Characterisation of Metalloprotease-mediated EGFR Signal Transactivation after GPCR Stimulation

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Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation mit dem Titel

„Characterisation of Metalloprotease-mediated EGFR Signal Transactivation after GPCR Stimulation“

selbständig am Max-Planck-Institut für Biochemie in Martinsried unter der Anleitung und Betreuung durch Herrn Prof. Dr. Axel Ullrich (MPI für Biochemie, Martinsried) und Herrn Prof. Dr. Georg Krohne (Julius-Maximilians-Universität, Würzburg) angefertigt habe und dabei keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet habe.

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Martinsried, den 21.03.2011

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Matthias Schneider

Dedicated to my Parents

and Martina

Contents

1. Introduction

Coordinating complex physiological processes and responding to a large diversity of extracellular signals is an essential capability of mammalian cells during embryonic development and adult life of multicellular organisms. This is achieved through a number of pathways that receive and process signals, not only from the external environment but also from different compartments within a cell. Communication takes place either by direct cell-cell contact between neighbouring cells or in the case of distant cells by secretion of messenger molecules such as growth factors and hormones. Binding of these ligands to their respective receptors on target cells and subsequent intracellular signal transmission finally leads to distinct biological responses including cell proliferation, migration, differentiation and apoptosis.

That this kind of information transfer has also a very important role in cellular regulation becomes more and more evident as deregulation of this signal transmission can cause various diseases such as cancer, diabetes, immune deficiencies or cardiovascular diseases among others (Hanahan and Weinberg 2000; Schlessinger 2000; Shawver et al. 2002).

A widespread regulatory element of almost all signal transduction processes is the reversible phosphorylation of proteins consisting of phosphorylation mediated by protein kinases and dephosphorylation catalyzed by protein phosphatases (Cohen 2002). Together these two protein classes can reversibly modify protein functions in a number of ways, for example by increasing or decreasing its biological activity, by stabilization or targeting the protein for degradation, by affecting its localisation in the cell or altering its affinity towards interaction partners. Impressive facts are, that more than one third of the human proteins can be phosphorylated and, accounting for 2% of gene products, protein kinases represent the single largest family of enzymes in the human genome (Knebel et al. 2001).

The sequencing of the Human Genome Project revealed 518 putative protein kinase genes and 130 protein phosphatases (Blume-Jensen and Hunter 2001; Manning et al. 2002). In addition 20% of the human genes encode for signaling molecules like

transmembrane proteins, guanine-nucleotide binding proteins (G proteins), proteases, kinases and phosphatases (Blume-Jensen and Hunter 2001).
According to their localization and their substrate specifity, the two last mentioned classes can be subdivided into cellular and transmembrane molecules and into tyrosine or serine/threonine specific kinases and phosphatases. As deregulation of phosphorylation patterns by aberrant expression or activity of kinases and phosphatases leads to various malignancies (Lim 2005) targeting these signaling pathways and the involved players holds promise for treating malignant disorders (Arora and Scholar 2005).

1.1 Protein Tyrosine Kinases

The protein tyrosine kinases can be spilt up into two major classes: 32 cytosolic tyrosine kinases and 58 receptor tyrosine kinases represent the two subgroups that build the overall 90 member comprising protein tyrosine kinases encoded by the human genome (Hanks and Hunter 1995).

1.1.1 Receptor Tyrosine Kinases (RTKs)

One class of type I transmembrane proteins that contain an intrinsic tyrosine kinase activity are the RTKs. Their composition is divided in three major parts: a glycosylated extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic domain containing the tyrosine kinase domain and the carboxyterminal-tail with several tyrosine phosphorylation sites (Hubbard and Till 2000, Citri and Yarden 2006). Both, a highly conserved protein tyrosine kinase core and additional regulatory sequences, are located in the cytoplasmic domain. The latter ones are subjected to autophosphorylation and phosphorylation by heterologous protein kinases.
So far more than 50 different RTKs, organized in 20 subfamilies have been described. Different sequence motifs in the extracellular domain like immunoglobulin-like domains, fibronectin type III-like domains, EGF (epidermal growth factor)-like

domains, cysteine-rich domains or other domains are mainly responsible for the structural diversity of RTKs (Figure 1).

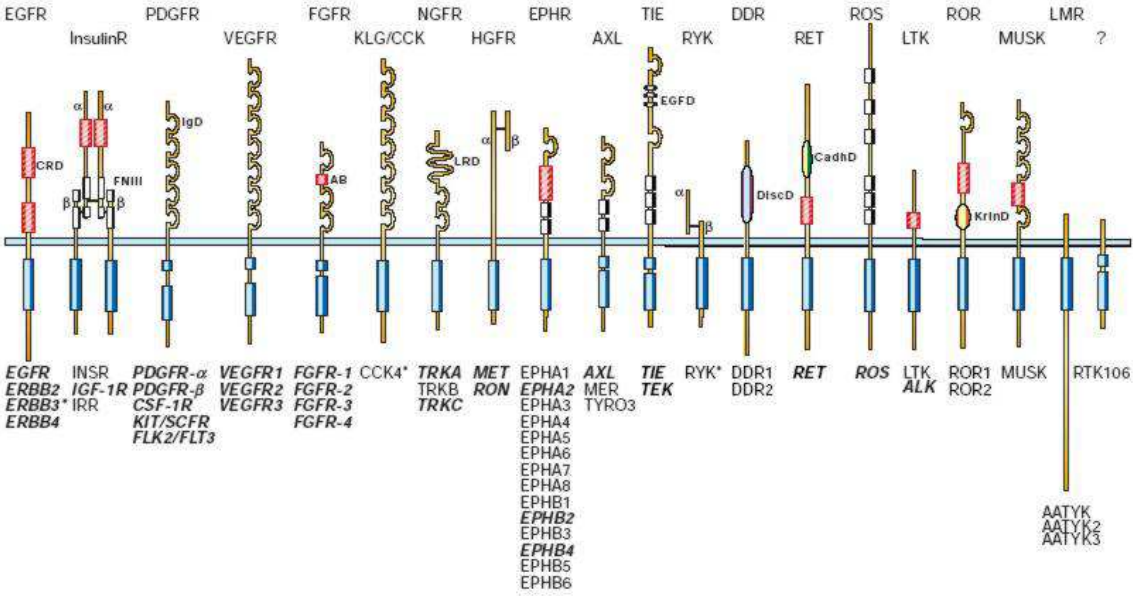


Figure 1. Subfamilies of receptor tyrosine kinases. Abbreviations: AB, acidic box; CadhD, cadherin-like domain; CRD, cysteine-rich domain; DiscD, discoidin-like domain; EGFD, epidermal growth factor-like domain; FNIII, fibronectin type III-like domain; IgD, immunoglobulin-like domain; KrinD, kringle-like domain; LRD, leucine-rich domain. The symbols α and β denote distinct RTK subunits (Blume-Jensen and Hunter, 2001).

Ligand induced dimerisation of monomeric receptors followed by transphosphorylation of selected tyrosine residues within the cytoplasmic domain represents the mechanism of activation of RTKs (Hubbard, Mohammadi et al. 1998; Schlessinger 2000). Docking sites for intracellular signal transducers containing phosphotyrosine interaction domains are formed hereby.

1.1.1.1 EGFR family

The epidermal growth factor receptor (EGFR) family consists of four members, including EGFR (Her1, erbB1) itself, Her2, Her3 and Her4 (erbB2, 3, 4). For every receptor several ligands have been described except for Her2 (Goldman et al.,

1990), and Her3 is the only one not showing kinase activity (Goldman et al. 1990; Ullrich and Schlessinger 1990).

The EGF-receptor, beeing the first cell surface signaling protein and protooncogene product to be characterized by molecular genetic methods, is the most prominent RTK and exhibits prototypical features of RTKs (Downward et al. 1984; Ullrich et al. 1984). Its implications in cancer development are due to its functional role as a proto-oncogene in viruses, the pathophysiological effects of EGFR mutants and its overexpression in several types of cancer. Epithelial cancer (Dong et al. 1999), lung and prostate cancer (Seth et al. 1999) as well as colon cancer (Damstrup et al. 1999) are examples where the EGFR plays important roles.

In 1997, ten years after Her2 overexpression in around one third of breast tumors was claimed (Slamon et al. 1987), it was shown that Her2 is the preferred dimerisation partner for the EGFR, HER3 and HER4 (Graus-Porta, Beerli et al.1997). One year later, the FDA approved trastuzumab (Herceptin), a humanized monoclonal antibody directed against the extracellular domain of Her2 for the treatment of metastatic breast cancer.

HER3, the third member of the EGFR family has several mutations in its kinase domain that result in an impaired kinase activity (Guy et al 1994; Stein and Staros 2000). Activation of Her3, either ligand-dependent or ligand-independent, is achieved by heterodimerisation followed by transphosphorylation. Despite of not being able to signal on their own, HER2/HER3 heterodimers represent the most potent mitogenic signalling complex that can be found in the ErbB network (Wallasch et al. 1995).

The last member of the family, HER4 shows almost the same characteristics as the EGFR: it can be activated by several ligands, has an active kinase domain and is therefore a fully functional receptor. It is capable of forming homo- as well as heterodimers but overall its implication in cancer is poorly understood.

1.1.1.2 EGF-like ligands and receptor activation

Direct activation of the EGFR was shown to be possible by eight different ligands: EGF, transforming growth factor alpha (TGFα), heparin-binding EGF-like growth

factor (HB-EGF), amphiregulin (AR), betacellulin (BC), epiregulin (Epi) (Riese and Stern, 1998), cripto (Salomon et al., 1999) and epigen (Strachan et al., 2001). In addition at least 26 different neuregulin (NRG) isoforms that are products of alternative splicing of the four neuregulin genes act as ligands of both HER3 and HER4 (Holmes et al. 1992; Zhang et al. 1997).

What all have in common is, that spatial and temporal expression of these ligands regulates the activation of the EGFR family RTKs (Peles and Yarden 1993), that they share the EGF like domain containing six conserved cysteine residues (Wingens et al. 2003) and possess a transmembrane domain. Furthermore all, except cripto, of the membrane anchored precursors are proteolytically cleaved at the cell surface to produce a mature soluble growth factor acting in an auto- or paracrine manner (Massague and Pandiella 1993; Harris, Chung et al. 2003). It is further reported, that some of the EGF family members act in a juxtacrine way in the membrane anchored form (Brachmann et al. 1989; Wong et al. 1989). EGF family members are either produced by tumor cells themselves or are provided by surrounding cells of the stroma. That leads in both cases to a more aggressive disease state via constitutive EGFR activation (Salomon et al. 1995). A 2:2 stoichiometry for ligand-receptor complexes generated from stable 1:1 ligand-receptor intermediates was revealed by biophysical investigations (Lemmon et al. 1997). Rather than to the bridging of receptor monomers by ligand molecules, ligand binding leads to conformational changes in the receptor (Schlessinger, Garrett 2002; Jorissen et al. 2003).

The EGFR family-unique dimerisation loop is responsible for receptor dimerisation, but in contrast to the majority of kinases, phosphorylation of the EGFR activation loop is not critical for its activation (Stamos 2002). Only after ligand binding the dimerisation arm becomes exposed and dimerisation of receptors can occur, leading to the activation of the intrinsically autoinhibited kinase domain. HER2 constitutively has to adopt this configuration with the exposed dimerisation arm due to not having a ligand (Burke and Stern 1998; Cho, Garrett et al. 2003). Figure 2 shows an overview of the preferred binding specificities of the EGF-like ligands and of HER2.

An enhanced tyrosine kinase activity going along with subsequent recruitment of downstream signaling molecules prerequisite an open conformation of the activation

loop. This in turn is caused by intermolecular autophosphorylation of cytoplasmic tyrosine residues in the activation loop of the catalytic tyrosine kinase domain resulting from receptor dimerisation (Schlessinger 2002).

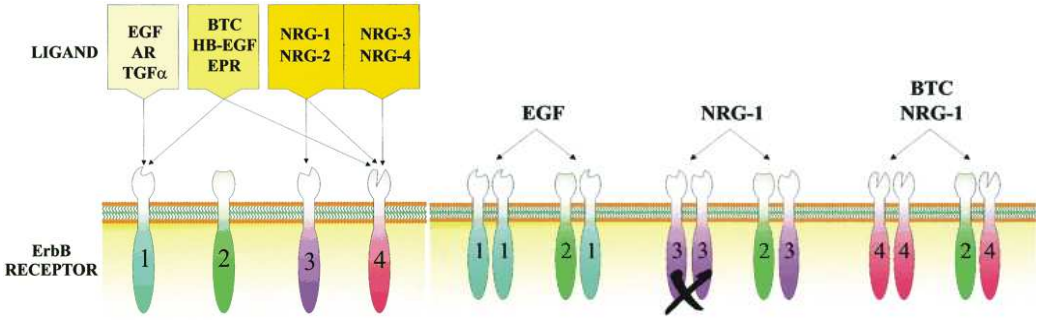


Figure 2. Binding specificities of the EGF-related peptide growth factors. There are four categories of ligands that bind ErbB family receptors. EGF, AR and TGFα bind ErbB1; BTC, HB-EGF and EPR bind ErbB1 and ErbB4; NRG-1 and NRG-2 bind ErbB3 and ErbB4; and NRG-3 and NRG-4 bind ErbB4.

ErbB2 is the preferred dimerisation partner for the other ErbB receptors. Ligand binding to ErbB1 (EGF), ErbB3 (NRG-1) or ErbB4 (NRG-1, BTC) induces the formation of receptor homodimers and ErbB2-containing heterodimers. ErbB3 homodimers do not signal (indicated by the X), since the receptor has impaired kinase activity. Only some of the possible ligand–receptor-induced combinations are indicated in Figure 2 for the sake of simplicity (Olayioye et al. 2000).

1.1.2 Cytoplasmic tyrosine kinases

Cytoplasmic tyrosine kinases, also named cytoplasmic non-receptor tyrosine kinases (NRTKs) represent the other class of PTKs besides the membrane-anchored receptor tyrosine kinases. Based on their kinase domain sequence they can be subdivided into 10 subfamilies: Abl, Ack, Csk, Fak, Fes, Frk, Jak, Src, Tec and Syk (Blume-Jensen and Hunter 2001). In contrast to RTKs they have neither a transmembrane-spanning region, nor an extracellular ligand binding domain. They are able to bind cell surface receptors. Although, through amino-terminal modifications such as myristoylation and palmitoylation some are bound to the cell

membrane, most of the NRTKs are localized in different intracellular compartments including the endoplasmic reticulum, the nucleolus, mitochondria and the cytoplasm (Hantschel and Superti-Furga 2004).

In addition to the tyrosine kinase domain – joint possessed with RTKs, they uniquely contain domains for mediating interactions with different classes of molecules like proteins, DNA and lipids. Tyrosine phosphorylation is the most common theme in NRTK regulation. An increased enzymatic activity is gained by tyrosine-phosphorylation by a number of other NRTKs or by *trans*-autophosphorylation within the activation loop. The direct opposite, a decrease in activity, occurs when phosphorylation of tyrosines outside of the activation loop takes place – thus showing a nice example of negative regulation (Hubbard and Till 2000).

The nine members containing Src family represents the largest subfamily and includes besides Src itself also Blk, Fgr, Fyn, Hck, Lck, Lyn, Yes and Yrk (Blume-Jensen and Hunter 2001). They play important roles in a variety of signaling processes, including T- and B-cell activation, cytoskeleton remodeling and mitogenesis (Parsons and Parsons 2004). The EGFR, the platelet derived growth factor receptor (PDGFR) and the NRTK focal adhesion kinase Fak are some of the Src substrates. Four domains are conserved in the structure of Src kinases (Figure 3): a short carboxy-terminal tail, followed by a tyrosine kinase domain (SH1), a SH2 domain, a SH3 domain, a unique domain, and a myristoylated amino terminal segment (SH4) (Boggon and Eck 2004). The two most important tyrosine phosphorylation sites are Tyr-416 and Tyr-527. Tyr-416 is an autophosphorylation site in the activation loop that is required for full kinase activity, whereas phosphorylation of Tyr-527 in the carboxy-terminal tail of Src, mediated for example by C-terminal Src kinase (Csk) or Csk homologous kinase (Chk), represses kinase activity. Biological importance of this site is shown in v-Src, an oncogenic variant of Src which is a product of the Rous sarcoma virus. Because of a carboxyterminal truncation, v-Src lacks the negative regulatory site Tyr-527 and therefore is constitutively active. This leads to uncontrolled growth of infected cells.

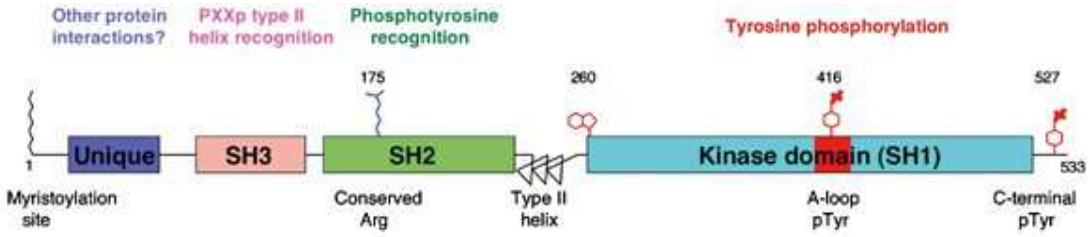


Figure 3. The Domain Structure of Src Family Kinases

The activation loop of the kinase domain is colored red, and the activating (Tyr416) and autoinhibitory (Tyr527) phosphorylation sites are indicated. Conserved residue Arg175 in the SH2 domain is critical for phosphotyrosine recognition; Trp260 at the extreme N-terminus of the kinase domain is important for autoinhibition. In the autoinhibited form of Src kinases, the SH2 domain binds the phosphorylated C-terminal tail, and the SH3 domain binds the linker segment between the SH2 and kinase domain, which forms a polyproline type II helix. By convention, amino-acid residues are numbered as in chicken Src. (Boggon and Eck 2004)

1.1.3 RTK Downstream Signaling and Protein Interaction Domains

Dependend on the stimulus and the cellular context, ligand-induced RTK activation leads to an onset of specific intracellular signal transduction pathways. Most intracellular signalling-proteins harbour domains that specifically interact with other protein domains, lipids, and nucleic acids in order to regulate many different cellular processes. Thereby proteins can be targeted to specific subcellular localizations, but the interaction domains can also be used as recognition-sites for posttranslational protein modifications or for chemical second messengers. Additionally they hold a variety of control-functions for example for activity, substrate specificity and conformation of enzymes (Pawson and Nash 2003).

In RTK signaling the most important domains are those which recognize the phosphorylated tyrosine itself (Schlessinger and Lemmon 2003). These are both Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains. As they can also bind to non-phosphorylated peptide sequences, the latter ones are not exclusively restricted to bind phosphotyrosine residues. Overall, SH2 represent the most prevalent binding domain (Schlessinger 2000).

Besides phosphotyrosine, phosphoserine is another common binding motiv where WW and 14-3-3 domains bind to, while phosphothreonine residues are recognized by FHA and WD40 domains. The proline-rich sequence motif PXXP represents an additional binding moiety which binds specifically to SH3 domains. Pleckstrin homology (PH), phox homology (PHOX), FERM and FYVE domains bind to phosphoinositides. Figure 4 shows an overview of different interaction domains and their binding specificities.

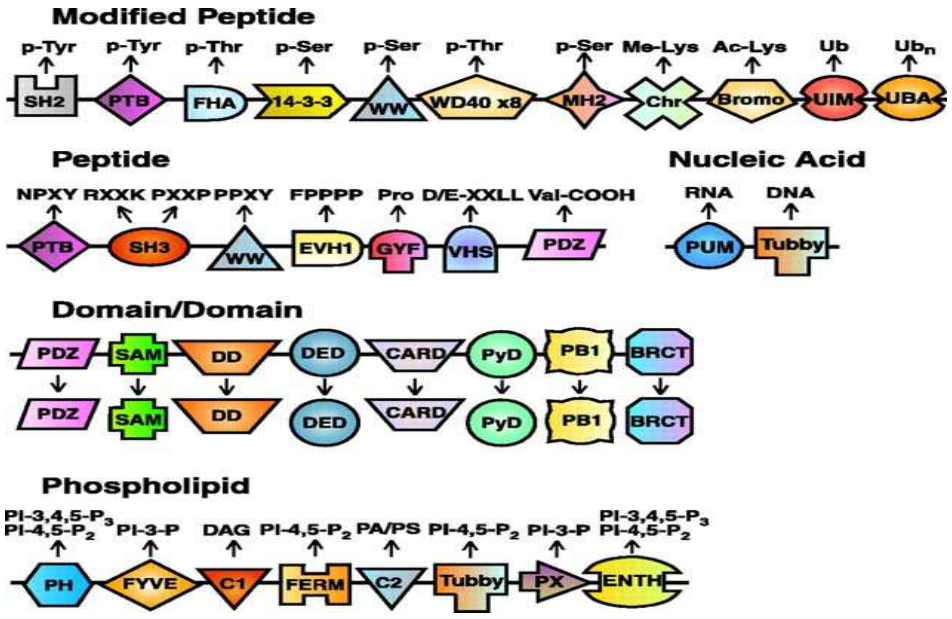


Figure 4. Modular interaction domains in signal transduction.

Interaction domains bind proteins, phospholipids, or nucleic acids. A subset of such domains is illustrated and their general binding functions are indicated (Pawson and Nash 2003).

Some signaling proteins exclusively consist of SH2 and SH3 domains and lack any catalytic domain. These adapter molecules act as some sort of scaffold whose function it is to facilitate interaction between two molecules. SH2 and SH3 domains containing Grb2, Crk and SHC proteins for example link activated RTKs to downstream signaling events such as the mitogen-activated protein kinases (MAPKs).

Other proteins in contrast are equipped with both enzymatic activity and interaction domains. Examples therefore include phospholipase C (PLC)-γ and Src. Both of them contain SH2 domains and additionally phosholipase- or tyrosine kinase activity.

1.2 G Protein Coupled Receptors

G Protein Coupled Receptors (GPCRs) are also called heptahelical or serpentine receptors as they contain a highly conserved structural motif of seven α-helical membrane-spanning regions, each consisting of 20-27 amino acids. Covering around 1% of the total genome of mammalians this superfamily of receptors represents the largest, more than 1000 members comprising, group of proteins (Hermans 2003). Besides their seven membrane spanning α-helices, they possess an extracellular N-terminus and an intracellular C-terminus which both can greatly vary in size.
The C-terminus as well as the intracellular loops interact with intracellular signaling partners, such as the associated heterotrimeric G proteins, but also with a wide variety of proteins containing structural interacting domains including PDZ, SH3 or PTB domains (Bockaert et al. 2003; Ji et al. 1998).

Based on their protein stucture, GPCRs can be divided into three major subfamilies:

type-A – receptors related to rhodopsin
type-B – receptors related to calcitonin receptor
type-C – receptors related to metabotropic receptor

The mechanisms of ligand binding depend on the sort of ligand. Smaller ligands bind to a hydrophobic core formed by transmembrane helices, whereas peptides bind via extracellular loops joining the transmembrane domains and the N-terminal tail. G proteins bind to domains located at the second and third cytoplasmic loop and at the C-terminus (Gether and Kobilka 1998). As GPCRs are involved in responses caused by a variety of external stimuli including neurotransmitters, hormones, lipids, photons,

odorants, taste ligands, nucleotides and calcium ions it was assumed that they are the targets for almost 60% of the drugs (Leurs et al. 1998). An overview of the complexity of GPCRs is shown in Figure 5.

A variety of downstream signalling pathways can be activated, depending on the type of G protein, to which the receptor is coupled. GPCR kinases (GRKs), that specifically phosphorylate activated GPCRs, attenuate signalling. Subsequent GPCR phosphorylation, arrestin binding is facilitated leading to receptor internalisation (Lutrell and Lefkowitz 2002). Complex interactions of intracellular domains of GPCRs with different intracellular proteins regulate receptor de- and eventually resensitization (Kroeze et al. 2003). Furthermore, GPCRs interact with a wide variety of proteins containing structural interacting domains including PDZ, SH3 or PTB domains, what can affect desensitization, location, trafficking and receptor signalling (Bockaert et al. 2003; Ji et al. 1998). To make the picture almost complete, these effects can also be achieved by homo- or hetero-oligomerisation among GPCRs (Breitwieser 2004).

