بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

قال الله تعالى

{وَلَقَدْ خَلَقْنَا الإِنسَانَ مِنْ سُلالَةٍ مِنْ طِينٍ * ثُمَّ جَعَلْنَاهُ نُطْفَةً فِي قَرَارٍ مَكِينٍ * ثُمَّ خَلَقْنَا النُّطْفَةَ عَلَقَةً فَخَلَقْنَا الْعَلَقَةَ مُضْغَةً فَخَلَقْنَا الْمُضْغَةَ عِظَامًا فَكَسَوْنَا الْعِظَامَ لَحْمًا ثُمَّ أَنشَأْنَاهُ خَلْقًا آخَرَ فَتَبَارَكَ اللَّهُ أَحْسَنُ الْخَالِقِينَ} [المؤمنون: 12-14].

(Englisch Translation)

And indeed We created man (Adam) out of an extract of clay (water and earth). Thereafter We made him (the offspring of Adam) as a Nutfah (mixed drops of the male and female sexual discharge) (and lodged it) in a safe lodging (womb of the woman). Then We made the Nutfah into a clot (a piece of thick coagulated blood), then We made the clot into a little lump of flesh, then We made out of that little lump of flesh bones, then We clothed the bones with flesh, and then We brought it forth as another creation. So blessed be Allah, the Best of creators.

Surat Al-Mu'minūn (The Believers) سورة المؤمنون *(The holy Koran).*

TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Genetik

Significance of the Negative Regulator of HER Receptor Tyrosine Kinase Family, mig-6 Protein, in Colon Cancer and Glioblastoma

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

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To my family and my wife

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I Introduction

1. Receptor tyrosine kinases (RTKs) as a regulation key

The regulation of important physiological processes by reversible protein phosphorylation is an important regulatory principle, which is based on the antagonistic action of kinases and phosphatases (Sun et al., 1994, Streuli et al., 1996, Weiss et al., 1998). Whereas protein kinases transfer phosphate groups of Adenotriphosphate (ATP) on the serine, threonine and tyrosine amino acids of proteins, which thereby get activated, the dephosphorylation of the corresponding proteins by protein phosphatases leads to the opposite effect. The Phosphorylation of proteins on tyrosine residues constitutes in fact less than 0, 01% of the total intracellular phosphorylation. It is however essential for the regulation of numerous processes such as proliferation, differentiation, cell adhesion and cell cycle regulation (Hunter et al., 1980, 1995, Levitzki et al., 1995). Indeed, tyrosine phosphorylation may be the primary, or even the exclusive, indicator of signal transduction in multicellular organisms (Ullrich and Schlessinger., 1990).

1.1 Structural properties

The family of receptor tyrosine kinases includes more then 50 transmembrane proteins that have a common topology. Starting at the N-terminus, the RTKs are characterized by an extracellular domain of several hundred amino acids that contains a distinctive pattern of Cys residues and having often a characteristic array of structural motifs. A transmembrane domain, which consists of a stretch of hydrophobic residues connecting the above described region to the intracellular domain. The latter consists of 3 successive domains: A juxtamembrabe region, which precedes the catalytic domain, the catalytic domain, which is about 250 to 350 residues in length, and the C-terminal tail (Van der Geer et al., 1994, Blume-Jensen et al., 2001, figure. 1). Most protein kinases share a conserved catalytic domain as sequence identity within this region ranges from 32% to 95%. Moreover Alignment of catalytic domain sequences revealed that there are 13 residues conserved in all 54 Sequence of RTKs (Ullrich et al., 1990, Van der Geer et al., 1994). This catalytic domain also known as a kinase domain is in fact responsible for tyrosine

phosphorylation of various RTK substrates as well as for the auto-phosphorylation of RTKs.

Despite the very similar topology, RTKs are characterized by some structural differences especially in the extracellular domain. Indeed, this receptor portion is responsible for ligand binding and differs from one RTK-family to another. According to these differences, RTKs were classified into 20 different families having different roles in the regulation of different biological and physiological processes (Van der Geer et al., 1994, Plowmann et al., 1994, Blume-Jensen et al., 2001; Figure. 1).

The activation of RTKs starts with a ligand induced conformational change that leads to the dimerization of both receptor-monomers. Such proximity of the catalytic domains of both receptor partners allows the mutual trans-phosphorylation of their tyrosine residues and the initiation of several various signalling pathways within the cell.

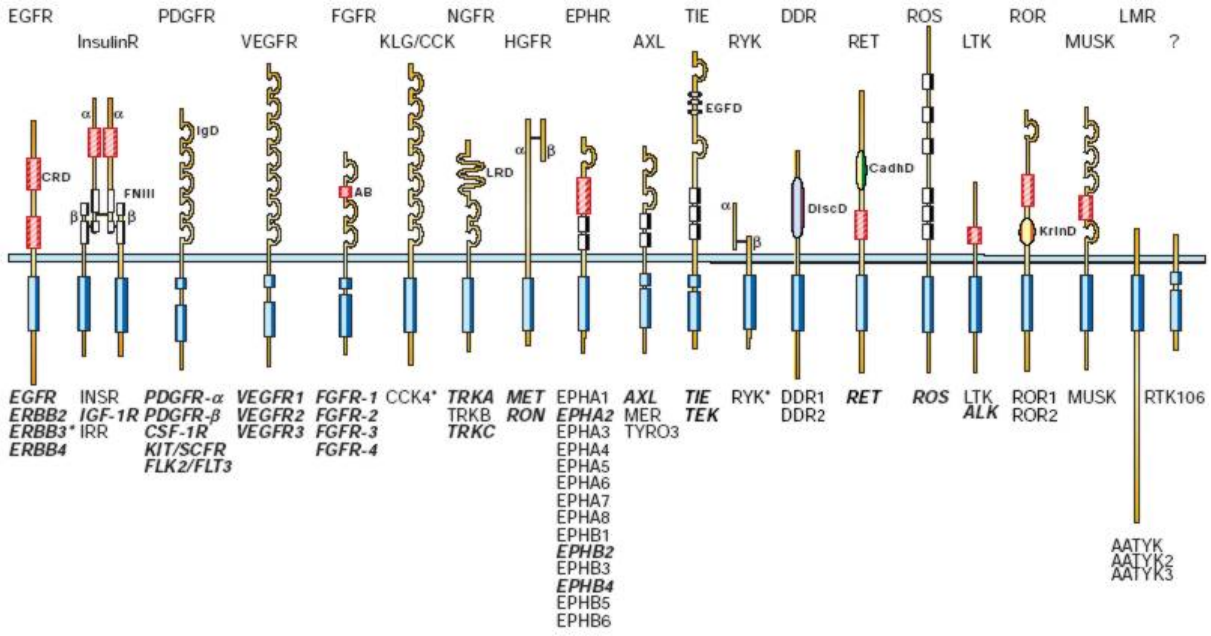


Figure 1. Schematic presentation of RTK families
The RTKs are classified in 20 families. The classification is mainly based on differences in the extracellular portion of each family, which is responsible for ligand-binding (The figure was taken from Blume-Jensen et al., 2001).

1.2 The EGFR receptor family

The EGFR family (ErbBs) consists of four closely related subtypes: EGFR (epidermal growth factor receptor) (erbB1), HER2 (erbB2/neu), HER3 (erbB3), and HER4 (erbB4) (Schlessinger et al., 2000, Simon et al., 2000). Structurally, these receptors are transmembrane glycoproteins sharing the same basic features and are composed from three regions namely the extracellular ligand-binding domain, the transmembrane domain, and the intracellular portion containing the catalytic also known as kinase domain (Olayioye et al., 2000, Figure. 2).

EGFR receptor ligands have been identified and classified into three different groups based on their binding to the various ErbB receptors (Fedi et al., 2000). Epidermal growth factor (EGF) and transforming growth factor-α (TGF- α) belong to the first group and bind exclusively to EGFR. In contrast, the second group binds to HER2 and HER4 and includes heparin-binding EGF (HB-EGF), betacellulin (BTC), and epiregulin (Shelly et al., 1998). The third and last group of ligands, which bind exclusively to HER3 and HER4, include a group of complex polypeptides called neuregulins (NRGs) (Salomon et al., 1995, Wen et al., 1994).

In general, binding of a ligand to the extracellular binding site of the EGFR, precedes dimerization and results in the activation of the intrinsic protein tyrosine kinase. The resultant phosphorylated tyrosine residues serve as docking sites downstream of signalling molecules and cytoplasmic messenger proteins, stimulating a cascade of signalling proteins through the ras-raf-mitogen-activated protein kinase and PI3K-AKT pathways, to the nucleus, culminating in gene expression, cell proliferation, -apoptosis, -migration and invasion (Gschwind et al., 2004, Figure. 2). Dysregulation of members of the HER family, either by activating mutations, receptor overexpression or aberrant ligand release triggers the development of a variety of human diseases including cancer (Yarden et al., 2001).

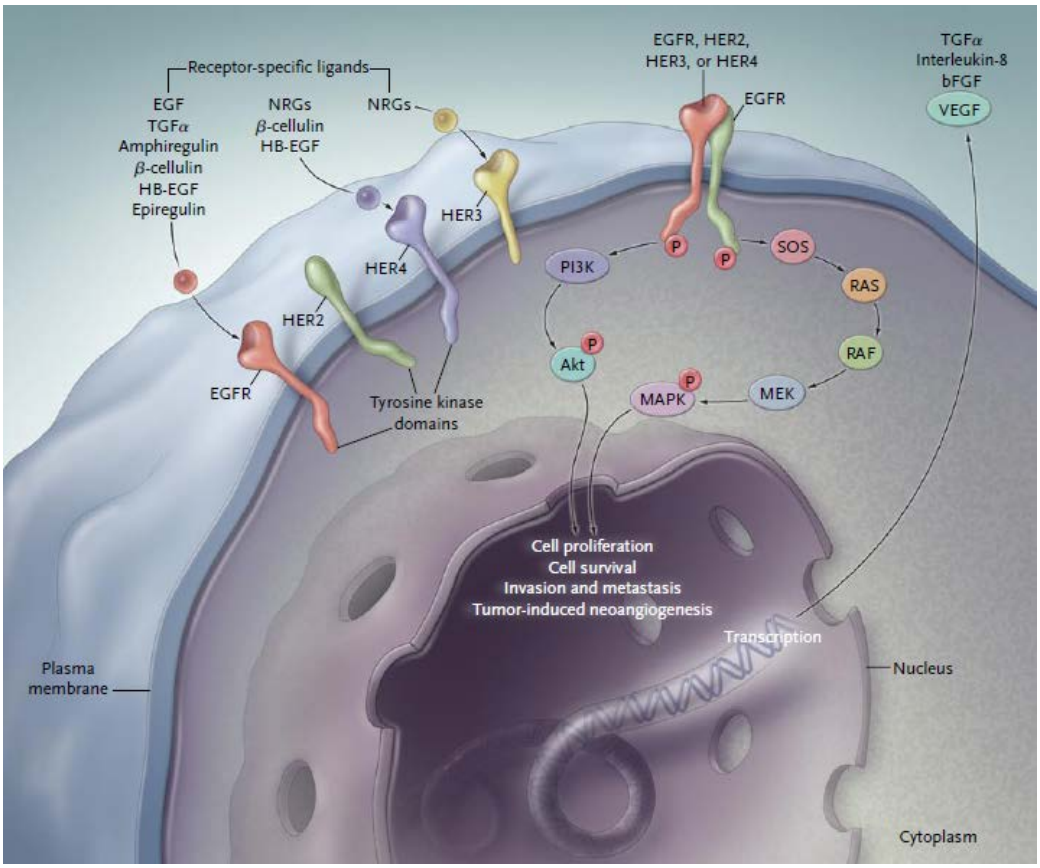


Figure 2. EGFR receptor family members and their signalling pathways

The EGFR receptors family consists of four members, the EGFR (HER1), HER2, HER3 and HER4. Specific binding of ligands including EGFR, TGFα,amphiregulin, beta-cellulin, epiregulin, heparin-binding EGF and the heregulins on their specific receptors leads to the activation of receptors and triggers subsequently the activation of signalling cascades, thereby regulating different biological outcomes including cell proliferation, -survival, -migration, and invasion (The figure was taken from Ciardiello et al., 2008).

1.2.1 EGFR (Epidermal growth factor receptor)

EGFR represents the first member of the ErbB receptors family. Its gene maps to the human chromosome 7p12, and results in a protein with a molecular weight of 170 Kda. EGFR was cloned the first time by Ullrich and colleagues in 1984 using molecular biological methods and was described as a tyrosine kinase possessing oncogenic properties (Ullrich et al., 1984). Upon ligand binding, EGFR becomes activated and forms either homo-dimers with another EGF-receptor or heterodimers by binding to another member of the family, mainly HER2 and HER3 (Schulze et al., 2005). Docking proteins, which play a pivotal role in the initiation of signalling into the cell nucleus, bind the phosphorylated tyrosine residues distal to the kinase domain of

the ErbB receptors and could initiate three main signalling pathways thereby regulating a wide range of cellular processes including cell proliferation, -migration, and -survival (Jorissen et al., 2003). EGFR activates mainly the mitogen-activated protein kinase (MAPK) signalling pathway through binding of growth factor receptor bound-2 (GRB2) and src-homology 2-containing (Shc) proteins to the phosphorylated docking sites of the receptor. Starting from these two adaptor proteins, the signal is then transduced to further proteins starting from son of sevenless (SOS) until extracellular regulated kinase 1/2 (ERK 1/2), which regulates transcription factors within the nucleus (Figure. 2). PI3K-AKT signalling pathway could be also stimulated upon EGFR activation (Figure. 2). Another direct adaptor protein of the EGFR is the signal transducer and activator of transcription 3 (STAT3), which activates downstream signalling events via the JAK/STAT pathway (Figure. 2).

A vast body of knowledge has been accumulating in recent years on the role of the EGFR in animal development and disease. The generation of genetically modified mouse models represents a useful tool employed in this regard. Indeed, knockout mice are severely affected, although to varying degrees depending on their genetic background (Threadgill et al., 1995, Sibilia et al., 1995, Miettinen et al., 1995). At its most severe, the lack of EGFR causes peri-implantation (CF-1 mouse strain) or mid-gestational death (129/Sv strain). In other strains (CD-1), however, the mice survive up to 3 weeks post birth. These animals show severe abnormalities of skin, lung, gastrointestinal tract, brain, and liver thereby confirming the importance of EGFR in epithelial cell regulation (Threadgill et al., 1995). On the other hand, a strain-independent progressive neurodegeneration was shown in EGFR-null mice survivors (Sibilia et al., 1998). Furthermore, mice expressing a naturally occurring dominant negative EGFR allele (*wa-2* strain) display reduced mammary glands in size and underdeveloped ducts suggesting the crucial role of EGFR in the development of these organs (Wiesen et al., 1999).

In the last years many studies have investigated the role of EGFR in the development and progression of human cancers. This phenotype is correlated with gain of function, which causes EGFR overexpression, or defined mutations.

Indeed, EGFR was described to be overexpressed in many solid tumors, including breast, ovarian, renal, lung and head and neck (Table. 1). Moreover, EGFR expression was shown to be greater in metastases compared to the primary tumors, and high EGFR expression has been associated with advanced tumor stage and

resistance to standard therapies (Arteaga et al., 2002). Furthermore, EGFR family over-expression and amplification was shown to be the main cause of cancer development compared to other RTKs. Indeed, normal cells contain about 20.000 to 200.000 receptor-copies per cell, but in malignant breast cells for instance, receptors can number 2 million per cell (Carpenter et al., 1978). Along with this, amplification of growth factor receptor genes can increase the number of cell surface receptors. EGFR gene amplification has been observed also in other tumors such as astrocytoma and non small cell lung cancer (NSCLC). Overproduction of EGFR thereby increases signalling in the presence of excess ligand, leading to uncontrolled cell division and growth (Collins et al., 1995, Reissmann et al., 1999). Aberrations in the EGFR gene such as deletions or point mutations, which maintain the receptor in its active status, were also described (Jorissen et al., 2003). The EGFRvIII also called EGFR-Δ is one naturally occurring example of such alterations. It is generated through an in-frame deletion of exons 2-7 from the extracellular domain of the receptor leading to a constitutively active form of the receptor (Ekstrand et al., 1992). More details about this mutation will be discussed below. Furthermore, several point mutations in the tyrosine kinase domain of the EGFR have been also investigated (Hynes et al., 2005, Cai et al., 2008). As by the EGFRvIII, these mutations also activate EGFR and are a direct cause for the initiation of cancer in various human organs.

1.2.2 HER2 (Human epidermal growth factor receptor2)

The second member of the HER family to be discovered was HER2 (erbB2/neu), which shows structural similarity to EGFR. Although HER2 is a more potent oncoprotein than other members of the EGFR family, it has no known high-affinity ligands. It is considered as a non-autonomous receptor, also known as orphan receptor (Hamid et al., 2004). Along with this, HER2 was shown to be the preferred hetero-dimerization partner of the other three EGFR-receptor family members (Tzahar *et al*., 1996) and is therefore called as the “non-autonomous amplifier”. Furthermore, HER2-containing dimers are endocytosis impaired and are therefore often recycled to the cell membrane (Baulida et al., 1996).

Like the EGFR receptor, HER2 was described to be crucial in healthy as well as in unhealthy tissues. On the one hand, HER2 was shown to play an essential role for

the maintenance of a healthy nervous system. Along this line, Britsch and colleagues show that mice harboring mutations in the *erbB2* gene display severe hypoplasia of the primary sympathetic ganglion chain (Britsch et al., 1998). Moreover, homozygous mutant *erbB2* embryos die at midgestation, and display a lack of trabecules in the heart ventricle and an abnormal appearance of cranial ganglia (Britsch et al., 1998; Lee et al., 1995). On the other hand, both in vitro and animal studies have shown that HER2 could play a pivotal role in oncogenic transformation and tumourigenesis. Indeed, overexpression of HER2 was observed in approximately one third of breast cancers and was found to be correlated with increased invasiveness, lack of response to endocrine suppression and chemotherapies, and poor prognosis (Revillion et al., 1998; Slamon et al., 1987; Torre et al., 1997; Tetu et al., 1994). Other malignancies in which the *neu* gene amplification or protein overexpression are found include ovarian, -lung, -gastric, -colorectal, -bladder, -kidney, -pancreatic, and -thyroid carcinomas as well as head and neck, and -mesenchymal malignancies (Watabe et al., 1998; Table. 1). The investigation of HER2 as a central target for the therapy of a subset of breast cancer patients is therefore essential and led to the discovery of the first genomic-research-based targeted anti-cancer therapeutic designed by humanizing the mouse monoclonal antibody 4D5, namely trastuzumab (Herceptin, Genetech Inc/Roche) (Hudziak et al., 1989; Fendly et al., 1990). This therapeutic was developed by Genetech, and was approved by the FDA (Food and Drug Administration) in 1998.

1.2.3 HER3 (Human epidermal growth factor receptor3)

HER3/ErbB3 is the third member of the human epidermal growth factor receptor (EGFR/HER) tyrosine kinases family. Its gene maps to human chromosome 12q13.2, is 23, 2 kb in size, consists of 28 exons, and results in a protein of 180 Kda (Kraus et al., 1989; Plowman et al., 1990; Zimonjic et al., 1995). HER3 is unique in the ErbB family members in that it has been shown to have weak or no tyrosine activity (Guy et al., 1994). Due to this property, HER3 phosphorylation after heregulins activation is dependent on physical association with other ErbB family members, to provide highly potent heterodimers (Sliwkowski et al., 1994; Carraway et al., 1994; Wallasch et al., 1995). HER3 phosphorylation may also occur without heregulins, via EGFR activation by its specific ligands (Pinkas et al., 1996). Complexation of HER3 with

EGFR plays thereby a prominent role. Indeed, it was shown in several different types of model systems that the expression of the EGF-receptor was sufficient to allow activation of HER3 in response to EGF (Soltoff et al., 1994, Kim et al., 1994, Kim et al., 1998). HER3 is therefore considered as a highly receptive substrate for EGFR tyrosine kinase activity (Sierke et al., 1997). Parallel to this, interaction of HER3 with HER2 receptor results in a complex with enhanced affinity for heregulins and increased HER3 phosphorylation (Sliwkowski et al., 1994, Yen et al., 2002). The HER3/HER2 complex contributes thereby synergistically to cell transformation and to malignant properties of cancer cells, and is considered as the most potent mitogenic signal generator among EGFR family members (Van der Horst et al., 2005). In fact, heregulin-induced formation of the HER3/HER2 complex results in conversion of HER2 from an inhibited to an active protein conformation (Zhang et al., 2006). Finally, an interaction between HER3 and HER4 has been also shown (Zscheppang et al., 2006). The obtained complex plays thereby an important role in stimulating cell division (Pinkas et al., 1998). Subsequent to its activation, HER3 effectively couples to the PI3K/Akt pathway thereby controlling different biological outcomes like proliferation, and cell survival (Figure. 3, Campbell et al., 2010). HER3 has in fact six tyrosine phosphorylation sites with YXXXM motifs that serve as excellent binding sites for PI3K (Cantley et al., 1991, Kim et al., 1994, Sithanandam et al., 2003, Schulze et al., 2005).

During the last years many reports have investigated the role of HER3 in normal development and maintenance of several organs. HER3 Knock-out mice showed indeed defects in valve formation characterized by a lack of sufficient connective tissues, resulting in death at E13.5 (Erickson et al., 1997). Moreover, the same mice line displays defects in cerebrellar plate differentiation as well as neural crest formation (Erickson et al., 1997). In human cancer, HER3 was described to be overexpressed in breast, -ovarian, -lung, -skin, -and prostate cancer and this genetic feature is correlated with poor prognosis (Sithanandam et al., 2005; Tanner at al., 2006; Soler et al., 2009; Reschke et al., 2008). In human breast cancer HER3 builds with HER2 a potent oncogenic receptor that promotes tumor development and progression (Wallasch et al., 1995).

As mentioned above, due to its kinase aberrant sequence characteristics in its kinase domain, HER3 is believed to be kinase impaired. However, recent work of Van der Horst and co-workers showed that heregulin-induced activation of HER3 in glioma-

derived cell line results in the Phosphorylation of a cytoplasmic kinase protein, PYK 2 (Van der Horst et al., 2005). Furthermore, monoclonal antibody targeting HER3 was shown to be able to reduce proliferative as well as migratory properties of breast cancer cell lines (Van der Horst et al., 2005). The importance of HER3 in promoting breast cancer was further supported by an additional investigation. Indeed, Sergina and his colleagues showed that treatment of HER2-amplified breast cancers with HER2-targeting TKIs (tyrosine kinase inhibitors) lead to a rapid compensatory increase in HER3 expression, localization, and signalling activity (Sergina et al., 2007). Furthermore, resistance to Gefitinib, an EGFR small molecule inhibitor, was shown to be connected to HER3 signal activation (Engelmann et al., 2007). The importance of HER3 as a suitable target for the therapy of different human cancers was further investigated. On the one hand, Sithanandam and colleagues showed that HER3 depletion by siRNA reduces cell proliferation, by both suppressing cell cycle and causing apoptosis and necrosis in the A549 human lung cancer cell line. Along with this, HER3 knockdown inhibits cell motility, migration, and invasion of the same cell line (Sithanandam et al., 2005). On the other hand, recent work of Spencer Liles and co-workers showed that HER3 expression promotes tumorigenesis in pancreatic adenocarcinoma (Liles et al., 2010).

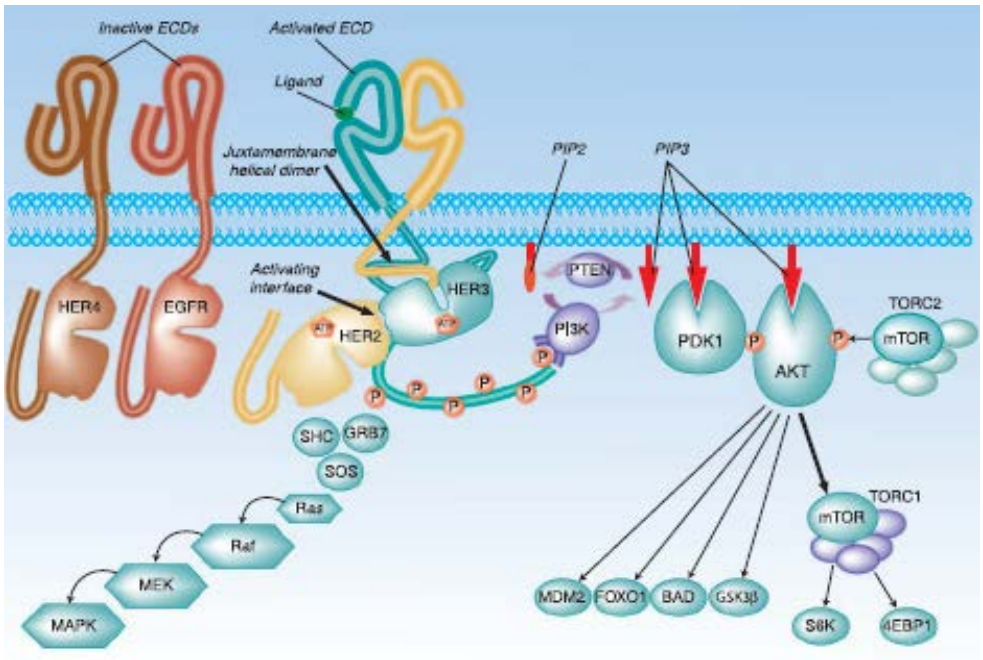


Figure 3. Schematic representation of HER3 signaling pathway
Through its activating interface, HER3 engages and activates its kinase partner(HER2 in this case). Phosphorylation of its C-tail leads to the activation of Ras but

principally PI3K and subsequently AKT thereby regulating cell survival and proliferation. (The figure was taken from Campbell et al., 2010).

1.2.4 HER4 (Human epidermal growth factor receptor4)

HER4 represents the last member of the EGF-receptor tyrosine kinases family (Plowman et al., 1993). This autonomous receptor shares recognition and signalling features with EGFR, as they both bind a large, distinct, and similar group of ligands including HB-EGF as well as epiregulin and epigen (Citri et al., 2006). Like EGFR, HER4 recruits GRB2 and STAT3 upon activation by ligand binding thereby regulating a series of different biological mechanisms into the cell. Interestingly, alternative mRNA splicing generates several isoforms of HER4 integrated in the regulation of different cellular functions. One isoform CYT-1- is able to signal via the PI3K-AKT pathway by direct binding to the PI3K protein (Elenius et al., 1997). As HER4 might not be able to directly recruit Cbl, the downregulation of this receptor is considered slow (Ni et al., 2001).

HER4 is implicated in different physiological processes, which are crucial for the normal development and differentiation of various tissues, including heart, neural system and mammary glands (Kay *et al*., 1994). Furthermore, *HER4* gene amplification was described in a large number of different tumors including those of the breast, colon, and brain ((Table. 1); Abd el rahim et al., 2004; Lee et al., 2004).

Cancer type	EGFR(%)	HER2(%)	HER3(%)	HER4(%)
Breast	14-91	10-37	Yes	Yes
Ovary	30-75	20-32	Yes	Yes
Renal	50-90	24-40	Yes	N.D.
Lung (NSCLC)	40-80	3-56	Yes	Yes
Head & neck	30-75	32-62	Yes	Yes
Colorectal	25-77	7	53	N.D.
Pancreas	30-50	N.D.	35	Yes
Glioma	40-50	N.D.	N.D.	N.D.

N.D., not enough data to support definitive statistics

Table 1. Incidence of the four members of the EGFR receptor tyrosine kinases family in different human cancer types (Hamid et al., 2003).

1.3 EGFR receptor family as a target for cancer therapy

Progress in identifying the biochemical and molecular causes of cancer has led to the discovery of abnormalities that characterize cancer cells and represent targets for the development of drug therapies. The family of EGFR receptor tyrosine kinases represents one such target when these are present in elevated quantities and/or aberrant forms. Abnormalities in these cell surface receptors have been correlated with development and progression of cancer, poor response to chemotherapeutic agents, and low survival rates. Thus, this family of RTKs represents a promising target for the selective treatment of a wide range of cancer types.

There are a variety of strategies under development for inhibiting the kinase activity of these receptors, targeting both the extracellular and intracellular domains. While small molecule inhibitors target the intracellular catalytic region by interfering with ATP binding, antibody-based approaches use the extracellular domain for targeted tumor therapy (Figure. 4).

Monoclonal antibodies specially bind to the extracellular portion of the receptor and thereby inhibit receptor function by two major mechanisms. They either act by blocking ligand binding and/or receptor dimerization, thereby enhancing receptor down-regulation or by recruiting the immune system (Fischer et al., 2003).

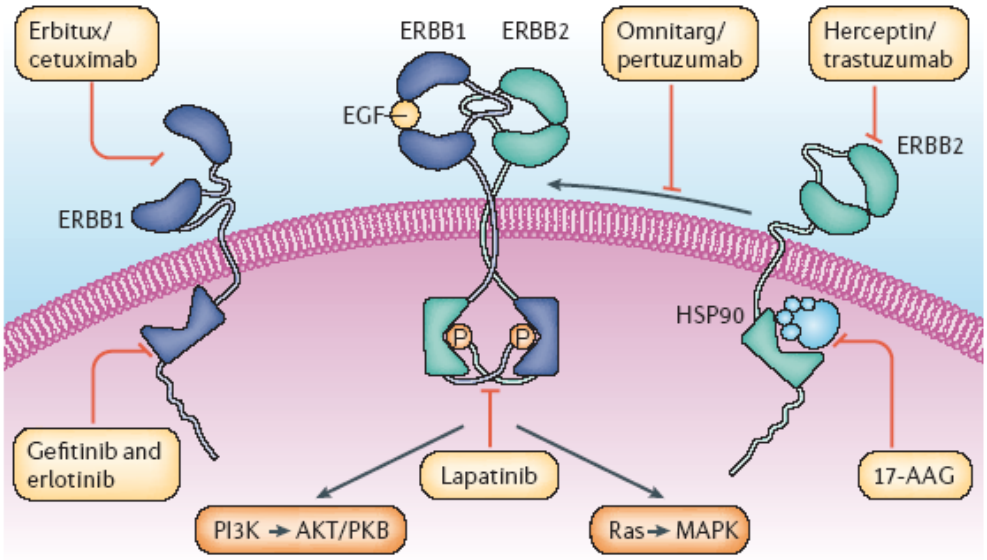


Figure 4. Agents targeting the EGF-receptor tyrosine kinase family
Due to their frequent occurrence in several human cancers, the family of the EGF-receptor tyrosine kinases family represents an essential therapy-target. Either by using monoclonal antibodies (Hereceptin, Cetuximab) which compete with natural

ligands on the binding on receptors or by employing small molecule inhibitors (Erlotinib, Gefitinib), members of the EGF-receptor tyrosine kinase family could be targeted. Other antagonists have the dual-specificy such as the monoclonal antibody Pertuzumab and are also promising therapeutic agents (The figure was taken from Citri et al., 2006).

