Technische Universität München

Lehrstuhl für Genetik

Mechanism of Receptor Tyrosine Kinase Transactivation in Skin Cancer Cell Lines

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat)

genehmigten Dissertation.

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Prüfer der Dissertation:	1. Univ.-Prof. Dr. rer. nat. Alfons Gierl
	2. Hon.-Prof. Dr. rer. nat. Axel Ullrich (Eberhard-Karls-Universität Tübingen)

Die Dissertation wurde am 04.12.2006 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 17.01.2007 angenommen.

Erklärung:

Ich erkläre an Eides statt, dass ich die der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München vorgelegte Dissertationsarbeit mit dem Titel:

“Mechanism of Receptor Tyrosine Kinase Transactivation in Skin Cancer Cell Lines”

angefertigt 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. Alfons Gierl (Institut für Genetik der TU München) ohne sonstige Hilfe verfasst und bei der Abfassung nur die gemäß § 6 Abs. 5 angegebenen Hilfsmittel benutzt habe.

München, den

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Bhuminder Singh

Once your mind gets stretched into a new idea,
it never gets back to its original dimension

A P J Abdul Kalam
11th President of India

Dedicated to my family

1 Introduction

Signal transduction is defined as the response of a cell to a change in extracellular environment. These changes could be brought about by chemical or physical agents, with typical examples being nutrients, light, oxygen, and hormones. All cells are equipped with elaborate systems for receiving signals from their environment. Multicellular organisms use signaling cascades to coordinate functions between different cells, and different compartments within a cell. Signaling is essential for the organism during embryonic development and adult life.

Signal transduction involves the following phenomena:

- Signal reception: Receptors receive the signal through a receiver domain (e.g. ligand binding domain for growth factor receptors, light absorbing chromophore in phototaxis) leading to their activation. A signaling domain then forwards the activation that may be present on the same or different polypeptide chain.
- Signal integration: At the signal integrator several stimuli (activation/inhibition) from different receptors converge and are relayed as a single signal downstream of it. In eukaryotic signal transduction networks, the cross talk between different systems adds another level of integration and complexity (Dumont et al. 2002).
- Signal amplification: Amplification typically consists of activation of a catalyst, such as a protein kinase, which amplifies the input of a single unit (photon or molecule) into the phosphorylation of many target molecules.
- Signal adaptation: Adaptation is one of the most important components of a signal transduction network. This enables the system to operate at different levels of sensitivity so as to respond to a varied form of stimuli.
- An Effector: A signal transduction chain finally ends in a biological readout. This could be in the form of induction of gene expression, cytoskeletal rearrangement, and organelle movement (lysosomes, flagella) etc.

Deregulated signal transduction events have been recognized as the underlying cause of many severe human diseases such as cancer, diabetes, immune deficiencies, and cardiovascular diseases, among many others (Hanahan and Weinberg 2000; Schlessinger

2000). Understanding signaling mechanisms also provides new targets and opportunities for treating various diseases (Shawver et al. 2002; Sausville et al. 2003).

The importance of signaling molecules is emphasized by the fact that around 20% of the human genes encode for signaling molecules (Venter et al. 2001). These signaling molecules belong to various classes such as transmembrane receptors, G protein subunits, kinases, phosphatases, and proteases etc. (Blume-Jensen and Hunter 2001).

Protein phosphorylation is one of the most important ways to modulate cellular signaling. More than one third of the human proteins can be phosphorylated; and protein kinases represent the single largest family of enzymes in the human genome accounting for 2% of gene products (Knebel et al. 2001). Phosphorylation and dephosphorylation, catalyzed by protein kinases and protein phosphatases, can modify the function of a protein in almost every conceivable way; by increasing or decreasing its biological activity, by stabilizing it or marking it for degradation, by facilitating or inhibiting movement between subcellular compartments, or by initiating or disrupting protein–protein interactions. The simplicity, flexibility, and reversibility of phosphorylation, coupled with the ready availability of ATP as a phosphoryl donor, explain its selection as the most general regulatory device adopted by eukaryotic cells.

The sequencing of the human genome identified 518 putative protein kinase genes and 130 protein phosphatase genes (Blume-Jensen and Hunter 2001; Manning et al. 2002). According to their localization and their substrate specificity, both protein kinases and phosphatases can be subdivided into cellular and transmembrane molecules and into tyrosine or serine/threonine specific kinases and phosphatases. Deregulation of phosphorylation patterns by aberrant expression or activity of kinases and phosphatases leads to various malignancies (Lim 2005). Thus targeting the signaling pathways regulated by phosphorylation and the kinases involved holds special promise for treating malignant disorders (Arora and Scholar 2005).

1.1 Receptor Tyrosine Kinases (RTKs)

RTKs are type I transmembrane proteins containing a glycosylated extracellular ligand binding domain, and an intracellular portion. The intracellular portion possesses catalytic activity, has regulatory sequences, and also acts as a scaffold for adaptor and regulatory proteins (Blume-Jensen and Hunter 2001). The presence of one or several copies of immunoglobulin-like domains, fibronectin type III-like domains, epidermal growth factor (EGF)-like domains, cysteine rich domains or other domains within the extracellular domains provides structural diversity (Figure 1).

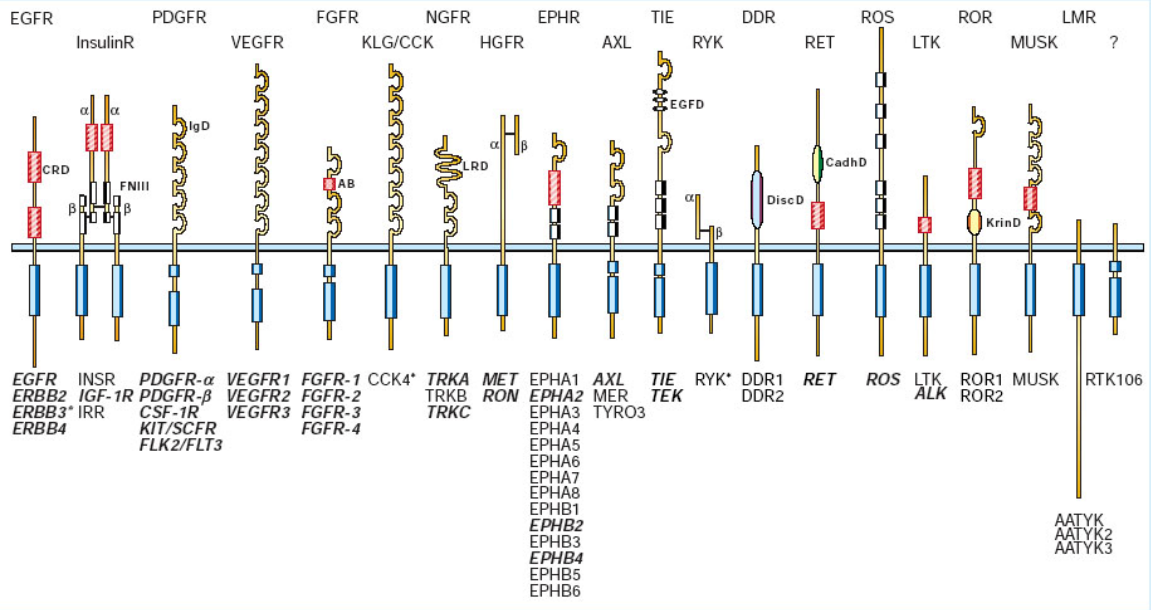


Figure 1. Subfamilies of Receptor Tyrosine Kinases (Blume-Jensen and Hunter 2001).

The RTK family can be broadly divided into two groups depending on the covalent organization of the receptor. Most RTKs possess a single polypeptide chain and are monomers in the absence of any ligand. Members of the insulin receptor subfamily, which includes insulin-like growth factor-I receptor, are disulfide-linked dimers of two polypeptide chains, forming a $\alpha_2\beta_2$ heterotetramer. Ligand binding to RTKs leads to dimerization of monomeric receptors or a rearrangement within the quaternary structure of heterotetrameric receptors, resulting in the autophosphorylation of specific tyrosine residues in the cytoplasmic portion (Hubbard et al. 1998).

1.1.1 Epidermal Growth Factor Receptor (EGFR) Family

EGFR was the first cell surface signaling protein and proto-oncogene product to be characterized by molecular genetic methods and exemplifies prototypical features of RTKs (Ullrich et al. 1984). The EGFR family consists of four members viz. EGFR (Her1, erbB1, v-erb-b), Her2 (neu, erbB2), Her3 (erbB3), and Her4 (erbB4). The four family members share an overall structure of two Cysteine-rich stretches in their extracellular region. Her2 does not bind to any of the known ligands so far, and depends on heterodimerization with other ligand bound family members for activation. Her2 is the preferred dimerization partner for EGFR, Her3, and Her4 (Graus-Porta et al. 1997). Her3 possesses an impaired kinase activity due to point mutations within the catalytic domain.

The EGFR signaling module has been highly conserved throughout the course of evolution with primordial signaling found in nematode *Caenorhabditis elegans* with one single receptor ligand pair to one receptor four ligand system in *Drosophila melanogaster* (Yarden and Sliwkowski 2001). The number of receptors in mammals have increased upto four and their ligands to eleven for a better signal diversification (Olayioye et al. 2000). The importance of EGFR family members in development is evident from analyses of genetically modified mice, as all knockdowns of individual receptors are lethal during embryonic development or early postnatally (Gschwind et al. 2004; Hynes and Lane 2005). EGFR family members are frequently overexpressed or mutated in various tumors, and are important in tumor initiation and development (Pao et al. 2004; Sunpaweravong et al. 2005). Thus therapeutic strategies targeting towards the inhibition of EGFR family members are a promising research field (Holbro and Hynes 2004).

1.1.2 EGF Like Ligands

EGF was first isolated from mouse submaxillary gland (Cohen 1962). The EGF family can be subdivided into four groups based on their receptor binding specificities. The first group includes EGF, transforming growth factor-α, and amphiregulin, which bind to EGFR alone. The second group includes betacellulin, heparin-binding EGF-like growth factor, and epiregulin, which bind to both EGFR and Her4. Neuregulin-3 forms the third group with specificity for Her4 alone. The last group is made of NRG1, which includes

heregulins (HRGs)/neu differentiation factors (NDFs), which bind to Her3 and Her4 (Sundaresan et al. 1998).

All members of the EGF family share six conserved cysteine residues forming an EGF like domain and also possess a transmembrane domain. The membrane-anchored precursor of the EGF family is enzymatically processed externally (except cripto) to release a mature soluble form that acts as autocrine or paracrine growth factor (Figure 2). Some members of the EGF family act as a juxtacrine growth factor in the membrane-anchored form (Brachmann et al. 1989; Wong et al. 1989).

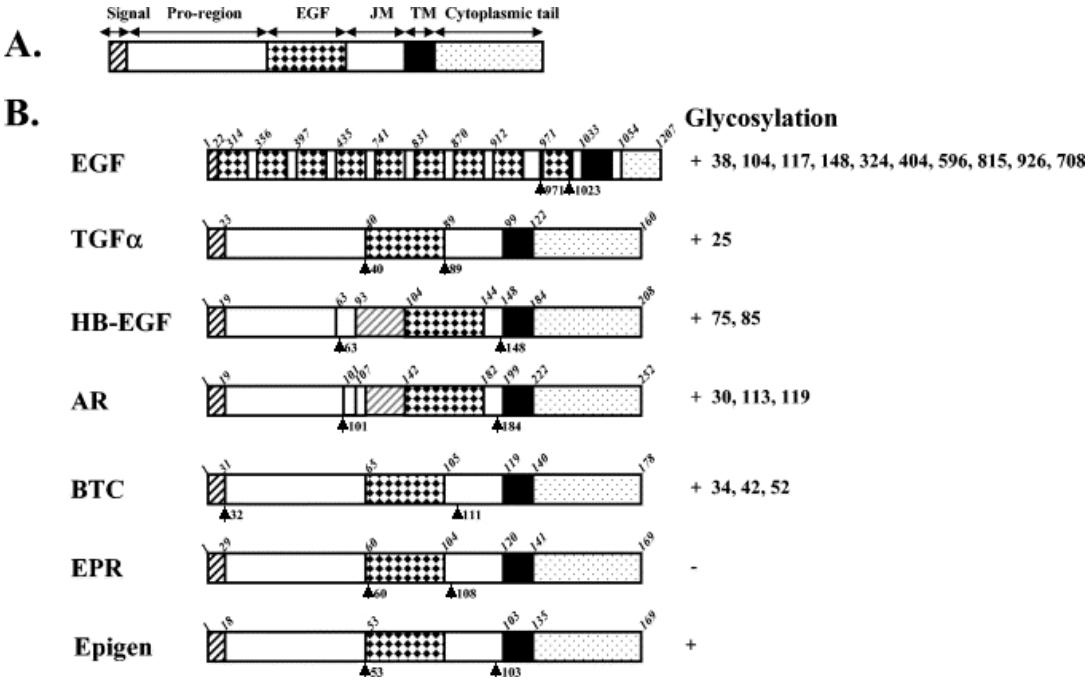


Figure. 2. Domain Organization of EGF Ligand Family (Harris et al. 2003).

(A) Signal peptide, pro region, mature EGF domain, juxtamembrane, transmembrane, and cytoplasmic tail. (B) Amino acid residues that make up these domains in the individual EGFR ligands are listed. EGF consists of 9 EGF-like repeats. Black arrows indicate proximal and distal sites of cleavage. Glycosylation sites are shown on the right.

EGF and related family members are important in normal physiological processes such as epidermal proliferation (COHEN and ELLIOTT 1963), embryonic development (Yamazaki et al. 2003), cardiac development (Jackson et al. 2003) etc. In many tumors EGF and its related growth factors are produced either by tumor cells themselves or are

available from surrounding stromal cells, leading to constitutive EGFR activation and thus to a more aggressive disease state (Salomon et al. 1995).

1.1.3 Ligand Induced Activation of Receptor Tyrosine Kinases

Biophysical investigations revealed a 2:2 stoichiometry for ligand-receptor complexes (Lemmon et al. 1997). Many reports support the hypothesis that a ligand-induced conformational change leads to receptor dimerization rather than bridging of receptor monomers by ligand molecules (Garrett et al. 2002; Ogiso et al. 2002; Schlessinger 2002; Jorissen et al. 2003).

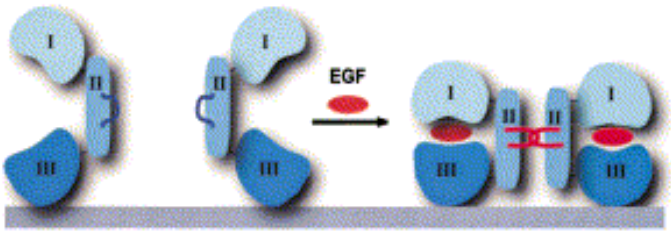


Figure 3. Mechanism of Ligand Induced Dimerization of erbB Receptor (Schlessinger 2002)

Receptor dimerization is mediated by a protrusion in domain II (dimerization loop, red color) that interacts with a specific region in the adjoining receptor to bring about receptor-receptor interactions. It is assumed that the dimerization loop in the unoccupied receptor adopts a conformation (colored blue) that does not facilitate receptor-receptor interactions. In addition, intramolecular domain II-IV interactions may also maintain the EGF receptor in an inactive state.

The kinase domain is intrinsically autoinhibited and an intermolecular interaction promotes its activation. The activation is not simply due to trans-phosphorylation of the activation loop because, in contrast to most kinases, phosphorylation of the EGFR activation loop is not critical to its activation (Burke and Stern 1998; Stamos et al. 2002). EGFR kinase domain can be activated by increasing its local concentration or by mutating a leucine (L834R) in the activation loop which mimics phosphorylated state (Zhang et al. 2006). EGFR activation results from the formation of an asymmetric dimer in which the C-terminal lobe of one kinase domain plays a role analogous to that of cyclin in activated CDK/cyclin complexes.

1.1.4 Cytoplasmic Tyrosine Kinases

Non-receptor tyrosine kinases (NRTKs) are divided into ten subfamilies viz. Src, Abl, Jak, Ack, Csk, Fak, Fes, Tec, and Syk (Blume-Jensen and Hunter 2001). They lack receptor-like features such as an extracellular ligand binding domain and a transmembrane spanning region. Most NRTKs are localized in the cytoplasm whereas some are anchored to the cell membrane through amino-terminal modifications, such as myristoylation or palmitoylation. In addition to a tyrosine kinase domain, NRTKs possess domains that mediate protein-protein, protein-lipid, and protein-DNA interactions. The most familiar theme in NRTK regulation, as in RTKs, is tyrosine phosphorylation. In particular, phosphorylation of tyrosines in the activation loop of NRTKs leads to an increase in enzymatic activity. Activation loop phosphorylation occurs via trans-autophosphorylation or phosphorylation by a number of other NRTKs (Hubbard and Till 2000). Phosphorylation of tyrosines outside of the activation loop can negatively regulate kinase activity.

The largest subfamily of NRTKs is the Src family with nine members. Src family members participate in a variety of signaling processes, such as mitogenesis, cell adhesion and migration, synaptic transmission and plasticity, T- and B-cell activation, and cytoskeleton remodelling (Parsons and Parsons 2004). Multiple *in vivo* substrates have been described for Src including the Platelet Derived Growth Factor Receptor (PDGFR) and EGFR. Src kinases share a conserved domain structure consisting of four domains in the following order: a myristoylated N-terminal segment (SH4), a unique domain, an SH3 domain, an SH2 domain, a tyrosine kinase domain (SH1), and a short C-terminal tail (Boggon and Eck 2004). Tyrosine-416 phosphorylation of Src members within a segment of the kinase domain called the activation loop is required for full kinase activity. Phosphorylation at tyrosine-527 in the short C-terminal segment leads to autoinhibition, and can be mediated by C-terminal Src kinase (Csk), or Csk homologous kinase (Chk). This phosphorylation at Tyr-527 leads to rearrangement of domain structure, where the domains fold on each other adopting a closed inactive conformation. v-Src is constitutively active as it lacks the carboxy terminal tail containing the negative regulatory Tyr-527, and thus leads to uncontrolled growth of infected cells.

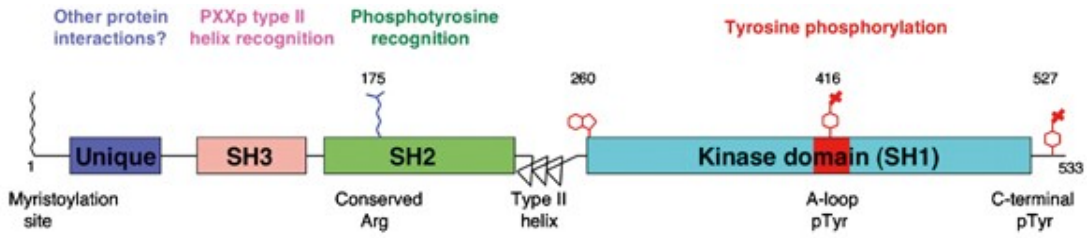


Figure 4. The Domain Structure of Src Family Kinases (Boggon and Eck 2004)

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.

1.2 Protein Interaction Domains and Downstream Signaling

Kinase activation upon ligand binding leads to a change in its conformation increasing its kinase activity. Conformational change also exposes various protein interaction domains or sites, which bind to signaling molecules in a stimulation dependent context (Hunter 2000). In a cell expressing a number of proteins at a given time, protein interaction domains help overcome the problem of molecular recognition.

Protein interaction domains recognizing phosphorylated tyrosine residues are the most important domains in RTK signaling (Schlessinger and Lemmon 2003). Both SH2 (Src homology region 2) and PTB (phosphotyrosine binding) domains are able to bind to phosphorylated tyrosines. The SH2 domain has strict requirement for phosphorylated tyrosine, but most PTB domains actually bind to their (non-phosphorylated) targets constitutively. SH2 and PTB domains target the host proteins to different cellular compartments, and regulate autoinhibition, activation and dimerization of their host proteins.

Adaptor molecules lack any catalytic domain and comprise of only interaction domains. Their major function is to promote interaction between two molecules via their interaction domains. Examples include Grb2, Crk, or Shc that contain SH2 and SH3 domains to link activated RTKs to downstream signaling pathways like mitogen activated

protein kinases (MAPKs). Another type of proteins possess both interaction domains and an enzymatic activity, such as the Src kinase, that contains SH2 domains and a tyrosine kinase activity, or phospholipase C (PLC)-γ, containing both an SH2 domain and a phospholipase activity.

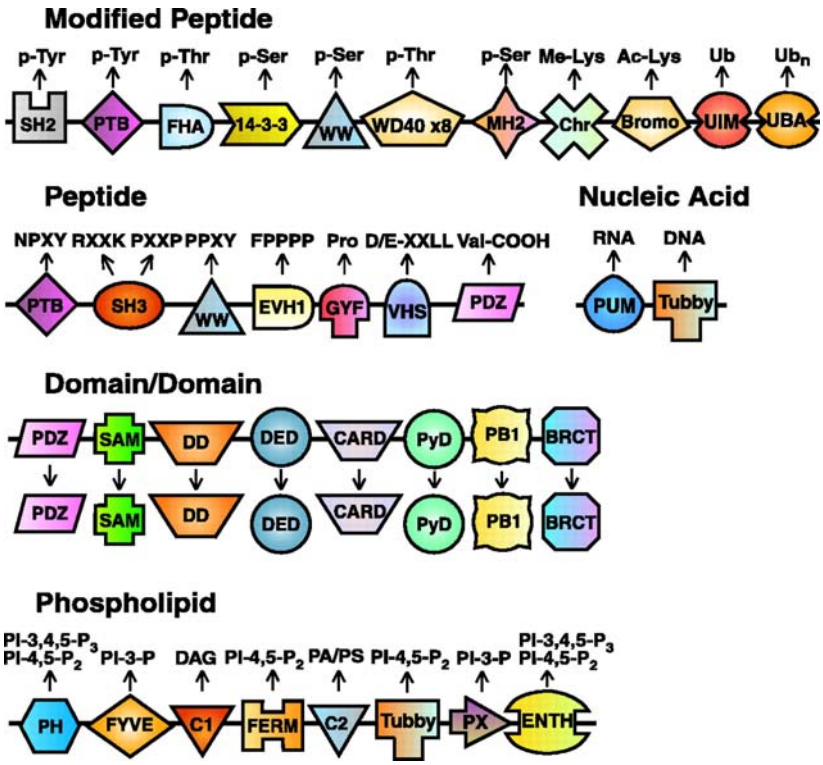


Figure 5. Protein Interaction Domains in Signal Transduction (Pawson and Nash 2003)

Each interaction domain (colored boxes) is responsible for recognizing and binding to a particular motif, as illustrated.

1.2.1 Mitogen Activated Protein (MAP) Kinase Pathways

MAP kinase pathways are one of the most extensively studied pathways and are present in all eukaryotes. MAP kinase pathways are important in various cellular functions including growth, differentiation, carcinogenesis, immune response, and apoptosis (Ip and Davis 1998; Schaeffer and Weber 1999). The core signaling unit consists of three protein kinases: a MAP kinase kinase kinase (MKKK), a MAP kinase kinase (MKK), and a MAP kinase, phosphorylating and activating the other in that sequence. Frequently MAP4K is also found to activate MAPKKK. The activated MAP kinases then phosphorylate appropriate substrates at serine and threonine residues, including other

protein kinases and transcription factors, to achieve the desired response to extracellular stimulation (Ip and Davis 1998; Schaeffer and Weber 1999).

Mammalian MAP Kinases are divided into five families: extracellular signal regulated kinases (ERK1 and ERK2), which are activated by mitogens, c-Jun NH_2-terminal kinases (JNK1, 2, and 3), p38 MAPKs (p38α, β, γ, and δ) that are primarily involved in cytokine signaling and in response to extracellular stress, ERK3/4, and ERK5 (Chang and Karin 2001; Weston et al. 2002). MAPKs are activated by dual phosphorylation of conserved residues within the activation loop (denoted T-X-Y) and phosphorylate targets on residues within a consensus PXT/SP motif (Chen et al. 2001). Pathway specificity is regulated at several levels, including kinase-kinase and kinase-substrate interactions, colocalization of kinases by scaffold proteins, and inhibition of cross talk by the MAPKs themselves. Scaffolds regulate MAPK signaling in multiple ways beyond simple tethering (Burack and Shaw 2000).

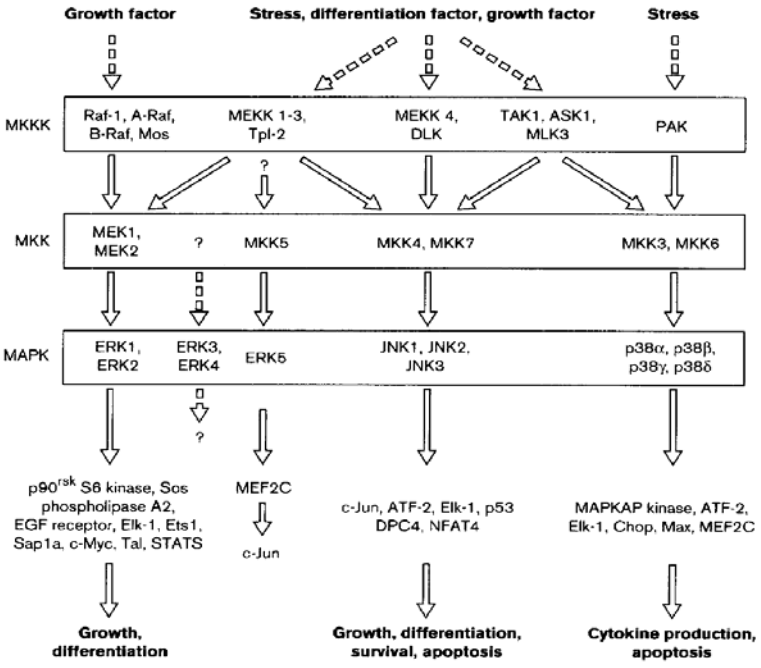


Figure 6. MAPK Pathways Interacting with each other (Garrington and Johnson 1999)

One prototypic example of MAPK cascade activation is ERK1/2 activation by RTKs. RTK stimulation leads to the recruitment of the adaptor protein Grb2 followed by

association and activation of the RasGEF son of sevenless (Sos), which in turn activates membrane accociated Ras GTPase. Ras in turn induces serine/threonine kinase activity of the MAPK kinase kinase Raf-1 that phosphorylates and activates the MAPK kinases 1/2 (MEK 1/2). MEK1/2 finally activate ERK1/2 by phosphorylation of threonine and tyrosine residues in the regulatory TEY motif (Burack and Shaw 2000).