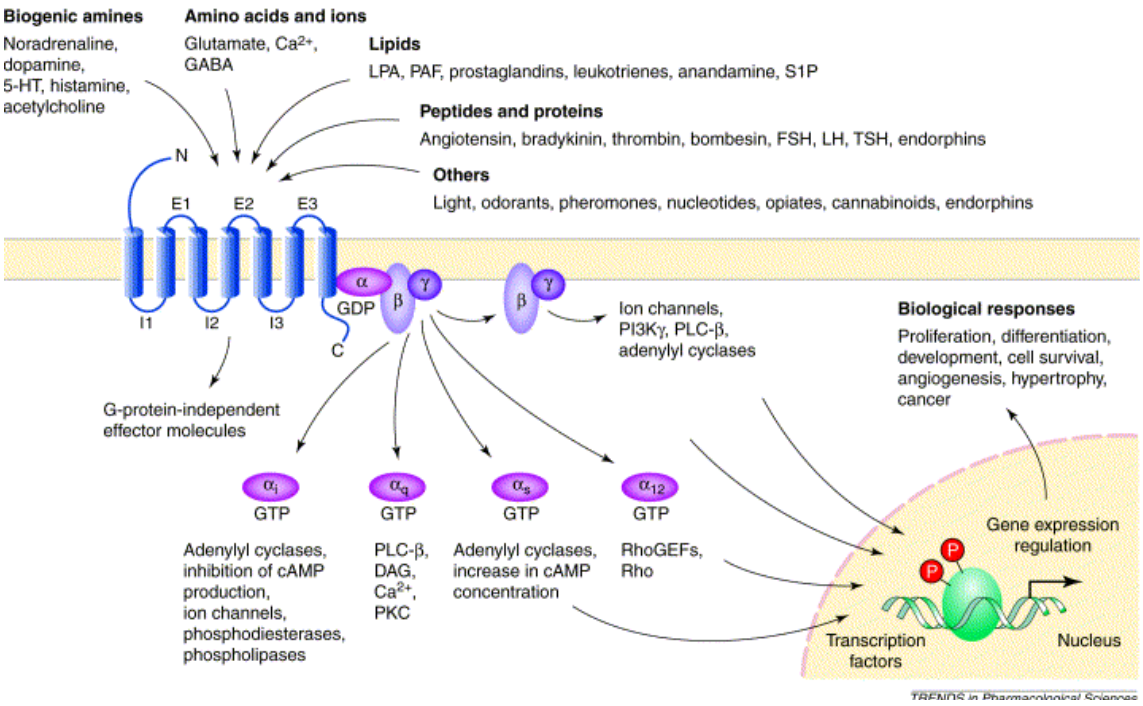


Figure 5. Diversity of G protein coupled receptors (GPCRs).

A wide variety of ligands, including biogenic amines, amino acids, ions, lipids, peptides and proteins use GPCRs to stimulate cytoplasmic and nuclear targets through heterotrimeric G protein dependent and independent pathways. Such signaling pathways regulate key biological functions such as cell proliferation, cell survival and angiogenesis. Abbreviations: DAG, diacylglycerol; FSH, follicle-stimulating hormone; GEF, guanine nucleotide exchange factor; LH, leuteinizing hormone; LPA, lysophosphatidic acid; PAF, platelet-activating factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; S1P, sphingosine-1-phosphate; TSH, thyroid-stimulating hormone (Marinissen and Gutkind 2001).

1.2.1 Heterotrimeric G-proteins

Although some studies reported G protein independent signaling through some GPCRs, the interaction and activation of downstream signaling cascades by heterotrimeric G proteins is a common characteristic of GPCRs (Hall et al. 1999; Bockaert and Pin 1999). Heterotrimeric G proteins are composed of a Gα subunit interacting with a Gβγ subunit.

Upon ligand binding the conformation of intracellular receptor domains is altered and the association with heterotrimeric G proteins is induced. Subsequently, GDP is exchanged for GTP in the active site of the Gα subunit, leading to the dissociation of the heterotrimeric complex. Gα as well as Gβγ subunits can activate cytoplasmic or membrane bound effector molecules (Hermans 2003).

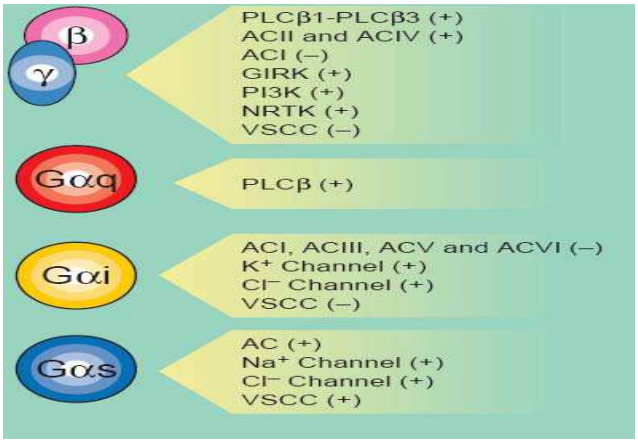


Figure 6. Downstream Signaling from Hetetrotrimeric G Proteins after GPCR Activation (Kroeze et al. 2003)

At least 23 Gα subunits derived from 17 different genes, 6 Gβ and 12 different Gγ subunits exist and are responsible for the specific and complex signaling induced by GPCRs. Broken down on the basis of amino acid similarities in four distinct families, namely $G\alpha_s$, $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12}$, G proteins are generally referred to by their Gα subunits (Pierce et al. 2001; Hermans 2003).

1.2.2 Mitogenic GPCR Signalling

Different potent mitogens such as acetylcholine, angiotensin, bombesin, bradykinin, endothelin-1, isoproterenol, lysophosphatidic acid (LPA), neurotensin, prostaglandin and thrombin showed oncogenic potential and induction of mitgenic responses in tissue culture systems (Daaka 2004).

The first connection between GPCRs and cellular transformation was detected with the discovery of the MAS1 oncogene, whose protein product displays a heptahelical structure, similar to GPCRs (Young et al. 1986). Contribution to malignant transformation and cancer is gained by sustained activation of GPCRs (Julius et al. 1989; Allen et al. 1991). Constitutively activated GPCRs, often found in transforming viruses are shown to induce cancer in animal models. Kaposi's sarcoma associated herpesvirus for example contains sequences encoding for a constitutive active GPCR (Montaner et al. 2003).

Not only GPCRs can carry oncogenic potential, also mutated G protein subunits like GTPase deficient forms of $G\alpha_i$, $G\alpha_q$, $G\alpha_0$, $G\alpha_{12}$, $G\alpha_{13}$ are found to be mitogenic in cellular systems. In addition activating mutants of the Gα subunits $G\alpha_{s,}$ $G\alpha_{i2}$ and $G\alpha_{12}$ encode for transforming oncogenes such as gsp, gip2 and gep (Landis et al. 1989; Lyons et al. 1990; Xu et al. 1993).

1.2.3 The Function of Arrestins

G protein coupled receptors can be regulated in many ways. Often, these receptors are adapted by phosphorylation and dephosphorylation. In addition to this, they can

also be regulated by changing their localisation in the cell, a process known as receptor redistribution. Factors binding to the receptors are another possibility to control them. It was shown for example that binding of calmodulin to the type 1 inositol 1,4,5-triphosphate receptor prevents binding of inositol 3-phosphate to this receptor (Sipma et al. 1999).

The family of arrestins also plays an important role in the process of receptor-regulation. After receptor activation lots of G protein coupled receptors rapidly get phosphorylated by G-protein coupled receptor kinases. Thereby the affinity of the receptor towards arrestins is increased significantly, which was first shown in the case of the ß2 adrenergic receptor (Lohse et al. 1992).

Up to now four arrestins have been described in mammals. Two of them are visual arrestins which are only expressed in the cone and rod photoreceptor cells of the retina respectively. In contrast to this the two other arrestins, named ß-arrestin1 and 2 are expressed ubiquitously (Sterne-Marr et al. 1995). Visual arrestins regulate photoreceptor-function by binding to and desensitising light-activated opsins, which were themselves phosphorylated by a G-protein coupled receptor kinase at their serine/threonine rich carboxy terminus.

Because of desensitising the ß-adrenergic receptor in a homologous manner, ß-arrestins 1 and 2 where named like this. Moreover, they extend this property to almost all members of the GPCR-superfamily. Different to visual arrestins ß-arrestins bind to proteins located in clathrin coated pits, thereby mediating internalisation of G-protein coupled receptors. The ß-adrenergic Receptor, a typical class A GPCR preferably binds to ß-arrestin 2 but dissolves already before endocytosis.

In contrast to this class B GPCRs, like the V2-vasopressin receptor for example, bind to ß-arrestin 1 and 2 and remains bound during internalisation as shown in Figure 7.

Dissociation from ß-arrestins during endocytosis promotes receptor dephosphorylation and thereby recycling to the plasma membrane. Degradation of internalised receptors by directing them to lysosomes happens when ß-arrestins are persistently bound to receptors during internalisation (Luttrell et al. 2002).

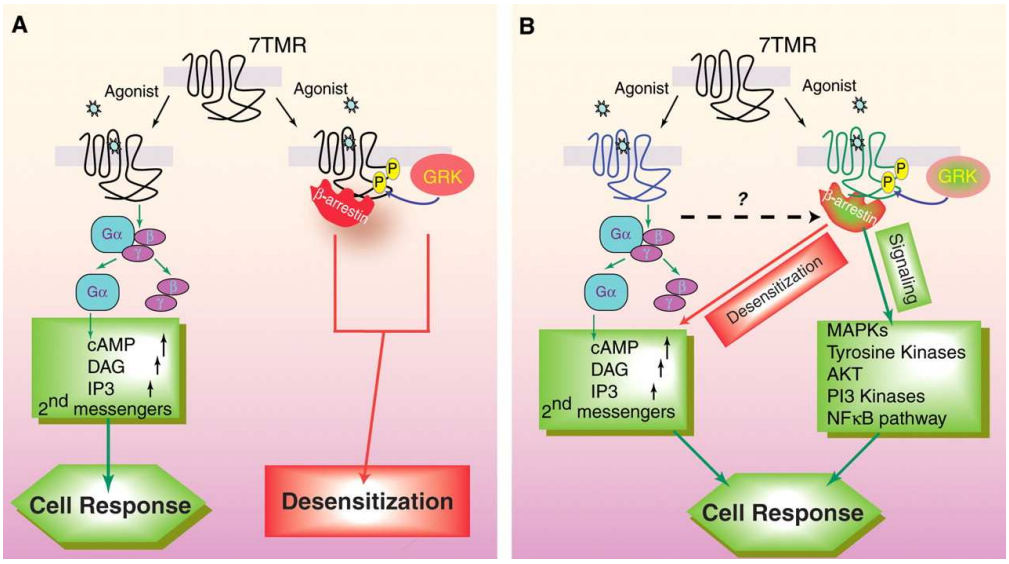


Figure 7. Signal transduction by G-protein coupled receptors

(**A**) Classical paradigm. The activated receptor stimulates heterotrimeric G proteins and gets phosphorylated by G protein–coupled receptor kinases (GRKs). This leads to ß-arrestin recruitment, desensitization of the receptor and termination of the signaling.

(**B**) New paradigm. Beside of mediating desensitization of G protein–signalling, ß-arrestins also act as signal transducers themselves (Lefkowitz and Shenoy 2005).

ß-arrestin mediated endocytosis of GPCRs was confirmed by a set of experiments with dominant-negative ß-arrestin constructs. Claing et al. postulated that over-expression of a ß-arrestin mutant unable binding to clathrin inhibits receptor-internalisation of the M1 muscarinic receptor (Claing et al. 2000). Another dominant-negative ß-arrestin variant, binding to clathrin but not to phosphorylated GPCRs anymore also led to an inhibition of receptor endocytosis when being overexpressed (Krupnick et al. 1997).

1.3 Mitogen-Activated-Protein-Kinase (MAPK) pathways

Connecting cell surface receptors to regulatory targets within the cell, Mitogen-activated protein kinases (MAPKs) represent a group of very important signal

transducing enzymes. Therefore their signaling pathways are one of the best characterised ones. MAPKs phosphorylate specific serine and threonine residues of target proteins and are implicated in several cellular function like cell migration, proliferation, differentiation, transformation and survival and apoptosis (Ip and Davis 1998; Schaeffer and Weber 1999). Three protein kinases form the signaling unit: a MAP kinase, a MAP kinase kinase (MKK) and a MAP kinase kinase kinase (MKKK) each of them being phosphorylated and activated by the successive one. A fourth kinase, MAP4K is often found and is responsible for activating MKKK.

Mammalian MAPKs can be split up into minimal four subclasses: extracellular-regulated kinase 1/2 (ERK1/2), Jun amino-terminal kinase (JNK1/2/3), p38 proteins (p38 α/β/γ/δ) and ERK 5 (Chang and Karin 2001). The organisation of the mammalian MAPK cascades is shown in Figure 8.

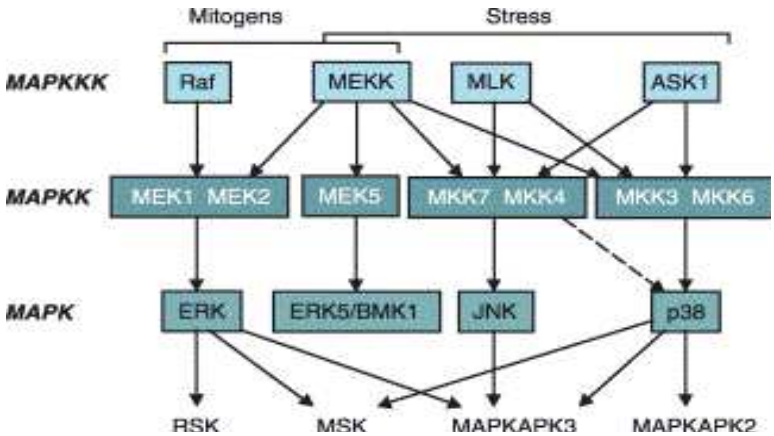


Figure 8. Organisation of mammalian MAPK cascades. MAPK cascades feature a core triple kinase module consisting of MAPKKKs, MAPKKs and MAPKs. There are a number of MAPKKK families and individual MAPKKKs are often components of more than one MAPK cascade. MAPKKs selectively target a particular MAPK. A potential exception is MKK4 which has been reported to activate p38 (dashed line) in addition to its major target JNK. MAPKs can phosphorylate transcriptional targets directly or this can occur via the indicated downstream protein kinases (Yang et al. 2003).

Regulation of gene expression either by direct phosphorylation of transcription factors, target co-activators or co-repressors demonstrates the major function of the

MAPK pathway (Yang et al. 2003). The pathway in which ERK1/2 is activated by RTKs is definitely the most prominent MAPK cascade: the adaptor protein Grb2 is recruited and associates with the RAS-GEF Sos complex upon ligand induced activation of RTKs. The RAS-GEF son of sevenless (Sos) complex is thereby activated and in turn activates membrane-associated Ras, a small monomeric GTP-binding protein (Schlessinger 2000). Serine/threonine kinase activity of the MAPK kinase kinase Raf-1 is induced by Ras, leading to phosphorylation and activation of the MAPK kinases 1/2 (MEK1/2) which finally activate ERK1/2 by phosphorylation of threonine and tyrosine residues in the regulatory TEY-motif (Burrack and Shaw 2000).

The MAPKKK, MAPKK, and MAPK signaling cassette is highly conserved in evolution and plays an important role in the control of metabolic processes, cell cycle, cell migration and cell shape as well as in cell proliferation and differentiation (Hunter 2000). Figure 9 shows an overview of the MAPKs, their pathways and the interactions in between. The specificity of MAPK interactions and of the effector molecules stimulated depends largely on the MAPK subtypes involved. In particular, extracellular signal-regulated kinases (ERK1/2)/MAPKs are primarily stimulated by growth factors and modulate cell growth and differentiation, whereas c-Jun N-terminal kinases (JNKs) and p38 MAPKs are most commonly activated by stress stimuli and are involved in cell growth, differentiation, survival, apoptosis, and cytokine production (Marinissen and Gutkind 2001).

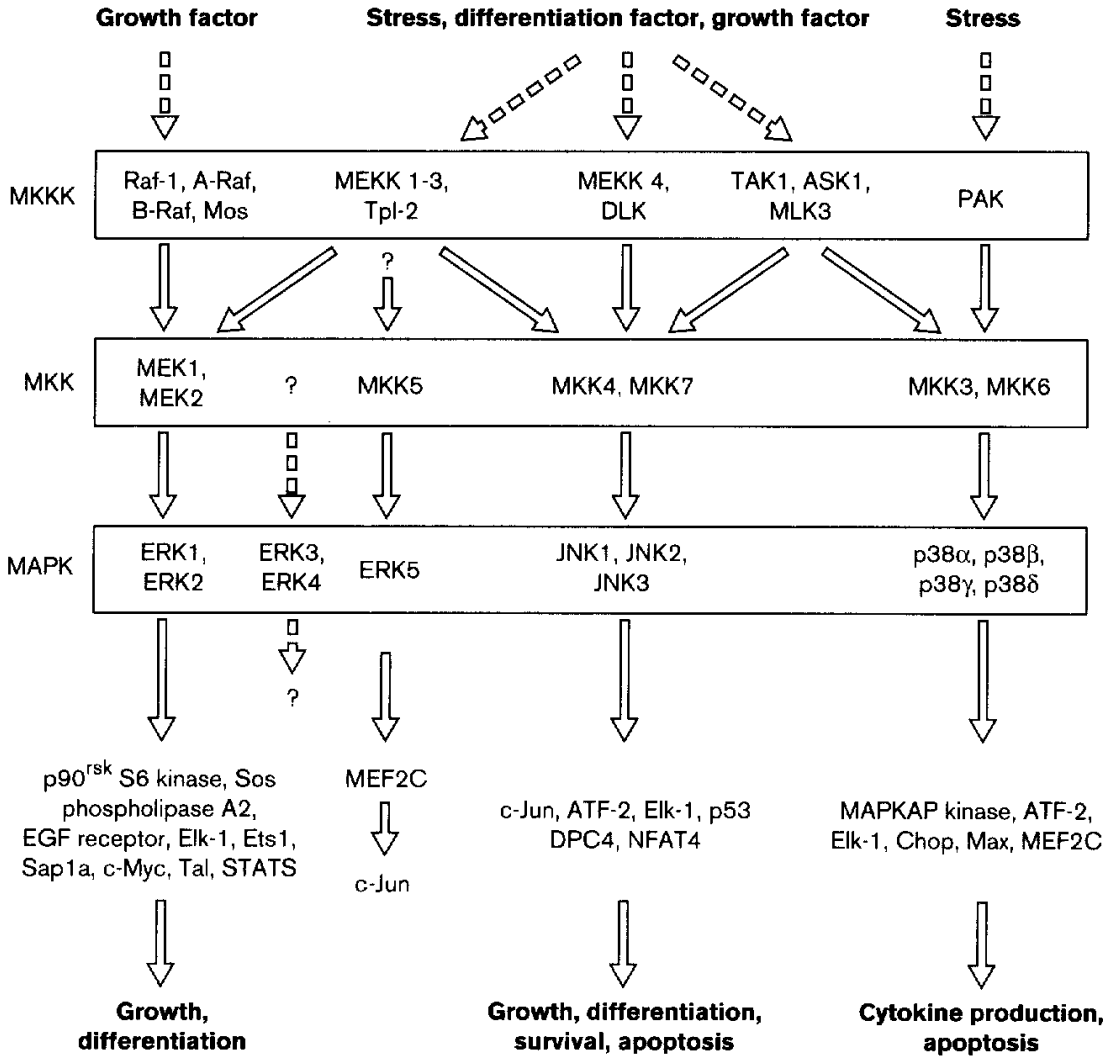


Figure 9. MAPK pathways interacting with each other

Mitogen-activated protein kinase modules. The MAPK module consists of an MKKK, an MKK, and a MAPK. MKKKs respond to a variety of extracellular signals, including growth factors, differentiation factors and stress. The activated MKKKs can then activate one or several MKKs. In contrast, the MKKs are relatively specific for their target MAPKs. Once activated, MAPKs can then phosphorylate transcription factors (for example ATF-2, Chop, c-Jun, c-Myc, DPC4, Elk-1, Ets1, Max, MEF2C, NFAT4, Sap1a, STATs, Tal, p53), other kinases (MAPKAP kinase, p90rsk S6 kinase), upstream regulators (EGF receptor, son of sevenless [Sos], Ras exchange factor), and other regulatory enzymes such as phospholipase A2. These downstream targets then control cellular responses including growth, differentiation, and apoptosis (Garrington and Johnson 1999)

1.4 Receptor tyrosine kinase transactivation

Upon ligand binding receptor tyrosine kinases transduce extracellular signals to the cytoplasm via activation and recruitment of effector and adaptor molecules, thereby activating various signaling cascades.

Apart from amplification, RTKs are also excellent molecules for signal integration and diversification. Over the last years, increasing interest focuses on interreceptor communication mechanisms both in normal physiology and in pathological states, not only because RTKs are key nodes in highly complex signaling networks that allow communication, coordination and adaptation of cellular signaling.

Also RTKs have been shown to be activated by a variety of different stimuli ranging from direct stimulation with their cognate ligands, GPCR agonists, integrin activation, cytokine receptors to ion channels (Gschwind et al. 2001; Prenzel et al. 2001; Fischer et al. 2004).

1.4.1 EGFR signal transactivation

Daub and colleagues where the first to identify the EGFR as an essential element in GPCR-induced mitogenesis of rat fibroblasts (Daub et al. 1996). Upon treatment of cells with lysophosphatidic acid (LPA), endothelin-1 (ET-1) and thrombin, representing G protein-coupled receptor agonists, both EGFR and its relative HER2 were rapidly tyrosine-phosphorylated.

This transactivation of a receptor tyrosine kinase couples GPCR ligands to ERK 1/2 activation and subsequent induction of *c-fos* gene expression. The GPCR-EGFR crosstalk pathways were further explored and they could be detected in a variety of other cell types such as primary mouse astrocytes, human keratinocytes, PC12 cells and vascular smooth muscle cells. A new pathway finally leading to the activation of the MAPK signal was established (Gschwind et al. 2001; Fischer et al. 2003; Hart et al. 2003).

As no EGF-like ligands could be detected in the cell culture medium after G protein activation and because of the short time-period from GPCR stimulation to EGFR phosphorylation its was assumed that this transactivation mechanism is occuring

intracellular (Daub et al. 1996; Zwick et al. 1999). However, few years later Prenzel and colleagues claimed the concept of a "Triple-Membrane-Passing-Signal" (TMPS) mechanism of EGFR signal transactivation (Figure10) involving a metalloprotease activity and processing of the transmembrane EGF-like growth factor precursor proHB-EGF (Prenzel et al. 1999).

Resultant from these findings, transmembrane metalloproteases turned out as the key enzymes of growth factor precursor shedding. Bombesin- and TPA-induced transactivation of the EGFR in PC-3 human prostate cancer cells as well as high constitutive levels of EGFR tyrosine phosphorylation in unstarved PC3 (Prenzel et al. 1999) and SCC25 squamous cell carcinoma cells (Gschwind et al. 2002) could be inhibited by the broadband metalloprotease inhibitor batimastat (BB94).

In 1999 Dong and collleagues reported that cell proliferation and cell migration of a human mammary epithelial cell line could be reduced by BB94, whose mode of action interfered with the release of EGFR ligands (Dong et al. 1999). LPA-induced proliferation and motility of head and neck cancer cells was also shown to be reduced by BB94 pointing out the involvement of metalloprotease-dependent transactivation of the EGFR (Gschwind et al. 2002). Since then several investigations revealed members of the ADAM subclass of metalloproteases to play a critical role in GPCR mediated transactivation of the EGFR (Gschwind et al. 2003; Sahin et al. 2004), resulting in an updated model of the triple membrane passing signal mechanism of EGFR signal transactivation (Figure 10).

To date it is known that all 7 ligands binding to EGFR are getting cleaved to form a soluble ligand and six out of them are cleaved by metalloproteases (Sahin et al. 2004; Kochupurakkal et al. 2005). Among these, also various ADAMs and matrix metalloproteases are responsible for the cleavage of EGF family ligands (Gschwind 2002, 2003, Hart et al. 2003). Biological importance of this mechanism was supported when EGFR function was reported to be critical for GPCR stimulated mitogenic signaling in several cancer cells (Castagliuolo et al. 2000; Venkatakrishnan et al. 2000).

