mM dithiothreitol (DTT) (ii) Hypertonic buffer contained 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 25% glycerol, 1 mM EDTA, 25mM β-glycerophosphate, 1 mM Na₃VO₄ and 1 mM DTT. To both, buffers protease and phosphatase inhibitors were added just before use.

Reporter gene assay

HeLa cells were seeded in 12-well-plates transfected with the SMAD2-dependent Activinresponse-element (ARE) Firefly Luciferase together with the FAST-myc co-factor, or the SMAD3-dependent CAGA Firefly luciferase reporter gene, increasing amounts of GPR50 Δ TTGH or WT and Renilla Luciferase as internal standard. Cells were left for 24 hrs, starved and stimulated with 2 ng/ml TGF β overnight. Lysis and measurement were performed with the Dual Luciferase Kit (Promega) according to manufacturer's advices. Experiments were performed in triplicates, figures show representative experiment.

Radioligand binding assay

MDA-MB-231 cells were plated in 6-well plates in incubated with ¹²⁵I-TGFß (100000 cpm/mL; NEX267) in DMEM, 20 mM Hepes, pH 7.4, 0.4% BSA for 4h at 4°C to determine the number of surface exposed receptors. Non-specific binding was determined in the presence of a 100 fold excess of unlabled TGFß. Cells were washed twice with ice-cold PBS and extracted in 1mL 1N NaOH and ¹²⁵I-TGFß quantified in a scintillation counter.

Soft agar assay/anchorage-independence assay

35mm dishes were coated with a layer of 0,5% Agar containing a DMEM/7% FBS solution. 1*10⁵ MDA-MB-231 cells were mixed with 0,3% agar containing DMEM-Media with 7% FCS and distributed upon the first layer. Colony formation was measured about 20 days after seeding.

Migration and wound healing

25.000 cells were seeded into $35\text{mm} \mu$ -dishes with a silicon insert (IBIDI), starved over night and the insert was removed the following day. Closure was assessed every 6 hrs for 36 hrs under a light microscope. Analysis of wound closure was performed using ImageJ.

Xenograft experiments

 1×10^{6} MDA-MB-231 cells were diluted 1:1 in a MatrigelTM (BD Biosciences) and subcutaneously injected into the right and left flank of nude mice. Tumor growth was

monitored every 3 days and measured using a caliper. Tumor volume was calculated with the $(width)^2 x \text{ length } x \pi/6 \text{ formula.}$

Statistical Analysis

Statistical analysis of data was performed with a two-tailed unpaired T-Test. A p value <0.05 was considered for statistical significance.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures that can be found with this article.

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Authors contributions:

S.W. participated in the project design, performed most of the experiments, interpreted the data and contributed to the writing of the manuscript; Z.B.-C. designed and participated in the soft agar and xenograft experiments and interpreted the data; R.A. designed and performed nuclear translocation assays; A-S.J. designed and performed all BRET experiments; A.D. designed and performed TAP experiments ; J-L.G. contributed to the management and to the work with MDA-MB-231 cells; A.S. performed experiments in the initial phase of the project;

N.C. conributed to the management in the initial phase of the project; T.C. performed control experiments; K.T. generated TBRI fusion proteins for BRET experiments; P.D. contributed to the management and design of the project. C.P. contributed to the management and design of the project, manuscript writing and performed control experiments in MDA-MB-231 cells; R.J. was responsible for the project supervision, experiment design, data interpretation, and manuscript writing and provided funding support.

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REFERENCES

Bagarova, J., Vonner, A.J., Armstrong, K.A., Borgermann, J., Lai, C.S., Deng, D.Y., Beppu, H., Alfano, I., Filippakopoulos, P., Morrell, N.W., *et al.* (2013). Constitutively active ALK2 receptor mutants require type II receptor cooperation. Mol Cell Biol *33*, 2413-2424.

Barrett, P., Ivanova, E., Graham, E.S., Ross, A.W., Wilson, D., Ple, H., Mercer, J.G., Ebling, F.J., Schuhler, S., Dupre, S.M., *et al.* (2006). Photoperiodic regulation of cellular retinol binding protein, CRBP1 [corrected] and nestin in tanycytes of the third ventricle ependymal layer of the Siberian hamster. J Endocrinol *191*, 687-698.

Bassing, C.H., Howe, D.J., Segarini, P.R., Donahoe, P.K., and Wang, X.F. (1994). A single heteromeric receptor complex is sufficient to mediate biological effects of transforming growth factor-beta ligands. The Journal of biological chemistry *269*, 14861-14864.

Batailler, M., Mullier, A., Sidibe, A., Delagrange, P., Prévot, V., Jockers, R., and Migaud, M. (2011). Neuroanataomical distribution of the orphan GPR50 receptor in adult sheep and rodent brains. J Neuroendocrinol.

Bavarva, J.H., Tae, H., Settlage, R.E., and Garner, H.R. (2013). Characterizing the Genetic Basis for Nicotine Induced Cancer Development: A Transcriptome Sequencing Study. PLoS One *8*, e67252.

Bhattacharyya, S., Luan, J., Challis, B., Keogh, J., Montague, C., Brennand, J., Morten, J., Lowenbeim, S., Jenkins, S., Farooqi, I.S., *et al.* (2006). Sequence variants in the melatonin-

related receptor gene (GPR50) associate with circulating triglyceride and HDL levels. J Lipid Res *47*, 761-766.

Buchholz, M., Braun, M., Heidenblut, A., Kestler, H.A., Kloppel, G., Schmiegel, W., Hahn, S.A., Luttges, J., and Gress, T.M. (2005). Transcriptome analysis of microdissected pancreatic intraepithelial neoplastic lesions. Oncogene *24*, 6626-6636.

Carcamo, J., Zentella, A., and Massague, J. (1995). Disruption of transforming growth factor beta signaling by a mutation that prevents transphosphorylation within the receptor complex. Mol Cell Biol *15*, 1573-1581.

Chen, G., Chen, X., Sukumar, A., Gao, B., Curley, J., Schnaper, H.W., Ingram, A.J., and Krepinsky, J.C. (2013). TGFbeta receptor I transactivation mediates stretch-induced Pak1 activation and CTGF upregulation in mesangial cells. J Cell Sci *126*, 3697-3712.

Chen, Y., Liu, F., and Massague, J. (1997). Mechanism of TGFbeta receptor inhibition by FKBP12. EMBO J *16*, 3866-3876.

Daulat, A., Maurice, P., Froment, C., Guillaume, J., Broussard, C., Monsarrat, B., Delagrange, P., and Jockers, R. (2007). Purification and identification of G proteincoupled receptor protein complexes under native conditions. Mol Cell Proteomics *6*, 835-844.

Drew, J., Barrett, P., Mercer, J., Moar, K., Canet, E., Delagrange, P., and Morgan, P. (2001). Localization of the melatonin-related receptor in the rodent brain and peripheral tissues. J Neuroendocrinol *13*, 453-458.

Dufourny, L., Levasseur, A., Migaud, M., Callebaut, I., Pontarotti, P., Malpaux, B., and Monget, P. (2008). GPR50 is the mammalian ortholog of Mel1c: evidence of rapid evolution in mammals. BMC Evol Biol *8*, 105.

Franzen, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C.H., and Miyazono, K. (1993). Cloning of a TGF beta type I receptor that forms a heteromeric complex with the TGF beta type II receptor. Cell *75*, 681-692.

Groppe, J.C., Wu, J., Shore, E.M., and Kaplan, F.S. (2011). In vitro analyses of the dysregulated R206H ALK2 kinase-FKBP12 interaction associated with heterotopic ossification in FOP. Cells, tissues, organs *194*, 291-295.

Grünewald, E., Kinnell, H., Porteous, D., and Thomson, P. (2009). GPR50 interacts with neuronal NOGO-A and affects neurite outgrowth. Mol Cell Neurosci *42*, 363-371.

Grunewald, E., Kinnell, H.L., Porteous, D.J., and Thomson, P.A. (2009). GPR50 interacts with neuronal NOGO-A and affects neurite outgrowth. Mol Cell Neurosci *42*, 363-371.

Grunewald, E., Tew, K.D., Porteous, D.J., and Thomson, P.A. (2012). Developmental expression of orphan g protein-coupled receptor 50 in the mouse brain. ACS Chem Neurosci *3*, 459-472.

Gubitz, A.K., and Reppert, S.M. (1999). Assignment of the melatonin-related receptor to human chromosome X (GPR50) and mouse chromosome X (Gpr50). Genomics *55*, 248-251.

Gunther, C.V., Georgi, L.L., and Riddle, D.L. (2000). A Caenorhabditis elegans type I TGF beta receptor can function in the absence of type II kinase to promote larval development. Development *127*, 3337-3347.

Hamouda, H., Chen, P., Levoye, A., Sözer-Topçular, N., Daulat, A., Guillaume, J., Ravid, R., Savaskan, E., Ferry, G., Boutin, J., *et al.* (2007). Detection of the human GPR50 orphan seven transmembrane protein by polyclonal antibodies mapping different epitopes. J Pineal Res *43*, 10-15.

Hanafusa, H., Ninomiya-Tsuji, J., Masuyama, N., Nishita, M., Fujisawa, J., Shibuya, H., Matsumoto, K., and Nishida, E. (1999). Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. J Biol Chem *274*, 27161-27167.

Heldin, C.H., and Moustakas, A. (2012). Role of Smads in TGFbeta signaling. Cell Tissue Res *347*, 21-36.

Huse, M., Chen, Y., Massagué, J., and Kuriyan, J. (1999). Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. Cell *96*, 425-436.

Huse, M., Muir, T., Xu, L., Chen, Y., Kuriyan, J., and Massagué, J. (2001). The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. Mol Cell *8*, 671-682.

Ikushima, H., and Miyazono, K. (2010). TGFbeta signalling: a complex web in cancer progression. Nature reviews. Cancer *10*, 415-424.

Ivanova, E., Bechtold, D., Dupré, S., Brennand, J., Barrett, P., Luckman, S., and Loudon, A. (2008). Altered metabolism in the melatonin-related receptor (GPR50) knockout mouse. Am J Physiol Endocrinol Metab *294*, E176-182.

Jockers, R., Maurice, P., Boutin, J.A., and Delagrange, P. (2008). Melatonin receptors, heterodimerization, signal transduction and binding sites: what's new? Br J Pharmacol *154*, 1182-1195.

Kang, J.S., Liu, C., and Derynck, R. (2009). New regulatory mechanisms of TGF-beta receptor function. Trends Cell Biol *19*, 385-394.

Kavsak, P., Rasmussen, R.K., Causing, C.G., Bonni, S., Zhu, H., Thomsen, G.H., and Wrana, J.L. (2000). Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. Mol Cell *6*, 1365-1375.

Levoye, A., Dam, J., Ayoub, M., Guillaume, J., Couturier, C., Delagrange, P., and Jockers, R. (2006a). The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. EMBO J *25*, 3012-3023.

Levoye, A., Dam, J., Ayoub, M.A., Guillaume, J.L., Couturier, C., Delagrange, P., and Jockers, R. (2006b). The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. EMBO J *25*, 3012-3023.

Levoye, A., Dam, J., Ayoub, M.A., Guillaume, J.L., and Jockers, R. (2006c). Do orphan Gprotein-coupled receptors have ligand-independent functions? New insights from receptor heterodimers. EMBO Rep *7*, 1094-1098.

Li, J., Hand, L.E., Meng, Q.J., Loudon, A.S., and Bechtold, D.A. (2011). GPR50 interacts with TIP60 to modulate glucocorticoid receptor signalling. PLoS One *6*, e23725.

Massague, J. (2008). TGFbeta in Cancer. Cell 134, 215-230.

Massague, J. (2012). TGFbeta signalling in context. Nat Rev Mol Cell Biol 13, 616-630.

Massagué, J. (2000). How cells read TGF-beta signals. Nat Rev Mol Cell Biol 1, 169-178.

Maurice, P., Daulat, A.M., Turecek, R., Ivankova-Susankova, K., Zamponi, F., Kamal, M., Clement, N., Guillaume, J.L., Bettler, B., Gales, C., *et al.* (2010). Molecular organization and dynamics of the melatonin MT(1) receptor/RGS20/G(i) protein complex reveal asymmetry of receptor dimers for RGS and G(i) coupling. EMBO J *29*, 3646-3659.

Maurice, P., Guillaume, J.L., Benleulmi-Chaachoua, A., Daulat, A.M., Kamal, M., and Jockers, R. (2011). GPCR-interacting proteins, major players of GPCR function. Advances in pharmacology *62*, 349-380.

Nakao, A., Afrakhte, M., Morén, A., Nakayama, T., Christian, J.L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N.E., Heldin, C.H., *et al.* (1997). Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. Nature *389*, 631-635.

Prevot, V., Bellefontaine, N., Baroncini, M., Sharif, A., Hanchate, N.K., Parkash, J., Campagne, C., and de Seranno, S. (2010). Gonadotrophin-releasing hormone nerve terminals, tanycytes and neurohaemal junction remodelling in the adult median eminence: functional consequences for reproduction and dynamic role of vascular endothelial cells. J Neuroendocrinol *22*, 639-649.

Prevot, V., Bouret, S., Croix, D., Takumi, T., Jennes, L., Mitchell, V., and Beauvillain, J.C. (2000). Evidence that members of the TGFbeta superfamily play a role in regulation of the GnRH neuroendocrine axis: expression of a type I serine-threonine kinase receptor for TGRbeta and activin in GnRH neurones and hypothalamic areas of the female rat. J Neuroendocrinol *12*, 665-670.

Prunier, C., Ferrand, N., Frottier, B., Pessah, M., and Atfi, A. (2001). Mechanism for mutational inactivation of the tumor suppressor Smad2. Molecular and cellular biology *21*, 3302-3313.

Rask-Andersen, M., Almen, M.S., and Schioth, H.B. (2011). Trends in the exploitation of novel drug targets. Nat Rev Drug Discov *10*, 579-590.

Shi, Y., and Massagué, J. (2003). Mechanisms of TGF-β Signaling from Cell Membrane to the Nucleus. Cell *113*, 685-700.

Sidibe, A., Mullier, A., Chen, P., Baroncini, M., Boutin, J., Delagrange, P., Prevot, V., and Jockers, R. (2010). Expression of the orphan GPR50 protein in rodent and human dorsomedial hypothalamus, tanycytes and median eminence. J Pineal Res *48*, 263-269.

Song, G.A., Kim, H.J., Woo, K.M., Baek, J.H., Kim, G.S., Choi, J.Y., and Ryoo, H.M. (2010). Molecular consequences of the ACVR1(R206H) mutation of fibrodysplasia ossificans progressiva. J Biol Chem *285*, 22542-22553.

Thomson, P.A., Wray, N.R., Thomson, A.M., Dunbar, D.R., Grassie, M.A., Condie, A., Walker, M.T., Smith, D.J., Pulford, D.J., Muir, W., *et al.* (2005). Sex-specific association between bipolar affective disorder in women and GPR50, an X-linked orphan G protein-coupled receptor. Mol Psychiatry *10*, 470-478.

Tsukazaki, T., Chiang, T.A., Davison, A.F., Attisano, L., and Wrana, J.L. (1998). SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell *95*, 779-791.

Wang, T., and Donahoe, P.K. (2004). The immunophilin FKBP12: a molecular guardian of the TGF-beta family type I receptors. Front Biosci *9*, 619-631.

Wang, T., Donahoe, P.K., and Zervos, A.S. (1994). Specific interaction of type I receptors of the TGF-beta family with the immunophilin FKBP-12. Science *265*, 674-676.

Wang, T., Li, B.Y., Danielson, P.D., Shah, P.C., Rockwell, S., Lechleider, R.J., Martin, J., Manganaro, T., and Donahoe, P.K. (1996). The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. Cell *86*, 435-444.

Watanabe, Y., Itoh, S., Goto, T., Ohnishi, E., Inamitsu, M., Itoh, F., Satoh, K., Wiercinska, E., Yang, W., Shi, L., *et al.* (2010). TMEPAI, a transmembrane TGF-beta-inducible protein, sequesters Smad proteins from active participation in TGF-beta signaling. Mol Cell *37*, 123-134.

Wieser, R., Wrana, J.L., and Massague, J. (1995). GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. EMBO J *14*, 2199-2208.

Wrana, J.L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994). Mechanism of activation of the TGF-beta receptor. Nature *370*, 341-347.

Xu, P., Liu, J., and Derynck, R. (2012). Post-translational regulation of TGF-beta receptor and Smad signaling. FEBS Lett *586*, 1871-1884.

Yi, J.J., Barnes, A.P., Hand, R., Polleux, F., and Ehlers, M.D. (2010). TGF-beta signaling specifies axons during brain development. Cell *142*, 144-157.

Yu, L., Hebert, M.C., and Zhang, Y.E. (2002). TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J *21*, 3749-3759.

Zhang, Y., Feng, X., We, R., and Derynck, R. (1996). Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. Nature *383*, 168-172.

Zhang, Y.E. (2009). Non-Smad pathways in TGF-beta signaling. Cell Res 19, 128-139.

Supplementary Figures

Supplementary Figure 1



S1 BRET experiments in absence and presence of ligand

(A and B) For BRET saturation curves, HEK293T cells were transfected with constant amount of T β RI-Luc GPR50 Δ 4-YFP (A) or GPR50wt-YFP (B). Cells were washed and coelenterazine was added. BRET signals were measured after addition of 2 ng/ml of TGF β or PBS as control.

Supplementary Figure 2



(A) The effect of GPR50 on ligand binding

To address binding of TGF β , MDA-MB-231 cells were transfected with GPR50 Δ 4 and GPR50wt. Binding of TGF β was assessed in a competion-radioligand-binding assay with radio-labeled and cold TGF β .

(B) Secretion of TGF β in presence of GPR50.

HEK 293T cells were starved and treated for one hour with the supernatant of HEK293T cells either transfected with Mock, GPR50 Δ 4 and GPR50 Δ WT. Stimulation with 2ng/ml of TGF β served as positive control. Cells were than analysed for their SMAD3 phosphorylation and total SMAD3. Cells providing supernatant were checked for GPR50 expression.

Supplementary Figure 3



S3 Co-IP of Smad2 and 4 with GPR50

Co-immunoprecipation was performed with HEK293T cells co-transfected with myc-SMAD2 or SMAD4 and either GPR50 Δ 4 or GPR50wt. Lysates were incubated with an anti-myc antibody to precipate the SMADs. Precipitates were blotted against GPR50 and total lysates were analysed with an anti-myc and an anti-GPR50 antibody for plasmid expression.

III. DISCUSSION

The initial aim of this project was to identify further binding partners for GPR50 by applying the TAP assay to the full-length receptor. After having identified the T β RI as a candidate binding partner, we successfully validated this interaction *in vitro* with different techniques. This prompted us to focus our attention on T β RI-dependent signal transduction and to investigate the impact of GPR50 on TGF β signaling. Unexpectedly, we observed that GPR50 has the capacity to induce a ligand-independent activation of the TGF β pathway, which is dependent on the T β RI kinase activity. The analysis of the molecular mechanism revealed that GPR50 competes with the negative regulator FKBP12 that keeps T β RI in an inactive conformation upon T β RI binding. Furthermore, we showed an activation of the T β RI in the absence of T β RII. The utilization of the MDA-MB-231 breast cancer cell lines overexpressing GPR50 or not, revealed pro-migratory and anti-proliferative effects of GPR50 that resembled the action of TGF β in this model.

We describe here a new regulatory mechanism of T β RI-dependent signaling upon complex formation with the orphan 7TM protein GPR50 with a possible role in breast cancer development. These findings can be integrated into the current knowledge under different aspects and give rise for future research, which are depicted in the following:

1. The complex of TβRI and GPR50

This work identified a novel regulator of TGF β signal transduction. The TGF β signal transduction pathway is a direct pathway that goes straight from the membrane through the cytosol into the nucleus. The core components of this pathway are the TGF β ligand, the serine/threonine kinase transmembrane receptors type I and type II and the signaling mediators, the Smads, that shuttle between cytosol and the nucleus, where they regulate gene expression (Massague, 1998). In order to establish signaling specificity and to allow cell context-specific signal transduction, proteins that interfere in a positive or a negative manner with activation or longevity of TGF β signaling are necessary (Schmierer & Hill, 2007). During recent years, many proteins that modulate TGF β signaling on the level of ligand (Moustakas & Heldin, 2009), receptor (Runyan et al, 2006) or the Smads (Xu et al, 2012) have been identified. With the discovery of GPR50 as a novel regulator of TGF β signaling, we not only identified another protein, that can fine-tune TGF β signaling, but that also adds previously unappreciated aspects to TGF β -dependent signaling.

1.1. A new mode of activation for the $T\beta RI$

The dogma for activation of the TGF β -dependent signaling consists in a well-defined signaling flow. The TGF β ligand binds to the T β RII, that recruits the T β RI into the ternary complex (Wrana et al, 1994) and activates T β RI via phosphorylation in its GS domain (Wieser et al, 1995). Subsequently, T β RI phosphorylates the Smads that transduce the signal into the nucleus and regulate gene expression (Nakao et al, 1997b) or may alternatively activate other non-canonical pathways as p38 (Zhang, 2009). Studies have proven the necessity of the presence of the T β RII for the activation of T β RI (Luo & Lodish, 1996; Okadome et al, 1994; Vivien et al, 1995) due to the transphosphorylation of the GS domain of T β RI by T β RII (Carcamo et al, 1995). Our findings in contrast, demonstrate a previously undescribed mode of activation for the T β RI, independent from ligand binding and T β RII-dependent phosphorylation. The question is, how GPR50 is able to induce the kinase activity of the T β RI without the normally preceding steps.

