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Mitogen-Inducible Gene-6 is a Negative Regulator of the HER-Family of Receptor Tyrosine Kinases

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To my family

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HER3 is a Determinant for Poor Prognosis in Melanoma

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1. Introduction

Signal transduction through the family of receptor tyrosine kinases (RTKs) and related signalling molecules is essential for the regulation of cell fate decisions, cell growth, differentiation and survival. Within a cell, receptor tyrosine kinases translate a variety of extracellular signals into different cellular responses. Activation of receptor tyrosine kinases by their cognate growth factors leads to the induction of several downstream signalling molecules which eventually activate transcription factors that regulate gene expression. The intensity and the duration of a signal transmitted by receptor tyrosine kinases determine which biological response will occur. Under normal physiological conditions, receptor tyrosine kinase signalling has to be precisely coordinated in order to generate normal cellular functions. This regulation is achieved by the interplay of a variety of positive and negative regulators that function at different levels of signal transduction cascades. Dysregulation of pathway components either by mutations or overexpression can lead to the development of a variety of human diseases including developmental defects, diabetes and cancer.

1.1 The EGF receptor family

The human EGF receptor (HER) family of receptor tyrosine kinases regulates a large variety of biological processes including cell proliferation, -migration, -invasion and -survival (Figure 1; Gschwind et al., 2004). The family consists of four members: EGFR (HER1), HER2 (ErbB2/neu), HER3 (ErbB3) and HER4 (ErbB4) (Figure 1; Ullrich and Schlessinger, 1990). To date, 13 extracellular ligands have been reported including epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor α (TGFα), amphiregulin (AR), epiregulin, beta-cellulin and the heregulins (Figure 1; Citri and Yarden., 2006). These ligands bind directly to their cognate receptors, which leads to the formation of receptor homo- or heterodimers that trigger the activation of multiple signalling pathways (Figure 1; Citri and Yarden, 2006). HER2 and HER3 are thought to be non-autonomous receptors. HER2 lacks the ability to bind extracellular ligands whereas HER3 has only weak kinase activity due to aberrant sequence characteristics in its kinase domain. Both receptors, however, are able to form heterodimers with other members of the family in order to induce cellular signalling events. The four HER receptor family members share an overall structure of two cysteine-rich stretches in their extracellular region, a transmembrane region and a kinase domain flanked by a carboxy-terminal tail with tyrosine

autophosphorylation sites (Downward et al., 1984; Riedel et al., 1987). Dysregulation of members of the HER-family either by activating mutations, receptor overexpression or aberrant ligand release leads to the development of a variety of diseases including human cancer (Yarden and Sliwkowski, 2001).

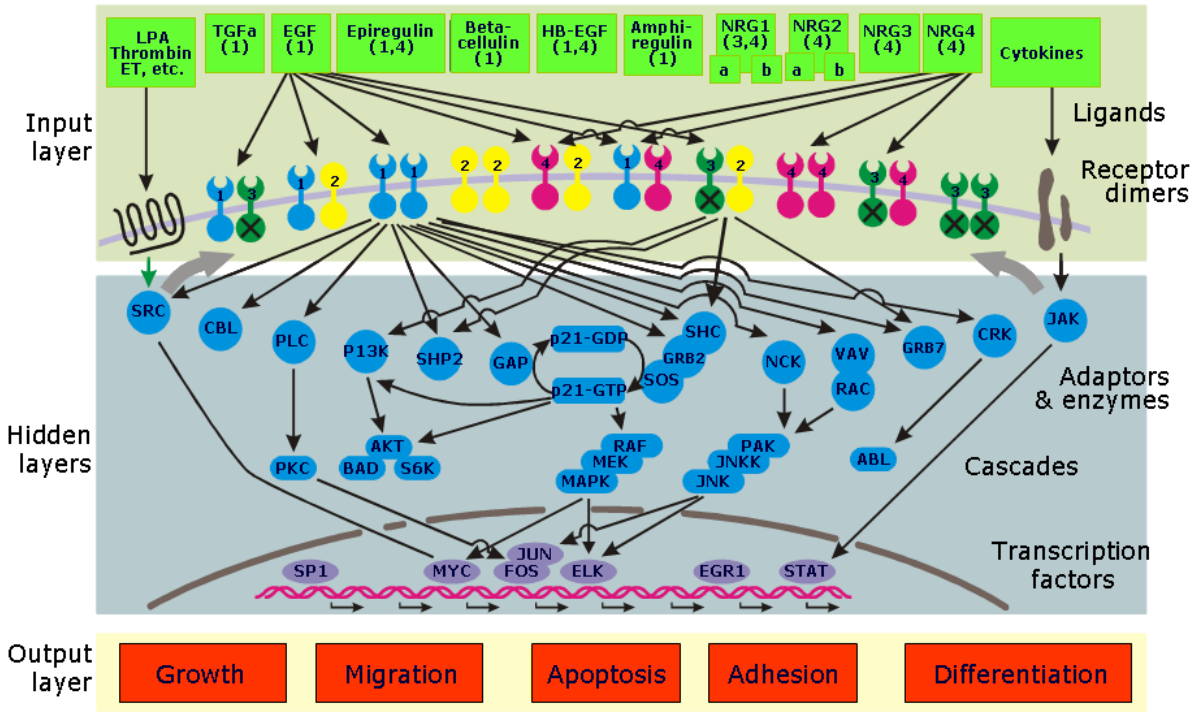


Figure 1. The human EGF receptor family
The human EGF receptor family consists of four members: the EGFR (HER1), HER2 (ErbB2/neu), HER3 (ErbB3) and HER4 (ErbB4). Thirteen ligands are known including EGF, TGFα, amphiregulin, epiregulin, beta-cellulin, heparin-binding EGF and the heregulins. Upon ligand binding, the receptors either form homo- or heterodimers that trigger the activation of multiple signalling cascades which translate the signal into various biological responses including cell proliferation, -migration and –survival (The figure was adapted from Yarden and Sliwkowski, 2001).