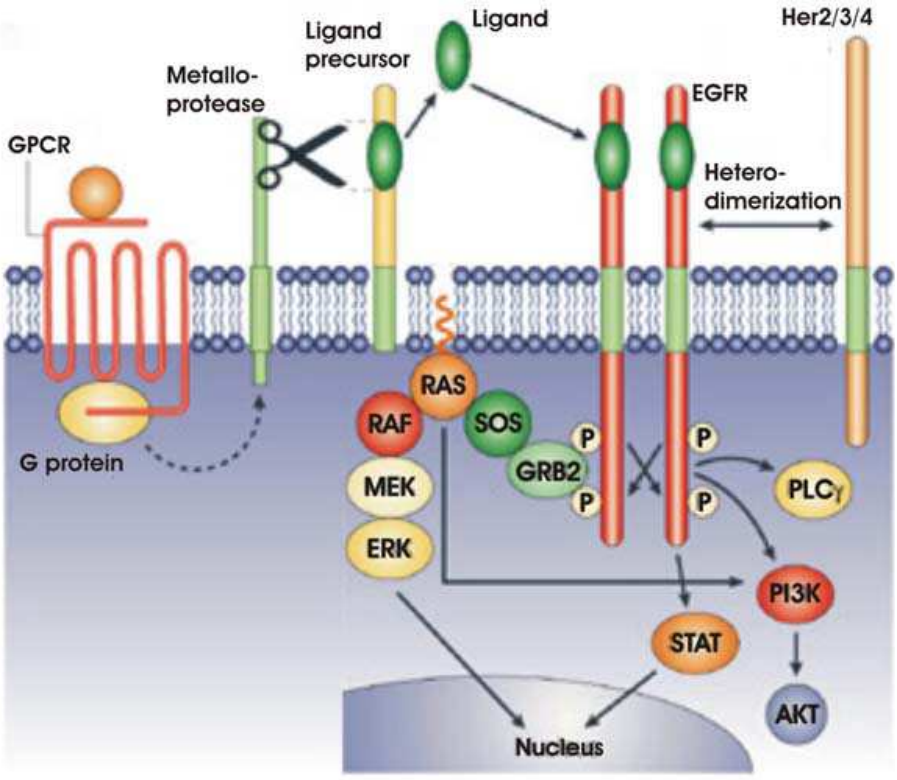


Figure 10. The triple-membrane-passing signal (TMPS) mechanism of EGFR transactivation. GPCR stimulation induces upregulation of a metalloprotease activity, therby leading to ectodomain cleavage of EGF-like growth factor precursors. Subsequent release of the mature growth factor stimulates EGFR kinase activity and transduces the GPCR signal inside the cell to stimulate characteristic EGFR downstream signaling pathways such as MAPK, PLC-γ, STAT or PI3K activation (Gschwind et al. 2004)

EGFR was not the only RTK to be involved in transactivation. Also other RTKs such as the insulin-like growth factor receptor (IGF-1R), hepatocyte growth factor receptor (Met-R), vascular endothelial cell growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR) and the Fibroblast growth factor receptor (FGFR)-1 showed activation upon GPCR stimulation (Belcheva et al. 2001; Endo et al. 2002; Fischer et al. 2004; Herrlich et al. 1998; Pai et al. 2003; Weiss et al. 1997).

1.4.1.1 Metalloproteases

Metalloproteases fullfill different functions including regulation of receptor cleavage to terminate migratory signaling, degradation of the extracellular matrix (ECM) to enable founder cells to move across tissues into nearby stroma and the proteolytic cleavage of growth factors therby generating soluble ligands for cells not in direct physical contact. They are therefore involved in many biological processes, ranging from cell migration, differentiation and remodelling of ECM to vascularization and cell proliferation, all of them occuring several times during organogenesis, in both normal development and during tumour progression. For all these important processes a fine-balanced regulation between the functions of matrix metalloproteases (MMPs) or metalloprotease-disintegrins (ADAMs) and natural tissue inhibitors of metalloproteases (TIMPs) is required.

According to the primary structure of their catalytic sites, zinc dependent proteases are differentiated into carboxypeptidase, DD carboxypeptidase, gluzincin, inuzincin and metzincin subgroups (Hooper 1994). The metzincins are further categorized in adamalysins, astacins, matrixins and serralysins (Stocker et al. 1995). The active site of zinc metalloproteases, responsible for hydrolytic processing of substrates, contains water ions and zinc which is coordinated by three conserved histidine residues and a downstream methionine. This forms a Met turn motif that loops around to face the consensus HEXXHXXGXXH site (Stocker and Bode 1995).

Most of the metalloproteases are synthesized as inactive precursors with a terminal prodomain, keeping the metalloprotease inactive by a cysteine switch (Becker et al. 1995). The prodomain of metalloproteases appears to be important for maturation, intracellular transport and acts as an inhibitor of the protease domain (Milla et al. 1999). It was proposed, that processing and activation of metalloproteases take place at the trans-Golgi network. It can either be executed by furin or other proprotein convertases (PCs) or happens in an autocatalytic manner (Anders et al. 2001; Kang et al. 2002).

1.4.1.1.1 ADAMs

Metalloprotease-disintegrins, forming a subgroup of metalloproteases are transmembrane glycoproteins which take part in cell-cell interaction and in processing of protein ectodomains (Wolfsberg et al. 1995).

These proteases are characterized by a conserved domain structure consisting of a N-terminal signal sequence followed by a prodomain, metalloprotease and disintegrin domains, a cysteine-rich region, an EGF-like domain and finally a transmembrane domain and cytoplasmic tail (Figure 11). ADAMs (a disintegrin and a metalloprotease domain) got their name due to the fact that they contain disintegrin and metalloprotease domains. Closely related to the ADAMs are the class III snake venom metalloproteases and the ADAM-TS family, which represent the other two subfamilies of adamalysins.

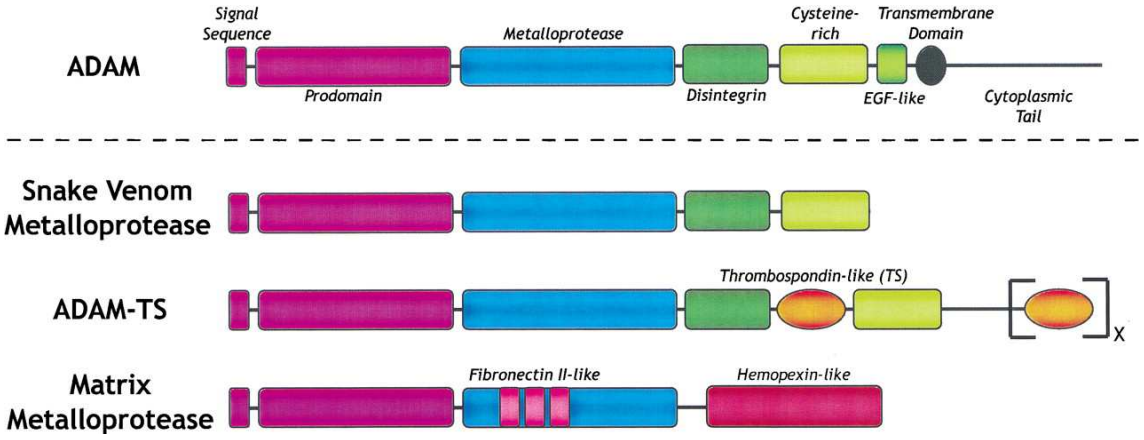


Figure 11. The topography of the ADAMs and related metalloproteases. Generalized domain structures of the ADAMs, SVMPs, ADAM-TS, and MMP families are shown. Note that ADAM-TS family members have a variable number of thrombospondin-like (TS) motifs. The MMP shown is of the gelatinase class. Other subclasses of MMPs lack hemopexin-like sequences and/or fibronectin type II-like sequences. The subclass of MT-MMPs has transmembrane domains and cytoplasmic tails in addition to the domains shown (Seals and Courtneidge 2003).

The zinc-binding catalytic-site consensus sequence (HEXXH) is only found in around 50% of the more than 30 ADAM cDNA sequences that are known, although ADAMs

having a relatively well-conserved metalloprotease domain. In humans,12 of the 19 known ADAMs show proteolytic activity (Kheradmand and Werb 2002).

They are implicated in various biological processes, including fertilization, cell fate determination and muscle development and play a role in cytokine, RTK and growth factor shedding as well as in the control of sperm-egg binding and fusion and of membrane fusion. Furthermore they are implicated in pathologies like inflammation and cancer (Schlondorff and Blobel 1999; Kheradmand and Werb 2002).

Sanderson and colleagues could show how inactive precursor ADAMs are activated via release of their prodomain (Figure12). However, it is only poorly understood how ADAM metalloprotease activity after prodomain removal is regulated. A prerequisite for processing of membrane proteins by ADAMs is that both the membrane-anchored enzyme and its substrate are in close proximity on the cell. Werb and colleagues showed that upon cell activation by PKC agonists, increases in cytoplasmic Ca^{2+} levels or tyrosine kinase stimulation, the proteases and substrates become co-clustered and can interact (Werb and Yan 1998).

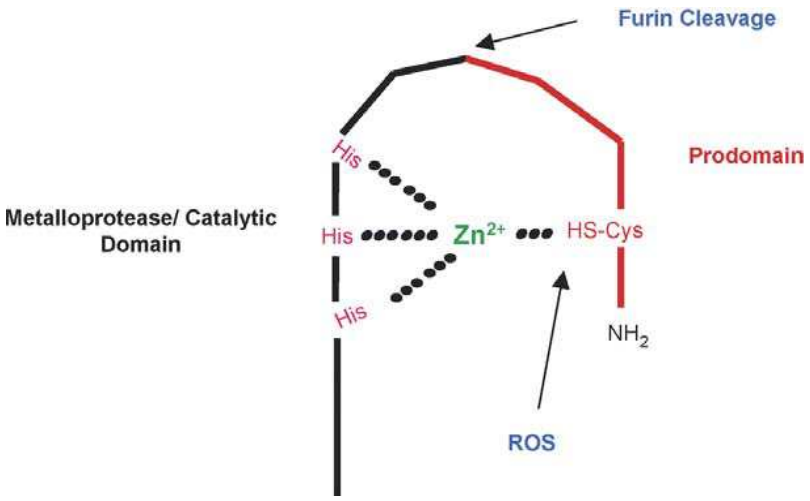


Figure 12. Mechanism of ADAM Activation

ADAM metalloproteases are produced as inactive precursors, with the inhibitory prodomain attached. Inhibition can be released either by furin cleavage or by oxidation of a susceptible cysteine in the prodomain by reactive oxygen (ROS) species leading to the opening up of the structure, dissociating the catalytic domain from the prodomain. (Sanderson et al. 2006)

One of the best characterized metalloproteases is Tumor necrosis factor-α convertase (TACE/ADAM17). It was originally identified as being responsible for the release of the inflammatory cytokine tumor necrosis factor (TNF)-α from its membrane-bound precursor proTNFα (Black et al. 1997; Moss et al. 1997). Since its identification it was reported that ADAM17 is not only involved in shedding of TNF-α but also of many other membrane bound proteins like L-selectin, p75 TNF receptor (Peschon et al. 1998), fractalkine (Garton et al. 2001), MUC1 (Thathiah et al. 2003), β-amyliod precursor protein (βAPP) (Buxbaum et al. 1998), p55 TNFR, interleukin-1 receptor II (IL-1R II) (Reddy et al. 2000), erbB4/HER4 (Rio et al. 2000), the Notch1 receptor (Brou et al. 2000), IL-6R (Althoff et al. 2000), growth hormone-binding protein (Zhang et al. 2000) and cellular prion protein (Vincent et al. 2001).
The implication of ADAM17 in the release and the constitutive availability of TGFα and other EGF-like ligands was first shown using fibroblasts derived from ADAM17 knock-out mice (Peschon et al. 1998; Sunnarborg et al. 2002). The epithelial defects observed in *tace*$^{\Delta Zn/\Delta Zn}$ fetuses are similar to those reported in mice lacking the epidermal growth factor receptor (EGFR) (Peschon et al. 1998). But in contrast to EGFR or TGFα deficient mice, Mice with *tace*$^{\Delta Zn/\Delta Zn}$ null mutation die at birth with phenotypic defects, including failure of eyelid fusion, hair and skin defects, and abnormalities of lung development. This makes it likely that ADAM17 has additional substrates implicated in the development of survival-required organs (Shi et al. 2003).

1.4.1.1.2 MMPs and TIMPs

Matrix metalloproteases (MMPs) belong to the matrixins and are mainly responsible for degradation and remodeling of extracellular matrix during development, wound healing and pathologies like cancer and arthritis (Chang and Werb 2001). Being closely related to the ADAMs, they have also been shown to be involved in EGFR-transactivation via cleaving members of the EGF ligand family (Suzukiet et al. 1997; Yu et al. 2002; Roelle et al. 2003). Based on their specificity for ECM components, MMPs can be divided into collagenases, gelatinases, stromelysins and matrilysins.

According to their structure the more then 20 MMPs were classified in a sequential numbering system structure (Nagase and Woessner 1999).

Members of five of the eight different classes of MMPs are secreted, whereas the remaining three classes are membrane-type MMPs (MT-MMPs). All of them are synthesized as prepro-enzymes and secreted as inactive pro-MMPs in most cases. The proteolytic activities of MMPs are tightly controlled by endogenous inhibitors like α-macroglobulins and tissue inhibitors of metalloproteinases (TIMPs). TIMPs do not only regulate MMPs but can also control ADAM activities. The characteristics of TIMP1-4 are shown in Table 1 (Crocker et al. 2004).

	Extracellular localization	*Proteases inhibited*
TIMP-1	Secreted	All but MT1-MMP ADAM10
TIMP-2	Secreted	Inhibits all MMPs tested
TIMP-3	Membrane	MMP-1,-2,-3,-7,-9,-14 ADAM10,-12S,-17 ADAM-TS4,-TS5
TIMP-4	Secreted	MMP-1,-2,-3,-7,9

Table 1: Characteristics of the mammalian TIMP protein family on inhibition of metalloproteases of the MMP and ADAM family (Crocker et. al. 2004)

1.4.2 Reactive Oxygen Species (ROS)

Superoxide (O_2^-), hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2) are the most prevalent forms of ROS produced inside a cell. ROS play a key role in signal

transduction as secondary messengers (Lambeth 2004; Rhee, Khang et al. 2005) and besides being constantly produced during metabolic reactions, they are also generated upon external stimuli like GPCR-stimulation, RTK activation and UV or ionizing radiation (Finkel 2003).

Assortments of proteins have been identified containing susceptible cysteine residues which are reversibly oxidized by ROS, leading to either activation or inhibition of their catalytic activity.

ADAMs, Src kinase (Giannoni, Buricchi et al. 2005) and G proteins of G alpha i/o subtype have been shown to be activated by reactive oxygen species (Nishida, Maruyama et al. 2000; Zhang, Oliver et al. 2001; Nishida, Schey et al. 2002).

Additionally, protein tyrosine phosphatases (Meng, Fukada et al. 2002), lipid phosphatases (Leslie, Bennett et al. 2003; Kwon, Lee et al. 2004), peroxiredoxins (Rhee, Kang et al. 2005), MAP kinase phosphatase (Kamata, Honda et al. 2005) and SUMO modifying proteins (Bossis and Melchior 2006) have been shown to be inhibited by ROS.

Imbalance of redox signaling in a cell or organism leads to numerous disease states including Alzheimer's (Butterfield and Boyd-Kimball 2004), aging (Storz 2006) and cancer (Benhar, Engelberg et al. 2002).

Therefore a balanced redox signalling is very important for maintaining normal biological function.

1.5 Aim of this study

The aim of the study is the investigation of metalloprotease-activation in the TMPS-pathway and the characterization of the proteins involved in this Receptor Tyrosine Kinase transactivation. Special attention was paid to the following aspects:

ADAM17 is one of the major sheddases involved in the TMPS-pathway via proligand-shedding of EGFR-ligands which then bind to the receptor, thereby activating it.

In the beginning several antibodies targeting ADAM17 were tested for their ability to block the metalloprotease's activity and subsequently block RTK transactivation. Different conditions were tested in well characterized cellular systems including kinetic and dose-dependent approaches.

Next, the involvement of molecules playing a role in metalloprotease activation was analysed and aimed to be characterized. It was intended to show that knock-down of these proteins would result in a reduced or even abrogated RTK-transactivation pathway.

Finally, the potential role of metalloproteases and their involvement in UV induced EGFR-transactivation was subjected to be analysed.

2 Materials and Methods

2.1 Materials

2.1.1 Laboratory chemicals and biochemicals

Acrylamide	Serva, Heidelberg
Agar	Difco, Detroit, USA
Agarose	BRL, Eggenstein
AG1478	Alexis, Grünberg
Ampicillin	Roche, Mannheim
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
ATP (Adenosine 3’-triphosphate)	Pharmacia, Freiburg
Batimastat (BB94)	British Biotech, Oxford, UK
Bisacrylamide	Roth, Karlsruhe
Bromphenol blue	Sigma, Taufkirchen
BSA (Bovine serum albumin)	Sigma, Taufkirchen
CHAPS	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
Dideoxynucleotides (ddG/A/T/CTP)	Pharmacia, Freiburg
Diphtheria toxin CRM mutant	List Biological Lab., CA, USA
DTT (Dithiothreitol)	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Fibronectin	Calbiochem, Bad Soden
Heparin	Sigma, Taufkirchen
HEPES (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid))	Serva, Heidelberg

IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Biomol, Hamburg
L-Glutamine	Gibco, Eggenstein
Lipofectamine®	Invitrogen, USA
Lipofectamine 2000	Invitrogen, USA
Lysozym	Sigma, Taufkirchen
LY 294002	Alexis, Grünberg
Mineral oil	Sigma, Taufkirchen
Na-DOC (Sodium-desoxycholat)	Sigma, Taufkirchen
Oligofectamine®	Invitrogen, USA
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
Polybrene (Hexadimethrine bromide)	Sigma, Taufkirchen
Ponceau S	Sigma, Taufkirchen
PP1	Calbiochem, Bad Soden
PP2	Calbiochem, Bad Soden
PTX (Pertussis toxin)	List Biological Lab., CA, USA
Scintillation cocktail (Rotiszint®ecoplus)	Roth, Karlsruhe
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sphingosine-1-phosphate, D-erythro	Biomol, PA, USA
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Aldrich, Steinheim
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
Triton X-100	Serva, Heidelberg
Tween 20, 40	Sigma, Taufkirchen
Tyrphostin AG1478	Alexis, Grünberg
Wortmannin	Sigma, Taufkirchen

All other chemicals were purchased from Merck (Darmstadt).

2.1.2 Enzymes

AMV reverse transcriptase	Roche, Mannheim
Alkaline Phosphatase (CIAP)	Roche, Mannheim
Restriction Endonucleases	Pharmacia, Freiburg
	Roche, Mannheim
	NEB, Frankfurt/ Main
	MBI Fermentas, St. Leon-Rot
T4-DNA Ligase	Roche, Mannheim
T7-DNA Polymerase	Pharmacia, Freiburg
Taq-DNA Polymerase	Roche, Mannheim
	Takara, Japan
Trypsin	Gibco, Eggenstein

2.1.3 "Kits" and other materials

Cell culture materials	Greiner, Solingen
	Nunclon, Dänemark
	Falcon, U.K.
Cellulose nitrate 0.45 µm	Schleicher & Schüll, Dassel
Concanavalin A-Sepharose 4B	Sigma, Taufkirchen
ECL Kit	PerkinElmer, Köln
Glutathione-Sepharose	Pharmacia, Freiburg
Hyperfilm MP	Amersham, USA
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Polyprep Chromatography columns	Biorad, München
Protein A-Sepharose	Pharmacia, Freiburg
Protein G-Sepharose	Pharmacia, Freiburg

QIAquick Gel Extraction Kit (50)	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
Random-Primed DNA Labeling Kit	Pharmacia, Freiburg
RNeasy Mini Kit	Qiagen, Hilden
Sephadex G-50 (DNA Quality)	Pharmacia, Freiburg
Sterile filter 0.22 µm, cellulose acetate	Nalge Company, USA
Sterile filter 0.45 µm, cellulose acetate	Nalge Company, USA
Whatman 3MM	Whatman, USA

2.1.4 Growth factors and ligands

Amphiregulin	R&D Systems
EGF (murine)	Toyoba, Japan
LPA	Sigma, Karlsruhe
Sphingosine 1-phosphate	UBI, Lake Placid
Thrombin	Sigma, Taufkirchen

All other growth factors and ligands were purchased from Sigma.

2.1.5 Media and buffers

2.1.5.1 Media for E. coli bacteria

LB-Medium	1.0 % Tryptone
	0.5 % Yeast extract
	1.0 % NaCl
	pH 7.2
2xYT-Medium	1.6 % Tryptone
	1.0 % Yeast extract

1.0 % NaCl
pH 7.2

When necessary the following antibiotics were added to the media after autoclavation:

Ampicillin 100 µg/mL
Kanamycin 100 µg/mL
Chloramphenicol 30 µg/mL

LB-plates additionally contained 1.5 % agar.

2.1.5.2 Cell culture media

All cell culture media and additives were from Gibco (Eggenstein), fetal calf serum (FCS) was purchased from Sigma and Gibco.

Dulbecco's modified eagle medium (DMEM) supplemented with 4.5 mg/mL glucose, 2 mM L-glutamine, 1 mM sodium pyruvate.

Eagle's minimum essential medium (MEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate.

RPMI 1640 medium supplemented with 2 mM L-glutamine and 1 mM sodium pyruvayte.

Nutrient mixture F12 (HAM) supplemented with 2 mM L-glutamine and DMEM supplemented with 4.5 mg/mL glucose, 2 mM L-glutamine, 1 mM sodium pyruvate mixed 1:1.

Freeze medium containing 90 % heat-inactivated FCS and 10 % DMSO.

2.1.6 Stock solutions and buffers

BBS (2x)	50 mM BES 280 mM NaCl 1.5 mM Na_2HPO_4 pH 6.96 (NaOH)
CHAPS lysis buffer	50 mM HEPES, pH 7.5 150 mM NaCl 1 mM EDTA 10 % Glycerol 10 mM $Na_4P_2O_7$ 10 mM CHAPS 2 mM VaO_5 10 mM NaF 1 mM PMSF 100 µg/L Aprotinin
DNA loading buffer (6x)	0.25 % Bromphenol blue 0.25 % Xylencyanol 30.0 % Glycerol 100.0 mM EDTA, pH 8.0
HBS (2x)	46 mM HEPES, pH 7.5 274 mM NaCl 1.5 mM Na_2HPO_4 pH 7.0
HNTG (1x)	20 mM HEPES, pH 7.5 150 mM NaCl 0.1 % Triton X-100

	10 % Glycerin
	10 mM $Na_4P_2O_7$
Laemmli buffer (2x)	187.5 mM Tris/ HCl, pH 6.8
	6.0 % SDS
	30.0 % Glycerol
	0.01 % Bromphenol blue
	5.0 % ß-Mercaptoethanol
NET (1x)	150.0 mM NaCl
	5 mM EDTA
	50 mM Tris/HCl, pH 7.4
	0.05 % Triton X-100
PBS (1x)	13.7 mM NaCl
	2.7 mM KCl
	80.9 mM Na_2HPO_4
	1.5 mM KH_2PO_4, pH 7.4 (HCl)
RIPA lysis buffer	1 % NP40
	1 % Na-DOC
	0.1 % SDS
	150 mM NaCl
	10 mM $NaPO_4$, pH 7.2
	2 mM EDTA
	5 mM β-Glycerolphosphat
	4 mM VaO_5
	10 mM NaF
	1 mM PMSF
	100 µg/l Aprotinin
	1mM DTT

SD-Transblot	50.0 mM Tris/ HCl, pH 7.5 40.0 mM Glycine 20.0 % Methanol 0.004 % SDS
“Strip” buffer	62.5 mM Tris/ HCl, pH 6.8 2.0 % SDS 100 mM ß-Mercaptoethanol
TAE (10x)	400 mM Tris/Acetate 10 mM EDTA pH 8.0 (Acetic acid)
TE10/0.1	10.0 mM Tris/ HCl, pH 8.0 0.1 mM EDTA pH 8.0
Tris-Glycine-SDS (10x)	248.0 mM Tris/ HCl, pH 7.5 1918.0 mM Glycine 1.0 % SDS
Triton X-100 lysis buffer	50 mM HEPES, pH 7.5 150 mM NaCl 1 mM EDTA 10 % Glycerin 1 % Triton X-100 10 mM $Na_4P_2O_7$ 2 mM VaO_5 10 mM NaF 1 mM PMSF 100 µg/L Aprotinin

2.1.7 Bacteria strains (E. coli)

E. coli	Description	Origin/ Reference
DH5aF'	F'/*end*A1 *hsd*17 (*rk-mk-*) *sup*E44, *rec*A1, *gyr*A (Nal) *thi*-1 (*lac*ZYA-*arg*F)	Genentech, San Francisco, USA
Rosetta™(DE3)	codonoptimized F', ompT, $hsdS_B$, gal, dcm	Novagen

2.1.8 Cell lines

Cell Line	Description	Origin/ Reference
A498	Human kidney carcinoma cell line	Sugen Inc., CA, USA
COS7	African green monkey, SV 40-transformed kidney fibroblasts	Genentech, San Francisco, USA
HEK293	Human embryonic kidney fibroblasts, transformed with adenovirus Typ V DNA	ATCC CRL-1573
MDA-MB 231	Human mammary carcinoma	ATCC HTB-26
Phoenix E, A	Retrovirus producer cell lines for the generation of helper free ecotropic and amphotropic retroviruses, based on HEK-293	Nolan, Stanford, USA
SCC9	Human squamous cell carcinoma of the tongue	ATCC CRL-1629

All other cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA) and grown as recommended by the supplier.