1.1.1. Competition of GPR50 for receptor binding with FKBP12

Our work could identify, that GPR50 competes with FKBP12 for T β RI binding. FKBP12 has been identified as negative regulator of TGF β signaling with a gate-keeping function. It stabilizes an inhibitory confirmation of the T β RI by promoting the formation of an inhibitory wedge of the GS region and thus prevents ligand-independent signaling (Wang & Donahoe, 2004) by locking the T β RI in a kinase-inactive conformation and blocking the access for Smad binding to the GS region. Ligand binding and subsequent phosphorylation by the T β RII is accompanied by dissociation of FKBP12, although it remains unclear, at which point the release of FKBP12 occurs. Removal of FKBP12 leads to a loss of the inhibitory wedge in the GS domain and favors T β RII-mediated phosphorylation of Ser/Thr residues in the GS region, which allows the binding of the Smads and further signal propagation. Thus, the depletion of FKBP12 from the receptor might explain how GPR50 is able to induce a ligand-independent signaling. But there are two points that indicate that the release of FKBP12 is not the only event that happens in the presence of GPR50:

First of all, by release of FKBP12, we only observe a weak basal signaling activity that increases further upon ligand stimulation (Charng et al, 1996). This stands in contrast with the high levels of activation we observed in the presence of GPR50, that did only show a small further increase of Smad phosphorylation after ligand stimulation (Figure 2 A,B), giving a first hint, that GPR50 might have further effects on the T β RI. Different studies have shown,

that the reason for leaky signaling in the absence of FKBP12 is a hypersensitivity to T β RII (Chen et al, 1997; Stockwell & Schreiber, 1998). In cells deficient in T β RII, an activity of T β RI mutants incapable for binding the FKBP12 was only observed after ectopic expression of a wildtype T β RII but not kinase inactive T β RII mutants. Hence, leaky signaling in the absence of FKBP12 is explained by incidental ligand-independent phosphorylation in the GS domain by the T β RII. In contrast, our results show ligand-independent signaling in the presence of GPR50 in T β RII-lacking SNU638 gastric cancer cells. Thus we can suggest an active participation of GPR50 through the stabilization of a constitutively active conformation of the T β RI that does not require the T β RII anymore. With regards to literature, there exist some hints, that a constitutive activity of the T β RI is possible.

1.2. Constitutive activity of the TβRI and ligand-independent signaling

Currently, there is only little evidence that the human wildtype $T\beta RI$ can be activated and phosphorylated by $T\beta RII$ in the absence of ligand. Nevertheless, some cases of constitutive activity of the $T\beta RI$ have been reported.

1.2.1. The TβRI T204D mutant

In a study, that claimed to investigate the different effects of mutations in the GS domain on activity and activation of the T β RI (Wieser et al, 1995), a mutation of Thr204 into Asp, resulted in a ligand-independent constitutive activitation of the receptor. Thr204 is located adjacent to the GS domain. Although it is most likely not directly phosphorylated it promotes phosphorylation of Ser/Thr residues in the GS domain. Exchange of Thr into a more spacious and negatively charged Asp might results in conformational changes that translate to the GS domain. The GS domain probably loses its inhibitory wedge and is able to exert positive allosteric effects on the kinase domain. However, this mutant is still sensitive to the T β RII and signaling increases further in the presence of ligand and intact T β RII. In the case of GPR50 and the T β RII and if additional effects are due to ordinarily activated T β RI or whether GPR50 and T β RII can exert mutually additive effects on T β RI activation. Results obtained in SNU638 cells argue that the GPR50/T β RI complex is completely ligand-insensitive and that residual TGF β effects are generated from classical T β RI/T β RII complexes in cells expressing both isoforms.

1.2.2. ACVR1 R206H and L196P mutants in fibrodysplasia ossificans progressiva

In the genetic disease *fibrodysplasia ossificans progressiva* (FOP) with a phenotype of exceeding and inappropriate bone growth, two different mutants are associated with constitutive ligand-independent type I receptor activation. These mutants are found in the activin A receptor 1 (ACVR1), another one of the seven family members of type I receptors, that binds the BMP ligand and signals via Smad 1, 5 and 8. In the case of the R206H mutant, the concerned amino acid is located, similar to the T β RI T204D mutant in proximity to the GS domain (Groppe et al, 2007), a conserved region among all the type I receptors. This mutant is displaying ligand-independent activation (Song et al, 2010) and shows reduced binding to FKBP12 (Groppe et al, 2011), which is similar to our results. This constitutive signaling in the absence of ligand results in permanent activation of BMP target genes even in the absence of external signals, explaining the excessive and undesired bone growth in these patients. Another mutant, which has been identified involves a Leu to Pro substitution at position 196, an amino acid directly located in the GS domain and important for the binding of FKBP12 (Ohte et al, 2011). This mutant also shows constitutive activity, probably due to the loss of FKBP12 binding.

It is noteworthy that, at least for the R206H mutant, constitutive signaling still depends on the presence of the T β RII (Le & Wharton, 2012) even though its kinase activity is not required for ligand-independent activity of the T β RI (Bagarova et al, 2013). Thus, a T β RII-mediated phosphorylation in the GS domain of the T β RI is not essential for type I receptor activation in the case of the ACVR1 R206H mutant. Nevertheless, the presence of T β RII might have a distinct role, like binding of other proteins necessary for activation. In the case of the T β RI/GPR50 complex, T β RII-dependent functions can probably be complemented by the presence of GPR50.

1.2.3. TβRII-independent signaling in *C.elegans*

Further evidence for the capacity of the T β RI to signal in the absence of an intact T β RII comes from evolutionary lower organisms like *C.elegans*. In this organism, the type I receptor corresponds to the DAF-1 protein (Georgi et al, 1990) that can signal together with DAF-4, the type II receptor homolog to transduce the signals of the DAF-7 growth factor. It has been reported, that this receptor is able to signal in the absence of DAF-4 kinase activity (Gunther et al, 2000), yet the presence of fully active DAF-4 further increases its signaling activity. Sequence alignment and comparison reveals, that DAF-1, compared to other type I receptors,

presents striking differences in the important region around the GS domain. Most of the phosphorylation targets are absent, the GS domain is reduced and also a Leu- and Pro-residue for a hypothetical FKBP12 binding are missing. These findings suggest that this altered sequence leads to a divergent structure of the GS domain that might result in a loss of the inhibitory wedge and its negative effects on the kinase domain of DAF-1, which eventually promotes constitutive activity. In contrast, the ligand-binding or at least the ligand-binding domain is required for this constitutive activity, suggesting either steric effects of the ligand binding domain or genuine ligand binding involved in the constitutive activity. This suggests, that at some point during evolution a constitutive active and ligand-independent type I receptor must have been useful. Probably, FKBP12 and the T β RII developed to provide control mechanisms for the T β RI activity that became necessary during evolution. Unfortunately it has not been studied whether, analogous to the ACVR1 R206H mutant, the presence of DAF-4 is required or whether DAF-1 can even signal in the absence of the type 2 receptor.

1.2.4. Stretch-induced activation of the TβRI

Recently, work has been published, that describes ligand-independent activation and signaling of the T β RI in case of cellular stretching (Chen et al, 2013). The mechanisms that act upstream and are responsible for activation have so far not been dissected, thus not excluding the involvement of other ligands than TGF β . Furthermore, no information is given about the involvement of the T β RII in activation.

The existence of constitutive active forms shows that under certain circumstances, a constitutive activity and a ligand-independent mechanism must have been advantageous that probably got lost during evolution. Taken together, the existence of this constitutive active forms, either early in evolution, artificial in laboratory mutations or in disease genotypes, support in coherence with our findings, that a type I receptor can signal in the absence of ligand and the absence of type II receptor kinase activity. Additionally, the examination of the behavior of this mutants showed, that a T β RII-mediated phosphorylation after ligand stimulation can have further increasing effects, but that it is not compulsory for an activation of the type I receptor. For the moment we have not figured out, whether, in the presence of GPR50, a GS domain phosphorylation is necessary for signaling or whether the T β RI can signal just by the allosteric alterations provided by GPR50. Further studies in a receptor-

deficient environment will hopefully help to gain information about the circumstances of a GPR50-mediated constitutive activity of the receptor.

1.3. The active role of GPR50 in inducing constitutive TβRI activity

Our work consists of the novelty, that GPR50 is able to activate the T β RI in absence of the T β RII. An interesting task for the future will be the investigation of additional effects of GPR50 making T β RII unnecessary. This research can be based on several hypotheses:

1.3.1. GPR50 acting as a kinase

GPR50 could be a kinase itself that phosphorylates the GS domain in order to induce kinase activity of the T β RI. Even though GPR50 has a characteristic long cytosolic C-terminus, modern *in silico* techniques of sequence alignment and motif identification did not identify any homology to any known kinase. Additionally, the C-terminus is an ancestor of the RNA Polymerase II, a nuclear protein without any intrinsic kinase activity.

1.3.2. GPR50 acting as a scaffold protein

GPR50 might be a scaffold for another kinases that can phosphorylate T β RI. Our results did so far not include the demonstration of direct association of the T β RI and GPR50. Thus, the activation might also pass through an intermediate. It can be imagined, that GPR50 recruits another cytosolic serine/threonine kinase that is capable of phosphorylating the T β RI to explain its activation in the absence of the T β RII. But yet, neither our TAP-assay, nor a Y2H assay from our group or others identified any kinase as a potential binding partner for GPR50. And with regards to T β RI, there is no indication that the T β RI gets phosphorylated by any other kinase then a type II receptor in the GS domain. Further experiments will address, whether GPR50 is in direct contact with the T β RI or whether additional proteins are involved.

1.3.3. Stabilisation of an active conformation of the TβRI

Our results indicate that GPR50 removes FKBP12 and stabilizes an active conformation of the T β RI. This observation is supported by the fact, that there is only a partial but sufficient competition between FKBP12 and GPR50. FKBP12 contacts the T β RI on two positions, with the 40s loop to the GS domain and with the 80s, which contains the ATGHP motif, also the L45 loop responsible for Smad binding (see Introduction, Chapter 3.2.3.4). We identified only the ⁸⁴ATGHP⁸⁸ of FKBP12 80's loop for being similar in GPR50, what proposes a partial

competition with FKBP12, that is yet sufficient to provoke its release and is consistent with our data that a loss of the the HP residues in FKBP12 is enough to disturb the binding to the T β RI. GPR50 probably takes place at the interface of Smad binding (L45 loop) but additional structural features of GPR50 might provoke a loss of the inhibitory wedge in the T β RI GS domain that usually gets stabilized by the 40's loop of FKBP12. Probably, an available crystal structure or structural prediction of GPR50 might allow a comparison of FKBP12 and GPR50 and their binding sites to T β RI.

1.3.4. Phosphorylation status in the presence of GPR50

We did so far not examine the phosphorylation status of the T β RI in presence of GPR50. A mutation of all the known putative phosphorylation residues in the GS region at once prohibits TGF β signaling, suggesting a role for downstream signaling (Wieser et al, 1995). An autophosphorylation in absence of the T β RII seems quite unlikely, even though it has been reported, that homodimers of the T β RI can complement each other if in one GS- and in the other the kinase domain are defective (Weis-Garcia & Massague, 1996). If we imagine GPR50 as an orchestrating molecule, that regroups T β RI and releases the inhibitory wedge in the T β RI, we cannot exclude an auto-transphosphorylation of T β RI within such clusters in the presence of GPR50. It will be an important question for the future, to identify the phosphorylation status of the T β RI in presence of GPR50 and to identify whether GPR50 still exerts its promoting effect on T β RI in the absence of known phosphorylation sites in the T β RI.

1.3.5. Importance of further FKBP12 similarity motifs

GPR50 contains four repetitive motifs, that are similar to the ⁸⁴ATGHP⁸⁸ sites in FKBP12 (as shown in Figure 3e). For the moment, we do not know, whether they all have the same influence or whether the effects we observed are restricted to the ⁴⁹⁵ATSHP⁴⁹⁹ sequence in proximity to the insertion/deletion of GPR50. The deletion might cause steric alterations that favor the interaction between T β RI and GPR50. Currently, it can not be excluded that all motifs regroup several receptors at once. Further mutational studies will surely provide insight into the role of this repetitive sequence in GPR50 with regards to T β RI activation.

1.3.6. Complex formation in the presence of GPR50

Another possibility is, to imagine GPR50 as a novel co-receptor for TGF β that might create, in complex with T β RI, a high affinity binding site for TGF β . It has been reported before that some receptors gain novel ligand binding capacities upon the formation of uncommon receptor combinations (ten Dijke & Hill, 2004). But according to our results, this hypothesis seems quite implausible. Our results show that the C-tail of GPR50 is involved in signal transduction and that the presence of the transmembrane part is not sufficient for downstream signaling. Thus, a role where GPR50 serves as a ligand-binding protein in the complex is difficult to imagine. Even though we cannot rule out the possibility that the different parts of GPR50 have separate functions: one that is responsible for the binding of a ligand and another that modulates the intracellular part to induce the activity of the T β RI, which is supported by the idea that full length GPR50 is required for being effective.

1.4. Regulation of complex formation GPR50/TβRI

In our case, the GPR50/T β RI complex seems to be formed constitutively. However, several ways to regulate this complex can be envisioned in a physiological context. According to existing principles in biology the following possibilities exist to regulate complex formation:

1.4.1. Regulation of cellular protein levels

Protein expression

The first possibility lies in the regulation of protein levels, either through gene expression, or half-life limitation by protein degradation. We know that T β RI is ubiquitously expressed, thus we can suppose its protein levels being more or less stable. With regards to GPR50, studies could show GPR50 expression levels are regulated during season (Barrett et al, 2006) and depending on the energy status (Ivanova et al, 2008). Furthermore, the existence of a miRNA target sequence in the promoter region (John et al, 2004) and the findings for a Dnmt-dependent regulation (Kotini et al, 2011) suggest a tight control of GPR50 expression and levels in the cell.

Protein stability

With regards to protein stability, other means of regulation of complex formation might consist in proteasomal or lysosomal degradation. Several mechanism that imply the ubiquitin-mediated degradation of the T β RI receptor complex have been described (Soond & Chantry,

2011) suggesting an involvement of such pathways to regulate the T β RI/GPR50 complex. GPR50, though being an orphan 7TM protein, might also be the subject to classical GPCR-desensitization pathways or regulations. The findings, that GPR50 is subject to proteolytic cleavage and subsequent nuclear translocation (Li et al, 2011) might add a possibility to regulate the activity of the complex, since the cytosolic part of GPR50 is indispensable for activation of the T β RI. Further investigation that addresses the proteolytic cleavage of GPR50 will surely provide more insight into possible regulation mechanisms of GPR50 activity.

Protein localisation

Regulation of the complex might also be established through a regulation of the subcellular localization of both proteins. It has been well described, that the T β RI is subject to internalization either in order to become recycled or in order to get lysosomally degradated (Di Guglielmo et al, 2003).

The interaction itself in contrast does not seem to interfere with localization of the T β RI; binding experiments in absence and presence of GPR50 did not show any changes in binding capacity, providing evidence that neither increased surface levels of receptor nor increased internalization or a change in receptor stability might occur for the complex in presence of GPR50.

1.4.2. Regulation by feedback loops

A quite frequent observation is, that most of the genes, that interfere with the activity of the TGF β signaling pathway and its receptor are part of feedback-loop mechanisms. Negative regulators as Smad7 (Nakao et al, 1997a) and DRAK2 (Yang et al, 2012) are often induced by TGF β signaling in order to attenuate signaling. This is a sophisticated mean of a cell to regulate signaling spatially and temporarily. For the moment, we did not investigate, if active TGF β signaling has any influence on the expression of GPR50. But future experiments will address the question whether there is a connection between GPR50 expression and active TGF β signaling.

Another question is, how the interaction itself deals with negative feedback loops. Usually, we would suggest, that the activation of the receptor in GPR50 goes along with an increased expression of negative feedback loop regulators as Smad7 that would terminate or weaken signaling by receptor complex degradation and a decrease in Smad phosphorylation. We did not address so far, whether GPR50 provides any protection against signaling arrest. Our

results do not indicate a decrease in signaling, as Smad phosphorylation remains constant. But we can assume, that there must be a way to cope with the habitual negative feedback loop that occurs after TGF β signaling. It might be interesting to see whether there is a competition between Smad7 and GPR50 on receptor binding or whether GPR50 also possesses the capacity to prevent binding of the negative Smad7 regulator and subsequent arrest of signaling if necessary. In turn, a signal induced by GPR50 that can endure long time would provide the cell with a constant amount of active TGF β signaling.

1.4.3. Regulation by other proteins

Another possibility lies in the fact, that accessory proteins might induce the interaction by functioning as scaffold proteins. Thus, distinct signals might induce a protein-mediated juxtaposition of GPR50 and the T β RI with the purpose of allowing a GPR50-induced constitutive activation in the absence of ligand. Such regulation would be a rapid way to induce TGF β -dependent signaling and is in favor of mechanism that involves gene expression of receptor and ligand and secretion.

1.5. Translation of our concept of activation to the TGFβ superfamily

In the present study, the main focus was lying on the prototypic T β RI/T β RII complex and its canonical downstream signaling through the Smads. After having concluded about the general aspects of complex formation and signaling mechanisms, an interesting task is now, to see whether this concept is applicable to the entire signaling spectrum of the TGF β superfamily of cytokines.

1.5.1. Extension to non-canonical signaling of receptors

The T β RI can not only signal via the Smads, but can also be directed to activate other downstream signaling pathways through non-canonical signaling (Zhang, 2009). Receptor associated proteins are often responsible for activating these pathways, as shown for TAK1 that activates p38 (Hanafusa et al, 1999) and TRAF6 that directs TGF β signals towards p38 and JNK (Yamashita et al, 2008) (see also Introduction Chapter 3.3 and 3.4). Whether GPR50-mediated activation of the T β RI results in activation of multiple possible downstream signaling pathways or whether GPR50 selectively activates distinct signaling pathways downstream of the T β RI has not been thoroughly studied. Our results indicate that GPR50 promotes also activation of p38, suggesting a more general effect of GPR50 on T β RI signaling. But additional experiments will provide understanding for the influence of GPR50 on downstream signaling.

1.5.2. Extension to other TGFβ receptor family members

The T β RI is one of seven members of type I receptors of the TGF β ligand superfamily. The other six members are four activin-like-receptors and two BMP-receptors. They display a strong sequence homology, especially in the cytoplasmic part that is relevant for signal transduction: they all comprise the GS domain and are capable for binding FKBP12 (Wang et al, 1994), suggesting that they underlie all the same activation mechanism, even though most information was gained upon investigating the T β RI and T β RII. Since our results suggest so far no mechanism, which seems to be unique for T β RI, the extension of this concept of activation towards other type I receptors seems likely. So far, with regards to the TAP assay, no further type I receptors interacting with GPR50, but this might depend on the relative expression levels of the different T β RI family members. One-by-one verification of these interactions by co-IP or BRET should resolve this issue and show whether activation of Smad 1,5 and 8 could be also regulated by GPR50. This in turn can also contribute to the understanding of the physiological relevance of such complexes (see below).

1.6. Crosstalk between GPCRs and TβRI

The proteins that regulate TGF β signaling are quite divers, ranging from proteins with enzymatic functions (phosphatases, kinases, ubiquitin ligases) to multidomain proteins with adaptor functions (see Chapter I – 3.3.). So far, there have been no reports describing 7TM proteins interfering with T β RI activation through direct receptor interaction. Nevertheless, some intersections have been described concerning a crosstalk between GPCR- and TGF β signaling at downstream levels during the last years.

It has been shown, that TGF β signaling can be regulated by proteins that usually are associated with regulation of GPCR signaling. The β -arrestin molecule is, amongst others, responsible for GPCR internalization and signal termination. A similar mechanism was described for the accessory T β RIII betaglycan (Chen et al, 2003), where interaction induces T β RII/T β RIII receptor complex internalization and signaling arrest. This data got support by the detection of a direct interaction between the T β RII and β -arrestin (McLean et al, 2013). Another link has been found for GPCR-specific kinases, the GRKs, that phosphorylate a

GPCR prior to β -arrestin binding, thus being part of a negative regulating mechanism. Analogous events have been described, where GRK2 had been identified as TGF β -response gene, which then associates with Smads and prevents their C-terminal phosphorylation, providing a mean for negative feedback loop regulation. Though these molecules have been identified originally to be responsible for the regulation of GPCRs it is now established, that their modes of action can also be extended to other signal transduction pathways (Gurevich et al, 2012) (Shenoy & Lefkowitz, 2011). Thus, in this case, we should not directly speak of a crosstalk, but of proteins with a regulatory function that initially have been identified in association with GPCRs, but are now employable for several other signal transduction pathways. Being an orphan receptor, no ligand-promoted pathway activation involving GRKs and β -arrestins have so far been described for GPR50.