The discovery of HER-2 gene amplification in human breast cancer by Dennis Salmon, Axel Ullrich and their colleagues led to the development of trastuzumab (Herceptin, Genetech Inc/Roche), the first genomic-research-based targeted anti-cancer therapeutic designed by humanizing the mouse monoclonal antibody 4D5 (Hudziak et al., 1989; Fendly et al., 1990), that was approved since 1998 by the FDA (Food and Drug administration). The binding of Hereceptin at the surface of tumour cells results in down-regulation of HER2 by inducing receptor internalization, inhibition of cell cycle progression through the induction of p27 expression (Lane et al., 2000) and into the initiation of the antibody dependent cellular cytotoxicity (ADCC) (Kumar et al., 1991). In 2004, The FDA approved Cetuximab (Erbitux, ImClone/Merck), a second blocking antibody targeting the EGF-receptor for the treatment of colon cancer patients. In this context Cetuximab was shown to bind with high affinity to the EGFR receptor and results in decreased receptor kinase activity, decreased cell proliferation, and cell cycle arrest (Goldstein et al., 1995). The mechanism for this growth arrest has been shown to involve up-regulation of cyclin-dependent kinase-2 (cdk-2) inhibitors and decreased cdk-2 activity leading to cell cycle arrest in the G1 phase (Wu et al., 1996). A decrease in angiogenesis may also contribute to the observed anti-proliferative effects (Perrotte et al., 1999).

Small molecule tyrosine kinase inhibitors (TKIs) characterize another class of selective therapeutics for the treatment of a variety of cancer types. Two major groups of inhibitors, which act in different ways, are approved for targeting the EGFR receptor family. Erlotinib (Tarceva, Genetech/Roche/OSI) and Gefitinib (Iressa, Astrazeneca) were first developed and approved for the treatment of non-small-cell lung cancer (NSCLC) (Kris et al., 2002, Fukuoka et al., 2002). They however only interact with EGFR thereby inhibiting its auto-phosphorylation (Carlos et al., 2001). Lapatinib (Glaxosmithkline) belongs to a second group of kinase inhibitors, which achieves improved tumor response rates. Indeed, Lapatinib is a dual kinase inhibitor, which targets both HER2 and the EGFR receptor. Lapatinib has demonstrated potent cytotoxic activity, which was shown effective towards proliferation of breast cancer cells in vitro (Rusnak et al., 2001; Xia et al., 2002; Xia et al., 2004). This dual inhibitor

markedly reduced tyrosine phosphorylation of EGFR and HER2, and inhibited activation of Erk1/2 and AKT, downstream effectors of proliferation and cell survival, respectively (Xia et al., 2002). Lapatinib was also shown to potentially overcome resistance to Herceptin if tumors develop compensatory mechanisms by other EGFR receptor family members (Esteva et al., 2004).

1.4 Negative regulators of the EGFR receptor tyrosine kinase family

Receptor activation in healthy cells is balanced by receptor down-regulation, which is mediated by a variety of negative regulatory molecules. These fall into several categories, including phosphatases and ubiquitin-ligases, and impinge upon both the magnitude and duration of RTK signaling (Sweeney et al., 2006; Kirisits et al., 2007). Several different negative regulators of receptor tyrosine kinases could be classified in two major groups, namely the pre-existing and the newly synthesized attenuators (Citri et al., 2006). Indeed, pre-existing attenuators primarily control receptor dephosphorylation and degradation. Receptor internalization coupled to degradation is considered as the most effective and irreversible process that robustly attenuates signalling by targeting surface receptors for degradation in lysosomes (Wiley et al., 2003). A second general mechanism of signal attenuation that functions at the receptor level is instigated by tyrosine phosphatases such as density-enhanced phosphatase-1 (DEP1), which dephosphorylates EGFR as well as other receptor tyrosine kinases (Berset et al., 2005), and also protein tyrosine phosphatase-1B (PTP1B), which dephosphorylates receptor tyrosine kinases in endosomes (Haj et al., 2002). Unlike pre-existing negative regulators, the synthesis of new attenuators occurs after receptor stimulation through ligand binding and reaches a peak within a short time. Transcriptional up-regulation of this group of attenuators is specific and affects multiple processes, which are indispensable for the maintenance of healthy tissues. LRIG1 (Leucine-rich repeats and immunoglobulin-like domains-1), the adaptor protein sprouty (SPRY), RALT (receptor-associated late transducer) also known as mig-6 and SOCS5 (Supressor of cytokine signalling-5) are the best known attenuators in this regard, and will be described below (Figure. 4 ,Kario et al., 2005, Hanafusa et al., 2002, Stutz et al., 2008, Hackel al., 2001).

1.4.1 SOCS-5 (Suppressor of cytokine signalling-5)

As the nomenclature indicates, SOCS proteins were first identified and described as negative regulators of cytokine signalling pathway. Indeed, they consist of 8 members characterized by a central SH2 domain, an amino-terminal domain of variable length and composition, and a carboxyl-terminal 40 amino-acid domain known as the SOCS box. This latter has been described recently to couple the SOCS proteins to an E3 ubiquitin ligase complex (Kamura et al., 1998, Zhang et al., 1999, Alexander et al., 2002). Recent evidence suggests that the action of the SOCS proteins may extend beyond the cytokine receptors to signalling initiated by members of the tyrosine kinase receptor family. Indeed, the function of the Drosophila SOCS36E gene, homologue of the mammalian SOCS-5 gene, was demonstrated to play an important role in regulating both JAK/STAT and EGFR signalling pathways (Callus et al., 2002). Interestingly, SOCS-5 was in new reports described as a negative regulator of the epidermal growth factor receptor (Sandra et al., 2005, Kario et al., 2005). The EGFR inhibition thereby occurs through enhanced proteasomal degradation of the receptor through SOCS box recruitment of E3 ubiquitin ligase activity (Sandra et al., 2005).

1.4.2 LRIG1 (Leucine-rich repeats and immunoglobulin-like domains-1)

LRIG1 is a second negative regulator of the receptor tyrosine kinases family, which was shown recently to have an inhibiting effect on EGFR-mediated signalling pathway (Gur et al., 2004, Stutz et al., 2008). Indeed, there are three known mammalian LRIG isoforms (two murine and one human), which share domain organisation with Kekkon proteins, described in previous studies as negative regulators of the EGFR receptor during oogenesis in Drosophila (Suzuki et al., 1996, Nilsson et al., 2001, Guo et al., 2004, Holmlund et al., 2004). Despite the structural similarity between mammalian and drosophila LRIG proteins, their modes of action during EGFR attenuation are totally different. Indeed, LRIG1 (mammalian) enhances both basal and ligand-stimulated receptor ubiquitination and degradation (Figure. 4; Rubin et al., 2005). Along this line, LRIG1 was shown to oppose tumor cell proliferation, motility, and invasion in vitro, and has been proposed to function as a tumor suppressor (Hedman et al., 2002). In addition, LRIG1 was described to be

under-expressed in a number of tumor types (Thomasson et al., 2003, Tanemura et al., 2005, Lindstrom et al., 2008). Interestingly, recent studies of Stutz and colleagues showed that LRIG1 regulates not only the wild type EGF-receptor but also the EGFRvIII mutant (Stutz et al., 2008).

1.4.3 Spry (Sprouty)

The Sprouty family of proteins was first identified in Drosophila as an antagonist of Breathless, the insect equivalent of the FGFR receptor and further as a negative regulator of DER, the Drosophila EGFR receptor (Hacohen et al., 1998, Reich et al., 1999). Subsequently, four mammalian genes (*mspry 1-4*) encoding protein homologues of the Drosophila Spry (dSpry) were identified and described (Minowada et al., 1999). Recent studies suggest the role of Spry as a conserved inhibitor of the RTK signalling in higher eukaryotes, thereby playing a role in signalling attenuation of several receptor tyrosine kinases mainly the FGFR receptor (fibroblast growth gactor receptor), and the VEGFR receptor (vascular endothelial growth factor receptor) in addition to EGFR receptor (Yusoff et al., 2002, Lee et al., 2001). Functionally, all sprouty proteins share a highly conserved cystein-rich domain at the carboxyl terminus, believed to be critical for its inhibitory effect on the MAPK pathway. It was in fact shown that once growth factor stimulation occurs, Spry1 and Spry2 translocate to the plasma membrane, they become tyrosine-phosphorylated, and interact with Grb2, thus inhibiting the recruitment of the Grb2-Sos complex to docking adaptor proteins such as FRS2 or Shp2 (Figure. 5 , Hanafusa et al., 2002).

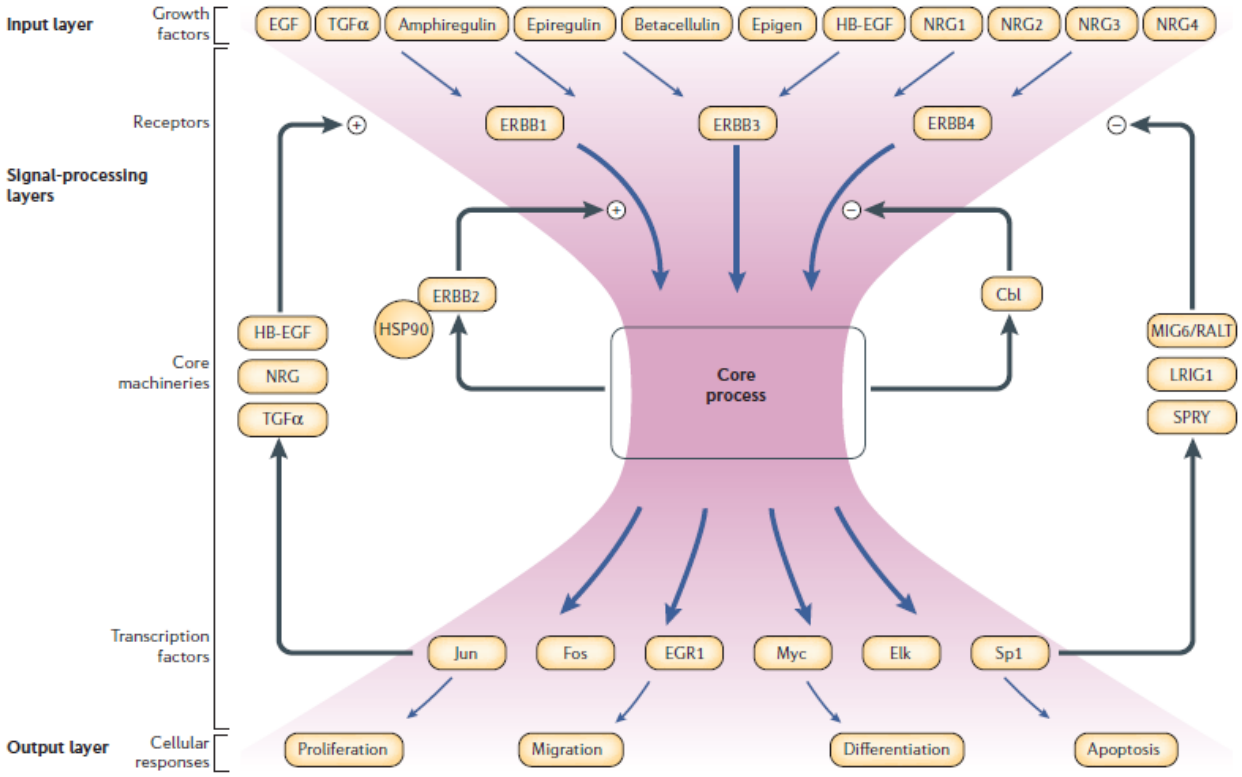


Figure 5. Negative and positive regulators of the EGFR family
Specific ligand binding to members of the EGF-receptor tyrosine kinase family regulates several biological processes which are essential for the maintenance of equilibrium in healthy tissues involving cell proliferation, migration, differentiation, and survival. The up-regulation of some negative regulator proteins ensures the maintenance of this balance inside healthy tissues through different attenuation mechanisms. On the one hand, an ubiquitin ligase that is involved in receptor degradation, Cbl, controls an important negative-feedback loop. Other attenuator-proteins act by a directly binding to members of the EGFR receptor tyrosine kinases family such as mig-6, LRIG1, and Spry (The figure was taken from Citri et al., 2006).

2. Mitogen-inducible gene-6 (Mig-6)

The mitogen-inducible gene-6, also known as gene 33, ralt and errfi 1, is a cytoplasmic multi-adaptor protein playing a prominent role in the negative regulation of the receptor tyrosine kinases family (Xu et al., 2005). Mig-6 was cloned the first time from a rat liver cDNA library (Lee et al, 1985), and was described as a mitogen-inducible gene which is regulated during cell cycle progression. The mig-6 gene was mapped to human chromosomal region 1p36 producing a protein with a molecular weight of approximately 54 KDa. Its modular structure on the one hand coupled with the absence of any catalytic activity on the other hand suggests the role of the mig-6 protein as a scaffold/adaptor protein having the ability to interact with a large number of proteins due to their protein-protein interaction domains (Figure. 6). Indeed, mig-6 contains an NH2-terminal cdc42/Rac interaction and binding domain (CRIB domain) through which it binds, in a GTP dependent manner, the small Rho family G protein Cdc42. The CRIB domain is followed by three SH3 domains and a domain for the binding of the phospho-serine binding adapter protein 14-3-3. The c-terminal tail of mig-6 is characterized by the presence of an EGF receptor (EGFR) binding domain through it mig-6 bind to EGFR and downregulates it by an unknown mechanism of action. This c-terminal has a high homology with the c-terminal of the ACK1 protein also implicated in the negative regulation of EGFR (Shen et al., 2007). In addition, mig-6 has two PEST sequences which may be responsible for mig-6 degradation and a putative nuclear localisation signal (Fiorini et al., 2002; Anastasi et al., 2003)

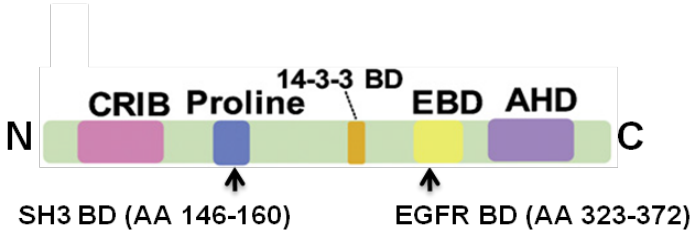


Figure 6. Schematic representation of the mig-6 protein structure
Mig-6 is an adaptor protein containing multiple domains potentially involved in protein-protein interactions. The interaction domain responsible for EGFR binding has been mapped between amino acids 337-361. (The figure was modified from Gotoh, 2009).

As an immediate early response gene, mig-6 expression was described to be up-regulated by a wide range of stimuli including hormones such as insulin, chemical agents like phorbol-ester, different stresses such as hypoxia as well as by growth factors like HGF/SF, PDGF and EGF (Zhang et al., 2007). The induction of mig-6 expression by the EGF is indeed a key point to start a very important mechanism during the regulation of mitogenic signals in healthy tissues. In this perspective mig-6 was described for the first time in 2001 as a negative regulator of the EGFR receptor tyrosine kinase (Hackel et al., 2001). This inhibition is exerted through a direct binding of mig-6 to the activated receptor thereby regulating EGFR activity, receptor stability and downstream signalling molecules like MAPK, AKT and JNK (Hackel et al., 2001; Anastasi et al., 2007; Figure. 8). The binding involves a ~25-residue epitope also known as segment 1 (Figure. 7).

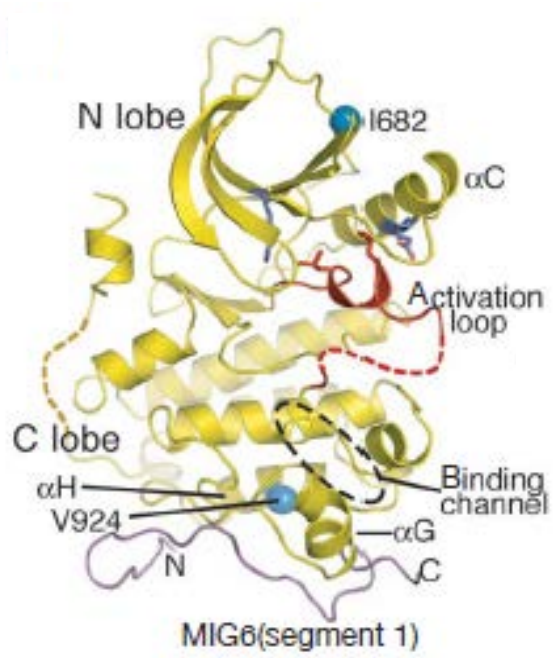


Figure 7. Binding-motif of mig-6 to EGFR
(The figure was taken from Zhang et al., 2007).

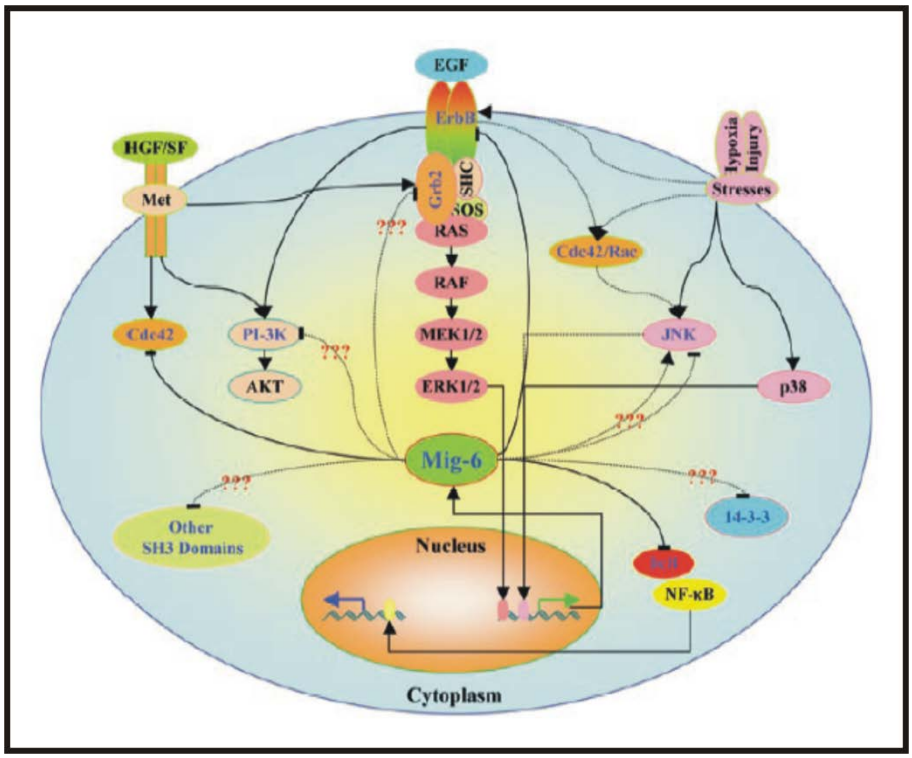


Figure 8. Role of mig-6 protein as an adaptor/scaffold protein
EGF/HGF binding on the respective receptors as well as other stimuli such as stresses and hypoxia induces up-regulation of mig-6 protein through the MAPK and JNK signalling pathways respectively. As a scaffold protein mig-6 possesses several protein interaction domains. Mig-6 can directly bind to EGFR and negatively regulates EGFR signalling. It inhibits HGF/SF-MET-mediated activation of the Cdc42 small GTP-ase through directly interaction with the GTP-bound Cdc42 protein. Furthermore, mig-6 can bind to IκBα which leads to the inhibition of the NF-κB transcription factor. Mig-6 binds also the 14-3-3 proteins as well as the PI3K in vitro (The figure was taken from Zhang et al., 2007).

Interestingly, recent work by Zhang and colleagues using crystallographic methods suggests that Binding of mig-6 to the EGFR kinase is sufficient for the inhibition of the EGFR activity (Zhang et al., 2007). Moreover mig-6 inhibits EGFR-mediated Rat1 cells transformation, suggesting the role of mig-6 as a tumor supressor of EGFR-dependent cell transformation. In addition to EGFR, mig-6 was described as a negative regulator of the other members of the EGFR receptor tyrosine kinase family, HER2 (Fiorentino et al., 2000), HER3 and HER4 (Anastasi et al., 2003). Furthermore, mig-6 can inhibit the HGF/Met-induced cell migration and proliferation in neurons as well as in cells of hepatic origin (Pante et al., 2005).
In the last years the function of mig-6 was extensively investigated. The generation of genetically modified mouse models is considered as a useful tool employed in this regard. Obvious development abnormalities were in fact observed as a result of

mig-6 disruption in vivo. Indeed, mig-6 deficiency was shown to induce hyperactivation of endogenous EGFR and sustained signalling through the mitogen-activated protein kinase (MAPK) pathway, resulting in over-proliferation and impaired differentiation of epidermal keratinocytes (Ferby et al., 2006). Moreover, mig-6 knock-out mice are highly susceptible to carcinogen-induced papilloma formation. Interestingly, the two phenotypes described above could be rescued by the replacement of the wild type EGFR with a kinase-deficient protein encoded by the hypomorphic waved-2 EGFR allele or by inhibition of EGFR signalling with the EGFR small molecule inhibitor, Gefitinib. These findings indicate the role of mig-6 as negative regulator of EGFR signalling in skin morphogenesis and as a novel tumor suppressor of EGFR dependent carcinogenesis (Ferby et al., 2006). The specific negative regulation of EGFR by mig-6 was further investigated. Recent in vivo studies show that ablation of mig-6 in liver leads to an increase in EGFR protein levels (Reschke et al., 2010). Along with this, it was shown that mig-6 is downregulated in human hepatocellular carcinoma and this correlates with increased EGFR expression. Moreover, mig-6 was shown to be an endogenous inhibitor of EGFR in liver as its suppression induced tumor cell migration in human liver cancer cell lines (Reschke et al., 2010). Parallel to these findings, mig-6 deficient mice display enhanced hepatocyte proliferation in the initial phases after partial hepatectomy compared to the wild type mice. This phenotype correlates with activation of endogenous EGFR signalling, predominantly through the protein kinase B pathway (Reschke et al., 2010). Additionally, mig-6 knockout mice develop a large number of tumors in a variety of different tissues including colon, ovary, gallbladder and bile duct (Table. 2; Ferby et al., 2006).

It was further shown, that the mig-6 expression is reduced in a large range of skin, breast, pancreatic and ovarian carcinomas as well as psoriasis suggesting its tumor suppressive role (Ferby et al., 2006). Mig-6 was shown to be also involved in maintaining normal joint function. Indeed, mig-6 deficient mice display bone and cartilage hyperplasia which leads to fixed joints, causing premature death of the mutants at the age of 5-7 months (Zhang et al., 2005).

Type of tumor or hyperplastic lesion		Number of mcie with described pathology of mice analyzed
Gastrointestinal cancer	Rectal tubular adenoma	15/27
	Rectal adenocarcinoma	7/27
	Gastric tubular adenoma	12/30
Bone/cartilage hyperplasia		
Lung	Alveolar septal thickening with epithelial hyperplasia	12/15
	Bronchoalveolar adenoma	4/15
	Papillomas one bare skin or eyelids	6/30
	SCC in perineal epidermis	7/27

Table 2. Incidence of tumors or hyperplastic lesions in mig-6 KO mice
(The figure was adapted from Ferby et al., 2006).

Interestingly, recent work by Ying and colleagues illustrated a novel mechanism used by mig-6 for the downregulation of EGFR in glioblastoma multiforme (GBM) cancer cells. In fact, mig-6 quells the malignant potential of GBM cells and dampens EGFR signalling by driving EGFR into late endosomes and lysosome-mediated degradation upon ligand stimulation (Ying et al., 2010). Mechanistically, this effect is mediated by the binding of mig-6 to a SNARE protein STX8, a protein known to be required for late endosome trafficking. Thus, mig-6 functions to ensure recruitment of internalized receptor to late endosomes and subsequently the lysosomal degradation compartment through its ability to specifically link EGFR and STX8 during ligand-stimulated EGFR trafficking (Ying et al., 2010).

3. Colon cancer

Colon cancer is a common type of bowel cancer that originates from the neoplastic transformation of epithelial cells of the colon through an adenoma precursor stage. Colon cancer is very abundant in the west-world and is considered as the fourth most common malignancy in the western world and the third most frequent cause of cancer-related mortality (Grivas et al., 2007; Jemal et al., 2010). There are several risk factors, which can lead to the development of such cancer type including eating and life habits, environmental conditions and especially genetic factors (Parkin, Bray, ferlay, & Piasani., 2005). Indeed, normal colonic epithelial cells undergo a variety of histo-pathological changes in order to form a malignant cell mass. The different phases of colon cancer formation are described by Markowitz et al., 2009 (Figure. 9). The disease begins as a benign adenomatous, which develops into an advanced adenoma with high-grade dyplasia and then progress to an invasive cancer. Invasive cancers that are confined within the wall of the colon (stages I and II) are curable, but if untreated, they start to dissociate from primary tumor sites and to spread to regional lymph nodes (stage III), and then metastasize to distant sites in the body (stage IV) (Markowitz et al., 2009).

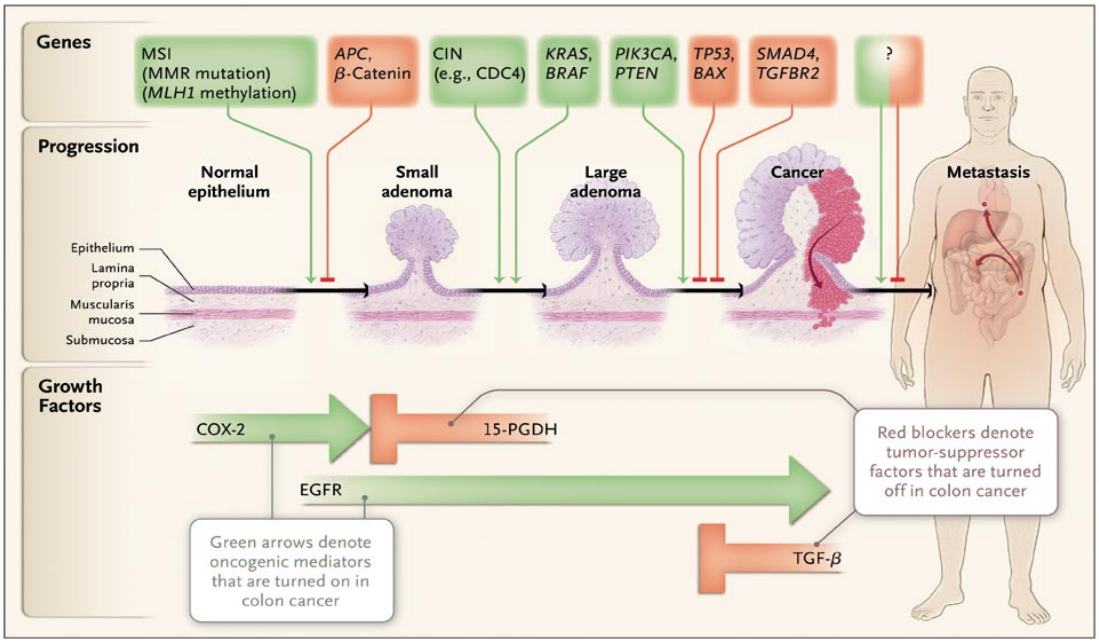


Figure 9. Biological and genetic events during colon cancer progression
(The figure was taken from Markowitz et al., 2009)

The underlying molecular mechanisms of colon cancer formation have been extensively studied over the years. Multiple genetic and epigenetic aberrations initiate the development of colon cancer and drive its progression. These alterations could be classified into two major tumor-genetic pathways according to their properties (A.M. Jiménez-Lara et al., 2007; Li et al., 2009). The first is driven by chromosomal instability (CIN), namely the Fearon and Vogelstein model supporting the theory that the transition from Adenoma to carcinoma tumor-state is controlled by the successive accumulation of mutations in *APC* (adenomatous polyposis coli) *K-Ras*, *TP53* (tumor protein P53), and *DCC* (deleted colorectal carcinoma) genes (Li et al., 2009; Fearon and Vogelstein., 1990; Ilyas et al., 1996). Indeed, mutations in the *APC* suppressor gene controlling the Wnt-signalling pathway and the *K-Ras* oncogene controlling the MAP-Kinase signalling pathway are relevant early molecular events in the development of colon cancer (A.M. Jiménez Lara et al., 2007; Figure. 10). Subsequent mutation in the *DCC* gene, encoding a cellular adhesion molecule, which has an important role in maintaining cell growth, differentiation and apoptosis, occurs as a late event by the formation of colon cancer prior to the acquisition of invasive properties (Ilyas et al., 1996). Microsatellite instability (MIN) is a second type of genetic defect in colon cancer, and results from epigenetic silencing or mutations in the mismatch repair genes (MMR) (Gervaz et al., 2001; Miyakura et al., 2001; Thibodeau et al., 1993).

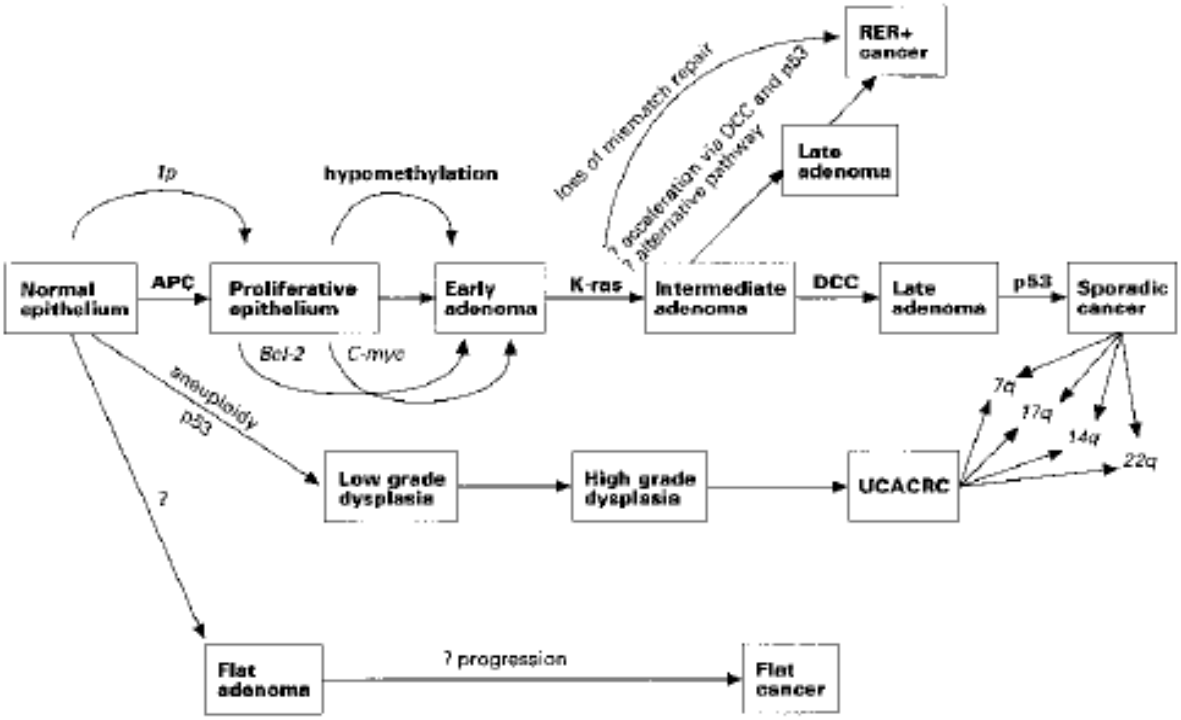


Figure 10. The fearon and Vogelstein model

The steps of the fearon and Vogelstein model are shown in bold with other candidate loci in italics. The generation and prpgression of colon cancer involves the initiation of mutations in *APC*, *K-RAS*, *DCC* and *p53* gens (The figure was taken from Ilyas et al., 1996).

So far, Surgery remains the primary treatment, while chemotherapy mainly and/or radiotherapy may be recommended depending on the individual patient's staging and other medical factors. The management of colon cancer using chemotherapy treatments including mainly 5-fluorouracil, and Leucovorin or Oxaliplatin have been shown in clinical trials to improve survival and/or reduce mortality rate, and have been approved for use by the US Food and Drug Administration. Nevertheless, targeted therapy designed towards specific markers has been hampered by the lack of known drugable key regulators of colon cancer initiation and progression. Thus, there is an urgent need to identify novel prognostic specific markers for the treatment of colon cancer patients.