2.1.9 Antibodies

The following antibodies were used in immunoprecipitation experiments or as primary antibodies in immunoblot analysis.

Antibody	Description/ Immunogen	Origin/ Reference
ADAM10	Rabbit, polyclonal/ AA732-748 of human ADAM10	Chemicon, Hofheim
ADAM17	Rabbit, polyclonal / AA807-823 of human ADAM17	Chemicon, Hofheim
Akt1/2	Rabbit, polyclonal/ AA 345-480 of human Akt1	Santa Cruz, USA
P-Akt/PKB	Rabbit, polyclonal/ phospho-Akt (Ser-473)	Cell Signaling Technologies
P-Akt/PKB	Rabbit, polyclonal/ phospho-Akt (Thr-308)	Cell Signaling Technologies
EGFR Sheep	polyclonal/ part of cytoplasmic domain of the human EGFR	UBI, Lake Placid
EGFR (108.1)	Mouse, monoclonal/ ectodomain of the human EGFR	(Daub et al., 1997)
ERK2 (C-14)	Rabbit, polyclonal/ peptide at C-terminus of rat ERK2	Santa Cruz, USA
ERK2 (K-23)	Rabbit, polyclonal/ peptide from sub-domain XI of rat ERK2	Santa Cruz, USA
P-ERK	Rabbit, polyclonal; recognizes phospho-p44/p42 (Thr-202/ Tyr-204) MAPK	NEB, Frankurt/M.
GFP	mouse, monoclonal; recognizes the green Fluorescent protein (GFP)	G. Gerisch
HA	Mouse, monoclonal; recognizes the influenza hemagglutinin epitope (HA)	Babco, CA, USA

SHC	Mouse, monoclonal	Santa Cruz, USA
SHC	Rabbit, polyclonal/ 220 AA at C-terminus of human SHC	(Daub et al., 1997)
P-Tyr (4G10)	Mouse, monoclonal; recognizes phospho- (3)-tyrosine residues	UBI, Lake Placid

For western blot secondary antibodies conjugated with horseradish peroxidase (HRP) were utilized.

Antibody	Dilution	Origin
Goat anti-mouse	1 : 10,000	Sigma, Karlsruhe
Goat anti-sheep	1 : 25,000	Dianova, Hamburg
Goat anti-rabbit	1 : 25,000	BioRad, München

2.1.10 Plasmids and oligonucleotides

2.1.10.1 Primary vectors

Vector	Description	Origin/ Reference
pcDNA3	Mammalian expression vector, Amp_r, CMV promotor, BGH poly A, high copy number plasmid	Invitrogen, USA
pLXSN	Expression vector for retroviral gene transfer, Amp_r, Neo_r, origin from pBR322, 5'-LTR and 3'-LTR from MoMuLV, SV40 promotor	Clontech, Palo Alto, USA
pLXSN-ESK	Modified pLXSN vector with multipe cloning site from pBluescript	J. Ruhe
pRK5	Expression vector, Amp_r, CMV promoter, SV 40 poly A, high copy number plasmid	Genentech, San Francisco, USA

pGEX5x-3	Bacterial expression vector for GST-fusion proteins, pBR322 origin, tac promo ter, Amp_r, *lac* I^q gene, protease recognition sites	Amersham, USA
pRetroSuper	Mammalian expression vector, also suitable for retroviral gene transfer, H1-RNA promoter for RNA transcription under control of PolIII, PGK promoter, $Puro_r$, Amp_r	(Brummelkamp et al., 2002)

2.1.10.2 Constructs

Vector	Description	Reference
pLXSN-ΔMPD	cDNA of human ADAM17 lacking the metalloprotease domain	S. Hart
pLXSN-T735A	cDNA of human ADAM17, T735A-mutation	S. Hart
pLXSN-Intra8	cDNA of human ADAM17 containing TMD and cytoplasmic domain	S. Hart
pLXSN-Δcyto	cDNA of human ADAM17 lacking aminoacids 695-825	S. Hart
PLXSN-WT	cDNA of human ADAM17	S. Hart
pcDNA3-HA-ßArr-V53D	cDNA of human ß-arrestin1 V53D-mutation	This study
pcDNA3-HA-ßArr-319	cDNA of human ß-arrestin1 lacking aminoacids 1-318	This study
pcDNA3-HA-ßArr-S412D	cDNA of human ß-arrestin1 S412D-mutation	This study
pcDNA3-HA-ßArr-WT	cDNA of human ß-arrestin1	This study

2.1.10.3 Important oligonucleotides

Sequence	Name
5’-ACAGATATCGCCACCATGTCCTACAAAGTGAAAGTGAAGCTG-3’	ß1ab319f
5’-ACATCTAGATCTGTTGTTGAGCTGTGGAGAGC-3’	Arrß1HArev
5’-ACAGATATCGCCACCARGGGCGACAAAGGGACGCG-3’	Arrß1f
5’-ACATCTAGATCTGTTGTTGAGCTGTGGATCGCCGGTACCATC-3’	ß1S412Dr
5’-GGCGCAGGTCAGCGTCACATAGTCTCTCCGCTCTTTGAGATACTC-3’	ß1V53Df

2.1.10.4 siRNA nucleotides

siRNA	Description/Sequence
GL2-sense	5’ CGUACGCGGAAUACUUCGAtt 3’
GL2-antisense	5’ UCGAAGUAUUCCGCGUACGtt 3’
ADAM9-sense	5’ AAUCACUGUGGAGACAUUUGCtt 3’
ADAM9-antisense	5’ GCAAAUGUCUCCACAGUGAUUtt 3’
ADAM9-sense	5’ AAACUUCCAGUGUGUAGAUGCtt 3’
ADAM9-antisense	5’ GCAUCUACACACUGGAAGUUUtt 3’
ADAM10-sense	5’ AAUGAAGAGGGACACUUCCCUtt 3’
ADAM10-antisense	5’ AGGGAAGUGUCCCUCUUCAUUtt 3’
ADAM10-sense	5’ AAGUUGCCUCCUCCUAAACCAtt 3’
ADAM10-antisense	5’ UGGUUUAGGAGGAGGCAACUUtt 3’
ADAM12-sense	5’ AACCUCGCUGCAAAGAAUGUGtt 3’
ADAM12-antisense	5’ CACAUUCUUUGCAGCGAGGUUtt 3’
ADAM12-sense	5’ AAGACCUUGAUACGACUGCUGtt 3’
ADAM12-antisense	5’ CAGCAGUCGUAUCAAGGUCUUtt 3’
ADAM15-sense	5’ AACUCCAUCUGUUCUCCUGACtt 3’
ADAM15-antisense	5’ GUCAGGAGAACAGAUGGAGUUtt 3’

ADAM15-sense	5’ AAAUUGCCAGCUGCGCCCGUCtt 3’
ADAM15-antisense	5’ GACGGGCGCAGCUGGCAAUUUtt 3’
ADAM17-sense	5’ AAGUUUGCUUGGCACACCUUtt 3’
ADAM17-antisense	5’ AAGGUGUGCCAAGCAAACUUtt 3’
ADAM17-sense	5’ AAGUAAGGCCCAGGAGUGUUtt 3’
ADAM17-antisense	5’ AACACUCCUGGGCCUUACUUtt 3’
ADAM17-sense	5’ AACAUAGAGCCACUUUGGAGAtt 3’
ADAM17-antisense	5’ UCUCCAAAGUGGCUCUAUGUUtt 3’
proAR-sense	5’ AACCACAAAUACCUGGCUAUAtt 3’
proAR-antisense	5’ UAUAGCCAGGUAUUUGUGGUUtt 3’
proAR-sense	5’ AAAUCCAUGUAAUGCAGAAtt 3’
proAR-antisense	5’ UUCUGCAUUACAUGGAUUUtt 3’
proHB-EGF-sense	5’ AAGUGAAGUUGGGCAUGACUAtt 3’
proHB-EGF-antisense	5’ UAGUCAUGCCCAACUUCACUUtt 3’
proHB-EGF-sense	5’ AUACAAGGACUUCUGCAUCCtt 3’
proHB-EGF-antisense	5’ GGAUGCAGAAGUCCUUGUAUtt 3’
proTGFα-sense	5’ AAAACACUGUGAGUGGUGCCGtt 3’
proTGFα-antisense	5’ CGGCACCACUCACAGUGUUUUtt 3’
proTGFα-sense	5’ AAGAAGCAGGCCAUCACCGCCtt 3’
proTGFα-antisense	5’ GGCGGUGAUGGCCUGCUUCUUtt 3’
ß-arrestin1-sense	5’ AAAGCCUUCUGCGCGGAGAAUtt 3’
ß-arrestin1-antisense	5’ AUUCUCCGCGCAGAAGGCUUUtt 3’
ß-arrestin1-sense	5’ GGCCUGCGGUGUGGAUUAUtt 3’
ß-arrestin1-antisense	5’ AUAAUCCACACCGCAGGCCtt 3’
ß-arrestin2-sense	5’ AAGGACCGCAAAGUGUUUGtt 3’
ß-arrestin2-antisense	5’ CAAACACUUUGCGGUCCUUtt 3’
ß-arrestin2-sense	5’ AGCCUUCUGUGCUGAGAACtt 3’
ß-arrestin2-antisense	5’ GUUCUCAGCACAGAAGGCUtt 3’
EVE1-sense	5’ CCUUCUGUAGCUCCCAAACtt 3’
EVE1-antisense	5’ GUUUGGGAGCUACAGAAGGtt 3’

SAP97-sense	5’ GAUAUCCAGGAACAUAAAUtt 3’
SAP97-antisense	5’ AUUUAUGUUCCUGGAUAUCtt 3’

2.2 Methods in molecular biology

2.2.1 Plasmid preparation for analytical purpose

Small amounts of plasmid DNA were prepared using Qiagen Mini-Kits (Qiagen, Hilden) according to the manufacturer’s recommendations.

2.2.2 Plasmid preparation in preparative scale

For transfection experiments of mammalian cells DNA of high quality was prepared using Qiagen Maxi-Kits (Qiagen, Hilden) according to the manufacturer’s recommendations.

2.2.3 Enzymatic manipulation of DNA

2.2.3.1 Digestion of DNA samples with restriction endonucleases

Restriction endonuclease cleavage was accomplished by incubating the enzyme(s) with the DNA in appropriate reaction conditions. The amounts of enzyme and DNA, the buffer and ionic concentrations, and the temperature and duration of the reaction were adjusted to the specific application according to the manufacturer's recommendations.

2.2.3.2 Dephosphorylation of 5'-termini with calf intestine alkaline phosphatase (CIAP)

Dephosphorylation of 5'-termini of vector DNA was performed in order to prevent self-ligation of vector termini. CIAP catalyzes the hydrolysis of 5'-phosphate residues from DNA, RNA, and ribo- and deoxyribonucleoside triphosphates. The dephosphorylated products possess 5'-hydroxyl termini.

For dephosphorylation 1-20 pmol DNA termini were dissolved in 44 µL deionized water, 5 µL 10x CIAP buffer (500 mM Tris/HCl pH 8.0, 1 mM EDTA pH 8.5) and 1 µL CIAP (1 U/µL). The reaction was incubated for 30 min at 37° C and stopped by heating at 85° C for 15 minutes.

2.2.3.3 DNA insert ligation into vector DNA

T4 DNA Ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA. T4 DNA Ligase thereby joins doublestranded DNA with cohesive or blunt termini.

The digested, dephosphorylated and purified vector DNA (200 ng), the foreign DNA insert, 1 µL 10x T4 DNA Ligase buffer (0.66 M Tris/HCl pH 7.5, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP) and 1 µL T4 DNA Ligase (2 U for sticky ends and 4 U for blunt ends) were added to a total volume of 10 µL. The reaction was incubated at 15° C overnight. T4 DNA Ligase was inactivated by heating the reaction mixture at 65° C for 10 min. The resulting ligation reaction mixture was directly used for bacterial transformation.

2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying, and purifying 0.5- to 25 kb DNA fragments. 1-2 % horizontal agarose gels with 1x TAE electrophoresis buffer were used for separation. The voltage was typically set to 1-10 V/cm gel length. Gels were stained by covering the gel in a dilute solution of ethidium bromide (0.5 µg/mL in water) and gently agitating for 30 min and destained by shaking in water for an additional 30 min.

2.2.4.1 Isolation of DNA fragments using low melting temperature agarose gels

Following preparative gel electrophoresis using low melting temperature agarose, the gel slice containing the band of interest was removed from the gel. This agarose slice was then melted and subjected to isolation using the QIAquick Gel Extraction Kit (Qiagen, Hilden).

2.2.5 Introduction of plasmid DNA into E.coli cells

2.2.5.1 Preparation of competent E. coli bacteria

Competent cells were made according to the procedure described by (Chung and Miller, 1988). For long-term storage competent cells were directly frozen at -70° C. Transformation frequency ranged between 10^6 and 10^7 colonies/µg DNA.

2.2.5.2 Transformation of competent E. coli bacteria

100 µL competent cells were added to 10 µL ligation mix and 20 µL 5x KCM (500 mM KCl, 150 mM $CaCl_2$, 250 mM $MgCl_2$) in 70 µL H_2O and incubated on ice for 20 min. Upon incubation at room temperature for 10 min, 1 mL LB medium was added and incubated for 1 h at 37° C with mild shaking to allow expression of the antibiotic resistance gene. Transformants were selected on appropriate LB-plates.

2.2.6 Enzymatic amplification of DNA by polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA (Mullis and Faloona, 1987). A multitude of applications have been developed including direct cloning from cDNA, *in vitro* mutagenesis and engineering of DNA, genetic fingerprinting of forensic samples, assays for the presence of infectious agents and analysis of allelic sequence variations. For long and accurate cDNA amplification LATaq™ polymerase (TaKaRa) and *Pfu* DNA polymerase (Fermentas) were used according to the manufacturer's recommendations:

0.5 µL template cDNA
2.0 µL "sense" oligonucleotide, 10 pmol/µL
2.0 µL "antisense" oligonucleotide, 10 pmol/µL
5.0 µL 10x LA PCR buffer II (without MgCl2)
5.0 µL 25 mM MgCl2
8.0 µL dNTP-Mix, 2.5 mM each

0.5 µL LA-Taq™ (5 U/µL)
ad 50 µL H2O

or 0.5 µL template cDNA
2.0 µL "sense" oligonucleotide, 10 pmol/µL
2.0 µL "antisense" oligonucleotide, 10 pmol/µL
5.0 µL 10x *Pfu*-buffer with $MgSO_4$
4.0 µL dNTP-Mix, 2.5 mM each
0.5 µL *Pfu* DNA Polymerase (2.5 U/µl)
ad 50 µL H2O

PCR reactions were performed in an automated thermal cycler („Progene“, Techne). The following standard protocol was adjusted to the specific application:

first denaturation:	3 min 94°C
amplification 20-35 cycles:	1 min 94°C (denatura tion)
	1 min 50-65°C (hybridization)
	1 min respective
	2 min/ kb product 72°C (extension)
final extension:	7 min 72°C

10 µL from each reaction were electrophoresed on an agarose gel appropriate for the PCR product size expected. PCR products were subjected to isolation using the PCR purification kit (Qiagen).

2.2.7 RT-PCR analysis

Expression of molecules of interest was confirmed by RT-PCR analysis. RNA isolated using RNeasy Mini Kit (Qiagen, Hilden) was reverse transcribed using AMV Reverse Transcriptase (Roche, Mannheim). 2-10 µg RNA and 1 µL random primer in a volume of 10 µL were incubated for 2 min at 68°C, followed by 10 min RT. After

addition of 0.5 µL RNase inhibitor, 4 µL 5x AMV RT buffer and 4 µL dNTPs (2.5 mM each) and 1µL AMV RT the volume was adjusted to 20 µL.
The reaction mix was incubated at 42°C for 1h.
PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ) and 1µL RT-PCR products were used for PCR amplification according to the manufacturer's recommendations. PCR products were subjected to electrophoresis on 1.5-2% agarose gels and DNA was visualized by ethidium bromide staining.

2.2.8 DNA sequencing

DNA sequencing was performed according to the "Big Dye Terminator Cycle Sequencing Protocol" (ABI). The following mix was subjected to a sequencing-PCR run:

0.5 µg DNA of interest
10.0 pmol oligonucleotide
4.0 µL Terminator Ready Reaction Mix
ad 20 µL H_2O
25 cycles: 30 sec 94°C
15 sec 45-60°C (annealing temperature)
4 min 60°C

The sequencing products were purified by sodium acetate/ EtOH precipitation, dissolved in 20 µL template suppression reagent, denatured for 2 min at 90°C and analyzed on a 310-Genetic Analyzer (ABI Prism).

2.3 Methods in mammalian cell culture

2.3.1 General cell culture techniques

Cell lines were grown in a humidified 93 % air, 7 % CO_2 incubator (Heraeus, B5060 Ek/CO_2) at 37° C and routinely assayed for mycoplasma contamination using a bisbenzimide staining kit (Sigma, Karlsruhe). Before seeding, cells were counted with a Coulter Counter (Coulter Electronics). Cells were cultured in the medium recommended by the manufacturer.

The following cell lines required special media additives:

SCC9 Dulbecco's modified Eagle's medium (DMEM)/ Ham's F12 medium 1:1 containing 0.5 mM sodium pyruvate, 2 mM L-glutamine, 400 mg/L Hydrocortisone and 10 % FCS.

MDA-MB-231 RPMI 1640 containing 10 % FCS, 2 mM L-glutamine and 2.67 U/L Insulin.

HEK-293, Cos-7 Dulbecco's modified Eagle medium (DMEM) containing 10 % FCS, 1 mM sodium pyruvate, 2 mM L-glutamine and 10µg/mL Insulin.

2.3.2 Transfection of cultured cell lines

2.3.2.1 Transfection of cells with calcium phosphate

HEK-293T cells in six-well dishes were transfected transiently at about 70% confluency with a total of 2 µg DNA by using a modified calcium phosphate precipitation method as described by (Chen and Okayama, 1987). In this protocol, a calcium phosphate-DNA complex is formed gradually in the medium during incubation with cells.

The transfection mix of DNA and $CaCl_2$ in water was prepared as follows:

Dish	6-well	6 cm	10 cm
Area	10 cm^2	21 cm^2	57 cm^2
Volume of medium	1 mL	2 ml	4 ml
DNA in H_2Obidest	2 µg in 90 µL	5 µg in 180 µL	10 µg in 360 µL
2.5 M $CaCl_2$	10 µL	20 µL	40 µL
2 x BBS (pH 6.96)	100 µL	200 µL	400 µL
Total volume	200 µL	400 µL	800 µL

To initiate the precipitation reaction the indicated volume of 2x BBS was added and mixed by vortexing. The reaction was incubated for 10 min at room temperature before being added to each well. Plates were placed in a humidified incubator at 3% CO_2 and 37℃ overnight. One day after transfection, cells were serum-starved for 24 hours in standard cell culture medium without FCS. Transfection efficiency was determined by LacZ staining after transfection of a LacZ-containing expression plasmid. For transfection of Phoenix cells HBS was used instead of BBS.

2.3.2.2 Transfection of cells with Lipofectamine®

COS7 cells were transiently transfected using Lipofectamine® (Gibco-BRL) essentially as described (Daub et al., 1997). For transfections in 6-well dishes, 90 µL of serum-free medium containing 10 µL of Lipofectamine and 1.5 µg of total plasmid DNA in 100 µL serum-free medium were mixed. After 20 min the transfection mixture was added to 800 µL serum-free medium per well.

After 4 h the transfection mixture was replaced by normal growth medium and 20 h later, cells were washed and cultured for a further 24 h in serum-free medium until lysis.

2.3.2.3 Transfection of cells with Lipofectamine 2000®

SCC9 cells were transiently transfected using Lipofectamine 2000® (Gibco-BRL) essentially according to the manufacturer's recommendations. For transfections in 6-well dishes, 2 µg of total plasmid DNA were diluted into 250 µL of serum-free

medium. 5 µL Lipofectamine 2000® (Gibco-BRL) were also diluted into 250µL of serum-free medium and allowed to incubate at room temperature for 5-10 min. After mixing of DNA and transfection reagent, the mixture was added to 2 mL of antibiotic-free, but serum containing medium per well. After 4 h the transfection mixture was removed and fresh media containing serum was added. After 20 h, cells were washed and cultured for a further 48 h in serum-free medium until lysis.

2.3.2.4 Transfection of siRNAs

Transfection of 21-nucleotide siRNA duplexes (Dharmacon Research, Lafayette, CO, USA) for targeting endogenous genes was carried out using Oligofectamine (Invitrogen) and 4.2 µg siRNA duplex per 6-well plate as described by (Elbashir et al., 2001).

24 h after transfection, cells were serum-starved and assayed 3 d after transfection. Highest efficiencies in silencing target genes were obtained by using mixtures of siRNA duplexes targeting different regions of the gene of interest.

2.3.3 Retroviral gene transfer in cell lines

The ecotropic packaging cell line Phoenix (Nolan, Stanford, USA) was transfected with pLXSN retroviral expression plasmids (Clontech, Palo Alto, CA) encoding genes of interest by the calcium phosphate/ chloroquine method as described previously (Kinsella and Nolan, 1996). 24 h after transfection the viral supernatant was collected and used to infect target cells (5×10^4 cells/6-well plate).

4 to 12 h later, retroviral supernatant was replaced with fresh medium. Selection for stable expression was started 48 h post infection with the respective antibiotic.

2.4 Protein analytical methods

2.4.1 Lysis of cells with triton X-100

Prior to lysis, cells grown to 80% confluency were treated with inhibitors and agonists as indicated in the figure legends. Cells were washed with cold PBS and then lysed for 10 min on ice in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/mL aprotinin. Lysates were precleared by centrifugation at 13000 rpm for 10 min at 4℃.

2.4.2 Determination of protein concentration in cell lysates

The „Micro BCA Protein Assay Kit" (Pierce, Sankt Augustin) was used according to the manufacturer's recommendations. For samples containing glycerol the BioRad Protein Assay (BioRad Laboratories GMBH, Munich) was used according to the manufacturer's recommendations.

2.4.3 Immunoprecipitation

An equal volume of HNTG buffer was added to the precleared cell lysates that have been adjusted for equal protein concentration. Proteins of interest were immunoprecipitated using the respective antibodies and 30 µL of protein A-Sepharose for 4 h at 4°C.

2.4.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted as described previously (Sambrook, 1990). The following proteins were used as molecular weight standards:

Protein	MW (kD)	Protein	MW (kD)
Myosin	205.0	Ovalbumin	42.7
ß-Galactosidase	116.25	Carboanhydrase	29.0
Phosphorylase b	97.4	Trypsin-Inhibitor	21.5

BSA	66.2	Lysozym	14.4

The detection of low-molecular weight proteins by a Tricine-SDS Gel electrophoresis was essentially performed as described by (Schagger and von Jagow, 1987). This method has a good resolution for small protein although utilizing 10 % agarose gels.