1.1.1 The EGF receptor (EGFR, HER1, ErbB1)

The epidermal growth factor receptor (EGFR) is the most prominent member of the HER receptor family. The EGFR was identified in 1978 as a membrane protein that showed increased phosphorylation upon EGF stimulation in A-431 epidermoid carcinoma cells (Carpenter et al., 1978). Two years later, it has been shown that the EGFR possesses tyrosine kinase activity (Ushiro et al., 1980). In 1984, the EGFR was the first cell surface receptor tyrosine kinase and proto-oncogene product to be cloned with molecular biological methods (Ullrich et al., 1984; Downward et al., 1984). Upon extracellular ligand binding the EGF receptor either forms homo- or heterodimers, which triggers the

phosphorylation of multiple tyrosines in the c-terminal receptor subunit in order to regulate a large variety of cellular processes including cell proliferation, angiogenesis and cell survival (Figure 2). The EGFR mainly activates the mitogen-activated protein kinase (MAPK) pathway through the binding of either growth-factor-receptor bound 2 (Grb2) or Src-homology 2-containing (Shc) proteins to its c-terminal tail which leads to the recruitment of son of sevenless (SOS) and subsequent activation of Raf-1 (Citri and Yarden, 2006). The signal is then transduced to MEK1/2 and extracellular regulated kinase 1/2 (ERK1/2). In addition, the EGFR can activate the PI3K-AKT signalling pathway through the small GTPase Ras (Figure 2). Another direct adaptor protein of the EGFR is the signal transducer and activator of transcription 5 (STAT5) which activates downstream signalling events via the JAK/STAT pathway (Figure 2; Citri and Yarden, 2006). In recent years, several negative regulators of the EGFR have been described. Receptor internalization is primarily controlled by the E3 ubiquitin ligase c-Cbl which targets the EGFR for lysosomal degradation (Levkowitz et al., 1998). Moreover, EGFR receptor signal attenuation is mediated by a group of signal-induced negative regulators which will be discussed below.

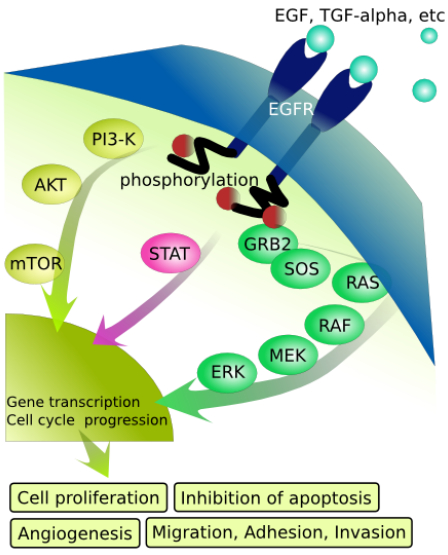


Figure 2. The EGF receptor signalling pathway
The EGF receptor signals via multiple downstream signalling pathways (e.g.: MAPK, PI3K-AKT, JAK/STAT) in order to regulate various biological processes including cell proliferation, cell survival, angiogenesis and cell motility (The figure was taken from www.weblio.jp).

The function of the EGFR in animal development and disease has been extensively studied in genetically modified mouse models. *EGFR* knock-out mice develop different phenotypes depending on their genetic background. The mice die either at mid-gestation (Sv-129 strain),

birth (C57BL/6 strain) or at postnatal day 20 (CD1, C3H and MF-1 strains; Sibilia et al., 1995; Miettinen et al., 1995; Threadgill et al., 1995). The observed phenotypes in *EGFR* mutant mice are mainly defects in the proliferation and migration of epithelial cells with different tissue origins including the skin, the lung and the placenta. Surviving *EGFR*-deficient mice display a strain-independent progressive neurodegeneration (Sibilia et al., 1998). In addition, mice humanized for the *EGFR* display defects in skin, lung and heart function (Sibilia et al., 2003). Moreover, recently published data suggest that the EGFR is a critical mediator of murine liver regeneration (Natarajan et al., 2007). On the other hand, an oncogenic role for the EGFR and its ligand TGFα has been demonstrated by the generation of transgenic animals. Ectopic expression of *TGFα* in the mammary gland leads to the development of breast cancer (Sandgren et al., 1995) whereas *EGFR* overexpression results in the preneoplastic conversion of multiple steroid hormone responsive tissues (Marozkina et al., 2007). Moreover, targeted *EGFR* expression in Schwann cells leads to neurofibromatosis-related peripheral nerve tumorigenesis (Ling et al., 2005).

The EGFR has been widely implicated in the development of human cancer. Overexpression of the EGFR, caused by gene amplification, has been reported for different human cancers including head and neck, ovarian, brain, breast, colon and lung cancer (Ullrich et al., 1984; Libermann et al., 1985). Furthermore, EGF-like ligands are often produced by human tumor cells or surrounding stromal cells which leads to an aberrant autocrine or paracrine activation of the EGFR (Slamon et al., 1995). Moreover, alterations in the *EGFR* gene such as deletions or point mutations which render the receptor hyperactive have been described. In glioblastoma, an in-frame deletion of the extracellular domain of the receptor, the EGFRvIII variant, has been described to contribute to tumor development (Malden et al., 1988). Several somatic point mutations in the tyroinse kinase domain of the EGFR have been discovered in non-small cell lung cancers (Hynes et al., 2005) whereas in prostate cancer, three novel missense mutations have been described which cause EGFR activation (Cai et al., 2008). Recently, defects in negative regulation of the EGFR in particular via the ubiquitin ligase c-cbl and the mitogen-inducible gene-6 (mig-6) have been linked to EGFR deregulation in human cancer (Ferby et al., 2006; Gschwind et al., 2004). Based on these investigations, the EGFR has been widely used as a target for therapeutic intervention in different human cancers.

1.1.2 HER2 (ErbB2/neu)

HER2 (ErbB2/neu) is the second member of the HER receptor family. It was discovered in 1985 and was shown to be the human homolog of the rat *neu* gene (Coussens et al., 1985; Schechter et al., 1985). HER2 represents a so called orphan receptor with none of the known EGF ligands able to bind and activate the receptor. However, HER2 is known to be the preferred dimerization partner for the other HER-family members thereby regulating cell proliferation and -differentiation (Tzahar et al., 1996). In addition, HER2-containing dimers are endocytosis impaired and are therefore often recycled to the cell membrane (Baulida et al., 1996). In rodents, HER2 is an important factor for normal brain and heart development. *HER2*-deficient mice die during embryonic development due to a lack of cardiac trabeculae (Lee et al., 1995).

Soon after its discovery by Axel Ullrich and colleagues in 1985 (Coussens et al., 1985) HER2 has been described as a proto-oncogene. It was shown that HER2 was amplified in human breast cancer cell lines and could transform normal mouse cells *in vitro*. In 1987 it has been shown that HER2 is overexpressed in 25-30% of primary lymph node-positive breast cancers (Slamon et al., 1987) and this genetic feature has been correlated with reduced patient survival and time to relapse. Moreover, HER2 serves as a prognostic marker for the response to various chemotherapeutic drugs in breast cancer therapy (reviewed in Fischer et al., 2003). Based on these observations HER2 became the first target for cancer therapy development in a subset of human breast cancers (further described in the section: *Targeting human EGF receptors for cancer therapy*).