Recent work from the group of Peter Little reported a crosstalk between the thrombin receptor (GPCR) and the T β RI, which represents the first example for such a crosstalk. The authors could show that ligand-dependent activation of the thrombin receptor PAR-1 can result in a T β RI-dependent activation of the Smads (Burch et al, 2010). This work provides first evidence of a crosstalk between GPCRs and the T β RI via transactivation on the receptor level (Burch et al, 2012). But so far a detailed mechanism, whether the crosstalk occurs directly through physical interaction on the receptor level or via other downstream intermediates that activate the T β RI, is missing. An indirect activation of a type I receptor has been shown once for serotonin-mediated activation of BMP-dependent Smads, that involve 5-HT_{2B/D} and Rho kinases (Liu et al, 2009b). The present work of the GPR50/T β RI complex is supported by literature evidence, that T β RI-dependent signaling might be affected by GPCRs. In comparison to the thrombin receptor, it seems, according to the current state of knowledge, implausible that we also have a ligand-dependent transactivation. GPR50 seems to have direct effects on the receptor and act as direct regulator of the T β RI. Thus, we report here for the first time a physical association of the T β RI and a 7TM protein on the membrane level.

GPCRs have a tendency to form higher order complexes, either with other GPCRs or GPCRregulating proteins (Maurice et al, 2011a; Maurice et al, 2011b), but also with other membrane receptors of the RTK family (Wetzker & Bohmer, 2003) or ion channels (Altier & Zamponi, 2011). A complex between the T β RI and GPR50 adds another element to the diversity of GPCR interactions and the crosstalk they can exert. Though we have to point out that a case of a constitutive, ligand-independent activation upon complex formation still stands out compared to the cases of ligand- or phosphorylation-dependent transactivation.

2. A new role for GPR50

With our findings of GPR50 forming a complex with the T β RI that induces its constitutive activation, we also established another role for the orphan 7TM protein GPR50. Initially, it was supposed, that transmembrane proteins can only have a function after activation by a ligand. The principal intention was their deorphanization, the discovery of their natural ligand. During the last decade, a slight shift from this doctrine arose with emerging findings proposing a ligand-independent function for orphan 7TM proteins (Levoye et al, 2006c). A prototypic example is GPR50, that has been associated with different ligand-independent functions that converge in the principle that GPR50 complex formation modulates activity of its binding partners, either in membrane, upon hetermerization with MT₁ (Levoye et al, 2006a), in the cytosol with Nogo-A (Grunewald et al, 2009) or upon partial proteolytic cleavage of the C-Tail in the nucleus with TIP60 (Li et al, 2011). Our findings provided another example for this ligand-independent function, that reinforce the concept of GPR50 being an interacting and activity-modulating protein. In contrast to existing publications, that identified Nogo-A and TIP60 as interacting partners, that were based on a Y2H assay with the C-terminal cytosolic portion of GPR50, we used a technique to identify proteins, that interact with the full-length receptor. The results of our TAP assay will probably help to identify further membrane proteins that might be regulated upon complex formation with GPR50.

2.1. Mechanism of function of GPR50

Further information about the of GPR50 and its regulation will surely also help to identify the conditions of these complexes. As GPR50 is most likely a product of genetic fusion of the *Mel1c* gene and an ancestor of the RNA polymerase II (Dufourny et al, 2008), it might also be divided in different functional units. The work from Li *et al.* concerning TIP60 provided evidence for a proteolytic cleavage with a subsequent nuclear translocation of the C-terminal part, which is consistent with the nuclear localization of the RNA Pol II. It will be an interesting task, to unravel the mechanism that induce the proteolytic cleavage of GPR50, thus proposing an important mode of regulation of its activity via its subcellular localization. On the one hand, we might suppose, that the transmembrane part and the cytosolic part function independently as a result of their different origins. But the results from our laboratory for MT₁ and the TβRI indicate a requirement for transmembrane and cytosolic part for being effective.

It is quite intriguing to see, how evolution was able to construct something functional by the fusion of two proteins, that probably originally had independent functions but obtain unique properties upon their combination. Further work will hopefully provide more clear information about the function of the different parts of GPR50, their association and proteolytic cleavage of GPR50, which might in turn also affect the complex formation and stability with the T β RI.

2.2. GPR50 activity

2.2.1. A ligand for GPR50?

An important remark must be made that over all we should be aware of the fact that (1) though it seems unlikely, we have to take in account that GPR50 might have an endogenous ligand that remains to be identified. Although currently, beside the proven inability for melatonin binding, no other possible ligand could have been identified. By employing *in silico* based methods and analysis of structural data of GPR50 a possible ligand might be identified. Under such conditions, we could also hypothesize a transactivation of the TGF β signaling pathway by GPR50's ligand, as for example shown for serotonin receptors 5-HT_{2B/D} and the BMP signaling pathway (Liu et al, 2009b). Especially in circumstances of overexpression, receptors often become hypersensitive to their ligands. But with regards to its restricted expression pattern, it seems not really likely, that a GPR50 ligand will be secreted or produced by HEK293T-, HeLa-, MDA-MB-231- and SNU638 cells that were used in our studies

2.2.2. Constitutive activity of GPR50?

Another possibility is the one of constitutive activity of GPR50. Being a 7TM protein, it might be possible, that GPR50 is constitutively coupled to G proteins, as it has been demonstrated for certain orphan GPCRs as GPR26 and GPR78 (Jones et al, 2007). Thus, an activation mechanism of GPR50 referring to "classical" G protein signaling cannot be completely excluded even so convincing evidence is currently lacking.

2.2.3. GPR50 as scaffold protein

As already pointed out above, the discovery of numerous putative interacting partners for GPR50 supports its function as a scaffold protein that recruits proteins which are responsible

for the effects we observe in the presence of GPR50. A function as a scaffolding protein, at least of the heptarepeats in the cytosolic part of GPR50, would be in accordance with the known function of the hepta-repeat motifs identified in the RNA pol II that can recruit a divers panel of other proteins.

2.3. Homology of GPR50 and FKBP12

During the dissection of the molecular mechanism of the interaction of GPR50 and the T β RI, we identified GPR50 amino acid motifs that are similar to a C-terminal motif in FKBP12. The question that arises is whether this motif in GPR50 might also conduct other functions that FKBP12 is associated with. The amino acids H87 and P88 of the 80s loop in FKBP12 are exposed, thus forming an interaction surface with other proteins as for the calcineurin phosphatase (Aldape et al, 1992; DeCenzo et al, 1996; Futer et al, 1995). In addition FKBP12 also regulates the activity of ryanodine- and IP3- calcium channels that trigger calcium release fom intracellular stores (Ivery, 2000). In turn, GPR50 has been identified to be regulated in dependency of the amount of plasma membrane calcium channels that depend on the activity of internal calcium stores (Zagranichnaya et al, 2005).

The actions of immunophilins as FKBP12 seem also to play an important role in the nervous system (Snyder et al, 1998), a fact of interest in relation to the GPR50 expression pattern in the brain.

2.4. A network of GPR50 interacting partners?

Beside the numerous binding partners of GPR50, which have been identified via Y2H- or TAP assay, we count four studies that describe the mode of action for GPR50 as an interacting protein. An interesting question is, whether they might all converge at some point, like going hand-in-hand or whether they all function independently in a different context and under different circumstances. Additionally these interacting partners are sometimes proteins that have a precise expression pattern, thus suggesting that GPR50 exerts this function like a chameleon in a cell- and tissue-dependent context.

2.5. Comparison of GPR50 variants

Two frequent human variants exist for GPR50, the GPR50wt and GPR50 Δ 4 variant, with the deletion of ⁵⁰²Thr Thr Gly His⁵⁰⁵ and a coupled amino acid exchange at position 532, with a frequency of 40% in the human population (Thomson et al, 2005). Though these variant have

been associated with different phenotypes in genetic association studies (see Introduction, Chapter 2.3.3.) as the tendency to develop mental disorders (Thomson et al., 2005) or lead to altered lipid parameters (Bhattacharyya et al., 2006), no study could so far provide any functional differences of both variants. Our study reports for the first time functional differences of both variants on the molecular level. First hints came up with the findings that both have a different strength to activate T β RI-dependent signal transduction. A deeper look revealed that one of the FKBP12 similarity motifs lies in direct proximity to the 4 amino acid ⁵⁰²TTGH⁵⁰⁵ insertion/deletion and might be the reason for a higher power of the GPR50 Δ 4 to induce the ligand-independent activation of the T β RI. Molecular evidence was also provided by the result, that the ⁴⁹⁵ATSHP⁴⁹⁹ motif is responsible for the competition with FKBP12 upon T β RI binding. The amino acids of the insertion/deletion at position 502 to 505, which contains two Thr residues might be subject to regulatory covalent modifications through phosphorylation. Addition of phosphate groups can cause structural changes effecting the conformation of the ⁴⁹⁵ATSHP⁴⁹⁹ motif. How far our findings can provide explanation for the associated phenotypes, has to be examined in a more physiologic context in the future.

2.6. A new mode of action for orphan GPCRs

Though in the recent years, some ligand-independent functions for orphan GPCRs have been identified, for the moment they have been nearly exclusively restricted to heteromer formation with related GPCRs. Literature data that shows the regulation of other proteins is still rare: only the long-time orphan GPR37 has been described to interact with ion channels and the dopamine active transporter DAT (Marazziti et al, 2007) upon physical interaction. The complex of GPR50 and T β RI describes a new field of action for an orphan GPCR in the regulation of core signaling pathways upon the interplay with the TGF β receptor serine/threonine kinase. Thus, GPR50 is part of the signaling network of a cell, which might be the case also for other orphan 7TM proteins. It will be interesting to see, whether we find similar mechanisms also for other signal transduction pathways as RTKs. For the moment, we still count about 100 orphan proteins that are homologous with GPCRs that wait for an elucidation of their cellular task.

3. Physiological importance of the TβRI-GPR50 complex

Our study mainly focused on the dissection of the molecular mechanisms that determine the interaction and its outcome in cellular models with exogenous expression. The future task is now, to identify the physiological importance of this crosstalk.

3.1. GPR50 and cancer

In order to have a first indication about the impact of GPR50 on TGFB signaling in a functional context, we decided to overexpress GPR50 in MDA-MB-231 breast cancer cells. As the TGF β signaling pathway is an important player in cancer development and progression (Massague, 2008), the MDA-MB-231 model appeared to be particularly attractive, as it is widely used to study the pro-migratory and anti-proliferative effects of TGF^β signaling. Accordingly, GPR50 induced cellular migration and decreased cellular proliferation in vitro and *in vivo*. It will be interesting to further investigate whether the diminished tumor growth in GPR50 expressing cells might be also related to a higher occurrence of metastatic events. TGFβ is known to promote metastasis into lung (Padua et al, 2008) and bone (Yin et al, 1999) in MDA-MB-231 cells. Thus, we might also imagine that cells with high GPR50 expression have higher metastasis rates. For these studies, appropriate experiments in vivo that monitor metastasis will provide more insight in the future. Models of spontaneous cancer development can give insight into GPR50's role in tumor formation: our intention is, to study the effect of GPR50 in MMTV/Neu transgenic mice (Taneja et al, 2009), that spontaneously develop breast cancer. A crossing of these mice with GPR50 KO mice can provide further in vivo evidence for the impact of GPR50 on cancer development. A protective effect of TGFB signaling has already been described (Siegel et al, 2003).

Additionally, it will be interesting to genotype cancer patients according to their GPR50 variant. This could give us an idea whether one genotype has a higher risk for cancer development to put our differential data for GPR50 Δ 4 and GPR50wt in a physiological context. Unfortunately, there are only few databases which align cancer risk and genetic variants. But the recent findings about the importance of genetic variants and their different functionality will hopefully drive interest about the correlation of germline gene variants and disease predisposition as recently demonstrated for the melatonin MT₂ receptor and diabetes risk (Bonnefond et al, 2012).

Some of the positive regulators of TGF β signaling are important tumorsuppressors. Thus, their upregulation could provide additional protective mechanisms against cancer development. There are two studies, which revealed a GPR50 upregulation in pancreatic neoplasms (Buchholz et al, 2005) and nicotine-induced transformation of lung cells (Bavarva et al, 2013). Possibly, an upregulation of GPR50 that goes along with a constitutive TGF β signaling activity might provide a protective mechanism for a cell against cancer in early stages. In contrast, in late cancer stages, TGF β signaling is either deficient or can have tumorpromoting effects. In a progressed cancer, an upregulation might be of negative influence, thus constant TGF β might increase the metastatic potential of a cell. A screening of human cancers concerning altered GPR50 expression can provide more insight for the role of GPR50 in cancer development.

SNU638 cells are cancer cells that have a mutation in the T β RII gene leading to the expression of a truncated extracellular variant (Ku & Park, 2005; Myeroff et al, 1995). Our results in SNU638 cells have shown that ectopic expression of GPR50 can restore T β RI-dependent signaling in these cells. These findings are promising and bear therapeutic potential for gene therapies of cancer with mutant T β RII or TGF β ligands.

In contrast, for late tumor stages, constitutive activation of T β RI might be undesired and should rather be blocked by appropriate pharmaceutical means.

3.2. Relation to known functions and the expression pattern of GPR50

Compared to the T β RI, GPR50 seems to have a more restricted expression pattern. Currently, most available data concern GPR50 expression patterns in the brain, where it has been found in several regions, comprising the area around the 3rd ventricle of median eminence and the tanycytes, hypothalamic regions as the DMH and other regions with different functions. Only few studies investigated the expression of GPR50 in peripheral tissue, demonstrating that GPR50 mRNA is present in various tissues as heart, kidney, testis, liver (Drew et al, 2001). KO mouse models will hopefully provide more information about tissues that contain GPR50 proteins. In addition, compared to the T β RI, the expression of GPR50 seems to be subject of regulation as it has been demonstrated for a season- and energy-status amount of GPR50 in the brain (Barrett et al, 2006; Ivanova et al, 2008). In order to find regions where the crosstalk might take place *in vivo*, tissues that express both receptors will help to gain information.

3.2.1. GPR50 expression in the brain

A region with a markedly high expression of GPR50 are tanycytes, that surround the 3^{rd} ventricle (Sidibe et al, 2010). The T β RI is also expressed in this brain area (Bouret et al, 2004; Bouret et al, 2002; Prevot et al, 2010; Prevot et al, 2000) and its expression can be induced by the gonadotropin-releasing hormone GnRH and subsequently downregulates GnRH activity. Probably, the GPR50/T β RI interaction might occur under certain conditions in order to orchestrate reproductive actions in organisms.

Furthermore, the expression of GPR50 displays different levels during embryogenesis, with the highest amount in late embryonal phase at E18 (Grunewald et al, 2012), when complex structures are formed and the development of brain compartments and neuronal connections takes place. This is coherent with the observation, that GPR50 promotes neurite outgrowth in number and size (Grunewald et al, 2009). Also TGF β signaling is implicated in axon specification during development (Yi et al, 2010) and plays a role in synaptogenesis in adults (Krieglstein et al, 2011; Poon et al, 2013). Probably, the crosstalk of both might have important function during brain development or in the adult brain in synaptic plasticity, which is often altered in mental disorders as depression (Marsden, 2013).

3.2.2. GPR50 and energy homeostasis

Another function of GPR50 is associated with energy homeostasis maintenance. Animals that lack GPR50 have a higher metabolic rate and less weight gain when fed a high fat diet (Ivanova et al, 2008). This is also consistent with the high levels of GPR50 expression in the DMH (Lee et al, 2012), a region important for regulation of the energy status of an organism. A recent study demonstrated an implication of TGF β signaling, notably Smad3, in the development of obesity and diabetes (Yadav et al, 2011).

3.2.3. GPR50 and wound healing

With its effect to promote cellular migration, epithelial-to-mesenchymal transition and the production of ECM components, TGF β has a positive influence on wound healing processes in the organism (O'Kane & Ferguson, 1997). Interestingly, also GPR50 has been found upregulated in hypertrophic scar (Zhang et al, 2010) during wound healing. Probably, we could expect synergistic effects of GPR50 and the T β RI in tissue repair in the organism. A more detailed analysis of wound healing and migration in the absence and presence of GPR50

in addition to our performed experiments and the establishment of relation to active TGF β signaling can tell, if this is an important pathway to influence tissue restoration after injury.

Further potential for physiological relevance of the crosstalk of GPR50 with T β RI dependent signaling will hopefully be gained by figuring out whether we can apply our concept to other members of the type I receptor family, whose expression levels are more precise and implicated in the regulation of specific functions in distinct organs.

An identification of *in vivo* relevance of this GPR50-mediated activation mechanism will provide insight where this alternative mode of T β RI activation takes place. In a (far) future, this might be the subject to therapeutic strategies that can directly target this crosstalk via genetic or chemical therapy.

4. Perspectives

The identification of a new regulatory mode for the T β RI by GPR50 bears therapeutic potential for the future. Even though GPR50 is an orphan receptor, synthetic ligands that act on GPR50 might be designed. These could probably regulate its activity and affect in turn the activity of their binding partners.

Our findings implicate also the possibility, that ectopic expression of GPR50 can restore T β RI activity in case of ligand or T β RII dysfunction. Strategies, related to gene therapies, either through vector based exogenous expression in target tissue or artificial modulation of gene expression are possible options to induce GPR50 expression when its actions might be advantageous for a cell.

In contrast, we can also think of cases, where a constitutive activity might be undesired, as in late stages of cancer or the fibrotic development of a tissue repair processes. In this case, strategies that target the activity of the complex, like inverse agonists or impede with complex formation like interfering peptides might be useful.

EPILOGUE

The exploration of regulatory mechanisms of signal transduction pathways has been one of the major interests in research during the last years. Regulatory proteins are often responsible for the establishment of flexibility and specificity and context-dependent signaling of a pathway. The work prepared for this thesis led to the discovery of a new regulator of TGF β signaling and showed for the first time of a 7TM protein and T β RI on the level of the plasma membrane. The orphan 7TM protein GPR50 can form a complex with the T β RI, which induces its constitutive activation. Surprisingly, this mechanism seems to be different from the commonly established activation mechanism for TGF β signaling, that implies signal propagation from extracellular ligand binding to T β RII then engaging the T β RI. In this work, we describe the previously unappreciated idea, that the T β RI can be activated in absence of ligand and T β RII. Thus, we not only identified another regulator for the TGF β signaling pathway that can assists in fine tuning of the TGF β signaling pathway, but we also describe a new mode of activation for the T β RI. Hence, we add a new facet to the various aspects of TGF β signaling which will hopefully help to better understand functioning of this signaling pathway in physiological and pathologicacal circumstances.

Moreover, we determine a new role for GPR50 that is comforting its ligand-independent function and strengthens its position as a modulatory protein in the signaling network of a cell. For the first time, we also provided evidence that the two frequent human variants of GPR50 have differential functional effects on a molecular level.

Future research that aims for further detailing of the interaction mechanism and where and when we might require a GPR50-induced ligand-independent TGF β signaling will surely provide more information on the formation, regulation and physiological relevance of the GPR50/T β RI complex.

Taken together, our work reveals new features of TGF β signaling, identifies another function for the orphan 7TM protein GPR50 and establishes a functional relevance for both human variants thus adding some new aspects to the understanding of cellular signal transduction concepts that open new ways for therapeutic strategies.