1.2.2 Protein Kinase B/Akt

PKB/Akt subfamily of serine/threonine kinases comprises three isoforms PKBα, PKBβ, and PKBγ (Akt1, 2, and 3, respectively). All three isoforms arising from distinct genes share a conserved structure namely an N-terminal pleckstrin homology (PH) domain, a central kinase domain, and a C-terminal regulatory domain containing the hydrophobic motif (HM) phosphorylation site [FxxF(S/T)Y] (Fayard et al. 2005). Akt activation is a multi step process and is initiated by phosphoinositide-3-kinase (PI3K) signaling. PI3K activation leads to phosphoinositol (3, 4, 5)-triphosphate (PIP_3) generation, which recruits Akt to the plasma membrane via its PH domain. Akt is then phosphorylated at thr308 in the activation loop of the kinase domain by phosphoinositide dependent kinase 1 (PDK1), which is also recruited to the plasma membrane by PIP_3 via its PH domain (Stephens et al. 1998). A second Akt phosphorylation event at ser473 within the HM of the regulatory domain by PDK2 is the key step in the activation of Akt because it stabilizes the active conformation (Yang et al. 2002). Activated Akt can then be translocated to the cytosol or the nucleus for further signaling.

Negative regulators of PKB signaling include the tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN) and SH2 domain containing inositol polyphosphate-5-phosphatase (SHIP). These phosphatases convert PIP_3 to PIP_2 and thus inhibit recruitment of PKB to plasma membrane and consequent activation. Other phosphatases like Protein Phosphatase 2A (PP2A) and PH domain leucin-rich repeat protein phosphatase (PHLPPα) do so by directly dephosphorylating Ser473 and/or Thr308 on PKB (Fayard et al. 2005).

PKB is involved in various cellular functions like cell survival, cell growth, protein synthesis, gene transcription, metabolism, and angiogenesis by positive or negative

regulation of downstream molecules. Among its negatively regulated substrates Bad, Bax, Caspase9, p21, p27, FOXO1, and GSK3 are major signaling proteins. In addition PKB has been shown to positively regulate Mdm2, eNOs, IRS1 etc. (Hanada et al. 2004). PKB activation is known to contribute to tumor development and metastasis as well as chemotherapeutic resistance (Nicholson and Anderson 2002).

1.3 G-Protein Coupled Receptors (GPCRs)

The G-protein coupled receptor superfamily is the largest and most diverse group of proteins comprising more than 1% of the human genes (Gutkind 2000). On the basis of homology with rhodopsin, these receptors are predicted to contain seven membrane spanning α-helices (20-27 amino acids), an extracellular N-terminus, and an intracellular C-terminus, and thus are also called 7-transmembrane (7-TM) receptors (Palczewski et al. 2000). GPCRs are involved in responses to a variety of stimuli, including mitogens, chemoattractants, neurotransmitters, hormones, polypeptides, photons, odorants, taste ligands, nucleotides, and calcium ions, and are estimated to be the targets for nearly 60% of all drugs available (Leurs et al. 1998). These proteins are conserved among various species ranging from yeast to mammals.

GPCRs can be divided into three major subfamilies based on certain key sequences: type-A related to rhodopsin; type-B related to calcitonin receptor; and type-C related to metabotropic receptor; with type A being the largest and most extensively investigated of all (Gether and Kobilka 1998). Ligand binding to GPCRs is quite diverse: smaller ligands bind to a hydrophobic core formed by the transmembrane helices, and peptide agonists bind via extracellular hydrophilic loops joining the transmembrane domains and the N-terminal tail. The domains critical for interaction with G proteins have been located at the second and third cytoplasmic loops, and at the C terminus (Gether and Kobilka 1998).

Agonist binding leads to a change in the conformation of the receptor that may involve disruption of a strong ionic interaction between the third and sixth transmembrane helices, which facilitates the activation of a G-protein heterotrimer. Depending on the type of G protein, to which the receptor is coupled, a variety of downstream signaling

pathways can be activated. Signaling is attenuated by GPCR kinases (GRKs), which specifically phosphorylate activated GPCRs, facilitating arrestin binding and subsequent internalization (Bunemann and Hosey 1999; Luttrell and Lefkowitz 2002). Receptor desensitization and eventual resensitization are regulated by complex interactions of various intracellular domains of GPCRs with numerous intracellular proteins (Kroeze et al. 2003). In addition, GPCRs interact with a variety of proteins containing PDZ, SH3, or PTB domains, like cytoplasmic scaffold proteins, which may change the receptor localization, trafficking, conformation, or association with effector molecules (Ji et al. 1998; Hall and Lefkowitz 2002). To add yet another layer of complexity, homo- or hetero-oligomerization among GPCRs influence ligand binding, receptor activation, desensitization, trafficking and receptor signaling (Breitwieser 2004).

1.3.1 Heterotrimeric G proteins

Heterotrimeric G proteins are composed of a Gα subunit interacting with a Gβγ subunit. Ligand binding to GPCRs alters the conformation of intracellular receptor domains and induces the association with heterotrimeric G proteins. This results in an exchange of GDP for GTP in the active site of Gα subunit, followed by the dissociation of the heterotrimeric complex. Both the Gα and Gβγ subunits activate cytoplasmic or membrane bound effector molecules (Hermans 2003).

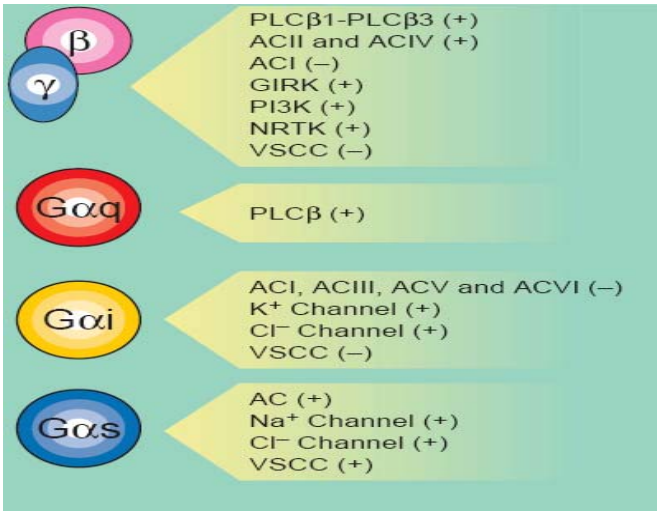


Figure 7. Downstream Signaling from Hetetrotrimeric G Proteins after GPCR Activation (Kroeze et al. 2003)

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

1.3.2 The Oncogenic Potential of GPCRs and G Proteins

Various GPCR ligands have been shown to be potent mitogens such as acetylcholine, angiotensin, bombesin, bradykinin, endothelin-I, isoproterenol, lysophosphatidic acid (LPA), neurotensin, prostaglandin, and thrombin etc. and are able to induce mitogenic responses in tissue culture systems (Daaka 2004).

Characterization of the MAS1 oncogene isolated from a human epidermoid carcinoma cell line, showed it to be having multiple transmembrane domains, a structure similar to GPCRs (Young et al. 1986). Persistent GPCR activation or activating mutations are shown to contribute to malignant transformation and cancer (Julius et al. 1989; Allen et al. 1991). Moreover, various transforming viruses e.g. Kaposi's sarcoma associated herpesvirus, contain sequences encoding constitutively active GPCRs shown to induce cancer in animal models (Montaner et al. 2003).

The oncogenic potential of mutated G protein subunits has also been shown. GTPase deficient mutants of $G\alpha_i$, $G\alpha_q$, $G\alpha_o$, $G\alpha_{12}$, and $G\alpha_{13}$ are found to be oncogenic in several cellular systems. In addition, naturally occurring activating mutations have been identified in various disease states, including cancer (Dhanasekaran et al. 1995). This led to the designation of several activated Gα mutants as oncogenes, including $G\alpha_s$, $G\alpha_{i2}$, and $G\alpha_{12}$, referred to as the gsp, gip2, and gep oncogenes, respectively (Landis et al. 1989; Lyons et al. 1990; Xu et al. 1993).

1.4 Receptor Tyrosine Kinase Transactivation

Receptor tyrosine kinases receive extracellular signals upon ligand binding and transduce to the cytoplasm, via activation and recruitment of effector and adaptor molecules, setting various signaling cascades in motion. However, RTKs are also excellent molecules for

signal integration and diversification apart from amplification. Multicelluar organisms employ RTKs as key nodes in the highly complex signaling networks allowing communication, coordination, and adaptation of cellular signaling. Therefore increasing interest focuses on interreceptor communication mechanisms in normal physiology and pathological states. EGFR has been shown to be activated by a variety of different stimuli in addition to direct stimulation with its cognate ligand, such as stress stimuli, GPCR agonists, integrin activation, cytokine receptors, or ion channels (Gschwind et al. 2001; Prenzel et al. 2001; Fischer et al. 2004). Cancer cells are able to couple various external stimuli (e.g. GPCR ligands) to EGFR and channel the signaling to various pathways helping in their better survival, proliferation, angionesis, or anti-apoptosis (Gschwind et al. 2002; Gschwind et al. 2003; Schafer et al. 2004).

1.4.1 EGFR Transactivation

EGFR transactivation was first described by Daub et al (Daub et al. 1996). In this report EGFR activation was shown upon treatment of rat fibroblasts with GPCR agonists. Subsequently, this phenomenon was shown in a variety of cell types with different GPCR agonists (Gschwind et al. 2002; Schafer et al. 2004). Initially, EGFR activation by GPCRs was believed to be occurring via intracellular signaling. However, Prenzel et al showed for the first time the metalloprotease mediated processing of the EGF like ligand HB-EGF and therefore a ligand dependent mechanism in EGFR transactivation (Prenzel et al. 1999). Blocking both proHB-EGF and metalloprotease function abrogated GPCR stimulated EGFR, Shc, and MAPK phosphorylation, revealing the involvement of metalloproteases and the EGF like ligand HB-EGF in the transactivation pathway. Since then all of the 7 ligands binding to EGFR have been shown to be cleaved to release a soluble form and six of them are shown to be cleaved by metalloproteases (Sahin et al. 2004; Kochupurakkal et al. 2005). Various metalloproteases including various ADAMs and matrix metalloproteases (MMPs) have also been shown to cleave EGF family ligands (Gschwind et al. 2002; Gschwind et al. 2003).

In addition to this pathway Src family kinases have been suggested as both upstream and downstream mediators of the GPCR induced EGFR transactivation. Besides Src kinases,

the serine/threonine kinase PKC has been frequently suggested to be involved in EGFR transactivation. Moreover, in different cellular systems the intracellular Ca^{2+} concentration and the Ca^{2+} regulated tyrosine kinase Pyk2 have been discussed as a mediator of EGFR signal transactivation (Zwick et al. 1997; Eguchi et al. 1998; Keely et al. 2000). These pathways could also activate EGFR in addition to the metalloprotease induced proligand shedding pathway. Furthermore, inhibiton of pathways negatively regulating RTK activity could also indirectly prolong the RTK signaling. Typical examples being inactivation of protein tyrosine phosphatases which dephosphorylate RTKs, reducing their activity and subsequent signaling (Knebel et al. 1996).

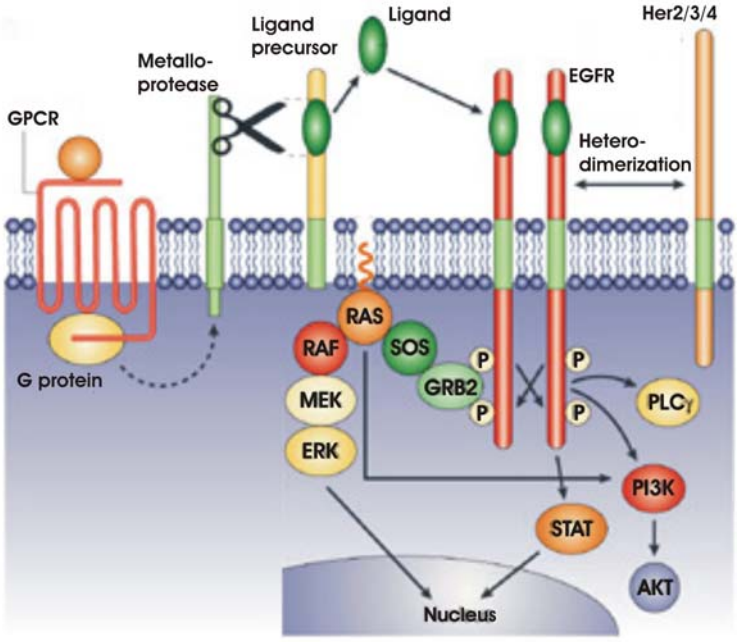


Figure 8. GPCR Induced EGFR Signal Transactivation (Gschwind et al. 2004)

GPCR stimulation leads to metalloprotease activation, proligand shedding, and EGFR activation.

1.4.2 ADAMs/Metalloproteases

Zinc dependent proteases are subdivided according to the primary structure of their catalytic sites and include gluzincin, metzincin, inuzincin , carboxypeptidase, and DD carboxypeptidase subgroups (Hooper 1994; Seals and Courtneidge 2003). The metzincin

subgroup is further divided into serralysins, astacin, matrixins, and adamalysins (Stocker et al. 1995). The matrixins are comprised of the matrix metalloproteases, or MMPs, principle agents responsible for extracellular matrix degradation and remodeling during development, wound healing, and pathology like arthritis and cancer (Chang and Werb 2001). Adamlysins are similar to the matrixins in their metalloprotease domain, but contain a unique integrin receptor binding disintegrin domain. The presence of these disintegrin and metalloprotease domains gives ADAMs their name (a disintegrin and a metalloprotease domain). The protein structure of ADAMs consists of a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine rich domain, an EGF like domain, a single transmembrane domain, and a cytoplasmic tail of variable length. The adamlysins subfamily also contains the class III snake venom metalloproteases and the ADAM-TS family, which, although similar to ADAMs, can be distinguished structurally.

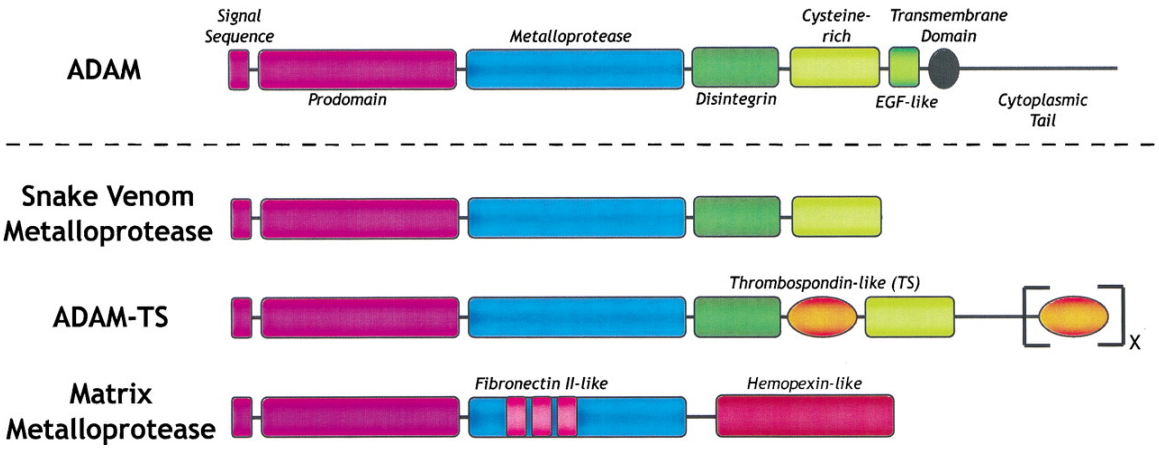


Figure 9. Topography of ADAMs and Related Metalloproteases (Seals and Courtneidge 2003).

12 of the 19 known human ADAMs show proteolytic activity (Kheradmand and Werb 2002). ADAMs are implicated in the control of membrane fusion, cytokine, RTK and growth factor shedding, cell migration and biological processes like muscle development, fertilization, cell fate determination, and pathologies such as inflammation and cancer (Schlondorff and Blobel 1999; Kheradmand and Werb 2002).

Tumor necrosis factor-α convertase (TACE/ADAM17) is the best characterised metalloprotease and was identified as the protease responsible for release of the inflammatory cytokine tumor necrosis factor (TNF)-α from its membrane bound precursor proTNFα (Black et al. 1997; Moss et al. 1997). Besides TNFα, ADAM17 mediates cleavage of diverse integral membrane proteins like L-selectin, p75 TNF receptor (Peschon et al. 1998), fractalkine (Garton et al. 2001), MUC1 (Thathiah et al. 2003), β-amyloid precursor protein (βAPP) (Buxbaum et al. 1998), p55 TNFR, interleukin-1 receptor (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). Studies using fibroblasts derived from ADAM17 knockout mice showed phenotypic defects, including failure of eyelid fusion, hair and skin defects, and abnormalities in lung development similar to the defects seen in EGFR or TGFα knockout mice (Peschon et al. 1998). However, perinatal lethality was seen in ADAM17 knockouts, indicating that TACE has additional substrates required for the development of some important organs that are necessary for survival (Shi et al. 2003).

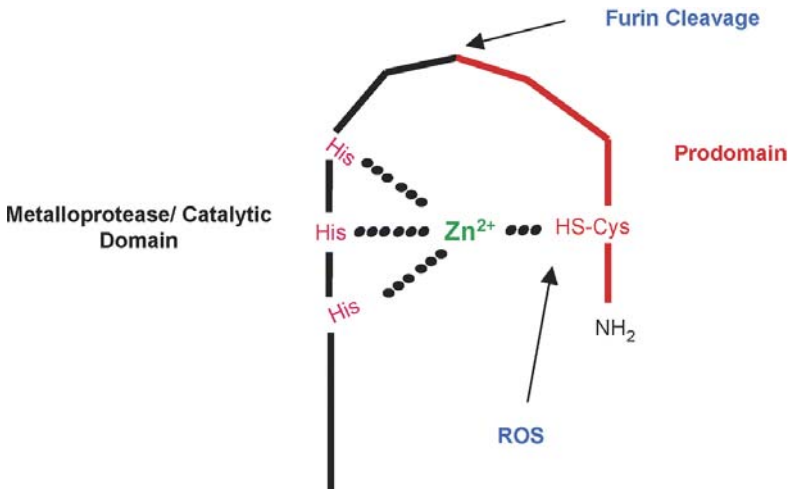


Figure 10. Mechanism of ADAM Activation (Sanderson et al. 2006)

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 species leading to the opening up of the structure, dissociating the catalytic domain from the prodomain.

A number of different subtypes of metalloprotease families including ADAMs, matrix metallproteases (MMPs), and membrane type matrix metalloproteases (MT-MMPs) have been implicated in the ectodomain shedding of transmembrane proteins including EGF like ligands. Metalloproteases are synthesized as catalytically inactive precursors that undergo maturation to an active form (Zhang et al. 2001; Sanderson et al. 2006).

MMPs belong to the matrixins family and are involved in degradation and remodeling of the extracellular matrix, development, wound healing, and in the pathology of hyperproliferative diseases such as arthritis and cancer (Chang and Werb 2001; Seals and Courtneidge 2003). MMPs have also been shown to cleave members of EGF ligand family and are therefore involved in EGFR transactivation (Suzuki et al. 1997; Yu et al. 2002; Roelle et al. 2003). The proteolytic activities of MMPs are controlled during activation from their precursors and are inhibited by the endogenous inhibitors α-macroglobulins and tissue inhibitor of metalloproteinases (TIMPs). TIMPs are the major endogenous regulators of MMPs, and also of ADAM activities in the tissue. Four homologous TIMPs have been identified to date (Crocker et al. 2004). Different reports show that TIMPs inhibit cell invasion *in vitro*, tumorigenesis, metastasis, and angiogenesis *in vivo* (Gomez et al. 1997).

1.4.3 Reactive Oxygen Species (ROS) in Signal Transduction

The most abundant forms of ROS produced inside a cell are superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (°OH). ROS are constantly produced during metabolic reactions and upon external stimuli like RTK activation, GPCR stimulation, and UV or ionizing radiation (Finkel 2003). ROS play a key role in signal transduction as secondary messengers (Lambeth 2004; Rhee et al. 2005). ROS susceptible proteins are reversibly oxidized at cysteine residues existing in the thiolate anion form (Cys-S^-) at physiological pH. A variety of proteins have been identified containing susceptible cysteine residues, and oxidation of cysteine can lead to activation or inhibition of their catalytic activity.

Protein tyrosine phosphatases (Meng et al. 2002), lipid phosphatases (Leslie et al. 2003; Kwon et al. 2004), peroxiredoxins (Rhee et al. 2005), SUMO modifying proteins (Bossis and Melchior 2006) and MAP kinase phosphatase (Kamata et al. 2005) have been shown to be inhibited by ROS. Additionally, ADAMs, Src kinase (Giannoni et al. 2005), and G proteins of $G\alpha_{i/o}$ subtype have been shown to be activated by reactive oxygen species (Nishida et al. 2000; Zhang et al. 2001; Nishida et al. 2002). Redox signaling is very important for normal functioning as imbalance in a cell or organism leads to numerous disease states including Alzheimer's (Butterfield and Boyd-Kimball 2004), aging (Storz 2006), and cancer (Benhar et al. 2002).

1.4.4 Growth Factor Stimulated ROS Production: NADPH Oxidases

Phagocytic NADPH oxidase catalyzes the production of ROS, which is used by phagocytic immune cells to kill pathogens. NADPH oxidase is a multicomponent enzyme, with the catalytic subunit gp91phox embedded in plasma membrane and various cytosolic regulatory units (Sumimoto et al. 2005). Recently, homologs of the catalytic subunit and regulatory subunits have been identified and are found to be expressed in various cell types (Cheng et al. 2001; Sumimoto et al. 2005). Activation of NADPH oxidases leads to an increase in the local concentration of O_2^- which could be further converted to H_2O_2 in an enzymatic or nonenzymatic way. H_2O_2 serves as an excellent secondary intracellular messenger because of its small size, ease of diffusion, ubiquitous presence, and rapid and transient nature of production. Since the initial observation that insulin induces the generation of H_2O_2 in adipose cells (May and de Haen 1979), the intracellular generation of this molecule has been detected in various nonphagocytic cells in association with receptor stimulation (Rhee et al. 2000; Lambeth 2002; Finkel 2003).

Recently, overexpression of Nox1 leading to increased ROS production has been demonstrated to induce transformation of NIH-3T3 cells and induced tumor formation in mice (Suh et al. 1999; Arnold et al. 2001; Arbiser et al. 2002). Additionally, other Nox isoforms have also been found to be overexpressed in various cancer types (Brar et al. 2003).

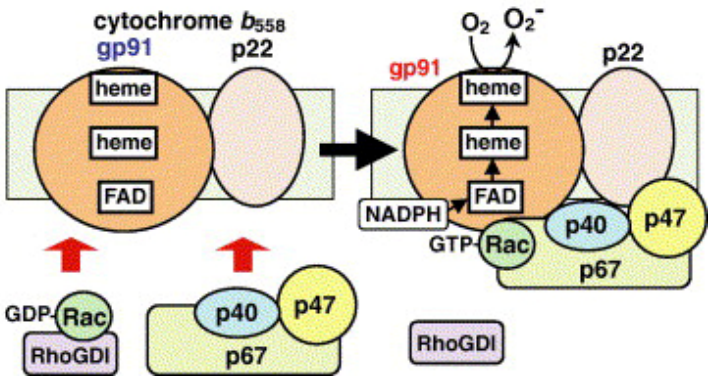


Figure 11. Subunit Organization of NADPH Oxidases.

Nox Catalytic unit is a membrane bound heterodimer, regulated by various cytosolic subunits.

1.5 Maintaining Mammalian Genome Integrity

Faithful copying of genetic material is very important both on individual and species levels as higher germline mutation rates are detrimental for species, and higher somatic mutations put an individual at risk to a variety of diseases like cancer. Internal sources of mutations are replication errors that arise from the inherent property of DNA bases to occasionally exist in isomeric (tautomers) forms, which have different base pairing properties; or misincorporation of nucleotides by DNA polymerases. External sources of mutations include environmental agents like ultraviolet radiation, cigarette smoke, incompletely defined dietary factors, reactive oxygen species, alkylating agents, and radiation etc. (Wood et al. 2001).

The repair mechanism can be classified into several distinct, if not completely independent, major pathways that differ with regard to the level at which the lesions in damaged DNA are reversed or removed by the repair machinery: (1) Reversal of base damage; (2) Excision of damaged, mispaired, or incorrect bases; (3) Strand break repair; and (4) Tolerance to DNA damage (Wolfram, Seide et al. 2006).

1.5.1 Direct Damage Reversal

O^6-methylguanine (O^6MeG) and O^4-methylthymine (O^4MeT) are the mutagenic lesions formed by alkylating agents like cisplatin or triethylenemelamine. These lesions are

repaired by specific methyltransferases, which accept methyl groups from nucleotides in a 1:1 non-catalytic ratio. Photoreactivation is another direct damage reversal mechanism repairing UV induced pyrimidine dimmers, which requires light (>300 nm) and DNA photolyase, but this activity is absent in placental mammals (Wood et al. 2001).

1.5.2 Excision of Damaged, Mispaired, or Incorrect Bases

Base Excision Repair (BER)

The BER pathway repairs deaminated, alkylated or oxidized base lesions induced by reactive oxygen species or by ionization through UV radiation (Slupphaug et al. 2003). Different DNA glycosylases recognize different oxidized bases and cleave them from the sugar-phosphate backbone. Most DNA glycosylases also have lyase activity and cleave the phosphodiester bond. Human AP endonuclease (APE1) then generates 5'-P and 3'-OH required for further steps of polymerization and ligation. During "short patch repair", this single nucleotide gap is filled by DNA polymerase β followed by ligation by ligase III/XRCC1 complex (Ide and Kotera 2004). Alternatively, in "long patch repair", 2-8 nucleotides are added by polymerase δ via strand displacement followed by incision of a flap structure containing lesion by FEN1, and the nick is sealed by ligase I. Spontaneous depurination is fairly common in mammals and is frequently repaired by the BER pathway.