1.1.3 HER3 (ErbB3)

HER3 (ErbB3) is the third member of the HER-family of receptor tyrosine kinases (Kraus et al., 1989; Plowman et al., 1990). Upon activation by heregulins, HER3 dimerizes with EGFR, HER2 and HER4 to form potent oncogenic receptor heterodimers (Sliwkowski et al., 1994; Carraway et al., 1994; Wallasch et al., 1995). Within this complex, HER3 recruits PI3-kinase to six distinct binding sites in its cytoplasmic domain thereby regulating proliferation and cellular survival (Figure 3; Schulze et al., 2005; Sithanandam et al., 2005). HER3 is endocytosis impaired and often escapes ligand-induced degradation (Baulida et al., 1996; Waterman et al., 1999); however, Cao and colleagues have shown recently that heregulin-

induced downregulation of HER3 can be mediated by the E3 ubiquitin ligase Nrdp1 (Cao et al., 2007).

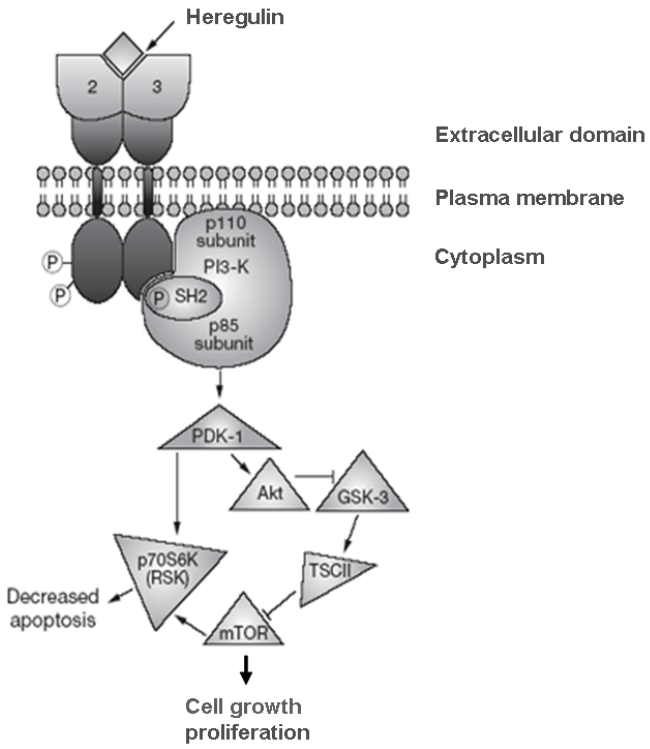


Figure 3. The HER3-PI3K-AKT signalling pathway
Upon activation by heregulin, HER3 dimerizes with the other members of the HER-family to form a functional receptor heterodimer. Within this complex HER3 recruits the PI3-Kinase to its cytoplasmic tail which leads to the activation of AKT and subsequent regulation of cell proliferation and -survival.

In rodents, HER3 is a critical mediator of normal cardiac development. *HER3* knock-out mice die at embryonic day 13.5 due to fatal defects of the heart valves (Erickson et al., 1997). In addition, these mice display defects in cerebellar plate differentiation, neural crest formation and show reduced numbers of Schwann cells (Erickson et al., 1997).

In human cancer, HER3 is overexpressed in breast, ovarian, lung and colorectal cancer and this genetic feature has been correlated with poor prognosis (Sithanandam et al., 2008; Tanner et al., 2006; Witton et al., 2003; Yi et al., 1997). In human breast cancer, HER3 preferentially dimerizes with HER2 to form a potent oncogenic receptor heterodimer that drives tumor development and progression (Wallasch et al., 1995; Sliwkowski et al., 1994). Furthermore, it has been shown that breast cancer cell motility and intravasation are dependent on HER3 activity (Xue et al., 2006). Thus far, it was assumed that HER3 is kinase-inactive due to apparently aberrant sequence characteristics in its kinase domain and that it requires heterodimerization with a kinase-intact member of the HER-family in order to initiate

signalling events (Guy et al., 1994). Consistent with this, it was shown that HER2 requires HER3 to drive breast tumor cell proliferation (Holbro et al., 2003). However, recent findings of Htun van der Horst and co-workers showed that HER3 is able to phosphorylate Pyk2 which results in the activation of the MAPK pathway in human glioma cells (van der Horst et al., 2005) suggesting that HER3 possesses selective kinase activity. Furthermore, monoclonal antibodies specific for HER3 can inhibit the proliferation and migration of cancer cell lines (van der Horst et al., 2005). Interestingly, it was shown recently that cancer cells escape HER-family inhibitor therapy by upregulation of HER3 signalling (Sergina et al., 2007) and that HER3 inhibition abrogates HER2-driven tamoxifen resistance in breast cancer cells (Liu et al., 2007). Moreover, HER3 contributes to tamoxifen resistance in estrogen receptor ß1-negative breast tumors (Folgiero et al., 2008). Furthermore, resistance to Gefitinib, an EGFR small molecule inhibitor, was shown to be connected to HER3 signal activation (Engelman et al., 2007). Based on these results HER3 represents an attractive target for the development of target directed therapies for the treatment of various human cancers.

1.1.4 HER4 (ErbB4)

HER4 (ErbB4) is the fourth member of the HER-family of receptor tyrosine kinases (Plowman et al., 1993). Upon activation mainly by heregulins, HER4 forms heterodimers with other members of the family to regulate downstream signalling events (Plowman et al., 1993). HER4 can be activated by a large set of EGF-like ligands including beta-cellulin, HB-EGF, epiregulin and epigen. Upon activation, HER4 can recruit Grb2, Shc and STAT5 to its cytoplasmic tail thereby activating similar intracellular signalling pathways like the EGFR (reviewed in Citri and Yarden, 2006). In addition, alternative mRNA splicing produces several HER4 isoforms which can fullfill different cellular functions. One isoform is able to translocate to the nucleus thereby regulating STAT5 expression (Ni et al., 2001; Williams et al., 2003). Furthermore, HER4 can be processed by a presenilin-dependent γ-secretase which regulates different receptor activities (Lee et al., 2002; Vidal et al., 2005).

In rodents, HER4, like HER2, is essential for brain and cardiac development. *HER4*-deficient mice display the same embryonic lethal phenotype like *HER2*-deficient mice suggesting certain redundant functions for HER2 and HER4 in these developmental processes (Gassmann et al., 1995).

HER4 is upregulated in many human cancers including colon, brain and prostate cancer and has been correlated with poor prognosis (reviewed in Stove et al., 2004).