BIBLIOGRAPHY

Abassi Y, Vuori K (2002) Tyrosine 221 in Crk regulates adhesion-dependent membrane localization of Crk and Rac and activation of Rac signaling. *EMBO J* **21**: 4571-4582

Abdalla SA, Letarte M (2006) Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. *Journal of medical genetics* **43**: 97-110

Abdollah S, Macias-Silva M, Tsukazaki T, Hayashi H, Attisano L, Wrana JL (1997) TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. *J Biol Chem* **272**: 27678-27685

Abreu JG, Ketpura NI, Reversade B, De Robertis EM (2002) Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. *Nat Cell Biol* **4:** 599-604

Achour L, Labbe-Jullie C, Scott MG, Marullo S (2008) An escort for GPCRs: implications for regulation of receptor density at the cell surface. *Trends Pharmacol Sci* **29**: 528-535

Adorno M, Cordenonsi M, Montagner M, Dupont S, Wong C, Hann B, Solari A, Bobisse S, Rondina MB, Guzzardo V, Parenti AR, Rosato A, Bicciato S, Balmain A, Piccolo S (2009) A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. *Cell* **137**: 87-98

Aghdasi B, Ye K, Resnick A, Huang A, Ha HC, Guo X, Dawson TM, Dawson VL, Snyder SH (2001) FKBP12, the 12-kDa FK506-binding protein, is a physiologic regulator of the cell cycle. *Proc Natl Acad Sci U S A* **98**: 2425-2430

Agnati LF, Ferre S, Burioni R, Woods A, Genedani S, Franco R, Fuxe K (2005) Existence and theoretical aspects of homomeric and heteromeric dopamine receptor complexes and their relevance for neurological diseases. *Neuromolecular medicine* **7**: 61-78

Akekawatchai C, Holland JD, Kochetkova M, Wallace JC, McColl SR (2005) Transactivation of CXCR4 by the insulin-like growth factor-1 receptor (IGF-1R) in human MDA-MB-231 breast cancer epithelial cells. *J Biol Chem* **280**: 39701-39708

Akhurst RJ, Hata A (2012) Targeting the TGFbeta signalling pathway in disease. *Nat Rev Drug Discov* **11**: 790-811

Alaerts M, Venken T, Lenaerts A, De Zutter S, Norrback K, Adolfsson R, Del-Favero J (2006) Lack of association of an insertion/deletion polymorphism in the G protein-coupled receptor 50 with bipolar disorder in a Northern Swedish population. *Psychiatr Genet* **16**: 235-236

Alarcon C, Zaromytidou AI, Xi Q, Gao S, Yu J, Fujisawa S, Barlas A, Miller AN, Manova-Todorova K, Macias MJ, Sapkota G, Pan D, Massague J (2009) Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. *Cell* **139**: 757-769

Albizu L, Cottet M, Kralikova M, Stoev S, Seyer R, Brabet I, Roux T, Bazin H, Bourrier E, Lamarque L, Breton C, Rives ML, Newman A, Javitch J, Trinquet E, Manning M, Pin JP,

Mouillac B, Durroux T (2010) Time-resolved FRET between GPCR ligands reveals oligomers in native tissues. *Nature chemical biology* **6:** 587-594

Aldape RA, Futer O, DeCenzo MT, Jarrett BP, Murcko MA, Livingston DJ (1992) Charged surface residues of FKBP12 participate in formation of the FKBP12-FK506-calcineurin complex. *J Biol Chem* **267**: 16029-16032

Alexandrow MG, Kawabata M, Aakre M, Moses HL (1995) Overexpression of the c-Myc oncoprotein blocks the growth-inhibitory response but is required for the mitogenic effects of transforming growth factor beta 1. *Proc Natl Acad Sci U S A* **92:** 3239-3243

Allen JA, Roth BL (2011) Strategies to discover unexpected targets for drugs active at G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **51:** 117-144

Altenbach C, Kusnetzow AK, Ernst OP, Hofmann KP, Hubbell WL (2008) High-resolution distance mapping in rhodopsin reveals the pattern of helix movement due to activation. *Proc Natl Acad Sci U S A* **105**: 7439-7444

Altier C, Zamponi GW (2011) Analysis of GPCR/ion channel interactions. *Methods Mol Biol* **756:** 215-225

Andersson EA, Holst B, Sparso T, Grarup N, Banasik K, Holmkvist J, Jorgensen T, Borch-Johnsen K, Egerod KL, Lauritzen T, Sorensen TI, Bonnefond A, Meyre D, Froguel P, Schwartz TW, Pedersen O, Hansen T (2010) MTNR1B G24E variant associates With BMI and fasting plasma glucose in the general population in studies of 22,142 Europeans. *Diabetes* **59**: 1539-1548

Angers S, Salahpour A, Joly E, Hilairet S, Chelsky D, Dennis M, Bouvier M (2000) Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc Natl Acad Sci U S A* **97:** 3684-3689

Annes JP, Munger JS, Rifkin DB (2003) Making sense of latent TGFbeta activation. *J Cell Sci* **116:** 217-224

Atfi A, Baron R (2008) p53 brings a new twist to the Smad signaling network. *Sci Signal* 1: pe33

Atfi A, Djelloul S, Chastre E, Davis R, Gespach C (1997) Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor beta-mediated signaling. *J Biol Chem* **272**: 1429-1432

Atfi A, Dumont E, Colland F, Bonnier D, L'Helgoualc'h A, Prunier C, Ferrand N, Clement B, Wewer UM, Theret N (2007) The disintegrin and metalloproteinase ADAM12 contributes to TGF-beta signaling through interaction with the type II receptor. *J Cell Biol* **178**: 201-208

Attisano L, Carcamo J, Ventura F, Weis FM, Massague J, Wrana JL (1993) Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* **75:** 671-680

Audet M, Bouvier M (2012) Restructuring G-protein- coupled receptor activation. *Cell* **151:** 14-23
Ayoub MA, Couturier C, Lucas-Meunier E, Angers S, Fossier P, Bouvier M, Jockers R (2002) Monitoring of ligand-independent dimerization and ligand-induced conformational changes of melatonin receptors in living cells by bioluminescence resonance energy transfer. *J Biol Chem* **277**: 21522-21528

Ayoub MA, Levoye A, Delagrange P, Jockers R (2004) Preferential formation of MT1/MT2 melatonin receptor heterodimers with distinct ligand interaction properties compared with MT2 homodimers. *Molecular pharmacology* **66**: 312-321

Baba K, Benleulmi-Chaachoua A, Journe AS, Kamal M, Guillaume JL, Dussaud S, Gbahou F, Yettou K, Liu C, Contreras-Alcantara S, Jockers R, Tosini G (2013) Heteromeric MT1/MT2 Melatonin Receptors Modulate Photoreceptor Function. *Sci Signal* **6**: ra89

Bagarova J, Vonner AJ, Armstrong KA, Borgermann J, Lai CS, Deng DY, Beppu H, Alfano I, Filippakopoulos P, Morrell NW, Bullock AN, Knaus P, Mishina Y, Yu PB (2013) Constitutively active ALK2 receptor mutants require type II receptor cooperation. *Mol Cell Biol* **33**: 2413-2424

Bai Y, Yang C, Hu K, Elly C, Liu YC (2004) Itch E3 ligase-mediated regulation of TGF-beta signaling by modulating smad2 phosphorylation. *Mol Cell* **15:** 825-831

Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL (2000) Phosphatidylinositol 3-kinase function is required for transforming growth factor betamediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* **275**: 36803-36810

Bakkebo M, Huse K, Hilden VI, Forfang L, Myklebust JH, Smeland EB, Oksvold MP (2012) SARA is dispensable for functional TGF-beta signaling. *FEBS Lett* **586**: 3367-3372

Baldwin JM (1993) The probable arrangement of the helices in G protein-coupled receptors. *EMBO J* **12**: 1693-1703

Barrett P, Conway S, Morgan PJ (2003) Digging deep--structure-function relationships in the melatonin receptor family. *J Pineal Res* **35:** 221-230

Barrett P, Ivanova E, Graham ES, Ross AW, Wilson D, Ple H, Mercer JG, Ebling FJ, Schuhler S, Dupre SM, Loudon A, Morgan PJ (2006) Photoperiodic regulation of cellular retinol binding protein, CRBP1 [corrected] and nestin in tanycytes of the third ventricle ependymal layer of the Siberian hamster. *J Endocrinol* **191**: 687-698

Barroso R, Martinez Munoz L, Barrondo S, Vega B, Holgado BL, Lucas P, Baillo A, Salles J, Rodriguez-Frade JM, Mellado M (2012) EBI2 regulates CXCL13-mediated responses by heterodimerization with CXCR5. *FASEB J* **26**: 4841-4854

Bassing CH, Howe DJ, Segarini PR, Donahoe PK, Wang XF (1994) A single heteromeric receptor complex is sufficient to mediate biological effects of transforming growth factor-beta ligands. *The Journal of biological chemistry* **269**: 14861-14864

Batailler M, Mullier A, Sidibe A, Delagrange P, Prévot V, Jockers R, Migaud M (2011) Neuroanataomical distribution of the orphan GPR50 receptor in adult sheep and rodent brains. *J Neuroendocrinol*

Bavarva JH, Tae H, Settlage RE, Garner HR (2013) Characterizing the Genetic Basis for Nicotine Induced Cancer Development: A Transcriptome Sequencing Study. *PLoS One* **8**: e67252

Bechtold DA, Sidibe A, Saer BR, Li J, Hand LE, Ivanova EA, Darras VM, Dam J, Jockers R, Luckman SM, Loudon AS (2012) A role for the melatonin-related receptor GPR50 in leptin signaling, adaptive thermogenesis, and torpor. *Curr Biol* **22**: 70-77

Beedle AM, McRory JE, Poirot O, Doering CJ, Altier C, Barrere C, Hamid J, Nargeot J, Bourinet E, Zamponi GW (2004) Agonist-independent modulation of N-type calcium channels by ORL1 receptors. *Nat Neurosci* **7**: 118-125

Beinborn M, Blum A, Hang L, Setiawan T, Schroeder JC, Stoyanoff K, Leung J, Weinstock JV (2010) TGF-beta regulates T-cell neurokinin-1 receptor internalization and function. *Proc Natl Acad Sci U S A* **107:** 4293-4298

Benovic JL (2012) G-protein-coupled receptors signal victory. Cell 151: 1148-1150

Bergelin N, Lof C, Balthasar S, Kalhori V, Tornquist K (2010) S1P1 and VEGFR-2 form a signaling complex with extracellularly regulated kinase 1/2 and protein kinase C-alpha regulating ML-1 thyroid carcinoma cell migration. *Endocrinology* **151**: 2994-3005

Bettler B, Kaupmann K, Mosbacher J, Gassmann M (2004) Molecular structure and physiological functions of GABA(B) receptors. *Physiol Rev* 84: 835-867

Bhattacharyya S, Luan J, Challis B, Keogh J, Montague C, Brennand J, Morten J, Lowenbeim S, Jenkins S, Farooqi IS, Wareham NJ, O'Rahilly S (2006) Sequence variants in the melatonin-related receptor gene (GPR50) associate with circulating triglyceride and HDL levels. *J Lipid Res* **47**: 761-766

Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL (2001) Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* **12**: 27-36

Bitzer M, von Gersdorff G, Liang D, Dominguez-Rosales A, Beg AA, Rojkind M, Bottinger EP (2000) A mechanism of suppression of TGF-beta/SMAD signaling by NF-kappa B/RelA. *Genes Dev* **14**: 187-197

Blahna MT, Hata A (2012) Smad-mediated regulation of microRNA biosynthesis. *FEBS Lett* **586:** 1906-1912

Blakely BT, Rossi FM, Tillotson B, Palmer M, Estelles A, Blau HM (2000) Epidermal growth factor receptor dimerization monitored in live cells. *Nature biotechnology* **18**: 218-222

Bobik A (2006) Transforming growth factor-betas and vascular disorders. *Arteriosclerosis, thrombosis, and vascular biology* **26:** 1712-1720

Bockaert J (2012) [G-protein coupled receptors. Nobel Prize 2012 for chemistry to Robert J. Lefkowitz and Brian Kobilka]. *Med Sci (Paris)* **28:** 1133-1137

Bockaert J, Pin JP (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J* **18**: 1723-1729

Bonnefond A, Clement N, Fawcett K, Yengo L, Vaillant E, Guillaume JL, Dechaume A, Payne F, Roussel R, Czernichow S, Hercberg S, Hadjadj S, Balkau B, Marre M, Lantieri O, Langenberg C, Bouatia-Naji N, Meta-Analysis of G, Insulin-Related Traits C, Charpentier G, Vaxillaire M, Rocheleau G, Wareham NJ, Sladek R, McCarthy MI, Dina C, Barroso I, Jockers R, Froguel P (2012) Rare MTNR1B variants impairing melatonin receptor 1B function contribute to type 2 diabetes. *Nat Genet* **44**: 297-301

Bonni S, Wang HR, Causing CG, Kavsak P, Stroschein SL, Luo K, Wrana JL (2001) TGFbeta induces assembly of a Smad2-Smurf2 ubiquitin ligase complex that targets SnoN for degradation. *Nat Cell Biol* **3**: 587-595

Bouatia-Naji N, Bonnefond A, Cavalcanti-Proenca C, Sparso T, Holmkvist J, Marchand M, Delplanque J, Lobbens S, Rocheleau G, Durand E, De Graeve F, Chevre JC, Borch-Johnsen K, Hartikainen AL, Ruokonen A, Tichet J, Marre M, Weill J, Heude B, Tauber M, Lemaire K, Schuit F, Elliott P, Jorgensen T, Charpentier G, Hadjadj S, Cauchi S, Vaxillaire M, Sladek R, Visvikis-Siest S, Balkau B, Levy-Marchal C, Pattou F, Meyre D, Blakemore AI, Jarvelin MR, Walley AJ, Hansen T, Dina C, Pedersen O, Froguel P (2009) A variant near MTNR1B is associated with increased fasting plasma glucose levels and type 2 diabetes risk. *Nat Genet* **41:** 89-94

Bouret S, De Seranno S, Beauvillain JC, Prevot V (2004) Transforming growth factor beta1 may directly influence gonadotropin-releasing hormone gene expression in the rat hypothalamus. *Endocrinology* **145**: 1794-1801

Bouret S, Prevot V, Takumi T, Beauvillain JC, Mitchell V (2002) Regulation by gonadal steroids of the mRNA encoding for a type I receptor for TGF-beta in the female rat hypothalamus. *Neuroendocrinology* **76**: 1-7

Bouvier M (2001) Oligomerization of G-protein-coupled transmitter receptors. *Nature reviews Neuroscience* **2:** 274-286

Bouvier M (2013) Unraveling the structural basis of GPCR activation and inactivation. *Nature structural & molecular biology* **20:** 539-541

Boyer SB, Slesinger PA (2010) Probing novel GPCR interactions using a combination of FRET and TIRF. *Communicative & integrative biology* **3**: 343-346

Bridges TM, Lindsley CW (2008) G-protein-coupled receptors: from classical modes of modulation to allosteric mechanisms. *ACS chemical biology* **3**: 530-541

Brown CB, Boyer AS, Runyan RB, Barnett JV (1999a) Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. *Science* **283**: 2080-2082

Brown JD, DiChiara MR, Anderson KR, Gimbrone MA, Jr., Topper JN (1999b) MEKK-1, a component of the stress (stress-activated protein kinase/c-Jun N-terminal kinase) pathway, can selectively activate Smad2-mediated transcriptional activation in endothelial cells. *J Biol Chem* **274**: 8797-8805

Buchholz M, Braun M, Heidenblut A, Kestler HA, Kloppel G, Schmiegel W, Hahn SA, Luttges J, Gress TM (2005) Transcriptome analysis of microdissected pancreatic intraepithelial neoplastic lesions. *Oncogene* **24**: 6626-6636

Burch ML, Ballinger ML, Yang SN, Getachew R, Itman C, Loveland K, Osman N, Little PJ (2010) Thrombin stimulation of proteoglycan synthesis in vascular smooth muscle is mediated by protease-activated receptor-1 transactivation of the transforming growth factor beta type I receptor. *J Biol Chem* **285**: 26798-26805

Burch ML, Osman N, Getachew R, Al-Aryahi S, Poronnik P, Zheng W, Hill MA, Little PJ (2012) G protein coupled receptor transactivation: extending the paradigm to include serine/threonine kinase receptors. *Int J Biochem Cell Biol* **44**: 722-727

Cabello N, Gandia J, Bertarelli DC, Watanabe M, Lluis C, Franco R, Ferre S, Lujan R, Ciruela F (2009) Metabotropic glutamate type 5, dopamine D2 and adenosine A2a receptors form higher-order oligomers in living cells. *J Neurochem* **109**: 1497-1507

Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, Hamm HE (2003) Insights into G protein structure, function, and regulation. *Endocrine reviews* **24**: 765-781

Calebiro D, Rieken F, Wagner J, Sungkaworn T, Zabel U, Borzi A, Cocucci E, Zurn A, Lohse MJ (2013) Single-molecule analysis of fluorescently labeled G-protein-coupled receptors reveals complexes with distinct dynamics and organization. *Proc Natl Acad Sci U S A* **110**: 743-748

Calvo JR, Gonzalez-Yanes C, Maldonado MD (2013) The role of melatonin in the cells of the innate immunity: a review. *J Pineal Res* **55:** 103-120

Canals M, Milligan G (2008) Constitutive activity of the cannabinoid CB1 receptor regulates the function of co-expressed Mu opioid receptors. *J Biol Chem* **283**: 11424-11434

Cao TT, Brelot A, von Zastrow M (2005) The composition of the beta-2 adrenergic receptor oligomer affects its membrane trafficking after ligand-induced endocytosis. *Molecular pharmacology* **67:** 288-297

Cao W, Luttrell LM, Medvedev AV, Pierce KL, Daniel KW, Dixon TM, Lefkowitz RJ, Collins S (2000) Direct binding of activated c-Src to the beta 3-adrenergic receptor is required for MAP kinase activation. *J Biol Chem* **275:** 38131-38134

Carcamo J, Zentella A, Massague J (1995) Disruption of transforming growth factor beta signaling by a mutation that prevents transphosphorylation within the receptor complex. *Mol Cell Biol* **15**: 1573-1581

Chambers JC, Zhang W, Zabaneh D, Sehmi J, Jain P, McCarthy MI, Froguel P, Ruokonen A, Balding D, Jarvelin MR, Scott J, Elliott P, Kooner JS (2009) Common genetic variation near

melatonin receptor MTNR1B contributes to raised plasma glucose and increased risk of type 2 diabetes among Indian Asians and European Caucasians. *Diabetes* **58**: 2703-2708

Chang J, Park K, Bang YJ, Kim WS, Kim D, Kim SJ (1997) Expression of transforming growth factor beta type II receptor reduces tumorigenicity in human gastric cancer cells. *Cancer research* **57**: 2856-2859

Charng MJ, Kinnunen P, Hawker J, Brand T, Schneider MD (1996) FKBP-12 recognition is dispensable for signal generation by type I transforming growth factor-beta receptors. *J Biol Chem* **271**: 22941-22944

Chaste P, Clement N, Mercati O, Guillaume J, Delorme R, Botros H, Pagan C, Périvier S, Scheid I, Nygren G, Anckarsäter H, Rastam M, Ståhlberg O, Gillberg C, Serrano E, Lemière N, Launay J, Mouren-Simeoni M, Leboyer M, Jockers R, Bourgeron T (2010) Identification of pathway-biased and deleterious melatonin receptor mutants in autism spectrum disorders and in the general population. *PLoS One* **5**: e11495

Cheifetz S, Bellon T, Cales C, Vera S, Bernabeu C, Massague J, Letarte M (1992) Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. *J Biol Chem* **267**: 19027-19030

Chen CR, Kang Y, Massague J (2001) Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program. *Proc Natl Acad Sci U S A* **98:** 992-999

Chen G, Chen X, Sukumar A, Gao B, Curley J, Schnaper HW, Ingram AJ, Krepinsky JC (2013) TGFbeta receptor I transactivation mediates stretch-induced Pak1 activation and CTGF upregulation in mesangial cells. *J Cell Sci* **126**: 3697-3712

Chen RH, Chang TY (1997) Involvement of caspase family proteases in transforming growth factor-beta-induced apoptosis. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* **8:** 821-827

Chen RH, Su YH, Chuang RL, Chang TY (1998) Suppression of transforming growth factorbeta-induced apoptosis through a phosphatidylinositol 3-kinase/Akt-dependent pathway. *Oncogene* **17:** 1959-1968

Chen SJ, Yuan W, Mori Y, Levenson A, Trojanowska M, Varga J (1999) Stimulation of type I collagen transcription in human skin fibroblasts by TGF-beta: involvement of Smad 3. *The Journal of investigative dermatology* **112:** 49-57

Chen W, Kirkbride KC, How T, Nelson CD, Mo J, Frederick JP, Wang XF, Lefkowitz RJ, Blobe GC (2003) Beta-arrestin 2 mediates endocytosis of type III TGF-beta receptor and down-regulation of its signaling. *Science* **301**: 1394-1397

Chen X, Rubock MJ, Whitman M (1996) A transcriptional partner for MAD proteins in TGFbeta signalling. *Nature* **383**: 691-696

Chen X, Xu H, Yuan P, Fang F, Huss M, Vega VB, Wong E, Orlov YL, Zhang W, Jiang J, Loh YH, Yeo HC, Yeo ZX, Narang V, Govindarajan KR, Leong B, Shahab A, Ruan Y,

Bourque G, Sung WK, Clarke ND, Wei CL, Ng HH (2008) Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. *Cell* **133**: 1106-1117

Chen Y, Liu F, Massague J (1997) Mechanism of TGFbeta receptor inhibition by FKBP12. *EMBO J* 16: 3866-3876

Cherezov V, Abola E, Stevens RC (2010) Recent progress in the structure determination of GPCRs, a membrane protein family with high potential as pharmaceutical targets. *Methods Mol Biol* **654**: 141-168

Chow JY, Quach KT, Cabrera BL, Cabral JA, Beck SE, Carethers JM (2007) RAS/ERK modulates TGFbeta-regulated PTEN expression in human pancreatic adenocarcinoma cells. *Carcinogenesis* **28**: 2321-2327

Chu J, Zheng H, Zhang Y, Loh HH, Law PY (2010) Agonist-dependent mu-opioid receptor signaling can lead to heterologous desensitization. *Cell Signal* **22**: 684-696

Ciruela F, Escriche M, Burgueno J, Angulo E, Casado V, Soloviev MM, Canela EI, Mallol J, Chan WY, Lluis C, McIlhinney RA, Franco R (2001) Metabotropic glutamate 1alpha and adenosine A1 receptors assemble into functionally interacting complexes. *J Biol Chem* **276**: 18345-18351