2.4.5 Transfer of proteins on nitrocellulose membranes

For immunoblot analysis proteins were transferred to nitrocellulose membranes (Gershoni and Palade, 1982) for 2 h at 0.8 mA/cm^2 using a "Semidry"-Blot device in the presence of Transblot-SD buffer. Following transfer proteins were stained with Ponceau S (2 g/L in 2% TCA) in order to visualize and mark standard protein bands. The membrane was destained in water.

2.4.6 Immunoblot detection

After electroblotting the transferred proteins are bound to the surface of the nitrocellulose membrane, providing access for reaction with immunodetection reagents. Remaining binding sites were blocked by immersing the membrane in 1x NET, 0.25 % gelatine for at least 4 h. The membrane was then probed with primary antibody (typically overnight at 4°C). Antibodies were diluted 1:500 to 1:2000 in NET, 0.25 % gelatin. The membrane was washed 3x 20 min in 1x NET, 0.25 % gelatin, incubated for 1 h with secondary antibody and washed again as before. Antibody-antigen complexes were identified using horseradish peroxidase coupled to the secondary anti-IgG antibody. Luminescent substrates were used to visualize peroxidase activity. Signals were detected with X-ray films. Membranes were stripped of bound antibody by shaking in strip-buffer for 1 h at 50°C.

Stripped membranes were blocked and reprobed with different primary antibody to confirm equal protein loading.

2.5 Biochemical and cell biological assays

2.5.1 Stimulation of cells

Cells were seeded in cell culture dishes of appropriate size and grown overnight to about 80% confluence. After serum-starvation for 24 or 48 h cells were treated with inhibitors and agonists as indicated in the Figure legends, washed with cold PBS and then lysed for 10 min on ice.

2.5.2 Erk 1/2 and Akt/PKB phosphorylation

For determination of Erk 1/2 and Akt phosphorylation, approximately 20 µg of whole cell lysate protein/lane was resolved by SDS-PAGE and immunoblotted using rabbit polyclonal phospho-specific Erk/MAPK antibody. Akt phosphorylation was detected by protein immunoblotting using rabbit polyclonal anti-phospho-Akt antibody. Quantification of Erk 1/2 was performed using the Luminescent Image Analyis System (Fuji). After quantification of Erk 1/2 phosphorylation, membranes were stripped of immunoglobulin and reprobed using rabbit polyclonal anti-Erk 1/2 or rabbit polyclonal anti-Akt antibody to confirm equal protein loading.

2.6 Statistical analysis

Student's t-test was used to compare data between two groups. Values are expressed as mean ± standard deviation (s. d.) of at least triplicate samples. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Testing of monoclonal ADAM17-antibodies for blocking

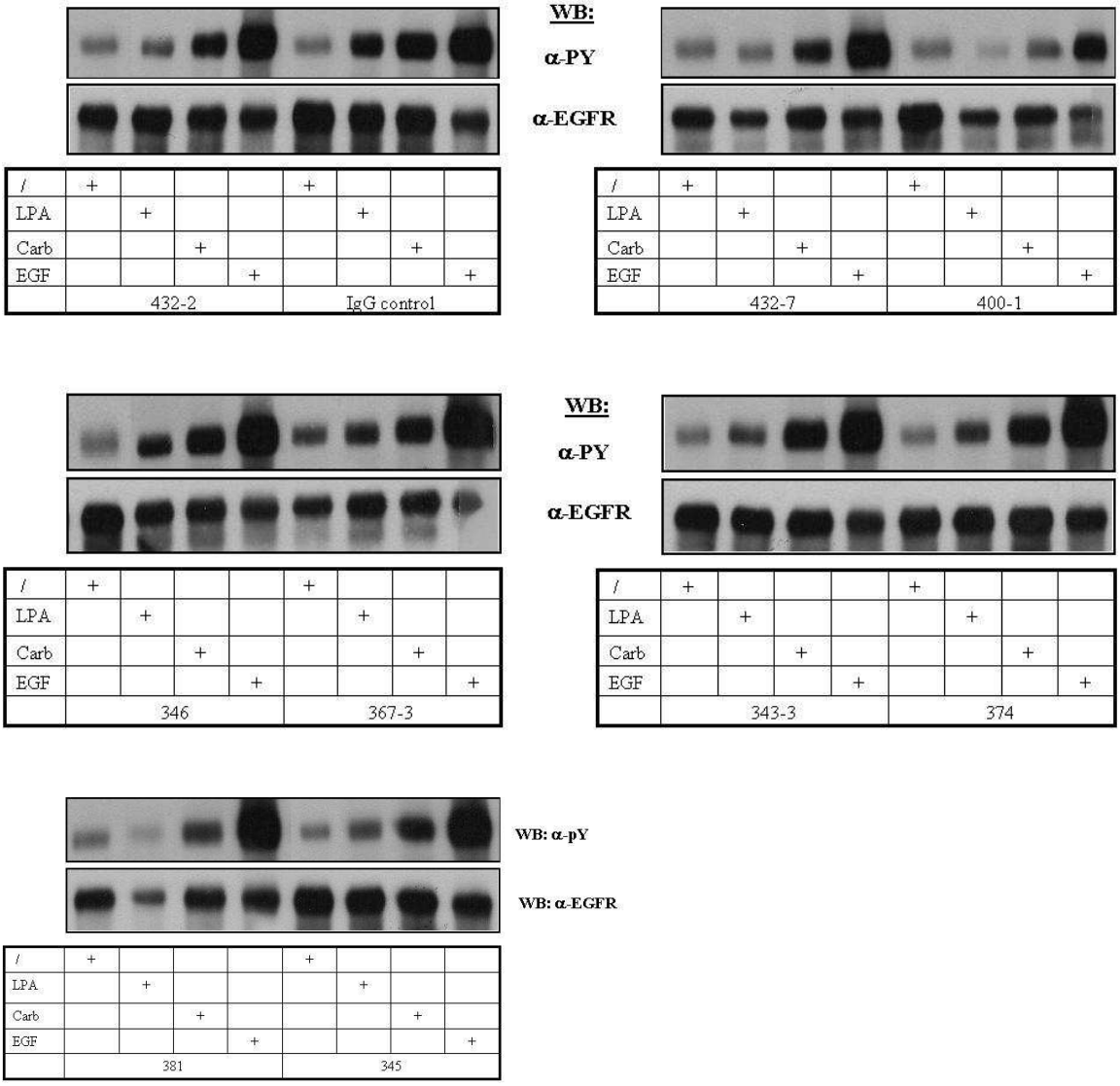
Metalloproteases both of the MMP- and the ADAM- family are widely expressed in different cancer types including breast, lung and head and neck (Izumi et al. 1998; Asakura et al. 2002; Yan et al. 2002). Especially TACE/ADAM17, a prominent member of the metalloprotease-disintegrin family, was shown to be involved in shedding of EGF-like growth factors like for example proAmphiregulin (proAR) (Peschon et al. 1998; Sunnaborg et al. 2002). Tissue inhibitors of metalloprotesases are endogenous inhibitors that tighly regulate the proteolytic activity of MMP and, at least some of them also of ADAMs. ADAM17 is one of the Metalloproteases inhibited by TIMP-3 (Amour et al. 1998). To clearify the involvement of ADAM17 in the TMPS pathway of EGFR-transactivation, blocking antibodies against the metalloprotease domain of TACE were raised in the departement as described before (Hart 2004). Preliminary tests of some of these antibodies showed blocking potential in the TMPS pathway. However to elucidate their characteristics, they were subjected to extensive investigations. In order to test their blocking activity SCC9 cells, a well characterised model for EGFR-transactivation, were preincubated with these antibodies, stimulated with GPCR-ligands and phospho-EGFR levels were measured. To cover a broad range of conditions a variety of different antibody concentrations and incubation times were investigated.

3.1.1 Concentration-dependent blocking activity of ADAM17-antibodies

In order to determine at which concentrations the antibodies show inhibitory effects, cells were preincubated with different amounts of antibodies (2µg and 10µg per ml)

for 30 minutes prior to stimulation with Lysophosphatidic acid (LPA), Carbachol (Carb), Thrombin (Thr) or Epidermal Growth Factor (EGF) for 10 minutes.
After that, cells where lysed and EGFR was immunoprecipitated. Following PAGE-analysis, gels were blotted, membranes were tested for phospho-tyrosine and reprobed for total EGFR. Figure 13A shows nine different TACE antibodies and an IgG-control antibody used at a concentration of 2µg/ml. In Figure 13B eight of these nine were tested at 10µg/ml.

A



B

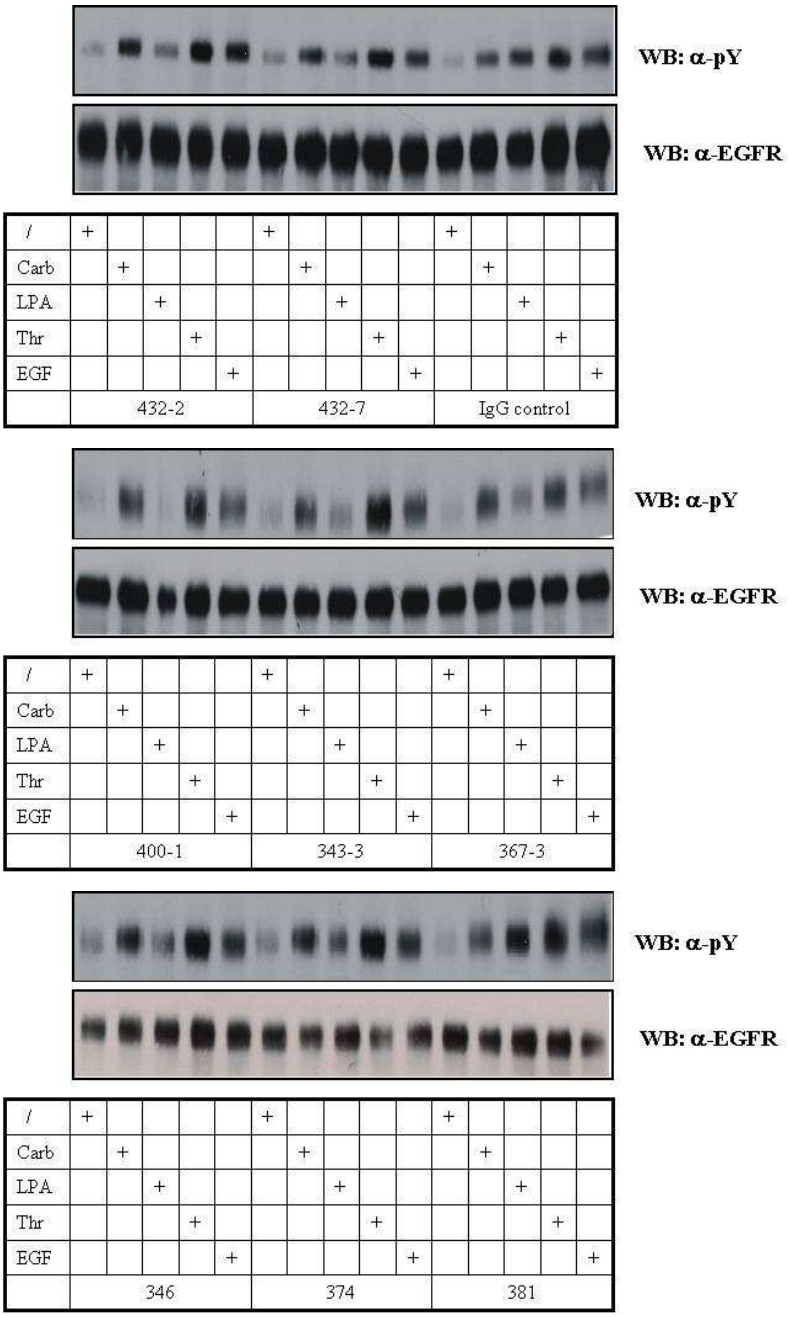


Figure 13. Concentration-dependent blocking activity of ADAM17 antibodies

A) SCC9 cells were preincubated for 30 minutes with 2µg/ml of the indicated TACE antibodies. After that cells were stimulated with the indicated GPCR-ligands or EGF. Lysates were immunoprecipitated for EGF receptor, blotted for phosphotyrosine and reprobed for total EGFR.

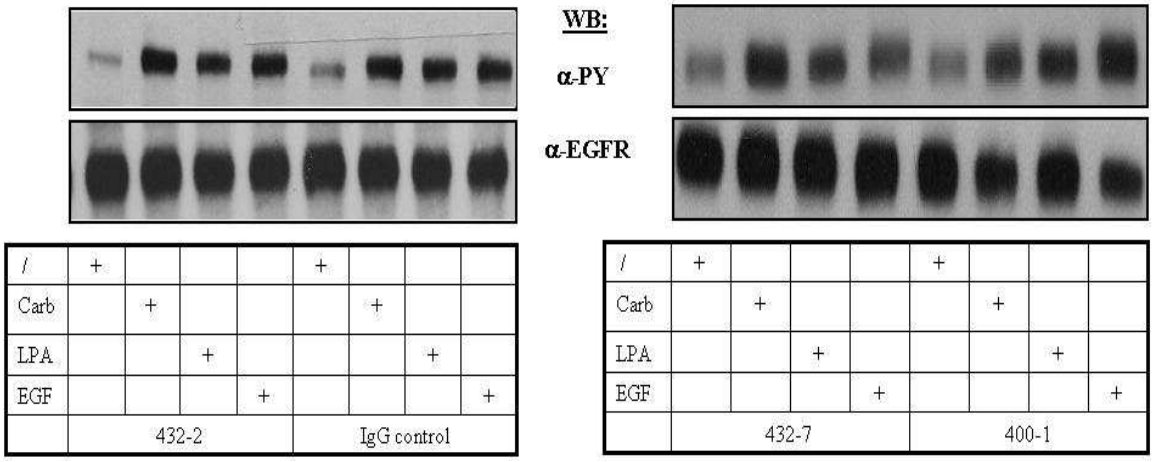
B) Cells were preincubated for 30 minutes with 10µg/ml of the different antibodies and afterwards treated as described in A

Antibodies 432-2, 432-7 and 400-1 showed slight reduction of EGFR-phosphorylation, mainly when cells where treated with LPA at a concentration of 2µg/ml. At 10µg/ml 432-2, 432-7, 400-1 and 343-3 showed inhibition of EGFR-phosphorylation again essentially after LPA-stimulation. None of the other antibodies tested showed an effect at the doses tested.

3.1.2 Time-dependent blocking activity of ADAM17-antibodies

As some of the antibodies showed effects in dose titration, the next investigation was to clearify the kinetics needed for their blocking activity. Therefore SCC9 cells were preincubated with 10µg of antibodies per milliliter for different periods of time (10 to 90 minutes), before being stimulated for 10 minutes and finally being lysed. Lysates were then treated in the same manner as described under 3.1.1.

A



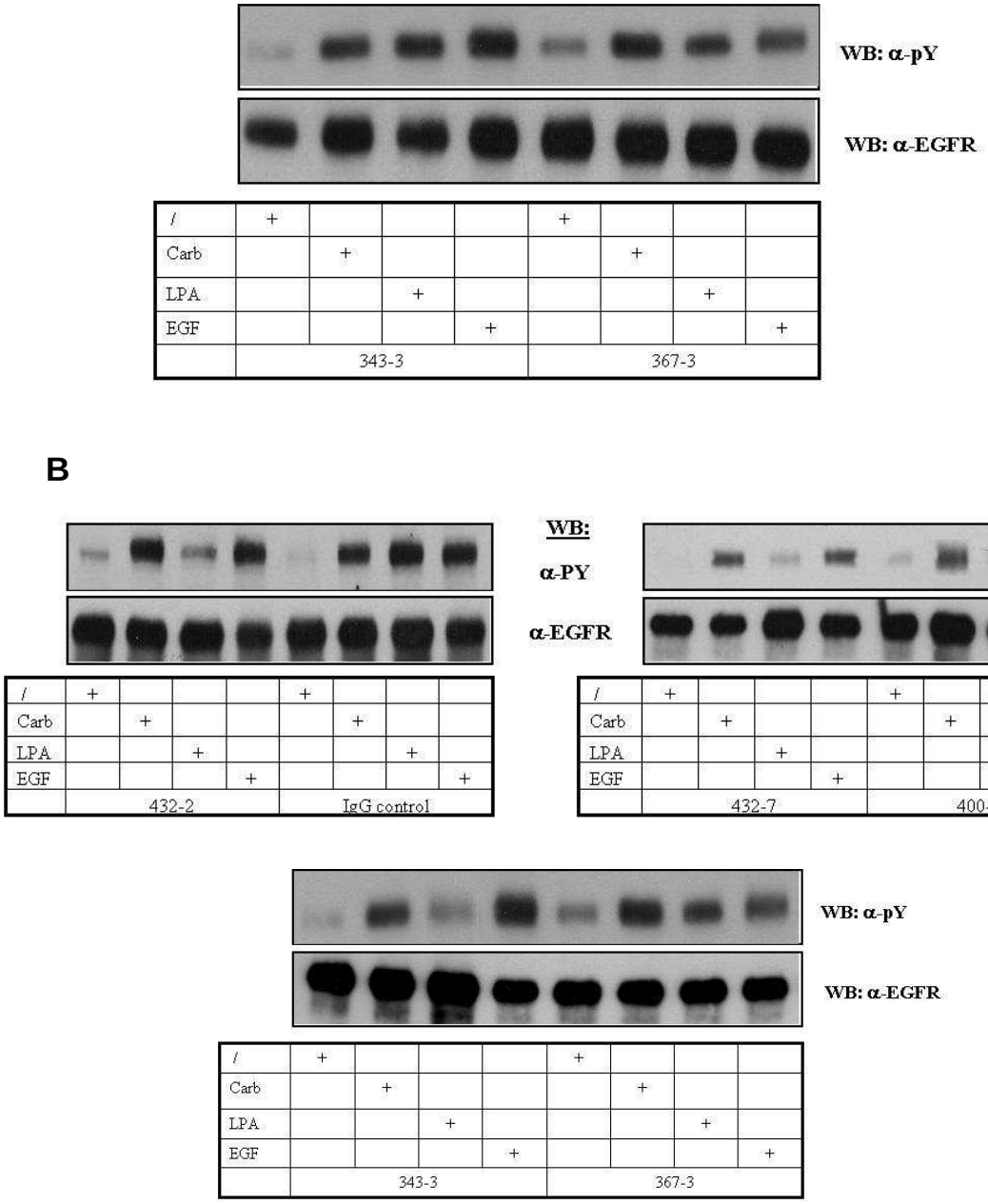


Figure 14. Kinetic analysis of ADAM17 blocking antibodies

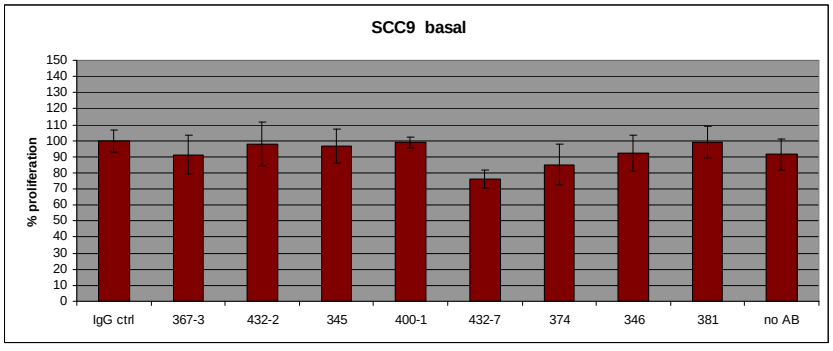
A) SCC9 cells were preincubated for 10 minutes with 10µg/ml of the indicated TACE antibodies. After stimulation with the indicated GPCR-ligands or EGF cells were lysed, lysates were immunoprecipitated for EGF receptor, blotted for phosphotyrosine and reprobed for total EGFR.

B) Cells were preincubated with 10µg/ml of the different antibodies for 90 minutes and afterwords treated as described in A

When cells were preincubated with different ADAM17 antibodies for 10 minutes, none of the tested ones showed any reduction of EGFR-phosphorylation. Treating cells with the antibodies for longer time periods revealed their ability to interfere in EGFR-transactivation. Cells preincubated for 90 minutes with 10µg/ml of TACE antibodies 432-2, 432-7, 400-1 and 343-3 had decreased EGFR-phosphorylation levels after GPCR-ligand stimulation. None of the antibodies could however completely block EGFR-transactivation in SCC9 cells probably due to the fact that ADAM17 is not the only metalloprotease responsible for proligand shedding in this cell line.

3.1.3 ADAM17 inhibition results in reduced proliferation activity

After having shown that TACE antibodies could reduce phospho-EGFR levels in some cases it is therefore likely, that they could also affect downstream signalling of the EGF-receptor. A well characterized example of an EGFR-downstream pathway results in ERK1/2 activation and represents a prototypical MAPK-pathway. The activation cascade links activated RTKs via RAF and MEK to ERK1/2. Since the latter is generally known to mediate proliferation in a variety of cells, the question was addressed, whether the antibodies could reduce proliferation in cancer cell lines. Because showing an effect on selected signalling molecules does not necessarily lead to biological effects. Proliferation was measured 72 hours after cells had been treated with ADAM17 antibodies, stimulated with LPA, Sphingosine-1-phosphate (S1P) and Thrombin or left untreated for analysing basal conditions.



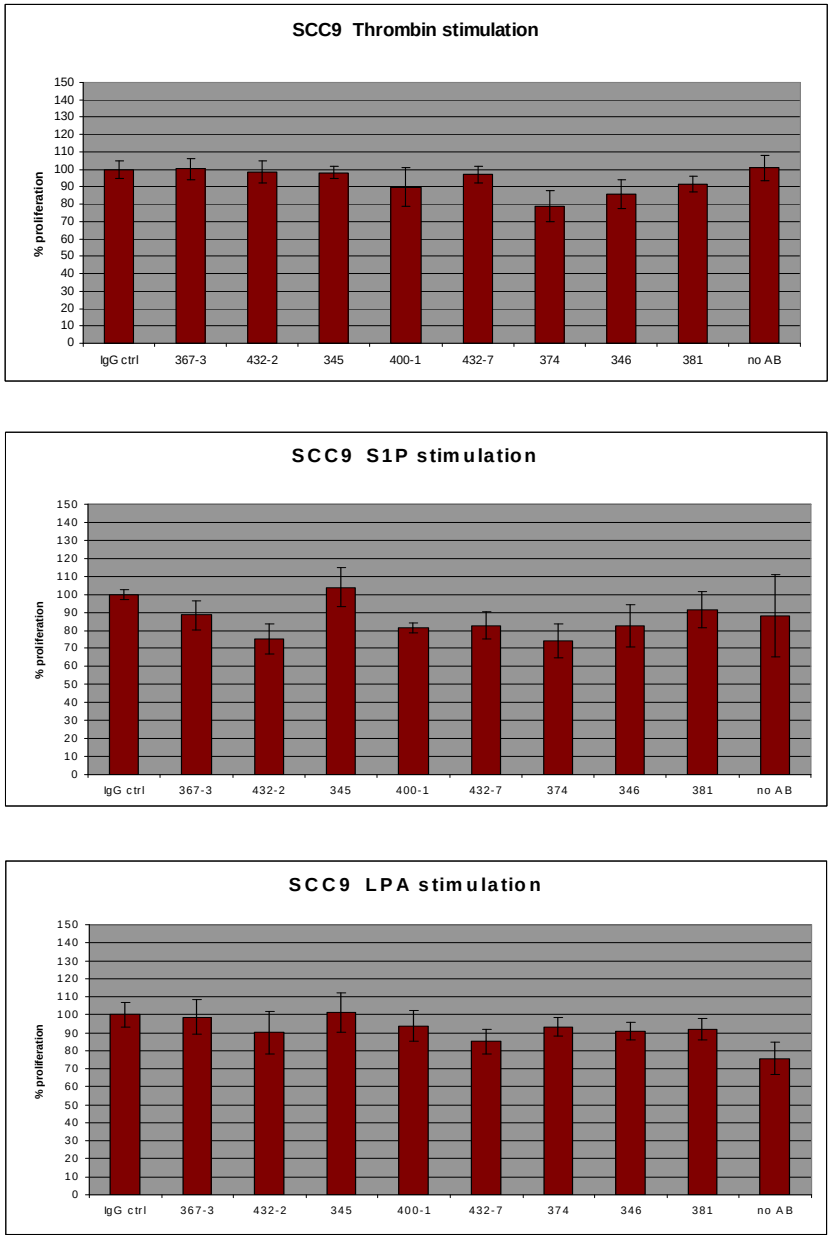


Figure 15. ADAM17-antibodies reduce proliferation activity in SCC9 cells

SCC9 cells were seeded in 96-wells in quadruplicates. 24h later, cells were incubated with 10µg/ml of the indicated TACE antibodies or IgG control and stimulated as indicated. After 72h proliferation was measured.