4. The epidermal growth factor receptor vIII (EGFRvIII)

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that plays an important role in growth and differentiation of normal cells. Over-expression or mutations of this receptor lead to the development of cancer. EGFRvIII is one of the most common and known EGFR mutations. The mutated receptor is characterized by the deletion of exons 2-7 in the wild type EGFR mRNA, which correspond to cDNA nucleotides 275-1075 encoding for amino acids 6 to 276, presumably through alternative splicing or rearrangements (Ekstrand et al., 1992; Yamazaki et al., 1998; Wong et al., 1992). Deletion of 801 bp within the extracellular domain of the EGFR gene results in an in-frame truncation of the normal EGFR protein, resulting in a 145 KDa big receptor, the EGFRvIII receptor (Figure. 11). In contrast to the wild type form (EGFR) the activation of the mutant form (EGFRVIII) is ligand independent, since EGFRvIII is constitutively phosphorylated thereby activating different downstream signalling pathways (Koji et al., 2007).

Many reports have illustrated the expression of EGFRvIII in a wide range of cancer types. EGFRvIII exists at high frequency in glioblastomas, and according to new reports, imparts a worse prognosis and confers therapeutic resistance (Nishikawa et al., 1994; Aldape et al., 2004; Heimberger et al., 2002; Lammering et al., 2004; Staverosky et al., 2005). Previous reports demonstrated also that EGFRvIII is frequently detected in other human cancers, including breast, ovarian, lung, and medulloblastoma tumors (Moscatello et al., 1995; Garcia et al., 1993), but has not been detected in normal adult tissue (Moscatello et al., 1995; Wikstrand et al., 1995).

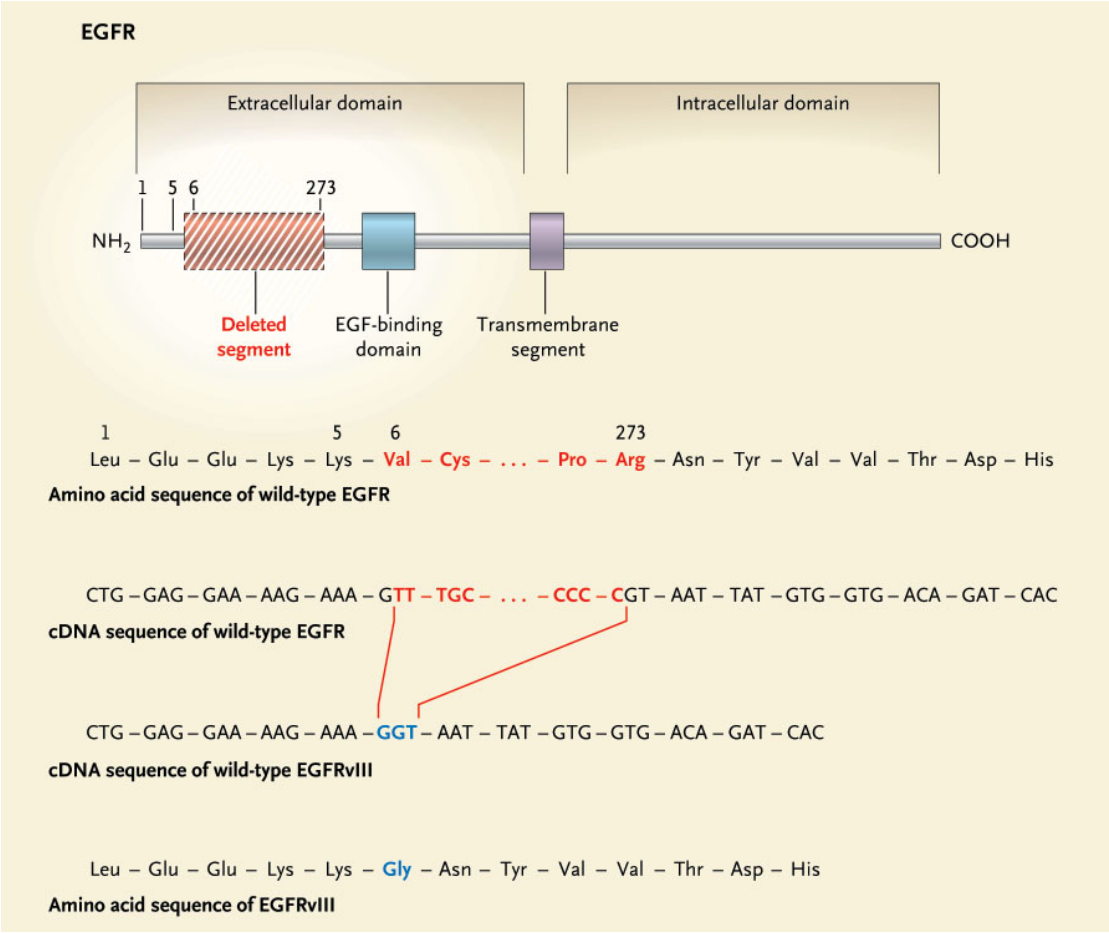


Figure 11. cDNA and amino acid sequences of Wild-type and mutant EGFR
The deletion of exons 2-7, shown in red, results in the creation of the glycine amino-acid inside the extracellular domain. This new generated amino-acid is specific for the mutated receptor (EGFRvIII) (The figure was taken from Friedman et al., 2005).

In the last years many studies have investigated the role of the EGFRvIII receptor in promoting different biological mechanisms, which ensure the initiation, development, and progression of tumors like proliferation, migration, invasion and others (Gan et al., 2009). Indeed Prigent and co-workers showed that EGFRvIII promotes a growth advantage when stably expressed in U87 cells (U87-EGFRvIII) in comparison to parental cells. The increase in the proliferation was shown to be mediated through the MAP-kinase signalling pathway (Prigent et al., 1996, Figure. 12). The PI3K-Akt signalling pathway is however, the dominant pathway regulating cell proliferation downstream of EGFRvIII (Huang PH et al., 2007; Mizoguchi et al., 2006). In fact, the stable expression of EGFRvIII in the same model system cited above (U87-EGFRvIII) increases the phosphorylation levels of Akt and reduced levels of $p27^{kip1}$ protein expression, a cell cycle regulator that inhibits G1-S phase transition (Narita et al.,

2002, Figure. 12). EGFRvIII signalling may also induce a decrease in apoptosis, since U87- EGFRvIII glioblastoma cell line showed an up-regulation in the expression of the anti-apoptotic molecule BcL-XL. The expression of this apoptosis-inhibitor is 3-fold higher in U87-EGFRvIII compared to parental U87 glioblastoma cells (Boise et al., 1993, Figure. 12). Furthermore, EGFRvIII was also shown to play a role in tumor invasion (Lal et al., 2002, Figure. 12) and tumor angiogenesis (Wu et al., 2007, Figure. 12). Indeed, the EGFRvIII transfected D54MG glioblastoma multiforme cell line displayed up-regulation of genes that promote an invasive phenotype of the cell such as matrix metalloproteinase *MMP-13*, which is a collagenase primarily expressed by malignant tumors. These cells showed greater migration ability and were shown to be more invasive compared to the parental glioma cell line in *in vitro*-assays (Lal et al., 2002). EGFRvIII-mediated increase of tumor cell invasion was however shown not only in glioma cell lines. Indeed, a recent study of Rahimi and colleagues in 2010 cited that the expression of EGFRvIII in breast cancer enhances the CXCL12/CXCR4-mediated invasion (Rahimi et al., 2010). Additionally, another study of Damstrup at al., 2002 demonstrated that the expression of EGFRvIII increases the *in vitro* invasiveness of small-cell lung cancer cell line (Damstrup et al., 2002). Enhanced invasiveness of EGFRvIII expression cells thereby was shown to be also associated with enhanced angiogenesis (Wu et al., 2004).

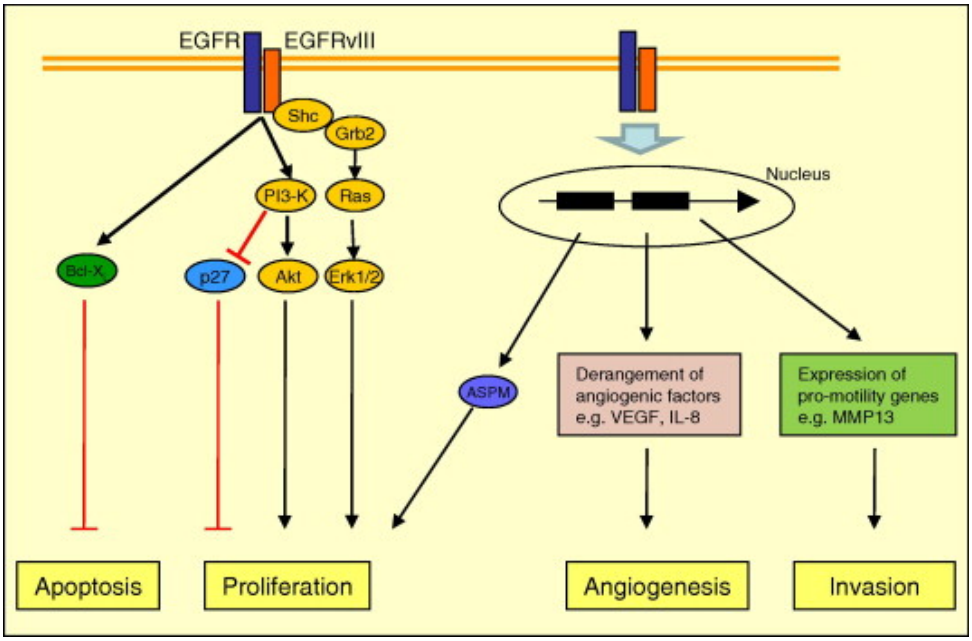


Figure 12. EGFRvIII confers enhanced tumorigenecity

EGFRvIII enhances cell proliferation by promoting PI3K-AKT signalling pathway. In addition, EGFRvIII promotes cell survival by increasing expression of anti-apoptic proteins such as BcL-XL, and enhances cell invasion and angiogenesis by up-regulating of the VEGF receptor, interleukin-8 (IL-8) and MMP13 expression (The figure was taken from Gan et al., 2009).

The absence of the ligand binding domain in the EGFRvIII receptor, due to the deletion renders the therapy of cancers expressing it through targeted therapies using monoclonal antibodies almost impossible and the use of small molecule inhibitors targeting the ATP binding sites of the intracellular domain of the receptor remains the best therapeutic approach. Nevertheless, recent studies showed the efficacy of cetuximab, a monoclonal antibody directed towards EGFR, as a prominent tool to target EGFRvIII. *In vitro* data showed that cetuximab is able to target EGFRvIII and results in receptor internalization, a reduction in EGFRvIII phosphorylation and an inhibition of EGFRvIII-mediated proliferation (Patel et al., 2007; Wu et al., 2007). However, *in vivo* studies have been less encouraging (Horvath et al., 2006; Yang et al., 2008).

Like the wild type receptor (EGFR), EGFRvIII could be negatively regulated through EGFR negative regulators. Indeed two different studies during the last 4 years established the downregulation of EGFRvIII though Cbl and LRIG1 regulators (Davies et al., 2006; Stutz et al., 2008). Nevertheless, there is no informations about the interaction between EGFRvIII and the EGFR-negative regulator, mig-6 protein.

II Objectives

The ErbB receptors tyrosine kinases family are fundamental by the enhancement of a series of cellular outputs including proliferation, cell survival, and metastasis through variable signalling loops. Modulation of ErbB receptors-mediated signalling pathways through specific antagonists is essential in this regard to prevent the occurrence of cancer. Different negative regulators including mig-6 were shown to play a crucial role by disrupting several signalling pathways. Loss of mig-6 in mice was shown to be accompanied by an activation of EGFR and tumor formation.

In the first part of the thesis we aimed to determine the role of mig-6 in human colon cancer. We intended to analyze the expression of mig-6 in primary human colon, and colon cancer cell lines and its relationship to HER3.

We further intended to analyze the role of mig-6, negative regulator of the wild type EGFR, as an antagonist of EGFRvIII *in vitro* in the second part of the thesis. Interaction and negative regulation of EGFRvIII by mig-6 in two different model systems were the first analyzed aspects. In addition we planned to investigate the impact of mig-6 on the regulation of the EGFRvIII-mediated cell signalling pathways and cellular functions including proliferation, migration and apoptosis in the glioblastoma cell line U87-EGFRvIII.

III Materials and methods

1. Materials

1.1 Chemicals

5-Fluorouracil	Sigma, Taufkirchen
Acrylamide	Serva, Heidelberg
Agar	Difco, USA
Agarose	BRL, Eggenstein
Ampicillin	Roche, Mannheim
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, Munich
ß-mercaptoethanol	Sigma, Taufkirchen
Bromophenolblue	Sigma, Taufkirchen
ATP (Adenosine triphosphate)	Amersham Pharmacia, Freiburg
BSA (Bovine serum albumin)	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Crystal Violet	Sigma, Taufkirchen
Desoxynucleotides	Roche, Mannheim
DTT (Dithiothreitol)	Sigma, Taufkirchen
EGF	Sigma, Taufkirchen
Ethidiumbromid	Sigma, Taufkirchen
Formaldehyde	PolySciences, Eppenstein
Geneticin (G418)	Invitrogen, Eggenstein
HEPES (4-(2-hydroxylethyl)-1-piperazin-ethansulfonicacid	Serva, Heidelberg
L-Glutamine (Gibco)	Invitrogen, Eggenstein
Lipofectamine® (Gibco)	Invitrogen, Eggenstein
Lipofectamine® RNAi Max	Invitrogen, Eggenstein
MTT	Sigma, Taufkirchen
Oligofectamine®	Invitrogen, Eggenstein
Penicillin/Streptomycin	Gibco, Eggenstein
Phenol	Roth, Karlsruhe
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
Ponceau S	Sigma, Taufkirchen
Propidium iodide	Sigma, Taufkirchen
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Sigma, Taufkirchen
TEMED (N,N,N´N´-Tetraethylmethylenediamine)	Serva, Heidelberg
Triton X-100	Serva, Heidelberg
Tween 20	Sigma, Taufkirchen

1.2 Enzymes

Alkaline phosphatase	MBI Fermentas, St. Leon-Rot
DNAse I, RNAse free	Roche, Mannheim
Restriction enzymes	MBI Fermentas, St. Leon Rot NEB, Frankfurt, Main Boehringer, Mannheim Biolabs, New England
Pfu DNA polymerase	MBI Fermentas, St. Leon-Rot
Platinium Taq polymerase	Invitrogen, Eggenstein
RED Taq® ReadyMix	Sigma, Taufkirchen
Reverse transciptase	Roche, Mannheim
RNase A	Sigma, Taufkirchen
T4-DNA ligase	Roche, Mannheim
Taq DNA polymerase	MBI Fermentas, St. Leon-Rot
Trypsin	Invitrogen, Eggenstein

1.3 Growth factors

EGF (mouse)	Toyoba, Japan
Heregulin ß1	R&D Systems Inc., USA

1.4 Kits and diverse materials

BigDye Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems, Forster City
Cell culture plates	Greiner, Solingen Nunclon, Dänemark Falcon, UK Corning Incorporated, USA
Cellulose nitrate 0,45µm	Schleicher & Schüll, Dassel
ECL-kit	PerkinElmer/NEN, Köln
Hyperfilm MP	Amersham Pharmacia, Freiburg
Matrigel	BD Biosciences, Pharmingen
MicroBCA protein assay kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Protein A-sepharose	Amersham Pharmacia, Freiburg
Protein G-sepharose	Amersham Pharmacia, Freiburg
QIAquick Gel Extraction kit (50)	Qiagen, Hilden
QIAquick PCR purification kit (50)	Qiagen, Hilden
QIAGEN plasmid Mini kit	Qiagen, Hilden
QIAGEN plasmid Maxi kit	Qiagen, Hilden
QIAGEN RNeasy Mini kit	Qiagen, Hilden
Sterile filter 0, 22/0, 45µm, cellulose acetate	Nalge Company, USA
Whatman 3MM	Whatman, Rotenburg, Fulda

1.5 Media and buffers

1.5.1 Bacterial media

LB-medium	1.0 % tryptone 0.5 % yeast extract 1.0 % NaCl pH 7.2
2x YT-medium	1.6 % tryptone 1.0% yeast extract 1.0% NaCl pH 7.2

The following concentrations of the antibiotics were used if required:

Ampicillin	100 µg/ml
Kanamycin	100 µg/ml
Chloramphenicol	30 µg/ml

1.5 % Agar was added to the media for production of agar plates.

1.5.2 Cell culture media

All media for the cell culture and additives were purchased from Invitrogen (Eggenstein). Freeye medim contained 90 % heated inactivated FCS and 10 % DMSO.

Dulbecco´s Modified Eagle Medium (DMEM) with 4.5 mg/ml glucose, 10 % FCS, 2 mM L-Glutamin, 1 mM sodiumpyruvated, 1 % Penicillin/Streptomycin

PRMI 1640, 10 % FCS, 2 mM L-Glutamine, 1 % Penicillin/Streptomycin

McCoy´s 5a, 10 % FCS, 2 mM L-Glutamine, 1 % Penicillin/Streptomycin

Ham´s F12, 20 % FCS, 2 mM L-Glutamine, 1 % Penecillin/Streptomycin

MEM, 10 % FCS, 2 mM L-Glutamine, 1 % Penicillin/Streptomycin

L-15, 10 % FCS, 2 mM L-Glutamine, 1 % Penecillin/Streptomycin

1.6 Stock solutions and buffers

Acrylamide solution (30/0.8 %)	30 % (w/v) Acrylamid 0.8 % (w/v) Bisacrylamid
HNTG	20 mM HEPES pH 7.5 150 mM NaCl 0.1 % TritonX-100 10 % Glycerol 10 mM $Na_4P_2O_7$
DNA loading buffer (6x)	0.05 % Bromophenol blue 0.05 % Xylencyanol 30 % Glycerol 100 mM EDTA pH 8.0
Laemmli buffer	65 mM Tris/HCl pH 6.8 2 % SDS 30 % Glycerol 0.01 % Bromophenol blue 5 % ß-mercaptoethanol
NET (10x)	50 mM Tris/HCl pH 7.4 5 mM EDTA 0.05 % TritonX-100 150 mM NaCl
PBS	137 mM NaCl 27 mM KCl 80 mM Na_2HPO_4 1.5 mM KH_2PO_4 pH 7.4
SD-Transblot	50 mM Tris/HCl 40 mM Glycine 20 % Methanol 0.004 % SDS
Strip buffer	62.5 mM Tris/HCl 2 % SDS 100 mM ß-mercaptoethanol
TAE	40 mM Tris/Acetate pH 8.0 1 mM EDTA
TE10/0.1	10 mM Tris/HCl pH 8.0 0.1 mM EDTA pH 8.0
Tris Glycine SDS	25 mM Tris/HCL pH 7.5 200 mM Glycine 0.1 % SDS

TritonX-100 lysis buffer	50 mM HEPES pH 7.5 150 mM NaCl 1 mM EDTA 10 % Glycerin 1 % Tritonx-100 100 mM Na_2HPO_4 2 mM VaO_5 10 mM NaF 1 mM PMSF 100 µg/l Aprotinin

1.7 Bacteria and cell lines

1.7.1 Bacteria

Strain	genotype	reference
DH5α	F´ endA1 hsd17 (rk-mk+) supE44 recA1 Gyr (Nal) thi-1 Δ (lacZYA-argF196)	Genetech, USA
XL-1Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´proAB lacIqZ ΔM15 Tn10 (Tetr)]	Stratagene, NL

1.7.2 Cell lines

Cell line	origin	reference
CaCO-2	Human colorectal adenocarcinoma	ATCC
Colo 320 dm	Human colorectal adenocarcinoma	ATCC/ Sugen
DLD-1	Human colorectal adenocarcinoma	ATCC
HC-2998	Human colorectal adenocarcinoma	NCI
HCT-116	Human colorectal adenocarcinoma	ATCC
HCT-15	Human colorectal adenocarcinoma	ATCC
HT-29	Human colorectal adenocarcinoma	ATCC
KM-12	Human colorectal adenocarcinoma	NCI
LoVo	Human colorectal adenocarcinoma	ATCC
LS-174T	Human colorectal adenocarcinoma	ATCC
LS-180	Human colorectal adenocarcinoma	ATCC

LS-123	Human colorectal adenocarcinoma	ATCC
RKO	Human colorectal adenocarcinoma	ATCC
SkCo-1	Human colorectal adenocarcinoma	ATCC
SW-48	Human colorectal adenocarcinoma	ATCC/ Sugen
SW-480	Human colorectal adenocarcinoma	ATCC
SW-620	Human colorectal adenocarcinoma	ATCC
SW-116	Human colorectal adenocarcinoma	ATCC
T-84	Human colorectal adenocarcinoma	ATCC
Wi-Dr	Human colorectal adenocarcinoma	ATCC/ Sugen
U-87 MG	Human malignant glioblastoma	ATCC
U-87 MG-EGFRvIII	Human malignant glioblastoma	gift from Dr. W. Cavenee

1.8 Antibodies

1.8.1 Primary antibodies

Antibody	immunogen origin	reference
Akt1/2	rabbit, polyclonal; AA 345-480 of human Akt1	Santa Cruz, USA
p-Akt/PKB	rabbit, polyclonal; phosphor-Akt (Ser-473); recognizes p-Akt of human, rabbit and rat origin	NEB, Frankfurt/M.
ß-actin	rabbit, polyclonal; directed against a C-terminal peptide	Sigma, Taufkirchen
Cyclin B1	mouse, monoclonal; peptide of murine cyclin B1	Cell signaling, MA
EGFR	sheep, polyclonal; part of cytoplasmic domain of the human EGFR	UBI, Lake Placid

EGFR (108.1)	mouse, monoclonal; ecto-domain of the human EGFR	(Daub et al., 1997)
p-EGFR(Y-1173)	rabbit, monoclonal; recognizes endogenous EGFR phosphorylated at Y1173	Cell signaling, MA
ERK(C-14)	rabbit, polyclonal; peptide at C-terminus of rat ERK2	Santa Cruz, USA
p-ERK	rabbit, polyclonal; recognizes phospho-p44/p-42 (Thr-202/ Tyr-204) MAPK	NEB, Frankfurt/M
HER2	rabbit, polyclonal; peptide between AA 1243-1255 of human HER2	Upstate, NY
p-HER2(Y-1248)	rabbit, monoclonal; synthetic phosphor-peptide between AA 1243-1255	Upstate, NY
Mig-6	rabbit, polyclonal; 124 AA at C-terminus of rat mig-6	(Hackel et al., 2001)
p-mTOR	rabbit, polyclonal; recognizes phospho-S2418 of human mTOR	Cell signaling, MA
$P27^{kip1}$	mouse, monoclonal; recognizes the full length p27 protein	Transduction Labs
PI3Kp85	rabbit, polyclonal; recognizes the SH2 domain f human p85	UBI, Lake Placid
Tubulin	mouse, monoclonal, ascites	Sigma,Taufkirchen
p-Tyr(4G10)	mouse, monoclonal; recognizes phosphor(3)-tyrosine residues	UBI, Lake Placid
VSV(P5D4)	mouse, monoclonal; recognizes an epitope of eleven amino acids derived from the vesicular stomatitis virus glycoprotein VSV-G	Roche, Mannheim

1.8.2 Secondary antibodies

The secondary antibodies that were use in western blots were coupled to horse radish peroxidase (HRP).

Antibody	dilution	reference
Goat anti-mouse-HRP	1:10.000	Sigma, Taufkirchen
Goat anti-rabbit-HRP	1:50.000	Bio-rad, Munich
Goat anti-sheep-HRP	1:25.000	Jackson Immunoresearch Labs, USA

1.9 Plasmids and oligonucleotides

1.9.1 Primary vectors

Plasmid	description	reference
pRK5	expression vector, Amp^r CMV promoter, SV40 poly A, high copy plasmid	Genetech, USA
pcDNA3	expression vector, Amp^r CMV promoter, BGH poly A, high copy plasmid	Invirogen, USA

1.9.2 Constructs

Vector description	origin	reference
pcDNA3-mig6-VSV	cDNA of human mig-6 in pcDNA3; c-terminal VSV tag	This study
pRK5-EGFR	cDNA of human HER erbBH	(Wallasch et al., 1995)
pH ß Apr-1-neo/EGFRvIII		gift from Dr. D. Bigner

1.9.3 Oligonucleotides

1.9.3.1 siRNAs

HER3 sense-1	5´-GGCUAUGUCCUCGUGGCCAtt-3´
HER3 antisense-1	5´UGGCCACGAGGACAUAGCCtg-3´
HER3 sense-2	5´GGCAGUGUGUCCUGGGACUtt-3´
HER3 antisense-2	5´AGUCCCAGGACACACUGCCtg-3´
GL-2 sense	5´-CGUACGCGGAAUACUUCGAtt-3´
GL-2 antisense	5´-UCGAAGUAUUCCGCGUACGtt-3´
Mig-6 sense	5´-CGAUAAUAGAACUAGUGACtt-3´
Mig-6 antisense	5´-GUCACUAGUUCUAUUAUCGtt-3´

1.9.3.2 RT-PCR primers

HER3 fwd	5´CTCCGCCCTCAGCCATCAC 3´
HER3 rev	5´TGCTCCGGCTTCTACACATTGACA 3´
Mig-6 fwd	5´GGAAGACCTACTGGAGCAGTCGCAG 3´
Mig-6 rev	5´TGAGATGGACCATTTTCTGCAAAGC 3´
GAPDH fwd	5´ACCACAGTCCATGCCATCAC 3´
GAPDH rev	5´TCCACCACCCTGTTGCTGTA 3´

2. Methods

2.1 Methods of molecular biology

2.1.1 Plasmid preparation

A pure quality of plasmid DNA was needed for transfection into mammalian cells. The plasmid purification kit of QIAGEN was used to purify plasmid DNA for this purpose according to the manufacturer's protocols.

2.1.2 Enzymatic manipulation of DNA

2.1.2.1 Restriction digestions of DNA fragments

10-20 units of restriction enzyme were incubated with 1-3 µg DNA in 30µl of the recommended incubation buffer at 37°C for 2 hours.

2.1.2.2 Dephosphorylation of 5´ends of DNA fragments

Dephosphorylation of the 5´ends prevented the re-ligation of well digested plasmids. 1-3 µg of cut plasmids was incubated in 10-20 µl of 1 x phosphatase buffer (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.5) for 10 minutes at 37°C.

2.1.2.3 Gel electrophoresis of DNA fragments

Double stranded DNA molecules between 0.5 and 10 Kb were separated in 0.5-2% horizontal agarose gels. The running buffer was 1 x TAE. The DNA-sample was mixed with 1/6 volume of 6 x DNA loading buffer. The electrophoresis was performed between 50-100 volts for 1-2 hours. After the separation, DNA fragments were stained by gently agitating gels in TAE containing 0.5g/ml ethidium bromide and were subsequently viewed under UV light.

2.1.2.4 Isolation of DNA fragments from agarose gels

DNA fragments were isolated from agarose gels with the QIAquick gel extraction kit of QIAGEN according to the manufacturer's instructions. The principle of the kit is the adsorption of DNA fragments (100 bp-10 kb) to silica-gel membranes in the presence of high concentration of chaotropic salts, which modify the structure of water. The elution is performed under basic conditions and low salt concentrations.

2.1.2.5 Ligation of vector and insert DNA

Purified, digested and dephosphorylated vector DNA (40 ng), the designated insert DNA, 1 µl 10 x T4 DNA ligase buffer (0.6 Tris/HCl pH 7.5, 50 mM $MgCl_2$ 50 mM DTT, 10 mM ATP) and 1 unit T4 DNA ligase were combined. A molar ratio between insert

and vector of 3 to 1 was usually chosen. Reactions were either left on 16°C overnight or at 37°C for 2 hours and subsequently transformed into component bacteria.

2.1.3 Introduction of plasmid-DNA into E-coli

2.1.3.1 Preparation of components cells

The preparation of component cells was according to the procedure described by Chung and Miller (Chung and Miller, 1998). Component cells were shock frozen in liquid nitrogen and stored for up to one year at -70°C. Transformation frequency ranged between 10 and 10^7 colonies/ g DNA.

2.1.3.2 Transformation of component bacteria

A 50µl aliquot of component bacteria was added to a 50µl mixture of DNA, usually ligation cocktails, 10µl 5x KCM solution (500mM KCl, 150mM $CaCl_2$, and 250mM $MgCl_2$) and water. After thoroughly mixing, samples were incubated on ice for 20 minutes, 10 minutes at room temperature and after addition of 300µl LB broth at 37°C for 1 hour constantly shaking. Bacteria were streaked out on appropriate agar plates containing ampicillin for the selection of the transformants.

2.1.4 Sequencing of plasmids

DNA sequencing was performed following the “Big Dye terminator cycle sequencing protocol” (ABI). Pellets were dissolved in 20µl template suppression reagent, briefly boiled and analysed on a 310-genetic analyser (ABI Prism).

2.1.5 Analysis of RNA

The ubiquity of contaminating RNAases and the concomitant difficulties in ensuring that an RNA preparation remains reasonably un-degraded requires specific precautions. All the solutions were prepared with milliporeTM-water and the glassware was baked at 200°C for two hours. Gloves were used for all the procedures. The gloves were changed frequently. If necessary, the deionized water was treated with diethylpyrocarbonate (DEPC) to inhibit RNAase activity.

2.1.5.1 Preparation of total RNA

Total RNA from cell cultures was isolated with the RNeasy mini kit of QIAGEN according the manufacturer’s instructions. The cells were lysed in a denaturing guanidinium isothiocyanate containing buffer and homogenized with a QIAshredder column. The RNA was then purified in spin columns containing special silica-gel membranes.

2.1.5.2 cDNA synthesis

Purified mRNA can be used to produce cDNA with the help of the AMV reverse transcriptase enzyme (Roche, Mannheim), which plays a crucial role in the replication of retroviruses. 2-10µg of RNA and 1µl of random primer in a volume of 10µl were incubated for 2 minutes at 68°C, followed by 10 minutes incubation at room temperature. After addition of 0, 5µl RNase inhibitor, 4µl 5 x AMV RT buffer, 4µl dNTPs (2, 5 MM each) and 1µl AMV RT, the volume was adjusted to 20µl. The reaction-mix was incubated at 42°C for 1 hour and thereafter cDNA was purified using the Qiagen PCR purification kit (Qiagen, Hilden).

2.1.6 PCR-amplification of DNA and c-DNA fragments

The method of the polymerase chain reaction (PCR) techniques allows the exponential amplification of a specified DNA sequence in vitro. In a repeating cycle, doubled stranded DNA is separated by heat denaturation, the primers anneal to their binding sites that flank the target region and a polymerase can synthesize new strands of DNA, complementary to the template. Each PCR reaction was performed in a total reaction volume of 50µl containing 0, 1 µg template DNA, 1x PCR buffer, 2, 5µM $MgCl_2$, 0,5µM of each primer, 200µM dNTP and 0,1-0,75 units Pfu DNA polymerase. PCR reactions were carried out using an automated thermal cycler (Eppendorf). The following standard protocol was adjusted to each specific application:

3 minutes 95°C (initial denaturation)
25-35 cycles
1 minute 95°C (denaturation)
1 minute Tm (hybridization at appropriate temperature)
5 minute 72°C (extension)

The amplification was verified by running an analytical agrose gel with an aliquot of the reaction.