Nucleotide Excision Repair (NER)

Nucleotide excision repair pathway removes a broad range of structurally unrelated bulky lesions (Friedberg Walker et al. 2006). NER consists of four basic steps: (1) recognition of DNA damage; (2) excision of an oligonucleotide of 24-32 residues containing the damaged DNA; (3) filling of the resulting gap by DNA polymerase; and (4) ligation of the nick (Sancar 1996). Global genome repair (GGR), a form of NER, repairs severe distortions like 6-4 photoproducts immediately in transcriptionally silent DNA. A second form of NER associated with stalled RNA polymerase II mediated transcription is referred to as transcription coupled repair (TCR). TCR removes lesions in transcriptionally silent DNA by making incisions on either side of it (bimodal incision).

In alternative excision repair (AER), only one incision is formed near the lesion (unimodal incision) (Wolfram, Seide et al. 2006).

Mismatch Repair (MMR)

MMR primarily removes mismatched bases and insertion-deletion loops formed during replication. In the *E. coli* MMR pathway, MutH cleaves the newly synthesized strand at a GATC site that can be located on either side of the mismatch. MutS and MutL help load helicase II in a biased fashion so that it unwinds towards the mismatch. Excision subsequently removes the mismatched DNA spanning the two sites, with ensuing repair synthesis initiated near the GATC site or the mismatch, depending on the polarity of the unmodified strand (Grilley et al. 1993). A majority of proteins of MMR are conserved, except MutH, suggesting an alternative recognition mechanism for the newly synthesized strand.

1.5.3 Repair of Strand Breaks

Single Strand Break (SSB) Repair

Sources of SSBs are ROS mediated base damage, destruction of deoxyribose residues, and accidental uncoupling of topoisomerase I from DNA (Wood et al. 2001). Unprotected SSBs are highly mutagenic and recombigenic, and are protected by poly(ADP-ribose) polymerase-1 (PARP1) binding (Wood et al. 2001). SSBs are processed and rejoined by enzymes involved in later stages of BER, sometimes with the additional steps of exonucleolytic removal of frayed ends and phosphorylation of 5'-termini by DNA kinase. The XRCC1 protein plays an important role in SSBR by recruiting various enzymes involved in end processing and gap filling at the lesion site like mammalian polynucleotide kinase, PARP1, Tdp1, APE1, DNA polymerase β, and DNA ligase IIIα (Kubota et al. 1996; Cappelli et al. 1997; Masson et al. 1998; Whitehouse et al. 2001; Plo et al. 2003).

Double Strand Break Repair

Homologous recombination (HR) is a highly conserved and error free pathway for the repair of double strand breaks. HR occurs during and after DNA replication, when the sister chromatid is available as a template to repair the damaged DNA strand. Double

strand breaks activate Ataxia Telangiectasia Mutated (ATM) kinases, which leads to activation of a set of exonucleases that generate single stranded 3' overhangs. Rad51 covers 3' overhangs depending on BRCA1 (breast cancer related antigen) and BRCA2. This nucleoprotein filament then with the help of accessory proteins, searches for the homologous duplex DNA sequence on the sister chromatid, and invades it. The later steps of the process include polymerization of nucleotides to restore degraded DNA strands and resolution of the recombination intermediates (van Gent et al. 2001).

1.5.4 Tolerance to DNA Damage

Translesion DNA Synthesis (TLS)

DNA polymerase η, ζ, κ, and ι lack endonuclease subunits and are able to synthesize DNA through distorted DNA without the need for normal hydrogen bonding, and are important mediators of translesion DNA synthesis (Kunkel et al. 2003). TLS polymerases are involved in DNA synthesis opposite to pyrimidine dimers (Polη and Polζ), 8-oxoG DNA lesions (Polη), AP sites (Polη, Rev1, and Polδ), and mismatched primer termini (Prakash and Prakash 2002; Larsen et al. 2005). TLS presents a fast alternative to the classical error free but slower NER, BER, and MMR pathways.

Postreplicative Gap Filling

Another class of damage tolerance mechanism is variously referred to as postreplicational repair, postreplicative gap filling, or postreplication recombinational repair. This mechanistic class is characterized by DNA template switching and/or recombination strategies that obviate the need to replicate directly across sites of base damage, hence avoiding generation of mutations. (Friedberg et al. 2005). This pathway can also bypass interstrand cross-links where excision repair cross complementation group1 (ERCC1) and xeroderma pigmentosum complementation group F (XPF) play important roles in mammals.

Replication Fork Regression

In this model a replication fork arrested at a lesion migrates backwards (fork regression) rearranging the strands into a variant of Holliday junction often referred to as chicken foot structure (Postow et al. 2001). Pairing with the newly synthesized strand permits the

extension of the daughter strands that had previously been blocked by the lesion. Following this, migration of the fork in the opposite direction (reverse regression) could restore the normal replication cycle, leaving the lesion on one of the replicated strands (Friedberg Wolfram et al 2006).

Non-Homologous End Joining

Highly toxic double strand breaks are removed very soon by joining DNA ends with the deletion of several base pairs. Restricted DNA movement in the nucleus generally favors joining of correct ends; occasionally, however, broken ends from different chromosomes are joined together, leading to chromosomal translocation. Shortly, DSBs are complexed with the KU70/80 heterodimer, and DNA PKc, followed by unwinding and end processing by removal of a few nucleotides probably by the RAD50-MRE11-NBS1 complex. This is followed by an end-to-end ligation performed by the DNA ligase IV-XRCC4 complex (van Gent et al. 2001). The balance between competing error free homologous recombination and error prone NHEJ is apparently influenced by the relative amounts of RAD52 and Ku (Van Dyck et al. 1999).

1.5.5 DNA Damage Checkpoints

DNA damage checkpoints are associated with biochemical pathways that end, delay, or arrest cell cycle progression. Proteins involved in DNA damage checkpoints can be classified on the basis of their functions into sensors, mediators, transducers, and effectors (Niida and Nakanishi 2006).

DNA damage checkpoints engage damage sensor proteins, such as the Rad9-Rad1-Hus1 PCNA like complex, or Rad17-RFC complex in an ATM independent manner. Mediators serve as protein-phosphorylation interaction modules and are recruited to lesion sites depending on phosphorylation of γH2AX by ATM (Niida and Nakanishi 2006). In mammals, signals initiated by the sensors are transduced rapidly to ATM, ATR, Chk1, and Chk2 kinases. These transducer molecules phosphorylate a great number of substrates including BRCA1, NBS1, Chk2, p53, Cdc25A, B, C, and Wee1 etc. Chk1 and Chk2 kinases regulate the effectors Cdc25, Wee1, and p53 that ultimately inactivate

cyclin-dependent kinases (Cdks), leading to cell cycle blockage thus giving time for repair.

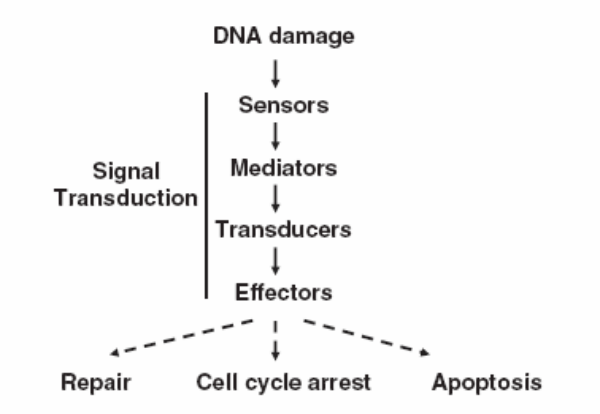


Figure 12. Conceptual Organization of DNA Damage Signaling (Niida and Nakanishi 2006)

Sensor proteins recognize DNA damage. The signals are transmitted to tranducers (mainly kinases) and the regulated transducer molecules suppress effector kinases, such as Cdks and Cdc7, thereby arresting the cell cycle at the specific phases.

1.5.6 Damaging Effects of UV

It has been estimated that under the strong sunlight typically encountered on a beach, an exposed cell in the human epidermis develops about 40,000 damaged sites in one hour, primarily from absorption of UV radiation by DNA (200–320 nm) (Ura and Hayes 2002). UV exposure accounts for approximately 65% of melanomas and 90% of basal and squamous cell carcinomas (Armstrong and Kricker 1993; Ziegler et al. 1996). UV directly leads to formation of mutagenic cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts (6–4PPs) in a 3:1 ratio under UVC radiation (Mitchell and Nairn 1989). UV also leads to production of ROS, which further induce oxidation of bases, single strand break formation, DNA cross-links and purine photoproducts (Marmur and Grossman 1961; Rosenstein and Ducore 1983; Duker and Gallagher 1988; Black et al. 1997).

The UV radiation spectrum has been divided into three segments designated UVA (320-400 nm), UVB (295-320 nm), and UVC (100-295 nm). UVC cannot penetrate the ozone layer, so only UVA and UVB reach the earth surface. However, most studies with UV are done with UVC light sources which are readily available and are more efficient in inducing lesions in DNA compared to longer wavelength radiations (Setlow 1974).

Aim of the Study

The aim of this study is to investigate Receptor Tyrosine Kinase transactivation and cross talk with other signaling molecules and their potential role in human skin cancer. To address this question, three different objectives are of special interest.

1. UV exposure leads to skin cancer. Additionally, a variety of skin cancers show overexpression of EGFR family members. Different cell lines covering majority of the cell types from skin were used to study the ligand dependent EGFR transactivation upon UV irradiation. We also studied UV induced EGFR transactivation dependent cellular responses, which are associated with a malignant phenotype such as proliferation or anti-apoptosis.

2. ROS production has been shown both in response to GPCR stimulation, RTK activation, and UV irradiation. ROS involvement in GPCR and UV induced RTK transactivation was investigated. Both upstream and downstream RTK signaling was observed in the context of reactive oxygen species.

3. In the end, various strategies aimed at blocking UV induced EGFR transactivation were compared for their effectiveness to kill cancer cells under UV irradiation. Evolution of RTK transactivation during cancer progression was investigated using primary and secondary melanoma pairs.

2 Materials and Methods

2.1 Materials

2.1.1 Laboratory Chemicals and Biochemicals

Acrylamide	Serva, Heidelberg
Agar	Difco, Detroit, USA
Agarose	BRL, Eggenstein
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
Coomassie G250	Serva, Heidelberg
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
Dideoxynucleotides (ddG/A/T/CTP)	Pharmacia, Freiburg
Diphenyleneiodonium chloride (DPI)	Alexis, Grünberg
DTT (Dithiothreitol)	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Fibronectin	Calbiochem, Bad Soden
GF-109203X	LC Laboratories, Grünberg
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®	Gibco, Eggenstein
MBP (Myelin basic protein)	Sigma, Taufkirchen
Mineral oil	Sigma, Taufkirchen
MOPS (3-Morpholinopropanesulfonic acid)	Biomol, Haub
Oligofectamine®	Invitrogen, Karlsruhe
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
Polybrene (Hexadimethrine bromide)	Sigma, Taufkirchen
PD98059	Alexis, Grünberg
Ponceau S	Sigma, Taufkirchen
PP1	Calbiochem, Bad Soden
PTX (Pertussis Toxin)	List, Campbell, USA
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

Scintillation cocktail (Rotiszint®ecoplus)	Roth, Karlsruhe
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
TPA (Tetradecanoyl-phorbol-13-acetate)	Sigma, Taufkirchen
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

Alkaline Phosphatase	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 Radiochemicals

[γ-^{32}P] ATP >5000 Ci/mmol

[a-33P] dATP 2500 Ci/mmol

L-[35S] Methionine >1000 Ci/mmol

All radiochemicals were obtained from PerkinElmer Life Sciences, Köln.

2.1.4 "Kits" and Other Materials

Cell culture materials	Greiner, Solingen
	Nunclon, Denmark
	Falcon, U.K.
Cellulose nitrate 0.45 µm	Schleicher & Schuell, Dassel
ECL Kit	PerkinElmer, Köln

Glutathione-Sepharose	Pharmacia, Freiburg
Hyperfilm MP	Amersham, USA
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
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
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
Transwells	Corning, New York, USA
Whatman 3MM	Whatman, USA

2.1.5 Growth Factors and Ligands

Anisomycin	Calbiochem
Angiotensin II	
Amphiregulin	R&D Systems
Bradykinin	Calbiochem
Carbachol	
EGF (murine)	Toyoba, Japan
Endothelin I	
LPA	Sigma, Taufkirchen
Neurotensin	
Sphingosine-1-phosphate	
Thrombin	Sigma, Taufkirchen
TPA	

All other growth factors and ligands were purchased from Sigma.

2.1.6 Media and Buffers

2.1.6.1 Media for E. coli

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 autoclaving:

Ampicillin 100 µg/mL

Kanamycin 100 µg/mL

Chloramphenicol 30 µg/mL

LB-plates additionally contained 1.5% Agar.

2.1.6.2 Cell culture media

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

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

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

Nutrient mixture F12 (HAM) with L-glutamine.

MDCB153 medium

L-15 medium

MEM alpha medium

Freeze medium: 90% heat-inactivated FCS, 10% DMSO.

2.1.7 Stock Solutions and Buffers

BBS (2x)	50	mM	BES

	280	mM	NaCl
	1.5	mM	Na_2HPO_4
			pH 6.96 (NaOH)
HBS (2x)	46	mM	HEPES pH 7.5
	274	mM	NaCl
	1.5	mM	Na_2HPO_4
			pH 7.0
DNA loading buffer (6x)	0.25	%	Bromphenol blue
	0.25	%	Xylenecyanol
	30.0	%	Glycerol
	100.0	mM	EDTA pH 8.0
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
	0.05	%	Triton X-100
			pH 7.4 (HCl)
PBS	13.7	mM	NaCl
	2.7	mM	KCl
	80.9	mM	Na_2HPO_4
	1.5	mM	KH_2PO_4, pH 7.4 (HCl)

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
SSC (20x)	3.0	M	NaCl
	0.3	M	Sodium citrate
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

2.1.8 Bacterial Strains (E. coli)

E. coli	Description	Origin/Reference
DH5aF'	F'/endA1 hsd17 (rk-mk-),supE44,recA1, gyrA (Nal), thi-1, (lacZYA-argF)	Genentech, San Francisco, USA

2.1.9 Cell Lines

Cell Line	Description	Origin/Reference
C8161	Human metastatic amelanotic melanoma	R. Gillies

HaCaT	Spontaneously immortalized human keratinocytes	
HEK-293	T Human embryonic kidney fibroblasts, transformed with adenovirus type V DNA	ATCC CRL-1573
Hs 294T	Lymph node melanoma	ATCC, HTB-140
Hs 695T	Lymph node amelanotic melanoma	ATCC, HTB-137
RPMI 7951	lymph node malignant melanoma	ATCC, HTB-66
SCC-9	Human squamous cell carcinoma of the tongue	ATCC CRL-1629
WM 115	melanoma derived from primary tumor site	ATCC, CRL-1675
WM 1205	cell line established from WM 793	M. Herlyn
WM 239A	secondary melanoma	M. Herlyn
WM 266-4	secondary melanoma	M. Herlyn
WM 793	primary melanoma	M. Herlyn

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

2.1.10 Antibodies

The following antibodies were used in immunoprecipitation experiments, as primary antibodies in immunoblot analysis or for staining of cell surface proteins in FACS analysis.

Antibody	Description/Immunogen	Origin/Reference
P-Tyr (4G10)	Mouse, monoclonal; recognizes phospho-(3)-tyrosine residues	UBI, Lake Placid
EGFR	Sheep, polyclonal/part of cytoplasmic domain of the human EGFR	UBI
EGFR (108.1)	Mouse, monoclonal/ectodomain of the human EGFR	(Daub et al., 1997)
HER2/neu	Rabbit, polyclonal/C-terminal peptide of human HER2/neu	(Daub et al., 1996)
Akt1/2	Rabbit, polyclonal/AA 345-480 of human Akt1	Santa Cruz, USA
	SHC Mouse, monoclonal	Santa Cruz

P-ERK	Rabbit, polyclonal; recognizes phospho-p44/p42 (Thr-202/Tyr-204) MAPK	NEB, Frankurt/M.
P-p38	Rabbit, polyclonal; recognizes phospho-p38 (Thr-180/Tyr-182) MAPK	NEB
P-Akt/PKB	Rabbit, polyclonal; recognizes phospho-Akt (Ser-473)	NEB
HB-EGF	Goat, polyclonal/recombinant, human HB-EGF	R&Dsystems, Weisbaden
AR	Goat, polyclonal/recombinant, human AR	R&D Systems
TGFα	Goat, polyclonal	R&D Systems
EREG	Goat, polyclonal	R&D Systems
Betacellulin	Goat, polyclonal	R&D Systems
ERK2 (C-14)	Rabbit, polyclonal/peptide at C-terminus of rat ERK2	Santa Cruz
ERK2 (K-23)	Rabbit, polyclonal/peptide from sub-domain XI of rat ERK2	Santa Cruz
Pan-ERK	Mouse monoclonal/AA 219-358 of human ERK2	Transduction Lab.
HA	Mouse, monoclonal; recognizes the influenza hemagglutinin epitope	Babco, California, USA
VSV (P5D4)	Mouse, monoclonal; recognizes an epitope of eleven AA derived from the vesicular stomatits virus glycoprotein VSV-G	Roche, Mannheim
Cyclin D1	Mouse, IgM, monoclonal, corresponding to amino acids 1-200	Transduction Lab
p38 (C-20)	Rabbit, polyclonal/peptide at C-terminus of murine p38	Santa Cruz
p21	Mouse, monoclonal	Santa Cruz
p27	Rabbit, polyclonal	Santa Cruz
PARP	Rabbit, polyclonal	Santa Cruz
Caspase3	Rabbit, polyclonal	Santa Cruz

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

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

The FITC-conjugated rabbit anti-goat secondary antibody for flow cytometry was obtained from Sigma.

2.2 Methods in Mammalian Cell Culture

2.2.1 General Cell Culture Techniques

Squamous cell carcinoma, melanoma cell lines, and keratinocytes 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). For subculturing cells were washed once with PBS and trypsinized for appropriate time in incubator. Cells were harvested by spinning in a tabletop centrifuge at 2000 rpm. Before seeding cells were counted with a Coulter Counter (Coulter Electronics). Cells were cultured in the medium recommended by the manufacturer.

2.2.2 Transfection of Cultured Cell Lines

2.2.2.1 Transfection of Cells with Calcium Phosphate

HEK-293 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 previously (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_2O	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 adequate 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 container at 3% CO_2 overnight. One day following 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.

2.2.2.2 RNA Interference

Transfection of 21 nucleotide siRNA duplexes (Dharmacon Research, Lafayette, CO; and Ambion inc. Austin, Texas) for targeting endogenous genes was carried out using Lipofectamine 2000 (Invitrogen) and 4,2 µg of siRNA duplex per 6-well plate as previously described (Elbashir, Harborth et al. 2001). Transfected SCC-9 cells were serum starved and assayed 2 days after transfection. Sequences of siRNAs used have been described before (Gschwind et al. 2003). siRNAs against ADAM9 and ADAM12 were purchased from Ambion. Specific silencing of targeted genes was confirmed by western blot (TACE) and RT-PCR analysis.

2.3 Protein Analytical Methods

2.3.1 Lysis of Eukaryotic Cells with Triton X100

Prior to lysis, cells grown to 80% confluence were treated with inhibitors and agonists as indicated in the figure legends. Cells were washed with cold PBS and then lysed for 30 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 15 min at 4°C.

2.3.2 Lysis of Eukaryotic Cells with RIPA Buffer

Cells were grown to 80% confluence and treated with inhibitors and agonists as indicated in figure legends. Cells were washed with cold PBS and then lysed for 30 minutes on ice in RIPA buffer with the composition Tris-HCl: 50 mM, pH 7.4; NP-40: 1%; Na-deoxycholate: 0.25%; NaCl: 150 mM; EDTA: 1 mM; PMSF: 1 mM; aprotinin, leupeptin, pepstatin: 1 µg/ml each; sodium orthovanadate: 1 mM; sodium fluoride: 1 mM. Lysates were precleared by centrifugation at 13000 rpm for 15 minutes at 4°C.

2.3.3 Determination of Protein Concentration in Cell Lysates

The "Micro BCA Protein Assay Kit" (Pierce, Sankt Augustin) was used according to the manufacturer´s recommendations.

2.3.4 Immunoprecipitation and *in vitro* Association with Fusion Proteins

An equal volume of HNTG buffer was added to the precleared cell lysates that had been adjusted for equal protein concentration. Proteins of interest were immunoprecipitated using the respective antibodies and 20 µL of protein-A-Sepharose for atleast 4 h at 4°C with continuous gentle rotation. Precipitates were washed three times with 0.5 mL of HNTG buffer, suspended in 2× SDS sample buffer, boiled for 3 min, spun down to remove the beads, and subjected to SDS-PAGE analysis.

2.3.5 SDS Polyacrylamide Gel Electrophoresis

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	Carbonic anhydrase	29.0
Phosphorylase b	97.4	Trypsin-Inhibitor	21.5

BSA	66.2	Lysozyme	14.4

2.3.6 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.

2.3.7 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% gelatin for at least 4 h. The membrane was then probed with primary antibody 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 or a digital camera unit. 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.

2.4 Biochemical and Cell Biological Assays

2.4.1 Stimulation of Cells

Cells were seeded in cell culture dishes of appropriate size and grown overnight to about 80% confluence. After serum-starvation for 48 h bladder and kidney cancer cells were treated with inhibitors and agonists as indicated in the figure legends, washed with cold PBS and then lysed for 30 min on ice.

2.4.2 ERK1/2 and AKT/PKB Phosphorylation

For determination of ERK1/2 and Akt phosphorylation, approximately 20 μg of whole cell lysate per 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. Quantitation of ERK1/2 was performed using the Luminescent Image Analyis System (Fuji). After quantitation of ERK1/2 phosphorylation, membranes were stripped of immunoglobulin and reprobed using rabbit polyclonal anti-ERK1/2 or rabbit polyclonal anti-Akt antibody to confirm equal protein loading.

2.4.3 ERK/MAPK Activity

Endogenous ERK2 was immunoprecipitated from lysates obtained from six-well dishes using 0.4 μg of anti-ERK2 antibody. Precipitates were washed three times with HNTG buffer, and washed once with kinase buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 200 μM sodium orthovanadate). Kinase reactions were performed in 30 μL of kinase buffer supplemented with 0.5 mg/mL myelin basic protein, 50 μM ATP and 1 μCi of [γ-^{32}P]ATP for 10 min at room temperature. Reactions were stopped by addition of 30 μL of Laemmli buffer and subjected to gel electrophoresis on 15% gels. Labeled MBP was quantitated using a Phosphoimager (Fuji).

2.4.4 FACS Analysis for Cell Cycle Distribution and Apoptosis Detection

FACS analysis was performed as described before (Prenzel et al. 1999). In brief, cells were seeded, grown for 20 h. Overnight starved or unstarved cells were stimulated with indicated doses of UV and harvested 20 hrs post irradiation by trypsinization. Cells were collected by spinning them down at 2000 rpm in tabletop cooling centrifuge. Cells were resuspended in hypotonic citrate buffer (0.1% Triton X-100, 0.1% sodium citrate, 20 μM propidium iodide) and incubated in dark for 2 hr. FACS analysis was done with FACScalibur (Becton Dickinson Biosciences). Sub G_0 population was counted as the apoptotic population and represented as fraction of the total cells counted. For cell cycle distribution different phases of cell cycle were separated on the basis of their nuclear content.

2.4.5 Incorporation of 3H-thymidine into DNA

Kidney or bladder cancer cells were seeded into 12-well plates (60000 cells per well). Upon serum deprivation for 48 h, cells were subjected to preincubation with inhibitors before ligand treatment. After 18 h incubation, cells were pulse-labelled with 3H thymidine (1 µCi/mL) for 4 h, and thymidine incorporation was measured by trichloroacetic acid precipitation and subsequent liquid-scintillation counting.

2.4.6 *In vitro* Wound Closure

The assay was performed as previously described (Fishman et al. 2001) with some modifications. Confluent monolayers of melanoma and carcinoma cells were wounded with a uniform scratch, the medium was removed and cells were washed twice with PBS. Medium without FCS was added and cells were subjected to 20 min preincubation with either DMSO (control), 250 nM AG1478 or 10 µM batimastat before ligand treatment. Cells were permitted to migrate into the area of clearing for 24 h. Wound closure was monitored by visual examination using a Zeiss microscope.

2.4.7 Migration and Invasion

Cell migration assays were performed using a modified Boyden chamber (Sieuwerts et al. 1997). Serum free medium containing LPA/serum as a chemoattractant was added to the lower well of a Boyden chamber. A polycarbonate filter (6.5 mm in diameter, 8 µm pore size) was placed over the lower well of the Boyden chamber and was secured with a gasket. $1x10^5$ cells in exponential growth were harvested and then preincubated with the inhibitor for 20 min and added to the upper well of the chamber in serum free medium. The chambers were incubated for 6 h in a humidified 7% CO_2, 37°C incubator. Finally, the cells that had migrated to the lower surface of the membrane were stained with crystal violet and counted under the microscope.

Analysis of cell motility was performed in 24-transwell dishes. Cells were permitted to migrate for 24h. Cells that had migrated to the lower surface were fixed with methanol and stained with crystal violet. The stained cells were solubilized in 10 % acetic acid, and the absorbance at 570 nm was measured in a micro-plate reader. Experiments done with several, individual clones showed similar results. Cell invasion assays were also

performed in modified Boyden chambers containing a polycarbonate filter coated with Matrigel on the upper surface (Sieuwerts et al. 1997).

As described above the chemoattractant was added to the lower well and $1x10^6$ cells were preincubated with the inhibitor and then added to the upper well. The chambers were incubated overnight. Finally cells were wiped from the upper surface with a cotton tip swab and the cells on the other side were stained and counted under the microscope.