Cismowski MJ (2006) Non-receptor activators of heterotrimeric G-protein signaling (AGS proteins). *Seminars in cell & developmental biology* **17:** 334-344

Civelli O (1998) Functional genomics: the search for novel neurotransmitters and neuropeptides. *FEBS Lett* **430**: 55-58

Civelli O (2005) GPCR deorphanizations: the novel, the known and the unexpected transmitters. *Trends Pharmacol Sci* **26**: 15-19

Civelli O (2012) Orphan GPCRs and neuromodulation. Neuron 76: 12-21

Civelli O, Reinscheid RK, Zhang Y, Wang Z, Fredriksson R, Schioth HB (2013) G proteincoupled receptor deorphanizations. *Annu Rev Pharmacol Toxicol* **53**: 127-146

Civelli O, Saito Y, Wang Z, Nothacker H, Reinscheid R (2006) Orphan GPCRs and their ligands. *Pharmacol Ther* **110**: 525-532

Conery AR, Cao Y, Thompson EA, Townsend CM, Jr., Ko TC, Luo K (2004) Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. *Nat Cell Biol* **6:** 366-372

Conway S, Drew JE, Mowat ES, Barrett P, Delagrange P, Morgan PJ (2000) Chimeric melatonin mt1 and melatonin-related receptors. Identification of domains and residues participating in ligand binding and receptor activation of the melatonin mt1 receptor. *J Biol Chem* **275**: 20602-20609

Cox B, Sharma P, Evangelou AI, Whiteley K, Ignatchenko V, Ignatchenko A, Baczyk D, Czikk M, Kingdom J, Rossant J, Gramolini AO, Adamson SL, Kislinger T (2011)

Translational analysis of mouse and human placental protein and mRNA reveals distinct molecular pathologies in human preeclampsia. *Mol Cell Proteomics* **10**: M111 012526

Datta PK, Chytil A, Gorska AE, Moses HL (1998) Identification of STRAP, a novel WD domain protein in transforming growth factor-beta signaling. *J Biol Chem* **273**: 34671-34674

Daub H, Wallasch C, Lankenau A, Herrlich A, Ullrich A (1997) Signal characteristics of G protein-transactivated EGF receptor. *EMBO J* **16**: 7032-7044

Daulat A, Maurice P, Froment C, Guillaume J, Broussard C, Monsarrat B, Delagrange P, Jockers R (2007) Purification and identification of G protein-coupled receptor protein complexes under native conditions. *Mol Cell Proteomics* **6**: 835-844

Daulat A, Maurice P, Jockers R (2013) Techniques for the discovery of GPCR-associated protein complexes. *Methods Enzymol* **521**: 329-345

de Caestecker MP, Parks WT, Frank CJ, Castagnino P, Bottaro DP, Roberts AB, Lechleider RJ (1998) Smad2 transduces common signals from receptor serine-threonine and tyrosine kinases. *Genes Dev* **12**: 1587-1592

de Jong JS, van Diest PJ, van der Valk P, Baak JP (2001) Expression of growth factors, growth factor receptors and apoptosis related proteins in invasive breast cancer: relation to apoptotic rate. *Breast cancer research and treatment* **66**: 201-208

DeCenzo MT, Park ST, Jarrett BP, Aldape RA, Futer O, Murcko MA, Livingston DJ (1996) FK506-binding protein mutational analysis: defining the active-site residue contributions to catalysis and the stability of ligand complexes. *Protein engineering* **9**: 173-180

DeFea KA, Zalevsky J, Thoma MS, Dery O, Mullins RD, Bunnett NW (2000) beta-arrestindependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol* **148**: 1267-1281

Delavest M, Even C, Benjemaa N, Poirier MF, Jockers R, Krebs MO (2012) Association of the intronic rs2072621 polymorphism of the X-linked GPR50 gene with affective disorder with seasonal pattern. *Eur Psychiatry* **27**: 369-371

Delcourt N, Bockaert J, Marin P (2007a) GPCR-jacking: from a new route in RTK signalling to a new concept in GPCR activation. *Trends Pharmacol Sci* **28**: 602-607

Delcourt N, Thouvenot E, Chanrion B, Galeotti N, Jouin P, Bockaert J, Marin P (2007b) PACAP type I receptor transactivation is essential for IGF-1 receptor signalling and antiapoptotic activity in neurons. *EMBO J* **26**: 1542-1551

Dennler S, Prunier C, Ferrand N, Gauthier JM, Atfi A (2000) c-Jun inhibits transforming growth factor beta-mediated transcription by repressing Smad3 transcriptional activity. *J Biol Chem* **275**: 28858-28865

Derynck R, Gelbart WM, Harland RM, Heldin CH, Kern SE, Massague J, Melton DA, Mlodzik M, Padgett RW, Roberts AB, Smith J, Thomsen GH, Vogelstein B, Wang XF (1996) Nomenclature: vertebrate mediators of TGFbeta family signals. *Cell* **87**: 173

Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JL (2003) Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol* **5**: 410-421

Dixon RA, Kobilka BK, Strader DJ, Benovic JL, Dohlman HG, Frielle T, Bolanowski MA, Bennett CD, Rands E, Diehl RE, Mumford RA, Slater EE, Sigal IS, Caron MG, Lefkowitz RJ, Strader CD (1986) Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. *Nature* **321**: 75-79

Dorsam RT, Gutkind JS (2007) G-protein-coupled receptors and cancer. *Nature reviews Cancer* **7**: 79-94

Dorsch S, Klotz KN, Engelhardt S, Lohse MJ, Bunemann M (2009) Analysis of receptor oligomerization by FRAP microscopy. *Nat Methods* **6:** 225-230

Drew J, Barrett P, Mercer J, Moar K, Canet E, Delagrange P, Morgan P (2001) Localization of the melatonin-related receptor in the rodent brain and peripheral tissues. *J Neuroendocrinol* **13:** 453-458

Drew J, Barrett P, Williams L, Conway S, Morgan P (1998) The ovine melatonin-related receptor: cloning and preliminary distribution and binding studies. *J Neuroendocrinol* **10**: 651-661

Dufourny L, Levasseur A, Migaud M, Callebaut I, Pontarotti P, Malpaux B, Monget P (2008) GPR50 is the mammalian ortholog of Mel1c: evidence of rapid evolution in mammals. *BMC Evol Biol* **8**: 105

Ebisawa T, Karne S, Lerner MR, Reppert SM (1994) Expression cloning of a high-affinity melatonin receptor from Xenopus dermal melanophores. *Proc Natl Acad Sci U S A* **91:** 6133-6137

Edlund S, Landstrom M, Heldin CH, Aspenstrom P (2002) Transforming growth factor-betainduced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA. *Mol Biol Cell* **13**: 902-914

Eichhorn PJ, Rodon L, Gonzalez-Junca A, Dirac A, Gili M, Martinez-Saez E, Aura C, Barba I, Peg V, Prat A, Cuartas I, Jimenez J, Garcia-Dorado D, Sahuquillo J, Bernards R, Baselga J, Seoane J (2012) USP15 stabilizes TGF-beta receptor I and promotes oncogenesis through the activation of TGF-beta signaling in glioblastoma. *Nat Med* **18**: 429-435

Felici A, Wurthner JU, Parks WT, Giam LR, Reiss M, Karpova TS, McNally JG, Roberts AB (2003) TLP, a novel modulator of TGF-beta signaling, has opposite effects on Smad2- and Smad3-dependent signaling. *EMBO J* **22**: 4465-4477

Feng X, Derynck R (2005) Specificity and versatility in tgf-beta signaling through Smads. *Annu Rev Cell Dev Biol* **21:** 659-693

Feng XH, Derynck R (1997) A kinase subdomain of transforming growth factor-beta (TGFbeta) type I receptor determines the TGF-beta intracellular signaling specificity. *EMBO J* 16: 3912-3923 Ferguson SS, Caron MG (2004) Green fluorescent protein-tagged beta-arrestin translocation as a measure of G protein-coupled receptor activation. *Methods Mol Biol* **237**: 121-126

Ferrand N, Atfi A, Prunier C (2010) The oncoprotein c-ski functions as a direct antagonist of the transforming growth factor-{beta} type I receptor. *Cancer research* **70:** 8457-8466

Ferrari G, Cook BD, Terushkin V, Pintucci G, Mignatti P (2009) Transforming growth factorbeta 1 (TGF-beta1) induces angiogenesis through vascular endothelial growth factor (VEGF)mediated apoptosis. *J Cell Physiol* **219:** 449-458

Ferre S, von Euler G, Johansson B, Fredholm BB, Fuxe K (1991) Stimulation of high-affinity adenosine A2 receptors decreases the affinity of dopamine D2 receptors in rat striatal membranes. *Proc Natl Acad Sci U S A* **88**: 7238-7241

Ferrigno O, Lallemand F, Verrecchia F, L'Hoste S, Camonis J, Atfi A, Mauviel A (2002) Yesassociated protein (YAP65) interacts with Smad7 and potentiates its inhibitory activity against TGF-beta/Smad signaling. *Oncogene* **21**: 4879-4884

Fischer OM, Hart S, Ullrich A (2006) Dissecting the epidermal growth factor receptor signal transactivation pathway. *Methods Mol Biol* **327:** 85-97

Franzen P, ten Dijke P, Ichijo H, Yamashita H, Schulz P, Heldin CH, Miyazono K (1993) Cloning of a TGF beta type I receptor that forms a heteromeric complex with the TGF beta type II receptor. *Cell* **75**: 681-692

Fribourg M, Moreno JL, Holloway T, Provasi D, Baki L, Mahajan R, Park G, Adney SK, Hatcher C, Eltit JM, Ruta JD, Albizu L, Li Z, Umali A, Shim J, Fabiato A, MacKerell AD, Jr., Brezina V, Sealfon SC, Filizola M, Gonzalez-Maeso J, Logothetis DE (2011) Decoding the signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. *Cell* **147**: 1011-1023

Fukuchi M, Imamura T, Chiba T, Ebisawa T, Kawabata M, Tanaka K, Miyazono K (2001) Ligand-dependent degradation of Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins. *Mol Biol Cell* **12**: 1431-1443

Furuhashi M, Yagi K, Yamamoto H, Furukawa Y, Shimada S, Nakamura Y, Kikuchi A, Miyazono K, Kato M (2001) Axin facilitates Smad3 activation in the transforming growth factor beta signaling pathway. *Mol Cell Biol* **21**: 5132-5141

Futer O, DeCenzo MT, Aldape RA, Livingston DJ (1995) FK506 binding protein mutational analysis. Defining the surface residue contributions to stability of the calcineurin co-complex. *J Biol Chem* **270**: 18935-18940

Galandrin S, Oligny-Longpre G, Bonin H, Ogawa K, Gales C, Bouvier M (2008) Conformational rearrangements and signaling cascades involved in ligand-biased mitogenactivated protein kinase signaling through the beta1-adrenergic receptor. *Molecular pharmacology* **74**: 162-172

Galliher AJ, Schiemann WP (2007) Src phosphorylates Tyr284 in TGF-beta type II receptor and regulates TGF-beta stimulation of p38 MAPK during breast cancer cell proliferation and invasion. *Cancer research* **67:** 3752-3758

Galvez T, Duthey B, Kniazeff J, Blahos J, Rovelli G, Bettler B, Prezeau L, Pin JP (2001) Allosteric interactions between GB1 and GB2 subunits are required for optimal GABA(B) receptor function. *EMBO J* **20**: 2152-2159

Georgi LL, Albert PS, Riddle DL (1990) daf-1, a C. elegans gene controlling dauer larva development, encodes a novel receptor protein kinase. *Cell* **61**: 635-645

Gomis RR, Alarcon C, Nadal C, Van Poznak C, Massague J (2006) C/EBPbeta at the core of the TGFbeta cytostatic response and its evasion in metastatic breast cancer cells. *Cancer Cell* **10:** 203-214

Gonzalez-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub NV, Lopez-Gimenez JF, Zhou M, Okawa Y, Callado LF, Milligan G, Gingrich JA, Filizola M, Meana JJ, Sealfon SC (2008) Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature* **452**: 93-97

Gordon KJ, Blobe GC (2008) Role of transforming growth factor-beta superfamily signaling pathways in human disease. *Biochim Biophys Acta* **1782:** 197-228

Goto D, Nakajima H, Mori Y, Kurasawa K, Kitamura N, Iwamoto I (2001) Interaction between Smad anchor for receptor activation and Smad3 is not essential for TGF-beta/Smad3-mediated signaling. *Biochem Biophys Res Commun* **281**: 1100-1105

Granier S (2012) [Structure of mu and delta opioid receptors]. Med Sci (Paris) 28: 870-875

Gray PC, Harrison CA, Vale W (2003) Cripto forms a complex with activin and type II activin receptors and can block activin signaling. *Proc Natl Acad Sci U S A* **100:** 5193-5198

Griswold-Prenner I, Kamibayashi C, Maruoka EM, Mumby MC, Derynck R (1998) Physical and functional interactions between type I transforming growth factor beta receptors and Balpha, a WD-40 repeat subunit of phosphatase 2A. *Mol Cell Biol* **18**: 6595-6604

Groppe J, Greenwald J, Wiater E, Rodriguez-Leon J, Economides AN, Kwiatkowski W, Affolter M, Vale WW, Izpisua Belmonte JC, Choe S (2002) Structural basis of BMP signalling inhibition by the cystine knot protein Noggin. *Nature* **420**: 636-642

Groppe JC, Shore EM, Kaplan FS (2007) Functional modeling of the ACVR1 (R206H) mutation in FOP. *Clinical orthopaedics and related research* **462**: 87-92

Groppe JC, Wu J, Shore EM, Kaplan FS (2011) In vitro analyses of the dysregulated R206H ALK2 kinase-FKBP12 interaction associated with heterotopic ossification in FOP. *Cells, tissues, organs* **194**: 291-295

Gruendler C, Lin Y, Farley J, Wang T (2001) Proteasomal degradation of Smad1 induced by bone morphogenetic proteins. *J Biol Chem* **276**: 46533-46543

Grünewald E, Kinnell H, Porteous D, Thomson P (2009) GPR50 interacts with neuronal NOGO-A and affects neurite outgrowth. *Mol Cell Neurosci* **42:** 363-371

Grunewald E, Kinnell HL, Porteous DJ, Thomson PA (2009) GPR50 interacts with neuronal NOGO-A and affects neurite outgrowth. *Mol Cell Neurosci* **42:** 363-371

Grunewald E, Tew KD, Porteous DJ, Thomson PA (2012) Developmental expression of orphan g protein-coupled receptor 50 in the mouse brain. *ACS Chem Neurosci* **3:** 459-472

Gubitz A, Reppert S (1999a) Assignment of the melatonin-related receptor to human chromosome X (GPR50) and mouse chromosome X (Gpr50). *Genomics* **55**: 248-251

Gubitz AK, Reppert SM (1999b) Assignment of the melatonin-related receptor to human chromosome X (GPR50) and mouse chromosome X (Gpr50). *Genomics* **55**: 248-251

Gubitz AK, Reppert SM (2000) Chimeric and point-mutated receptors reveal that a single glycine residue in transmembrane domain 6 is critical for high affinity melatonin binding. *Endocrinology* **141:** 1236-1244

Guillaume JL, Daulat AM, Maurice P, Levoye A, Migaud M, Brydon L, Malpaux B, Borg-Capra C, Jockers R (2008) The PDZ protein mupp1 promotes Gi coupling and signaling of the Mt1 melatonin receptor. *J Biol Chem* **283**: 16762-16771

Gunther CV, Georgi LL, Riddle DL (2000) A Caenorhabditis elegans type I TGF beta receptor can function in the absence of type II kinase to promote larval development. *Development* **127**: 3337-3347

Guo X, Wang XF (2009) Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell Res* **19:** 71-88

Gupte J, Swaminath G, Danao J, Tian H, Li Y, Wu X (2012) Signaling property study of adhesion G-protein-coupled receptors. *FEBS Lett* **586**: 1214-1219

Gurevich EV, Tesmer JJ, Mushegian A, Gurevich VV (2012) G protein-coupled receptor kinases: more than just kinases and not only for GPCRs. *Pharmacol Ther* **133**: 40-69

Hahn SA, Hoque AT, Moskaluk CA, da Costa LT, Schutte M, Rozenblum E, Seymour AB, Weinstein CL, Yeo CJ, Hruban RH, Kern SE (1996) Homozygous deletion map at 18q21.1 in pancreatic cancer. *Cancer research* **56:** 490-494

Hamouda H, Chen P, Levoye A, Sözer-Topçular N, Daulat A, Guillaume J, Ravid R, Savaskan E, Ferry G, Boutin J, Delagrange P, Jockers R, Maurice P (2007a) Detection of the human GPR50 orphan seven transmembrane protein by polyclonal antibodies mapping different epitopes. *J Pineal Res* **43**: 10-15

Hamouda HO, Chen P, Levoye A, Sozer-Topcular N, Daulat AM, Guillaume JL, Ravid R, Savaskan E, Ferry G, Boutin JA, Delagrange P, Jockers R, Maurice P (2007b) Detection of the human GPR50 orphan seven transmembrane protein by polyclonal antibodies mapping different epitopes. *J Pineal Res* **43**: 10-15

Hanafusa H, Ninomiya-Tsuji J, Masuyama N, Nishita M, Fujisawa J, Shibuya H, Matsumoto K, Nishida E (1999) Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. *J Biol Chem* **274**: 27161-27167

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100: 57-70

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144: 646-674

Hanks SK, Hunter T (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J* **9**: 576-596

Hanyu A, Ishidou Y, Ebisawa T, Shimanuki T, Imamura T, Miyazono K (2001) The N domain of Smad7 is essential for specific inhibition of transforming growth factor-beta signaling. *J Cell Biol* **155**: 1017-1027

Hardeland R, Cardinali DP, Srinivasan V, Spence DW, Brown GM, Pandi-Perumal SR (2011) Melatonin--a pleiotropic, orchestrating regulator molecule. *Prog Neurobiol* **93:** 350-384

Hart PJ, Deep S, Taylor AB, Shu Z, Hinck CS, Hinck AP (2002) Crystal structure of the human TbetaR2 ectodomain--TGF-beta3 complex. *Nature structural biology* **9**: 203-208

Hay DL, Poyner DR, Sexton PM (2006) GPCR modulation by RAMPs. *Pharmacol Ther* **109**: 173-197

Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Jr., Wrana JL, Falb D (1997) The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* **89:** 1165-1173

Hebert TE, Bouvier M (1998) Structural and functional aspects of G protein-coupled receptor oligomerization. *Biochem Cell Biol* **76:** 1-11

Heikkinen PT, Nummela M, Leivonen SK, Westermarck J, Hill CS, Kahari VM, Jaakkola PM (2010) Hypoxia-activated Smad3-specific dephosphorylation by PP2A. *J Biol Chem* **285**: 3740-3749

Heldin CH, Landstrom M, Moustakas A (2009) Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. *Curr Opin Cell Biol* **21**: 166-176

Heldin CH, Moustakas A (2012) Role of Smads in TGFbeta signaling. *Cell Tissue Res* 347: 21-36

Heldin CH, Vanlandewijck M, Moustakas A (2012) Regulation of EMT by TGFbeta in cancer. *FEBS Lett* **586**: 1959-1970

Hillion J, Canals M, Torvinen M, Casado V, Scott R, Terasmaa A, Hansson A, Watson S, Olah ME, Mallol J, Canela EI, Zoli M, Agnati LF, Ibanez CF, Lluis C, Franco R, Ferre S, Fuxe K (2002) Coaggregation, cointernalization, and codesensitization of adenosine A2A receptors and dopamine D2 receptors. *J Biol Chem* **277**: 18091-18097

Hirono M, Yoshioka T, Konishi S (2001) GABA(B) receptor activation enhances mGluRmediated responses at cerebellar excitatory synapses. *Nat Neurosci* **4:** 1207-1216 Ho J, Cocolakis E, Dumas VM, Posner BI, Laporte SA, Lebrun JJ (2005) The G proteincoupled receptor kinase-2 is a TGFbeta-inducible antagonist of TGFbeta signal transduction. *EMBO J* **24:** 3247-3258

Hocevar BA, Brown TL, Howe PH (1999) TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. *EMBO J* **18**: 1345-1356

Hocevar BA, Smine A, Xu XX, Howe PH (2001) The adaptor molecule Disabled-2 links the transforming growth factor beta receptors to the Smad pathway. *EMBO J* **20**: 2789-2801

Hollenstein K, Kean J, Bortolato A, Cheng RK, Dore AS, Jazayeri A, Cooke RM, Weir M, Marshall FH (2013) Structure of class B GPCR corticotropin-releasing factor receptor 1. *Nature* **499:** 438-443

Huang J, Chen S, Zhang JJ, Huang XY (2013) Crystal structure of oligomeric beta1adrenergic G protein-coupled receptors in ligand-free basal state. *Nature structural & molecular biology* **20**: 419-425

Huse M, Chen Y, Massagué J, Kuriyan J (1999) Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. *Cell* **96:** 425-436

Huse M, Muir T, Xu L, Chen Y, Kuriyan J, Massagué J (2001) The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. *Mol Cell* **8:** 671-682