2.2 Methods of biochemistry

2.2.1 Cell lysis

To discard rests of the culture medium, cells were washed first with cold PBS (4°C). Afterwards, they were lysed for 13 minutes on ice in lysis-buffer containing 50 mM HEPES pH 7.2, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 10 % glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium floride, 1 mM Phenymethylsulfonyl fluoride and 10 µg/ml aprotinin. Lysates were pre-cleared by centrifugation at full speed (13000 rpm) for 5 minutes at 4°C. For later applications, lysates were stored at -80°C.

2.2.2 Estimation of the total protein concentration in lysates

The overall protein concentration was determined using the Micro BCA protein assay Kit (Pierce, Sankt Augustin) according to the manufacturer's protocol.

2.2.3 Immunoprecipitation of proteins

For immunoprecipitations, specific antibodies at corresponding amounts and 50 µl of protein A- or G- Sepharose (Pharmacia, Piscataway, NJ) were added to the cleared lysate and incubated overnight at 4°C. Immunoprecipitates were washed with HNTG buffer [20 mM HEPES (pH 7.5), containing 150 mM NaCl, 1 mM EDTA, 1 mM sodium fluoride,10% (v/v) glycerol, 0.1% (v/v) Triton X-100]. Sample buffer containing SDS and 2-mercaptoethanol was added, and samples were denatured by heating at 95°C for 8 min. Proteins were fractionated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters.

2.2.4 SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE)

The SDS-polyacrylamide-gel electrophoresis allows the separation of proteins according to their molecular weight (Laemmli et al., 1970). The detergent sodium-dodecyl-sulfate binds and denatures proteins. The binding of the dodecyl-sulfate results in a negative, total charge of the protein independent of the specific amino acid composition, but correlated to its molecular weight. The negative charge determines the migration of the protein through the gel. SDS-PAGE was conducted as described previously (Sambrook et al., 1990). Briefly, 50 µg to 100 µg of denatured proteins were separated using vertical Atto-gels (Atto Corporation, Japan). Depending on the proteins of interest 7.5 % to 12 % polyacrylamide gels were used for protein fractioning. An overview of the different elements present in two gels is given in the following table.

Separating gel	7.5 %	10 %	12 %
H_2O	20 ml	16.7 ml	13.3 ml
4x low-tris Buffer	10 ml	10 ml	10 ml
Acrylamide (30 %/0.8 %)	10 ml	13.3 ml	16.7 ml
TEMED	25 µl	25 µl	25 µl
APS	270 µl	270 µl	270 µl

Stacking gel (4 %)	
H_2O	9.6 ml
4X upper-tris Buffer	3.25 ml
Acrylamide (30 %/0.8 %)	1.8 ml
TEMED	25 µl
APS	270 µl

The following proteins were used as molecular standards:

Protein	MW (KD)	Protein	MW (KD)
Myosin	205	Ovalbumin	42.7
ß-galactosidase	116.25	Carboanhydrase	29
Phosphorylase	97.4	Trypsin-inibitor	21.5
BSA	66.2	Lysozym	14.4

2.2.5 Transfer of proteins on nitrocellulose membrane

For immuno-detection, the proteins were blotted onto nitrocellulose membranes. The transfer was performed with transblot-SD-buffer (50.0 mM Tris/HCL pH 7.5, 40.0 mM glycine, 20 % methanol, 3.75X 10^{-5} % SDS) at 0.8 mA/cm^2 nitrocellulose membrane in a semidry blot aperture for 2 hours. After the transfer, the proteins were stained with Ponceau S (2 g/lietr in 2 % TCA), the bands of the molecular weight standard were marked and the membrane was destained and blocked with 1X NET-gelatine (50.0 mM Tris/HCL pH 8.0, 0.5 mM EDTA pH 8.0, 150 mM NaCl, 0.5 % Triton X-100 0.25 % gelatine) for at least 1 hour.

2.2.6 Immunodetection of proteins (Western blot analysis)

Proteins that are immobilized on nitrocellulose membranes can be detected with antibodies. In a first step the immobilized protein (antigen) is recognized by a specific monoclonal or polyclonal antibody (primary antibody). In a second step the primary antibody is recognized by a species specific secondary antibody, to which a horse radish peroxidase is coupled. In alkaline medium luminal is oxidized by horse radish peroxidase and hydrogen peroxidase. Immediately following oxidation, the luminal is in an excited state which then decays to ground state via a light emitting pathway, which can be detected by autoradiography. The nitrocellulose membrane was incubated for one hour with three changes of 1 X NET-gelatine (50.0 mM Tris/HCl pH 8.0, 5 mM EDTA pH 8.0, 150 mM NaCl, 0.5 % Triton X-100. 0.25 % gelatine) to block unspecific binding sites of antibodies. The membrane was incubated with the primary antibody (1:500 to 1:1000 dilution in 1 X NET-gelatine) at 4°C overnight. After removal the primary antibody, the membrane was washed three times for 20 minutes with 1 X NET-gelatine at room temperature and incubated with the secondary antibody (rabbit, mouse or sheep) for one hour. The membrane was then washed three times for 20 minutes with 1 X NET-gelatine before detection with ECL-kit. The membrane was incubated in a 1:1 mixture of both ECL-reagents for three minutes. Afterwards, the membrane was wrapped with SaranWrap, and the air bubbles between the membrane and the SaranWrap were removed by gently smoothing with a tissue paper. The membrane was exposed to the film for different time periods. When the membrane should be re-probed with another antibody, the bound antibodies were removed by stripping for one hour at 50°C.

2.3 Methods of mammalian cell culture

2.3.1 General cell culture techniques

All used cell lines were cultured in incubators (Heraeus, B5060 EK/CO_2) at 5-7% CO_2, 37°C and a saturated water vapour atmosphere. All cell culture work was performed aseptic in a sterile hood (Heraeus, laminair). The cells were passaged regularly and the media was changed every other day. In addition, the cultures were checked routinely for infection with mycoplasma. The cells were frozen in a media containing 90% FCS and 10% DMSO for long term storage in liquid nitrogen. A coulter counter (Beckton Dickinson) was used for counting the cells.

2.3.2 Stimulation of cells

Cells were seeded in corresponding cell culture dishes and grown overnight to about 80 % confluence. After serum-starvation for 24 hours cells were stimulated with appropriate growth factors, washed with cold PBS and then lysed for 13 minutes on ice. In some cases cells were transfected 24 hours after seeding and serum-starved one day following transfection before being stimulated as indicated above.

2.3.3 Transfection of mammalian cells by calcium phosphate-DNA co-precipitation

A solution of calcium chloride, plasmid DNA and N-N-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid (BES) buffer is added to a plate of cells. During an overnight incubation under an atmosphere of 3% CO_2 a calcium phosphate-DNA precipitate forms. This precipitate adheres to the cell surface and is taken up by the cells.Transfections were carried out using a modified calcium phosphate method. Briefly, $0{,}5x10^6$ cells were incubated overnight in 3ml of growth medium the day before transfection. 20 µg of plasmid DNA were diluted in, 500 µl $CaCl_2$ and 500 µl 2xBBS were added dorpwise while swirling vortex under the hood. The solution was then incubated at room temperature for 20 minutes. The calcium phosphate-DNA solution was added dropwise onto the medium-containing plate. The cells were then incubated at 3% CO_2 atmosphere for 12 to 24 hours.

2.3.4 Transfection of mammalian cells using lipofectamine®

Lipofectamine® (Gibco-BRL) is a polykationic transfection reagent. It binds DNA and its lipophilic property allows the transfer of DNA across the cell membrane into cells. Target cells were transiently transfected as described previously (Daub et al., 1997). Briefly, cells were seeded in 6cm plates. 350µl of serum-free medium containing 10µl of Lipofectamine and 2µg of total plasmid DNA per plate were used. After 4 hours the transfection mixture was supplemented with an equal volume of medium containing 10 % FCS. Then, cells were either stimulated or left untreated, lysed and subjected to Western blot analysis.

2.3.5 Transfection of mammalian cells using Lipofectamine RNAiMAX®

HER3, and *GL-2* siRNAs were transiently transfected in cells using Lipofectamine RNAiMAX® (Gibco-BRL) according to the manufacturer's recommendations. This new art of transfection is in fact called as reverse-transfection. Briefly, 20pmol of siRNA was mixed with the appropriate amount of OPTI-MEM medium, mixed with the Lipofectamine RNAiMAX® reagent and incubated for 10 to 15 minutes at room temperature. During this time cells were trypsinized and counted for the further transfection. A mixture containing the cells added by the siRNA, OPTI-MEM and the Lipofectamine RNAiMAX® containing reagent was incubated with the cells for 6 to 8 hours and thereafter a 20% FCS medium was added to transfected cells. The day after, the medium was replaced by normal growth medium containing 10 % FCS. Silencing efficiency was tested at different time-points after transfection by Western blot analysis.

2.4 Methods of cell biology

2.4.1 Proliferation assay

Cells were transfected with *HER3* or *GL-2* siRNA using Lipofectamine® RNAiMax (Invitrogen). The cells were either grown in their appropriate medias or serum starved in medium containing 1% FCS and stimulated with 100 ng/ml heregulinß1. The cell number of cells was counted (Coulter Counter, Beckton Dickinson) at the indicated time points. The data are shown as mean ± SD.

2.4.2 Cell cycle analysis

To analyze the cell cycle, HER3-knockdown cells as well as control cells were harvested and fixed with 70% ethanol in PBS. Cells were then resuspended in 0.5mL PBS containing propidium iodide and DNase-free RNase for 2 hours and subsequently subjected to flow cytometric analysis (Becton Dickinson Biosciences) to determine cell-cycle profiles. At least 10,000 cells were analyzed from each sample.

2.4.3 Migration and invasion assays

Cells transfected with *HER3* or *GL-2* siRNA using Lipofectamine® RNAiMax (Invitrogen) were serum starved in medium containing 0, 1% FCS for 24h and seeded either onto a membrane or onto a growth factor-reduced Matrigel-coated membrane with 8 µM pores of a modified Boyden chamber (Schubert and Weiss) containing 500 µl serum-free medium; 10% FCS medium served as a chemo-attractant. After 20 h or 24 h incubation at 37°C, migrated and invaded cells respectively were stained with crystal violet and washed in PBS. The cells in the insert were removed by wiping gently with a cotton swab. Cells on the reverse side of the insert were examined and photographed by microscopy (Zeiss Axiovert 300 Microscope). At least 10 random fields were counted per filter for quantification. The data are shown as mean ± SD.

2.4.4 Apoptosis assay and cell cycle analysis by propidium Iodide staining

HER3 or *GL-2* transfected cells were treated with either 10 or 20µmol/L 5-fluorouracil or DMSO as a negative control. 72h after, floating cells were collected and adherent cells were trypsinized. After centrifugation, cells were incubated for 2 h in a propidium iodide buffer (0.1% sodium citrate, 0.1% Triton X-100, and 20 µmol/L propidium iodide) and subsequently subjected to flow cytometric analysis (Beckton Dickinson Biosciences). Apoptotic cells were identified as the sub-G0-G1 peak and quantified using the CellQuest Pro software (Beckton Dickinson).

2.4.5 HER3 blocking antibody: Apoptosis, migration, and invasion experiments

The HER3 blocking antibody was purchased from Upstate. To assess the HER3 phosphorylation state, cells were serum starved for 24 h, incubated with 10 µg/ml blocking antibody for 1 h, stimulated with 100 ng/ml heregulin-ß1 for 4h, lysed, and subjected to immunoprecipitations using a specific home-made HER3 antibody (clone. 1B4C3).
The MDA-MB-468 breast cancer cells were already described to be inhibited by the anti-HER3 antibody (clone 105.5; albini et al., 1987) and served as a positive control.
The migration and invasion assays were done as described previously (Albini et al., 1987, Van der horst et al., 2005; Reschke et al., 2008). Briefly, cells were seeded in 10-cm plates and serum starved in medium containing 0.1% FCS for 24 h. Cells (400.000/ml) were then incubated with 10 µg/ml HER3 blocking antibody or an isotype control antibody for 1 h. In both assays, cells were plated in triplicate at a density of 100,000 cells per well either onto a membrane or onto a growth factor-reduced Matrigel-coated membrane with 8 µM pores of a modified Boyden chamber (Schubert and Weiss) containing 500 µl serum-free medium. Conditioned NIH3T3 medium containing 100 ng/ml heregulin-ß1, and 0.01 % ascorbic acid served as a chemoattractant. The cells were allowed to migrate or invade for 20 or 24 h, respectively. Migrated and invaded cells were stained by crystal violet, washed in PBS, and analyzed using a Zeiss Axiovert 300 microscope. For quantification, 10 random fields at least were counted per filter. The data are shown as mean ± SD.

2.4.6 Immunofluorescence

In order to test the expression and mainly the emplacement of some protein markers an immunofluorescence staining was established. Briefly, confluent *HER3* or *GL-2* transfected cells were washed three times with 1 x PBS in order to totally eliminate all media and media-containing supplements (growth factors, FCS …etc). Cells were then fixed in a 4 % PFA solution for 8 to 10 minutes at 37°C and then washed three times with a PBST (PBS-Tween) solution. For permeabilisation, cells were treated for 20 minutes with Triton X-100 at a concentration of 0, 5 M. Cells were then blocked using a 37°C warm wash-solution containing 5 % (BSA/PBS). Blocked cells were, by a further step, incubated for 30 to 40 minutes with the first antibody (1:100), which is dissolved in a 2 % (BSA/PBST) solution. In order to remove unspecific binding, cells were washed three times, 5 minutes each in a warm PBST solution. Thereafter, cells were incubated with the second antibody coupled to a fluorophore for 30 to 40 minutes. After washing, cells were incubated with DAPI diluted in PBST solution (1:80) for the DNA staining. By a last step, cells were washed for a short time in a

warm PBST solution. Using a fluorescence microscope at the indicated wave lengths, proteins-markers were then visualized.

2.5 Colon cancer patients

2.5.1 Clinical samples and statistical analyses

HER3 expression was evaluated using formalin-fixed, paraffin-embedded (FFPE) colon cancer samples of patients who underwent surgical tumour resection at the Ludwig-Maximilians-Universität München between 1994 and 2005. Follow-up data were recorded by the Tumorregister München. Paraffin embedded tissue samples of primary tumours were available in 110 cases. The median age of the patients at the time of diagnosis was 67 years. Clinicopathological characteristics of the study population are summarized in Table. 3. For inclusion models, age, gender, tumor grade, and T-category were considered as co-variables. Frequency data were analysed using the $\chi 2$ test or Fisher's exact test. Cancer specific survival was calculated from the date of primary surgery to the date of cancer associated death. Survival data of patients whose death was not attributable to colorectal cancer or whose follow-up ended before death were censored. Survival analysis was done with the Kaplan-Meier method and the groups were compared with the log-rank test. Univariate and multivariate analyses were based upon the Cox regression model. Cases with missing data were excluded from multivariate analyses. Statistical procedures were done using SPSS version 15.0 (SPSS Inc.). $P < 0.05$ was considered as statistically significant.
The study was approved by the ethics committee of the Medical Faculty of the Ludwig-Maximilians-Universität München.

2.5.2 Immunohistochemistry, scoring of HER3 expression, and statistical analyses

All tumors were arrayed in quadruplicate on tissue microarrays using 1.0 mm diameter punch sets (Beecher instruments). 5µm sections were prepared for immunohistochemical staining. As primary antibodies we used anti-HER3 (clone C-17, 1:50; Santa Cruz). Staining was performed on a Ventana Benchmark autostainer with the XT ultraView DAB Kit (Ventana Medical Systems). All slides were counterstained with Hematoxylin. To exclude unspecific staining, isotype and system controls were included. HER3 expression was scored semiquantitatively, considering membranous immunostaining of the tumor cells only. Depending on the intensity of staining, HER3 expression was classified as weak, intermediate, or strong.

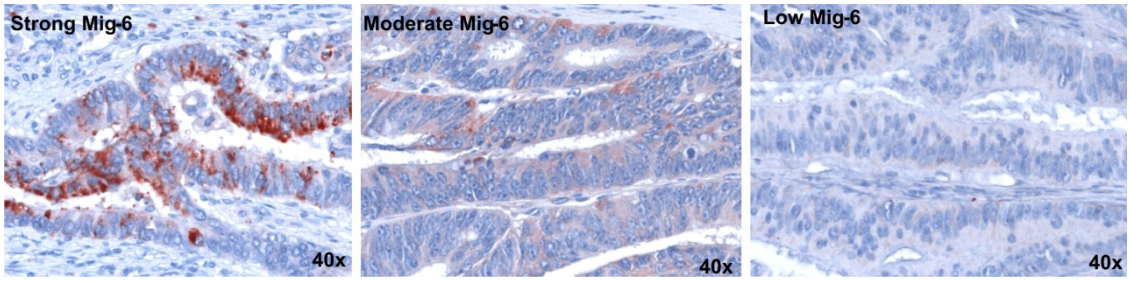
IV Results

1. HER3/mig-6 crosstalk in colon cancer

1.1 Mig-6 expression in primary tumors from colon cancer patients

We first analysed the expression levels of mig-6 immunohistochemically using a tissue micro array of 110 colon cancer patients. As mig-6 was described in previous studies as a cytoplasmic adaptor-protein (Reschke et al., 2010), a mig-6 protein staining, which is localised in the cytoplasm was expected in our study. Indeed, all three expression levels ranging from low, moderate to strong were observed exclusively in the cytoplasm of the examined primary tumor tissues (Figure. 13A). Percentages of the different expression intensities are summarized in the following figure (Figure. 13B).

A



B

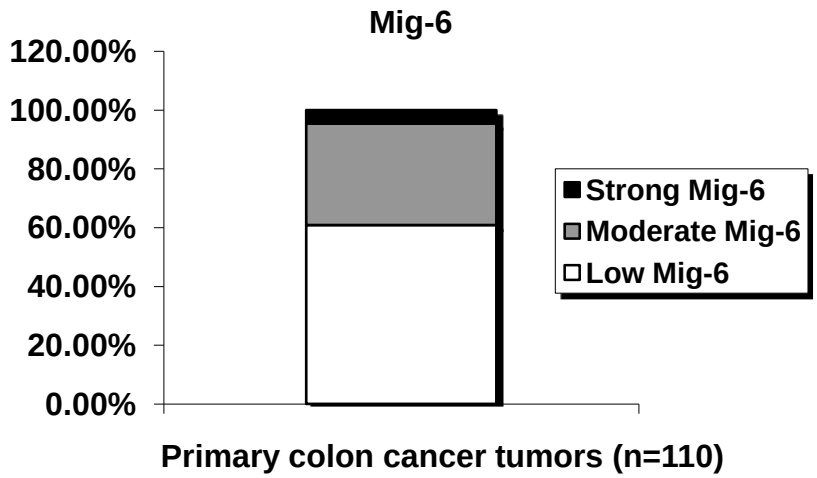
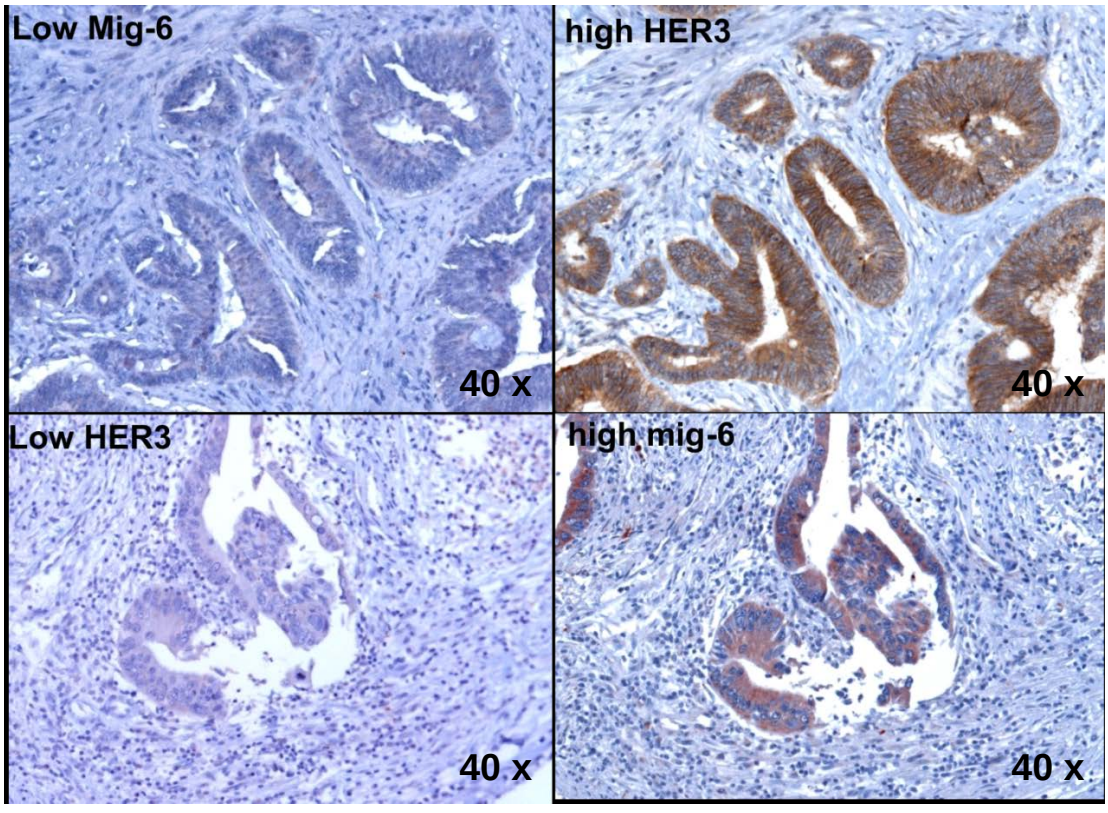


Figure 13. Mig-6 is under-expressed in primary tumors from colon cancer patients
(A) Strong, moderate and high mig-6 protein expression in primary colon cancer tumors.
(B) Mig-6 expression frequencies ranging from low, moderate to strong in primary tumors of colon cancer patients.

1.2 HER3 inversely correlates with mig-6 in primary colon cancer tumors and in colon cancer cell lines

Since mig-6 was already defined as a negative regulator of EGFR and even of HER3 in melanoma (Hackel al., 2000; Ferby et al., 2006), we asked the question, whether there is any correlation between mig-6 and HER3 expression in colon cancer primary tumors. Indeed, the negative correlation between HER3 and mig-6 was investigated in this model system. As shown in the figure below, tumor-areas with low mig-6 expression levels show high HER3 staining levels (Figure. 14A). The same observation was identified in tumors arrays characterised by strong mig-6 staining. They in fact show similarly low HER3 expression levels (Figure. 14A). This negative regulation was observed in 62, 27 % of the cases (Figure. 14B).

A



B

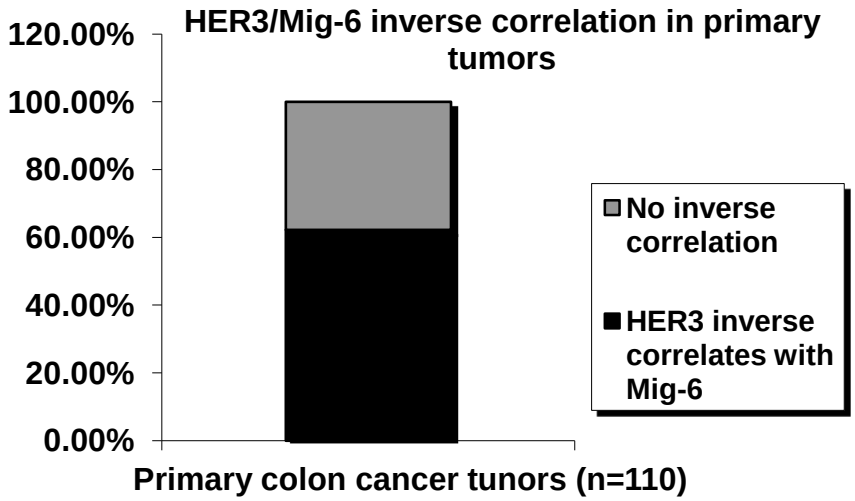


Figure 14. HER3 expression inversely correlates with mig-6 in primary tumors of colon cancer patients
(A) Immunohistochemical staining of HER3 and mig-6 in colon cancer primary tumors showing inverse protein expression.
(B) Statistical evaluation of the cases showing the HER3/mig-6 inverse correlation (black).

The same negative correlation was then investigated in colon cancer cell lines. HER3 and mig-6 protein expression levels were in fact examined in six different colon cancer cell lines including DLD-1, LoVo, CaCO2, RKO, HT-29, and HCT-116 using Western-Blot analysis (Figure. 14). In addition to the considerable differences in the HER3 protein expression levels observed in the tested cells, a clear inverse correlation between HER3 and mig-6 expression in CaCO2, DLD-1, HCT-116 and HT-29 could be detected (Figure. 15). The last observations suggest that mig-6 inverse correlates with HER3 also in colon cancer cell lines and may be a negative regulator of HER3 in this model system.

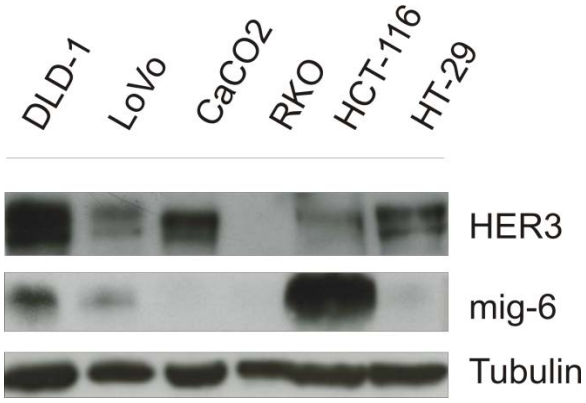


Figure 15. HER3 expression inversely correlates with mig-6 in colon cancer cell lines
Total lysates form lysed DLD-1, LoVo, CaCO2, RKO, HCT-116, and HT-29 colon cancer cell lines were subjected to Western blot analysis for HER3, and mig-6. Tubulin was used as a loading control.

1.3 Increased HER3 expression is associated with decreased survival of colon cancer patients

We analysed HER3 protein expression in 110 primary colon carcinoma using tissue microarrays. Immuno-staining revealed differential expression of HER3 among these cases with 25.5%, 53.6%, and 20.9% showing low, intermediate, and high expression, respectively (Fig. 16A-E). Using Kaplan-Meier statistics, we observed that patients with intermediate or high HER3 expression levels had significantly shorter survival times than cases with low HER3 expression (Fig. 17). Because intermediate and high HER3 expression did not show a significant difference in this survival analysis, we next categorized all cases into two groups only. Low expressing cases were defined as HER3-low while intermediate and high expressing cases were defined as HER3-high (Fig 17). In order to learn about the dependence of these two survival groups on other clinical factors, we next analysed their association with variables such as age, gender, T-stage, tumor grade, and tumor location and observed no significant correlation (Table 3). To determine whether these survival groups thus are independent of these clinical variables, we then used multivariate Cox-regression analysis. In this analysis, we found HER3-high to be an independent prognostic marker for low survival in our study collection, associated with a relative risk of 3.29 (Table 4). Taken together our data suggests that HER3 expression is of clinical relevance when assessed in colon cancer samples and indicates a higher risk of tumor associated death when highly expressed.

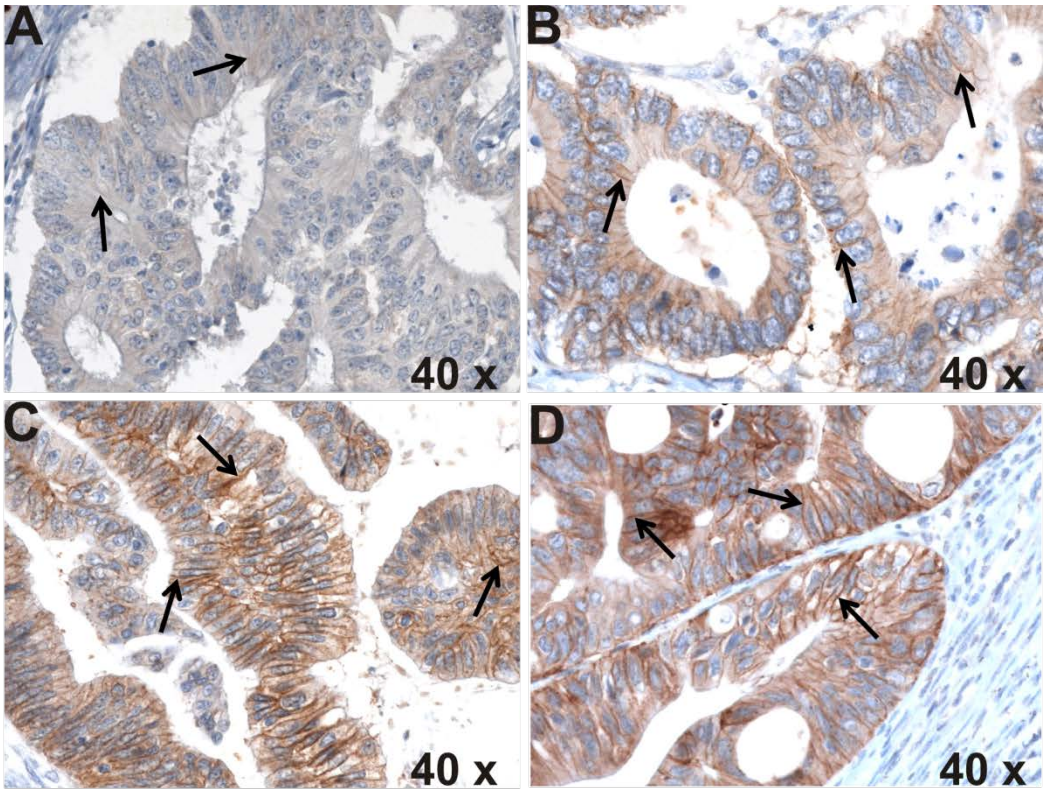
Variables	No. Patients	HER3		
		low	high	*P*
All patients	110 (100)	28 (25.5)	82 (74.5)	
Age (median 67y)				
≤ 67	54 (49.1)	16 (14.5)	38 (34.5)	0.32
≥ 68	56 (50.9)	12 (10.9)	44 (40)	
Gender				
Male	64 (58.2)	16 (14.5)	48 (43.6)	0.90
Female	46 (41.8)	12 (10.9)	34 (30.9)	
T stage				
pT1	1 (0.9)	0 (0)	1 (0.9)	0.84
pT2	37 (33.6)	11 (10)	26 (23.6)	
pT3	64 (58.2)	15 (13.6)	49 (44.5)	
pT4	8 (7.3)	2 (1.8)	6 (5.5)	
Tumor grade				
G2	94 (85.5)	24 (21.8)	70 (63.6)	0.96
G3	16 (14.5)	4 (3.6)	12 (10.9)	
Tumor location				
right	41 (37.3)	10 (9.1)	21 (19.1)	0.46
left	31 (28.2)	8 (7.3)	33 (30)	
rectum	38 (34.5)	10 (9.1)	28 (25.5)	

Percent values are given in parentheses

Table 3. Clinicopathological variables and correlation with membranous HER3

HR	HR	(95% confidence interval)	p
Age (≥ 68 vs. ≤ 67)	1.72	(0.91-3.22)	0.09
Gender (M vs. F)	0.93	(0.47-1.81)	0.82
Tumor grade	0.89	(0.37-2.18)	0.80
Tumor location (rectum vs. colon)	1.75	(0.88-3.46)	0.11
T stage	3.95	(2.04-7.66)	<0.001
HER3 (high vs. low)	3.29	(1.28-8.45)	0.014

Table 4. Multivariate analysis of factors possibly influencing overall survival.