1.2 Targeting human EGF receptors for cancer therapy

The human EGF receptor family of receptor tyrosine kinases has been widely implicated in the development of various human cancers. Human EGF receptors undergo a wide range of activating alterations in different tumour types thereby contributing to tumor development and progression. Thus, the EGF receptor family represents a promising target for selective cancer therapeutics (Figure 4). Thus far, there are two strategies which are used to target receptor tyrosine kinases: humanized monoclonal therapeutic antibodies and small molecule tyrosine kinase inhibitors. Monoclonal antibodies specifically bind to the extracellular portion of the receptor and thereby inhibit receptor function. The discovery of *HER2* gene amplification in human breast cancer by Denis Slamon, Axel Ullrich and colleagues led to the development of trastuzumab (Herceptin, Genentech Inc/Roche), the first humanized therapeutic anti-cancer antibody. Trastuzumab binds to HER2 at the cell surface of tumor cells and leads to receptor internalization, inhibition of cell cycle progression through the induction of p27 expression and to the recruitment of immune cells (Gschwind et al., 2004). Trastuzumab was approved by the US Food and Drug Administration (FDA) for the treatment of HER2-overexpressing metastatic breast cancer in 1998. In 2004, the FDA approved Cetuximab (Erbitux, ImClone/Merck), a monoclonal antibody targeting the EGFR for the treatment of patients with colorectal cancer. The fact that HER2 is the preferred dimerization partner for the other HER receptors led to the development of the monoclonal antibody 2C4 (Omnitarg, Pertuzumab). Importantly, 2C4 blocks HER2 dimerization with other members of the HER-family thereby preventing the activation of downstream signalling cascades (Figure 4; Hudziak et al., 1989).

Small molecule tyrosine kinase inhibitors (TKI's) are another form of targeting agents and function by competing with ATP to the receptor tyrosine kinase domain. For the EGFR two inhibitors, Erlotinib (Tarceva, Genentech/Roche/OSI) and Gefitinib (Iressa, AstraZeneca) have been developed and were approved for the treatment of non-small-cell lung cancer (NSCLC).

The so far described anti-HER receptor agents have all shown clinical efficacy; however, in many cases tumors do not fully respond to these treatments or acquire drug resistance.

Therefore, combination therapies or inhibitors which specifically recognize multiple members of the family are being developed to achieve improved tumor response rates. Lapatinib (GlaxoSmithKline), CI-1033 (Pfizer) and EKB-569 (Wyeth-Ayerst Research) are dual- or multi-specific inhibitors which target the EGFR and HER2 at the same time and show potent anti-tumor activity (Figure 4; Xia et al., 2002).

As mentioned above, one major problem associated with targeted therapies is resistance formation. Tumor cells can acquire resistance towards certain therapeutic drugs through different mechanisms including the occurrence of mutations in the targeted oncogene or the activation of compensatory tyrosine kinase pathways (Daub et al., 2004). Recently, it has been shown that breast and lung cancer cells escape EGFR and HER2 targeted therapy by upregulation of HER3 signalling (Sergina et al., 2007; Engelman et al., 2007). Moreover, recent work suggests that the PI3K signalling pathway as well as the receptor tyrosine kinase MET contribute to Herceptin resistance in HER2-overexpressing breast cancer cells (Berns et al., 2007; Shattuck et al., 2008). Thus, interfering with such resistance-associated pathways either by monoclonal antibodies, small molecule inhibitors or in combination with established anti-cancer agents may provide novel opportunities towards the extended treatment of cancer with targeted agents.

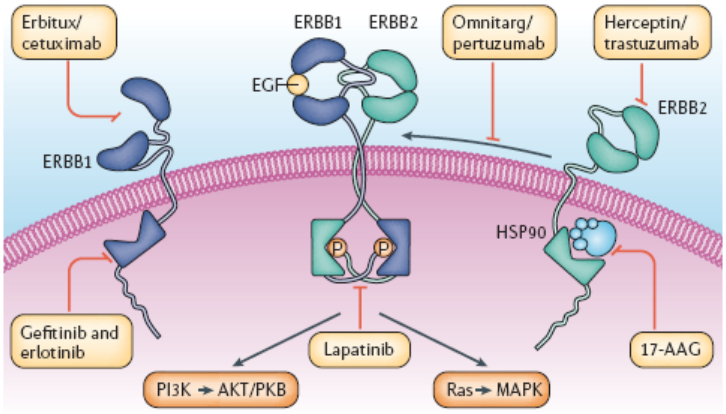


Figure 4. Cancer therapies targeted to human EGF receptor tyrosine kinases
The human EGF receptor tyrosine kinases are widely implicated in the development of human cancer and, therefore, represent excellent targets for targeted cancer therapies. Therapeutic antibodies (Erbitux and Trastuzumab) as well as small molecule inhibitors (Erlotinib and Gefitinib) have been approved for cancer therapy. Several other inhibitors like the dual-specific kinase inhibitor Lapatinib, the HSP90 inhibitor 17-AAG and the monoclonal antibody 2C4 (Omnitarg, Pertuzumab) are currently in clinical development (The figure was taken from Citri and Yarden, 2006).

1.3 Negative regulators of receptor tyrosine kinase signalling

Deregulation of receptor tyrosine kinase signalling leads to the development of a variety of human diseases including diabetes and cancer. Several mechanisms are used by the cells to ensure proper signal transduction. One such mechanism is the upregulation of negative regulators of receptor tyrosine kinases which act in negative feedback loops to control signal attenuation. During the last years it has become increasingly clear that negative regulators of receptor tyrosine kinases are critical for normal cellular function. In addition, most negative regulators are regarded as potential tumor suppressors. Along these lines, it has been shown that negative regulators are lost in human cancers leading to the activation of receptor tyrosine kinase signalling (Ferby et al., 2006; Kwabi-Addo et al., 2004; Lo et al., 2004).

Negative regulators either exist prior to receptor activation or are induced upon signal initiation. Existing regulators are mainly involved in the control of receptor dephosphorylation and degradation. Receptor dephosphorylation is mediated by a variety of receptor tyrosine phosphatases whereas receptor degradation is controlled by E3 ubiquitin ligases which target the receptors for lysosomal degradation. Signal-induced negative regulators have been first described in the fruit fly *Drosophila melanogaster*. Here, receptor tyrosine kinase activation leads to the expression of the proteins Sprouty, Argon and Kekkon which are all implicated in receptor tyrosine kinase regulation (Casci et al., 1999; Musacchio et al., 1996; Golembo et al., 1996). In human cells, growth factor-induced negative regulators have been mainly described for the EGFR (Figure 5). EGF stimulation induces the expression of various proteins like suppressor of cytokine signalling 4 and 5 (SOCS4, SOCS5), leucine-rich repeats and immunoglobulin-like domains-1 (LRIG-1), the serine/threonine kinase GAK, Sprouty proteins and the mitogen-inducible gene-6 (mig-6) which are all known to negatively regulate EGFR signalling through different mechanisms (Figure 5; Kario et al., 2005; Gur et al., 2004; Zhang et al., 2004; Hackel et al., 2001; Hanafusa et al., 2002). In addition, it has been shown that the protein tyrosine kinase Syk can control EGFR activity (Figure 5; Ruschel et al., 2004).