2.5 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 UVC Induces Epidermal Growth Factor Receptor (EGFR) Transactivation and Activates Downstream Signaling

3.1.1 UV Induces EGFR Phosphorylation in a Time Dependent Manner

To investigate the effect of UV irradiation on skin cells, we started with two cell lines: C8161 (secondary melanoma), and HaCaT (immortalized keratinocytes), originating from melanocytes and keratinocytes, respectively, representing the major constituent cells of the epidermis. In C8161 cells (Fig. 13A) we found an increase in EGFR phosphorylation upon UV treatment compared to untreated control cells. pEGFR levels were seen as early as 10 minutes post-stimulation and remained high for upto 2 hours, returning to lower levels after 6 hours. HaCaT cells showed similar effects (Fig. 13B) where UV irradiation led to EGFR phosphorylation starting at 10 minutes post UV irradiation, and returning to basal levels after 6 hours.

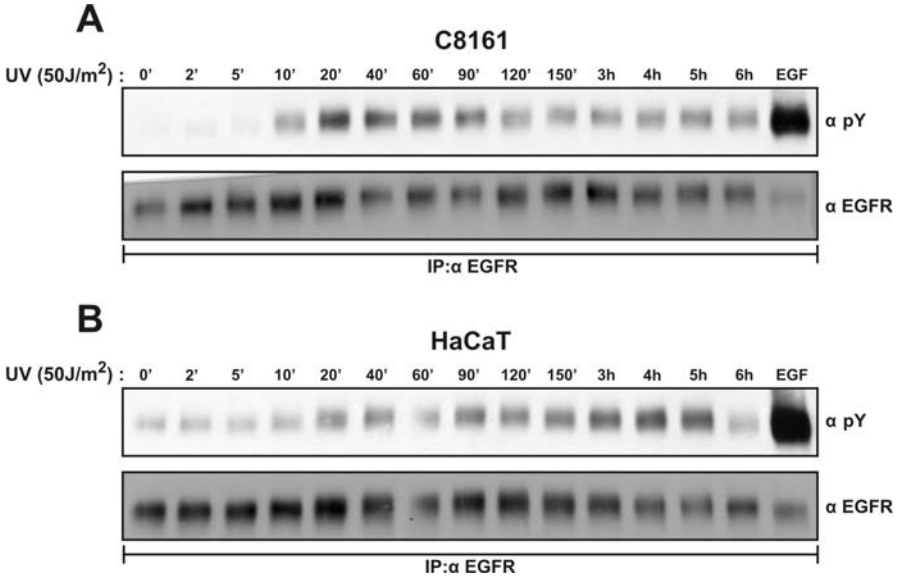


Figure 13. UVC Irradiation Induces Phosphorylation of EGFR in a Time Dependent Manner.

(A) C8161 cells were seeded at 180,000 cells/6cm plate. Cells were treated with 50J/m^2 UV 24 hours after serum starvation, and lysed at the indicated time points. Lysates were immunoprecipitated (IP) for EGFR, blotted, probed for pY and reprobed for EGFR. EGF treatment (5 ng/ml) was taken as positive phosphorylation control. (B) HaCaT cells were seeded at 400,000 cells/6cm plate and treated as in (A).

3.1.2 UVC Induces EGFR Phosphorylation in a Dose Dependent Manner

C8161 and HaCaT cells were treated with increasing doses of UVC irradiation (0-500 J/m^2) for 15 minutes. In both C8161 (Fig. 14A) and HaCaT cell lines (Fig. 14B) we observed that EGFR phosphorylation increases with increasing dose of UV irradiation at lower doses of up to 100 J/m^2. For doses above 100 J/m^2 the increase in EGFR phosphorylation was much less prominent. So in our study 0-100 J/m^2 of UV dose represents the most responsive phase. Notably, the 10-20 minutes time period is also the most dynamic range for observing EGFR phosphorylation under UV stress.

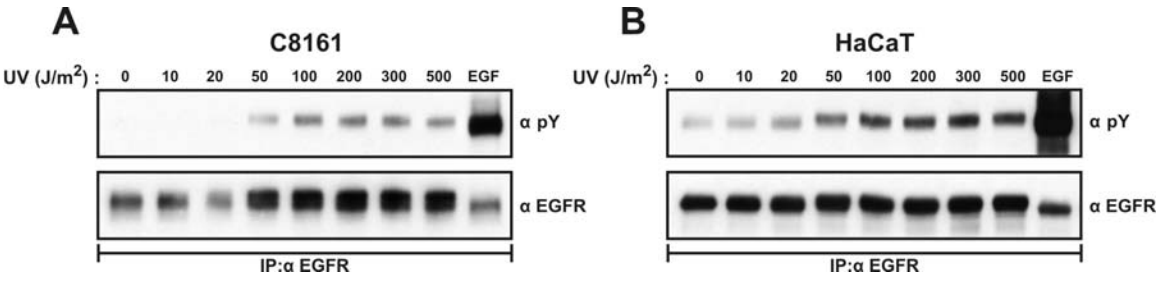


Figure 14. UVC Induces EGFR Phosphorylation in a Dose Dependent Manner

C8161 and HaCaT cells were seeded at 180,000 and 400,000 cells/6cm plate, respectively. Serum starved cells were treated with indicated doses of UVC (J/m^2), and lysed 15 minutes after irradiation. Lysates were immunoprecipitated (IP) for EGFR, blotted, probed for pY and reprobed for EGFR.

3.1.3 UVC Irradiation Leads to the Activation of Signaling Molecules Downstream of EGFR

EGFR activation is coupled to the activation of downstream signaling molecules, which mediate most of the cellular processes, with Erk and Akt being the most frequent downstream molecules. The activation of downstream signaling molecules Erk and Akt was observed in a UV dependent time course. C8161 cells also showed similar phosphorylation of Erk and Akt (Fig. 15A). In HaCaT cells, Erk and Akt phosphorylation appeared after 15 minutes, with Erk phosphorylation visible up to 1 hour and Akt phosphorylation up to 4 hours (Fig. 15B). In another experiment, UV led to a dose dependent increase in phosphorylation of Erk and Akt in HaCaT cells (Fig. 15C), with their phosphorylation appearing at 20 J/m^2 and 50 J/m^2, respectively.

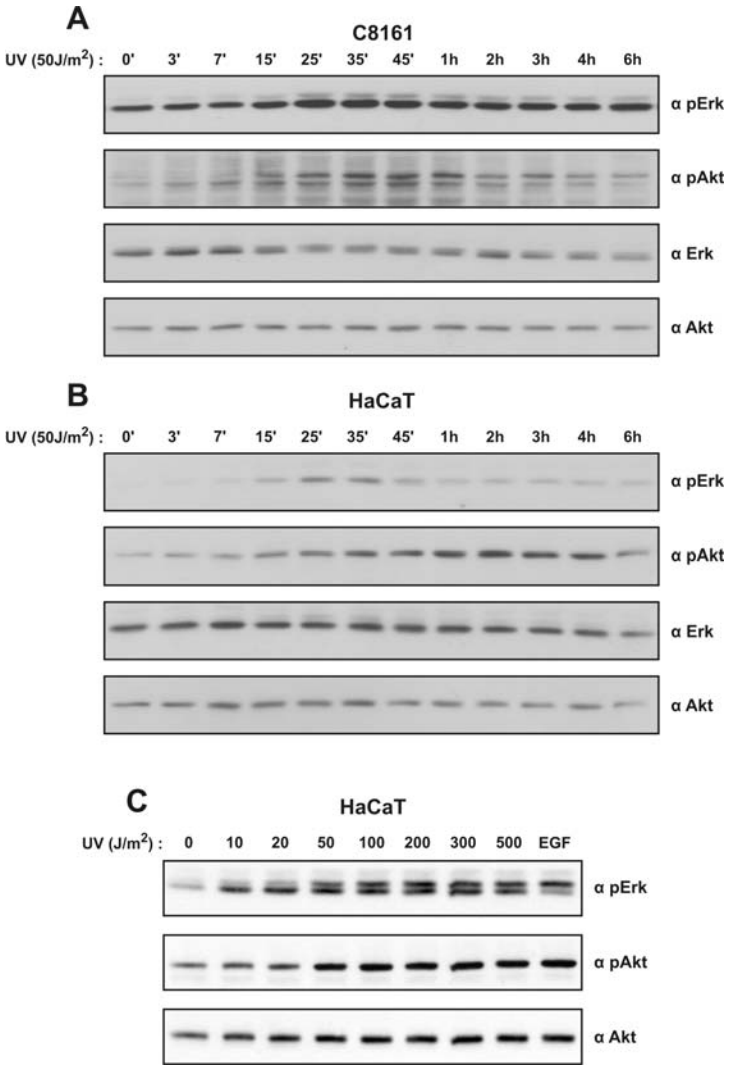


Figure 15. UVC Induced Erk and Akt Phosphorylation in a Time and Dose Dependent Manner.

(A) C8161 cells were seeded at 180,000 cells/6cm plate. Cells were treated with 50J/m^2 UV after serum starvation for 24 hours, and lysed at the indicated time points. Equal amounts of lysates were blotted and probed for pErk, pAkt and reprobed for total Erk and Akt. (B) HaCaT cells were seeded at 400,000 cells/6cm plate and treated as in (A). (C) HaCaT cells were seeded and starved as in (B) and irradiated with indicated UVC doses, and lysed 15 minutes after stimulation. Samples were further processed for immublotting as in (A).

3.1.4 Phosphorylation of EGFR by UV can be Blocked by the Metalloprotease Inhibitor BB94

The metalloprotease inhibitor BB94 is a broad range inhibitor of the ADAM subfamily of metalloproteases. ADAMs have been involved in EGFR transactivation. They are

responsible for cleavage of the EGF family proligands releasing the active form of ligand, which binds to and activates EGFR. Preincubation of C8161 and HaCaT cells with BB94 followed by UV treatment led to decreased phosphorylation of EGFR as compared to UV treatment alone (Fig. 16A, C). Solvent control DMSO did not show any effect on UV induced phosphorylation (Fig. 16A, C). Another secondary melanoma cell line, RPMI7951 (Fig. 16B) and a squamous cell carcinoma cell line, SCC-9 (Fig. 16D), showed a similar inhibition of UV induced phosphorylation of EGFR upon BB94 preincubation. Phosphorylation of EGFR could be brought down to basal levels in RPMI7951 (Fig. 16B) and HaCaT (Fig. 16C) cells, demonstrating a metalloprotease mediated mode of EGFR activation in these cell lines under these experimental conditions. For C8161 (Fig. 16A) and SCC-9 (Fig. 16D) cells, where EGFR phosphorylation was not completely inhibited by BB94, other mechanisms like PTP inactivation may also be involved (Gross et al. 1999).

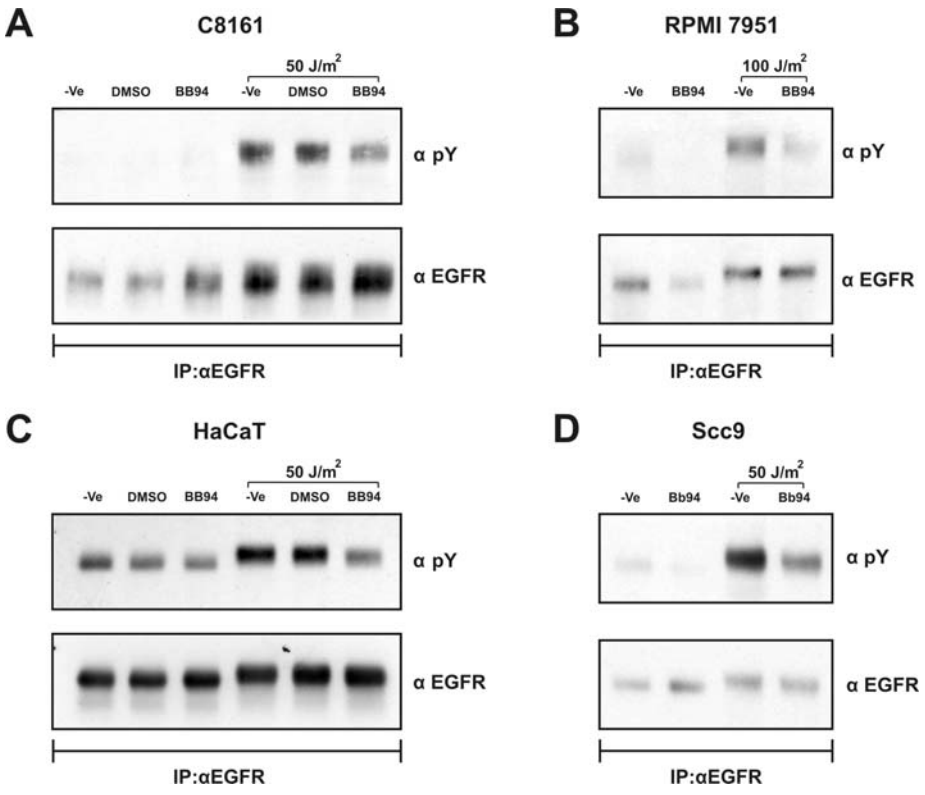


Figure 16. Metalloprotease Inhibitor (BB94) Reduces Phosphorylation of EGFR Induced by UV Irradiation

C8161 (A), RPMI7951 (B), HaCaT (C), and SCC-9 (D) cells were preincubated with BB94 (10 µM, 30 minutes) and treated with the indicated UV doses. Cells were lysed 15 minutes post irradiation, lysates were immunoprecipitated for EGFR, blotted and probed for pY and EGFR levels. DMSO was taken as solvent control.

3.1.5 UV Induced Activation of EGFR Downstream Signaling Molecules Can be Inhibited by BB94

Activation of the PI3K/Akt pathway and MAPK1/2 pathway has been shown to be linked to activation of members of EGFR family, either by direct binding to activated receptors or via adaptor molecules (Schulze et al. 2005). Fig. 15A, B, C showed phosphorylation of Erk and Akt in response to UV with activation seen shortly after phosphorylation of EGFR was observed. We further investigated if these pathways were linked to UV induced EGFR transactivation. Preincubation with BB94 reduced both pErk and pAkt levels in HaCaT (Fig. 17A) and SCC-9 (Fig. 17B) cells induced by UV. This experiment shows that the phosphorylation of Erk and Akt is dependent on the activation of EGFR after metalloprotease activation.

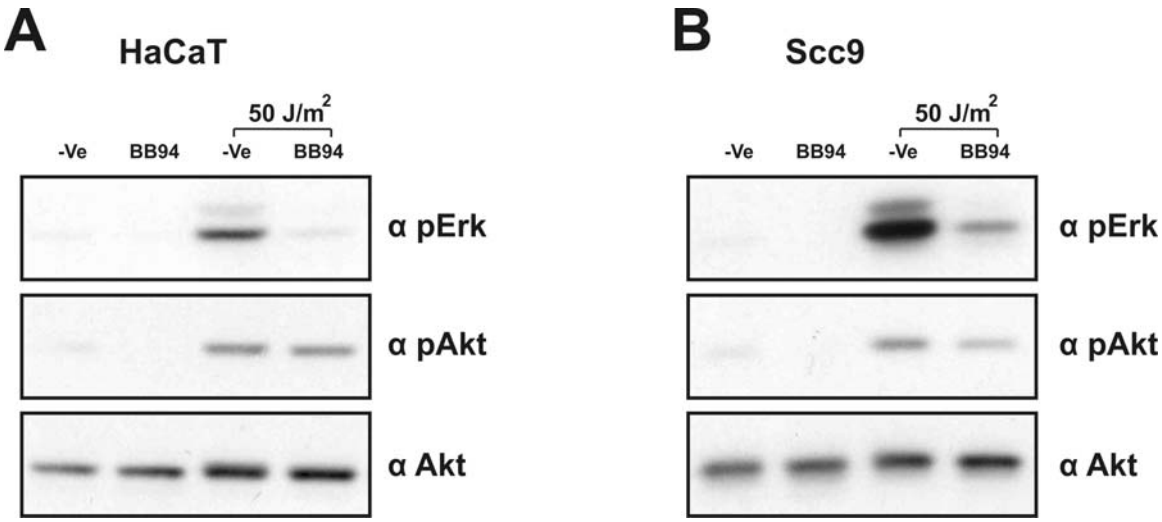


Figure 17. BB94 Abrogates EGFR Downstream Signaling Induced by UV

(A) Starved HaCaT and (B) SCC-9 cells were preincubated with BB94 (10 µM, 30 min) and treated with 50J/m^2 UV and lysed after 15 minutes. Lysates were blotted and membranes were probed for pErk and pAkt and reprobed for Akt as loading control.

Table 1 UV Induced EGFR Transactivation Sensitivity to BB94 (Immunoblot Analysis)

Cell line	Origin	pEGFR	pErk	pAkt
C8161	Melanocyte	+	+	+
HaCaT	Keratinocyte	+	+	+
RPMI7951	Melanocyte	+	+	+
SCC-9	Keratinocyte	+	+	+
WM1205	Melanocyte	+	+	+
HS 695T	Melanocyte	+	+	+

3.2 EGFR Transactivation Induced by UV is Dependent on Ligand Binding to the EGFR Extracellular Ligand Binding Domain

3.2.1 EGFR Extracellular Ligand Binding Domain is Required for UV Induced EGFR Transactivation

In EGFR transactivation pathway, metalloprotease activation leads to cleavage of membrane anchored proforms of ligands of the EGF ligand family, which then diffuses in the extracellular matrix and bind to the EGFR extracellular ligand binding domain (Prenzel et al. 1999). We investigated the role of an extracellular ligand binding event for EGFR activation under UV stress, to show it to be a pathway involving extracellular components. Ligand binding to the extracellular domain of monomeric EGFR leads to a conformational change exposing the dimerization domain, which bridges two monomers to form an active dimer (Schlessinger 2002). Antibodies directed against the extracellular domain of EGFR compete with EGFR ligand binding, shutting down the extracellular pathway of ligand induced EGFR activation (Goldstein et al. 1995). C8161 and HaCaT cells were preincubated with blocking antibodies followed by irradiation with 50 J/m^2 UV for 15 minutes. Preincubation with blocking antibodies led to a reduction in UV induced EGFR phosphorylation in both C8161 (Fig. 18A) and HaCaT (Fig. 18B) cell lines, showing that extracellular ligand binding is necessary for EGFR activation. Treatment with the control antibody did not affect the basal EGFR phosphorylation level

(Fig 18A, B, and cAb lane). Similarly, UV induced phosphorylation of EGFR was also unaffected by treatment with control antibody.

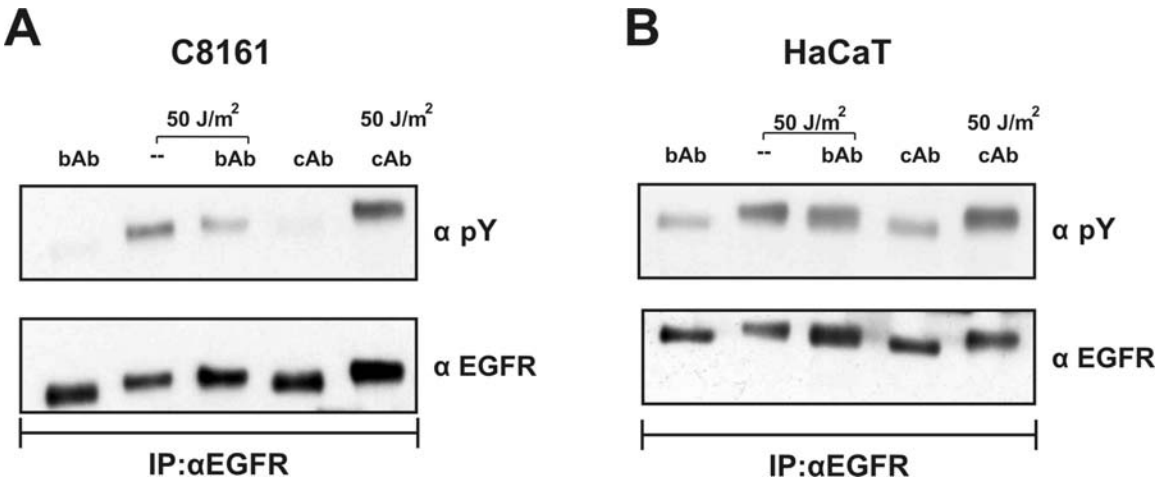


Figure 18. Effect of EGFR Blocking Antibodies on UV Induced EGFR Transactivation.

(A) C8161 cells were preincubated (1h) with blocking antibodies (bAbs) against the EGFR ligand binding domain, and control goat antibody (cAb). Cells were then treated with 50 J/m^2 and lysed after 15 minutes. Lysates were immunoprecipitated for EGFR, probed for pY, and reprobed for EGFR. (B) HaCaT cells were preincubated and treated as in (A), probed for pY and reprobed for total EGFR levels after EGFR immunoprecipitation.

3.2.2 UV induced EGFR transactivation and downstream signaling is dependent on the Proligand Amphiregulin

Seven of the eight EGF ligand family members are synthesized as membrane bound precursors and have been shown to be cleaved by metalloproteases to produce soluble ligands (Harris et al. 2003; Sahin et al. 2004). Expression levels of different members of the EGF ligand family were checked by RT-PCR in C8161 and HaCaT cells (Fig. 19A). Cells were preincubated with a cocktail of neutralizing antibodies against amphiregulin, TGFα, Hb-EGF, and a control polyclonal anti-goat antibody for 1 hour. There was a decrease in EGFR phosphorylation upon UV stimulation in samples pretreated with neutralizing antibody cocktail compared to samples pretreated with control anti-goat antibody or UV treated alone both in C8161 (Fig 19. B) and HaCaT (Fig. 19C) cells. These results show that EGFR phosphorylation by UV irradiation depends on proligand binding to EGFR in C8161 and HaCaT cells.

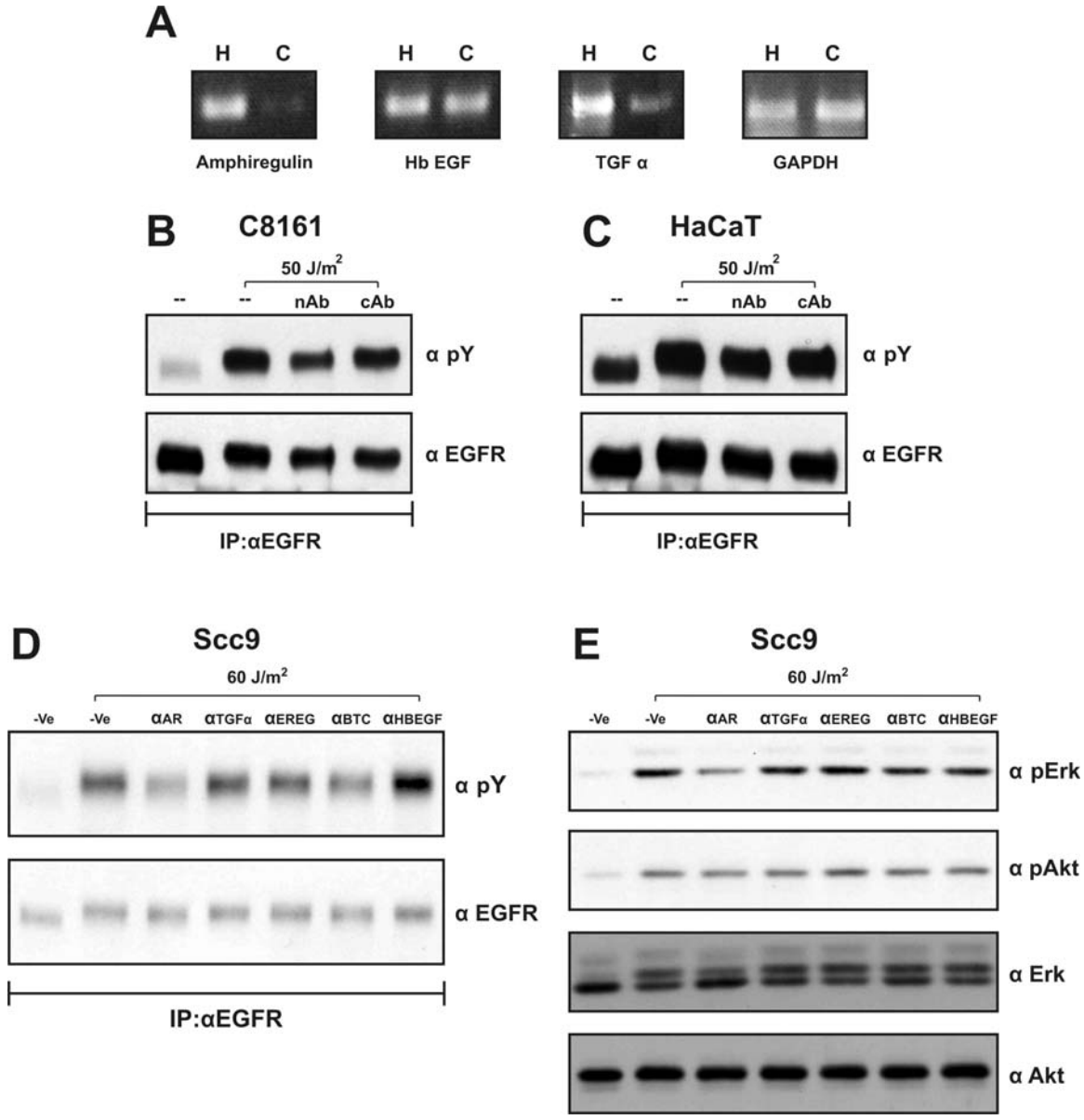


Figure 19. Finding the Proligand Involved in UV Induced EGFR Transactivation.