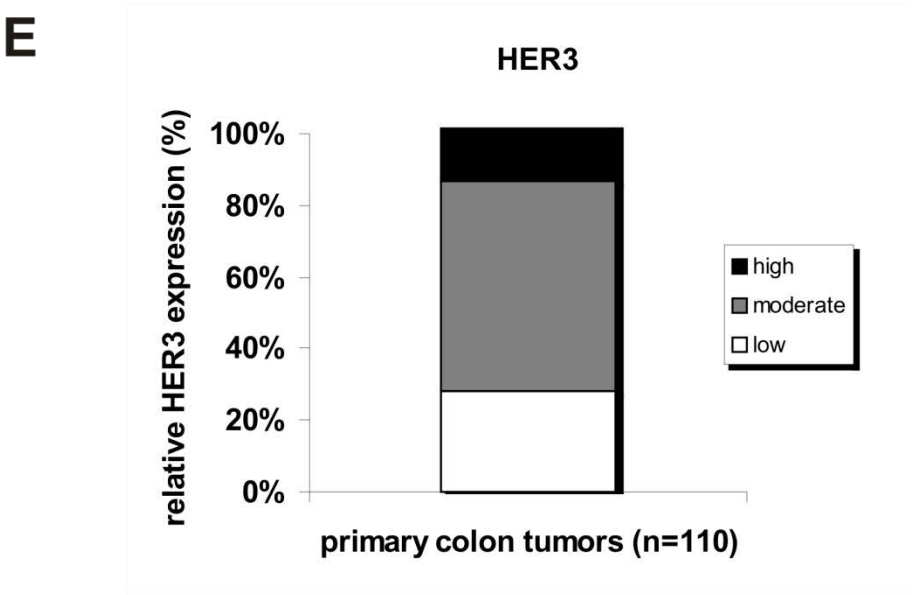


Figure 16. HER3 is frequently expressed in primary colon cancer tumors
HER3 protein expression was analysed by immunohistochemistryy in 110 primary tumors from colon cancer patients using specific antibodies and colon carcinoma tissue micro arrays.
(A-D) Low, moderate and high HER3 expression in primary colon cancer tumors. HER3 immuno reactivity is totally presented at the cell membrane (Arrows).
(E) HER3 expression frequencies in primary colon cancer tumors.

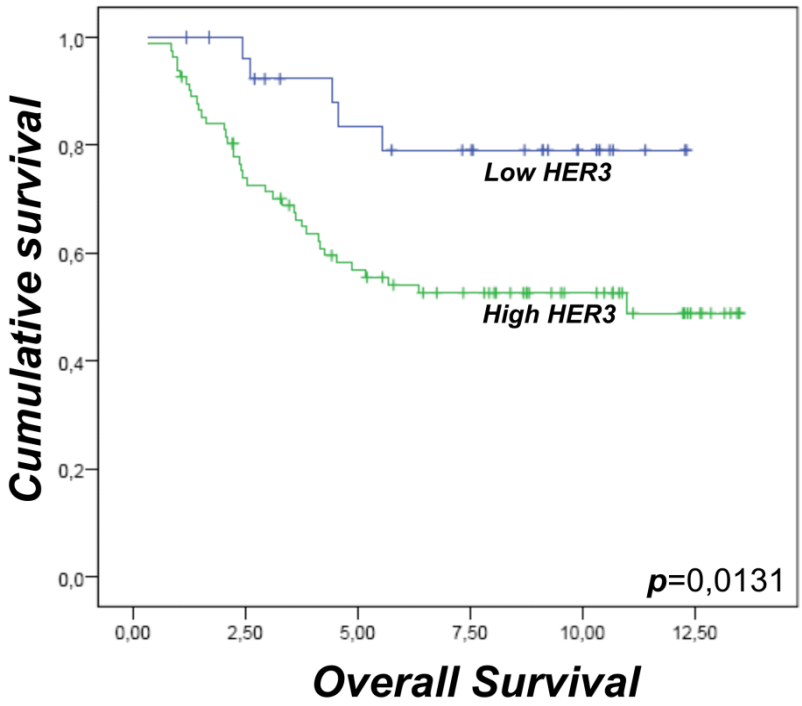


Figure 17. HER3 expression confers poor prognosis in colon cancer patients
High HER3 expression is significantly associated with reduced overall survival of colon cancer patients (p=0,0131). The overall survival of colon cancer patients was estimated by the Kaplan Meier method and the groups were compared with the log-rank test. Statistical procedures were performed using SPSS version 15.0 (SPSS Inc.). $p < 0,05$ was considered as statistically significant.

1.4 HER family member expression in colon cancer cell lines

In order to select a model system which is suitable for our investigation, we screened a panel of 5 colon cancer cell lines (DLD1, LoVo, CaCO2, RKO, and T-84) for the protein expression levels of three members of the HER family, namely HER3, HER2, and EGFR. HT-29 and HCT-116 colon cancer cell lines were tested previously (Choi et al., 2010) and considered as positive and negative control for HER3 expression, respectively (Fig. 18). A considerable difference by the HER3 expression was observed among the five tested cell lines. Whereas DLD-1 and T-84 cell lines are characterized by a very high to a high HER3 levels respectively, CaCO2 and LoVo express HER3 moderately. No detectable HER3 was observed in the RKO cell line. While HER2 expression levels vary from one cell line to the other, all tested cell lines showed moderate EGFR expression levels (Fig. 18).

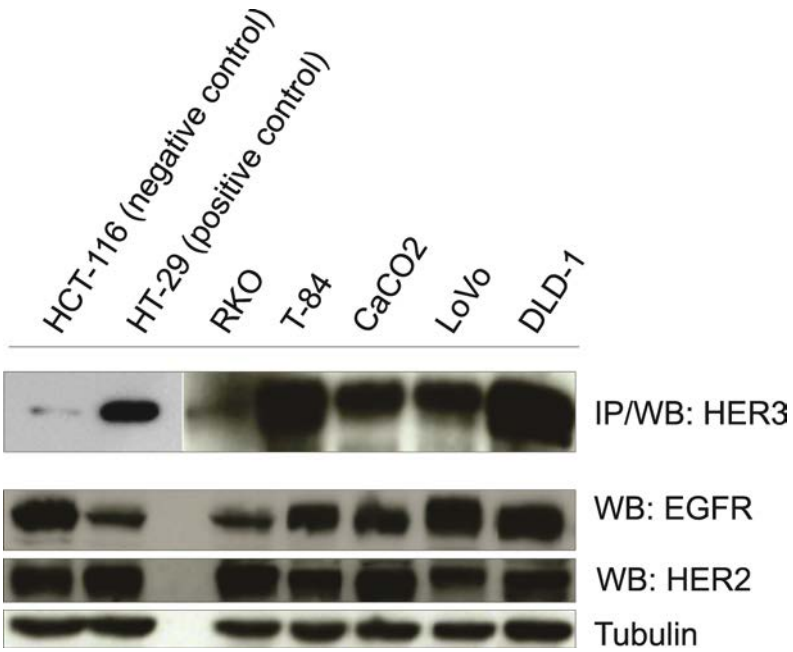


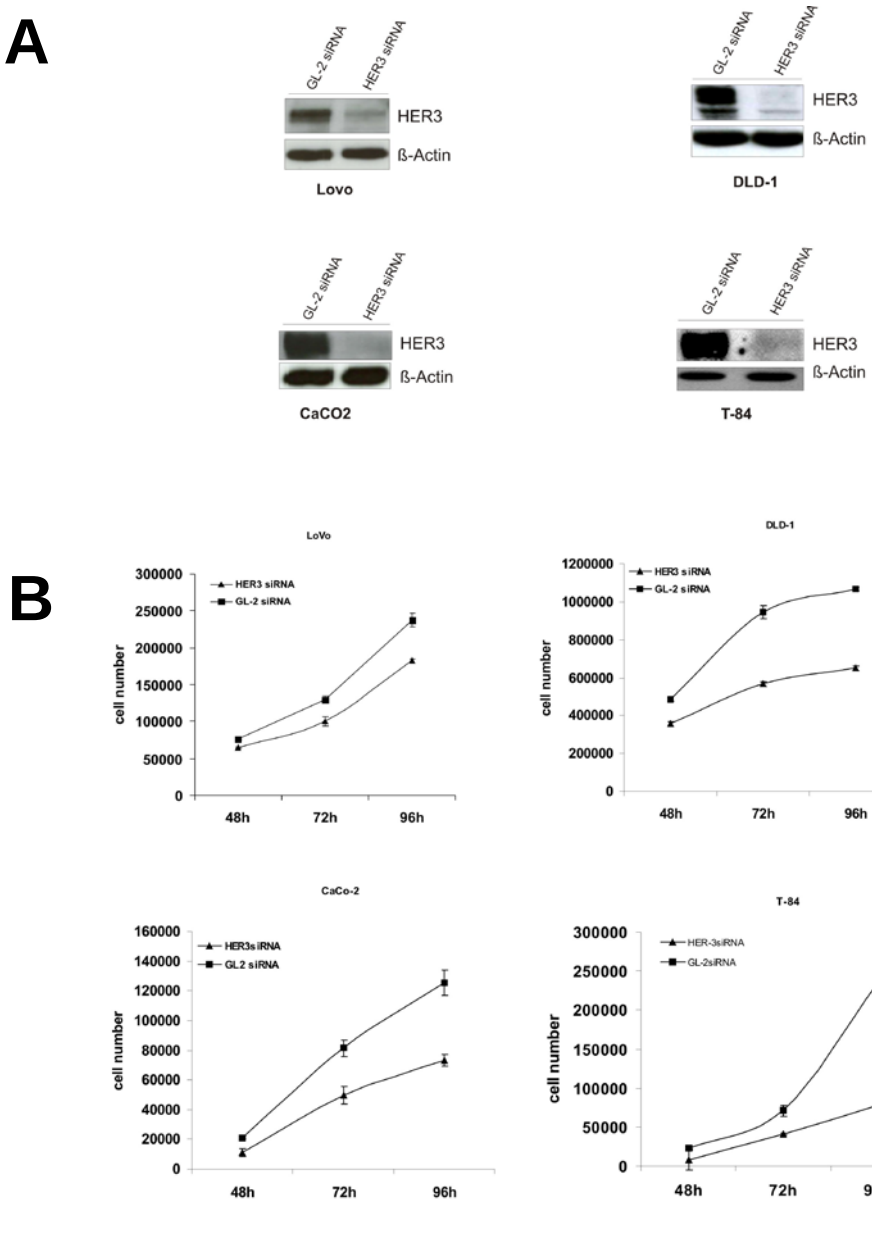
Figure 18. EGFR, HER2 and HER3 protein expression in colon cancer cell lines
EGFR, HER2 and HER3 protein expression levels were analysed by Western blot in a panel of 5 colon cancer cell line. HCT-116 and HT-29 cells were taken as a negative and positive control for the HER3 expression, respectively.

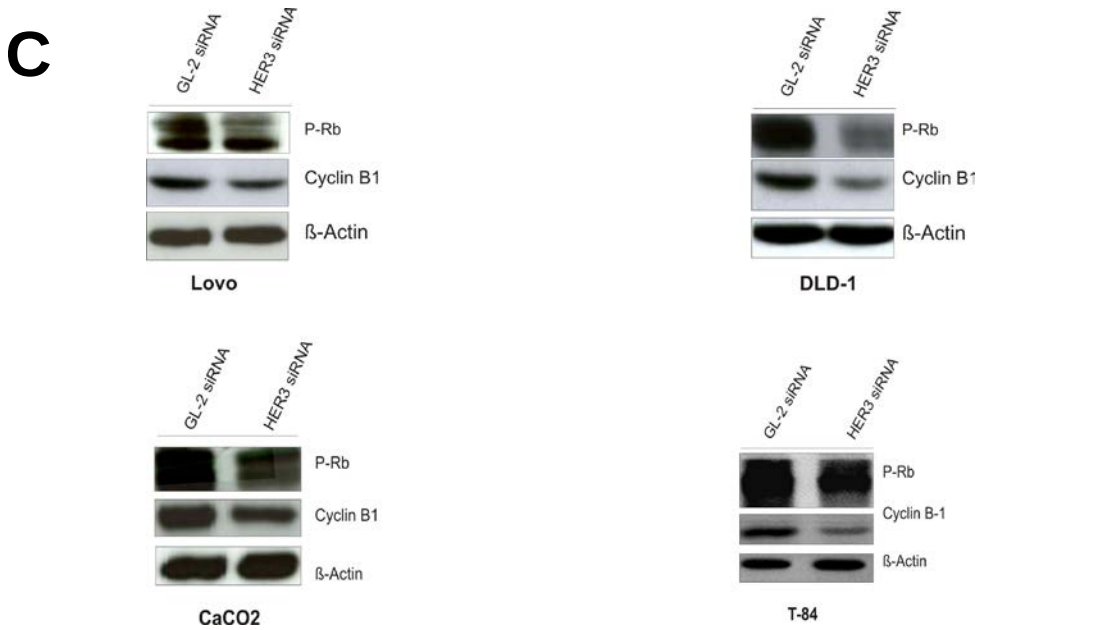
1.5 HER3 knockdown affects proliferation of colon cancer cell lines and interferes with activation of its downstream targets

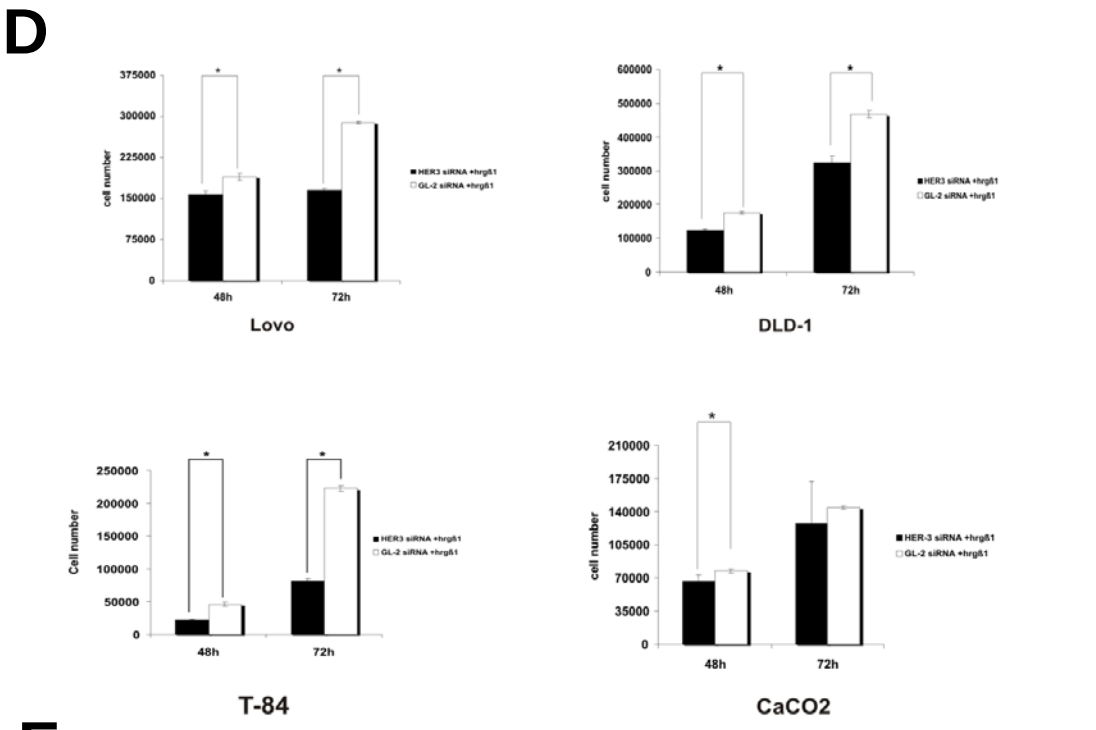
To validate our clinical *in vivo* results, we focused on the function of HER3 receptor tyrosine kinase *in vitro*, thereby testing its intervention by the modulation of diverse biological processes including proliferation, motility, invasion, and survival. We therefore reasoned to down-regulate *HER3* in DLD-1, LoVo, CaCO2, and T-84 colon cancer cell lines by small interfering RNA (siRNA) (Figure. 19A).

First, we addressed the question whether HER3 could be involved in the regulation of colon cancer cell proliferation. As anticipated, depletion of *HER3* strongly inhibited proliferation in all four tested cell lines (Figure. 19B). To further characterize the molecular mechanism behind this growth inhibition, we checked expression levels of main proteins implicated in the regulation of the cell cycle before and after *HER3* knockdown. As shown in Figure. 19C, HER3 suppression leads to a significant decrease in cyclin B1 expression levels as well as to decreased Rb phosphorylation, suggesting that HER3 controls cell proliferation by interfering with key cell cycle regulators. In addition, HER3 knockdown significantly inhibited heregulin-ß1-induced

cell proliferation (Figure. 19D). We subsequently asked whether *HER3* knockdown has an effect on known downstream substrates, namely ERK1/2 and Akt, which are, respectively, effector proteins responsible for MAPK cascade activation and lipid signalling (Citri et al., 2003; Wallasch et al., 1995). Akt activation as well as phospho-mTOR levels were impaired subsequent to *HER3* knockdown in both heregulin-ß1 stimulated and non-stimulated cells, indicating that HER3 may signal via the PI3K-Akt pathway in colon cancer cells (Figure. 19E). Moreover, HER3 knockdown cells showed increased p27 protein expression levels in comparison to control cells (Figure. 19E). In contrast, phospho-extracellular signal-regulated kinase remained unchanged (Figure. 19E).




C
GL-2 siRNA
HER3 siRNA
P-Rb
Cyclin B1
ß-Actin
Lovo
DLD-1
CaCO2
T-84
Cyclin B-1


D
cell number
48h
72h
HER3 siRNA +hrgß1
GL-2 siRNA +hrgß1
Lovo
DLD-1
T-84
CaCO2

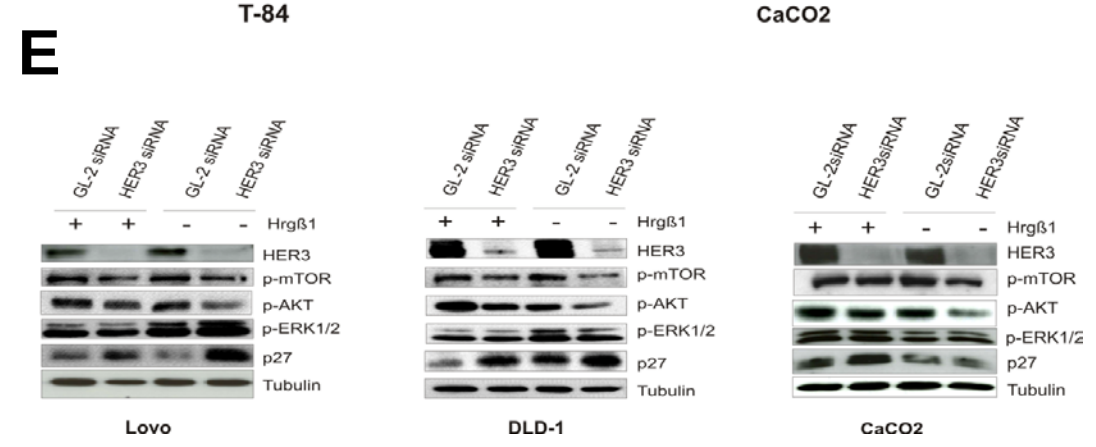

E
GL-2 siRNA
HER3 siRNA
Hrgß1
HER3
p-mTOR
p-AKT
p-ERK1/2
p27
Tubulin
Lovo
DLD-1
CaCO2

Figure 19. *HER3* knockdown inhibits colon cancer cell proliferation

(A) *HER3* knockdown in LoVo, DLD-1, CaCO2, and T-84 colon cancer cells. The cells were transfected with *HER3* or *GL-2* siRNAs as described in Material and Methods. After 72 hours, cells were lysed and subjected to Western blot analysis. Immunoblots for HER3 and ß-actin are shown.
(B) Inhibition of LoVo, DLD-1, CaCO2, and T-84 cell proliferation by *HER3* siRNA. The cells were transfected with *HER3* or *GL-2* siRNAs and counted at the indicated time points using a coulter counter (Beckton Dickinson). Differences between groups were assessed with the Students T-test and data are shown as ± SDM. P-values below 0, 05 were considered statistically significant.
(C) *HER3* knockdown induces a decrease in the cyclin B1 expression as well as in the Rb phosphorylation.
(D) *HER3* knockdown inhibits heregulin-ß1-stimulated proliferation of LoVo, DLD-1, CaCO2, and T-84 cells. The cells were transfected with *HER3* or *GL-2* siRNAs, straved for 24 hours in a medium containing 0, 01 % FCS and stimulated with 100 ng/ml heregulin-ß1 at the indicated time points. The cell number was determined as described in **B**.
(E) *HER3* knockdown impairs AKT and mTOR activities in LoVo, DLD-1, and CaCO2 colon cancer cell lines. Western blot analysis for HER3, phospho-AKT, p27, pERK1/2, and p-mTOR are shown. Tubulin served as a loading control.

1.6 *HER3* knockdown induces a G2-M cell cycle arrest in colon cancer cell lines

Since *HER3* suppression by siRNA leads to inhibition of Rb phosphorylation as well as to decreased cyclin B1 protein expression levels, we asked whether the absence of HER3 could influence progression of the cell cycle. In order to address this question, we performed a cell cycle analysis and checked elements of the cell cycle before and subsequent to *HER3* knockdown. Interestingly significant increased amounts of cells by the G2-M phase were observed in all 4 *HER3* knockdown cells when compared to the controls (*GL-2* siRNA as well as untransfected cells). Parallel to this, a shift in the G1 and S phases was detected (Fig. 20A-B).

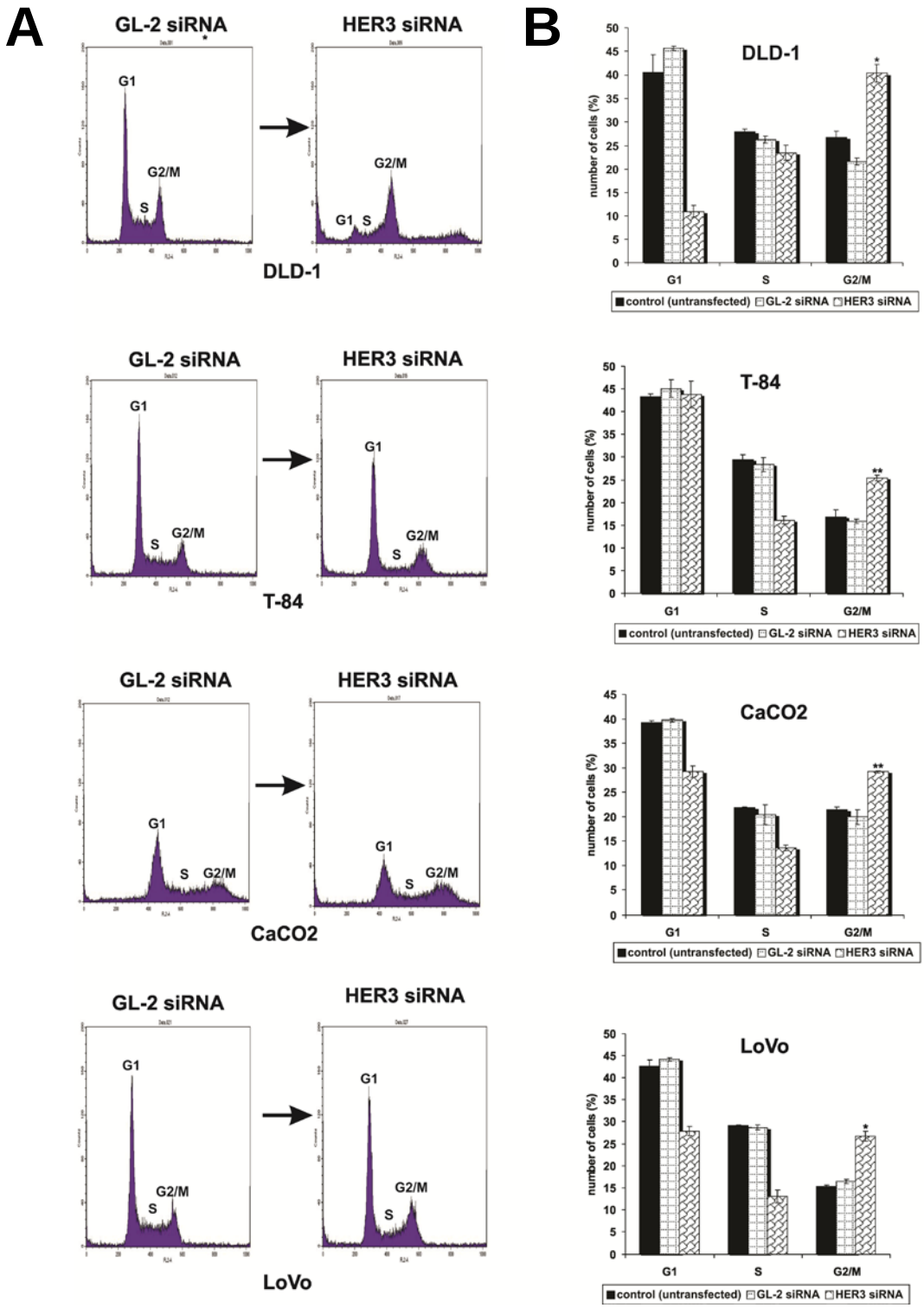


Figure 20. HER3knockdown inducesa G2–M cell-cycle arrest in colon cancer cell lines.
(A) Representative flow cytometric histograms of DLD-1, T-84, CaCO2, and LoVo cell lines. Forty-eight hours posttransfection, cell-cycle kinetics of HER3 knockdown cells and control cells were determined by propidium iodide staining.
(B) data represent means of triplicate experiments.

1.7 *HER3* depletion by siRNA induces a morphology change in colon cancer cell lines

To further characterize the role of HER3 in colon cancer cell lines, we addressed the question whether HER3 has any impact on the morphology of cells. Interestingly, knockdown experiments show that the deletion of *HER3* expression leads to a clear morphology change of the tested cell lines. Knock-down colon cancer cell lines show a flat appearance with a big cytoplasm towards a compact and a fibroblast-like shape, which was shown by the control cells (Figure. 21A-B).

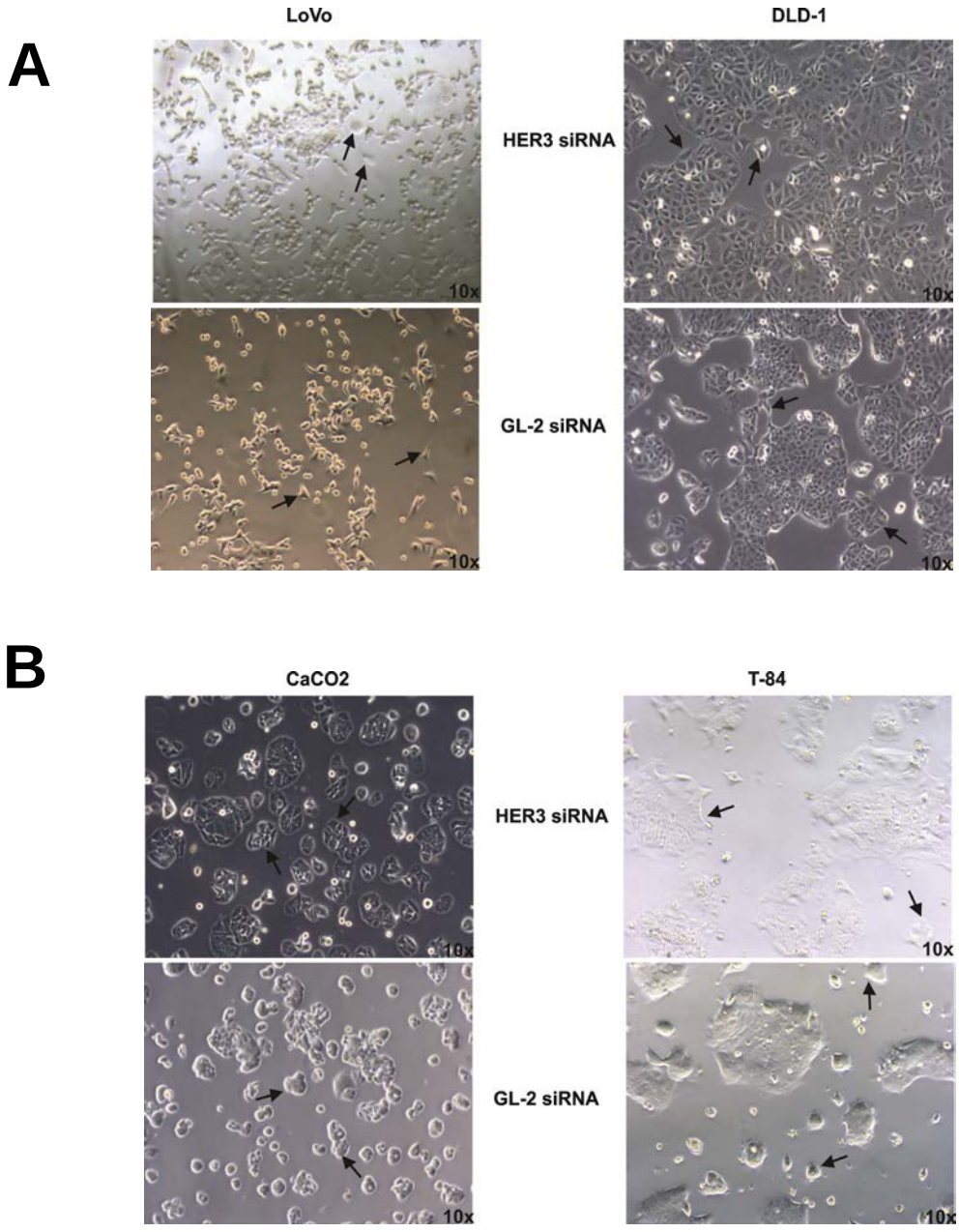
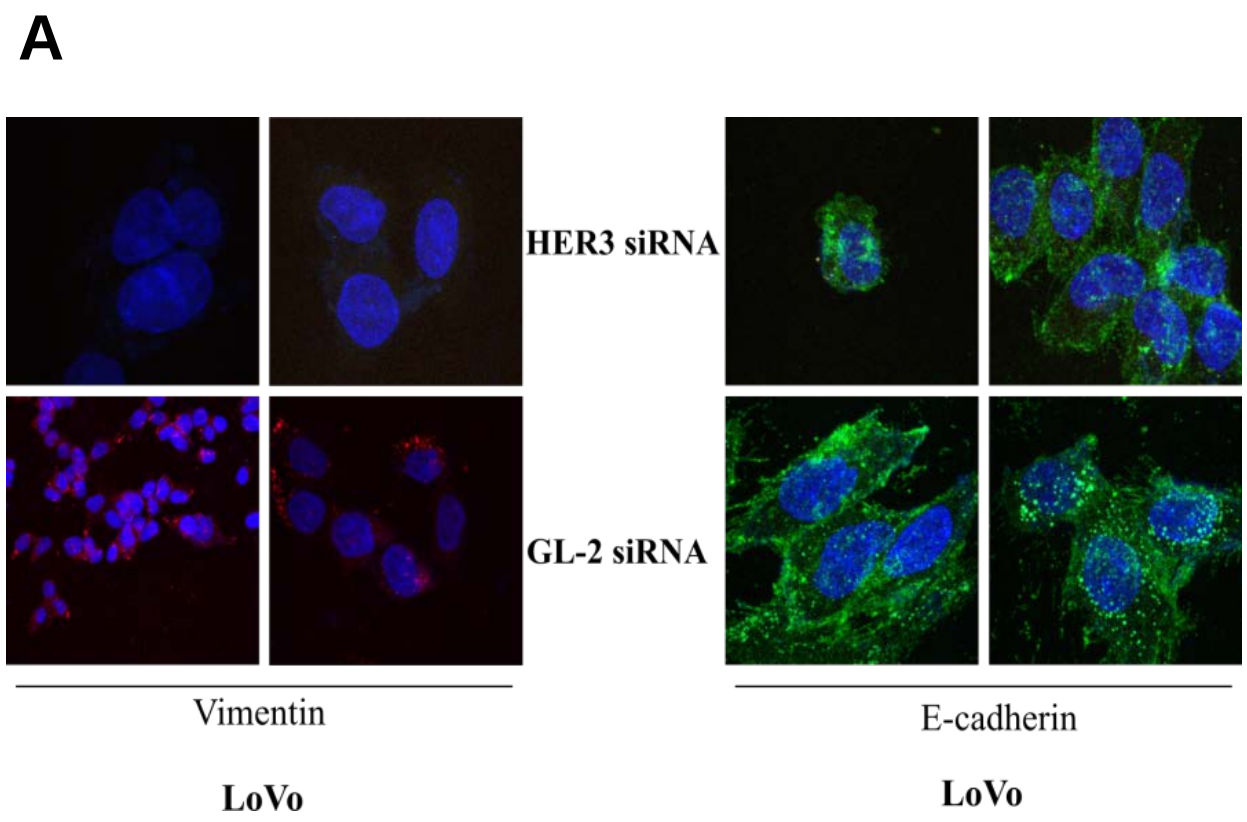


Figure 21. *HER3* knockdown induces a morphology change in colon cancer cells
(A), (B) DLD-1, LoVo, T-84, and CaCO2 colon cancer cell lines were transfected with *HER3* or *GL-2* siRNAs as described in Materials and Methods. Brightfield pictures were taken at the indicated time points using a Zeiss Axiovert 300 mciroscope at a 10 x magnification.