Under S1P-stimulated conditions, six of the antibodies showed inhibition of proliferation that was greater than 10 percent. Three antibodies showed reduction after Thrombin and one after LPA-stimulation. Two of the antibodies tested were able to reduce proliferation under basal conditions. Candidates 432-7 and 374 showed the best activity with reducing proliferation in three out of four conditions tested. In Table 2 the activities of the different TACE-blocking antibodies are summarized. Inhibition activity is shown in percent reduction and only activities of more than ten percent are indicated.

		Stimulation			
		/	Thr	S1P	LPA
TACE-Antibody	367-3			12%	
	432-2			25%	
	345				
	400-1		10%	18%	
	432-7	23%		17%	15%
	374	15%	21%	25%	
	346		14%	17%	
	381				

Table 2. Inhibition of proliferation activity in SCC9 cells by percentage
Overview of inhibition activity of tested antibodies in proliferation assay.
Only inhibitions of more than 10% are indicated.

3.2 The Role of beta Arrestin1 in EGFR-Transactivation

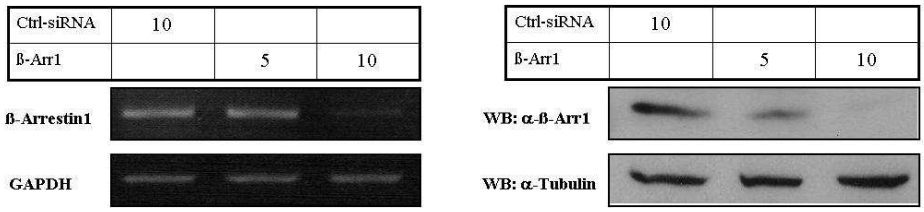
ß-Arrestins are versatile adapter proteins forming complexes with G-protein-coupled receptors (GPCRs) after these were phosphorylated by G-protein-coupled receptor

kinases (GRKs) upon agonist binding. Their classical major role is being involved in desensitization and GPCR sequestration, leading to the termination of G protein activation. ß-Arrestin binding to GPCRs both uncouples receptors from heterotrimeric G proteins and targets them to clathrin coated pits for endocytosis. However growing support indicates that ß-Arrestins also function as GPCR signal transducers by forming complexes with several signaling proteins. By recruiting these proteins directly to the GPCR, ß-arrestins can confer distinct enzymatic activities upon the receptor, which may lead to signals that are important for the regulation of cellular growth or differentiation. If ß-Arrestins are involved in transactivation of the EGF receptor and if so, what exactly their role in the TMPS pathway is is not clear yet.

3.2.1 siRNA-Downregulation of beta Arrestin1 blocks EGFR-Transactivation

To address this question RNA interference was used to knock down endogenous expression of ß-Arrestin1. First, Head and Neck squamous cell carcinoma cell line SCC9 was transfected with different amounts (5µg and 10µg per well of a 6-well plate) of ß-arrestin1 siRNA and a control siRNA targeting firefly luciferase, not known to have any targets in mammalian cells. Knockdown specificity and efficiency was determined both on RNA-level via RT-PCR and on protein-level via Western blotting.

A



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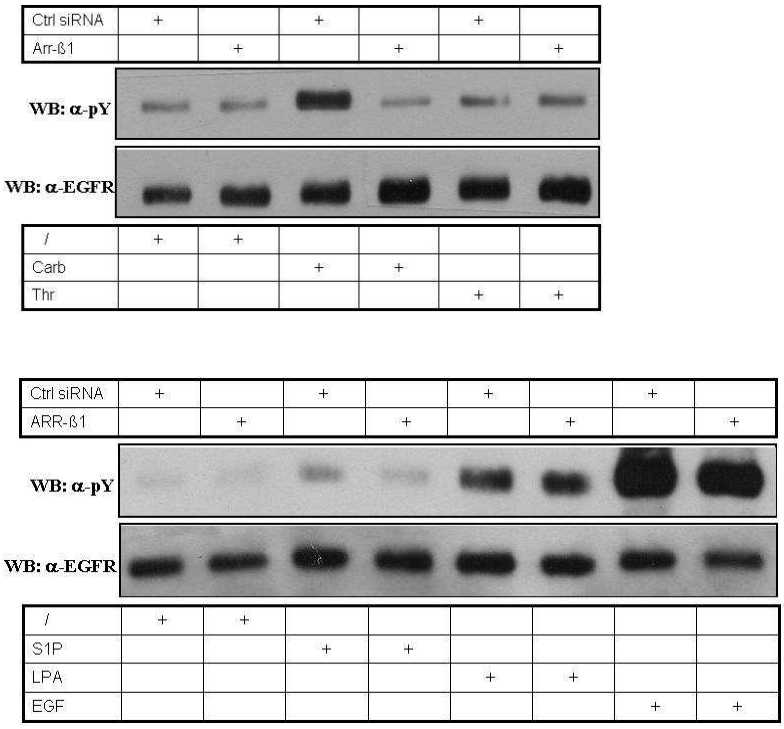


Figure 16. ß-Arrestin1 knockdown in SCC9 cells

SCC9 cells were transfected with ß-Arrestin1 (ß-Arr1) or control (Ctrl) siRNA.

A) Gene expression was analysed by RT-PCR (left panel) or immunoblotting with ß-Arrestin1 antibody (right panel).

B) Following siRNA transfection cells were serum-starved, stimulated with Carbachol (Carb), Thrombin (Thr), Sphingosine-1-phosphate (S1P), Lysophosphatydic acid (LPA) and Epidermal growth factor (EGF) for 10 min and assayed for EGFR tyrosine phosphorylation.

Following siRNA transfection, cells were serum-starved and stimulated with GPCR-ligands in order to activate EGFR-transactivation. SiRNA-directed inhibition of ß-Arrestin1 resulted in reduction of EGFR-phosphorylation to basal levels, after cells had been treated with Carbachol and Spinghosine-1-phosphate.

EGF-treated cells however showed no difference upon ß-Arrestin1 knockdown, supporting the view that ß-Arrestin1 is involved in transactivation of the EGFR.

3.2.2 S1P stimulation of SCC9 cells leads to reduced level of phospho-ß-Arrestin

To further investigate the role of ß-Arrestin1 in the TMPS pathway we tested the effects of GPCR-ligands on ß-Arrestin protein in an EGFR-transactivation model system. SCC9 cells were serum starved and stimulated with GPCR agonists to activate EGFR-transactivation. Lysates were then analysed for ß-Arrestin1 phosphorylation and total ß-Arrestin1 protein levels.

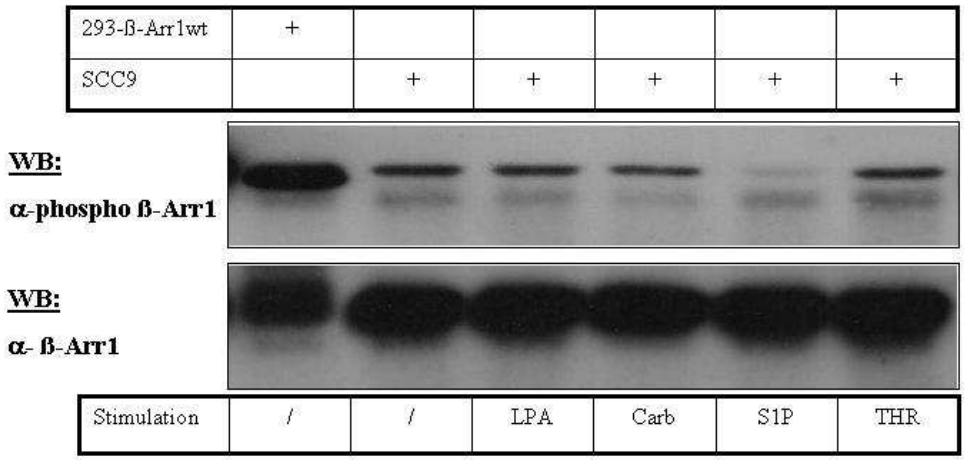


Figure 17. Reduction of ß-Arrestin1 phosphorylation after S1P stimulation

SCC9 cells were stimulated with Lysophosphatydic acid (LPA), Carbachol (Carb), Sphingosine-1-phosphate (S1P) and Thrombin (Thr) for 10 min and assayed for ß-Arrestin1 phosphorylation (upper panel) and total ß-Arrestin1 (lower panel). HEK293 cells transiently transfected with ß-Arrestin1 wild type (293-ß-Arr1wt) served as positive control.

Stimulation of SCC9 cells with Sphingosine-1-phosphate resulted in a complete abrogation of ß-Arrestin1 phosphorylation. In contrast, neither LPA, Carbachol nor Thrombin could reduce the phosphorylation of this protein. As a positive control, lysates of HEK293 cells, transiently transfected with full length ß-Arrestin1, were likewise loaded on the gel. They showed increased phosphorylation compared to the other lanes, probably due to the fact that ß-Arrestin1 is overexpressed in this cell line.

3.2.3 Dominant-negative beta Arrestin 1 mutants

Overexpressing both proteins and their dominant-negative versions served as an additional approach to understand the function of ß-Arrestin in EGFR-transactivation. Therefore mutants with different binding characteristics to phosphorylated GPCRs and clathrin where cloned into pcDNA3-HA-vector. The first mutant, ß-Arrestin1 S412D is a point mutation where Serine 412 is substituted by Aspartic Acid. This dominant-negative mutant is described being capable of binding to phosphorylated GPCRs with unchanged affinity but has lost its ability to bind to clathrin (Claing et al. 2002).

The second mutant is a truncated version containing only the last 99 carboxy-terminal amino acids of ß-Arrestin1. It has complete opposite characteristics than the first one, resulting in binding to clathrin but not being able to bind GPCRs. Therefore it acts as a dominant negative inhibitor of clathrin-mediated GPCR endocytosis. To compare these mutants to the wild type form, full length ß-Arrestin1 was also cloned into pcDNA3-HA-vector.

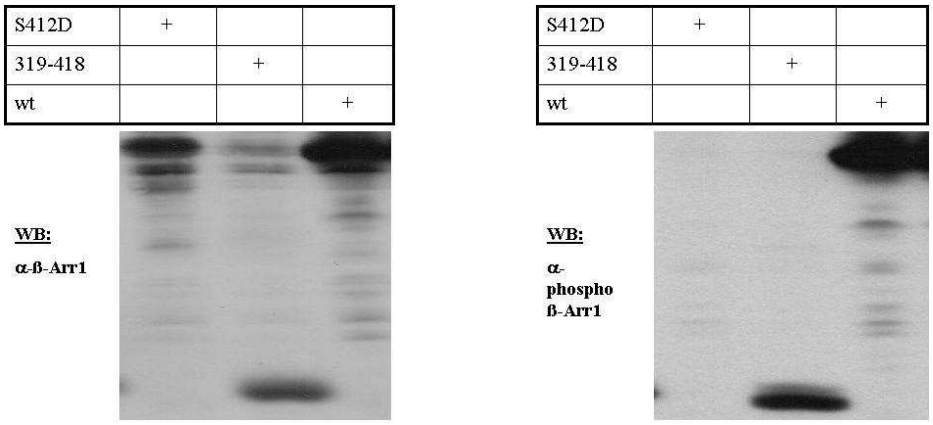


Figure 18. Characteristics of ß-Arrestin1 mutants

HEK293 cells were transfected with ß-Arrestin mutant S412D, c-terminal ß-Arrestin 319-418 and ß-Arrestin wildtype (wt). Lysates were analysed for total ß-Arrestin1 (left side) and phospho ß-Arrestin1 (right side).

The constructs were transfected into HEK293 cells and lysates were subjected to Western blot analysis. All three constructs could be detected with an antibody against total ß-Arrestin1, whereas only wild type ß-Arrestin1 and the c-terminal version 319-418 showed up when an antibody was used that only detects ß-Arrestin1 when it is phosphorylated at Serine 412.

3.2.4 Interaction of ß-Arrestin1 and ADAM17

3.2.4.1 ADAM17 in ß-Arrestin1 overexpressing cell system

Testing in which way the dominant-negative ß-Arrestin1 mutants influence the proteins involved in the TMPS pathway was the next step in trying to gain insides into EGFR-transactivation.

Signal transduction from activated metalloprotease to the EGF-receptor is well characterized, but how metalloproteases are regulated themselves is poorly understood. As ADAM17 is a very prominent and well studied metalloprotease in EGFR-transactivation we set out to explore if there is evidence that ß-Arrestin1 could somehow interfere with this sheddase. HEK293 cells were transfected with the different ß-Arrestin1-HA-tagged constructs and lysates were immunoprecipitated with an antibody against the HA-tag. After PAGE analysis and Western blotting, membranes were probed for ADAM17.

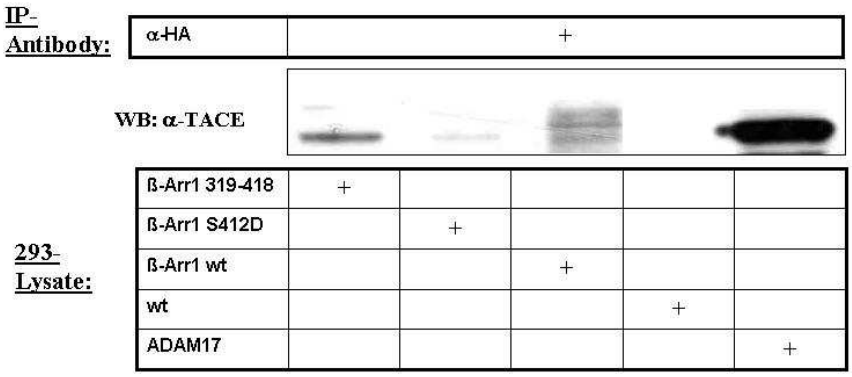


Figure 19. Co-immunoprecipitation of ADAM17 and phospho-ß-Arrestin1

HEK293 cells were transfected with c-terminal ß-Arrestin 319-418 (ß-Arr1 319-418), ß-Arrestin mutant S412D (ß-Arr1 S412D), ß-Arrestin wildtype (ß-Arr1 wt) and full length ADAM17 (ADAM17), all of which are HA-tagged. Lysates were immunoprecipitated with anti HA (α-HA) antibody. Following PAGE and Western blotting, membranes were probed for ADAM17 with anti-TACE antibody.

ADAM17 could be Co-immunoprecipitated in three of the five settings. When HEK293 lysates overexpressing the truncated c-terminal 319-418 form of ß-Arrestin1 were used, a clear band appeared after ADAM17 immunoblotting. The same was true for the lysates overexpressing ß-Arrestin1 wild type and as being expected the strongest band came up in lysates that overexpressed ADAM17. These were used as a positive control. However, lysates obtained from cells overexpressing the ß-Arrestin1 S412D mutant showed no co-immunoprecipitation, similar to the wild type HEK293 cells used as a negative control. These findings indicate that phosphorylation at Serine 412 is essential for the interaction of ADAM17 and ß-Arrestin1.

3.2.4.2 ß-Arrestin1 in ADAM17 overexpressing cell system

Trying to find more evidence that there is a relationship between ADAM17 and ß-Arrestin1 was the mission of subsequent investigations. Therefore the prerequisites of the experimental setup were turned the other way round. Now, HEK293 cells were transiently transfected with a full length ADAM17 construct and different ß-Arrestin1 antibodies were used for immunoprecipitation.

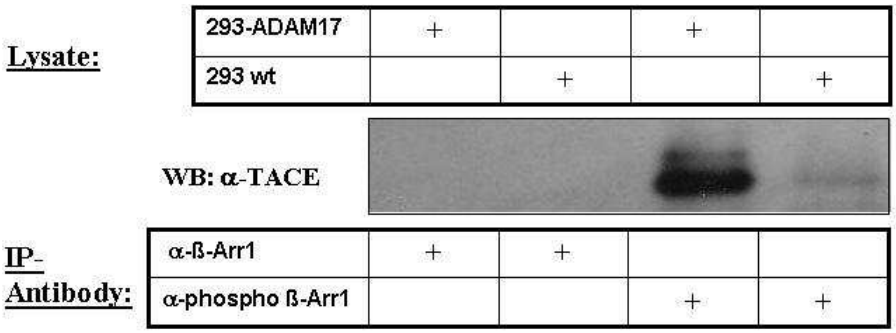


Figure 20. Co-immunoprecipitation of phospho-ß-Arrestin1 and ADAM17

HEK293 cells were transfected with full length ADAM17 (293-ADAM17). Lysates were immuno-precipitated with either ß-Arrestin1 (α-ß-Arr1) or phospho-ß-Arrestin1 (α-phospho ß-Arr1) antibodies. Following PAGE and Western blotting, membranes were probed with anti-TACE antibody.

This time a very strong band appeared when cells overexpressing ADAM17 were immunoprecipitated with an antibody only recognizing phosphorylated ß-Arrestin1. A second, very faint band showed up in the HEK293 wild type lysates probably due to the considerable lower level of ADAM17. When lysates were immunoprecipitated with an antibody against total ß-Arrestin1 no co-immunoprecipitation was observed. This nicely supported the previous finding that only phosphorylated ß-Arrestin1 seems to interact with ADAM17.

3.3 Involvement of EVE1 and Sap97 in EGFR-Transactivation

EVE1, the human counterpart of mouse Sh3d19-protein, is described as an intracellular binding partner of different ADAMs including ADAM9, 10, 12, 15 and 17 and was shown to have an effect on ectodomain shedding of several EGFR ligands in HT1080 cells. It is expressed in various human tissues including skeletal muscle, heart, kidney, and placenta and was also shown to be expressed in different cancer cell lines (Tanaka et al. 2004). The scaffolding protein synapse associated protein 97 (SAP97) is described as a binding partner of ADAM17. Hence both proteins were tested for a potential involvement in transactivation of the EGF-receptor. To address this question, first their expression in the cancer cell lines SCC9 and COS7 was tested by RT-PCR.

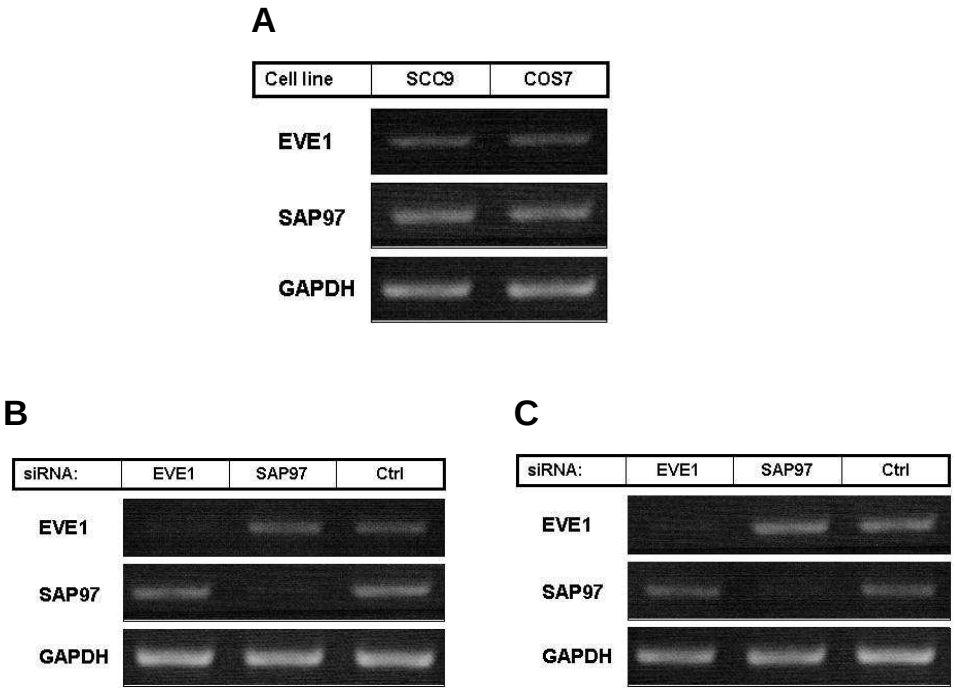


Figure 21. EVE1 and SAP97 expression and knockdown in cancer cells

A) SCC9 and COS7 cells were analysed for EVE1 and SAP97 gene expression by RT-PCR.
COS7 cells (B) and SCC9 cells (C) were transfected with EVE1, SAP97 or control (Ctrl) siRNA and knockdown efficiency was analysed by RT-PCR.

Thereby the two proteins showed expression both in SCC9 and COS7 cells. Next, a siRNA-mediated knockdown of these proteins was established in both cell lines. Knockdown efficiency and specificity was also determined by RT-PCR.

After having optimised conditions for the knockdown, SCC9 and COS7 cells were transfected with siRNAs against EVE1 and SAP97. Again a control siRNA against firefly luciferase was used to exclude off target effects. Subsequently the cells were serum-starved and stimulated with GPCR-ligands to check for the involvement of EVE1 and SAP97 in the TMPS pathway.

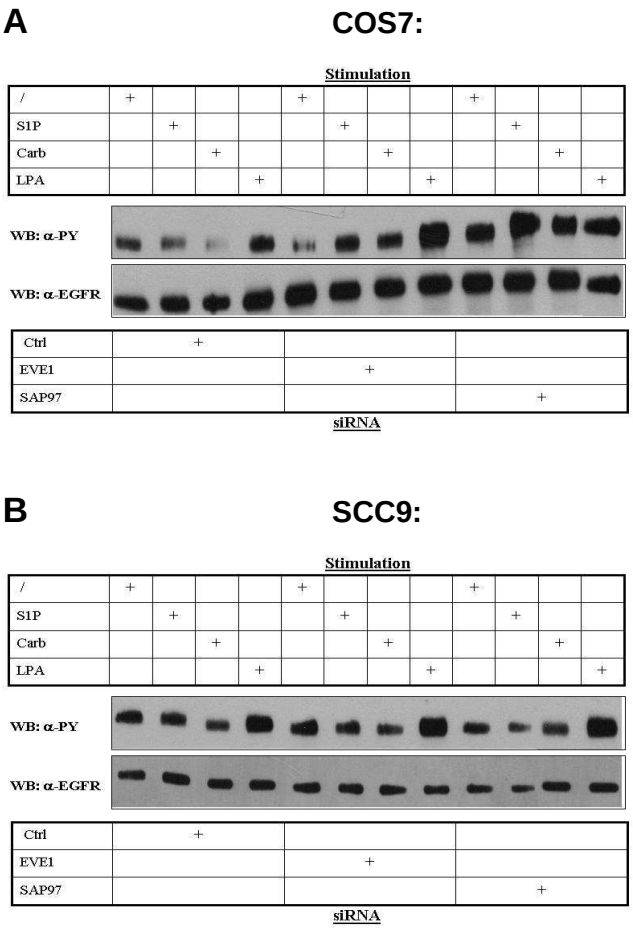


Figure 22. EVE1 and SAP97 knockdown in cancer cells

COS7 (A) and SCC9 (B) cells were transfected with EVE1, SAP97 or control (Ctrl) siRNA. Following siRNA transfection cells were serum-starved, stimulated with Sphingosine-1-phosphate (S1P), Carbachol (Carb), and Lysophosphatydic acid (LPA) for 10 min and assayed for EGFR tyrosine-phosphorylation.

In SCC9 cells no reduction of EGFR-phosphorylation was observed neither following EVE1 nor SAP97 knockdown. Different stimulation conditions using Sphingosine-1-phosphate, Carbachol and LPA showed no alteration in signal intensity compared to the control siRNA. COS7 cells showed in principle the same picture. In none of the set ups knockdown of the two proteins resulted in decreased EGFR phosphorylation. Therefore it seems that neither EVE1 nor SAP97 are involved in EGFR-transactivation in the cellular model systems analysed.

3.4 UV induced EGFR-Transactivation in SCC9 cells

After the original discovery, that UV irradiation induces EGFR-transactivation on various cell lines from the keratinocyte and melanocyte lineages, by Singh (Singh 2007) we investigated the possibility of EGFR signal transactivation in the squamous cell carcinoma cell line SCC9.