(A) cDNA extracted from C8161, C and HaCaT, H cells was subjected to RT-PCR analysis for amphiregulin, TGFα, and Hb-EGF. GAPDH was used as loading control. C8161 (B) and HaCaT (C) cells were preincubated with a cocktail of neutralizing antibodies against amphiregulin, TGFα, and Hb-EGF (nAb) and treated with 50 J/m^2 UV for 15 minutes. Secondary goat antibody was used as specificity control (cAb). (D) SCC-9 cells were pretreated for 1 hour with neutralizing antibodies against amphiregulin (AR), Transforming growth factor alpha (TGFα), epiregulin (EREG) betacellulin (BTC), and heparin binding EGF like growth factor (Hb-EGF). Cells were then treated with 60 J/m^2 UV and lysed after 15 minutes to test for pY and EGFR levels after EGFR immunoprecipitation. (E) SCC-9 cells were pretreated with blocking antibodies, stimulated with UV and lysed as in (D). Equal amounts of lysates were blotted and probed for pErk and pAkt; and reprobed for Erk and Akt.

We preincubated SCC-9 cells with neutralizing antibodies against individual proligands of the EGF family, followed by irradiation with 60 J/m^2 UV for 15 minutes. Preincubation with a neutralizing antibody against amphiregulin (AR) led to a reduction in UV induced EGFR phosphorylation (Fig.19D) compared to samples treated with other neutralizing antibodies of the EGF ligand family. Furthermore, UV induced Erk phosphorylation was also reduced upon preincubation with neutralizing antibodies against amphiregulin (Fig.19E). These results confirm that binding of amphiregulin to the EGFR extracellular ligand binding domain is responsible for UV induced EGFR transactivation in SCC-9 cells. Activation of downstream signaling molecules Erk and Akt is also dependent on amphiregulin binding to EGFR in SCC-9 cells.

3.3 Finding the Metalloprotease Responsible for Proligand Shedding During UV Induced EGFR Transactivation

Metalloproteases of the ADAM family are shown to shed EGF ligand family members. ADAM 9, -10, -12, 15, and -17 have been shown to be involved in EGFR transactivation (Fischer et al. 2003; Schafer et al. 2004). To identify possible candidates responsible for cleavage of proligand, expression levels of different ADAMs in C8161 and HaCaT were determined. RNA extracted from cells was used to synthesize cDNA which was analyzed for the expression of individual ADAMs by RT-PCR analysis. All members of ADAM family observed were found to be expressed in C8161 and HaCaT cells with only a very weak expression of ADAM12 in both the cell lines observed (Fig. 20A). Depletion of survivin protein leads to cell cycle arrest and cells accumulate in the G2 phase of the cell cycle, ~30% of the population shift from G1 to G2 phase implies almost complete depletion (>90% reduction) of survivin. siRNA against survivin were used to optimize transfection conditions and good transfection efficiency was obtained with G1 population shifts found upto 34% (Fig. 20B).

Furthermore, SCC-9 cells were depleted of individual ADAMs by siRNA transfection. ADAM9 knockdown resulted in decreased EGFR phosphorylation upon UV treatment (Fig. 20C). Mock controls were treated with transfection reagents only and siGl2 (siRNA against firefly luciferase gene) as a specificity control.

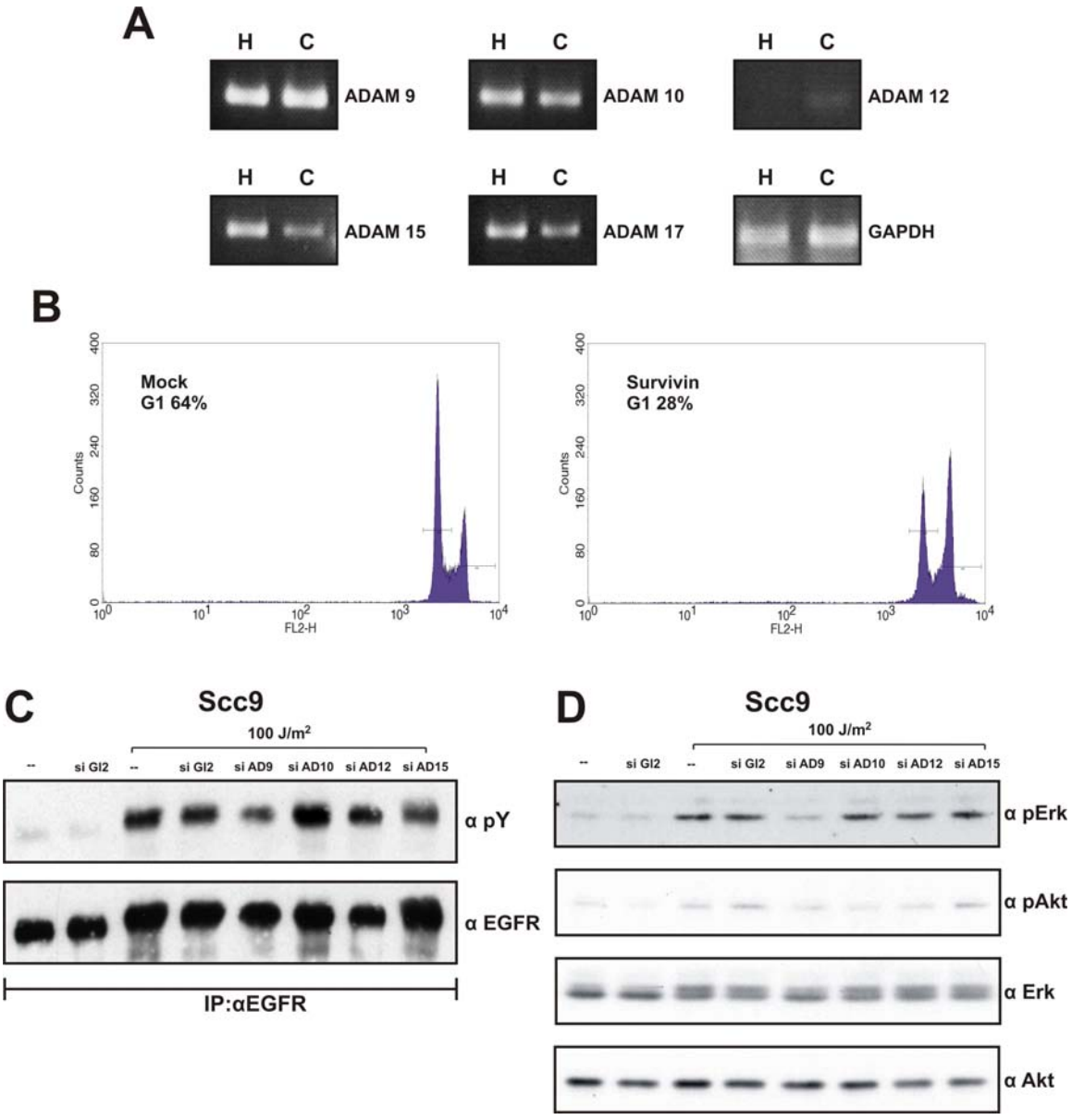


Figure 20. Finding the ADAM Responsible for EGF Family Proligand Shedding Under UV Irradiation Leading to EGFR Activation.

(A) cDNA was prepared from C8161, (C) and HaCaT, (H) cells and RT-PCR was performed for ADAM9, 10, 12, 15, and 17. (B) FACS analysis of siRNA against survivin and mock treated cells to check for nuclear content, percentage written is fraction of cells residing in the G1 subpopulation. (C) SCC-9 cells were treated with mock (--), control (siGl2), and individual ADAMs (siAD9, 10, 12, and 15) siRNA and stimulated with 100J/m^2 UV irradiation and lysed after 15 minutes. IPs were done for EGFR and processed to check for phospho-tyrosine and total EGFR levels. (D) SCC-9 cells were treated as in (C) and total lysates were probed for pErk, pAkt and reprobed for total Erk and Akt.

ADAM9 knockdown was also able to reduce UV induced pErk and pAkt levels compared to mock and siGl2 treated cells (Fig. 20D). These results confirm ADAM9 activity to be necessary for proligand shedding under UV irradiation for activation of EGFR and its downstream signaling.

3.4 Biological Significance of UV Induced EGFR Transactivation

3.4.1 EGFR Transactivation by UV Irradiation Confers an Anti-apoptotic Advantage to Cells Under UV Stress

Various transformed cells exploit the EGFR transactivation pathway to achieve different biological advantages e.g. proliferation, invasion, migration, and anti-apoptosis . We tried to find out whether UV induced EGFR transactivation could provide any biological advantage to the cells under UV stress. We analyzed a possible anti-apoptotic advantage mediated by EGFR transactivation. C8161 and HaCaT cells were treated with 25 and 50 J/m^2 UV, respectively, in the presence or absence of BB94 for 20 hours, and analyzed for nuclear fragmentation by Flow cytometry. Cells in the apoptotic subpopulation were counted as having sub-Go nuclear DNA content. We could show that metalloprotease inhibition did not significantly change the sub-Go nuclear population under untreated conditions in C8161 (Fig. 21A). The same was true for HaCaT cells (Fig. 21B). UV irradiation initiated apoptosis in C8161 (Fig. 21C, E) and HaCaT (Fig. 21D, F) cells which was monitored by an increase in sub-Go nuclear population. This result is in line with the well known phenomenon of UV induced lethality, primarily mediated by formation of irreparable DNA lesions, and subsequent apoptosis.

However, upon preincubation with BB94, UV irradiation further increased the number of apoptotic cells in both C8161 (Fig. 21C, E) and HaCaT cells (Fig. 21D, F). The increase in apoptosis rates was 10% and 20% in C8161 (Fig. 21E) and HaCaT (Fig. 21F) cells, respectively. This experiment shows that UV induced EGFR transactivation allows cells to survive for longer periods of time under UV stress, minimizing the apoptotic response to DNA damage induced by UV.

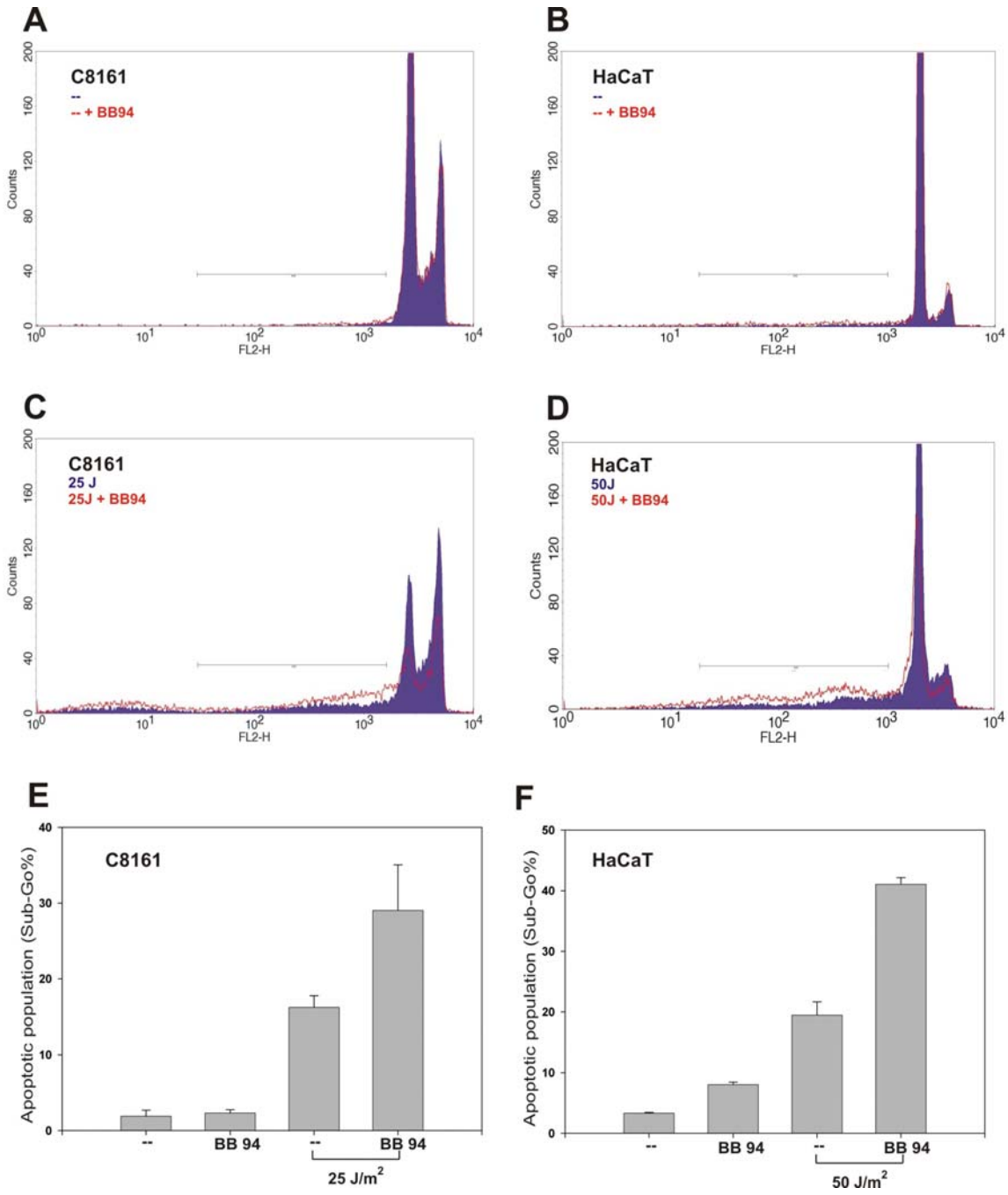


Figure 21. Blocking EGFR Transactivation Pathway Leads to Increase in UV Induced Apoptosis.

C8161 (A) and HaCaT (B) were starved and treated with the metalloprotease inhibitor BB94 for 20 hours and analyzed for nuclear DNA content by flow cytometry. Starved C8161 and HaCaT cells were preincubated with BB94 for 30 minutes and irradiated with indicated doses of UV and analyzed by flow cytometry 20 hours after irradiation. Statistical representation of the apoptotic fraction of C8161 (E) and HaCaT (F) population, results are average of triplicates ± S.D.

3.4.2 UV Induced EGFR Transactivation Leads to Increased Stability of the DNA Repair Enzyme PARP

UV induces apoptosis in cells by inducing irreparable DNA damage. Poly(ADP-ribose) Polymerase, PARP, is a nuclear enzyme which is cleaved into an inactive form by caspases during apoptosis (Scovassi and Poirier 1999). PARP is also involved in DNA repair processes in single strand break repair (SSB), base excision repair (BER), and nucleotide excision repair (NER) (Flohr et al. 2003; Masutani et al. 2003; Parsons et al. 2005). The cleavage of PARP was assessed upon UV irradiation. We could show that UV induced cleavage of PARP at various time points, starting at 12 hours in C8161 (Fig. 21B) and 6 hours in HaCaT cells (Fig. 21B) indicating PARP inactivation and initiation of apoptosis. Cleavage of PARP upon UV irradiation could be further increased upon BB94 preincubation in both C8161 (Fig. 21A) and HaCaT (Fig. 21B) cells. These experiments indicate that UV induced EGFR transactivation allows higher concentration of active PARP molecules to be maintained in the nucleus, thus prolonging PARP mediated repair of damaged DNA.

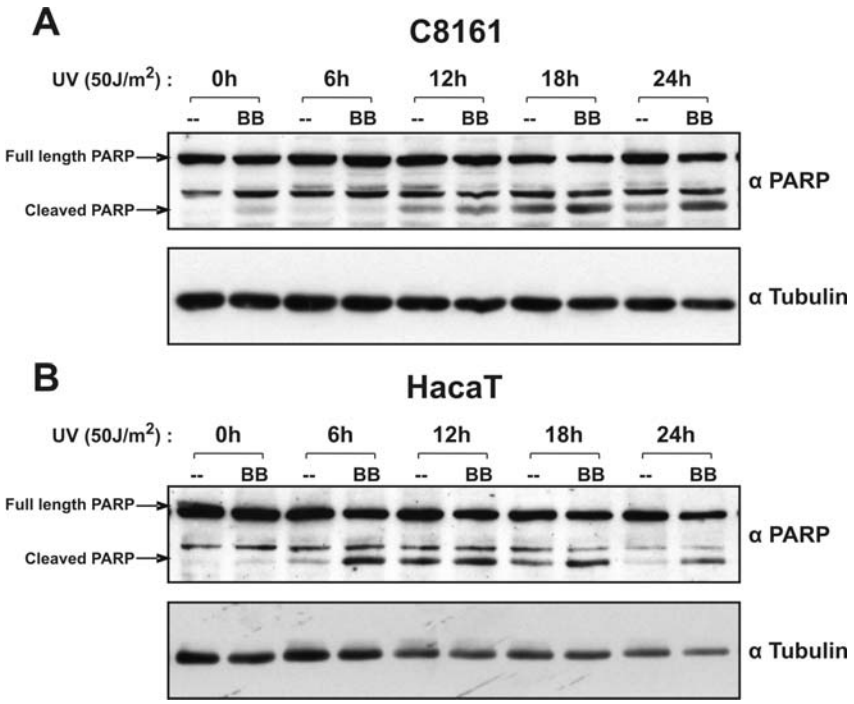


Figure 21. UV Induced PARP Cleavage is Reduced by EGFR Transactivation Pathway.

(A) C8161 and (B) HaCaT cells were pretreated with BB94 (10 uM, 30') and stimulated with UV. Cells were lysed with RIPA buffer at indicated time points and probed for PARP, its cleavage product, and for tubulin as loading control.

3.5 Src Family Kinases are Involved in UV Induced EGFR Transactivation

Src family kinases are shown to be involved in GPCR induced EGFR transactivation (Roelle et al. 2003). Src family kinases have also been shown to activate EGFR by phosphorylation (Fischer et al. 2003). To find the possible role of the Src family kinases in UV induced EGFR transactivation we used a Src family inhibitor PP1 and observed its effect on UV induced EGFR phosphorylation in C8161 and HaCaT cells. In both the cell lines C8161 (Fig. 22A) and HaCaT (Fig 22B) under study we were able to find inhibition of EGFR phosphorylation by UV when the cells were preincubated with PP1. These results show that Src family members are also involved in UV induced EGFR transactivation.

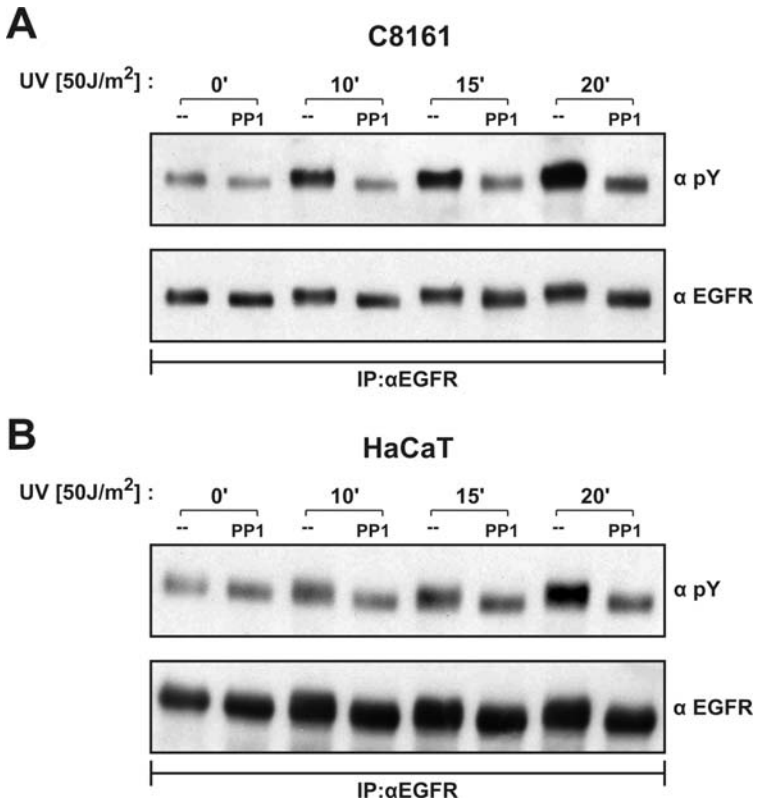


Figure 22. Effect of Src Inhibitor PP1 on UV Induced EGFR Transactivation.

Starved C8161 (A) and HaCaT (B) cells were preincubated with PP1 (10 μM) for 30 minutes. Cells were then stimulated with UV and lysed at the indicated time points. Lysates were processed to monitor pEGFR and EGFR levels.

3.6 Reactive Oxygen Species Signaling in EGFR Transactivation

3.6.1 GPCR Ligands Phosphorylate EGFR and Downstream Molecules in C8161 and HaCaT Cells

GPCR agonists administration has been shown to induce RTK phosphorylation in a variety of cell lines (Fischer et al. 2003). We set out to find which of the GPCR agonists induce EGFR phosphorylation in cell lines of skin lineage. C8161 and HaCaT originating from melanocytes and keratinocytes, respectively, were used in this experiment to observe EGFR phosphorylation in response to GPCR agonist treatment. Endothelin-I and thrombin were able to induce EGFR phosphorylation (Fig. 23A) in C8161 cells. HaCaT cells showed EGFR phosphorylation in response to LPA, thrombin, and bradykinin (Fig. 23B).

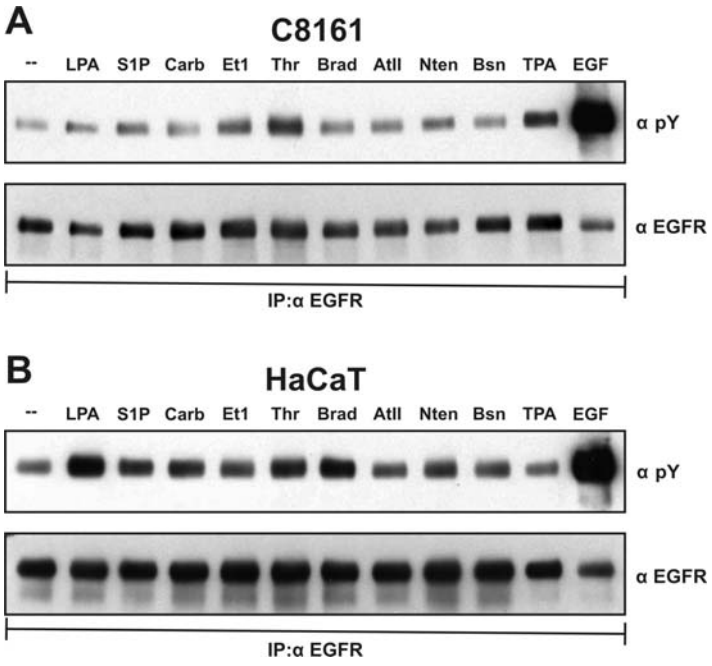


Figure 23. Various GPCR Ligands Lead to EGFR Phosphorylation.

C8161 (A) and HaCaT (B) cells were seeded at 180,000 cells/6cm and 400,000 cells/6 cm plates, respectively, and starved in serum free medium for 24 hours. Various GPCR agonists were then added to the medium and incubated for 5 minutes. Cells were then lysed and immunoprecipitated (IP) for EGFR, blotted, probed for pY and reprobed for EGFR. EGF treatment (5 ng/ml) was taken as positive phosphorylation control. Abbreviations: LPA = Lysophosphatidic acid, S1P = Sphingosine-1-phosphate, Carb = Carbachol, Et1 = Endothelin I, Thr = Thrombin, Brad = Bradykinin, AtII = Angiotensin II, Nten = Neurotensin, Bsn = Bombesin, TPA = 12-O-tetradecanoyl-phorbol-13-acetate, EGF = Epidermal Growth Factor.

3.6.2 EGFR Transactivation is Dependent on EGFR Kinase Activity and Metalloprotease Activity

In previous experiments we established that UV induced EGFR transactivation depends on proligand shedding by metalloproteases (Fig. 16, 18, and 19). To analyse whether GPCR agonist induced EGFR transactivation is dependent on a similar pathway, we preincubated the cells with the metalloprotease inhibitor BB94, prior to thrombin administration. Thrombin induced EGFR phosphorylation could be reduced upon preincubation with BB94 in both C8161 (Fig. 24A) and HaCaT (Fig. 24B) cells. Preincubation with the EGFR kinase inhibitor AG1478 also had the same effect in both of the cell lines (Fig. 24A, B). EGF induced phosphorylation of EGFR however could only be blocked by AG1478 as expected (Fig. 24A, B).

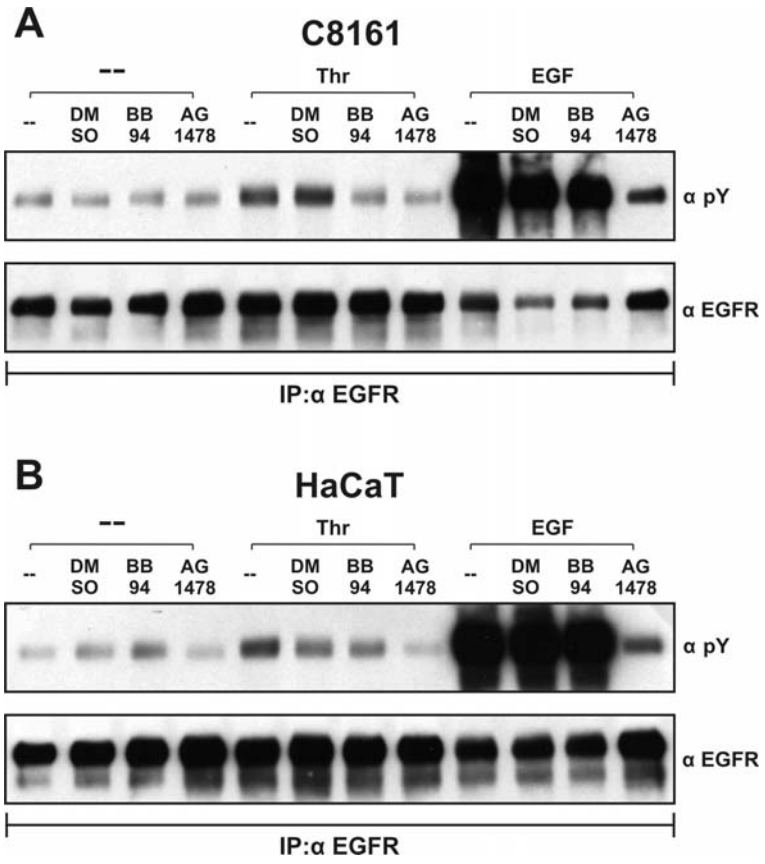


Figure 24. Thrombin Induced EGFR Phosphorylation is Blocked by BB94 and AG1478.