The last observations support the mesenchymal to epithelial transition hypothesis and allows us to strengthen it further. By using on the one hand immunofluorescence we checked the expression of key proteins implicated by the regulation of the cell morphology namely E-cadherin and Vimentin proteins. Indeed, cell-staining show a down-regulation in the Vimentin expression after HER3 knockdown (Figure. 22A). Nonetheless, E-cadherin expression levels remains unchanged before and after *HER3* deletion (Figure. 22A). Western blotting analysis on the other hand further supports our above observations. Indeed, a decrease in the protein expression level of Vimentin was identified after *HER3* deletion in the LoVo colon cancer cell line (Figure. 22B), suggesting the certainty of our hypothesis at least in one unique cell line. However, further investigation of this hypothesis using other colon cancer cell lines and by checking further cell-morphology markers is indispensable.



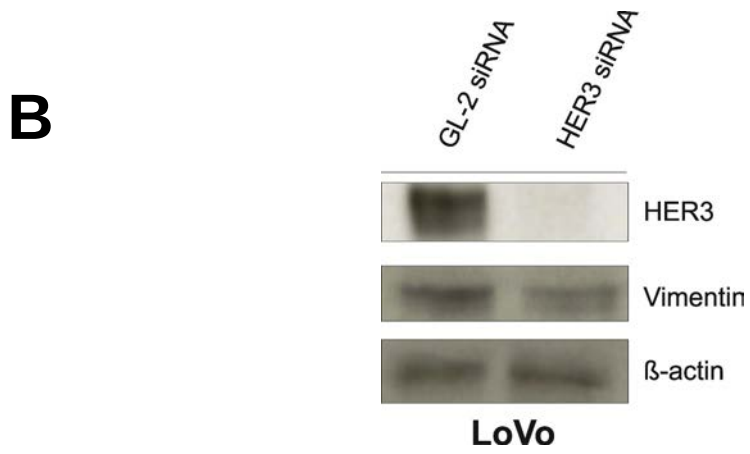


Figure 22. *HER3* knockdown induces a morphology change in colon cancer cells
(A) LoVo colon cancer cell line was transfected with *HER3* or *GL-2* siRNAs. After 48 hours, immunofluorescence staining for Vimentin and E-cadherin was performed as described in Material and Methods.
(B) LoVo colon cancer cell line was transfected with *HER3* or *GL-2* siRNAs. After 48 hours, cells were lysed and subjected to Western blot analysis. Immunoblots for Vimentin and Tubulin are shown.

The shift in the cell morphology induced subsequent to HER3 depletion prompted us to check HER3 impact on cell migration and invasion.

1.8 *HER3* knockdown suppresses colon cancer cell migration and invasion

HER3 has been shown to be implicated in the regulation of several oncogenic processes, particularly migration and invasion in different cancer types including those of the breast, lung and skin (Van der Horst et al., 2005; Sithnandam et al 2005; Reschke et al., 2008). To address the question, whether HER3 is required for colon cancer motility and invasion, we performed migration and invasion assays with DLD-1 and LoVo cell lines in the presence or absence of small interfering RNA (siRNA) targeting *HER3*. To exclude the possibility of measuring inhibition of proliferation, we used serum-starved conditions and we monitored migration and invasion after 20 and 24 h, respectively. As shown in Figure. 23A, *HER3* siRNA effectively abrogated migration of both cell lines, under proliferation conditions. Furthermore, the invasion amplitude of both DLD-1 and LoVo was also markedly inhibited upon *HER3* suppression (Figure. 23B). Taken together, these results suggested a prominent role of HER3 in regulating migration as well as invasion of colon cancer cell lines.

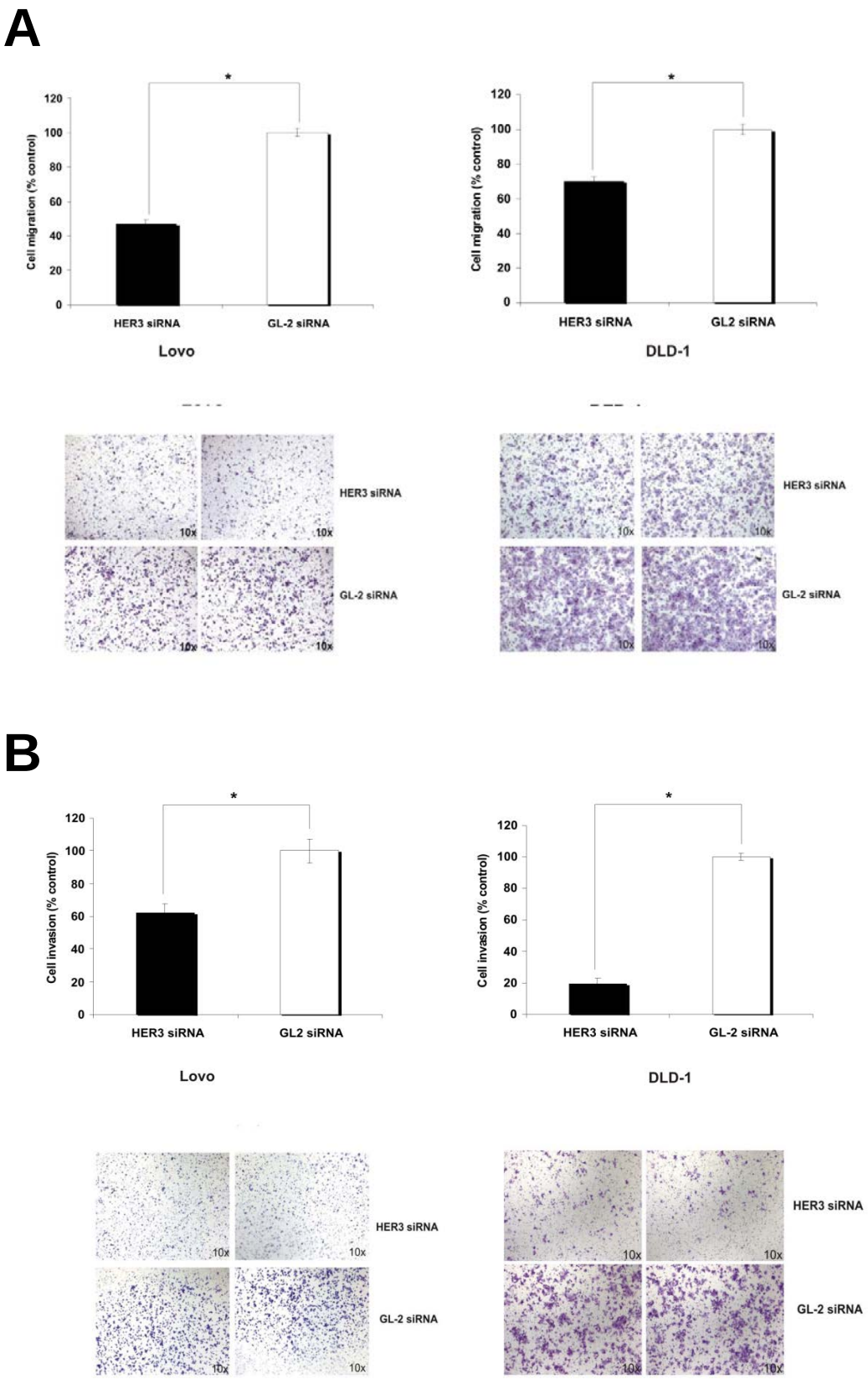


Figure 23. HER3 knockdown inhibits colon cancer cell line migration and invasion

(A) *HER3* suppression by siRNA induces an inhibition of DLD-1 and LoVo colon cancer cell lines migration. The cells were transfected with *HER3* or *GL-2* siRNAs. A culture of 100.000 cells was starved for 24h in medium containing 0, 1 % FCS medium, seeded on to a membrane with 8 µM pores of a modified boyden chamber containing 500 µl serum-free medium. The lower chamber was filled with 700 µl 10 % FCS medium serving as a chemoattractant. Cells were allowed to migrate for 24 hours and were then fixed and stained using a cristal-violet buffer. Stained cells were washed in PBS and pictures were taken on a Zeiss Axiovert 300 microscope. For quantification at least 10 randomly selected microscopic fields were counted. The values for control cells were set to 100% and results are shown relative to controls. Mean ± SD. P-values were calculated using the paired Students T-test and values below 0,05 were considered statistically significant.
(B) *HER3* suppression by siRNA blocks invasion of DLD-1 and LoVo colon cancer cell lines. The cells were transfected with *HER3* or *GL-2* siRNAs.100.000 cells were starved for 24h in medium containing 0, 1 % FCS medium, seeded on to a membrane coated with growth factor-reduced matrigel of a modified boyden chamber containing 500 µl. serum-free medium. The lower chamber was filled with 700 µl 10 % FCS medium serving as a chemoattractant. Cells were allowed to invade for 24 hours and were then fixed and stained using a cristal-violet buffer. Stained cells were washed in PBS and pictures were taken on a Zeiss Axiovert 300 microscope. For quantification at least 10 random microscopic fields were counted. The values for control cells were set to 100% and results are shown relative to controls. Mean ± SD. P-values were calculated using the paired Students T-test and values below 0,05 were considered statistically significant.

1.9 HER3 depletion induces apoptosis in colon cancer cell lines

To further characterize the role of HER3 *in vitro*, we asked whether *HER3* down-regulation by small interference RNA induces cell death in colon cancer cell lines. Interestingly, in line with previously published data in lung cancer cell lines, which undergo apoptosis in the absence of *HER3*, suppression of *HER3* lead to a moderate induction of apoptosis which varies from one cell line to the other in our model system (Figure. 24A). We next checked whether 5-fluorouracil, a common chemotherapeutic used for the treatment of colon cancer patients, could increase the already obtained apoptotic effect in cells with *HER3* knockdown. Importantly, *HER3* inhibition by knockdown synergizes with chemotherapy in apoptosis induction. Treatment of *HER3* knockdown cells with 5-fluorouracil but not of control cells gives rise to a significant increase in the apoptosis rate of the treated colon cancer cell lines, which reaches ~45% and ~32% for DLD-1 and LoVo cell lines respectively (Figure. 24B). The same effect was also observed in both other colon cancer cell lines CaCO2 (~45%) and T-84 (~30%) (Figure. 24B). These results suggest that a

combinatorial therapy with anti HER3 directed and 5-fluorouracil-like drugs might be useful for the treatment of malignant colon cancer.

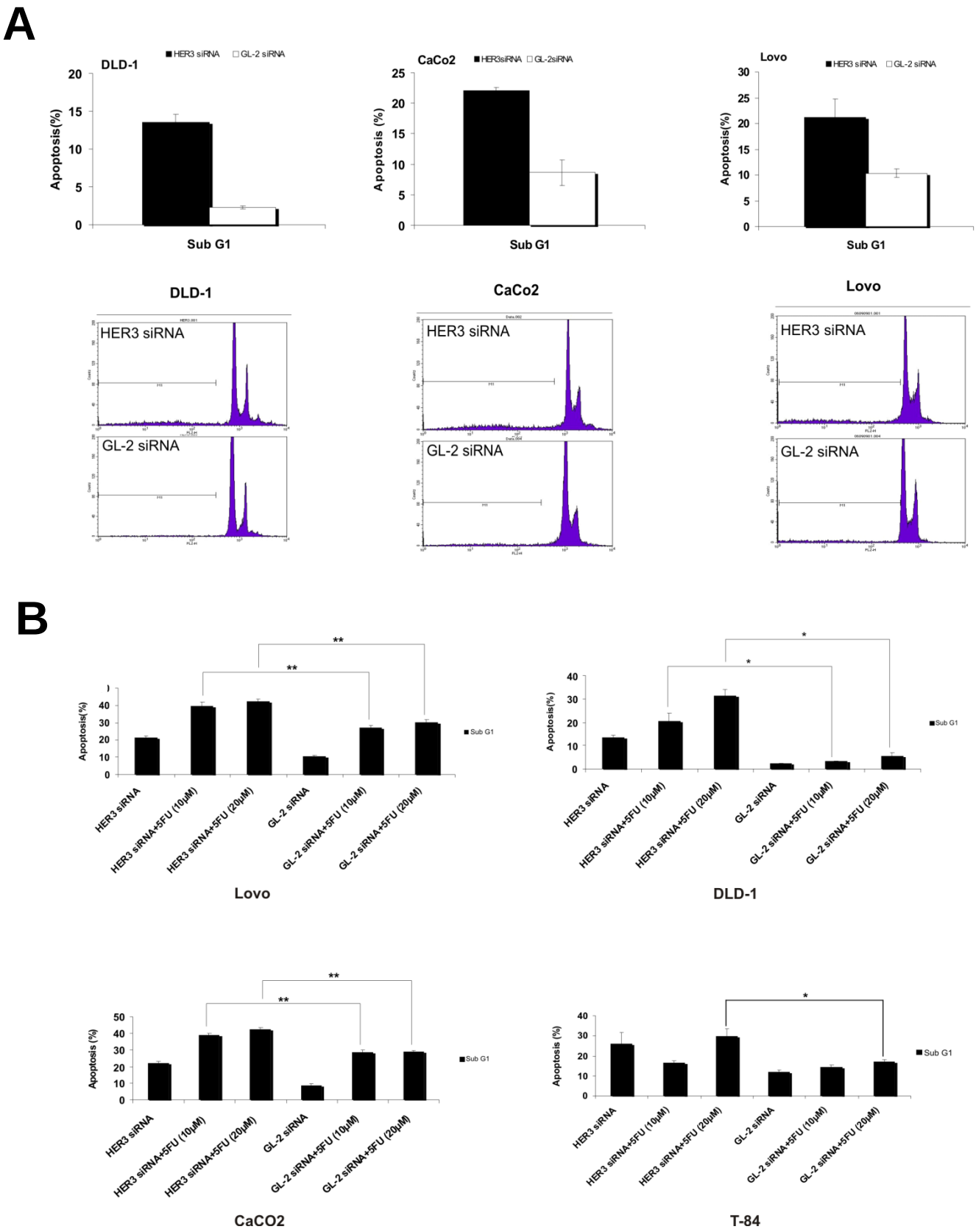


Figure 24. HER3 knockdown induces apoptosis in colon cancer cell lines

(A) DLD-1, LoVo, and CaCO2 colon cancer cell lines were transfected with *HER3* or *GL-2* siRNAs and grown for 72 hours. Apoptosis was measured by propidium iodide staining on a flow cytometer and quantified using the Cell Quest Pro software (Beckton Dickinson).
(B) Treatment of HER3 Knockdown DLD-1, LoVo, CaCO2, and T-84 colon cancer cell line with increased 5-fluorouracil concentration (10 and 20 µmol/L) increases the apoptosis rate. Mean ± SD.

1.10 Anti-HER3 monoclonal antibody blocks heregulin-ß1-induced HER3 activation, inhibits migration and invasion, and induces apoptosis in colon cancer cell lines

To test the hypothesis that HER3 is a potential target for colon carcinoma therapy, we analysed the effects of a HER3 monoclonal blocking antibody (clone. 105.5), which specifically antagonizes the heregulin-ß1 binding to HER3 in breast and skin cancer cell lines (Van der Horst et al., 2005; Reschke et al., 2008). We, therefore immunoprecipitated HER3 in cells treated with either HER3 mAB 105.5 or a control antibody and stimulated with heregulin-ß1. We then assessed the phosphorylation of HER3 and the expression levels of the regulatory subunit of the PI3K, the p85 protein. Remarkably, anti-HER3 monoclonal antibody interferes with the activation of heregulin-ß1-induced activation of the HER3 receptor and its association with the PI3K subunit, p85 in DLD-1 and LoVo cells. However, inhibition was incomplete in the CaCO2 cell line (Figure. 25A). Similar to previous reports (Van der Horst et al., 2005; Reschke et al., 2008), HER3 seems to be degraded or internalized through the anti–HER3 monoclonal antibody in DLD-1 and LoVo colon cancer cell lines (Figure. 25A). Interestingly, the HER3-blocking antibody was able to block migration and invasion and to induce apoptosis in the DLD-1 and LoVo colon cancer cell lines (Fig. 25B–D), indicating that such antibodies may be effective for the treatment of patients with colon cancer. Taken together, these results have potential therapeutic signifi-cance of HER3 as new promising target for colon cancer treatment.

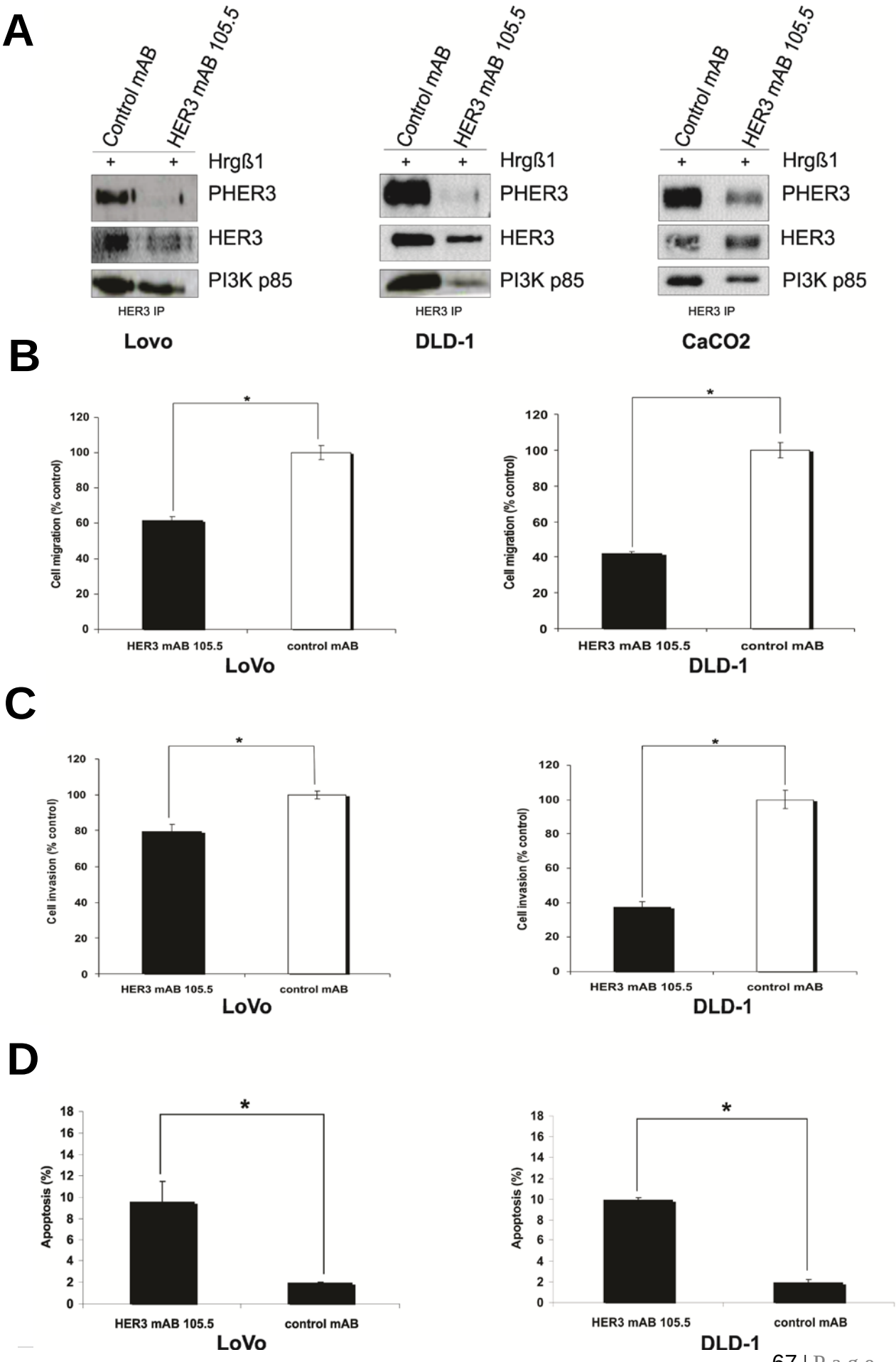

A
Control mAB
HER3 mAB 105.5
Hrgß1
PHER3
HER3
PI3K p85
HER3 IP
Lovo
DLD-1
CaCO2
B
Cell migration (% control)
HER3 mAB 105.5
control mAB
LoVo
DLD-1
C
Cell invasion (% control)
D
Apoptosis (%)

Figure 25. An anti-HER3 monoclonal antibody (clone 105.5) inhibits HER3-mediate signalling and blocks colon cancer cell migration and invasion
(A) Anti-HER3 antibody treatment blocks heregulin-ß1-induced HER3 activation and its association with p85 and leads to receptor internalization or degradation.
Serum-starved DLD-1, LoVo, and CaCO2 colon cancer cell lines were pre-treated with 10µg/ml of either an isotyp control antibody or HER3 blocking antibody (clone 105.5) for 60 minutes. Cell stimulation with heregulinß-1 was carried out for 120 minutes. Cell lysates were subjected to immunoprecipitation using a specific HER3 antibody. Western blots of phospho-HER3 (Tyr 1289), HER3, and p85 are shown.
(B) An anti-HER3 antibody (clone 105.5) inhibits colon cancer cell migration. Serum-starved DLD-1 and LoVo colon cancer cell lines were detached using 10 mM EDTA, incubated with 10µg/ml of either an isotyp control antibody or HER3 blocking antibody (clone 105.5) for 60 minutes, and transferred to the upper well of a Boyden chamber. Conditioned NIH3T3 medium containing 100 ng/ml heregulinß-1, and 0, 01% ascorbic acid was used as a chemoattractant. The quantification was done as described in Materials and Methods. Mean ± SD.
(C) An anti HER3 antibody (clone 105.5) blocks colon cancer cell invasion. The assay was done as in **B** using growth factor-reduced Matrigel in a modified Boyden chamber.
(D) An anti-HER3 antibody (clone 105.5) induces the apoptosis in DLD-1 and LoVo colon cancer cell lines. Apoptosis rates were determined by a propidium iodide staining as described in Materials and Methods.

2. Mig-6 negatively regulates the oncogenic EGF receptor mutant, EGFRvIII

2.1 Mig-6 interacts with members of the HER family as well as with EGFRvIII (EGFRΔ), a constitutively active mutant of the EGFR receptor in an overexpression system

We begann our study by investigating the interaction of mig-6 protein with members from the EGFR family as well as with the EGFRvIII receptor. Two different cell lines were selected to perform overexpression experiments with HEK293 (human embryonic kidney) or CHO (Chinese hamster ovary) cells. Indeed, these cell lines were selected mainly due to their high transfection-efficiency and handling facility.

2.1.1 Mig-6 interaction with members of the EGFR receptor tyrosine kinase family

Mig-6 also known as RALT was first described as an adaptor protein harbouring numerous protein-protein interaction domains. As this protein was described in 2000 by Hackel and co-workers as a negative regulator of EGFR (Hackel et al., 2000), the first member of the HER receptor family, the interest to investigate the interaction of mig-6 with further members of the same family was increased. We therefore transfected the Human Embryonic Kidney HEK293 cell line with plasmid-constructs coding for HER2, HER3, as well as for mig-6-VSV and checked possible interaction partners by using co-immunoprecipitation. While HEK293 cell line transfected with an EGFR-construct was used as a positive control, cells transfected with a GFP construct were taken as a negative control as well as a control for the transfection efficiency. By using a simple western-blotting analysis, we first checked the expression of the transfected plasmid-constructs. As shown in Figure 26B, 72h after transfection, we were able to detect the protein expression of all four transfected constructs.

On the other hand, by applying antibodies directed against EGFR, HER2, and HER3 receptors we were able to co-immunoprecipitate mig-6 (Figure. 26A). In order to further validate these interactions between mig-6 and members of the EGFR family, we asked whether antibodies directed against mig-6 could also co-immunoprecipitate the EGFR, HER2, and HER3 receptors. In contrast to the above observed results, the mig-6 antibody was able to pull-down only HER2 (Figure. 26A). By using a

second mig-6 antibody, a clear interaction between mig-6 and EGFR as well as HER3 was detected (Figure. 26C).

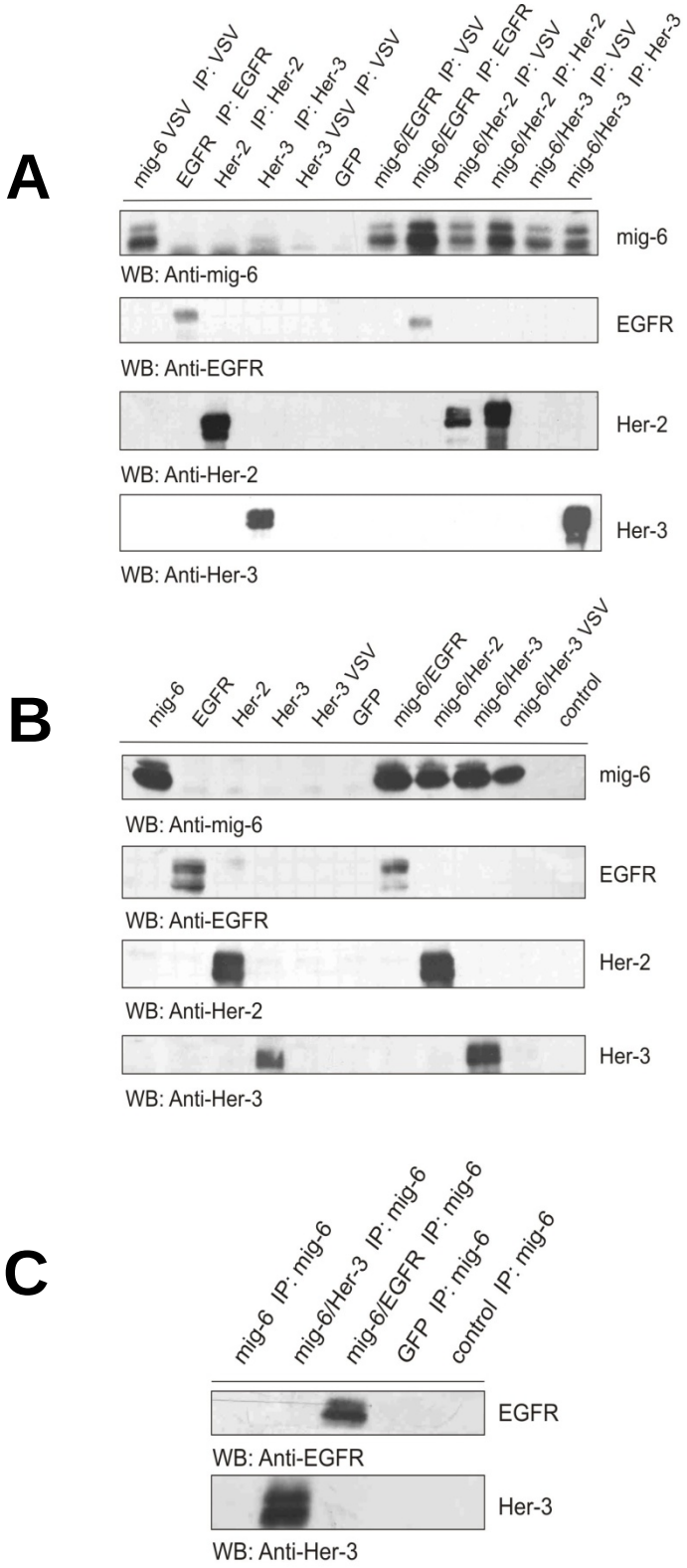
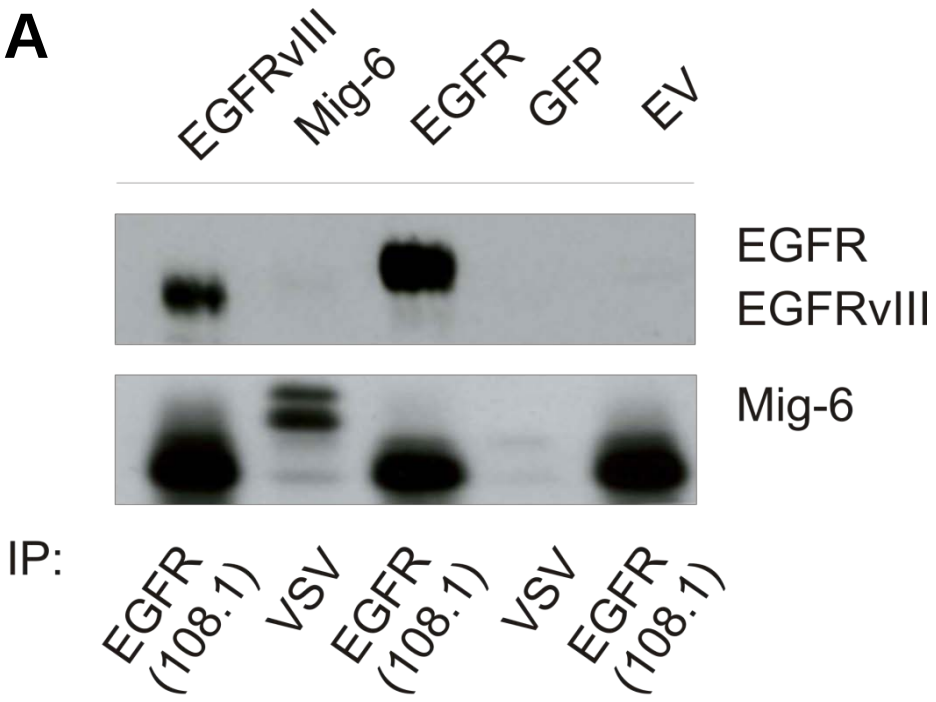


Figure 26. Mig-6 interacts with the EGFR, HER2, and HER3 members of the HER family in an overexpresson model system
HEK293 cells (300.000) were transfected and/or co-transfected with plasmid-constructs coding for EGFR, HER2, HER3, and mig-6VSV. Cells transfected by the GFP construct were taken as a negative control and to test the trasfection-efficiency.
(A) Mig-6/HER2 interaction. Home-made anti-HER2 (clone. 13D1B1) and anti-VSVtag (clone. p5d4) were used.
(B) Total lysate.
(C) Interactions between mig-6/HER3 and mig6/EGFR were further confirmed using a second home-made anti-mig-6 antibody (clone. 1575) by the immunoprecipitation. In contrast to the first antibody, the second mig-6 antibody was able to pull-down EGFR and HER3 in the same model system.