Ikushima H, Miyazono K (2010) TGFbeta signalling: a complex web in cancer progression. *Nature reviews Cancer* **10**: 415-424

Inman GJ (2011) Switching TGFbeta from a tumor suppressor to a tumor promoter. *Current opinion in genetics & development* **21**: 93-99

Inman GJ, Nicolas FJ, Hill CS (2002) Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. *Mol Cell* **10**: 283-294

Ivanova E, Bechtold D, Dupré S, Brennand J, Barrett P, Luckman S, Loudon A (2008) Altered metabolism in the melatonin-related receptor (GPR50) knockout mouse. *Am J Physiol Endocrinol Metab* **294:** E176-182

Ivery MT (2000) Immunophilins: switched on protein binding domains? *Med Res Rev* 20: 452-484

Iwasaki H, Suda T (2009) Cancer stem cells and their niche. *Cancer science* **100:** 1166-1172

Jang CW, Chen CH, Chen CC, Chen JY, Su YH, Chen RH (2002) TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. *Nat Cell Biol* **4**: 51-58

Janssens K, ten Dijke P, Janssens S, Van Hul W (2005) Transforming growth factor-beta1 to the bone. *Endocrine reviews* **26:** 743-774

Jin W, Kim BC, Tognon C, Lee HJ, Patel S, Lannon CL, Maris JM, Triche TJ, Sorensen PH, Kim SJ (2005) The ETV6-NTRK3 chimeric tyrosine kinase suppresses TGF-beta signaling by inactivating the TGF-beta type II receptor. *Proc Natl Acad Sci U S A* **102**: 16239-16244

Jin W, Yun C, Kwak MK, Kim TA, Kim SJ (2007) TrkC binds to the type II TGF-beta receptor to suppress TGF-beta signaling. *Oncogene* **26**: 7684-7691

Jockers R, Maurice P, Boutin JA, Delagrange P (2008) Melatonin receptors, heterodimerization, signal transduction and binding sites: what's new? *Br J Pharmacol* **154**: 1182-1195

Jockers R, Petit L, Lacroix I, de Coppet P, Barrett P, Morgan PJ, Guardiola B, Delagrange P, Marullo S, Strosberg AD (1997) Novel isoforms of Mel1c melatonin receptors modulating intracellular cyclic guanosine 3',5'-monophosphate levels. *Molecular endocrinology* **11**: 1070-1081

John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS (2004) Human MicroRNA targets. *PLoS Biol* **2:** e363

Jones PG, Nawoschik SP, Sreekumar K, Uveges AJ, Tseng E, Zhang L, Johnson J, He L, Paulsen JE, Bates B, Pausch MH (2007) Tissue distribution and functional analyses of the constitutively active orphan G protein coupled receptors, GPR26 and GPR78. *Biochim Biophys Acta* **1770**: 890-901

Jordan BA, Devi LA (1999) G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* **399:** 697-700

Jordan BA, Trapaidze N, Gomes I, Nivarthi R, Devi LA (2001) Oligomerization of opioid receptors with beta 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. *Proc Natl Acad Sci U S A* **98:** 343-348

Josso N, Belville C, di Clemente N, Picard JY (2005) AMH and AMH receptor defects in persistent Mullerian duct syndrome. *Human reproduction update* **11:** 351-356

Kamal M, Marquez M, Vauthier V, Leloire A, Froguel P, Jockers R, Couturier C (2009) Improved donor/acceptor BRET couples for monitoring beta-arrestin recruitment to G protein-coupled receptors. *Biotechnology journal* **4**: 1337-1344

Kang JS, Liu C, Derynck R (2009) New regulatory mechanisms of TGF-beta receptor function. *Trends Cell Biol* **19**: 385-394

Kang JS, Saunier EF, Akhurst RJ, Derynck R (2008) The type I TGF-beta receptor is covalently modified and regulated by sumoylation. *Nat Cell Biol* **10**: 654-664

Kang Y, Chen CR, Massague J (2003a) A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol Cell* **11**: 915-926

Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordón-Cardo C, Guise TA, Massagué J (2003b) A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* **3**: 537-549

Karamitri A, Renault N, Clement N, Guillaume JL, Jockers R (2013) Minireview: Toward the Establishment of a Link between Melatonin and Glucose Homeostasis: Association of

Melatonin MT2 Receptor Variants with Type 2 Diabetes. *Molecular endocrinology* **27:** 1217-1233

Katritch V, Cherezov V, Stevens RC (2012) Diversity and modularity of G protein-coupled receptor structures. *Trends Pharmacol Sci* **33**: 17-27

Katritch V, Cherezov V, Stevens RC (2013) Structure-function of the G protein-coupled receptor superfamily. *Annu Rev Pharmacol Toxicol* **53:** 531-556

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B (1998) GABA(B)-receptor subtypes assemble into functional heteromeric complexes. *Nature* **396**: 683-687

Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL (2000) Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* **6**: 1365-1375

Kenakin T, Christopoulos A (2013) Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat Rev Drug Discov* **12**: 205-216

Kern A, Albarran-Zeckler R, Walsh HE, Smith RG (2012) Apo-ghrelin receptor forms heteromers with DRD2 in hypothalamic neurons and is essential for anorexigenic effects of DRD2 agonism. *Neuron* **73**: 317-332

Kirsch T, Sebald W, Dreyer MK (2000) Crystal structure of the BMP-2-BRIA ectodomain complex. *Nature structural biology* **7:** 492-496

Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, Gastel JA, Zatz M, Iuvone PM, Rodriguez IR, Begay V, Falcon J, Cahill GM, Cassone VM, Baler R (1997) The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. *Recent progress in hormone research* **52**: 307-357; discussion 357-308

Kniazeff J, Galvez T, Labesse G, Pin JP (2002) No ligand binding in the GB2 subunit of the GABA(B) receptor is required for activation and allosteric interaction between the subunits. *J Neurosci* **22**: 7352-7361

Koinuma D, Shinozaki M, Komuro A, Goto K, Saitoh M, Hanyu A, Ebina M, Nukiwa T, Miyazawa K, Imamura T, Miyazono K (2003) Arkadia amplifies TGF-beta superfamily signalling through degradation of Smad7. *EMBO J* **22**: 6458-6470

Kolakowski LF, Jr. (1994) GCRDb: a G-protein-coupled receptor database. *Receptors & channels* 2: 1-7

Komuro A, Imamura T, Saitoh M, Yoshida Y, Yamori T, Miyazono K, Miyazawa K (2004) Negative regulation of transforming growth factor-beta (TGF-beta) signaling by WW domaincontaining protein 1 (WWP1). *Oncogene* **23**: 6914-6923

Kostenis E, Waelbroeck M, Milligan G (2005) Techniques: promiscuous Galpha proteins in basic research and drug discovery. *Trends Pharmacol Sci* **26**: 595-602

Kotini AG, Mpakali A, Agalioti T (2011) Dnmt3a1 upregulates transcription of distinct genes and targets chromosomal gene clusters for epigenetic silencing in mouse embryonic stem cells. *Mol Cell Biol* **31:** 1577-1592

Krakowski AR, Laboureau J, Mauviel A, Bissell MJ, Luo K (2005) Cytoplasmic SnoN in normal tissues and nonmalignant cells antagonizes TGF-beta signaling by sequestration of the Smad proteins. *Proc Natl Acad Sci U S A* **102**: 12437-12442

Kretzschmar M, Doody J, Timokhina I, Massague J (1999) A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. *Genes Dev* **13**: 804-816

Kretzschmar M, Liu F, Hata A, Doody J, Massague J (1997) The TGF-beta family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. *Genes Dev* **11**: 984-995

Krieglstein K, Zheng F, Unsicker K, Alzheimer C (2011) More than being protective: functional roles for TGF-beta/activin signaling pathways at central synapses. *Trends in neurosciences* **34**: 421-429

Ku JL, Park JG (2005) Biology of SNU cell lines. *Cancer research and treatment : official journal of Korean Cancer Association* **37:** 1-19

Kuratomi G, Komuro A, Goto K, Shinozaki M, Miyazawa K, Miyazono K, Imamura T (2005) NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) negatively regulates TGF-beta (transforming growth factor-beta) signalling by inducing ubiquitin-mediated degradation of Smad2 and TGF-beta type I receptor. *Biochem J* **386**: 461-470

Lagerstrom MC, Schioth HB (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov* **7:** 339-357

Laschinger CA, Bellows CG, Wasi S (1991) Modulation of plasminogen activators and plasminogen activator inhibitors by TGF-beta, IL-1 alpha and EGF in fetal rat calvaria cells at different times of culture. *Bone and mineral* **13**: 23-34

Lawler S, Feng XH, Chen RH, Maruoka EM, Turck CW, Griswold-Prenner I, Derynck R (1997) The type II transforming growth factor-beta receptor autophosphorylates not only on serine and threonine but also on tyrosine residues. *J Biol Chem* **272**: 14850-14859

Le VQ, Wharton KA (2012) Hyperactive BMP signaling induced by ALK2(R206H) requires type II receptor function in a Drosophila model for classic fibrodysplasia ossificans progressiva. *Dev Dyn* **241**: 200-214

Lebon G, Warne T, Edwards PC, Bennett K, Langmead CJ, Leslie AG, Tate CG (2011) Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation. *Nature* **474:** 521-525

Ledent C, Demeestere I, Blum D, Petermans J, Hamalainen T, Smits G, Vassart G (2005) Premature ovarian aging in mice deficient for Gpr3. *Proc Natl Acad Sci U S A* **102:** 8922-8926 Lee KH, Kim SH, Lee HR, Kim W, Kim DY, Shin JC, Yoo SH, Kim KT (2013) MicroRNA-185 oscillation controls circadian amplitude of mouse Cryptochrome 1 via translational regulation. *Mol Biol Cell* **24**: 2248-2255

Lee MK, Pardoux C, Hall MC, Lee PS, Warburton D, Qing J, Smith SM, Derynck R (2007) TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. *EMBO J* **26**: 3957-3967

Lee S, Bookout AL, Lee CE, Gautron L, Harper MJ, Elias CF, Lowell BB, Elmquist JK (2012) Laser-capture microdissection and transcriptional profiling of the dorsomedial nucleus of the hypothalamus. *J Comp Neurol* **520**: 3617-3632

Lerner AB, Case JD, Takahashi Y, Lee TH, Mori W (1958) Isolation of Melatonin, the Pineal Gland Factor That Lightens Melanocytes. *Journal of the American Chemical Society* **80**: 2587-2587

Levoye A, Dam J, Ayoub M, Guillaume J, Couturier C, Delagrange P, Jockers R (2006a) The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *EMBO J* **25:** 3012-3023

Levoye A, Dam J, Ayoub MA, Guillaume JL, Couturier C, Delagrange P, Jockers R (2006b) The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *EMBO J* **25:** 3012-3023

Levoye A, Dam J, Ayoub MA, Guillaume JL, Jockers R (2006c) Do orphan G-proteincoupled receptors have ligand-independent functions? New insights from receptor heterodimers. *EMBO Rep* **7**: 1094-1098

Levoye A, Jockers R (2008) Alternative drug discovery approaches for orphan GPCRs. *Drug Discov Today* **13:** 52-58

Li J, Hand LE, Meng QJ, Loudon AS, Bechtold DA (2011) GPR50 interacts with TIP60 to modulate glucocorticoid receptor signalling. *PLoS One* **6**: e23725

Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA (2006) Transforming growth factorbeta regulation of immune responses. *Annual review of immunology* **24:** 99-146

Liang M, Liang YY, Wrighton K, Ungermannova D, Wang XP, Brunicardi FC, Liu X, Feng XH, Lin X (2004) Ubiquitination and proteolysis of cancer-derived Smad4 mutants by SCFSkp2. *Mol Cell Biol* **24**: 7524-7537

Libert F, Vassart G, Parmentier M (1991) Current developments in G-protein-coupled receptors. *Curr Opin Cell Biol* **3:** 218-223

Lin HK, Bergmann S, Pandolfi PP (2004) Cytoplasmic PML function in TGF-beta signalling. *Nature* **431:** 205-211

Lin X, Duan X, Liang YY, Su Y, Wrighton KH, Long J, Hu M, Davis CM, Wang J, Brunicardi FC, Shi Y, Chen YG, Meng A, Feng XH (2006) PPM1A functions as a Smad phosphatase to terminate TGFbeta signaling. *Cell* **125**: 915-928

Lindsay ME, Dietz HC (2011) Lessons on the pathogenesis of aneurysm from heritable conditions. *Nature* **473:** 308-316

Little P, Burch M, Getachew R, Al-Aryahi S, Osman N (2010) Endothelin-1 Stimulation of Proteoglycan Synthesis in Vascular Smooth Muscle is Mediated by ET Receptor Transactivation of the Transforming Growth Factor beta-Type I Receptor. *J Cardiovasc Pharmacol*

Liu C, Xu P, Lamouille S, Xu J, Derynck R (2009a) TACE-mediated ectodomain shedding of the type I TGF-beta receptor downregulates TGF-beta signaling. *Mol Cell* **35**: 26-36

Liu W, Rui H, Wang J, Lin S, He Y, Chen M, Li Q, Ye Z, Zhang S, Chan SC, Chen YG, Han J, Lin SC (2006) Axin is a scaffold protein in TGF-beta signaling that promotes degradation of Smad7 by Arkadia. *EMBO J* **25**: 1646-1658

Liu Y, Ren W, Warburton R, Toksoz D, Fanburg BL (2009b) Serotonin induces Rho/ROCK-dependent activation of Smads 1/5/8 in pulmonary artery smooth muscle cells. *FASEB J* 23: 2299-2306

Lo RS, Wotton D, Massague J (2001) Epidermal growth factor signaling via Ras controls the Smad transcriptional co-repressor TGIF. *EMBO J* **20**: 128-136

Lopez-Casillas F, Payne HM, Andres JL, Massague J (1994) Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites. *J Cell Biol* **124**: 557-568

Lopez-Casillas F, Wrana JL, Massague J (1993) Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* **73**: 1435-1444

Lopez-Ilasaca M, Gutkind JS, Wetzker R (1998) Phosphoinositide 3-kinase gamma is a mediator of Gbetagamma-dependent Jun kinase activation. *J Biol Chem* **273**: 2505-2508

Lowe LA, Supp DM, Sampath K, Yokoyama T, Wright CV, Potter SS, Overbeek P, Kuehn MR (1996) Conserved left-right asymmetry of nodal expression and alterations in murine situs inversus. *Nature* **381:** 158-161

Lu Z, Murray JT, Luo W, Li H, Wu X, Xu H, Backer JM, Chen YG (2002) Transforming growth factor beta activates Smad2 in the absence of receptor endocytosis. *J Biol Chem* **277**: 29363-29368

Luo K, Lodish HF (1996) Signaling by chimeric erythropoietin-TGF-beta receptors: homodimerization of the cytoplasmic domain of the type I TGF-beta receptor and heterodimerization with the type II receptor are both required for intracellular signal transduction. *EMBO J* **15**: 4485-4496

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ (1999) Beta-arrestindependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* **283**: 655-661 Luttrell LM, Lefkowitz RJ (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* **115**: 455-465

Ma YC, Huang J, Ali S, Lowry W, Huang XY (2000) Src tyrosine kinase is a novel direct effector of G proteins. *Cell* **102:** 635-646

Macias-Silva M, Abdollah S, Hoodless PA, Pirone R, Attisano L, Wrana JL (1996) MADR2 is a substrate of the TGFbeta receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* **87:** 1215-1224

Macintyre D, McGhee K, Maclean A, Afzal M, Briffa K, Henry B, Thomson P, Muir W, Blackwood D (2010) Association of GPR50, an X-linked orphan G protein-coupled receptor, and affective disorder in an independent sample of the Scottish population. *Neurosci Lett* **475**: 169-173

Magalhaes AC, Dunn H, Ferguson SS (2012) Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. *Br J Pharmacol* **165**: 1717-1736

Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**: 704-715

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. *Science* **298**: 1912-1934

Marazziti D, Mandillo S, Di Pietro C, Golini E, Matteoni R, Tocchini-Valentini GP (2007) GPR37 associates with the dopamine transporter to modulate dopamine uptake and behavioral responses to dopaminergic drugs. *Proc Natl Acad Sci U S A* **104**: 9846-9851

Marchese A, Docherty JM, Nguyen T, Heiber M, Cheng R, Heng HH, Tsui LC, Shi X, George SR, O'Dowd BF (1994) Cloning of human genes encoding novel G protein-coupled receptors. *Genomics* **23**: 609-618

Marianayagam NJ, Sunde M, Matthews JM (2004) The power of two: protein dimerization in biology. *Trends Biochem Sci* **29:** 618-625

Marinissen MJ, Gutkind JS (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol Sci* 22: 368-376

Markus RP, Ferreira ZS, Fernandes PA, Cecon E (2007) The immune-pineal axis: a shuttle between endocrine and paracrine melatonin sources. *Neuroimmunomodulation* **14**: 126-133

Marsden WN (2013) Synaptic plasticity in depression: molecular, cellular and functional correlates. *Progress in neuro-psychopharmacology & biological psychiatry* **43**: 168-184

Massague J (1998) TGF-beta signal transduction. Annual review of biochemistry 67: 753-791

Massague J (2008) TGFbeta in Cancer. Cell 134: 215-230

Massague J (2012) TGFbeta signalling in context. Nat Rev Mol Cell Biol 13: 616-630

Massagué J (2000) How cells read TGF-beta signals. Nat Rev Mol Cell Biol 1: 169-178

Massague J, Blain SW, Lo RS (2000) TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* **103**: 295-309

Massagué J, Wotton D (2000) Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J* **19:** 1745-1754

Matsuura I, Denissova NG, Wang G, He D, Long J, Liu F (2004) Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature* **430**: 226-231

Maurel D, Comps-Agrar L, Brock C, Rives ML, Bourrier E, Ayoub MA, Bazin H, Tinel N, Durroux T, Prezeau L, Trinquet E, Pin JP (2008) Cell-surface protein-protein interaction analysis with time-resolved FRET and snap-tag technologies: application to GPCR oligomerization. *Nat Methods* **5**: 561-567

Maurel D, Kniazeff J, Mathis G, Trinquet E, Pin JP, Ansanay H (2004) Cell surface detection of membrane protein interaction with homogeneous time-resolved fluorescence resonance energy transfer technology. *Analytical biochemistry* **329**: 253-262

Maurice P, Daulat AM, Turecek R, Ivankova-Susankova K, Zamponi F, Kamal M, Clement N, Guillaume JL, Bettler B, Gales C, Delagrange P, Jockers R (2010) Molecular organization and dynamics of the melatonin MT(1) receptor/RGS20/G(i) protein complex reveal asymmetry of receptor dimers for RGS and G(i) coupling. *EMBO J* **29**: 3646-3659

Maurice P, Guillaume JL, Benleulmi-Chaachoua A, Daulat AM, Kamal M, Jockers R (2011a) GPCR-interacting proteins, major players of GPCR function. *Advances in pharmacology* **62**: 349-380

Maurice P, Kamal M, Jockers R (2011b) Asymmetry of GPCR oligomers supports their functional relevance. *Trends Pharmacol Sci* **32:** 514-520

McLean S, Bhattacharya M, Di Guglielmo GM (2013) betaarrestin2 interacts with TbetaRII to regulate Smad-dependent and Smad-independent signal transduction. *Cell Signal* **25:** 319-331

Mehlmann LM, Saeki Y, Tanaka S, Brennan TJ, Evsikov AV, Pendola FL, Knowles BB, Eppig JJ, Jaffe LA (2004) The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. *Science* **306**: 1947-1950

Mercier JF, Salahpour A, Angers S, Breit A, Bouvier M (2002) Quantitative assessment of beta 1- and beta 2-adrenergic receptor homo- and heterodimerization by bioluminescence resonance energy transfer. *J Biol Chem* **277**: 44925-44931

Meyer RC, Giddens MM, Schaefer SA, Hall RA (2013) GPR37 and GPR37L1 are receptors for the neuroprotective and glioprotective factors prosaptide and prosaposin. *Proc Natl Acad Sci U S A* **110**: 9529-9534

Milasta S, Pediani J, Appelbe S, Trim S, Wyatt M, Cox P, Fidock M, Milligan G (2006) Interactions between the Mas-related receptors MrgD and MrgE alter signalling and trafficking of MrgD. *Molecular pharmacology* **69**: 479-491

Milligan G (2006) G-protein-coupled receptor heterodimers: pharmacology, function and relevance to drug discovery. *Drug Discov Today* **11**: 541-549

Milligan G (2009) G protein-coupled receptor hetero-dimerization: contribution to pharmacology and function. *Br J Pharmacol* **158:** 5-14

Milligan G, Kostenis E (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol* **147 Suppl 1:** S46-55

Mills A, Duggan MJ (1994) Orphan seven transmembrane domain receptors: reversing pharmacology. *Trends in biotechnology* **12:** 47-49

Miura S, Takeshita T, Asao H, Kimura Y, Murata K, Sasaki Y, Hanai JI, Beppu H, Tsukazaki T, Wrana JL, Miyazono K, Sugamura K (2000) Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. *Mol Cell Biol* **20**: 9346-9355