3.4.1 UVC irradiation induces phosphorylation of EGFR and downstream signaling molecules in a dose-dependent manner

To find out if EGFR in this cell line gets phosphorylated after cells were irradiated with UVC, we compared different treatment conditions with untreated control cells. SCC9 cells were exposed to increasing doses of UVC irradiation ranging from 0 to 500 J/m^2 for 15 minutes. Following lysis and immunoprecipitation of the EGFR, membranes were probed for phospho-tyrosine and reprobed for total EGFR to ensure that equal protein amounts have been loaded.

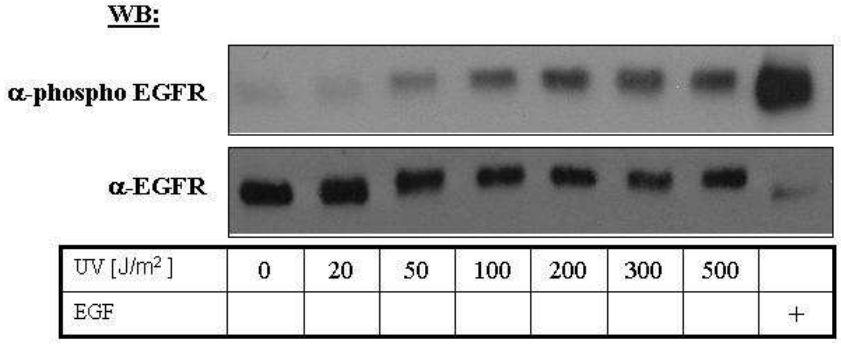


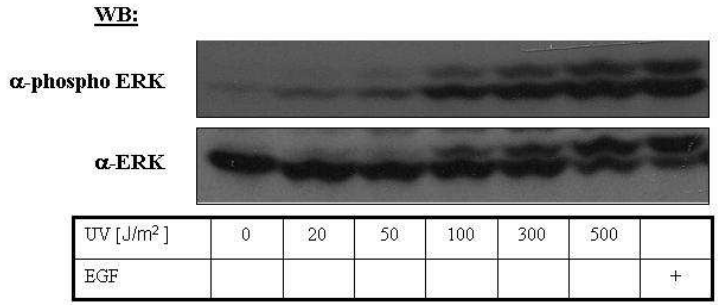
Figure 23. UVC induces EGFR phosphorylation in a dose dependent manner

Serum starved SCC9 cells were treated with indicated doses of UVC (J/m^2) or stimulated with EGF and lysed 15 minutes after irradiation. Lysates were immunoprecipitated for EGFR, transferred to nitrocellulose membrane, probed for pY and reprobed for EGFR.

In the squamous cell carcinoma cell line we observed that EGFR was phosphorylated after irradiation with 50 J/m^2 of UVC. Increased phosphorylation was observed for 100 J/m^2 ^{that} remained unaltered for doses up to 500 J/m^2. No phosphorylation was observed in untreated control cells as well as in cells treated with 20 J/m^2 UV, whereas the positive control, EGF-treated cells, showed the highest phosphorylation.

To further analyse if this EGFR phosphorylation transmits to other proteins, EGFR-downstream signalling was checked. The most prominent candidates of these downstream signaling molecules are certainly extracellular regulated kinase (Erk) and protein kinase B (Akt). Therefore cells were treated as before and total lysates were analysed for ERK- and AKT-phosphorylation as well as for total ERK- and AKT-levels.

A



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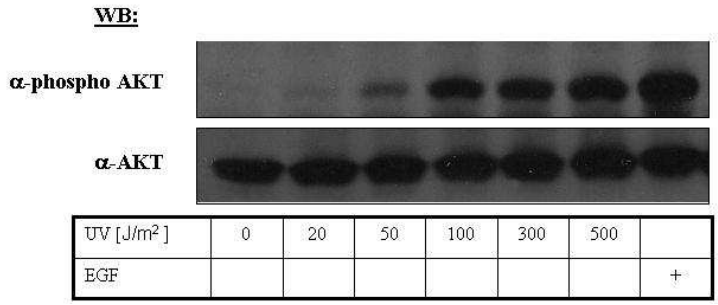


Figure 24. UVC induces Erk and Akt phosphorylation in a dose dependent manner

(A) SCC9 cells were serum starved for 24 hours, treated with indicated doses of UVC (J/m^2) and lysed 15 minutes after irradiation. Equal amounts of lysates were blotted, probed for pErk and reprobed for total Erk. (B) Cells were treated and samples were blotted as in (A). Samples were then probed for pAkt and reprobed for total Akt.

In SCC9 cells both ERK- and AKT-phosphorylation were observed after UVC irradiation in a dose dependent manner.In line with the EGFR-phosphorylation seen, the activation of ERK and AKT also started at UV doses around 50 J/m^2 and remained stable from 100 to 500 J/m^2.

3.4.2 EGFR-phosphorylation in SCC9 cells by UV is metalloprotease dependent

Many of the metalloproteases of the ADAM subfamily have been shown to be involved in EGFR signal transactivation. Upon activation by a GPCR agonist they cleave members of the EGF family of proligands releasing their active forms, which bind to and activate EGFR (Prenzel et al, 1999). To test if UV mediated EGFR signal transactivation is metalloprotease dependent, cells were preincubated with the metalloprotease inhibitor BB94, which is a broad range inhibitor of the ADAM subfamily of metalloproteases.

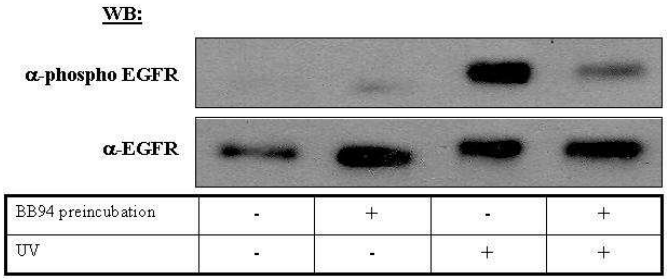


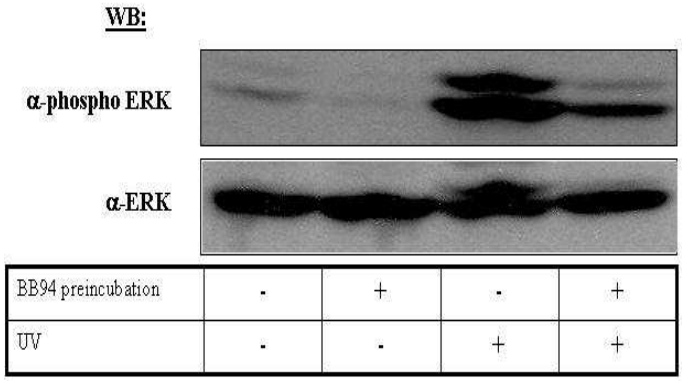
Figure 25. Metalloprotease inhibitor BB94 decreases UV-induced EGFR phosphorylation
SCC9 cells were serum starved for 24 hours, preincubated with BB94 (10µM, 30 minutes) and treated with 50J/m^2 UVC. Cells were lysed 15 minutes after irradiation. Lysates were immunoprecipitated for EGFR, transferred to nitrocellulose membrane, probed for pY and reprobed for total EGFR.

A decreased phosphorylation of the EGFR was observed upon preincubation of SCC9 cells with BB94 followed by UV exposure as compared to UV exposure alone. These results demonstrate a metalloprotease-dependent activation of the EGFR after UV irradiation. However, EGFR phosphorylation could not be completely inhibited by BB94 in SCC9 cells.

3.4.3 BB94 inhibits UV-induced EGFR-downstream signaling in SCC9 cells

Having seen that UV-induced phosphorylation transmits on EGFR downstream signalling, we wanted to test if preincubation with the metalloprotease inhibitor BB94 could reduce the phospho-Erk or the phospho-Akt signal in a similar manner than it did with the phospho-EGFR signal.

A



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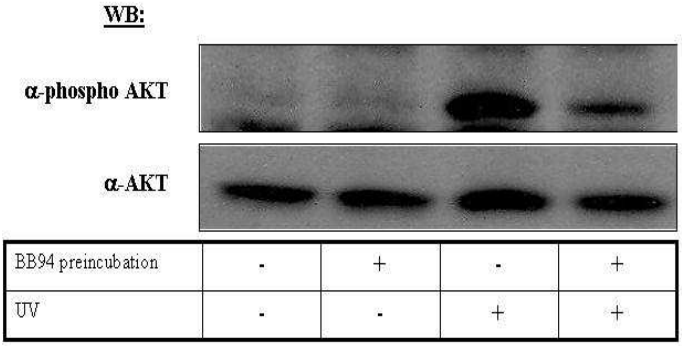


Figure 26. Metalloprotease inhibitor BB94 decreases UV-induced EGFR downstream signaling
(A) SCC9 cells were serum starved for 24 hours, preincubated with BB94 (10µM, 30 minutes) and treated with 50J/m^2 UVC. Cells were lysed 15 minutes after irradiation, equal amounts of lysates were blotted, probed for pErk and reprobed for total Erk. (B) Cells were treated and samples were blotted as in (A). Samples were then probed for pAkt and reprobed for total Akt.

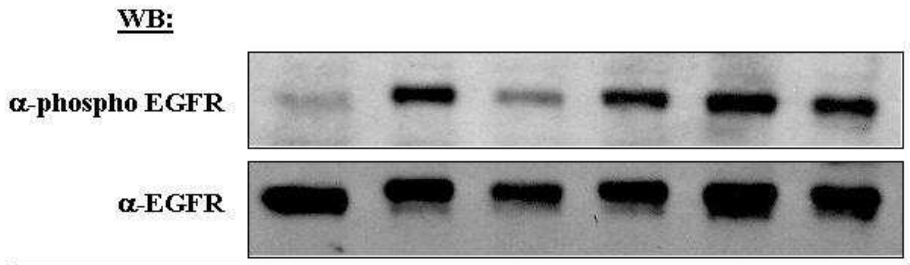
After preincubation with BB94 we observed reduced phospho Erk-and phospho Akt-levels in SCC9 cells after UV stimulation compared to BB94 untreated control cells. This indicates that activation of both Akt and Erk is dependent on metalloprotease-mediated EGFR-phosphorylation induced by UVC irradiation.

3.4.4 Finding the proligand responsible for UV-induced EGFR Transactivation

In his studies about UV induced EGFR-transactivation, Singh could also show that the EGFR extracellular binding domain is required for this novel transactivation pathway (Singh 2007). By blocking the extracellular domain of EGFR with antibodies and therefore inhibiting the extracellular pathway of ligand induced EGFR activation, he could also block UV-induced phosphorylation in C8161 and HaCaT cells. We then wanted to know if this translates to SCC9 cells and which of the proligands of the EGFR is responsible for UV-induced EGFR-transactivation in squamous cell carcinoma.

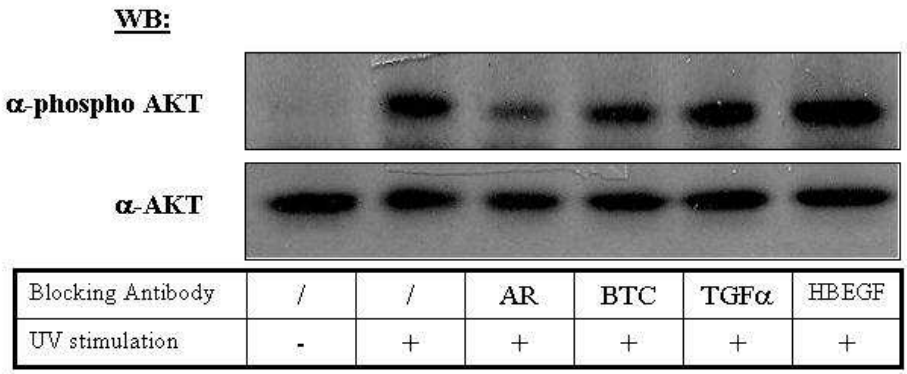
SCC9 cells were therefore preincubated with neutralizing antibodies against Amphiregulin, Betacellulin, Transforming Growth Factor-alpha and Heparin Binding EGF-like growth factor, followed by UV irradiation with 100 J/m^2 for 15 minutes.

A



Blocking Antibody	/	/	AR	BTC	TGFα	HBEGF
UV stimulation	-	+	+	+	+	+

B



Blocking Antibody	/	/	AR	BTC	TGFα	HBEGF
UV stimulation	-	+	+	+	+	+

Figure 27. Neutralizing antibodies against EGF family ligands inhibit UV-induced EGFR signal transactivation

(A) SCC9 cells were preincubated for 1 hour with neutralizing antibodies against Amphiregulin (AR), Transforming Growth Factor-alpha (TGFα), Betacellulin (BTC) and Heparin Binding EGF-like Growth Factor (HB-EGF). Cells were then irradiated with 100 J/m^2 UV, lysed after 15 minutes, immunopreciptated for EGFR, tested for phospho-tyrosine and reprobed for total EGFR levels.
(B) SCC9 cells were pretreated with blocking antibodies, treated and lysed as in (A). Equal amounts of lysates were blotted and probed for phospho-Erk and phospo-Akt and reprobed for total Erk and Akt.

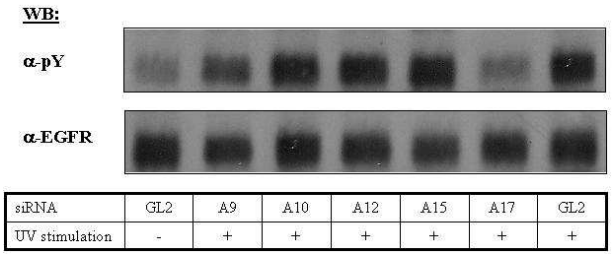
Phosphorylation of EGFR was observed to be reduced after cells were preincubated with a blocking antibody against Amphiregulin. None of the other antibodies used showed an effect on EGFR phosphorylation (Figure 27A). In addition, phosphorylation of EGFR downstream signaling molecules Erk and Akt was also reduced upon application of the neutralizing antibody against amphiregulin in SCC9 cells.
This indicates that Amphiregulin is involved in UV-induced EGFR-transactivation in this squamous cell carcinoma cell line.

3.4.5 Finding the metalloprotease responsible for UV-induced EGFR Transactivation

When Singh set out to explore which metalloproteases play a role in proligand-shedding, he started with determing the expression levels of individual ADAMs in

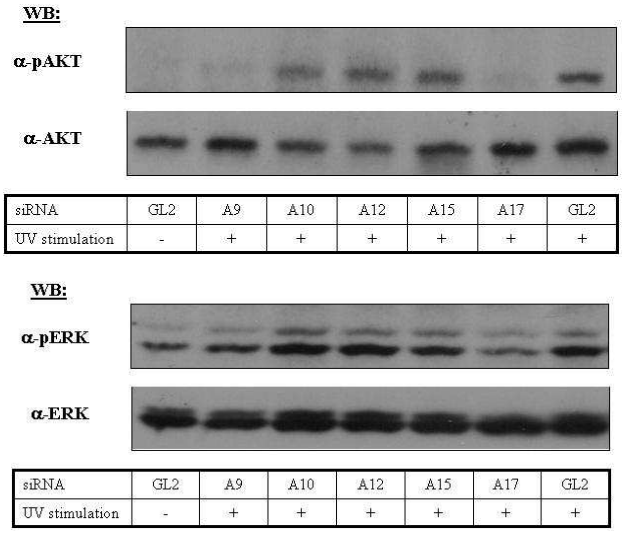
SCC9 cells. Therefore cDNA, synthesized from cell-extracted RNA, was analyzed for the expression of different ADAMs by RT-PCR analysis. By knocking down ADAMs 9, 10, 12 and 15 he found out that after ADAM9 knockdown in the UV-induced Transactivation system, a reduction of phospho-EGFR was observed. We extended the set up and also included ADAM17 to be checked for involvement in UV-mediated shedding in SCC9 cells.

A



siRNA	GL2	A9	A10	A12	A15	A17	GL2
UV stimulation	-	+	+	+	+	+	+

B



siRNA	GL2	A9	A10	A12	A15	A17	GL2
UV stimulation	-	+	+	+	+	+	+

siRNA	GL2	A9	A10	A12	A15	A17	GL2
UV stimulation	-	+	+	+	+	+	+

Figure 28. Knockdown of different ADAMs in SCC9 cells

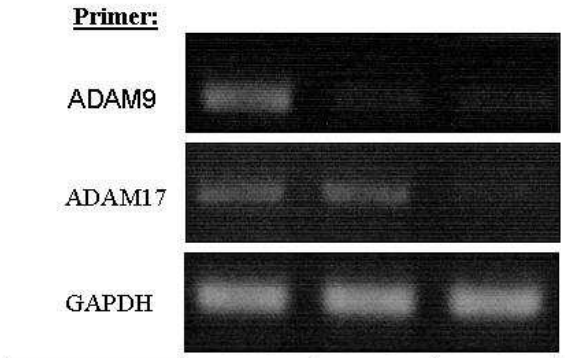
(A) SCC9 cells were transfected with siRNAs against ADAM9, ADAM10, ADAM12, ADAM15, ADAM17 and control GL2. Cells were irradiated with 100 J/m^2 UV, lysed after 15 minutes, immunopreciptated for EGFR, tested for phospho-tyrosine and reprobed for total EGFR levels

(B) SCC9 cells were transfected, treated and lysed as in (A). Equal amounts of lysates were blotted and probed for phospho-Erk and phospo-Akt and reprobed for total Erk and Akt.

The results from Singh could nicely be reproduced as ADAM9-knockdown also resulted in decreased phosphorylation of the EGFR.
Downstream signalling was affected in the same manner, as both ERK and AKT showed reduced phosphorylation after ADAM9 knockdown. Beside this we could observe an even stronger reduction of phospho EGF-receptor after knocking down ADAM17. This again translated on phospho-ERK and phospho-AKT levels. Thereby having shown that not only ADAM9 but also ADAM17 is responsible for UV-induced proligand shedding in SCC9 cells.

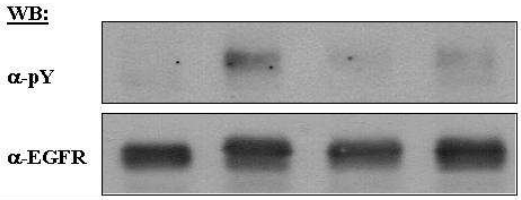
To further strengthen this finding, we made a double knock-out of ADAM9 and ADAM17 to check if we could see additive effects compared to knocking down one metalloprotease only. Knockdown efficiency of the conditions was scrutinized on RNA-level via RT-PCR.

A



si GL2	+		
si ADAM9		+	
si ADAM9 + 17			+

B



siRNA	GL2	GL2	A9 + A17	A9
UV stimulation	-	+	+	+

C

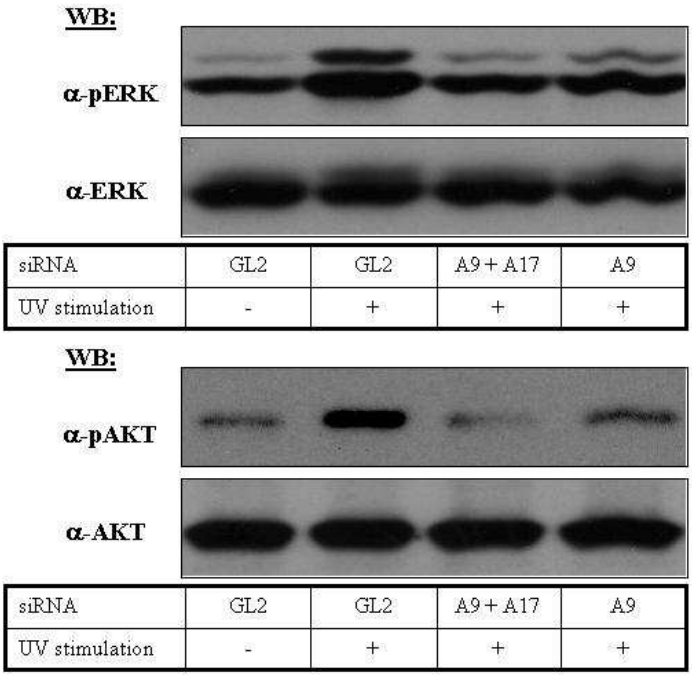


Figure 29. ADAM9 and ADAM17 are responsible for UV-induced EGFR-transactivation

A) SCC9 cells were transfected with ADAM9, combined ADAM9 and ADAM17 or control (GL2) siRNA and knockdown efficiency was analysed by RT-PCR . B) SCC9 cells were transfected with control (GL2) siRNA, ADAM9 siRNA and combined ADAM9 and ADAM17 siRNA. Cells were irradiated with 100 J/m^2 UV, lysed after 15 minutes, immunopreciptated for EGFR, tested for phospho-tyrosine and reprobed for total EGFR levels. C) SCC9 cells were transfected, treated and lysed as in (B). Equal amounts of lysates were blotted and probed for phospho-Erk and phospo-Akt and reprobed for total Erk and Akt.

Knockdown of single ADAM proteins revealed two of them, namely ADAM 9 and ADAM 17 being responsible for UV-induced transactivation in SCC9 cells. Not only on EGFR- but also on ERK- and AKT-level we observed strongly reduced phospho signals in both cases. When we knocked down both ADAM9 and ADAM17 at once, we could observe a complete inhibition of EGFR-phosphorylation after UV-stimulation. This again transmitted to EGFR-downstream signalling as we thereby could reduce both phospho-ERK and phospho-AKT levels to basal condition. This nicely supported the finding that both metalloproteases, ADAM9 and ADAM17, are responsible for UV-induced shedding of EGFR-proligands in SCC9 cells.

4. Discussion

Receptor tyrosine kinases have been shown to play a critical role in different cellular processes like migration, differentiation, proliferation, survival and cell cycle regulation. Screening of the human genome revealed 58 genes that encode for transmembrane receptor kinases (Blume-Jensen and Hunter 2001). According to their structural differences, they are split up into 20 subfamilies. The EGFR family is one of these and consists of four closely related receptor tyrosine kinases: EGFR (HER1), HER2, HER3 and HER4 with EGFR certainly being the most prominent RTK. Members of this family and their ligands are known to play crucial roles in the regulation of cell proliferation and differentiation, and in the survival of many types of cancer (Krause and Van Etten 2005). In many cancers HER family members are activated, due to for example by overexpression, mutation or aberrant signaling of upstream molecules and are therefore considered as targets for cancer therapy. Monoclonal antibodies and small-molecule inhibitors represent currently clinically used drugs that target HER family members for the treatment of different tumors.

Producing soluble and functional EGFR ligands is mediated by sheddases of the ADAM family. The ligand then binds to the EGFR and induces rapid transient EGFR phosphorylation and subsequent activation of downstream signaling events such as mitogen-activated protein kinase phosphorylation, leading to the transcription of various genes. In parallel, upon shedding of EGFR ligands by ADAMs, the carboxy-terminal fragments of EGFR ligands in the cytoplasm are translocated to the nucleus and induce cell cycle progression and cell proliferation by binding and exporting repressors and activating cyclin A and c-Myc (Nanba et al. 2003; Shimura et al. 2008; Tanida et al. 2010). These findings support ADAM inhibitors as potentially drugs for cancer treatment because targeting HER members alone might not be sufficient.

The G protein-coupled receptor family is the largest protein family, consisting of more than 1000 receptors and representing up to 1% of the total mammalian genome (Hermanns 2003). Various findings link GPCRs to the induction of mitogenic responses and to the involvement in regulation of pathologic proliferation.

Mitogenic responses in tissue culture systems have been shown to be induced by potent mitogens including acetylcholine, angiotensin, bombesin, LPA and thrombin (Daaka 2004). The enormous quantity of family members and their mitogenic potential makes GPCRs representing the most common target of drugs on the market.

Given the significance of each of these families, GPCRs, metalloproteases, Receptor tyrosine kinases and their ligands alone strengthens the view that the triple membrane passing signaling pathway of EGFR transactivation, as a molecular cluster for all of them, often seen in pathophysiological disorders holds therapeutic potential. An overview of the intervention sites of the TMPS pathway is shown in Figure 30.