Protein	Inhibition Targets	Mechanism
Ack1	EGFR	Unknown
LRIG-1	EGFR family	Enhances receptor ubiquitylation
SOCS 4 and 5	EGFR	Enhance receptor dephosphorylation
GAK	EGFR	Enhances receptor dephosphorylation
Sprouty	Several RTKs	Inhibits Ras-MAPK signalling
Mig-6	EGFR family	Enhances receptor dephosphorylation
c-Cbl	Several RTKs	Mediates receptor ubiquitylation
DEP1 and PTP1B	EGFR	Mediate receptor dephosphorylation
Syk	EGFR	Modulates receptor activity

Figure 5. Negative regulators of the EGFR and their mechanism of action

1.3.1 Mitogen-inducible gene-6 (Mig-6)

The mitogen-inducible gene-6 (mig-6; also known as ralt, gene33 and errfi1) is a multi-adaptor protein implicated in the negative regulation of receptor tyrosine kinases (Zhang et al., 2007). Mig-6 was cloned from a rat liver cDNA library (Lee et al., 1985) and was described to be a mitogen-inducible gene which is regulated during cell cycle progression (Wick et al., 1995). The *mig-6* gene was mapped to the human chromosomal region 1p36 and contains 4 coding exons. Mig-6 contains a large number of protein-protein interaction domains (Figure 6). In its n-terminus, mig-6 has a cdc42/Rac interaction and binding (CRIB) domain followed by three SH3 domains and a 14-3-3 binding site. Furthermore, mig-6 contains an EGFR binding domain and in its c-terminus a PDZ domain. Moreover, the c-terminus of mig-6 shares high homology to the non-receptor protein tyrosine kinase Ack1 which was previously implicated in EGFR regulation (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).

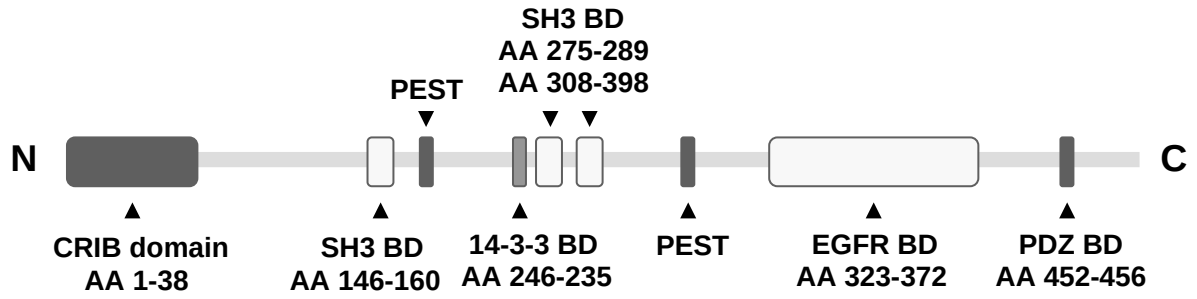


Figure 6. Schematic representation of the mig-6 protein structure
Mig-6 is a multi-adaptor protein implicated in the regulation of receptor tyrosine kinases. It is localized in the cytoplasm and has a primary structure of 459 amino acids. Mig-6 can interact with various proteins through its multiple protein binding domains including a CRIB domain, three SH3 binding domains, a PDZ domain and a 14-3-3 binding motif. The interaction domain responsible for EGFR binding has been mapped between amino acids 323-372.

Mig-6 expression is inducible by a large variety of different stimuli including growth factors, hormones, chemical agents and different stresses (Zhang et al., 2007). Mig-6 can be transcriptionally induced by EGF, HGF as well as Insulin (Hackel et al., 2001; Pante et al., 2005; Chu et al., 1988) which is mainly mediated by the Ras-Raf-MEK-ERK pathway since pharmacological inhibition of MEK1/2 blocks growth factor-induced mig-6 expression (Hackel et al., 2001). In recent years, mig-6 has been implicated in the negative regulation of receptor tyrosine kinases. It was shown that mig-6 is a negative regulator of EGFR signalling (Figure 7; Hackel et al., 2001). Here, mig-6 directly binds to the activated receptor thereby regulating EGFR activity, receptor stability and downstream signalling molecules like MAPK, AKT and JNK (Figure 7; Hackel et al., 2001; Anastasi et al., 2007). Interestingly, recent work by Zhang and colleagues using crystallographic methods suggests that binding of mig-6 to the EGFR kinase domain is sufficient to inhibit EGFR activity (Zhang et al., 2007). Furthermore, mig-6 can inhibit EGFR-mediated transformation of Rat-1 fibroblasts (Hackel et al., 2001) suggesting that mig-6 is a potential tumor suppressor of EGFR-dependent cell transformation. To date, the exact mechanism of EGFR regulation by mig-6 and possible mig-6 interaction partners are still not completely understood. Mig-6 is able to bind to Grb2 and thereby potentially blocks EGFR receptor downstream signalling. In addition, mig-6 can bind to other downstream modulators like Src, p85 PI3K, PLC-γ and 14-3-3 (Fiorentino et al., 2000; Benzinger et al., 2005); however, so far no biological significance could be shown for these interactions.