2.1.2 Mig-6 adaptor protein interacts with EGFRvIII receptor in CHO cells

After establishing the interaction between mig-6 and three members of the HER receptor family, we asked whether this interaction persists with a constitutively active mutant form of the EGFR receptor, EGFRvIII. We, therefore, followed the same strategy as used before. Plasmid-constructs coding for the wild type EGFR receptor as well as for mig-6VSV and EGFRvIII were overexpressed at equal concentrations in CHO cell lines. Indeed, Cells were either transfected or co-transfected with 1µg plasmid-DNA from each construct. While cells transfected with empty-vector were used as a negative control, GFP construct-transfected cell lines were taken as a control for the transfection efficiency. In order to check the expression of the transfected constructs, we first performed a western-blotting analysis using antibodies towards EGFR as well as mig-6. As we can see in the figure below, clear protein bands at the molecular weight of ~145 Kda and of ~170 Kda corresponding to EGFRvIII and EGFR receptors respectively were observed. Moreover, a protein band which corresponds to the mig-6 protein was also observed with a molecular weight of ~54 Kda (Figure. 27A).



Further, we used the same model system to investigate the interaction between mig-6 and EGFRvIII. Indeed, CHO cells were co-transfected with either EGFR-mig6VSV (positive control) or EGFRvIII-mig6VSV constructs. By a co-immunoprecipitation assay using a VSV antibody, we were not able to detect any interaction even with the wild type receptor, EGFR (Figure. 27B). Only mig-6 was detectable. Nonetheless, the use of a homemade EGFR antibody (clone 108.1) looked more efficient, thereby pulling-down mig-6 in both cases described above (Figure. 27B). These findings suggest for the first time, that mig-6 could interact not only with the wild type EGFR receptor but also with its constitutively phosphorylated mutant form, the EGFRvIII receptor.

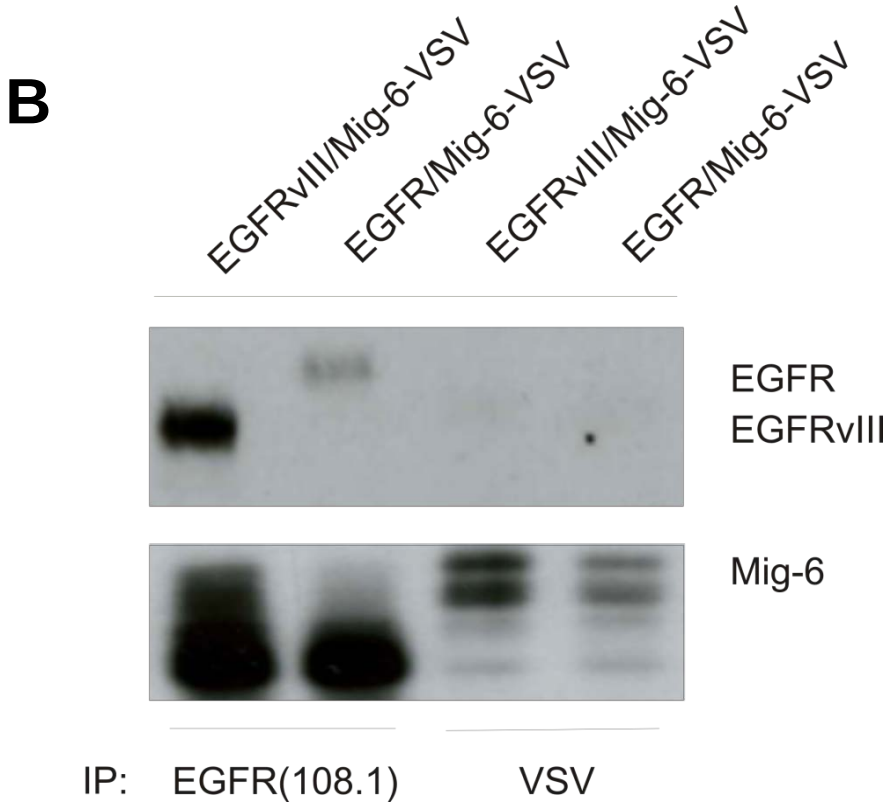


Figure 27. EGFRvIII/mig-6 interaction in an overexpression model system
(A) CHO (300.000) cells were transfected with constructs expressing EGFR (positive control), EGFRvIII, and mig-6-VSV. While cells transfected with GFP were used to test the transfection-efficiency, cells transfected with the empty vector (EV) were taken as a negative control. After lysis, equal amounts of protein were subjected to immunoprecipitations using a home-made EGFR antibody (clone. 108.1) as well as an anti-VSVtag antibody. Western blots for EGFR, EGFRvIII and mig-6 are shown.
(B) EGFRvIII interacts with mig-6 protein. CHO cells were transfected With EGFR/mig-6 (positive control) and EGFRvIII/mig-6 plasmid-constructs and lysed after 72 hours. After lysis, equal amounts of protein were subjected to immunoprecipitations using a home-made EGFR antibody (clone. 108.1) as well as an anti-VSVtag antibody. Western blots for EGFR, EGFRvIII and mig-6 are shown.

2.1.3 Mig-6 is a negative regulator of EGFRvIII in an overexpression model system (CHO cells)

As mig-6 adaptor protein was already described as a negative regulator of the wild type EGF-receptor, and after establishing for the first time the interaction between mig-6 and EGFRvIII, we asked whether this mutant EGFR form could also be negatively regulated by the mig-6 protein. In order to test this hypothesis, we either co-transfected CHO cells with EGFR-mig6VSV, and EGFRvIII-mig6VSV constructs or transfected them with EGFR, and EGFRvIII constructs alone. After performing a

co-immunoprecipitation assay using the EGFR antibody (clone 108.1), we checked the phosphorylation levels of the EGFR and EGFRvIII receptors by using a phosphotyrosine antibody (P-Y: 4G10). As expected, cells co-transfected with EGFR and mig-6 plasmid-DNA (positive control) displayed low phosphorylation levels of the EGFR receptor when compared with cells transfectd with the EGFR construct alone (Figure. 28). Interestingly, this was also the case for cells transfected with the other two constructs. As shown in the figure below, phosphorylation levels of the EGFRvIII receptor were decreased when cells are co-transfected with mig-6 and EGFRvIII plasmid-DNAs compared to cells transfected only with EGFRvIII (Figure. 28). Indeed, these findings permit us to confirm for the first time that mig-6 is a negative regulator of the wild type EGFR as well as of its mutant form EGFRvIII. Notably, phosphorylation levels of the mutant EGFRvIII receptor was higher when compared to that of the EGFR due to its constitutively active property.

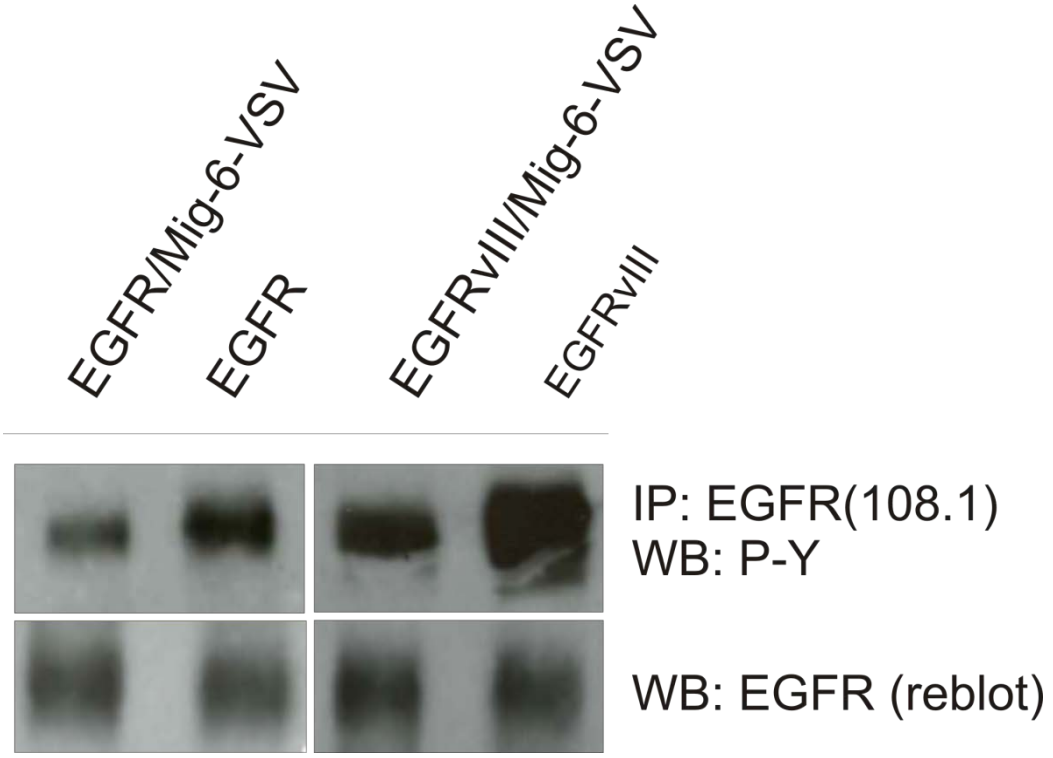


Figure 28. Mig-6 is a negative regulator of EGFRvIII in an overexpression model system
CHO cells were co-transfected with EGFR/mig-6VSV (positive control) and EGFRvIII/mig-6VSV constructs. After 72 hours, cells were lysed and equal amounts of protein were subjected to immunoprecipitations using the anti-EGFR antibody (clone. 108.1). Western blots for p-Y (p-tyrosine), and EGFR are shown.

2.2 Mig-6 and EGFRvIII in glioblastoma as an endogenous model system

After exploiting the link between mig-6 adaptor protein and the mutant EGFRvIII receptor in the CHO cell line as an over-expression model system, we asked whether these findings could be reproduced in one more real system (endogenous system). As many studies report the over-expression of EGFRvIII mutant receptor in glioblastoma tumors, we selected the U87-EGFRvIII cell line as a model for our investigation.

2.2.1 Mig-6 interacts with EGFRvIII in the U87-EGFRvIII glioblastoma cell line

Three more main reasons are behind the selection of the U87-EGFRvIII as model for the further extension of our study. On the one hand, maternal U87 glioblastoma cell lines (U87-MG) are characterized by a very low to no expression of the wild type EGF-receptor. On the other hand, the U87-EGFRvIII cell line displays a high expression level of the mutant EGFRvIII receptor. Besides EGFRvIII expression, the U87-EGFRvIII glioblastoma cell line also showed an endogenous basal expression level of the mig-6 protein, thereby providing us a favorable work-system.

To answer on our first question, whether mig-6 interacts with the EGFRvIII mutant receptor in glioblastoma, we performed a co-immunoprecipitation assay using the EGFR antibody (clone 108.1) used before in our previous experiments. Indeed, western blotting analysis revealed the ability of the EGFR antibody to bind EGFRvIII, which in turn couples to the mig-6 protein (Figure. 29). These results are in fact in line with our previous findings and further confirm the EGFRvIII-mig-6 interaction observed previously in an over-expression model system (CHO cell line).

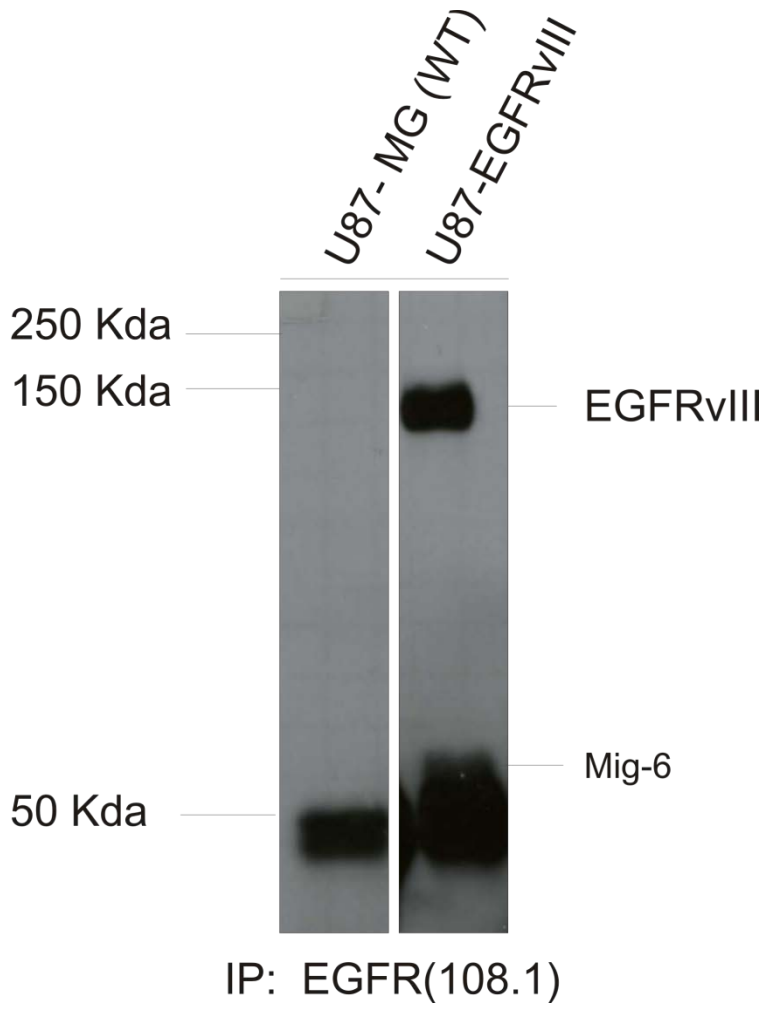


Figure 29. Mig-6 interacts with EGFRvIII mutant receptor in the U87-EGFRvIII glioblastoma cell line (endogenous model system)
Equal protein amounts of the U87-EGFRvIII cell line as well as the U87-WT (wild type: negative control) glioblastoma cell lines were subjected to immunoprecilpitations using the home-made anti-EGFR antibody (clone. 108.1). Western blots of EGFRvIII and mig-6 proteins are shown.

2.2.2 Mig-6 negatively regulates EGFRvIII receptor in glioblastoma

We further asked, whether the negative regulatory effect exerted through mig-6 on the EGFRvIII mutant receptor is also reproducible in glioblastoma. Two different strategies were followed to answer this question. Indeed, we first checked EGFRvIII as well as mig-6 expression levels in primary tumors from glioblastoma patients by RT-PCR. The effect of mig-6 knockdown on the phosphorylation levels of EGFRvIII and on related signaling pathways in the U87-EGFRvIII glioblastoma cell line was performed in a further step as a second strategy.

2.2.2.1 Mig-6/EGFRvIII expression levels in primary tumors from glioblastoma patients

By using RT-PCR as an investigation method, EGFR/EGFRvIII as well as mig-6 gene expression levels was measured in a panel of 48 different primary human tumors (Figure. 30). More than 60% of the studied cases (33 samples) illustrate a dominant expression level of the wild type EGFR receptor. Only in some few samples (~17%) a co-expression of the wild type EGFR receptor and his mutant form, EGFRvIII, was observed. Considering all tumor samples, an almost totally absence of any mig-6 gene expression was shown, confirming first the already known negative regulation of the wild type EGFR receptor through mig-6 protein and secondly suggesting that mig-6 could be also a negative regulator of the EGFRvIII mutant receptor.

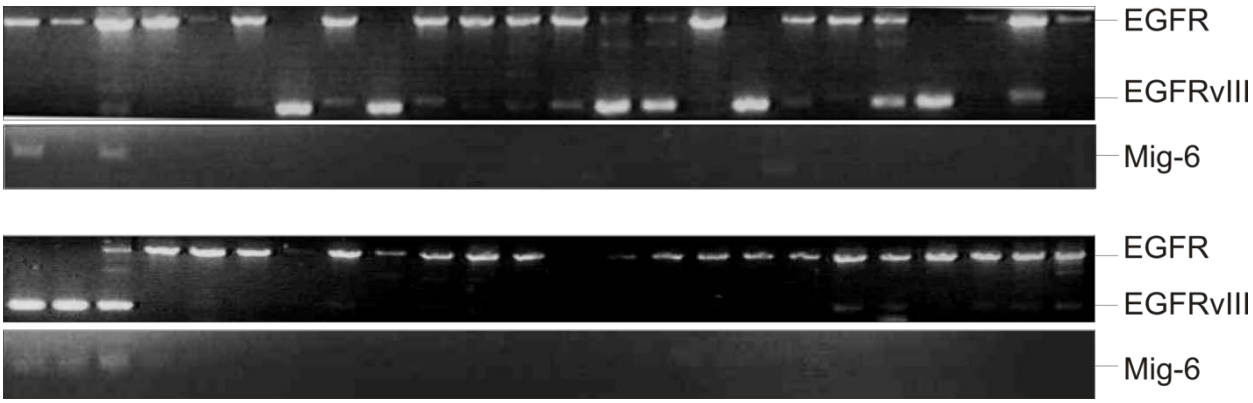


Figure 30. Mig-6 is a negative regulator of EGFRvIII in primary tumors
Reverse transcription polymerase chain reactions for EGFR (positive control), EGFRvIII and mig-6 are shown.

2.2.2.2 Mig-6/EGFRvIII negative regulation in U87-EGFRvIII glioblastoma cell line

In order to confirm our previous suggestions concerning the negative regulation of EGFRvIII through mig-6, the impact of mig-6 knockdown on EGFRvIII phosphorylation was investigated. Indeed, knockdown experiments were performed in the U87-EGFRvIII cell line. The same experiment was performed in the wild type U87-MG glioblastoma cell line as a negative control. Up to 48h after siRNA transfection a clear downregulation of the mig-6 expression could be observed. *Mig-6* ablation by siRNA was persisting even 72h after knockdown (Figure. 31). After establishing the knockdown conditions, phosphorylation levels of the EGFRvIII

mutant receptor was analyzed. Western blotting analysis revealed an easily detectable increase in the P-EGFRVIII expression levels after 48h as well as 72h of mig-6 knockdown (Figure. 31). Indeed, these findings are in accordance with previous results, thereby permitting us to confirm the role of mig-6 adaptor protein as a negative regulator of the mutant EGFRvIII receptor.

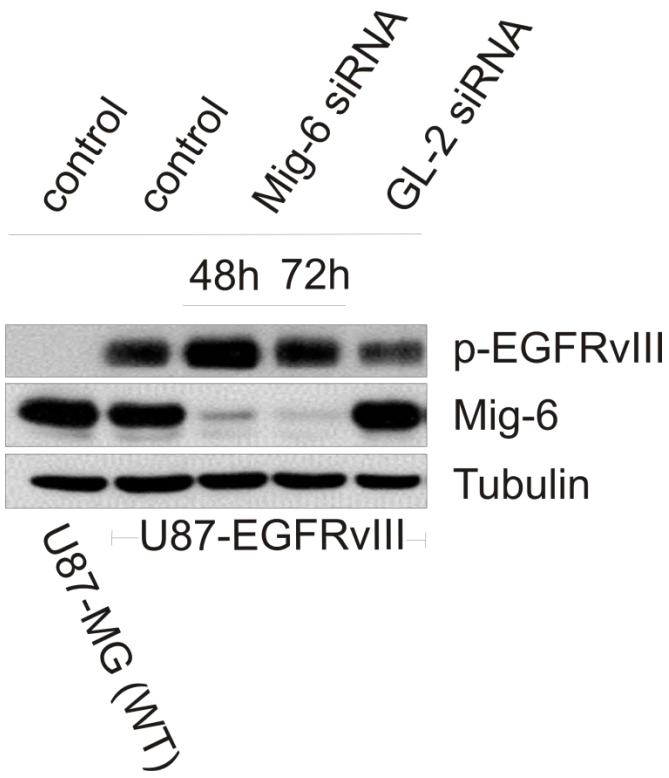


Figure 31. Mig-6 is a negative regulator of EGFRvIII in glioblastoma cells
U87-EGFRvII glioblastoma cell line was transfected with *mig-6* or *GL-2* siRNAs as described in Materials and Methods. Western blot analysis for p-EGFR (Y-1173) and mig-6 are shown. Tubulin served as a loading control. Two different controls were taken including the wild type U87-MG (WT) cell line as well as the non-transfected U87-EGFRvIII glioblastoma cell line.

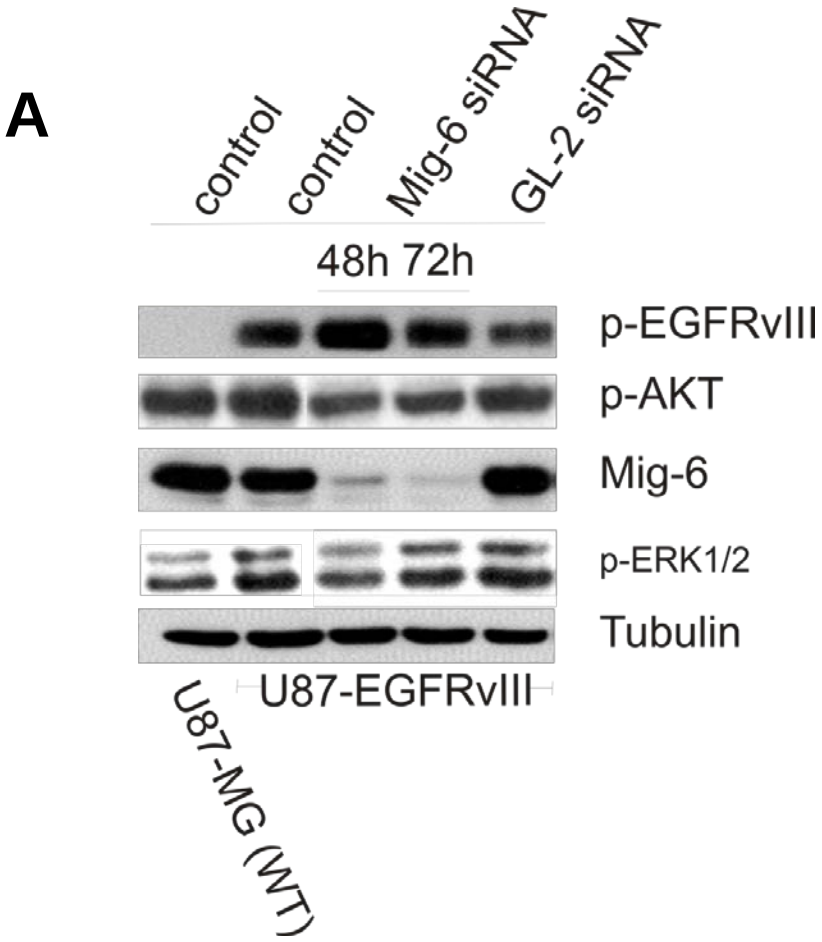
2.2.3 Role of EGFRvIII/mig-6 complex in glioma-genesis regulation

As the EGFRvIII expression was shown to be very abundant in glioma, an indispensable role of this receptor is thought to be present during the regulation of glioma-genesis. Indeed, several previous clinical studies indicate the intervention of this receptor during the initiation as well as the progression of brain tumors. Nevertheless, the role of mig-6 protein as a crucial part of the EGFRvIII-mig-6 loop in

regulating glioma-genesis events is until nowadays not exploited. It's therefore an urgent need to more investigate this field.

2.2.3.1 Mig-6 is an endogenous inhibitor of the EGFRvIII signaling pathway in the U87-EGFRvIII cell line glioblastoma cell line

After establishing mig-6 adaptor protein as a negative regulator of EGFRvIII in the U87-EGFRvIII glioblastoma cell line, many open questions related to the role of this protein in regulating EGFRvIII-initialized signaling pathway have to be answered. We therefore checked the activation of three main signaling pathways thought to be indispensable during tumor development and progression. Indeed, phosphorylation levels of ERK, AKT, and STAT3 proteins were examined before and after *mig-6* knockdown in the U87-EGFRvIII cell line. The wild type U87-MG cell line was used as a negative control. As we can see in the figure below (Figure. 32A), mig-6 knockdown clearly induces an increase in the phosphorylation of EGFRvIII, but doesn't have any obvious effect on the activation of ERK as well as of AKT (Figure. 32A).



However, further independent analysis of the effect of the knockdown indicates a clear increase in the phosphorylation level of STAT3 signaling protein (Figure. 32B). These findings indicate that mig-6 is crucial by the modulation of the EGFRvIII-STAT3 signaling pathway, thereby regulating related biological outcomes.

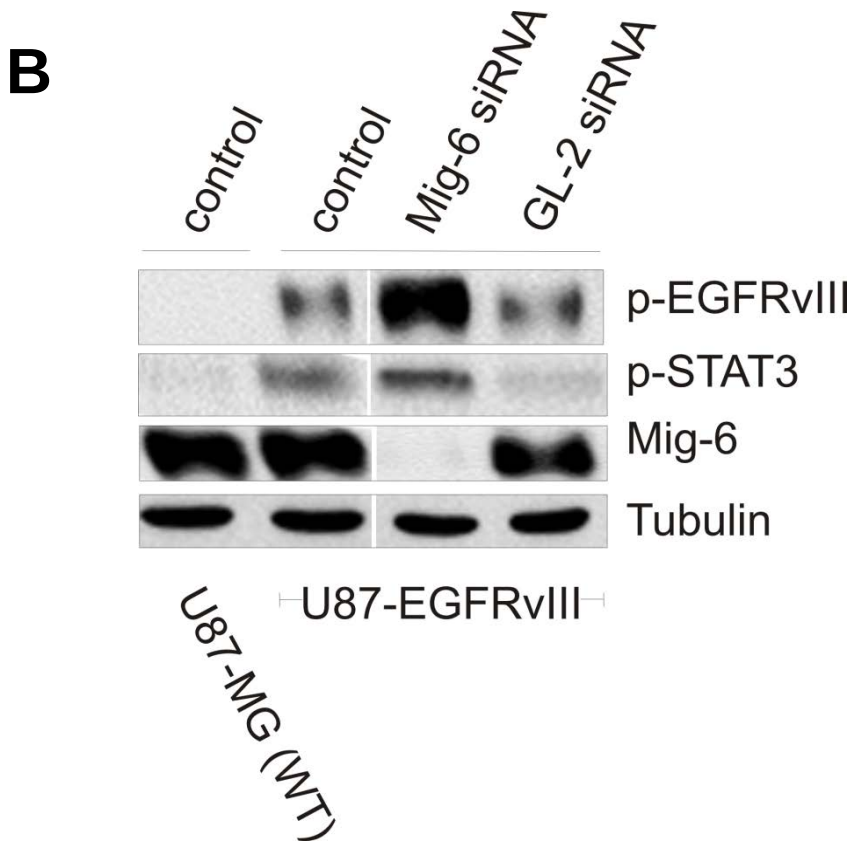
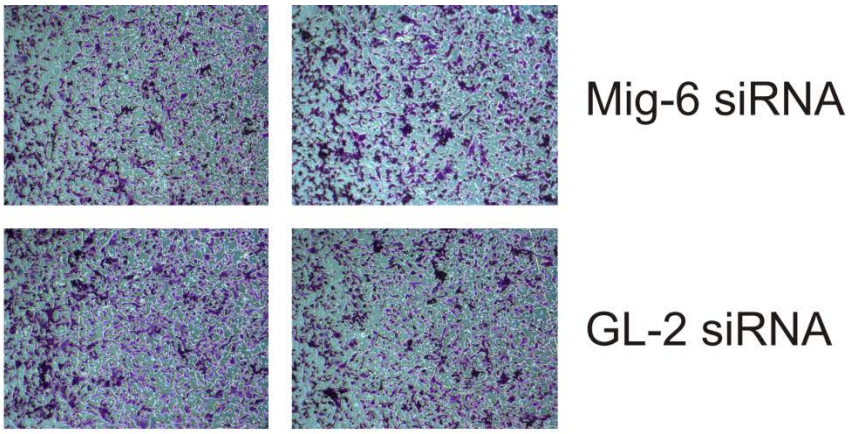


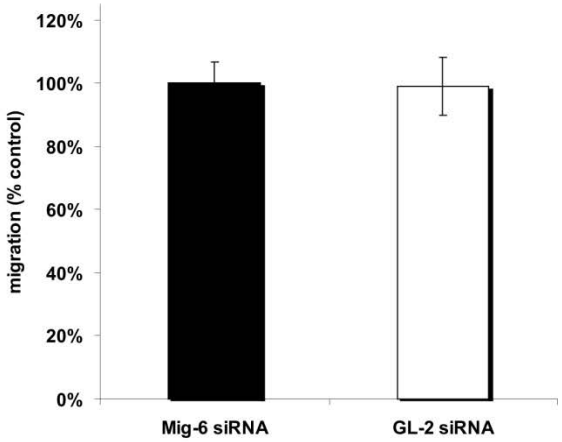
Figure 32. Mig-6 is a negative regulator of the EGFRvIII-STAT3 signalling pathway in a glioblastoma cell line
(A) U87-EGFRvIII glioblastoma cell line was transfected with *mig-6* or *GL-2* siRNAs as described in Materials and Methods. Western blot analysis for p-EGFR (Y-1173), p-AKT (R 1473), p-ERK1/2, and mig-6 are shown. Tubulin served as a loading control. Two different controls were taken including the wild type U87-MG (WT) cell line as well as the non-transfected U87-EGFRvIII glioblastoma cell line.
(B) U87-EGFRvIII glioblastoma cell line was transfected with *mig-6* or *GL-2* siRNAs as described in Materials and Methods. Western blot analysis for p-EGFR (Y-1173), p-STAT3 and mig-6 are shown. Tubulin served as a loading control. Two controls were taken as described in **(A)**.

2.2.3.2 Suppression of mig-6 leads to a decrease in the doxorubicin induced apoptosis in the U87-EGFRvIII cell line

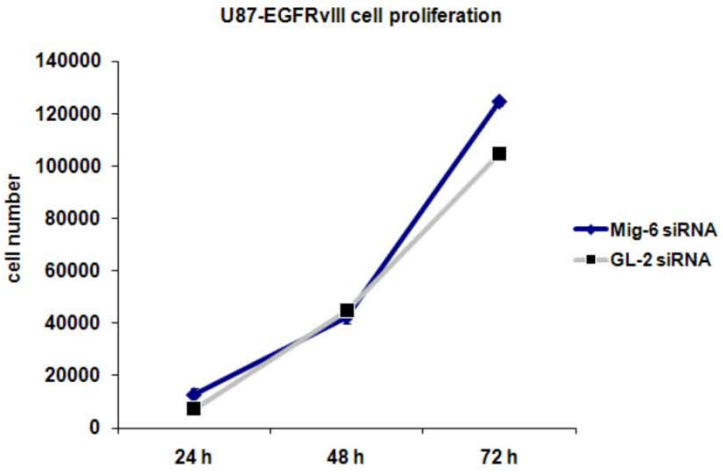
In order to explore the functional consequence of the increased EGFRvIII/STAT3 signaling pathway in the mig-6 knockdown U87-EGFRvIII glioblastoma cell line, we first checked its impact on proliferation and migration. However, we didn´t observed any difference in proliferation and migration between *mig-6* knockdown and control cells (figure. 33A,B). Since the EGFR-STAT signaling pathway was demonstrated in previous reports to be responsible for the modulation of apoptotic events within the cell, we tested the impact of *mig-6* knockdown on this cellular output. In order to do this, U87-EGFRvIII cells were treated with 1 µM doxorubicin subsequent to *mig-6* Knockdown. Apoptosis rates were then measured after 48 hours by propidium iodide staining on a flow cytometer and quantified using the Cell Quest Pro software (Beckton Dickinson). As expected, *mig-6* knockdown cells display resistance towards doxorubicin treatment in comparison to control cells (figure. 33C). In fact, down-regulation of *mig-6* results in a reduction of the induced apoptosis by almost
20 %, suggesting that the EGFRvIII-mig-6 loop is involved mainly by the regulation of apoptosis but not of proliferation or migration in the U87-EGFRvIIII glioblastoma cell line at least (Figure. 33C).