Starved C8161 (A) and HaCaT (B) cells are preincubated with BB94 (10 μM), and AG1478 (250 nM) or solvent alone (DMSO) for 30 minutes. Cells were then treated with Thrombin (2 U/ml) or EGF (5 ng/ml) for 5 minutes, lysed and processed to monitor pEGFR and EGFR levels.

3.6.3 Thrombin Induced EGFR Transactivation is Dependent on Hb-EGF Proligand Shedding in C8161 Cells

Next, we explored for the proligand which is responsible for the activation of thrombin induced EGFR transactivation. The diphtheria toxin mutant CRM197 specifically binds to membrane anchored proHb-EGF and stops its processing, thus making it unavailable for the EGFR transactivation pathway (Prenzel et al. 1999). We preincubated starved C8161 cells with CRM197, irradiated with UVC and monitored pEGFR and EGFR levels. We found that UV induced EGFR phosphorylation could be reduced upon preincubation with CRM197 in a dose dependent manner (Fig. 25). These results show that UVC induced EGFR stimulation depends on metalloprotease induced shedding of proHb-EGF.

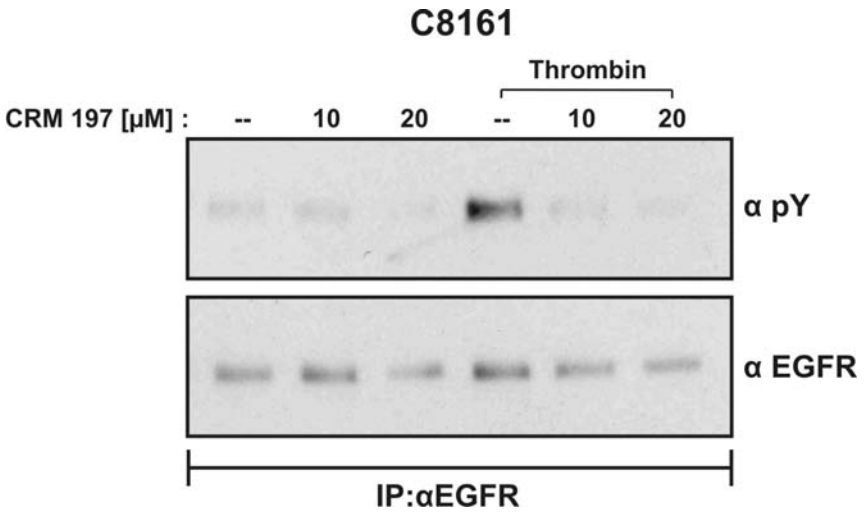


Figure 25. Effect of CRM197 on Thrombin Induced EGFR Transactivation.

Starved C8161 cells were preincubated with indicated concentrations of CRM197, stimulated with thrombin (2U/ml), and lysed after 5 minutes. Lysates were immunoprecipitated (IP) for EGFR, blotted, probed for pY and reprobed for EGFR.

3.6.4 EGFR Transactivation Leads to Production of Reactive Oxygen Species

EGFR transactivation is a complex process and only few molecules involved have been identified and characterized. Mechanism of the metalloprotease activation under GPCR or UV stimulation is not well understood. On the other hand reactive oxygen species have been shown to be produced in response to RTK activation after ligand stimulation (Bae et

al. 1997; Finkel 2003; Fischer et al. 2004). ROS production has also been reported under UV irradiation and GPCR agonist treatment (Griendling et al. 1994; Huang et al. 1996) in a number of observations.

Next, we explored the potential role of ROS in the EGFR transactivation pathway. We started by investigating if ROS are produced upon UV stimulation, as we already showed that this stimulation leads to EGFR transactivation. To measure ROS production, we used a cell permeable dye, DCFDA, which is desacetylated by cellular esterases into a cell impermeable form. This form then oxidizes to a fluorescent form upon reacting with ROS (Fig. 26) (LeBel et al. 1992).

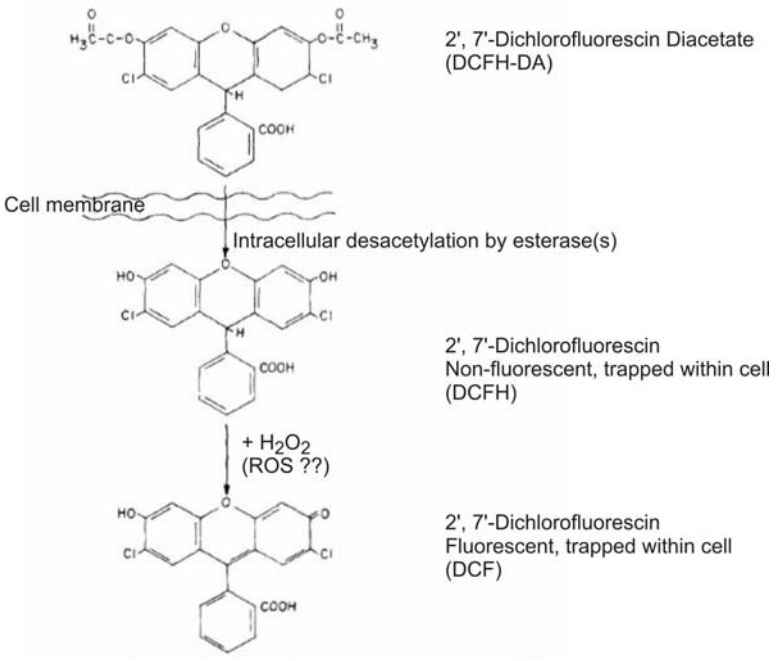


Figure 26. Mechanism of DCFDA Fluorescence Upon Reacting with Intracellular ROS (LeBel et al. 1992).

Non-fluorescent DCFH-DA crosses the cell membrane and is trapped inside cells by desacetylation by intracellular esterase(s) in DCFH form. Intracellular ROS react with DCFH and oxidize it to fluorescent DCF, which is measured spectrophotometrically.

Starved C8161 cells were preincubated with DCFDA dye and irradiated with 100 and 300 J/m^2 UV and DCF fluorescence was measured after 5 minutes. There was an increase in DCF fluorescence intensity after 100 J/m^2 UVC stimulation as compared to untreated

cells (Fig. 27A). This showed that UV irradiation led to an increase in intracellular ROS levels. ROS levels could be further increased with an increase in the intensity of UV irradiation as 300 J/m^2 UV led to even higher DCF fluoresecence levels than 100 J/m^2 UVC irradiation (Fig. 27A). HaCaT cells showed a similar dose dependent increase in UV induced DCF fluorescence, indicating a dose dependent production of ROS in them (Fig. 27B).

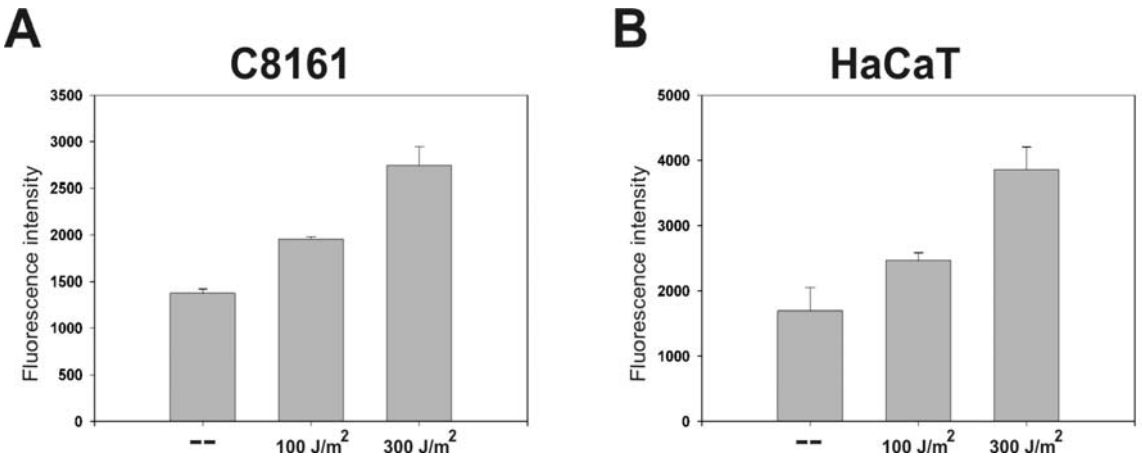


Figure 27. UV Induced ROS Production in C8161 and HaCaT Cells.

Starved C8161 (A) and HaCaT (B) cells were preincubated with DCFDA (10 μM) for 30 minutes and then stimulated with 100 and 300 J/m^2 UVC. Production of ROS was measured as increase in DCF fluorescence 5 minutes after UV irradiation. Values are plotted as mean fluorescence intensity ± S.D. (triplicates).

3.6.5 UV and GPCR Induced ROS Production is Dependent on EGFR Kinase Activity and Metalloprotease Activity

Reactive oxygen species have been shown to be produced upon UV irradiation or GPCR agonist stimulation (Fig. 27A, B). To examine whether EGFR transactivation could be involved in stimulation dependent ROS production, we preincubated the cells with an EGFR kinase inhibitor and a metalloprotease inhibitor and then stimulated the cells with UVC and observed for the generation of ROS. C8161 cells showed a decrease in ROS production when preincubated with BB94 or AG1478 compared to cells treated with UV alone (Fig. 28A). HaCaT cells also showed a similar reduction in ROS production when EGFR kinase activity or metalloprotease activity was inhibited (Fig. 28B). These

experiments show that ROS production in response to UV irradiation depends on EGFR transactivation pathway.

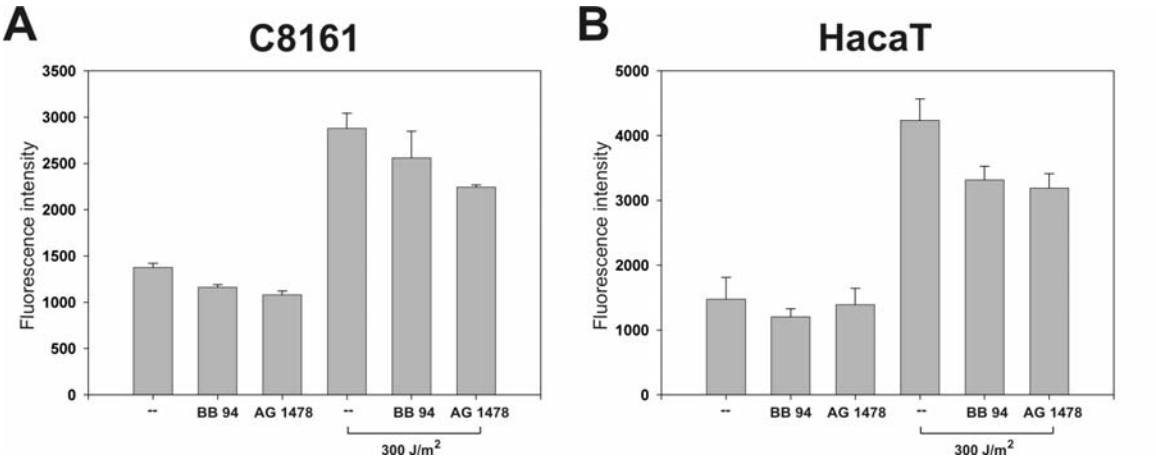


Figure 28. UV Induced ROS Production can be Inhibited by BB94 and AG1478.

Starved C8161 (A) and HaCaT (B) cells were preincubated with DCFDA, BB94, and AG1478 for 30 minutes and stimulated with 300 J/m^2 UVC. Fluorescence intensity was measured 5 minutes after irradiation. Values are plotted as mean fluorescence intensity ± S.D. (triplicates).

3.6.6 GPCR and UV Induced EGFR Transactivation Can be Inhibited by the ROS Scavenger NAC in C8161 and HaCaT Cells

ROS production has been shown to activate various RTKs including EGFR. ROS reversibly inactivate protein tyrosine phosphatases, which are negative regulators of RTK signaling. PTP1B directly associates with and dephosphorylates EGFR and therefore acts as its negative regulator (Tomic et al. 1995). UV enhances EGFR signaling by inactivating PTP1B (Gross et al. 1999). Furthermore, irreversible inactivation of PTPs via calpain mediated degradation shows another mechanism of UV induced RTK activation (Gulati et al. 2004). We further analysed if ROS production during EGFR transactivation is involved in EGFR activation. To investigate the involvement of ROS in EGFR transactivation we used the ROS scavenger N-acetyl cysteine (NAC) and observed if NAC preincubation can lead to a decrease in UV/GPCR induced EGFR phosphorylation. Both thrombin and UV induced EGFR phosphorylation could be reduced upon preincubation with increasing concentrations of NAC in C8161 cells (Fig. 29A). On the other hand EGFR phosphorylation by EGF administration could not be reduced by NAC

preincubation. HaCaT cells also showed a similar reduction in thrombin induced EGFR phosphorylation upon NAC preincubation (Fig. 29B). These experiments show that in the EGFR transactivation pathway reactive oxygen species play a critical role in the phosphorylation of EGFR. NAC preincubation however, had no effect on UV induced EGFR transactivation in HaCaT cells (Fig. 29B).

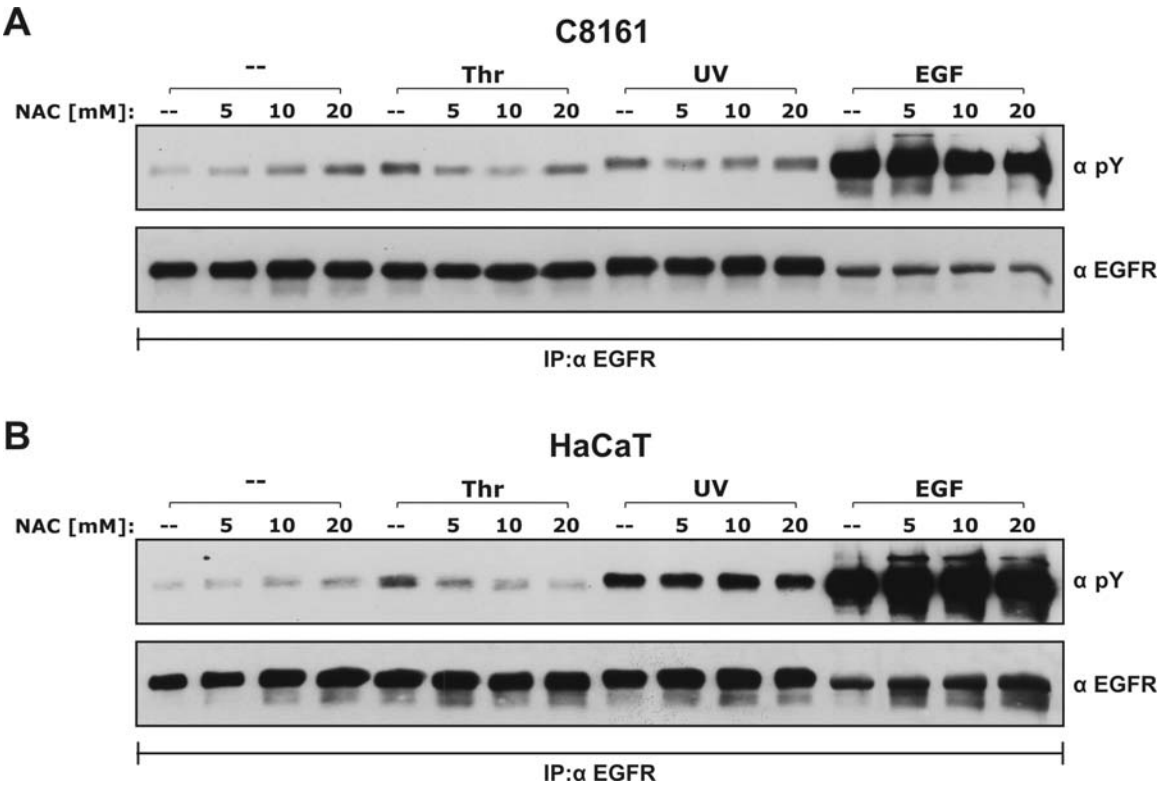


Figure 29. Inhibition of Stimulation Dependent EGFR Phosphorylation by ROS Scavenger.

Starved C8161 (A) and HaCaT (A) cells were preincubated with the indicated NAC concentrations, and stimulated with thrombin (2U/ml, 5'), UV (50 J/m^2, 15'), and EGF (5 ng/ml, 5'). Cells were then lysed and immunoprecipitated (IP) for EGFR, blotted, probed for pY and reprobed for EGFR.

3.6.7 EGFR Transactivation Can be Inhibited by the NADPH Oxidase Inhibitor DPI

One major source of production of intracellular reactive oxygen species, which can be regulated by various mechanisms, are the multi-subunit enzymes called NADPH oxidases (Nox). Diphenylene iodonium chloride (DPI) inhibits members of the Nox family, and the subsequent ROS generation. To investigate the source of ROS generation we

inhibited Nox proteins through DPI preincubation and tested if this leads to inhibition of EGFR phosphorylation after stimulation. We found that DPI preincubation led to a decrease in EGFR phosphorylation after UV stimulation in HaCaT cells (Fig. 30). Doubling the concentration of DPI inhibitor to 20 µM decreased UV induced EGFR phosphorylation to an even greater extent as compared to 10 µM concentration (Fig. 30).

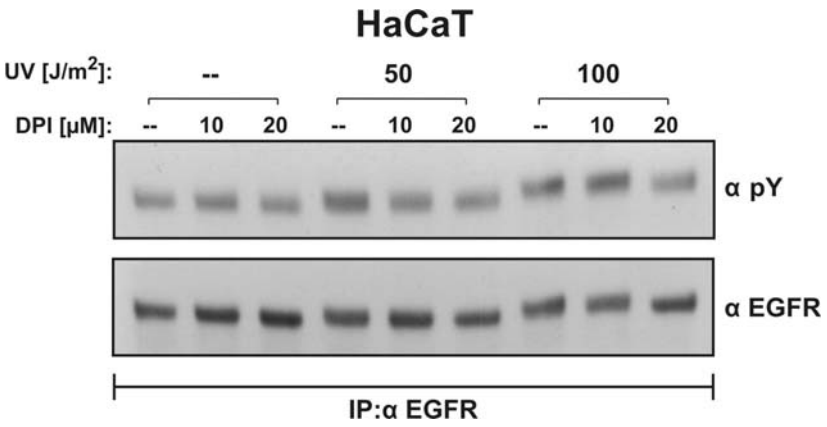


Figure 30. Effect of the Nox Inhibitor DPI on UV Induced EGFR Phosphorylation

Starved HaCaT cells were preincubated with indicated concentrations of DPI for 30 minutes, and irradiated with indicated doses of UVC. The cells were lysed 15 minutes after stimulation and immunoprecipitated (IP) for EGFR, blotted, probed for pY and reprobed for EGFR.

This experiment shows that the members of the Nox family are involved in the UV induced phosphorylation of EGFR. Members of the Nox family thus could be the possible source of ROS production under UV irradiation leading to EGFR activation.

3.6.8 EGFR Downstream Signaling Can be Inhibited by the NADPH Oxidase Inhibitor DPI in C8161 and HaCaT Cells

Activation of the PI3K/Akt and MAPK1/2 pathways has been shown to be linked to the activation of members of EGFR family, either by direct binding to the activated receptor or via adaptor molecules (Schulze et al. 2005). We already showed that UV induced EGFR activation is dependent on the production of reactive oxygen species, and that ROS production is dependent on the activity of proteins belonging to the Nox family (Fig. 27, 29). To test whether EGFR downstream signaling is also dependent on the production of ROS by Nox proteins, we preincubated cells with the Nox inhibitor DPI

and observed UV induced Erk and Akt phosphorylation. In both C8161 and HaCaT cells Erk and Akt phosphorylation increased after UV irradiation in a dose dependent manner (Fig. 31A, B). This phosphorylation of Erk and Akt however could be reduced upon preincubation of the cells with the Nox inhibitor, DPI in a dose dependent manner (Fig. 31A, B). These experiments show that similar to UV induced EGFR activation, the activation of downstream signaling molecules Erk and Akt also depends on the activity of Nox proteins, which could be the possible source of ROS production.

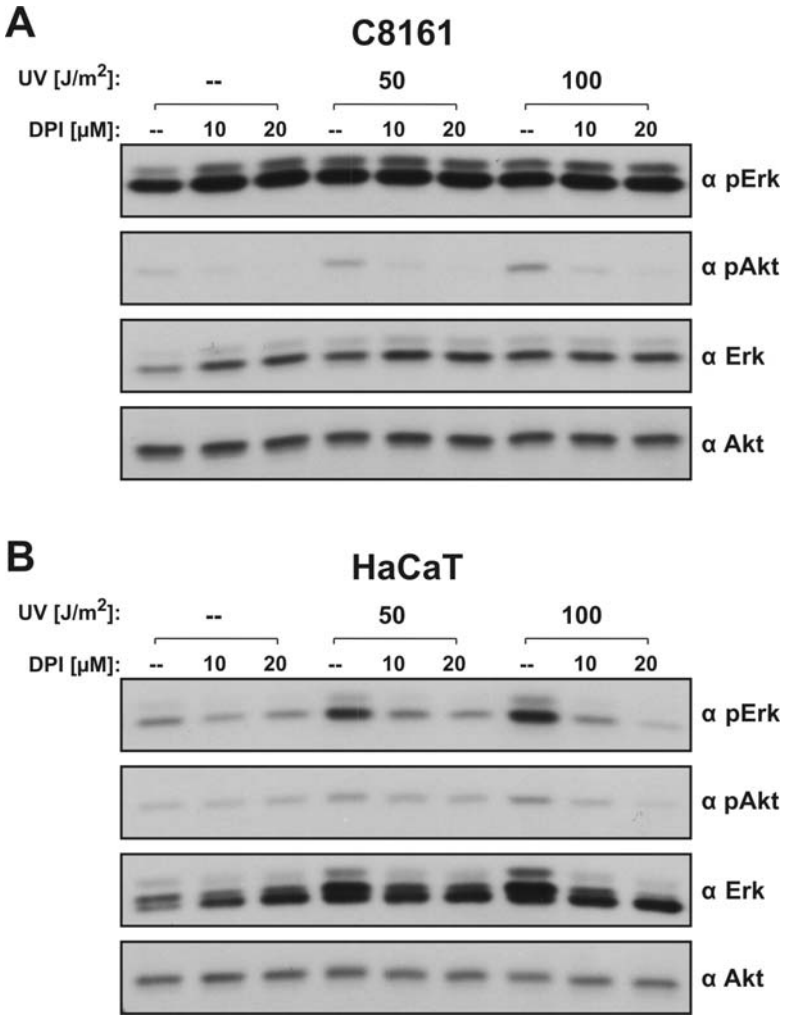


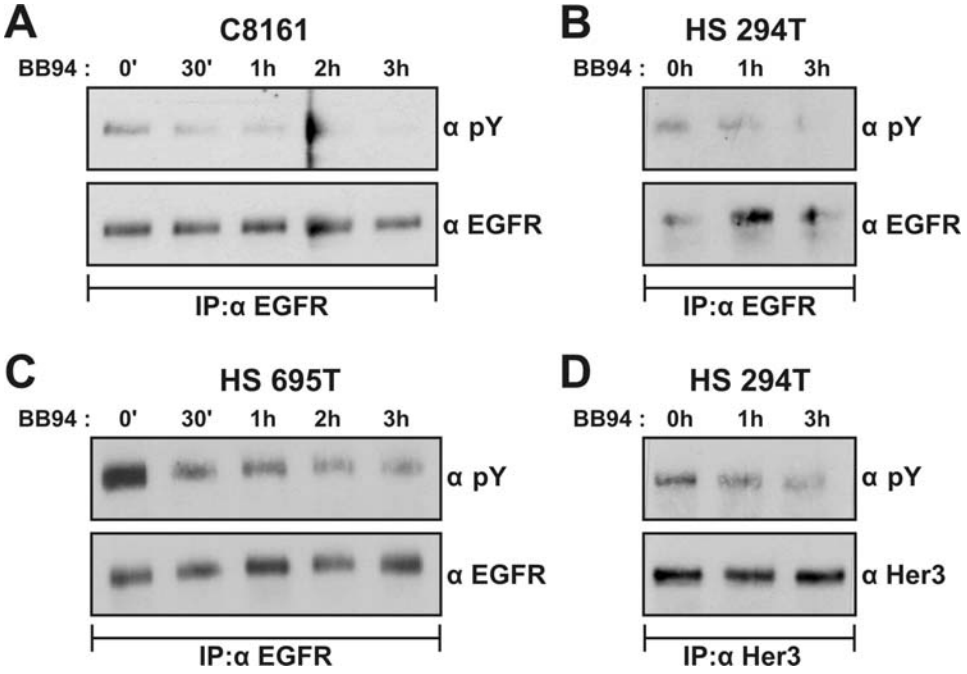
Figure 31. UV Induced Erk and Akt Phosphorylation Depends on Nox Activity

Starved C8161 (A) and HaCaT (B) cells were pretreated with various concentrations of the Nox inhibitor DPI for 30 minutes. Cells were then irradiated with the indicated doses of UVC and lysed after 15 minutes. Equal amounts of lysates were blotted and probed for pErk and pAkt. The same membranes were later reprobed for total Erk and Akt levels as loading control.

3.7 Therapeutic Potential of Blocking EGFR Transactivation Pathway in Cancer of Skin Lineage

3.7.1 EGFR Transactivation is Responsible for Basal Levels of RTK Phosphorylation in Unstarved Cells.

Cells propagated in serum supplemented medium resemble a state close to that of cells in the human body, where there is a continuous supply of growth factors and other GPCR agonists. EGFR transactivation imparts various biological advantages to cancer cells, like migration, invasion, proliferation, and/or anti-apoptosis etc. Thus blockage of EGFR transactivation pathway in cancer cells is a potential therapeutic strategy. In this study we analysed the presence of EGFR transactivation pathway in transformed cells of skin lineage. Cells were seeded in serum-supplemented medium and incubated with the metalloprotease inhibitor BB94 for various time points. We found a reduction in EGFR phosphorlyation levels in various cell lines including C8161, RPMI7951, HS695T, and HS294T with an increase in the time of incubation with BB94 (Fig. 32A, B, C, and E). We also observed a decrease in Her3 phosphorylation levels in the HS294T cell line upon incubation with BB94 (Fig. 32D). These results show that transformed skin cells use the transactivation pathway for maintaining higher phosphorylation among the EGFR family.