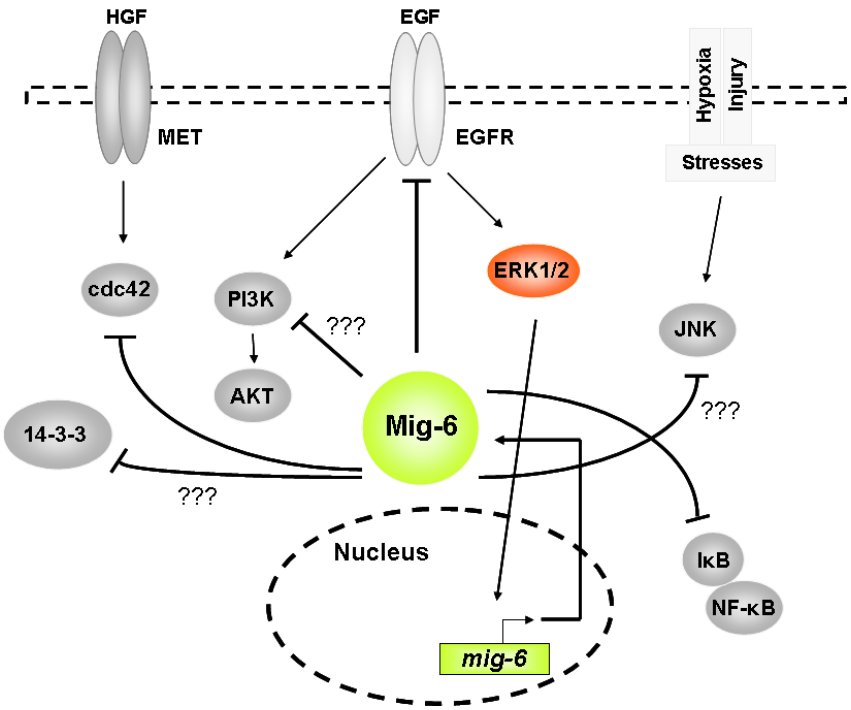


Figure 7. The role of mig-6 in growth factor- and stress-induced signal transduction
Mig-6 can be transcriptionally induced by a variety of external stimuli including growth factors and stress. EGF and HGF can induce mig-6 through their respective receptors and subsequent activation of the MAPK pathway. In addition, mig-6 expression can be induced by hypoxia or other stress stimuli most likely through the JNK pathway which in turn may be regulated by mig-6. The mig-6 protein can interact with many different molecules to regulate a variety of signalling cascades. Mig-6 can directly bind to the HER-family of receptor tyrosine kinases thereby negatively regulating HER receptor signal transduction. Moreover, mig-6 can inhibit HGF/MET receptor functions by directly modulating the activity of cdc42. In addition, mig-6 can bind to IκBα which leads to the inhibition of NF-κB. Mig-6 can bind to 14-3-3 proteins and the PI3K *in vitro*; however, so far no functional significance was shown for these interactions (The figure was adapted from Zhang et al., 2007).

Besides its inhibitory activity towards the EGFR, mig-6 was shown to be a negative regulator of HER2 (Fiorentino et al., 2000), HER3 and HER4 (Anastasi et al., 2003). Furthermore, mig-6 can inhibit HGF/MET-induced proliferation and migration through a direct modulation of cdc42 (Figure 7; Pante et al., 2005).

The function of mig-6 in development and disease has been extensively studied in genetically modified mouse models. These studies established mig-6 as an essential regulator of EGFR-dependent skin morphogenesis. Mice with targeted *mig-6* expression in epidermal keratinocytes develop a waved-like phenotype which resembles the skin abnormalities observed in mice carrying an *EGFR* hypomorphic allele (Ballaro et al., 2005; Luetteke et al.,

1994). In contrast, targeted disruption of *mig-6* in the mouse genome led to a psoriasis-like phenotype (Ferby et al., 2006) which is characterized by hyperproliferation and impaired differentiation of epidermal keratinocytes. It was further shown that *mig-6*-deficient keratinocytes show hyperactivation of the EGFR and sustained signalling through the MAPK pathway (Ferby et al., 2006). Moreover, *mig-6* knock-out mice are highly susceptible to carcinogen-induced papilloma formation. Importantly, both the epidermal hyperproliferation as well as the papilloma formation could be rescued by the EGFR small molecule inhibitor gefitinib or by crossing *mig-6* knock-out mice to *EGFR waved2* animals. These data clearly demonstrate that mig-6 is a negative regulator of EGFR-dependent skin morphogenesis and skin tumor formation (Ferby et al., 2006).

In addition, it was shown that *mig-6* knock-out mice develop a degenerative joint disease which possibly causes the premature death observed in these mice (Zhang et al., 2005; Ferby et al., 2006).

As mentioned above, mig-6 is a potential tumor suppressor of HER receptor-mediated carcinogenesis. Along these lines, *mig-6* knock-out mice develop a large number of tumors or hyperplasia in a variety of different tissues including skin, lung, colon, ovary, gallbladder and bile duct (Figure 8; Ferby et al., 2006; Zhang et al., 2007). Due to the limited life span of *mig-6* knock-out mice it is difficult to examine all the mentioned phenotypes in detail. However, recently Jin and colleagues generated a floxed *mig-6* allele which allows conditional inactivation of *mig-6* using Cre recombinase (Jin et al., 2007). This mouse model will significantly improve our understanding of the role of mig-6 in receptor tyrosine kinase regulation as well as tumor suppression.

Occurrence of tumors or hyperplasia in *mig6*$^{-/-}$ mice

Type of tumor or hyperplasia		# of mice with described pathology of mice analyzed.
GIT	Rectal mucinous tubular adenoma	15/27
	Rectal mucinous adenocarcinoma	7/27
	Gastric adenomatous polyps	12/30
Bone/cartilage hyperplasia		30/30
Lung	Alveolar epithelial thickening	12/15
	Bronchoalveolar adenoma	4/15
Skin	Papillomas on bare skin or eyelids	6/30
	SCC in perineal epidermis	7/27

Indicated number of mutant mice were analyzed for tumors or hyperplasia between 5 to 7 months of age near expected or at death.

Figure 8. Occurrence of tumors or hyperplasia in *mig-6* knock-out mice
(The figure was taken from Ferby et al., 2006)

Along with its tumor suppressive function in mice, mig-6 was found to be downregulated in various human cancers including breast, skin, brain and prostate cancer and this genetic feature correlates with increased expression and activation of HER-family receptor tyrosine kinases (Ferby et al., 2006). Along these lines, it was shown that mig-6 is downregulated in HER2-overexpressing breast tumors and may contribute to Herceptin resistance (Anastasi et al., 2005). In fact, overexpression studies revealed that mig-6 can inhibit HER-family activation and EGFR- or HER2-mediated cell transformation (Hackel et al., 2001; Fiorentino et al., 2000; Xu et al., 2005). These data strongly indicate that mig-6 is a potential tumor suppressor of HER receptor-mediated carcinogenesis.

1.4 Melanoma

Melanoma is a common type of skin cancer, which develops from the malignant transformation of melanocytes and accounts for approximately 80% of deaths arising from skin cancer (Miller et al., 2006). There are several risk factors which can lead to the development of melanoma including sun exposure, family history of melanoma, multiple benign or atypical nevi or immunosuppression. Melanocytes undergo a variety of histopathological changes in order to form a malignant melanoma. The different phases of melanoma formation are described in the so called Clark Model (Miller et al., 2006). In the initial phase of melanoma development melanocytes form a benign nevus, which, however, rarely progresses to cancer (Figure 9; Miller et al., 2006). In some cases such benign nevi can acquire aberrant cell growth leading to the formation of dysplastic nevi which eventually progress to the radial-growth phase (Figure 9; Miller et al., 2006). Here, the melanocytes acquire the ability to grow in the epidermis and to invade the dermis as single cells. Melanoma cells which then progress to the vertical-growth phase are able to fully cross the basement membrane and to invade the dermis. Finally, in late stage disease, melanoma cells start to dissociate from primary tumor sites and form metastases by invading distant organs like the lung, the liver or the brain (Figure 9; Miller et al., 2006). The underlying molecular mechanisms of melanocyte transformation and melanoma progression have been studied extensively in the past (Chin et al., 2006; Fecher et al., 2007). Multiple molecular changes occur during melanoma progression (Figure 9). Early events involve activating BRAF mutations (deLuca et al., 2008) as well as the loss of tumor suppressor genes like p16, p19, Cyclin-dependent kinase 4 (CDK4; Kamb et al., 1994; Nobori et al., 1994) and PTEN (Steck et al., 1997). At later stages during melanoma progression tumor cells acquire prominent changes in cell adhesion molecules like loss of E-cadherin and gain of N-cadherin expression