A


Mig-6 siRNA
GL-2 siRNA


120%
100%
80%
60%
40%
20%
0%
migration (% control)
Mig-6 siRNA
GL-2 siRNA

B


U87-EGFRvIII cell proliferation
140000
120000
100000
80000
60000
40000
20000
0
cell number
24 h
48 h
72 h
Mig-6 siRNA
GL-2 siRNA

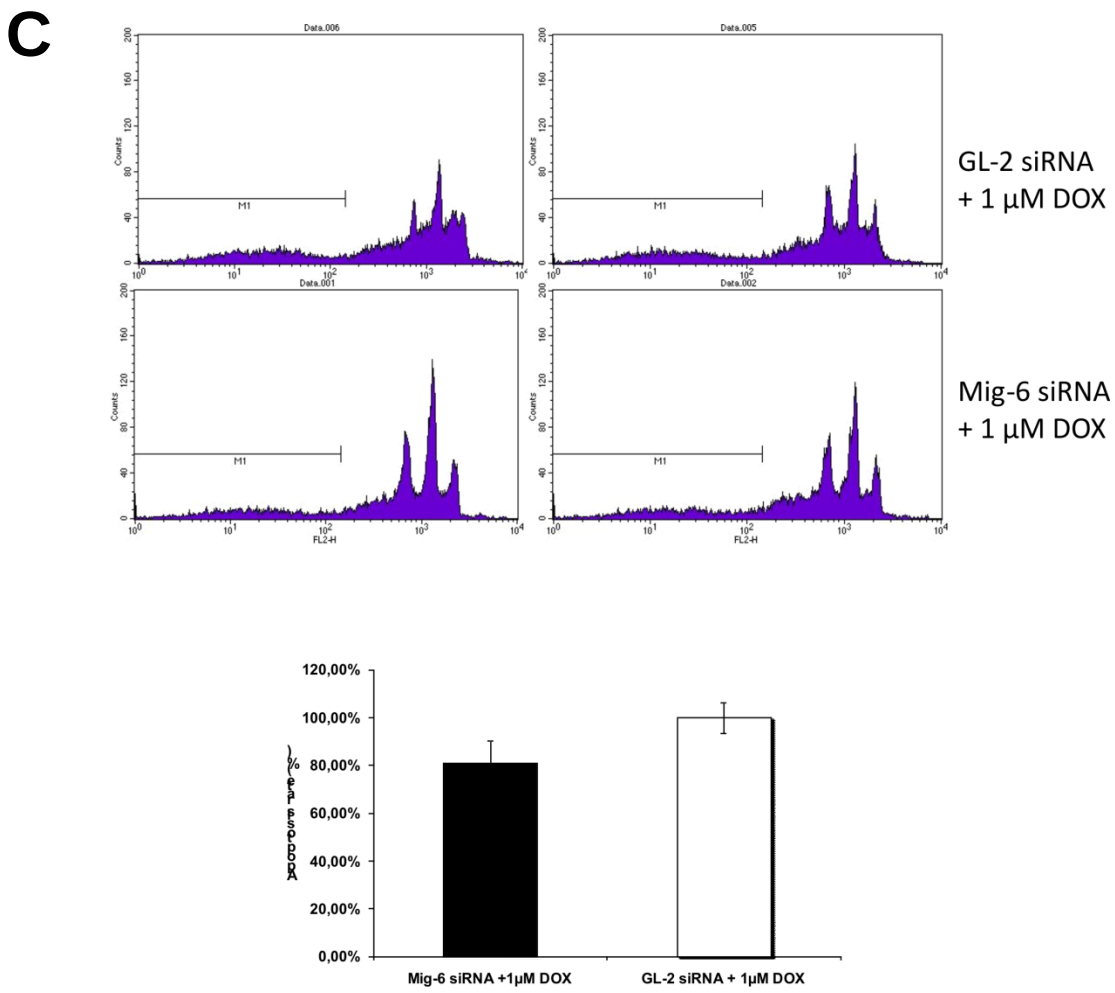


Figure 33. Mig-6 depletion by siRNA induces resistance towards doxorubicin treatment in a glioblastoma cell line.
(A) Mig-6 knockdown in the U87-EGFRvIII glioblastoma cell lines doesn't affect the migratory potential of cells. U87-EGFRvIII glioblastoma cell line was transfected with *mig-6* or *GL-2* siRNAs as described in Materials and Methods. A cell number of 20.000 were starved for 24h in medium containing 0, 1 % FCS medium, seeded on to a membrane with 8 µM pores of a modified boyden chamber containing 500 µl serum-free medium. The lawer chamber was filled with 700 µl 10 % FCS medium serving as a chemoattractant. Cells were allowed to migrate for 24 hours and were then fixed and stained using a cristal-violet buffer. Stained cells were washed in PBS and pictures were taken on a Zeiss Axiovert 300 microscope. For quantification at least 10 random microscopic fields were counted. The values for control cells were set to 100% and results are shown relative to controls. Mean ± SD.
(B) *Mig-6* knockdown in the U87-EGFRvIII glioblastoma cell lines doesn't affect the mproliferative potential of cells. U87-EGFRvIII glioblastoma cell line was transfected with *mig-6* or *GL-2* siRNAs as described in Materials and Methods and counted at the indicated time points using a coulter counter (Beckton Dickinson). Data are shown as ± SDM.
(C) *Mig-6* suppression by siRNA induces a resistance towards doxorubicin treatment (1µM) in the U87-EGFRvIII cell line. Apoptosis was measured by propidium iodide staining on a flow cytometer and quantified using the Cell Quest Pro software (Beckton Dickinson).

V Discussion

1. The Role of HER3 and mig-6 in colon cancer

1.1 HER3 as a target for the treatment of colon cancer patients

Pseudo-kinases such as HER3 are emerging as crucial regulators of diverse cellular functions, despite lacking the ability to directly phosphorylate substrates (Boudeau et al., 2006). Recent studies highlighted HER3 as a fundamental signalling receptor involving by the modulation of different biological processes in a wide range of cancer types including those of pancreatic, -breast, -prostate, -gastric, ovarian and skin cancer (J.Spencer et al., 2010; Wallasch et al., 1995; Koumakpayi et al., 2007; Zhang et al., 2009; Tanner et al., 2006; Reschke et al., 2008). In some of these tumors, HER3 expression was even shown to be correlated with poor prognosis (Tanner et al., 2006; Witton et al., 2003; Yi et al., 1997; Reschke et al., 2008). Additionally, recent works demonstrated that tumors escape from EGFR and HER2 targeted therapy by a rapid compensatory increase in HER3 expression, and in a PI3K-Akt signaling pathway (Sergina et al., 2007; Engelmann et al., 2007), suggesting that agents targeting HER3 could provide a novel and promising approach towards the treatment of such cancers.

HER3 represents a trans-membrane receptor that is activated by ligand binding. Therefore, one might assume that the predominant localization of HER3 should be the cell membrane. Nevertheless, several publications using different anti-HER3 antibodies for staining of tumor samples demonstrated a cytoplasmic rather than a membrane staining pattern for this receptor (Tanner et al., 2006; Grivas et al., 2007). In our study we detected a clear membrane localization of HER3 in all 110 analysed paraffin embedded specimens of primary colorectal tumors as well as a strong correlation of HER3 expression with overall survival of patients, establishing HER3 as a novel independent prognostic marker in colon cancer. This is of great importance as several therapeutic antibodies aimed at targeting the membrane localized HER3 receptor are currently being tested in clinical trials and will hopefully be available as treatment modality soon. Indeed, our results are in accordance with previous studies, where HER3 expression is tightly correlated with poor survival in colorectal cancer patients (Kapitanovic et al., 2000; Baiocchi et al., 2009). Nevertheless, further

investigations concerning the HER3 status versus the genetic classification of the CRC tumors, namely, LOH, microsatellite instability, hereditary nonpolyposis colorectal cancer would be of interest to get further insights into the relevance of HER3 expression in different colon cancer subtypes.

To specifically address the function of HER3 in colon cancer development and progression, human colon cancer cell lines were analyzed by siRNA interference with HER3 expression. As described above, down-regulation of *HER3* induced a reduction in proliferation as well as a G2–M cell cycle arrest in all 4 tested colon cancer cell lines. On the molecular signaling level, *HER3* knockdown resulted in increased p27 expression levels. In addition, phosphorylation of Rb and cyclin B1 expression were reduced upon *HER3* knockdown in comparison with the control cells. Indeed, these molecules are well established and known as essential cell-cycle regulators. Their modulation seemed to be the direct cause of the observed growth inhibition and the cell-cycle arrest in colon cancer cells.

HER3 down-regulation in both heregulin-ß1 stimulated and non stimulated cells results in a reduction of the Akt phosphorylation (Fig. 19E). In fact, the observed up-regulation of p27 protein expression may be a direct consequence of the detected impairment of the Akt activity since Akt is known to trigger p27 degradation and cell cycle progression (Viglietto et al., 2002; Shin et al., 2002; Liang et al., 2002; Liang et al., 2003). Furthermore, it is for great importance to note that several already published reports have demonstrated the well-established potential of HER3 to activate downstream PI3K-Akt pathway signalling by virtue of six consensus phosphotyrosine sites, which are absent in EGFR or HER2 (Soltoff et al., 1994; Prigent et al., 1994; Fedi et al., 1994; van der Horst et al., 2005; Reschke et al., 2008). Interestingly, the impaired Akt activity shown in cells upon *HER3* knockdown is accompanied by a reduction in the phosphorylation of mTOR at Ser^{2448}, which is known to be mediated by the PI3K-AKT signalling pathway (Huang et al., 2009). However, *HER3* ablation did not affect the extracellular signal-regulated kinase 1/2, suggesting that inhibition of other downstream pathways seems to be sufficient to block colon cancer cell proliferation. Taken together, our results suggest that HER3 expression and Phosphorylation control colon cancer cell proliferation majorly through the PI3K-AKT signalling pathway.

We subsequently checked the role of HER3 as a regulator of cell survival in colon cancer. Interestingly, *HER3* depletion in all four tested colon cancer cell lines resulted

in an induction of apoptosis, which varies from one cell line to another. Our observations are in fact in accordance with previous studies demonstrating the involvement of HER3 receptor in regulating cell survival. Indeed, Lee and co-workers showed that deletion of HER3 induces a tumor-specific apoptosis in mouse intestinal epithelium (Lee et al., 2009). Additionally, *HER3* down-regulation in lung cancer cells did lead to a significant induction of apoptosis (Sithanandam et al, 2005). Moreover, *HER3* knockdown synergistically enhanced dacarbazine-induced apoptosis in melanoma cell lines (Reschke et al., 2008). We next asked, whether a combination of HER3 down-regulation with chemotherapeutic drug treatment would increase percentages of the observed induction of cell death in our model system. To date, 5-Fluorouracil is the frequently chemotherapeutic used for the treatment of colon cancer patients. 5-Fluorouracil was also approved to be effective towards different colon cancer cell lines (Thant et al., 2008; Li et al., 2009). Interestingly the treatment of colon cancer cells using increasing 5-Fluorouracil concentrations upon *HER3* down-regulation results in increased rates of apoptosis, suggesting that HER3 might prove effective in the treatment of malignant colon cancer.

Notably established here for the first time is also the importance of HER3 in modulating migration, and invasion of colon cancer cell lines. Both processes were markedly inhibited by *HER3* siRNA interference in DLD-1 and LoVo cell lines. Indeed, regulation of tumor migration and invasion appears to be an essential function of HER3 in some other model systems since *HER3* down-regulation was shown to abolish migration and migration/invasion in breast and melanoma cancer cell lines respectively (Van der Horst et al., 2005; Reschke et al., 2008). The involvement of HER3 in regulating motility and migration was additionally demonstrated in the A549 lung cancer cell line (Sithanandam et al., 2005). It will be therefore of importance to elucidate the exact role of HER3 in these processes and to identify factors, which mediate HER3 responses during colon cancer metastasis like (e.g.,metalloproteinases).

Interestingly,HER3-knockdown cells display a shift in the cell morphology, which is characterized by the change of cells toward a flat and round appearance. We believe that HER3 knockdown shifted the cells toward an epithelial-like cell morphology. To address this hypothesis in more detail, additional experiments were carried out. We investigated changes in expression of themesenchymalmarker vimentin after HER3 downregulation in LoVo colon cancer cells as an example. Decreased expression

levels of vimentin were observed by Western blot analysis and by immunofluorescence (Supplementary Fig. S3). Because these findings indicate HER3 as a potential regulatory factor for mesenchymal marker expression within colon cancer, it is possible that HER3 expression is linked to epithelial-to-mesenchymal transition (EMT), a common principle during colon cancer progression.

The use of blocking antibodies for the treatment of cancers over-expressing pseudo-kinases such as HER3 looks to be the ideal therapy-way, since the use of small molecules inhibitors is thought to be inefficient in this case due to the absence of phosphotyrosine sites (Ross et al., 2003). We therefore treated human colon cancer cell lines using anti-HER3 monoclonal antibody, thereby testing the aptitude of HER3 to be considered as a novel target in colon cancer therapy. Our results indicate that an antibody directed against the extracellular domain of HER3 efficiently inhibits its phosphorylation after heregulin-ß1 stimulation. Anti-HER3 monoclonal antibody accelerates the endocytosis of HER3, resulting from its clearance from the cell surface, and inhibits the activation of downstream effectors since the binding of p85, the regulatory subunit of PI3K, to HER3 was abrogated. Remarkably, colon cancer cell proliferation was shown to be unaffected after treatment of cells using the HER3 blocking antibody. A possible explanation for this observation is that *K-ras gene* might be constitutively activated in our model system. Mutations of *K-ras* have been found in fact in 30-60% of colorectal carcinomas and are believed to be associated with tumor initiation, progression and metastasis formation (Vidic et al., 2010). Nonetheless, migration as well as invasion were considerably reduced in antibody-treated cells when compared to controls. Indeed, our results represent a loop in a chain of previous studies, which established the efficiency of the same blocking antibody towards HER3-mediated migration and migration/invasion in breast and skin cancer, respectively. (Van der Horst et al., 2005; Reschke et al., 2008). Taken together, previous as well as our present data indicate that anti-HER3 antibodies can inhibit HER3 signalling most likely through the PI3K-Akt pathway in breast, melanoma, and colon cancer thereby blocking several cellular functions. It will necessary in the future to test the efficacy of anti-HER3 blocking antibodies on colon cancer development and metastasis in preclinical animal models to further validate HER3 as a possible target for colon cancer therapy.

Collectively our data confirm that HER3 emerges as an important determinant of colon cancer behaviour as well as a novel target for drug design.

1.2 Mig-6 is a negative regulator of HER3 in colon cancer

The mitogen-inducible gene-6 (mig-6) is a multi-adaptor protein implicated by the regulation of the HER receptors tyrosine kinases family (Hackel et al., 2001; Anastasi et al., 2003; Reschke at al., 2010). Its role as an antagonist of the third member of this receptors-family, HER3 receptor, was the first time described by Anastasi and co-workers (Anastasi et al., 2003). Interestingly, recent *in vitro* studies reported on an inverse correlation between HER3 and mig-6 in melanoma cell lines (Ferby et al., 2006). In line with this, recent work of Reschke and co-workers report on a significant inverse correlation between HER3 and mig-6 in primary melanoma and metastases (unpublished data). According to these observations, we checked the mig-6 expression in respect to HER3 in our tumor samples. In primary colon cancer tumor-arrays samples, mig-6 was shown to be down-regulated in a significant number of cases, and this correlates with an increase in HER3 expression. These data suggest that loss of mig-6 in human colon cancer tumors seems to be sufficient to generate increased HER3 signaling which may lead to tumor formation and progression. Additionally, in HER3-overexpressing tumors mig-6 expression is lost (Figure. 14A). Interestingly, the observed HER3/mig-6 negative correlation was not limited on tumor samples, since it was also observed in human colon cancer cell lines. Indeed, according to our western blotting analysis HER3 expression was at the most of the cases inverse correlated with mig-6. Several mechanisms including loss of heterozygosity, epigenetic silencing and mutations can in fact account for *mig-6* repression in primary colon cancer tumors. Interestingly, missense and nonsense mutations in the *mig-6* coding region were observed in human lung cancer cell lines (Zhang et al., 2007). In addition, loss of heterozygosity (LOH) at the *mig-6* locus was identified to be associated with different solid human tumors including colon and rectum, kidney, breast and ovary (Ragnarsson et al., 1999). It will be the aim of future studies to investigate the exact mechanism of *mig-6* regulation in colon cancer and whether it can serve as a marker for HER3-mediated colon carcinogenesis.

A model comprising the role of HER3, its activation through different pathways and its attenuation through mig-6 could be illustrated in the following figure (Figure. 34).

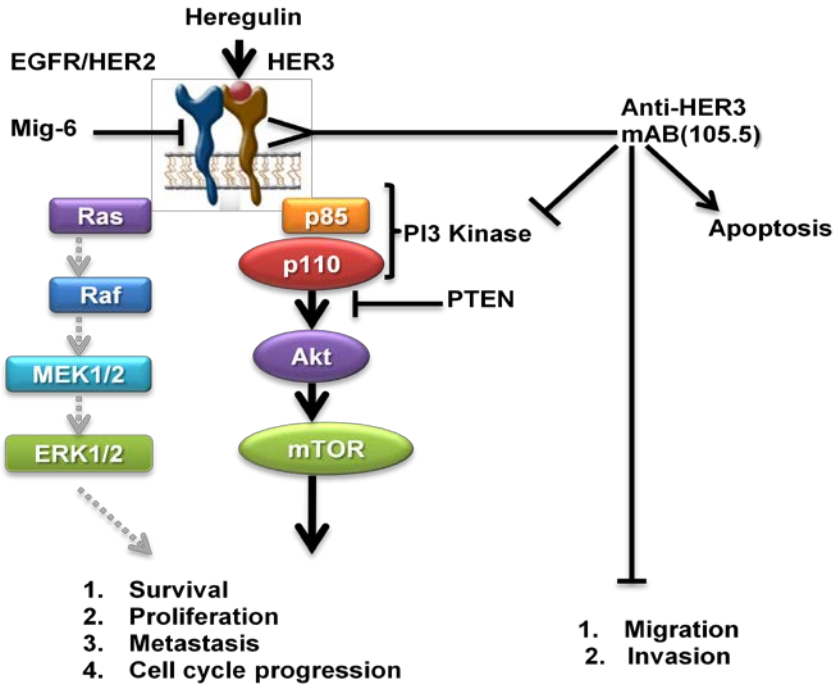


Figure 34 . Model for the role of HER3 and mig-6 in colon cancer

2. Mig-6 negatively regulates the oncogenic EGF receptor mutant EGFRvIII

2.1 Mig-6 is a negative regulator of EGFRvIII receptor

As described above, EGFRvIII is the best characterized and most common mutation of the *EGFR* wild type gene consisting in an in frame deletion of the extracellular amino acids 6 to 273, which constitute the dimerization arm and an essential part of the ligand-binding pocket. This deletion results in a smaller receptor form with a molecular weight of only ~145 KD. As EGFRvIII receptor was described in all reports to be a constitutively active receptor, which is continually phosphorylated, its involvement by the initiation and the progression of different cancer types is believed to be crucial (Pedersen et al., 2004, 2005). Indeed, since its description for the first time in 1990 by Sugawa and co-workers (Sugawa et al., 1990) several studies in rodents, using multiple molecular approaches, have investigated the high potential of this mutated receptor in tumor formation through activation of multiple signalling networks. In line with this, Grandal and colleagues demonstrated recently that EGFRvIII is characterized by a long lifetime in human tumors. Thereby, EGFRvIII was shown to escape downregulation due to impaired internalization and sorting to lysosomes (Grandal et al., 2007). This latter observation in addition to several previous reports rends the identifying of new molecular mechanisms that antagonize EGFRvIII of great importance.

Activity modulation of EGFRvIII through receptor tyrosine kinases negative regulators is considered as one of the best strategies to control the high potential of this oncogene. In the last years two different reports described the downregulation as well as the negative regulation of EGFRvIII through Cbl proteins and LRIG1 respectively (Davies et al., 2006; Stutz et al., 2008). However, the role of mig-6 adaptor-protein in the negative regulation of EGFRvIII remains unknown.

It has been shown previously shown that mig-6 is a negative regulator of members of the EGFR receptor tyrosine kinases family (Hackel et al., 2001; Ferby et al., 2006; Reschke et al., 2010; Anastasi et al., 2003). Here we show that mig-6 negatively regulates EGFRvIII. Three different model systems were in fact considered during our study. On the one hand CHO (Chinese hamster ovary) cells were used for an overexpression model system. These cells don't show in fact any endogenous expression neither of wild type EGFR nor of mig-6. On the other hand, the U87-

EGFRvIII glioblastoma cell line characterized by a high EGFRvIII expression as well as by the presence of endogenous mig-6 protein expression was taken as an endogenous model system. Finally the relationship between EGFRvIII and mig-6 was investigated in primary tumors of glioblastoma patients by using RT-PCR. An interaction between EGFRvIII mutated receptor and mig-6 negative regulator was observed in both first described model systems (CHO, U87-EGFRvIII) suggesting a direct interaction between these two proteins. On the other hand, almost the totality of analyzed primary tumors indicates an absence of any mig-6 expression. This absence is indeed correlated with the expression of the wild type EGFR at the most of the cases and also of EGFRvIII in some others. Such expression pattern is due to the already known negative correlation between wild type EGFR and mig-6 and most likely to the negative regulation of EGFRvIII through mig-6. This latter hypothesis was further confirmed. Indeed, a decrease in the phosphorylation levels of EGFRvIII after co-transfection of CHO cells with mig-6 and EGFRvIII in comparison to cells uniquely transected with EGFRvIII was observed. Moreover, down-regulation of *mig-6* expression in U87-EGFRvIII glioblastoma cell lines by knockdown results in an increase in the p-EGFRvIII expression levels. In summary, we have demonstrated for the first time that mig-6 could directly interact with EGFRvIII and that mig-6 is a new negative regulator of the constitutively active EGFRvIII receptor.

2.2 Mig-6 is a negative regulator of the EGFRvIII mediated glioma-genesis

Given its role as an antagonist of EGFRvIII in the U87-EGFRvIII glioblastoma cell line, we analyzed the role of mig-6 negative regulator by the modulation of crucial oncogenic events initiated by EGFRvIII in this model system. We demonstrated that depletion of *mig-6* in glioblastoma cell line over-expressing the EGFRvIII mutated receptor doesn´t affect neither the proliferation nor the migration of cells. Nevertheless, *mig-6* knockdown cells display a resistance towards treatment with chemotherapeutic (doxorubicin) in apoptotic events when compared to control cells. These data are in fact in accordance with our results and support them, since we have show that the down-regulation of *mig-6* only affects the STAT3 but not the ERK or AKT mediated signaling pathways. Interestingly, numerous previous studies report on the role of the STAT3 mediated signalling by the repression of apoptosis in

glioblastoma and other human cancers (Fuh et al., 2009; Wang et al., 2008; Aoki et al., 2003).

These data suggest that mig-6 acts in a negative feedback loop, thereby controlling EGFRvIII signalling pathway in human glioblastoma.

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VI Summary

In this thesis we investigated the role of mig-6 and its relation to the EGF-receptors family in colon cancer as well as in glioblastoma.

In the first part of the study, a negative correlation between HER3 (ErbB3) and mig-6 (errfi1) in primary colon cancer tumors as well as in colon cancer cell lines was shown. Along with this, we demonstrated that HER3 could be considered as a target for colon cancer therapy, since HER3 expression was shown to confer poor prognosis in colon cancer patients. In line with this, *In-vitro* studies employing 4 different colon cancer cell lines revealed the involvement of HER3 by the regulation of the proliferation, migration, and invasion as well as by the induction of apoptosis. Furthermore, an anti-HER3 monoclonal antibody directed against the extra-cellular portion of the receptor inhibits its activation, its binding to p85, the subunit of the PI3K kinase protein, thereby blocking its signaling network and accelerating its endocytosis into cell compartments. Anti-HER3 blocking antibody also inhibits cell migration and invasion of colon cancer cell lines.

In the second part of this work we investigated the link between the mig-6 adaptor protein and EGFRvIII, the mutant EGF-receptor tyrosine kinase. An interaction between these two proteins was established in over-expression (CHO cell line) as well as in endogenous model system (U87-EGFRvIII glioblastoma cell line). As an already established negative regulator of the EGFR receptor tyrosine kinases family, mig-6 was shown to also negatively regulate the mutant EGFRvIII receptor. Indeed, an inverse correlation between both proteins was demonstrated in primary tumors from glioblastoma patients. Moreover, down-regulation of mig-6 by siRNA in the U87-EGFRvIII glioblastoma cell line or its absence in co-transfected CHO cells induces an increase in the phosphorylation of EGFRvIII. On the molecular level, *mig-6* knockdown doesn't have any impact on the AKT and ERK signaling pathways but induces an increase in the p-STAT3 phosphorylation levels in the U87-EGFRvIII glioblastoma cell line. As the EGFR-STAT3 pathway was characterized by its anti-apoptotic effect, we showed that *mig-6* knockdown results in a resistance towards doxorubicin treatment in the same cell line. In line with this, *mig-6* suppression by

siRNA doesn’t show any effect on neither the proliferation nor the migration of the cells.

VII Zusammenfassung

Im Rahmen der vorliegenden Doktorarbeit wurde die Funktion von mig-6 und dessen Einfluss auf die EGFR Rezeptoren sowohl im Kolonkarzinom als auch im Glioblastom untersucht.

Im ersten Teil der Studie wurde eine negative Korrelation zwischen mig-6 und HER3 (ErbB3) sowohl in Primärtumoren von Kolonkarzinom Patienten als auch in Kolonkarzinom Zelllinien nachgewiesen. Des Weiteren wurde die Expression des HER3 Rezeptors von Kolonkarzinom Patienten analysiert. Hierbei zeigte sich eine hohe HER3 Expression als negativ prognostischer Marker. In einer *In-vitro* Studie, unter Verwendung von 4 verschiedenen Kolonkarzinom Zelllinien konnte die Rolle von HER3 bei der Regulation des Zellwachstums, der Migration und der Invasion sowie bei der Induktion von Apoptose gezeigt werden. Außerdem hemmt ein Anti-HER3 monoklonaler Antikörper gegen den extrazellulären Teil des Rezeptors, sowohl die HER3 Aktivierung, als auch die p85 Bindung und blockiert dadurch sein Signalisierungsnetzwerk und beschleunigt die Endozytose in Zellkompartimente. Der HER3 blockierende Antikörper hemmte außerdem sowohl die Migration als auch die Invasion von Kolonkarzinom Zelllinien. Derselbe Antikörper hat jedoch keinen Einfluss auf das Zellwachstum.

Im zweiten Teil dieser Arbeit wurde den Zusammenhang zwischen dem mig-6 Adapter-Protein und EGFRvIII (mutierter EGFR) erforscht. Hierbei konnte eine Interaktion zwischen den beiden Proteinen sowohl in einem Überexpressions -(CHO-Zelllinie) als auch in einem endogenen Modellsystem (U87-EGFRvIII Glioblastoma-Zelllinie) gezeigt werden. Als ein bekannter negativer Regulator des EGF-Rezeptors, kann mig-6 den mutierten EGFRvIII Rezeptor auch inhibieren. Eine inverse Korrelation zwischen den beiden Proteinen wurde in der Tat in Primärtumoren von Glioblastoma-Patienten nachgewiesen. Darüber hinaus induziert die Reduzierung der mig-6 Protein-Expression mittels siRNA in U87-EGFRvIII Glioblastoma-Zelllinie oder die mig-6 Abwesenheit in co-transfizierten CHO Zellen eine Erhöhung der Phosphorylierung von EGFRvIII. Auf einer molekularbiologischen Ebene übt mig-6 siRNA keine Auswirkung auf die AKT und ERK Signalwege aus, induziert aber eine

Erhöhung der p-STAT3 Phosphorylierung in der U87-EGFRvIII Glioblastoma-Zelllinie. Da der EGFR-STAT3 Signalweg durch seine anti-apoptotische Wirkung geprägt ist zeigten wir bei einem „apoptosis-assay“, dass die Unterdrückung der mig-6 Expression zu einer Insensitivität gegenüber Doxorubicin-Behandlung in der gleichen Zelllinie führt. Im Gegensatz dazu übt die Unterdrückung der mig-6 Expression keine Wirkung auf die Proliferation noch auf die Migration der Zellen aus.

IX Appendix

1. Abbreviations

AR	Amphiregulin
ATCC	American type culture collection
ATP	Adenotriphosphate
bp	Base pairs
BSA	Bovine serum albumin
°C	Degree celsius
cDNA	Copy DNA
CDK4	Cyclin dependent kinase 4
CIAP	Calf intestin alcaline phosphatase
CRIB	Cdc42/Rac interaction and binding
DMEM	Dulbecco´s modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dsDNA	Double-stranded DNA
E.coli	Escherichia coli
ECL	Enhanced chemiluminiscence
EDTA	Ethylenediamintetraacetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FDA	US Food and Drug Administration
Fig.	Figure
GRB2	Growth factor bound 2
GST	Glutathion-S-transferase
GTP	Guanosintriphosphate
h	Hour
HEPES	N-(2-Hydorxylethyl)-piperazin-N`-2-Ethansulfonic acid
HER 2, 3, 4	Human EGF receptor 2, 3, 4
Hrgß1	Heregulin ß1
IP	Immunoprecipitation
Kb	Kilobase
Kda	Kilodalton
KO	Knock-out
KD	Knock-down
LRIG-1	leucine-rich repeats and immunoglobulin-like domains-1
µ	Micro
m	Milli
M	Molar
mAB	Monoclonal antibody
MAP	Mitogen-activated protein

MAPK	MAP kinase
MEK	Mouse embryonic fibroblasts
Mig-6	Mitogen-inducible gene 6
Min	Minute
MMP	Matrix metallo proteinase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenyl-Methyl-Sulfonyl-Fluoride
PYK 2	Proline rich tyrosine kinase 2
Raf	Homologue to v-raf (murine sarcoma viral oncogene)
Ras	Homologue to v-rat sarcoma viral oncogene)
Rb	Retinoblastoma
RNA	Ribonucleic acid
Rpm	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
SH2	Src-homology 2
SH3	Src-homology 3
SHC	Src-homology 2-containing
siRNA	Short interfering RNA
Spry	Sprouty
Sos	Son of sevenless
SOCS	Supressor of cytokine signaling
Src	Homologue to v-src (sarcoma viral oncogene)
STAT	Signal transducer and activator of Transcription
Syk	Signal transducer and activator of Transcription
TEMED	N, N, N´,N´-Tetramethyletylendiamine
TGFα	Transforming growth factor alpha
TNFα	Tumor necrosis factor alpha
Tween 20	Polyoxyethylensorbitanmonolaureate
V	Volt
VEGFR	Vascular endothelial growth factor receptor
Vol	Volume
VSV	Vesicular stomatitis virus glycoprotein VSV-
WT	Wildtype

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