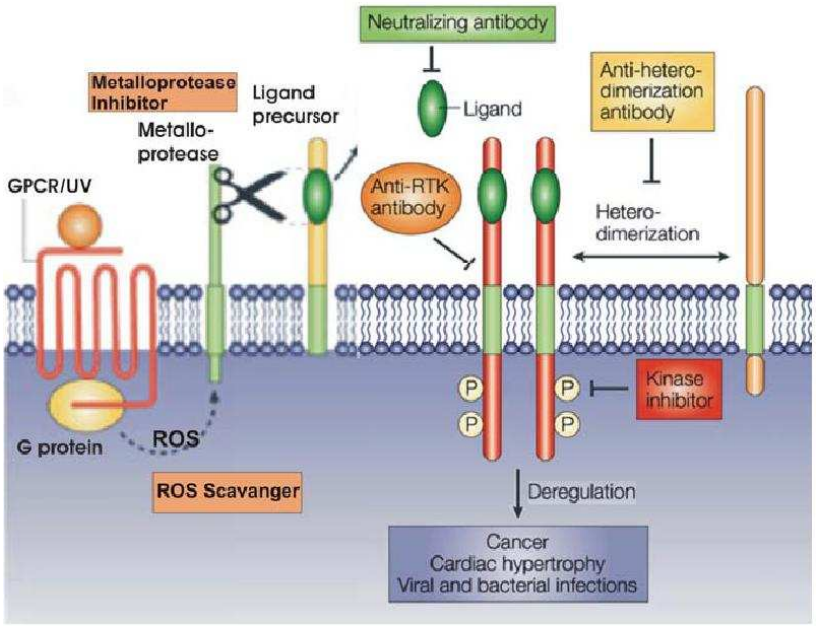


Figure 30. Sites of Therapeutic Intervention in Receptor Tyrosine Kinase Transactivation (Gschwind et al. 2004)

4.1 Targeting ADAM17 in cancer

ADAM-mediated transactivation of EGFR-signaling has been recognized as a physiologically relevant signaling mechanism in various cells and as a very important mechanism for causing abnormal cell growth for example in different cancers (Lautrette et al. 2005; Schäfer et al. 2004; Gschwind et al. 2003; Zhang et al. 2006). Various ADAMs including ADAM 9, 10, 15 and 17 have been implicated in the transactivation of EGF-receptor in cancer cells (Gschwind et al. 2003; Fischer et al. 2003) Evidence gathered from studies on the GPCR-mediated activation of ADAMs in different cells suggest that activation of different GPCRs can lead to ADAM activation in a celltype- and GPCR- specific manner. Furthermore, GPCR ligands can activate different ADAMs in different cells leading to the shedding of the same or different molecules (Reiss et al. 2006; Schlondorff et al. 1999; Arribas et al. 2006)

In various cancer cell lines this is nicely examplified by the shedding of amphiregulin, betacellulin or HB-EGF. Shedding of these proteins has been demonstrated both in cancerous and normal cells in an ADAM-mediated manner upon GPCR stimulation (Asakura et al. 2002; Lemjabbar and Basbaum 2002). Additionally, recent findings that there is a dual role for ADAM-cleaved EGF-like ligands (Figure 31), leading to downstream signaling in both an EGFR-dependent and independent manner, contribute to the importance of inhibiting the metalloproteases involved.

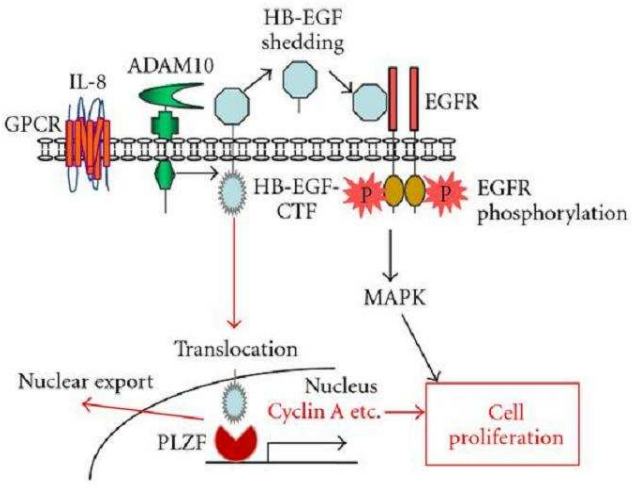


Figure 31. Dual HB-EGF signaling induced by IL-8

IL-8 binds to the GPCR, thereby inducing ADAM10-mediated cleavage of proHB-EGF, resulting in ectodomain shedding of its N-terminus and the generation of an intracellular C-terminal fragment (HB-EGF-CTF). The shed soluble HB-EGF binds to the EGFR and induces rapid transient EGFR phosphorylation and subsequent activation of downstream signaling events such as mitogen-activated protein kinase (MAPK) phosphorylation, leading to the transcription of various genes. In parallel, HB-EGF-CTF is transported into the nucleus, where it subsequently induces nuclear export of PLZF, which leads to cell cycle progression (Quoted and modified from Tanida et al. 2010).

Monoclonal antibodies, originated by Hart and colleagues were tested in this study for their ability to block ADAM17 activity and therefore interfere in EGFR transactivation by aborting the shedding of functional EGF-like ligands. In order to find a robust and well suited model system where the antibodies could be tested with, we concentrated on both COS7 and SCC9 cells. In these cells the TMPS pathway was extensively characterized and ADAM17 had been shown to be the major metalloprotease responsible for EGF-like ligand cleavage after GPCR stimulation. Inhibiting metalloprotease activity with the broadband metalloprotease inhibitor batimastat (BB94) as well as with TIMP3 could reduce GPCR-agonist induced EGFR phosphorylation to basal levels, indicating that a metalloprotease is involved. SiRNA knockdown subsequently revealed ADAM17 as the metalloprotease responsible for proligand-shedding in SCC9 cells. Having different antibodies against ADAM17 and not knowing their exact characteristics, we first tested them in a concentration dependent manner using concentrations from 2 to 10 µg/ml (Figure 13). Three of the antibodies tested showed reduction of EGFR phosphorylation when tested at 2µg/ml and one additionally showed slight activity when being used at 10µg/ml. Next we set out to explore the kinetics of the inhibitory antibodies. Treatment of cells with the antibodies for different time periods from 10 to 90 minutes resulted in no activity shown within the 10 minutes time range, whereas upon preincubation for 90 minutes inhibitory activity was seen in four cases (Figure14). Knowing that ADAMs are involved in the regulation of different cellular processes and being aware of the fact that inhibition of one single molecule doesn't necessarily transfer into biological effects, the antibodies were subjected to show their potential in inhibiting cell proliferation. Especially when cells where stimulated with sphingosine-1-phosphate

some of the tested antibodies showed inhibitory effects up to a maximum level of 25% (Figure15).
Taken together, the study revealed few antibody candidates targeting ADAM17 that have inhibitory potential on molecular level and show slight activity in cellular assays.

4.2 Involvement of ß-Arrestin1 in EGFR Transactivation

Receptors are the eyes and ears of the cell. They are present in all life forms and enable response to environmental changes. Besides this, especially in multicellular organisms they are also responsible for intracellular communication. Therefore a tight receptor regulation is essential for normal functioning as deregulations often lead to diseases. Receptors can be regulated in different ways, by altering their synthesis, by affecting their rate of degradation or changes of their properties. Regulatory factors are beside protein kinases, that mediate receptor phosphorylation and protein phosphatases, mediating dephosphorylation a third way of changing receptor properties. The family of arrestins is one example of regulatory proteins. So far, four mammalian arrestins are known: visual arrestin and cone arrestin are restricted to the phototransduction pathway, whereas ß-arrestin1 and ß-arrestin2 are ubiquitously expressed (Sterne-Marr et al. 1995). The classical role of ß-arrestins is regulating receptor desensitization. Upon agonist binding and GRK-mediated GPCR phosphorylation, ß-arrestin binding is promoted. Further G-protein coupling and G-protein-mediated second messenger signalling is thereby prevented. Following desensitization, receptors get internalized in general via clathrin-coated-pits (von Zastrow and Kobilka 1992). Recent findings however support the idea that ß-arrestins not only mediate desensitisation of GPCR-signaling but rather act as signal transducers themselves (Lefkowitz and Shenoy 2005). GPCRs underly multiple conformational changes upon agonist binding, mediating binding of signalling molecules. These therefore must be able to interact with the receptor in an activation dependent fashion. This attribute is hold by only three families of proteins to date:

GRKs, heterotrimeric G proteins and ß-arrestins. That is likely the reason that ß-arrestins have been shown to mediate a variety of regulatory processes, receptor signalling and are binding partners for a growing number of endocytic and signalling proteins (Shenoy et al. 2003). The activation of ERK mediated by ß-arrestin is one prominent example of ß-arrestin-dependent signalling (Figure 32). It is closely linked to the function of ß-arrestins in mediating endocytosis of receptors in clathrin-coated pits.

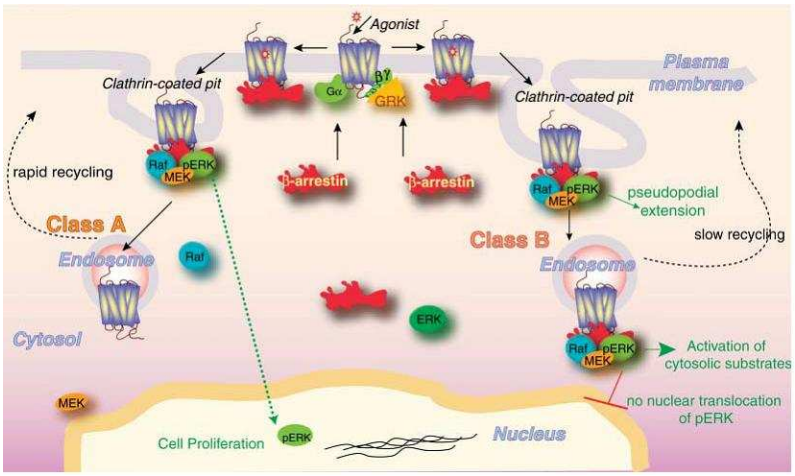


Figure 32. ß-Arrestin–dependent ERK activation.

ß-Arrestin binding to phosphorylated receptors interdicts G protein–dependent signaling, while initiating new waves of signal transduction, for example, by activating ERK1/2 (Lefkowitz et al. 2005)

4.2.1 ß-Arrestin1 is required for GPCR-induced EGFR transactivation

Due to the fact that ß-Arrestins are more and more being involved in signalling we investigated the potential implication of ß-Arrestins in the context of EGFR transactivation. Therefore we set up a siRNA based knockdown of ß-Arrestins in SCC9 cells which was confirmed in specificity and efficiency by RT-PCR (Figure 16A). Knocking down ß-Arrestin2 showed no alteration in EGFR phosphorylation after GPCR stimulation. In contrast, knocking down ß-Arrestin1 resulted in a strong reduction of phospho-EGFR levels although not being observed in all conditions

used. Upon stimulation of cells with Carbachol and Sphinghosine-1-phosphate, the signal was clearly reduced compared to unstimulated control cells (Figure 16B). EGF-treated cells however were not affected by the ß-Arrestin knockdown, leading to the assumption that the interference must take place in an earlier step of the TMPS pathway. This favored the presumption, that ß-Arrestin1 plays a role in the transactivation of the EGF-receptor. Notably, this reduction of EGFR phospho-levels was not seen in Thrombin or LPA treated cells, indicating that different GPCR agonist might use different ways in transactivating the EGFR.

4.2.2 Interaction of ß-Arrestin1 and ADAM17

Subsequently we wanted to gain deeper insights into the molecular characteristics of this ß-Arrestin1 involvement. We therefore cloned different mutants that were known to act as dominant-negative constructs for example in GPCR-endocytosis. Their predicted phosphorylation pattern was nicely observed by Western blotting (Figure18). Both the wild type form of ß-Arrestin and the carboxy-terminal truncated version showed clear phosphorylation when assayed with an antibody against phospho-Serine412. In contrast, the point-mutated S412D form of ß-Arrestin showed no specific phosphorylation as expected. Transfection of these constructs into SCC9 cells however showed no alteration of EGFR-phosphorylation after cells had been stimulated with GPCR ligands in order to induce the transactivation of the EGF receptor. One possible explanation that no differences with the constructs could have been detected, might be that the endogenous levels of ß-arrestin1, still present in the cells, compensated for the signalling mediated by the constructs.

We then went on to overexpress these constructs in HEK293 cells for having a cellular system were high transfection rates were easily possible. In principle we had two different settings. The first being a cellular system, where the different ß-Arrestin constructs are overexpressed and a second one where ADAM17 wildtype protein is overexpressed. Using the first system, we could co-immunoprecitate ADAM17 with ß-Arrestin1 in two cases. When we overexpressed ß-Arrestin wild type we obtained a nice band when blotting against ADAM17 and also when ß-Arrestin 319-418, the c-

terminal truncated version was overexpressed. We could not immunoprecipitate ADAM 17 when we overexpressed ß-Arrestin S412D (Figure 20). These findings led to the assumption, that phosphorylation at Serine 412 of ß-Arrestin1 is crucial for the interaction with ADAM17. To further strengthen this finding, we used the second model system, where ADAM17 is overexpressed. Immunoprecipitation with different ß-Arrestin1 antibodies resulted in one case in a pulldown of ADAM17. When an antibody that detects total ß-Arrestin1 was used for immunoprecipitation we could not observe any relation between ß-Arrestin1 and ADAM17. In contrast, when we used an antibody specific for phospho-ß-Arrestin1 we could co-immunoprecipitate ADAM17 (Figure 19). This nicely supported the findings from the first model system, and led to the notion that phosphorylation of ß-Arrestin1 is a prerequisite for the interaction with ADAM17.

4.3 UV-induced EGFR Transactivation

Being the biggest human organ with surface area up to two square meters, the skin is very important. It protects the body against pathogens and loss of water and overtakes functions like for example insulation and the regulation of temperature. In principle, skin is composed of three layers, the hypodermis, the dermis and epidermis. Being the outermost layer of the skin, the epidermis, that is mainly composed of Merkel cells, Langerhand cells, keratinocytes and melanocytes, it is also the site of the skin that is mostly exposed to sun light and therefore to ultraviolet (UV) radiation. There is evidence that each of the three main types of skin cancer, basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and malignant melanoma (MM), is caused by sun exposure. Singh and colleagues revealed a novel pathway, where the EGF receptor gets activated upon UV irradiation using cell lines of melanocyte and keratinocyte origin (Singh et al. 2009). Using squamous cell carcinoma cells we set out to explore the details of the novel mechanism.

We first started to treat SCC9 cells with increasing doses of UVC irradiation, starting with 20 J/m^2 and using up to 500 J/m^2 as a maximum dose. Upon stimulating cells

with 50 J/m^2 phosphorylation of the EGFR began to take place and reached a plateau at around 200 J/m^2. Additionaly, the level of EGFR-phosphorylation couldn't reach the level that was induced by stimulation with EGF (Figure 23), which also was consistent with the findings in C8161 and HaCaT cells by Singh. EGF receptor activation is closely linked to the activation of downstream signalling molecules, for example ERK and AKT kinases. We therefore analysed if UV induced EGFR activation would transmit to these kinases. Both ERK and AKT kinases turned out to be activated upon irradiating SCC9 cells with UVC. ERK-phosphorylation was onset at doses of 100 J/m^2 (Figure 23), whereas AKT phosphorylation was observed already at 50 J/m^2 (Figure 24). Both reached a maximum level which could not be exceeded with increasing doses of UV irradiation. Next, the metalloprotease-dependency of this mechanism was ruled out. Preincubation of SCC9 cells with the broad band metalloprotease inhibitor batimastat (BB94) resulted in decreased EGFR activation (Figure 25). This again affected EGFR downstream signalling, as ERK and AKT (Figure 26) activation could also be inhibited by the metalloprotease inhibitor.
Remarkably, in none of the three signalling molecules observed, the phosphorylation upon UV irradiation could be shut down to basal levels, probably due to the involvement of another UV induced EGFR activating pathway described by Xu and colleagues, where ROS produced by UV can inactivate PTPs (Xu et al. 2006) which are negative regulators of EGFR phosphorylation (Knebel et al. 1996). This was also shown for keratinocyte and melanocyte cells by Singh.
We then concentrated on finding the proligand involved in the UV-induced EGFR transactivation pathway. Blocking antibodies against different EGF-like ligands were used to address this and in the case of Amphiregulin a clear reduction of receptor- and downstream molecules-phosphorylation was shown (Figure 27). This again was not reduced to basal levels as seen when batimastat was used.

The next challenge was to find out which metalloprotease is responsible for cleaving the proligand upon UV irradiation. In SCC9 cells, ADAMs 9, 10, 12, 15 and17 were shown to be expressed and were already linked to GPCR mediated EGFR transactivation. Therefore we concentrated on these sheddases, knocking down the

single ADAMs by siRNA technique. As shown in Figure 28A, knockdown of ADAM9 resulted in a reduction of EGFR phosphorylation, leading to the assumption that ADAM9 is involved in shedding of amphiregulin in this cell line. Furthermore, siRNA mediated depletion of ADAM17 showed an even higher decrease of EGFR-phosphorylation compared to the one observed with ADAM9 knockdown. This extended the picture, revealing both ADAM9 and ADAM17 being involved in the shedding process. Phospho-AKT as well as phospho-ERK levels corresponded to the observed EGFR- levels further strengthening this finding (Figure 28B).
To finally complete the picture, a double knock out was performed, comparing the knockdown of ADAM9 together with ADAM17 to the single knockdown of each metalloprotease. We have observed a nice additive effect by the double knockdown, compared to the single one. On EGFR level, the phosphorylation could further be reduced upon ADAM9 / ADAM17 double knockdown, however did again not reach basal levels (Figure 29B). ERK and AKT behaved in a similar way also showing further reduced phosphorylation still above basal level (Figure29 C+D).

By detailed analysis of the squamous cell carcinoma SCC9 cell line we could show, that UV irradiation leads to EGFR phosphorylation. This mechanism was shown to happen in a dose dependent manner and required metalloprotease-activity and proligand-shedding. Amphiregulin turned out to be the ligand responsible for signal transduction and both ADAM9 and ADAM17 are the sheddases involved in this cell line.
This novel mechanism shown in cells originated from the second most common type of skin cancer, squamous cell carcinoma, emphasis once more the therapeutic potential of targeting the key players of the EGFR transactivation pathway.

5 Summary

In the context of metalloprotease-mediated transactivation of the epidermal growth factor receptor, different monoclonal antibodies against ADAM17 / TACE were characterized for their ability to block the sheddase. Activity of some of them was observed at doses between 2µg/mL and 10µg/mL. Kinetic analyses showed their activity starting at around 30 minutes.

In cellular assays performed with the antibodies, especially upon treatment of cells with sphingosine-1-phosphate a reduction in proliferation was observed with some candidates.

Moreover this study provides potential new roles for ß-Arrestins. Their involvement in the triple membrane-passing signal pathway of EGFR transactivation was shown. Furthermore, in overexpressing cellular model systems, an interaction between ADAM17 and ß-Arrestin1 could be observed. Detailed analysis discovered that phosphorylation of ß-Arrestin1 is crucial for this interaction.

Additionally, the novel mechanism of UV-induced EGFR transactivation was extended to squamous cell carcinoma. The mechanism happens in a dose dependent manner and requires a metalloprotease to shed the proligand Amphiregulin. The involvement of both ADAM9 and ADAM17, being the metalloproteases responsible for this cleavage, was shown for SCC9 cells.

6 Zusammenfassung

Im Rahmen dieser Arbeit wurden verschiedene monoklonale Antikörper gegen ADAM17 / TACE im Kontext der Metalloprotease-vermittelten Transaktivierung des Epidermalen Wachstumsfaktors auf ihre Fähigkeit hin untersucht, die Proteaseaktivität zu unterdrücken. Einige von Ihnen zeigten inhibitorische Aktivität bei Konzentrationen zwischen 2µg/ml und 10µg/ml. Die Untersuchung der Zeitabhängigkeit ihrer Wirkungsweise ergab eine Aktivität ab 30 Minuten Vorinkubation.

In zellulären Versuchen konnte eine Verminderung der Proliferation besonders nach Stimulation mit Sphingosin-1-Phosphat gezeigt werden.

Darüber hinaus konnten möglich neue Funktionen von ß-Arrestinen gezeigt werden. Eine Beteiligung am „triple membrane-passing“ Signalwegs der Transaktivierung des Epidermalen Wachstumsfaktors wurde dargestellt.

Zudem wurde eine Interaktion von ß-Arrestin1 und ADAM17 in überexprimierenden Zellsystemen gezeigt. Detaillierte Analysen belegten, dass die Phosphorylierung von ß-Arrestin1 eine notwendige Voraussetzung dafür ist.

Weiterhin wurde der neue Mechanismus der UV-vermittelten Aktivierung des epidermalen Wachstumsfaktors auf Plattenephithelkarzinom-Zellen ausgeweitet.

Er findet in einer dosisabhängigen Form statt und bedarf einer Metalloprotease zum Aktivieren des Liganden Amphiregulin. Sowohl ADAM9 als auch ADAM17 wurden als die verantwortlichen Metalloproteasen in den untersuchten SCC9 Zellen ermittelt.

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8. Appendix

Abbreviations

AA	Amino acid
Ab	Antibody
ADAM	A disintegrin and metalloprotease domain
Ampr	Ampicilline resistence
APS	Ammoniumpersulfate
AR	Amphiregulin
ATP	Adenosintriphosphate
Bp	Base pairs
BSA	Bovine serum albumin
℃	Degree celsius
cAMP	Cyclic adenosinmonophosphate
Ca^{2+}	Calcium Ions
cDNA	Complementary DNA
c-fos	Cellular homologue to v-fos (FBJ murine osteosarcoma viral oncogene)
c-jun	Cellular homologue to v-jun (avian sarcoma virus 17 oncogene)
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
DN	Dominant negative
DMSO	Dimethylsulfoxide
DNA	Desoxyribonukleic acid
dsDNA	Double-stranded DNA
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethlendiamintetraacetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
g	Gramm
GDP	Guanosindiphosphate
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor binding protein 2
GST	Glutathion-S-transferase
GTP	Guanosintriphosphate
h	Hour
HA	Hemagglutinin

HB-EGF	Heparin-binding EGF-like growth factor
H2Obidest	Twice-destilled, deionised Water
HEPES	N-(2-Hydroxyethyl)-piperazin-N‘-2-Ethansulfonic acid
HER	Human EGFR-related
HNSCC	Head and neck squamous cell carcinoma
Ig	Immunglobulin
IP	Immunoprecipitation
IP3	Inositol-1,4,5-trisphosphate
IPTG	Isopropyl-ß-thiogalactopyranoside
J	Joule
JNK	c-Jun N-terminal kinase
kb	Kilobase
kDa	Kilodalton
l	Liter
LPA	Lysophosphatydic acid
µ	Micro
m	Milli
m^2	Square meter
M	Molar
MAP	Mitogen-activated protein
MAPK	MAP kinase
MEK	MAPK/ERK Kinase
min	Minute
MMP	Matrix metalloprotease
n	Nano
NRG	Neuregulin
Nox	NADPH oxidase
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol-4,5-diphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl-fluoride
PTP	Protein tyrosine phosphatase
PTX	Pertussis toxin
PY	Phospho-tyrosine
Raf	Homologue to v-raf (murine sarcoma viral oncogene)
Ras	Homologue to v-ras (rat sarcoma viral oncogene)
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature

RTK	Receptor tyrosine kinase
S1P	Sphingosine-1-phosphate
SAP97	ynapse associated protein 97
S. D.	Standard deviation
SDS	Natriumdodecylsulfate
SDS-PAGE	SDS polyacrylamide gel elektrophoresis
Sec	Second
SH2, 3 domain	Src homology 2, 3 domain
SHP-2	SH2-containing PTP-2
Sos	Son of sevenless
SPHK	Sphingosine-Kinase
Src	Homologue to v-src (sarcoma viral oncogene)
TACE	TNFα-converting enzyme
TGFα	Transforming growth factor alpha
TEMED	N, N, N‘, N‘-Tetramethyletylendiamine
Timp	Tissue inhibitor of metalloproteases
TMPS	Triple-Membrane-Passing signal
TNFα	Tumor necrosis factor alpha
TPA	12-O-Tetradecanoyl-phorbol-13-acetate
Tris	Tris(hydroxymethyl)aminomethan
Tween 20	Polyoxyethylensorbitanmonolaureate
U	Enzymatic activity unit
O/N	Overnight
UV	Ultraviolett
UVC	Ultraviolett C
V	Volt
Vol	Volume
Wt	Wild type

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