Monod J, Changeux JP, Jacob F (1963) Allosteric proteins and cellular control systems. *Journal of molecular biology* **6:** 306-329

Moren A, Hellman U, Inada Y, Imamura T, Heldin CH, Moustakas A (2003) Differential ubiquitination defines the functional status of the tumor suppressor Smad4. *J Biol Chem* **278**: 33571-33582

Moughal NA, Waters C, Sambi B, Pyne S, Pyne NJ (2004) Nerve growth factor signaling involves interaction between the Trk A receptor and lysophosphatidate receptor 1 systems: nuclear translocation of the lysophosphatidate receptor 1 and Trk A receptors in pheochromocytoma 12 cells. *Cell Signal* **16**: 127-136

Moustakas A, Heldin CH (2009) The regulation of TGFbeta signal transduction. *Development* **136:** 3699-3714

Mu Y, Sundar R, Thakur N, Ekman M, Gudey SK, Yakymovych M, Hermansson A, Dimitriou H, Bengoechea-Alonso MT, Ericsson J, Heldin CH, Landstrom M (2011) TRAF6 ubiquitinates TGFbeta type I receptor to promote its cleavage and nuclear translocation in cancer. *Nature communications* **2**: 330

Murga C, Fukuhara S, Gutkind JS (2000) A novel role for phosphatidylinositol 3-kinase beta in signaling from G protein-coupled receptors to Akt. *J Biol Chem* **275**: 12069-12073

Muto T, Tsuchiya D, Morikawa K, Jingami H (2007) Expression, purification, crystallization and preliminary X-ray analysis of the ligand-binding domain of metabotropic glutamate receptor 7. Acta crystallographica Section F, Structural biology and crystallization communications **63**: 627-630

Myeroff LL, Parsons R, Kim SJ, Hedrick L, Cho KR, Orth K, Mathis M, Kinzler KW, Lutterbaugh J, Park K, et al. (1995) A transforming growth factor beta receptor type II gene

mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. *Cancer research* **55**: 5545-5547

Nakao A, Afrakhte M, Morén A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH, ten Dijke P (1997a) Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* **389:** 631-635

Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, ten Dijke P (1997b) TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. *EMBO J* 16: 5353-5362

Nelson MR, Wegmann D, Ehm MG, Kessner D, St Jean P, Verzilli C, Shen J, Tang Z, Bacanu SA, Fraser D, Warren L, Aponte J, Zawistowski M, Liu X, Zhang H, Zhang Y, Li J, Li Y, Li L, Woollard P, Topp S, Hall MD, Nangle K, Wang J, Abecasis G, Cardon LR, Zollner S, Whittaker JC, Chissoe SL, Novembre J, Mooser V (2012) An abundance of rare functional variants in 202 drug target genes sequenced in 14,002 people. *Science* **337**: 100-104

Nosjean O, Ferro M, Coge F, Beauverger P, Henlin JM, Lefoulon F, Fauchere JL, Delagrange P, Canet E, Boutin JA (2000) Identification of the melatonin-binding site MT3 as the quinone reductase 2. *J Biol Chem* **275:** 31311-31317

Nosjean O, Nicolas JP, Klupsch F, Delagrange P, Canet E, Boutin JA (2001) Comparative pharmacological studies of melatonin receptors: MT1, MT2 and MT3/QR2. Tissue distribution of MT3/QR2. *Biochemical pharmacology* **61**: 1369-1379

O'Kane S, Ferguson MW (1997) Transforming growth factor beta s and wound healing. *Int J Biochem Cell Biol* **29:** 63-78

Ohgushi M, Kuroki S, Fukamachi H, O'Reilly LA, Kuida K, Strasser A, Yonehara S (2005) Transforming growth factor beta-dependent sequential activation of Smad, Bim, and caspase-9 mediates physiological apoptosis in gastric epithelial cells. *Mol Cell Biol* **25**: 10017-10028

Ohte S, Shin M, Sasanuma H, Yoneyama K, Akita M, Ikebuchi K, Jimi E, Maruki Y, Matsuoka M, Namba A, Tomoda H, Okazaki Y, Ohtake A, Oda H, Owan I, Yoda T, Furuya H, Kamizono J, Kitoh H, Nakashima Y, Susami T, Haga N, Komori T, Katagiri T (2011) A novel mutation of ALK2, L196P, found in the most benign case of fibrodysplasia ossificans progressiva activates BMP-specific intracellular signaling equivalent to a typical mutation, R206H. *Biochem Biophys Res Commun* **407**: 213-218

Okadome T, Yamashita H, Franzen P, Moren A, Heldin CH, Miyazono K (1994) Distinct roles of the intracellular domains of transforming growth factor-beta type I and type II receptors in signal transduction. *J Biol Chem* **269**: 30753-30756

Onichtchouk D, Chen YG, Dosch R, Gawantka V, Delius H, Massague J, Niehrs C (1999) Silencing of TGF-beta signalling by the pseudoreceptor BAMBI. *Nature* **401**: 480-485

Overington JP, Al-Lazikani B, Hopkins AL (2006) How many drug targets are there? *Nat Rev Drug Discov* **5:** 993-996

Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL (2005) Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science* **307**: 1603-1609

Paavola KJ, Hall RA (2012) Adhesion G protein-coupled receptors: signaling, pharmacology, and mechanisms of activation. *Molecular pharmacology* **82:** 777-783

Padua D, Massague J (2009) Roles of TGFbeta in metastasis. Cell Res 19: 89-102

Padua D, Zhang XH, Wang Q, Nadal C, Gerald WL, Gomis RR, Massague J (2008) TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* **133**: 66-77

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289:** 739-745

Pardali E, Goumans MJ, ten Dijke P (2010) Signaling by members of the TGF-beta family in vascular morphogenesis and disease. *Trends Cell Biol* **20**: 556-567

Pardali K, Moustakas A (2007) Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. *Biochim Biophys Acta* **1775:** 21-62

Park JH, Scheerer P, Hofmann KP, Choe HW, Ernst OP (2008) Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature* **454**: 183-187

Patel N, Itakura T, Gonzalez JM, Schwartz SG, Fini ME (2013) GPR158, an orphan member of G protein-coupled receptor Family C: glucocorticoid-stimulated expression and novel nuclear role. *PLoS One* **8:** e57843

Patowary S, Alvarez-Curto E, Xu TR, Holz JD, Oliver JA, Milligan G, Raicu V (2013) The muscarinic M3 acetylcholine receptor exists as two differently sized complexes at the plasma membrane. *Biochem J* **452**: 303-312

Penheiter SG, Singh RD, Repellin CE, Wilkes MC, Edens M, Howe PH, Pagano RE, Leof EB (2010) Type II transforming growth factor-beta receptor recycling is dependent upon the clathrin adaptor protein Dab2. *Mol Biol Cell* **21**: 4009-4019

Perlman R, Schiemann WP, Brooks MW, Lodish HF, Weinberg RA (2001) TGF-betainduced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. *Nat Cell Biol* **3**: 708-714

Persson U, Izumi H, Souchelnytskyi S, Itoh S, Grimsby S, Engström U, Heldin CH, Funa K, ten Dijke P (1998) The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett* **434**: 83-87

Pessah M, Prunier C, Marais J, Ferrand N, Mazars A, Lallemand F, Gauthier JM, Atfi A (2001) c-Jun interacts with the corepressor TG-interacting factor (TGIF) to suppress Smad2 transcriptional activity. *Proc Natl Acad Sci U S A* **98**: 6198-6203

Petit L, Lacroix I, de Coppet P, Strosberg AD, Jockers R (1999) Differential signaling of human Mel1a and Mel1b melatonin receptors through the cyclic guanosine 3'-5'-monophosphate pathway. *Biochemical pharmacology* **58**: 633-639

Piek E, Van Dinther M, Parks WT, Sallee JM, Bottinger EP, Roberts AB, Ten Dijke P (2004) RLP, a novel Ras-like protein, is an immediate-early transforming growth factor-beta (TGF-beta) target gene that negatively regulates transcriptional activity induced by TGF-beta. *Biochem J* **383**: 187-199

Pierreux CE, Nicolas FJ, Hill CS (2000) Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus. *Mol Cell Biol* **20**: 9041-9054

Pin JP, Comps-Agrar L, Maurel D, Monnier C, Rives ML, Trinquet E, Kniazeff J, Rondard P, Prezeau L (2009) G-protein-coupled receptor oligomers: two or more for what? Lessons from mGlu and GABAB receptors. *The Journal of physiology* **587**: 5337-5344

Poon VY, Choi S, Park M (2013) Growth factors in synaptic function. Front Synaptic Neurosci 5: 6

Prevot V, Bellefontaine N, Baroncini M, Sharif A, Hanchate NK, Parkash J, Campagne C, de Seranno S (2010) Gonadotrophin-releasing hormone nerve terminals, tanycytes and neurohaemal junction remodelling in the adult median eminence: functional consequences for reproduction and dynamic role of vascular endothelial cells. *J Neuroendocrinol* **22**: 639-649

Prevot V, Bouret S, Croix D, Takumi T, Jennes L, Mitchell V, Beauvillain JC (2000) Evidence that members of the TGFbeta superfamily play a role in regulation of the GnRH neuroendocrine axis: expression of a type I serine-threonine kinase receptor for TGRbeta and activin in GnRH neurones and hypothalamic areas of the female rat. *J Neuroendocrinol* **12**: 665-670

Prezeau L, Rives ML, Comps-Agrar L, Maurel D, Kniazeff J, Pin JP (2010) Functional crosstalk between GPCRs: with or without oligomerization. *Current opinion in pharmacology* **10:** 6-13

Prunier C, Ferrand N, Frottier B, Pessah M, Atfi A (2001) Mechanism for mutational inactivation of the tumor suppressor Smad2. *Molecular and cellular biology* **21**: 3302-3313

Prunier C, Pessah M, Ferrand N, Seo SR, Howe P, Atfi A (2003) The oncoprotein Ski acts as an antagonist of transforming growth factor-beta signaling by suppressing Smad2 phosphorylation. *J Biol Chem* **278**: 26249-26257

Pyne NJ, Pyne S (2011) Receptor tyrosine kinase-G-protein-coupled receptor signalling platforms: out of the shadow? *Trends Pharmacol Sci* **32**: 443-450

Rajagopal S, Rajagopal K, Lefkowitz RJ (2010) Teaching old receptors new tricks: biasing seven-transmembrane receptors. *Nat Rev Drug Discov* **9:** 373-386

Rask-Andersen M, Almen MS, Schioth HB (2011) Trends in the exploitation of novel drug targets. *Nat Rev Drug Discov* **10:** 579-590

Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, Devree BT, Rosenbaum DM, Thian FS, Kobilka TS, Schnapp A, Konetzki I, Sunahara RK, Gellman SH, Pautsch A, Steyaert J, Weis WI, Kobilka BK (2011a) Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. *Nature* **469**: 175-180

Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, Schertler GF, Weis WI, Kobilka BK (2007) Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* **450**: 383-387

Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, Mathiesen JM, Shah ST, Lyons JA, Caffrey M, Gellman SH, Steyaert J, Skiniotis G, Weis WI, Sunahara RK, Kobilka BK (2011b) Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature* **477**: 549-555

Remy I, Montmarquette A, Michnick SW (2004) PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. *Nat Cell Biol* **6:** 358-365

Reppert SM, Godson C, Mahle CD, Weaver DR, Slaugenhaupt SA, Gusella JF (1995) Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor. *Proc Natl Acad Sci U S A* **92:** 8734-8738

Reppert SM, Weaver DR, Ebisawa T (1994) Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. *Neuron* **13**: 1177-1185

Reppert SM, Weaver DR, Ebisawa T, Mahle CD, Kolakowski LF, Jr. (1996) Cloning of a melatonin-related receptor from human pituitary. *FEBS Lett* **386:** 219-224

Rezgaoui M, Susens U, Ignatov A, Gelderblom M, Glassmeier G, Franke I, Urny J, Imai Y, Takahashi R, Schaller HC (2006) The neuropeptide head activator is a high-affinity ligand for the orphan G-protein-coupled receptor GPR37. *J Cell Sci* **119**: 542-549

Riobo NA, Lu K, Emerson CP, Jr. (2006) Hedgehog signal transduction: signal integration and cross talk in development and cancer. *Cell cycle* **5**: 1612-1615

Ritter SL, Hall RA (2009) Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat Rev Mol Cell Biol* **10:** 819-830

Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, et al. (1986) Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc Natl Acad Sci U S A* **83**: 4167-4171

Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Yao XJ, Weis WI, Stevens RC, Kobilka BK (2007) GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. *Science* **318**: 1266-1273

Rosenbaum DM, Rasmussen SG, Kobilka BK (2009) The structure and function of G-proteincoupled receptors. *Nature* **459:** 356-363 Rosenkilde MM, Benned-Jensen T, Andersen H, Holst PJ, Kledal TN, Luttichau HR, Larsen JK, Christensen JP, Schwartz TW (2006) Molecular pharmacological phenotyping of EBI2. An orphan seven-transmembrane receptor with constitutive activity. *J Biol Chem* **281**: 13199-13208

Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Heldin CH, Miyazono K (1995) Cloning and characterization of a human type II receptor for bone morphogenetic proteins. *Proc Natl Acad Sci U S A* **92:** 7632-7636

Ross EM, Wilkie TM (2000) GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annual review of biochemistry* **69**: 795-827

Rozenfeld R, Devi LA (2010) Receptor heteromerization and drug discovery. *Trends Pharmacol Sci* **31**: 124-130

Rozenfeld R, Devi LA (2011) Exploring a role for heteromerization in GPCR signalling specificity. *Biochem J* **433**: 11-18

Runyan CE, Poncelet AC, Schnaper HW (2006) TGF-beta receptor-binding proteins: complex interactions. *Cell Signal* **18**: 2077-2088

Safina A, Vandette E, Bakin AV (2007) ALK5 promotes tumor angiogenesis by upregulating matrix metalloproteinase-9 in tumor cells. *Oncogene* **26**: 2407-2422

Saha D, Datta PK, Beauchamp RD (2001) Oncogenic ras represses transforming growth factor-beta /Smad signaling by degrading tumor suppressor Smad4. *J Biol Chem* **276**: 29531-29537

Salahpour A, Angers S, Bouvier M (2000) Functional significance of oligomerization of G-protein-coupled receptors. *Trends in endocrinology and metabolism: TEM* **11:** 163-168

Sapkota G, Knockaert M, Alarcon C, Montalvo E, Brivanlou AH, Massague J (2006) Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor-beta pathways. *J Biol Chem* **281**: 40412-40419

Savaskan E, Jockers R, Ayoub M, Angeloni D, Fraschini F, Flammer J, Eckert A, Muller-Spahn F, Meyer P (2007) The MT2 melatonin receptor subtype is present in human retina and decreases in Alzheimer's disease. *Current Alzheimer research* **4**: 47-51

Savaskan E, Olivieri G, Brydon L, Jockers R, Krauchi K, Wirz-Justice A, Muller-Spahn F (2001) Cerebrovascular melatonin MT1-receptor alterations in patients with Alzheimer's disease. *Neurosci Lett* **308**: 9-12

Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP, Ernst OP (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature* **455**: 497-502

Schmierer B, Hill CS (2007) TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* **8:** 970-982

Schneider G, Schmid RM (2003) Genetic alterations in pancreatic carcinoma. *Molecular cancer* **2:** 15

Schuster N, Krieglstein K (2002) Mechanisms of TGF-beta-mediated apoptosis. *Cell Tissue Res* **307:** 1-14

Sebag JA, Pantel J (2012) [G-protein-coupled receptors targeting: the allosteric approach]. *Med Sci (Paris)* **28:** 845-851

Seifert R, Wenzel-Seifert K (2002) Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn-Schmiedeberg's archives of pharmacology* **366:** 381-416

Seoane J, Pouponnot C, Staller P, Schader M, Eilers M, Massague J (2001) TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. *Nat Cell Biol* **3**: 400-408

Shah BH, Catt KJ (2004) GPCR-mediated transactivation of RTKs in the CNS: mechanisms and consequences. *Trends in neurosciences* **27**: 48-53

Shenoy SK, Lefkowitz RJ (2011) beta-Arrestin-mediated receptor trafficking and signal transduction. *Trends Pharmacol Sci* **32:** 521-533

Shi W, Sun C, He B, Xiong W, Shi X, Yao D, Cao X (2004) GADD34-PP1c recruited by Smad7 dephosphorylates TGFbeta type I receptor. *J Cell Biol* **164:** 291-300

Shi Y, Massagué J (2003a) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113:** 685-700

Shi Y, Massagué J (2003b) Mechanisms of TGF- β Signaling from Cell Membrane to the Nucleus. *Cell* **113**: 685-700

Shibuya H, Yamaguchi K, Shirakabe K, Tonegawa A, Gotoh Y, Ueno N, Irie K, Nishida E, Matsumoto K (1996) TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. *Science* **272**: 1179-1182

Shou W, Aghdasi B, Armstrong DL, Guo Q, Bao S, Charng MJ, Mathews LM, Schneider MD, Hamilton SL, Matzuk MM (1998) Cardiac defects and altered ryanodine receptor function in mice lacking FKBP12. *Nature* **391:** 489-492

Sidibe A, Mullier A, Chen P, Baroncini M, Boutin J, Delagrange P, Prevot V, Jockers R (2010) Expression of the orphan GPR50 protein in rodent and human dorsomedial hypothalamus, tanycytes and median eminence. *J Pineal Res* **48**: 263-269

Siegel PM, Massague J (2003) Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nature reviews Cancer* **3**: 807-821

Siegel PM, Shu W, Cardiff RD, Muller WJ, Massague J (2003) Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci U S A* **100**: 8430-8435

Siu FY, He M, de Graaf C, Han GW, Yang D, Zhang Z, Zhou C, Xu Q, Wacker D, Joseph JS, Liu W, Lau J, Cherezov V, Katritch V, Wang MW, Stevens RC (2013) Structure of the human glucagon class B G-protein-coupled receptor. *Nature* **499**: 444-449

Smith NJ, Milligan G (2010) Allostery at G protein-coupled receptor homo- and heteromers: uncharted pharmacological landscapes. *Pharmacological reviews* **62**: 701-725

Snyder SH, Sabatini DM, Lai MM, Steiner JP, Hamilton GS, Suzdak PD (1998) Neural actions of immunophilin ligands. *Trends Pharmacol Sci* **19**: 21-26

Song GA, Kim HJ, Woo KM, Baek JH, Kim GS, Choi JY, Ryoo HM (2010) Molecular consequences of the ACVR1(R206H) mutation of fibrodysplasia ossificans progressiva. *J Biol Chem* **285**: 22542-22553

Soond SM, Chantry A (2011) How ubiquitination regulates the TGF-beta signalling pathway: new insights and new players: new isoforms of ubiquitin-activating enzymes in the E1-E3 families join the game. *BioEssays : news and reviews in molecular, cellular and developmental biology* **33**: 749-758

Sorrentino A, Thakur N, Grimsby S, Marcusson A, von Bulow V, Schuster N, Zhang S, Heldin CH, Landstrom M (2008) The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. *Nat Cell Biol* **10**: 1199-1207

Souchelnytskyi S, ten Dijke P, Miyazono K, Heldin CH (1996) Phosphorylation of Ser165 in TGF-beta type I receptor modulates TGF-beta1-induced cellular responses. *EMBO J* **15**: 6231-6240

Southern C, Cook JM, Neetoo-Isseljee Z, Taylor DL, Kettleborough CA, Merritt A, Bassoni DL, Raab WJ, Quinn E, Wehrman TS, Davenport AP, Brown AJ, Green A, Wigglesworth MJ, Rees S (2013) Screening β -Arrestin Recruitment for the Identification of Natural Ligands for Orphan G-Protein-Coupled Receptors. *J Biomol Screen* **18**: 599-609

Sparso T, Bonnefond A, Andersson E, Bouatia-Naji N, Holmkvist J, Wegner L, Grarup N, Gjesing AP, Banasik K, Cavalcanti-Proenca C, Marchand M, Vaxillaire M, Charpentier G, Jarvelin MR, Tichet J, Balkau B, Marre M, Levy-Marchal C, Faerch K, Borch-Johnsen K, Jorgensen T, Madsbad S, Poulsen P, Vaag A, Dina C, Hansen T, Pedersen O, Froguel P (2009) G-allele of intronic rs10830963 in MTNR1B confers increased risk of impaired fasting glycemia and type 2 diabetes through an impaired glucose-stimulated insulin release: studies involving 19,605 Europeans. *Diabetes* **58**: 1450-1456

Spiekerkoetter E, Tian X, Cai J, Hopper RK, Sudheendra D, Li CG, El-Bizri N, Sawada H, Haghighat R, Chan R, Haghighat L, de Jesus Perez V, Wang L, Reddy S, Zhao M, Bernstein D, Solow-Cordero DE, Beachy PA, Wandless TJ, Dijke PT, Rabinovitch M (2013) FK506 activates BMPR2, rescues endothelial dysfunction, and reverses pulmonary hypertension. *J Clin Invest*

Staller P, Peukert K, Kiermaier A, Seoane J, Lukas J, Karsunky H, Moroy T, Bartek J, Massague J, Hanel F, Eilers M (2001) Repression of p15INK4b expression by Myc through association with Miz-1. *Nat Cell Biol* **3**: 392-399