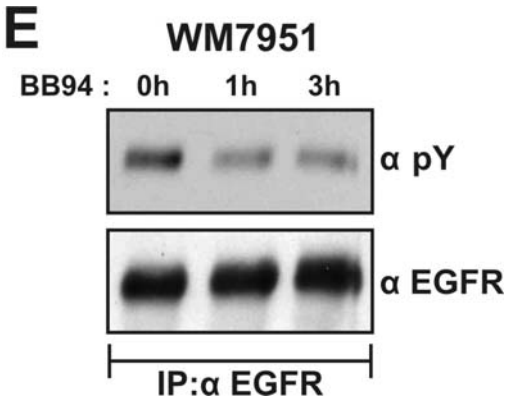


Figure 32. Basal Levels of RTKs Phosphorylation can be Reduced by BB94 Preincubation.

C8161 (A), Hs294T (B), Hs695T (C), and RPMI7951 (E) were seeded in 6 cm plates supplemented with serum containing medium and incubated with BB94 (10μM) for the indicated period of time. Cells were then lysed and immunoprecipitated (IP) for EGFR, blotted, probed for pY and reprobed for EGFR. Unstarved Hs294T cells were treated with BB94 (10μM) for the indicated time periods. Cells were lysed and immunoprecipitated (IP) for Her3, blotted, probed for pY and reprobed for Her3.

3.7.2 Differences Between Primary and Secondary Melanoma in UV Induced EGFR Transactivation

Primary melanocytes express low amounts of EGFR and its family members, thus excluding the possibility of EGFR transactivation pathway in them. However, the expression of EGFR and its family members has been reported to be increased in advanced melanomas and thus the occurrence of EGFR transactivation (Huang et al. 1996). We used primary (WM793) and secondary (WM1205) melanoma cell lines from the same patient and analysed for the differences in EGFR transactivation pathway in response to UV irradiation. We show that UV is unable to induce EGFR phopshorylation in primary melanoma WM793, whereas secondary melanoma WM1205 shows phosphorylation of EGFR upon UV irradiation (Fig. 33 left panel). Furthermore, EGFR phosphorylation in WM1205 cells was dependent on metalloprotease activity, as it could be attenuated upon preincubation with BB94 (Fig. 33 right panel). This experiment shows that the EGFR transactivation pathway is switched on in secondary melanomas, that is during the later stages of melanoma progression, and thus could be an adaptive process of the melanomas to survive under UV stress.

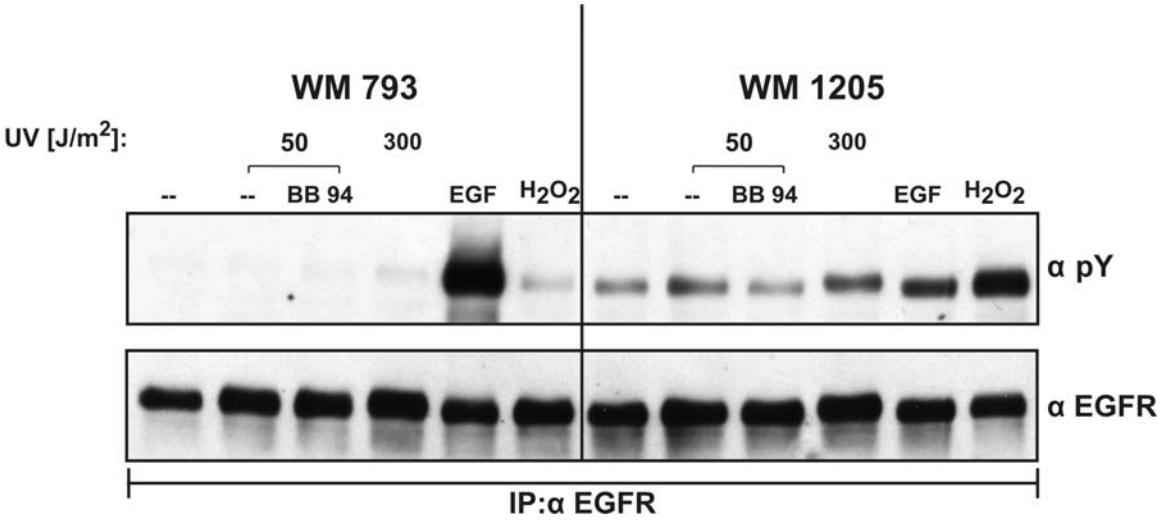


Figure 33. UV Induced EGFR Transactivation in WM793 and WM1205 Cells.

Starved WM793 and WM1205 cells were preincubated with BB94 (10µM, 30') and stimulated with indicated doses of UVC. Cells were lysed 15 minutes after UV irradiation and immunoprecipitated (IP) for EGFR, blotted, probed for pY and reprobed for EGFR. EGF and H_2O_2 are taken as positive phosphorylation controls.

3.7.3 Intervention of EGFR Transactivation With Chemical Inhibitors

UV or GPCR induced EGFR transactivation is very important in skin carcinogenesis as it provides the cancer cells with anti-apoptotic signals, which allow them to survive longer under UV stress and, thus, accumulate harmful mutations increasing their aggresiveness. The EGFR transactivation pathway can be targeted at various places, like reducing the concentration of reactive oxygen species using ROS scavengers, inhibition of metalloproteases, neutralizing antibodies against ligands of the EGF family, anti-RTK antibodies, kinase inhibitors, anti-heterodimerization antibodies, and inhibition of downstream signaling pathways (Fig. 34) (Gschwind et al. 2004).

In this study we compared two modes of EGFR transactivation inhibition and tried to find out which one would have better chances of directing the cancer cells to the apoptotic pathway. We compared two inhibitors BB94 and AG1478 targeting metalloprotease and EGFR kinase activity, respectively. The knowledge gained in this study could further be applied to design more effective therapeutic strategies for the treatment of cancer cells belonging to skin lineage deriving carcinogenic signals from UV irradiation.

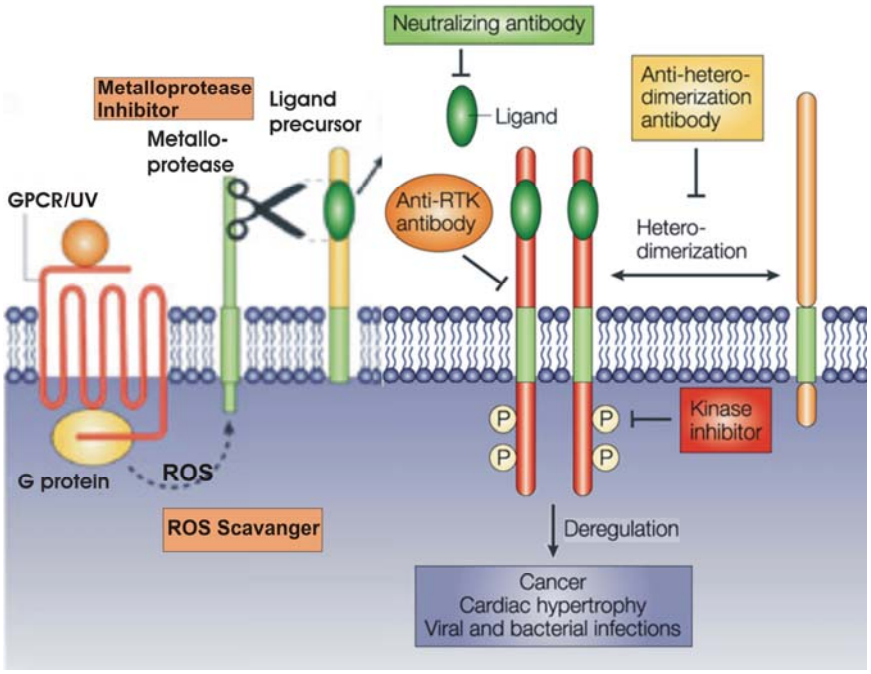


Figure 34. Sites of Therapeutic Intervention in Receptor Tyrosine Kinase Transactivation (Gschwind et al. 2004).

3.7.3.1 EGFR Phosphorylation Upon UV Stimulation is Inhibited by AG1478 to a Greater Extent Than by BB94

The EGFR kinase inhibitor, AG1478 would stop all the signaling through the EGFR in a stimulation dependent manner, and even the basal level of signaling in stimulation independent manner. The metalloprotease inhibitor, BB94 on the other hand would only inhibit stimulation dependent signaling and effectively stop only the EGFR transactivation pathway. Basal levels of EGFR signaling are not affected and occur uninterrupted. We used these two inhibitors, and compared for the differences in signaling and biological outcome of these different modes of inhibition. C8161, HaCaT, RPMI7951, and SCC-9 cells were stimulated with UV after preincubation with inhibitors separately and compared for differences in EGFR phosphorylation. UV induced EGFR phosphorylation could be reduced in all the cell lines upon preincubation with both the inhibitors (Fig. 35A, B, C, and D). But at the concentrations used, AG1478 was able to reduce UV induced EGFR phosphorylation to a greater extent than BB94 (Fig. 35A, B, C, and D). In addition, AG1478 was further able to reduce basal levels of EGFR

phosphorylation under unstimulated conditions in HaCaT, RPMI7951, and SCC-9 cells (Fig 35B, C, and D). These results show that AG1478 is better at inhibiting UV induced EGFR phosphorylation as compared to BB94.

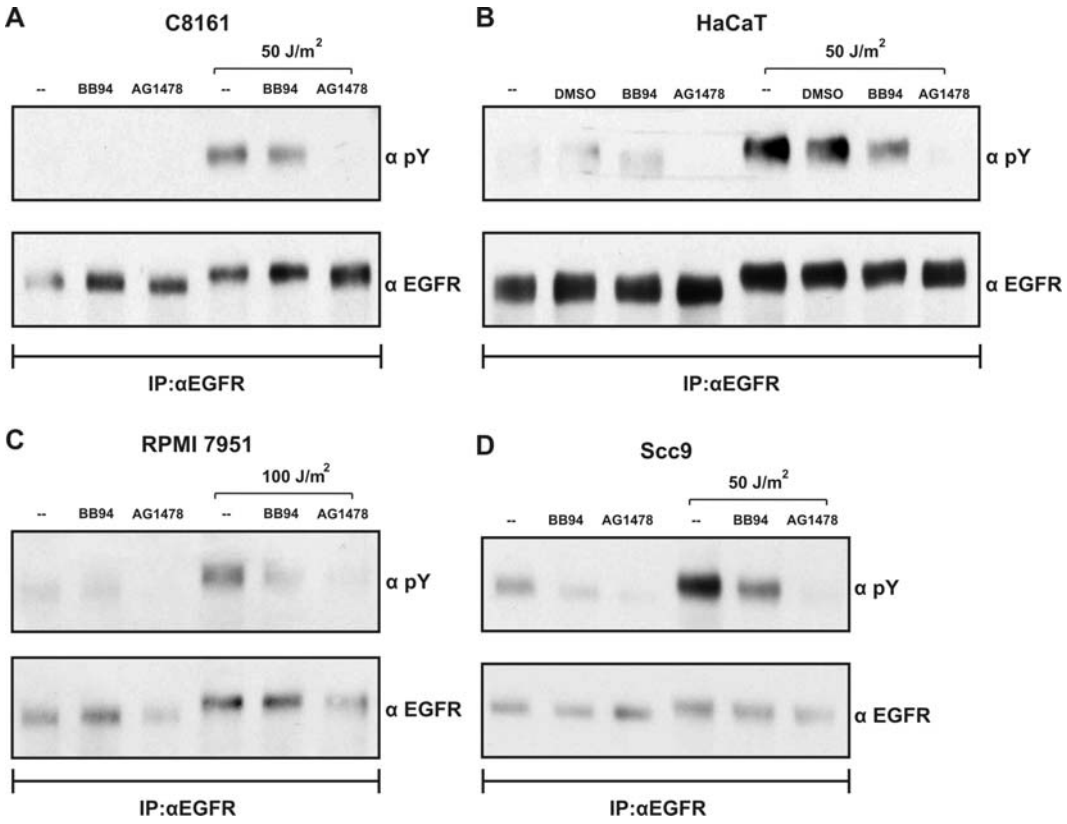


Figure 35. Inhibition of UV Induced EGFR Phosphorylation by AG1478, and BB94.

Starved C8161 (A), HaCaT (B), RPMI7951 (C), and SCC-9 (D) cells were preincubated with BB94 (10µM, 30') and AG1478 (250 nM, 30') and stimulated with indicated doses of UVC [J/m^2]. Cells were lysed after 15 minutes immunoprecipitated (IP) for EGFR, blotted, probed for pY and reprobed for EGFR. DMSO is the carrier control in HaCaT cells.

3.7.3.2 Erk and Akt Phosphorylation Upon UV Stimulation is Inhibited by AG1478 to a Greater Extent Than by BB94

In order to analyse the differences in the abilitiy to inhibit downstream signaling by the two chemical inhibitors, we observed Erk and Akt phosphorylation after UV irradiation upon AG1478 and BB94 preincubation. UV irradiation led to an increase in phosphorylation of Erk and Akt in HaCaT and SCC-9 cells, and this phosphorylation could be reduced on preincubation with both the inhibitors, AG1478 and BB94 (Fig. 36A, B). Here again we found AG1478 to be a better inhibitor of UV induced Erk and

Akt phosphorylation at both of the doses of UV administered in HaCaT and SCC-9 cell lines (Fig. 36A, B). These results show that the EGFR kinase activity inhibitor AG1478 diminishes EGFR downstream signaling more effectively as compared to the metalloprotease inhibitor BB94.

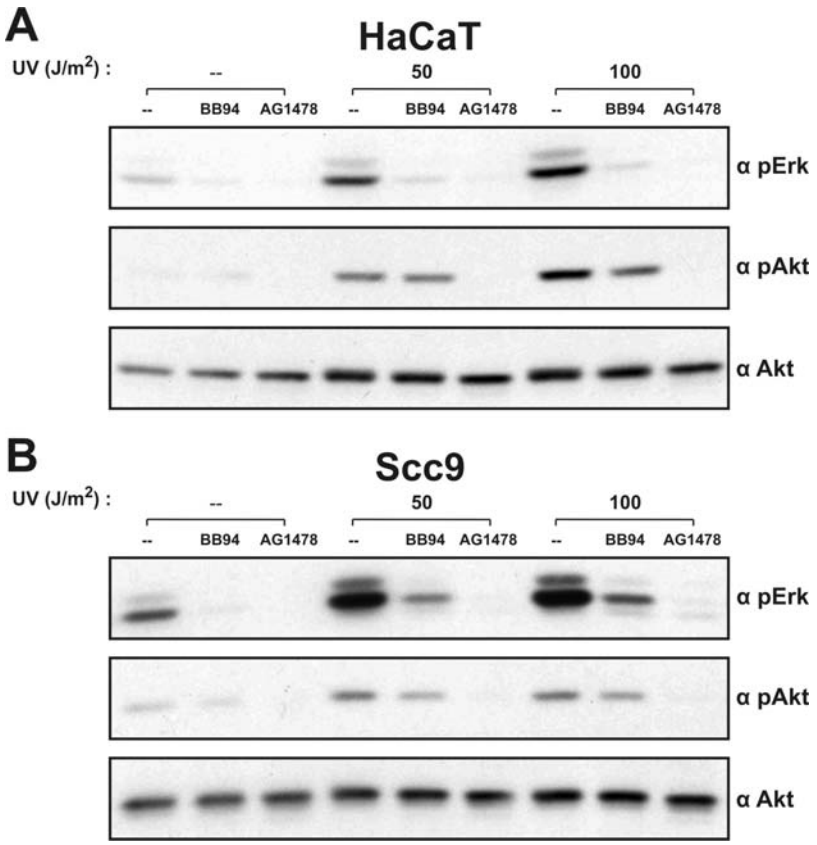


Figure 36. Inhibition of UV Induced Erk and Akt Phosphorylation by BB94 and AG1478.

Starved HaCaT cells were preincubated with DMSO (solvent control), BB94 (10μM, 30'), and AG1478 (250 nM, 30') and stimulated with the indicated doses of UVC [J/m^2] and lysed after 15 minutes. Equal amounts of lysates were blotted and probed for pErk and pAkt. The same membranes were later reprobed for total Erk and Akt levels as loading control.

3.7.3.3 Transactivation Block is More Efficient Than Direct Kinase Inhibition of EGFR in Inducing Apoptosis in Cancer Cells Under UV Stress

We have shown previously that EGFR transactivation upon UV irradiation gives an anti-apoptotic advantage to cancer cells of the skin lineage (Fig. 21). Here we analysed for the increase in apoptosis induction after inhibiting UV induced EGFR transactivation by

mechanistically different inhibitors. C8161 and HaCaT cells used in this study did not show any major apoptosis induction when unstimulated cells were treated with the inhibitors alone (Fig. 37A, B). UV led to apoptosis induction in both the cell lines, which was further increased upon preincubation with both the inhibitors. However, the increase in apopotosis rate was much more pronounced when cells were treated with BB94, compared to AG1478 preincubated ones, where increase in apoptosis was lying within error bars (Fig. 37A, B). These results show that BB94 is better at increasing UV induced apoptosis as compared to AG1478 inspite of the latter being a better inhibitor of UV induced EGFR phosphorylation and downstream signaling molecules Erk and Akt phosphorylation as compared to AG1478.

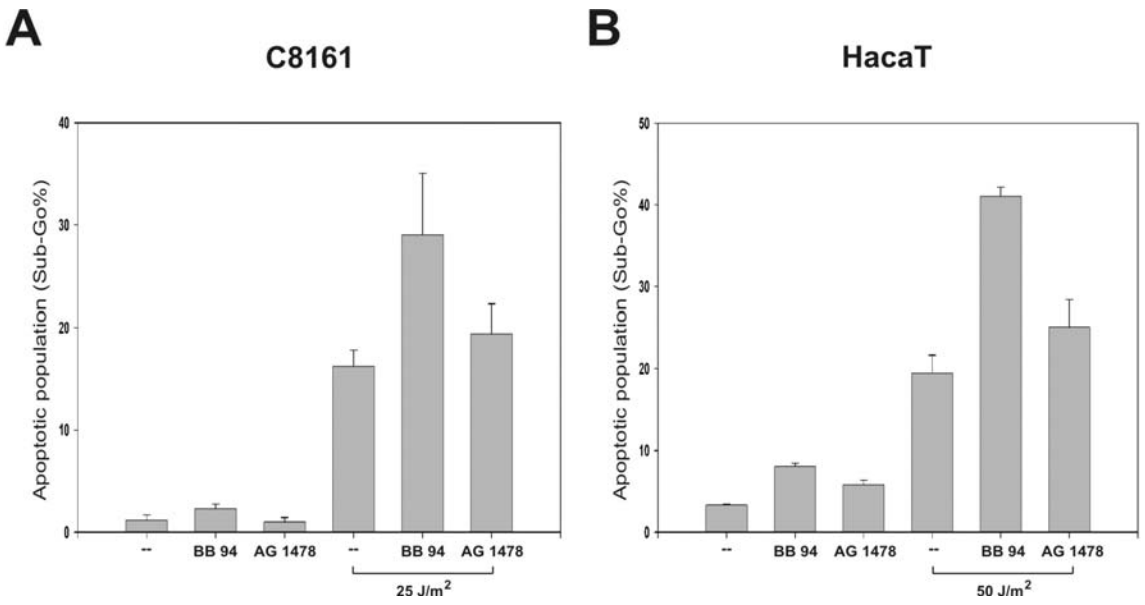


Figure 37. Effect of BB94 and AG1478 Preincubation on UV Induced Apoptosis.

Starved C8161 and HaCaT cells were preincubated with BB94 (10 μM) and AG1478 (250 nM) for 30 minutes, irradiated with the indicated doses of UV and analyzed by flow cytometry 20 hours after irradiation. Statistical representation of the apoptotic fraction of C8161 (A) and HaCaT (B) population, results are average of triplicates ± S.D.

3.7.3.4 BB94 Induces Higher PARP Cleavage Upon UV Stimulation as Compared to AG1478

Poly(ADP-Ribose) Polymerase (PARP) molecules are important nuclear proteins acting as DNA damage sensors (Masutani et al. 2003; Parsons et al. 2005). PARP binds at single strand break sites and recruits members of the DNA repair machinery to the site of

damage, facilitating repair. PARP is also a substrate for caspases and is cleaved into inactive fractions, compromising DNA repair processes (Scovassi and Poirier 1999). In the following experiment we analyzed if preincubation with BB94 or AG1478 leads to a change in the availability of PARP proteins for sites of UV induced DNA lesions. UV irradiation led to an increase in PARP cleavage in both C8161 and HaCaT cells lines as seen by the increased intensity of 89 kDa cleaved PARP fragment (Fig. 38A, B). Increase in UV induced PARP cleavage could be further increased upon preincubation with BB94, however preincubation with AG1478 led to lesser increase in the PARP cleavage in both C8161 and HaCaT cells (Fig. 38A, B). These experiments show that PARP activity can be decreased to a higher extent upon preincubation with BB94.

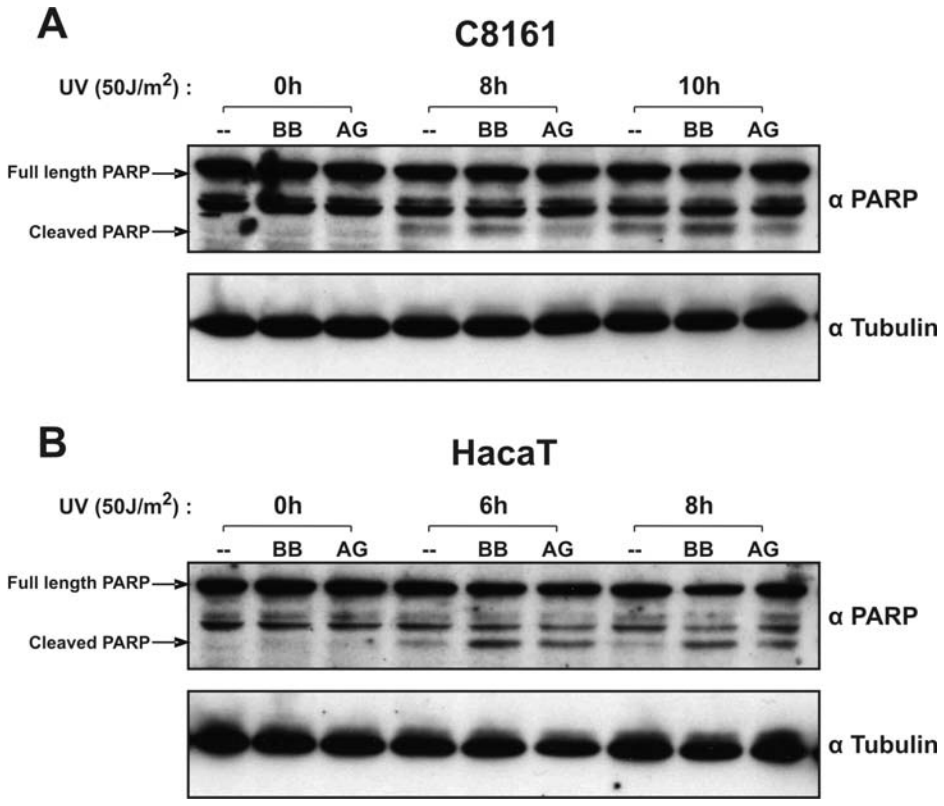


Figure 38. UV Induced PARP Cleavage can be Increased to Higher Levels upon BB94 Preincubation as Compared to AG1478.

Starved C8161 and HaCaT cells were preincubated with BB94 (10μM) and AG1478 (250 nM) for 30 minutes and stimulated with 50 J/m^2 for the indicated time points prior to lysis in RIPA lysis buffer. Equal amounts of lysates were blotted and probed for PARP and αTubulin.

3.7.3.5 AG1478 Induces G2/M Cell Cycle Arrest in Unstarved C8161 and HaCaT Cells

DNA damage leads to a cell cycle arrest, which facilitates the repair of lesions (Khanna and Jackson 2001). To investigate the role of AG1478 and BB94 affecting DNA repair processes and thus survival under UV stress, we observed the effect of these inhibitors on inducing cell cycle arrest. Unstarved C8161 and HaCaT cells were incubated with BB94 and AG1478 for 20 hours and subjected to flow cytometric cell cycle analysis. AG1478 preincubation led to an accumulation of the cell population in G1 phase (G1 arrest) in C8161 and HaCaT cells (Fig. 39A, B). Preincubation with BB94 however on the other hand did not lead to arrest in G1 phase (Fig. 39A, B), instead a slight release of G1 block was observed in C8161 cells measured as a decrease fo the cell population in G1 phase (Fig. 39A). These results show that AG1478 leads G1 arrest in C8161 and HaCaT cells.

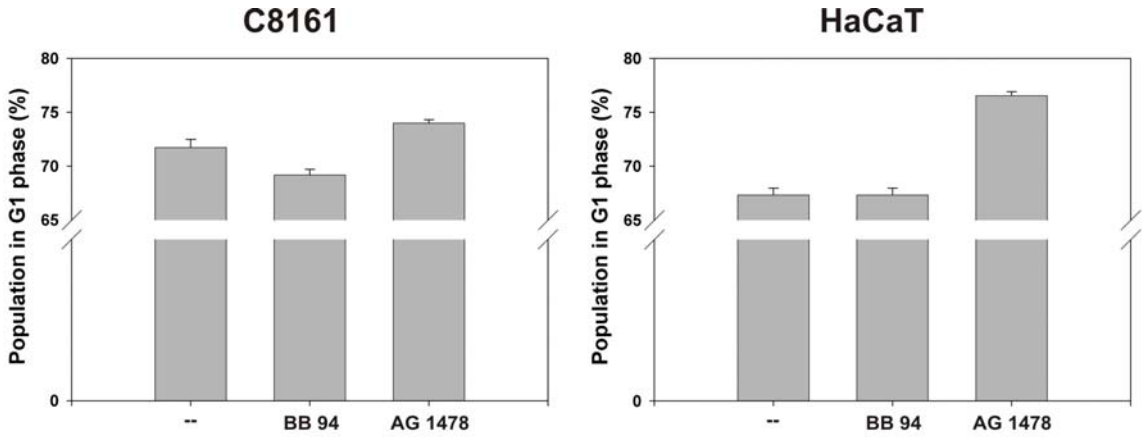


Figure 39. AG1478 Induced Cell Cycle Arrest in Unstarved C8161 and HaCaT Cells.