as well as the expression of matrix-metallo proteinases (MMPs) which facilitate melanoma cell invasion (Figure 9). Moreover, several other pathways including the PI3K-AKT and the MAPK pathway have been described to contribute to the malignant behaviour of melanoma cells (Gray-Schopfer et al., 2007). Thus far, surgery is the most efficient treatment for local melanomas. However, no therapies are available that significantly prolong patient survival once melanoma progresses to the metastatic state (Chudnovsky et al., 2005). Radio- and chemotherapy show only limited efficacy towards metastatic melanoma since melanoma cells appear to be highly resistant towards such conventional therapies. Moreover, the development of target-directed therapies has been hampered by the lack of known, drugable key regulators of melanomagenesis. Clinical trials with inhibitors targeting the MAPK signalling pathway have been stopped due to their limited anti-tumor activity (Eisen et al., 2006). Thus, there is an urgent need for novel therapeutic agents and prognostic markers in the treatment of melanoma patients.

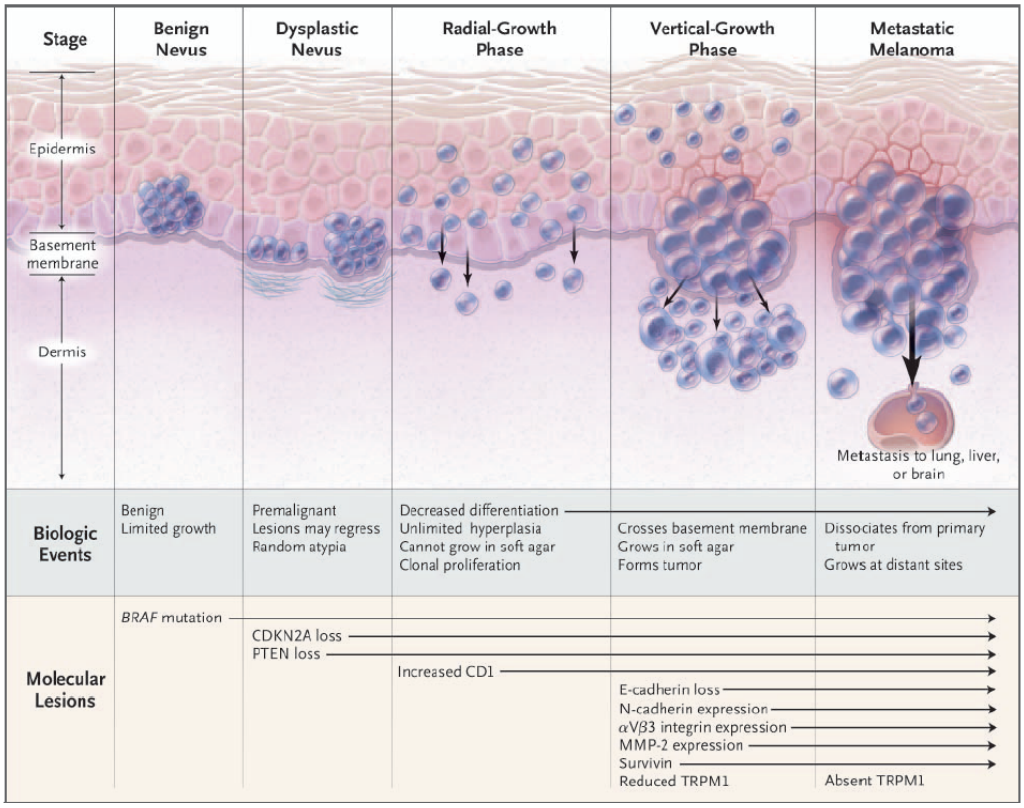


Figure 9. Biological and genetic events during melanoma progression
(The figure was taken from Miller et al., 2006)

1.5 Liver regeneration and human liver cancer

In rodents the liver has a tremendous capacity to regenerate. Normally, hepatocytes are in a quiescent state and rarely divide. In response to a two-third partial hepatectomy, however, 95% of all hepatocytes synchronously re-enter the cell cycle and restore the liver to its full size within one week (Taub et al., 2004). This rapid cell growth provides an excellent model to study the molecular mechanisms of cell cycle progression and cell proliferation *in vivo*. Importantly, defects in liver regeneration can contribute to the development of severe diseases like liver cirrhosis or liver cancer (Fausto et al., 2004). In order to achieve proper liver regeneration after liver injury, the process has to be tightly controlled and several cell types contribute to this regulation (Figure 10).

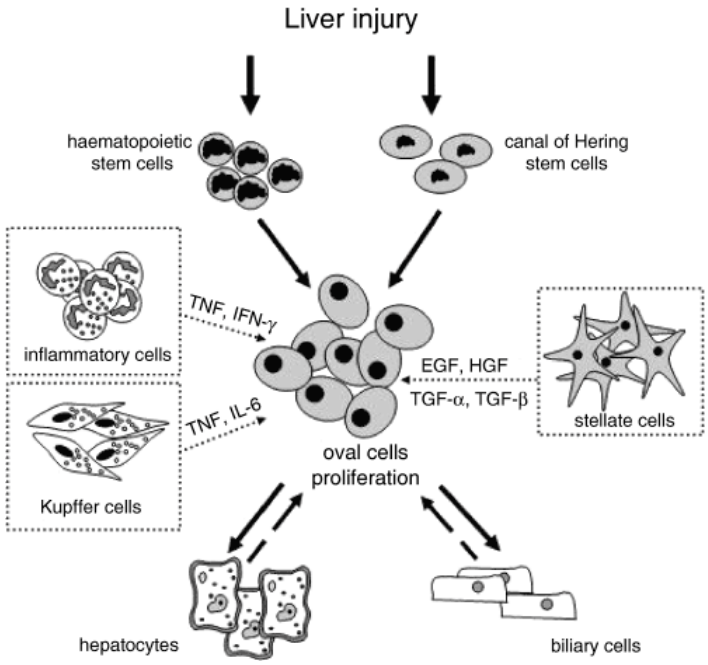


Figure 10. Multiple cell types are involved in the repair of injured liver tissue
Upon liver injury, Kupffer and inflammatory cells produce cytokines like TNFα or IL-6 which represent the priming phase of liver regeneration. Stellate cells produce growth factors like EGF and HGF which are essential for hepatocyte cell cycle progression. Additional cell types like haematopoietic stem cells or biliary cells contribute to proper liver regeneration (The figure was taken from www.medscape.com).