Stockwell BR, Schreiber SL (1998) TGF-beta-signaling with small molecule FKBP12 antagonists that bind myristoylated FKBP12-TGF-beta type I receptor fusion proteins. *Chemistry & biology* **5**: 385-395

Stoddart LA, Brown AJ, Milligan G (2007) Uncovering the pharmacology of the G proteincoupled receptor GPR40: high apparent constitutive activity in guanosine 5'-O-(3-[35S]thio)triphosphate binding studies reflects binding of an endogenous agonist. *Molecular pharmacology* **71**: 994-1005

Stroschein SL, Bonni S, Wrana JL, Luo K (2001) Smad3 recruits the anaphase-promoting complex for ubiquitination and degradation of SnoN. *Genes Dev* **15**: 2822-2836

Su JL, Yang CY, Zhao M, Kuo ML, Yen ML (2007a) Forkhead proteins are critical for bone morphogenetic protein-2 regulation and anti-tumor activity of resveratrol. *J Biol Chem* **282**: 19385-19398

Su Y, Zhang L, Gao X, Meng F, Wen J, Zhou H, Meng A, Chen YG (2007b) The evolutionally conserved activity of Dapper2 in antagonizing TGF-beta signaling. *FASEB J* **21:** 682-690

Sugita K, Yamamura C, Tabata K, Fujita N (2013) Expression of orphan G-protein coupled receptor GPR174 in CHO cells induced morphological changes and proliferation delay via increasing intracellular cAMP. *Biochem Biophys Res Commun* **430**: 190-195

Sun PD, Davies DR (1995) The cystine-knot growth-factor superfamily. *Annual review of biophysics and biomolecular structure* **24**: 269-291

Sun Y, Liu X, Eaton EN, Lane WS, Lodish HF, Weinberg RA (1999) Interaction of the Ski oncoprotein with Smad3 regulates TGF-beta signaling. *Mol Cell* **4**: 499-509

Suzuki C, Murakami G, Fukuchi M, Shimanuki T, Shikauchi Y, Imamura T, Miyazono K (2002) Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. *J Biol Chem* **277**: 39919-39925

Tadagaki K, Tudor D, Gbahou F, Tschische P, Waldhoer M, Bomsel M, Jockers R, Kamal M (2012) Human cytomegalovirus-encoded UL33 and UL78 heteromerize with host CCR5 and CXCR4 impairing their HIV coreceptor activity. *Blood* **119**: 4908-4918

Takahashi Y, Forrest AR, Maeno E, Hashimoto T, Daub CO, Yasuda J (2009) MiR-107 and MiR-185 can induce cell cycle arrest in human non small cell lung cancer cell lines. *PLoS One* **4**: e6677

Tanaka S, Ishii K, Kasai K, Yoon SO, Saeki Y (2007) Neural expression of G protein-coupled receptors GPR3, GPR6, and GPR12 up-regulates cyclic AMP levels and promotes neurite outgrowth. *J Biol Chem* **282**: 10506-10515

Taneja P, Frazier DP, Kendig RD, Maglic D, Sugiyama T, Kai F, Taneja NK, Inoue K (2009) MMTV mouse models and the diagnostic values of MMTV-like sequences in human breast cancer. *Expert review of molecular diagnostics* **9**: 423-440

ten Dijke P, Arthur HM (2007) Extracellular control of TGFbeta signalling in vascular development and disease. *Nat Rev Mol Cell Biol* **8:** 857-869

ten Dijke P, Franzen P, Yamashita H, Ichijo H, Heldin CH, Miyazono K (1994) Serine/threonine kinase receptors. *Progress in growth factor research* **5:** 55-72

ten Dijke P, Hill CS (2004) New insights into TGF-beta-Smad signalling. *Trends Biochem Sci* **29:** 265-273

Terrillon S, Durroux T, Mouillac B, Breit A, Ayoub MA, Taulan M, Jockers R, Barberis C, Bouvier M (2003) Oxytocin and vasopressin V1a and V2 receptors form constitutive homoand heterodimers during biosynthesis. *Molecular endocrinology* **17:** 677-691

Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. *Nature reviews Cancer* **2**: 442-454

Thiery JP, Acloque H, Huang RY, Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. *Cell* **139:** 871-890

Thomson PA, Wray NR, Thomson AM, Dunbar DR, Grassie MA, Condie A, Walker MT, Smith DJ, Pulford DJ, Muir W, Blackwood DH, Porteous DJ (2005) Sex-specific association between bipolar affective disorder in women and GPR50, an X-linked orphan G protein-coupled receptor. *Mol Psychiatry* **10**: 470-478

Tian X, Pascal G, Fouchecourt S, Pontarotti P, Monget P (2009) Gene birth, death, and divergence: the different scenarios of reproduction-related gene evolution. *Biol Reprod* 80: 616-621

Tiberi M, Caron MG (1994) High agonist-independent activity is a distinguishing feature of the dopamine D1B receptor subtype. *J Biol Chem* **269**: 27925-27931

Torre-Amione G, Beauchamp RD, Koeppen H, Park BH, Schreiber H, Moses HL, Rowley DA (1990) A highly immunogenic tumor transfected with a murine transforming growth factor type beta 1 cDNA escapes immune surveillance. *Proc Natl Acad Sci U S A* **87:** 1486-1490

Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL (1998) SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* **95**: 779-791

Tyndall JD, Sandilya R (2005) GPCR agonists and antagonists in the clinic. *Medicinal chemistry* **1**: 405-421

Ulloa L, Doody J, Massague J (1999) Inhibition of transforming growth factor-beta/SMAD signalling by the interferon-gamma/STAT pathway. *Nature* **397**: 710-713

Vakkuri O, Lamsa E, Rahkamaa E, Ruotsalainen H, Leppaluoto J (1984a) Iodinated melatonin: preparation and characterization of the molecular structure by mass and 1H NMR spectroscopy. *Analytical biochemistry* **142**: 284-289

Vakkuri O, Leppaluoto J, Vuolteenaho O (1984b) Development and validation of a melatonin radioimmunoassay using radioiodinated melatonin as tracer. *Acta endocrinologica* **106:** 152-157

Valant C, Robert Lane J, Sexton PM, Christopoulos A (2012) The best of both worlds? Bitopic orthosteric/allosteric ligands of g protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **52:** 153-178

van Rijn RM, Harvey JH, Brissett DI, DeFriel JN, Whistler JL (2013) Novel screening assay for the selective detection of G-protein-coupled receptor heteromer signaling. *The Journal of pharmacology and experimental therapeutics* **344**: 179-188

Vanecek J (1998) Cellular mechanisms of melatonin action. *Physiol Rev* 78: 687-721

Vanecek J, Vollrath L (1989) Melatonin inhibits cyclic AMP and cyclic GMP accumulation in the rat pituitary. *Brain research* **505:** 157-159

Vassilatis DK, Hohmann JG, Zeng H, Li F, Ranchalis JE, Mortrud MT, Brown A, Rodriguez SS, Weller JR, Wright AC, Bergmann JE, Gaitanaris GA (2003) The G protein-coupled receptor repertoires of human and mouse. *Proc Natl Acad Sci U S A* **100**: 4903-4908

Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF, Babu MM (2013) Molecular signatures of G-protein-coupled receptors. *Nature* **494:** 185-194

Verrecchia F, Mauviel A (2007) Transforming growth factor-beta and fibrosis. *World journal of gastroenterology : WJG* **13:** 3056-3062

Vischer HF, Watts AO, Nijmeijer S, Leurs R (2011) G protein-coupled receptors: walking hand-in-hand, talking hand-in-hand? *Br J Pharmacol* **163**: 246-260

Vivien D, Ali C (2006) Transforming growth factor-beta signalling in brain disorders. *Cytokine Growth Factor Rev* **17:** 121-128

Vivien D, Attisano L, Wrana JL, Massague J (1995) Signaling activity of homologous and heterologous transforming growth factor-beta receptor kinase complexes. *J Biol Chem* **270**: 7134-7141

von Gall C, Stehle JH, Weaver DR (2002) Mammalian melatonin receptors: molecular biology and signal transduction. *Cell Tissue Res* **309**: 151-162

Vrajová M, Peková S, Horácek J, Höschl C (2011) The effects of siRNA-mediated RGS4 gene silencing on the whole genome transcription profile: implications for schizophrenia. *Neuro Endocrinol Lett* **32:** 246-252

Wacker D, Wang C, Katritch V, Han GW, Huang XP, Vardy E, McCorvy JD, Jiang Y, Chu M, Siu FY, Liu W, Xu HE, Cherezov V, Roth BL, Stevens RC (2013) Structural features for functional selectivity at serotonin receptors. *Science* **340**: 615-619

Waldhoer M, Fong J, Jones RM, Lunzer MM, Sharma SK, Kostenis E, Portoghese PS, Whistler JL (2005) A heterodimer-selective agonist shows in vivo relevance of G proteincoupled receptor dimers. *Proc Natl Acad Sci U S A* **102**: 9050-9055 Waldhoer M, Kledal TN, Farrell H, Schwartz TW (2002) Murine cytomegalovirus (CMV) M33 and human CMV US28 receptors exhibit similar constitutive signaling activities. *Journal of virology* **76:** 8161-8168

Wang C, Wu H, Katritch V, Han GW, Huang XP, Liu W, Siu FY, Roth BL, Cherezov V, Stevens RC (2013) Structure of the human smoothened receptor bound to an antitumour agent. *Nature* **497:** 338-343

Wang T, Donahoe PK (2004) The immunophilin FKBP12: a molecular guardian of the TGFbeta family type I receptors. *Front Biosci* **9**: 619-631

Wang T, Donahoe PK, Zervos AS (1994) Specific interaction of type I receptors of the TGFbeta family with the immunophilin FKBP-12. *Science* **265**: 674-676

Wang T, Li BY, Danielson PD, Shah PC, Rockwell S, Lechleider RJ, Martin J, Manganaro T, Donahoe PK (1996) The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. *Cell* **86:** 435-444

Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R, Leslie AG, Tate CG, Schertler GF (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* **454**: 486-491

Watabe T, Miyazono K (2009) Roles of TGF-beta family signaling in stem cell renewal and differentiation. *Cell Res* **19**: 103-115

Watanabe Y, Itoh S, Goto T, Ohnishi E, Inamitsu M, Itoh F, Satoh K, Wiercinska E, Yang W, Shi L, Tanaka A, Nakano N, Mommaas AM, Shibuya H, Ten Dijke P, Kato M (2010) TMEPAI, a transmembrane TGF-beta-inducible protein, sequesters Smad proteins from active participation in TGF-beta signaling. *Mol Cell* **37**: 123-134

Weis-Garcia F, Massague J (1996) Complementation between kinase-defective and activation-defective TGF-beta receptors reveals a novel form of receptor cooperativity essential for signaling. *EMBO J* **15**: 276-289

Wetzker R, Bohmer FD (2003) Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nat Rev Mol Cell Biol* **4:** 651-657

White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM, Marshall FH (1998) Heterodimerization is required for the formation of a functional GABA(B) receptor. *Nature* **396**: 679-682

Whorton MR, Bokoch MP, Rasmussen SG, Huang B, Zare RN, Kobilka B, Sunahara RK (2007) A monomeric G protein-coupled receptor isolated in a high-density lipoprotein particle efficiently activates its G protein. *Proc Natl Acad Sci U S A* **104**: 7682-7687

Wicks SJ, Haros K, Maillard M, Song L, Cohen RE, Dijke PT, Chantry A (2005) The deubiquitinating enzyme UCH37 interacts with Smads and regulates TGF-beta signalling. *Oncogene* 24: 8080-8084

Wicks SJ, Lui S, Abdel-Wahab N, Mason RM, Chantry A (2000) Inactivation of smadtransforming growth factor beta signaling by Ca(2+)-calmodulin-dependent protein kinase II. *Mol Cell Biol* **20**: 8103-8111

Wieser R, Wrana JL, Massague J (1995) GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. *EMBO J* **14:** 2199-2208

Witt-Enderby P, Bennett J, Jarzynka M, Firestine S, Melan M (2003) Melatonin receptors and their regulation: biochemical and structural mechanisms. *Life Sci* **72**: 2183-2198

Wolf G (2006) Renal injury due to renin-angiotensin-aldosterone system activation of the transforming growth factor-beta pathway. *Kidney Int* **70**: 1914-1919

Wotton D, Lo RS, Lee S, Massague J (1999a) A Smad transcriptional corepressor. *Cell* **97:** 29-39

Wotton D, Lo RS, Swaby LA, Massague J (1999b) Multiple modes of repression by the Smad transcriptional corepressor TGIF. *J Biol Chem* **274:** 37105-37110

Wrana JL, Attisano L, Wieser R, Ventura F, Massague J (1994) Mechanism of activation of the TGF-beta receptor. *Nature* **370:** 341-347

Wrighton KH, Lin X, Feng XH (2008) Critical regulation of TGFbeta signaling by Hsp90. *Proc Natl Acad Sci U S A* **105:** 9244-9249

Wrighton KH, Lin X, Feng XH (2009) Phospho-control of TGF-beta superfamily signaling. *Cell Res* **19:** 8-20

Wrighton KH, Willis D, Long J, Liu F, Lin X, Feng XH (2006) Small C-terminal domain phosphatases dephosphorylate the regulatory linker regions of Smad2 and Smad3 to enhance transforming growth factor-beta signaling. *J Biol Chem* **281**: 38365-38375

Wu G (2000) Structural Basis of Smad2 Recognition by the Smad Anchor for Receptor Activation. *Science* **287**: 92-97

Wu JW, Fairman R, Penry J, Shi Y (2001) Formation of a stable heterodimer between Smad2 and Smad4. *J Biol Chem* **276:** 20688-20694

Wu MY, Hill CS (2009) Tgf-beta superfamily signaling in embryonic development and homeostasis. *Dev Cell* 16: 329-343

Wurthner JU, Frank DB, Felici A, Green HM, Cao Z, Schneider MD, McNally JG, Lechleider RJ, Roberts AB (2001) Transforming growth factor-beta receptor-associated protein 1 is a Smad4 chaperone. *J Biol Chem* **276**: 19495-19502

Xi Q, Wang Z, Zaromytidou AI, Zhang XH, Chow-Tsang LF, Liu JX, Kim H, Barlas A, Manova-Todorova K, Kaartinen V, Studer L, Mark W, Patel DJ, Massague J (2011) A poised chromatin platform for TGF-beta access to master regulators. *Cell* **147:** 1511-1524

Xiao Z, Liu X, Henis YI, Lodish HF (2000a) A distinct nuclear localization signal in the N terminus of Smad 3 determines its ligand-induced nuclear translocation. *Proc Natl Acad Sci U S A* **97:** 7853-7858

Xiao Z, Liu X, Lodish HF (2000b) Importin beta mediates nuclear translocation of Smad 3. *J Biol Chem* **275:** 23425-23428

Xu F, Wu H, Katritch V, Han GW, Jacobson KA, Gao ZG, Cherezov V, Stevens RC (2011) Structure of an agonist-bound human A2A adenosine receptor. *Science* **332:** 322-327

Xu H, Staszewski L, Tang H, Adler E, Zoller M, Li X (2004) Different functional roles of T1R subunits in the heteromeric taste receptors. *Proc Natl Acad Sci U S A* **101**: 14258-14263

Xu L, Chen YG, Massague J (2000) The nuclear import function of Smad2 is masked by SARA and unmasked by TGFbeta-dependent phosphorylation. *Nat Cell Biol* **2:** 559-562

Xu L, Kang Y, Col S, Massague J (2002) Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus. *Mol Cell* **10**: 271-282

Xu P, Liu J, Derynck R (2012) Post-translational regulation of TGF-beta receptor and Smad signaling. *FEBS Lett* **586**: 1871-1884

Yadav H, Quijano C, Kamaraju AK, Gavrilova O, Malek R, Chen W, Zerfas P, Zhigang D, Wright EC, Stuelten C, Sun P, Lonning S, Skarulis M, Sumner AE, Finkel T, Rane SG (2011) Protection from obesity and diabetes by blockade of TGF-beta/Smad3 signaling. *Cell metabolism* **14:** 67-79

Yakymovych I, Ten Dijke P, Heldin CH, Souchelnytskyi S (2001) Regulation of Smad signaling by protein kinase C. *FASEB J* **15:** 553-555

Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H, Matsumoto K (1999) XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. *EMBO J* **18**: 179-187

Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K (1995) Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. *Science* **270**: 2008-2011

Yamaguchi T, Kurisaki A, Yamakawa N, Minakuchi K, Sugino H (2006) FKBP12 functions as an adaptor of the Smad7-Smurf1 complex on activin type I receptor. *Journal of molecular endocrinology* **36**: 569-579

Yamashita M, Fatyol K, Jin C, Wang X, Liu Z, Zhang YE (2008) TRAF6 mediates Smadindependent activation of JNK and p38 by TGF-beta. *Mol Cell* **31**: 918-924

Yamazaki S, Iwama A, Takayanagi S, Eto K, Ema H, Nakauchi H (2009) TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. *Blood* **113:** 1250-1256

Yang KM, Kim W, Bae E, Gim J, Weist BM, Jung Y, Hyun JS, Hernandez JB, Leem SH, Park T, Jeong J, Walsh CM, Kim SJ (2012) DRAK2 participates in a negative feedback loop to control TGF-beta/Smads signaling by binding to type I TGF-beta receptor. *Cell reports* **2**: 1286-1299

Yang MY, Hilton MB, Seaman S, Haines DC, Nagashima K, Burks CM, Tessarollo L, Ivanova PT, Brown HA, Umstead TM, Floros J, Chroneos ZC, St Croix B (2013) Essential regulation of lung surfactant homeostasis by the orphan G protein-coupled receptor GPR116. *Cell reports* **3**: 1457-1464

Yao D, Dore JJ, Jr., Leof EB (2000) FKBP12 is a negative regulator of transforming growth factor-beta receptor internalization. *J Biol Chem* **275:** 13149-13154

Yao X, Parnot C, Deupi X, Ratnala VR, Swaminath G, Farrens D, Kobilka B (2006) Coupling ligand structure to specific conformational switches in the beta2-adrenoceptor. *Nature chemical biology* **2:** 417-422

Yi JJ, Barnes AP, Hand R, Polleux F, Ehlers MD (2010) TGF-beta signaling specifies axons during brain development. *Cell* **142**: 144-157

Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massague J, Mundy GR, Guise TA (1999) TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* **103**: 197-206

Yingling JM, Datto MB, Wong C, Frederick JP, Liberati NT, Wang XF (1997) Tumor suppressor Smad4 is a transforming growth factor beta-inducible DNA binding protein. *Mol Cell Biol* **17**: 7019-7028

Yoon JH, Choi YJ, Choi WS, Ashktorab H, Smoot DT, Nam SW, Lee JY, Park WS (2013) GKN1-miR-185-DNMT1 Axis Suppresses Gastric Carcinogenesis through Regulation of Epigenetic Alteration and Cell Cycle. *Clinical cancer research : an official journal of the American Association for Cancer Research*

Yoshida M, Miyazato M, Kangawa K (2012) Orphan GPCRs and methods for identifying their ligands. *Methods Enzymol* **514**: 33-44

Yu L, Hebert MC, Zhang YE (2002) TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. *EMBO J* **21**: 3749-3759

Zagranichnaya TK, Wu X, Danos AM, Villereal ML (2005) Gene expression profiles in HEK-293 cells with low or high store-operated calcium entry: can regulatory as well as regulated genes be identified? *Physiological genomics* **21**: 14-33

Zanello SB, Collins ED, Marinissen MJ, Norman AW, Boland RL (1997) Vitamin D receptor expression in chicken muscle tissue and cultured myoblasts. *Hormone and metabolic research* = *Hormon- und Stoffwechselforschung* = *Hormones et metabolisme* **29**: 231-236

Zhang JC, Xie YF, Liu SJ, Dai LB, Li JP (2010) [The expression of melatonin receptor in human hypertrophic scar]. *Zhonghua Zheng Xing Wai Ke Za Zhi* **26:** 203-207

Zhang S, Fei T, Zhang L, Zhang R, Chen F, Ning Y, Han Y, Feng XH, Meng A, Chen YG (2007) Smad7 antagonizes transforming growth factor beta signaling in the nucleus by interfering with functional Smad-DNA complex formation. *Mol Cell Biol* **27**: 4488-4499

Zhang Y, Chang C, Gehling DJ, Hemmati-Brivanlou A, Derynck R (2001) Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. *Proc Natl Acad Sci U S A* **98**: 974-979

Zhang Y, Feng X, We R, Derynck R (1996) Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature* **383**: 168-172

Zhang YE (2009) Non-Smad pathways in TGF-beta signaling. Cell Res 19: 128-139

Zhao GQ, Zhang Y, Hoon MA, Chandrashekar J, Erlenbach I, Ryba NJ, Zuker CS (2003) The receptors for mammalian sweet and umami taste. *Cell* **115:** 255-266

Zhu S, Wang W, Clarke DC, Liu X (2007) Activation of Mps1 promotes transforming growth factor-beta-independent Smad signaling. *J Biol Chem* **282**: 18327-18338
ANNEX