Unstarved C8161 (A) and HaCaT (B) cells were incubated with BB94 (10μM) and AG1478 (250 nM) for 20 hours. Cells were then harvested and analyzed by flow cytometry. The plotted results are average of triplicates ± S.D.

3.7.3.6 AG1478 Leads to Increase in the Concentration of Cell Cycle Inhibitors p21 and p27, Whereas BB94 Decreases Their Concentration

Members of the EGFR family have been shown to increase proliferation partly by reducing the concentration of cell cycle inhibitors p21 and p27. Therefore inhibition of

the kinase activity of EGFR family members leads to cell cycle arrest by an increase in the concentration of cell cycle inhibitors. We analysed if incubation with the metalloprotease inhibitor BB94 and EGFR kinase inhibitor, AG1478 has any effect on the concentration of the cell cycle inhibitor molecules, p21 and p27. In the cell lines C8161, HaCaT, and RPMI7951 we could see an increase in p27 concentration upon incubation with AG1478 as compared to untreated controls (Fig. 40A, B, C). p21 levels were also found to be increased upon AG1478 incubation as compared to untreated controls in all three cell lines (Fig. 40A, B, C). Incubation with BB94 did not lead to an increase in either p21 or p27 levels as compared to untreated controls, instead there was a slight decrease in p27 levels in C8161 and HaCaT cells (Fig. 40A, B); and a decrease in p21 levels in C8161 and RPMI7951 cells (Fig. 40A, C). These results show that the arrest in G1 phase of cell cycle upon incubation with AG1478 is because of the increase in the concentration of cell cycle inhibitors. BB94 on the other hand could not induce cell cycle arrest, an effect in line with its inability to increase the concentration of cell cycle inhibitors.

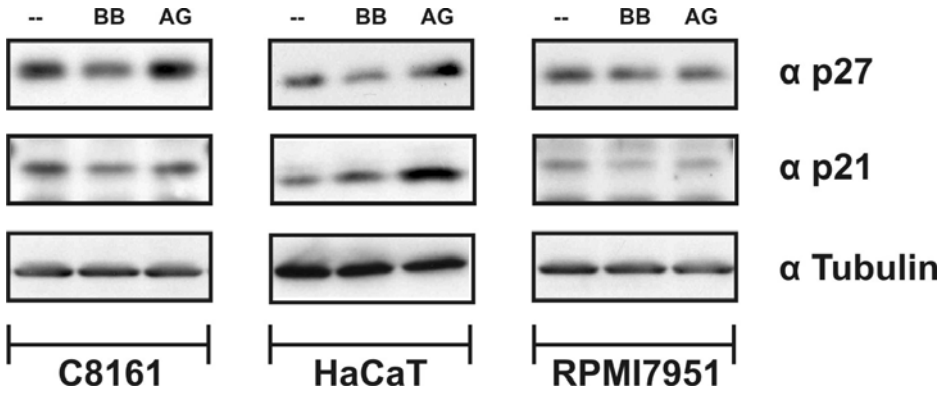


Figure 40. Effect of BB94 and AG1478 Incubation on Concentration of Cell Cycle Inhibitors.

Unstarved C8161 (A), HaCaT (B), and RPMI7951 (C) were incubated with BB94 (10μM) and AG1478 (250 nM) for 20 hours prior to lysis. Equal amounts of lysates were blotted and probed for p21, p27, and αTubulin.

4 Discussion

Protein phosphorylation is a major mode of rapid modulation of protein activity, regulating possibly more than one third of the human proteins (Knebel et al. 2001). Mammals possess 518 putative protein kinase genes, and 130 protein phosphatases to choose from in order to achieve regulated phosphorylation and thus activity of various proteins (Blume-Jensen and Hunter 2001; Manning et al. 2002). The Protein tyrosine kinase subfamily consists of 90 identified tyrosine kinases (TK) and 39 TK like genes in humans, their products regulate cellular proliferation, survival, differentiation, and motility (Krause and Van Etten 2005). The EGFR family belonging to the receptor tyrosine kinase (RTK) subset c4onsists of four members; Her1, Her2, Her3, and Her4. They are highly conserved and present in *Caenorhabditis elegans* to Drosophila to humans (Yarden and Sliwkowski 2001). Members of the EGFR family are essential for normal physiological functions and signaling aberrations lead to various kinds of hyperproliferative diseases (Hynes and Lane 2005; Sunpaweravong et al. 2005). Research strategies aimed at inhibiting signaling pathways involving EGFR family members in cancer hold therapeutic promise (Holbro and Hynes 2004).

4.1 EGFR Transactivation by UV Irradiation

Incidence of skin cancer is on the rise and much of it can be attributed to exposure to ultraviolet radiations from sunlight (Gloster and Brodland 1996). Members of the EGFR family have been shown to be overexpressed in skin cancer, but activating mutations have not been found to date (Krahn et al. 2001). In this study we show a novel mechanism of EGFR activation upon UVC irradiation, using various cell lines derived from skin cells. The EGFR activation is dependent on metalloprotease activity and proligand shedding, a pathway close in character to GPCR induced EGFR transactivation. Furthermore, at the low doses of UVC used in this study we found this pathway to be the predominant mode of EGFR activation. More importantly, the biological significance of this pathway can not be overemphasized by our findings that skin cells exploit this pathway to survive longer under UV irradiation, and repair potentially lethal DNA lesions, allowing more time to accumulate carcinogenic mutations.

4.1.1 UVC Induced EGFR Transactivation Depends on Metalloprotease Activity and Proligand Shedding

EGFR transactivation involves metalloprotease activity and proligand shedding of EGF ligand family (Daub et al. 1996; Prenzel et al. 1999; Fischer et al. 2004). We show a similar mechanism of UVC induced EGFR activation, where metalloprotease activity and proligand shedding are the necessary events. EGFR activation by UV has been described before, where ROS produced by UV can inactivate PTPs, which are negative regulators of EGFR phosphorylation (Knebel et al. 1996). However, we observed contrasting features of UV induced EGFR activation compared to the ones that have been described before. One of the first differences we observed was a delayed onset of EGFR phosphorylation in our study as compared to fast activation shown in previous study (10 minutes compared to 1-5 minutes) (Fig. 13A, B). Secondly, in our study UVC increased EGFR phosphorylation in a dose dependent manner, but only at low doses and reached a plateau at around 300 J/m^2 (Fig. 14A, B). This effect was not observed in the previous study, where UV continued to increase pEGFR levels over a range of 50-5000 J/m^2. Finally, in our observation the intensity of EGFR phosphorylation was at lower levels as compared to EGF (5ng/ml) treatment (Fig. 13A, B), whereas previous studies show much stronger EGFR phosphorylation. At higher doses UV induced ROS production leading to PTP inactivation might be the predominant pathway of EGFR activation. At lower doses, however, our findings show that EGFR activation depends more on the transactivation pathway, as BB94 inhibitor alone reduces the phosphorylation of EGFR to almost basal levels in HaCaT (Fig. 16C) and RPMI7951 (Fig. 16B) cells. But the PTP inactivation mechanism or another pathway could not be ruled out, because BB94 could not completely abort basal EGFR phosphorylation in C8161 (Fig. 16A) and SCC-9 cells (Fig. 16D). The possibility of interaction of different pathways and also their mechanism is an exciting topic for further investigation.

Upon observing the kinetics of activation of EGFR upon UV irradiation in both cell lines, we find that EGFR phosphorylation appears at about 10 minutes, which occurs considerably later as compared to direct EGF treatment (1 min) or GPCR agonist administration (3-5 min) (Fig 13 A, B). UV leads to photolysis of water producing

Reactive Oxygen Species (ROS); these ROS then mediate the biological effects that are associated with UV exposure. Delay in UV induced EGFR phosphorylation could thus be explained as delay in generation of ROS. ROS could participate in EGFR transactivation by modulating ADAM activity which leads to generation of EGF like ligands. ADAMs are synthesized as zymogens, which are autoinhibited by their prodomain. ROS mediated removal of the prodomain leading to ADAM activation has been shown previously and this could be a way of UV induced EGFR transactivation (Zhang et al. 2001).

Members of EGFR family exert their biological effect via various downstream signaling cascades. PI3K/Akt pathway and MAPK pathway are usually coupled to EGFR activation, and exert various biological effects like proliferation, migration, invasion, and anti-apoptosis. UV induced Erk and Akt phosphorylation has been shown before (Huang et al. 2001; Bode and Dong 2003). In this study, we show the mechanism of Erk and Akt phosphorylation to be EGFR transactivation dependent, as BB94 preincubation could reduce UV induced Erk and Akt phosphorylation (Fig 17A, B). Thus, the dependence of Erk and Akt activation on metalloprotease activity places the transactivation pathway in physiologically relevant perspective, as Akt provides a strong resistance to apoptosis by inactivation of proapoptotic proteins and Erk provides a strong proliferation signal.

We found out that EGFR activation upon UV irradiation is dependent on an extracellular ligand binding event as, UV induced EGFR phosphorylation could be blocked by antibodies binding to the extracellular EGFR domain (Fig. 18A, B). Neutralizing antibodies against amphiregulin blocked UV induced EGFR phosphorylation in SCC-9 cells, showing it to be the proligand required for EGFR activation by UV (Fig. 19). Interestingly, amphiregulin is an autocrine keratinocyte growth factor, and aberrant activation or overexpression results in rapidly growing keratinocytic tumors and melanoma (Tomic et al. 1995; Piepkorn et al. 1998; Bardeesy et al. 2005). The overexpression of amphiregulin observed in skin cancer and its role in UV induced EGFR transactivation identifies it as a key signaling component in skin cancer.

ADAM9 is absent in normal melanocytes but its expression is found in melanoma within the invasive front and in various melanoma cell lines (Zigrino et al. 2005). We show

ADAM9 to be responsible for proligand shedding upon UV irradiation activating EGFR in SCC-9 cells (Fig. 20). Altogether, overexpression of ADAM9 may allow efficient exploitation of UV induced EGFR transactivation pathway by skin cancer cells.

4.1.2 EGFR Transactivation Upon UVC Irradiation Provides Anti-apoptotic Advantage and Prolonged Activity of PARP

Preincubation with a metalloprotease inhibitor led to increased sensitivity of C8161 (Fig. 21A) and HaCaT (Fig. 21B) cells to UV induced apoptosis. EGFR transactivation led to the activation of PI3K/Akt pathway (Fig. 17A, B), which has been shown to inactivate several proteins in the pro-apoptotic pathway, like caspase9, Ask1 (Apoptosis signal-regulating kinase 1), and Bax-subfamily members of family like Bad leading to prolonged survival (Kandel and Hay 1999). Thus the dependence of PI3K/Akt pathway on EGFR transactivation could allow the cells to survive longer under UV stress.

Prolonged exposure to UV leads to highly damaged DNA, which if left unrepaired would direct the cells to apoptosis. But if UV induced lethality can be overcome by repairing potentially lethal DNA lesions, UV can be an excellent source for accumulating oncogenic mutations. PARP cleavage has been widely used as an early indicator of apoptosis (Lazebnik et al. 1994). However, numerous studies show PARP to be a key anti-apoptotic mediator owing to its DNA damage repair activity, rather than just an innocent bystander cleaved by caspases (Scovassi and Poirier 1999). EGFR transactivation leads to prolonged stability of PARP, prolonging the nucleotide excision repair to remove DNA lesions directly induced by UV; and single strand break repair and base excision repair to remove lesions induced by ROS (Wood et al. 2001; Flohr et al. 2003). UV induced EGFR transactivation also activated MAPK1/2 which have been shown to increase the expression of DNA repair proteins XRCC1 and ERCC1 (Yacoub et al. 2001). Thus EGFR transactivation provides a mechanism for cells to survive under UV stress and accumulate harmful mutations by facilitating them with antiapoptotic signals, and at the same time prolonging and activating DNA repair processes to remove potentially lethal lesions induced by UV and ROS.

In the end, EGFR transactivation under UV irradiation holds therapeutic potential in malignancies of skin origin. Upon blockage of this pathway transformed cells will undergo apoptosis during UV exposure, thereby interfering with oncogenesis. Blockage of EGFR transactivation will also reduce the chances of accumulating mutations which may lead to a more aggressive form of cancer.

4.2 Reactive Oxygen Species in EGFR Transactivation

In this study we found that various GPCR agonists induce EGFR transactivation (Fig. 22). GPCR induced EGFR phosphorylation was further found to be dependent on metalloprotease activity and EGFR kinase activity (Fig. 23). We could also show UV induced EGFR phosphorylation to be dependent on metalloprotease activity, EGF induced EGFR phosphorylation however was unaffected in presence of BB94 (Fig. 23). Thrombin induced EGFR activation and downstream signaling, could also be inhibited by metalloprotease and EGFR kinase inhibitors (Fig 24). We further showed Hb-EGF shedding to be involved in thrombin induced EGFR transactivation as preincubation with Hb-EGF inhibitor CRM197 could reduce thrombin induced EGFR phosphorylation (Fig 25). Together, these results show that both UV and GPCR stimulation depend on metalloprotease activition and proligand shedding in skin cancer cells.

4.2.1 ROS Production During EGFR Transactivation is Dependent on EGFR Kinase Activity and Metalloprotease Activity

Reactive oxygen species are shown to be produced during EGF induced EGFR activation. This ROS production assists the lateral propagation of EGFR activating signal (Bae et al. 1997; Fischer et al. 2004). Several reports also show ROS to be produced in response to GPCR agonist administration (Griendling et al. 1994). We could further find the generation of ROS during EGFR transactivation (Fig. 27). Furthermore, we showed that the production of ROS by UV irradiation is dependent on EGFR kinase activity and the metalloprotease activity, showing the dependence of ROS production on EGFR transactivation pathway. ROS are key intracellular secondary messengers, which act by oxidizing various proteins at susceptible cysteine residues altering their biological

activity positively or negatively (Nishida et al. 2000; Meng et al. 2002; Kamata et al. 2005). ROS involvement in EGFR transactivation increases the chances to interact with more pathways and signaling molecules. Cross talk between various pathways is important in signal amplification and diversification, which often leads to new biological functions and outcomes. Aberrant ROS signaling has been shown to be involved in various pathological processes including cancer (Benhar et al. 2002), thus investigating ROS signaling in EGFR transactivation would lead to a better understanding of the disease processes.

4.2.2 Nox Proteins Produce ROS Which Mediates EGFR Transactivation.

In this report we showed GPCR and UV induced EGFR transactivation to be dependent on ROS production (Fig. 29). Additionally, the source of ROS production was found to be proteins belonging to the NADPH oxidase family, as UV induced EGFR transactivation and downstream signaling was inhibited by the Nox family inhibitor, DPI (Fig. 30, 31). Stress induced EGFR transactivation has been shown to be dependent on ROS, and our study further increases our understanding of the role of ROS in EGFR transactivation (Fischer et al. 2004).

The mechanism of ROS induced EGFR activation is still not clear and a number of signaling molecules may be involved. PTP inactivation and/or degradation in response to high UV doses leading to prolonged EGFR activity has been previously reported (Gross et al. 1999; Gulati et al. 2004). At low UV doses used in our studies, however, the inhibition of metalloprotease activity almost completly abolished EGFR activation showing it to be the major mode of EGFR activation. Thus PTP inactivation plays only a marginal role if at all in EGFR transactivation at lower UV doses. We show that Src family members have been involved in UV induced EGFR transactivation (Fig. 22). Src is involved in GPCR induced EGFR transactivation (Roelle et al. 2003). Moreover, Src family members carry ROS susceptible cysteines leading to its activation upon oxidation, and thus could provide the link between ROS production and EGFR transactivation (Giannoni et al. 2005). Additionally, ROS could interact with EGFR transactivation via ADAMs as they have been shown to be activated by ROS via oxidation of cysteines in

the inhibitory loop (Sanderson et al. 2006). We also show that ROS production partially depends on EGFR kinase activity and metalloprotease activity. This observation hints at a positive feedback loop, where, EGFR activation leads to increased ROS production, possibly by regulating the activation of NADPH oxidases. Nox proteins are multisubunit proteins and thus provide the possibility of being regulated via interactions with the signaling proteins.

Combined together our study puts ROS production and Nox proteins into EGFR transactivation pathway. This study also opens another possibility to inhibit EGFR transactivation pathway either alone or in combination with other strategies aimed at skin cancer prevention and cure.

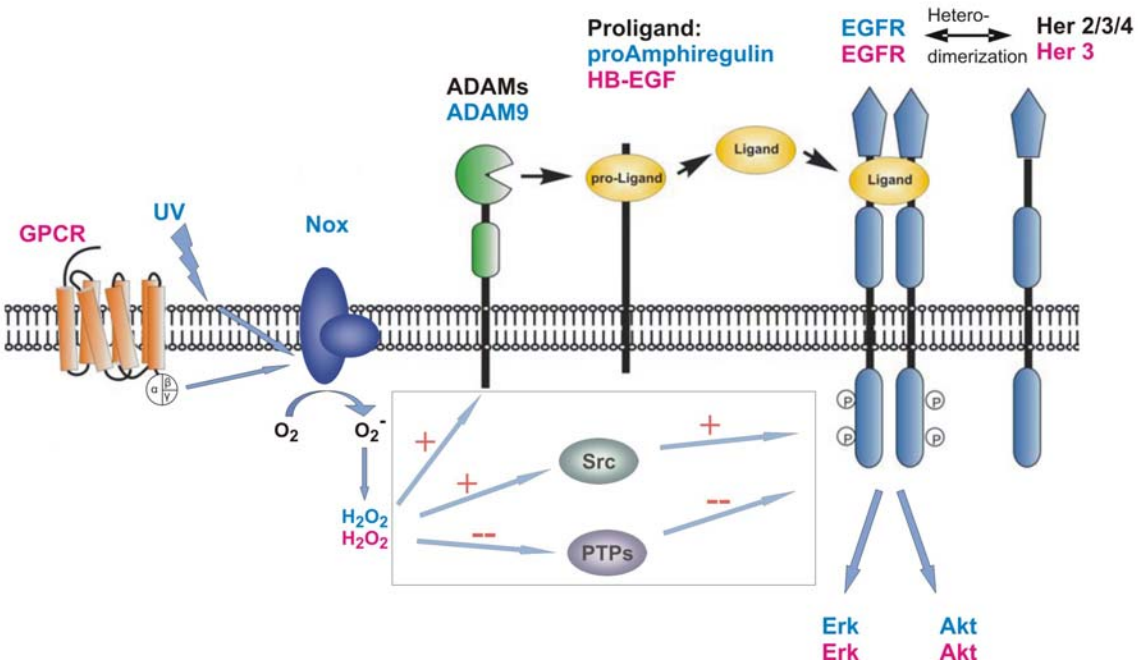


Figure 45. UV and GPCR induced EGFR transactivation in cancer cells of skin lineage, and the role of reactive oxygen species

UV induced EGFR transactivation leads to activation of ADAM9 which inturn cleaves proamphiregulin. Amphiregulin then diffuses and binds to EGFR leading to its activation and downstream signaling, which, then confers anti-apoptotic advantage to skin cancer cells.

Reactive oxygen species are produced during UV irradiation and GPCR agonist stimulation by activation of Nox proteins, which lead to EGFR transactivation, possibly involving ADAM/Src activation (shaded pathways in the box are possible mechanisms).

4.3 Therapeutic Potential of Blocking EGFR Transactivation in Skin Cancer by Metalloprotease Inhibition

The EGFR transactivation pathway has been exploited by various cancer cells to gain several advantages like invasion, migration, proliferation, and anti-apoptosis. Thus strategies to inhibit EGFR transactivation in cancer are promising therapeutic intervention points. In this report we further our understanding of EGFR transactivation pathway in cancer cells of skin origin in stimulation dependent and independent ways. We began by showing that EGFR transactivation occurs in skin cancer cells under UV irradiation and that this pathway gives anti-apoptotic advantage to these cells. We also observed higher basal activation of members of Her family in cancer cells and showed it to be dependent on the transactivation pathway under serum supplemented conditions. Furthermore, using the primary and secondary melanoma cell lines from the same patient, we showed that the EGFR transactivation pathway appears during late stages of cancer progression, and is absent in non-transformed melanocytes, and primary melanoma. Thus EGFR transactivation pathway is a very important point of intervention for preventing the initiation and propagation of skin cancer.

We employed two strategies to stop EGFR transactivation pathway one inhibiting the metalloprotease activity and the other inhibiting the EGFR kinase activity. BB94, a broadspecificity inhibitor of metalloproteases belonging to ADAM family, and AG1478, a specific EGFR kinase inhibitor were used in this study. We found out that metalloprotease inhibition by BB94 was more efficient than EGFR tyrosine kinase inhibiton by AG1478 in reducing the population of skin cancer cells under UV irradiation. The effect could partially be explained by our results which showed that AG1478 led to arrest in G1 phase of the cell cycle. The cell cycle arrest provides opportunity for the repair of DNA damage (Khanna and Jackson 2001). Moreover, among both the inhibitors BB94 treatment was more effective in inactivating DNA repair protein PARP involved in the repair of potentially lethal lesions induced by UV irradiation. AG1478 treatment leads to shutting down of all signals from EGFR, which

are required for proliferation, arresting DNA replication and inducing cell cycle arrest. UV leads to DNA lesions, which lead to stalling of replication forks in rapidly dividing cells, signaling them to undergo apoptosis. Skin cancer therapy with direct EGFR kinase inhibitor is thus not advisable as it induces cell cycle arrest and gives more time for cancer cells to repair UV induced potentially lethal lesions during this phase.

Metalloproteases like ADAM9 are associated strongly with melanoma and are absent in normal melanocytes (Zigrino et al. 2005). Overexpression and expression of aberrant form of ADAM9 has been implicated in cancer invasion and metastasis (Shintani et al. 2004; Zigrino et al. 2005). Skin cancer cells are dependent on the transactivation pathway for anti-apoptotic signaling via Akt and to repair their DNA lesions via PARP. The blockage of this pathway makes them hypersensitive to UV induced apoptosis. The metalloprotease inhibitor BB94 does not lead to cell cycle arrest, and cells proliferate normally. Thus BB94 blocks only stimulation dependent EGFR signaling in cancer cells and is more effective in inducing apoptosis than inhibiting all the signaling through EGFR. Additionally, ADAMs interact with many proteins which could lead to other anti-apoptotic effects in addition to the ones through EGFR signaling. Thus ADAM inactivation is a key therapeutic target in skin cancer.

5 Summary

The main findings of this study are:

1. UV led to EGFR transactivation in melanoma and squamous cell carcinoma cell lines. This pathway conferred cancer cells with survival advantage under UV irradiation.

2. UV/GPCR induced EGFR transactivation was found to be dependent on ROS production by the Nox protein family.

3. Inhibition strategies targeting ADAMs led to higher apoptosis by UV irradiation as compared to the direct EGFR inhibition, which could be explained by differences in the ability of inhibitors to induce cell cycle arrest.

Additionally, we demonstrated a selective presence of EGFR transactivation pathway in malignant melanoma, and absence in primary melanoma. Blocking EGFR transactivation pathway thus holds prophylactic and therapeutic potential in skin cancer.

Zusammenfassung

Die wichtigsten Erkenntnisse dieser Studie sind:

1. UV-Bestrahlung führte in Melanom- und Plattenepithelkarzinom-Zelllinien zur Transaktivierung des EGF-Rezeptors. Dieser Signalweg verlieh den Krebszellen einen Überlebensvorteil unter UV-Strahlung.

2. Es wurde gezeigt, dass die UV/GPCR- abhängige Transaktivierung des EGFR-Rezeptors von der Produktion von ROS durch die Familie der NOX-Proteine abhängig ist.

3. Die Inhibition von ADAMs führte im Vergleich zu einer direkten EGFR-Inhibition zu einer höheren UV-induzierten Apoptoserate. Dies kann erklärt werden durch Unterschiede in deren Vermögen, den Zellzyklus zu stoppen.

Zusätzlich konnten wir zeigen, dass der Signalweg für die EGFR-Transaktivierung nur in malignen Melanomen und nicht in primären Melanomen zu finden ist. Die Blockierung der EGFR-Transaktivierung beinhaltet daher ein prophylaktisches und therapeutisches Potential für die Bekämpfung von Hautkrebs.

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Acknowledgements

I am especially grateful to my supervisor Prof. Dr. Axel Ullrich for accepting me as his Ph.D student, for his invaluable suggestions and guidance throughout the training period, help with preparation of manuscripts, and help in planning future career moves.

I am indebted to Prof. Dr. Alfons Gierl for supporting and promoting this doctoral thesis at the Technische Universität, München. He helped me a lot with the university formalities.

I thank Pjotr for discussions and sharing array data and Tatjana for cDNA and RT-PCR help. I am grateful for the technical help I received from Uta and Renate to meet deadlines. Iris has been a great help with official and administrative support.

I thank my labmates and colleagues, for intellectual discussion and help and for making my stay enjoyable in lab. I especially thank Philip, Markus, Matthias, Jacqueline, Sushil, Anke, Martin, Nina, and Christian for careful reading of thesis and manuscripts, for calls in german, and translations. I thank my seniors Stefan, Oliver, Michael, and Beatrix for initial help and encouragement.

I am very grateful to all my teachers for guidance and inspiration. I also want to thank my friends for their unfailing support.

I especially want to thank my parents and my sister, for their unconditional love and support. Finally, I want to thank Shruti, my greatest ally and unyielding critique.

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Publications

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- **Singh B**, Knyazev P, and Ullrich A. UVC induced EGFR transactivation is dependent on proligand shedding induced by metalloprotease activation, which confers survival advantage to transformed skin cells (***manuscript under preparation***).
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