The process of liver regeneration is dependent on various growth factors, cytokines and metabolic processes. Cytokines like tumor necrosis factor α (TNFα) or interleukin-6 (IL-6), which are produced by Kupffer and inflammatory cells act as the priming factors of liver regeneration, driving quiescent hepatocytes into the cell cycle (Fausto et al., 2006). Several growth factors and receptor tyrosine kinases control hepatocyte cell cycle progression by

stimulating the S-phase entry of hepatocytes. The EGF receptor, the EGF receptor ligands HB-EGF, TGFα and amphiregulin as well as the MET receptor have been described to be essential regulators of liver regeneration (Figure 11; Fausto et al., 2006). The exact function of these proteins during liver regeneration has been studied in genetically modified mouse models. Targeted disruption of *AR*, *HB-EGF* or the *MET* receptor in mice results in a delay of liver regeneration after partial hepatectomy (Mitchell et al., 2005; Borowiak et al., 2004; Berasain et al., 2005). Recently, Natarajan and colleagues have shown for the first time that specific ablation of the *EGFR* in hepatocytes delays liver regeneration (Natarajan et al., 2007). *TGFα* knock-out mice have no defects in liver regeneration but transgenic mice overexpressing *TGFα* show constitutive hepatocyte proliferation and develop liver cancer (Webber et al., 1994).

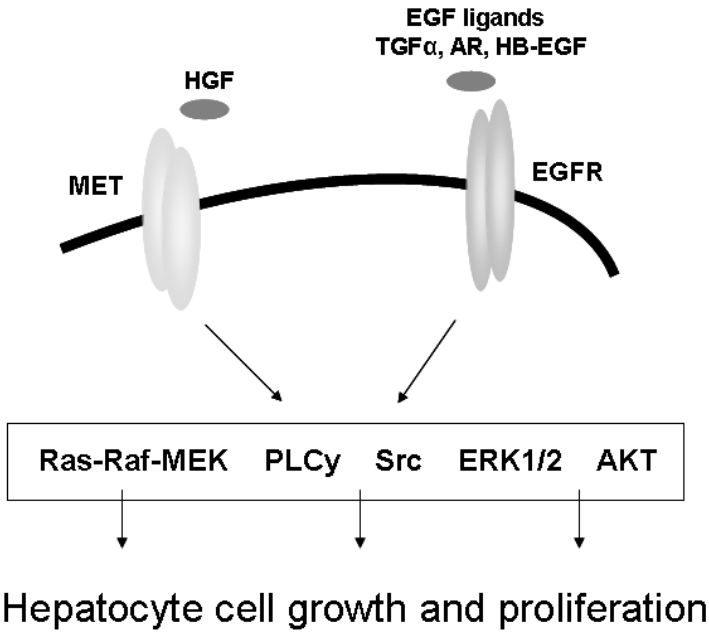


Figure 11. The EGF and MET receptor signalling pathways in liver regeneration
The activation of the receptor tyrosine kinases EGFR and MET by their respective ligands TGFα, amphiregulin, HB-EGF and HGF is essential for the control of proper hepatocyte cell growth and proliferation during liver regeneration (The figure was adapted from Fausto et al., 2006).

As mentioned above, defects in proper liver regeneration can lead to the development of liver cancer. Human hepatocellular carcinoma (HCC) is the fifth common cancer type worldwide and accounts for approximately 90% of all primary liver cancers (El-Serag et al., 2007). HCC can be caused by a variety of environmental factors including hepatitis B and C virus

infection, alcohol abuse or increased uptake of aflatoxin B1-contaminated food (Lupberger et al., 2007; Koike et al., 2007; Seitz et al., 2007). Liver injury by aforementioned factors leads to necrosis and hepatocyte proliferation which can manifest in chronic liver disease which eventually further progresses to the state of liver cirrhosis (Schuppan et al., 2008). The cirrhotic liver is characterized by increased liver nodule formation and extensive scarring of the liver which may lead to the development of hyperplastic nodules. These nodules can further progress ultimately leading to hepatocellular carcinoma formation (Figure 12). Various genetic changes and chromosomal instabilities have been implicated in the development and progression of HCCs. This includes the inactivation of several tumor suppressor genes like p53 as well as the aberrant activation of receptor tyrosine kinases like the EGFR or MET (Farazi et al., 2006). Patients with early stage liver cancer are treated by surgical removal of cancerous liver tissue followed by systemic chemotherapy. However, HCCs are highly resistant to conventional chemotherapeutics and to date there is no effective therapy which significantly prolongs patient survival (Chattopadhyay et al., 2007). Based on these facts there is an urgent need for novel therapeutics to improve patient outcome. Among them there are novel as well as established agents targeting a wide variety of different molecular pathways including the EGFR pathway, the VEGFR pathway as well as the MAPK and PI3K pathways (Farazi et al., 2006). It remains to be shown whether these approaches approve to be effective in HCC patients and whether such therapies can significantly prolong patient survival.

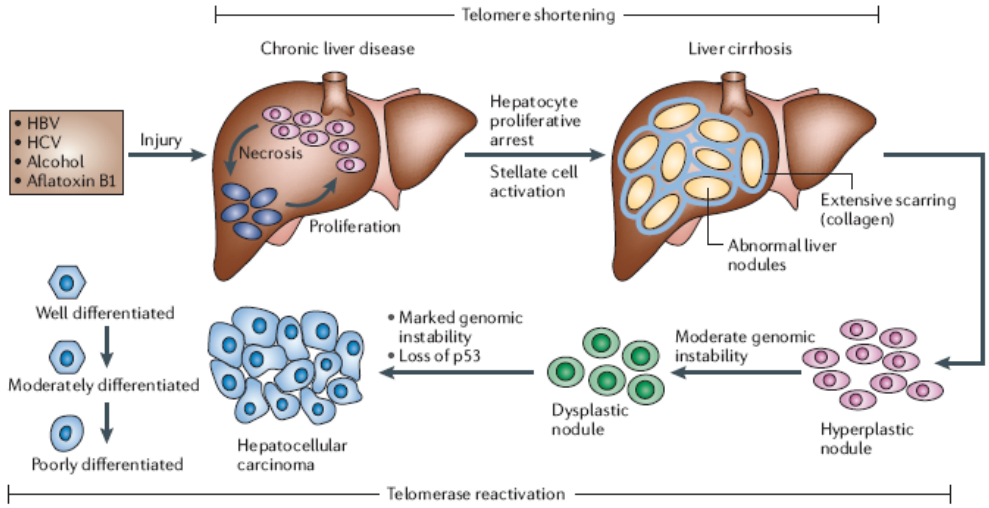


Figure 12. Schematic representation of human hepatocellular carcinoma development
(The figure was taken from Farazi and dePinho, 2006)

2. Specific Aims

Receptor tyrosine kinases (RTKs) regulate a large variety of cellular processes including cell proliferation, -migration and -survival. In recent years, several negative regulators of RTKs have been identified including the mitogen-inducible gene-6 (mig-6) which is a specific regulator of the HER-family of receptor tyrosine kinases. It has been shown that mig-6 is essential for the proper regulation of EGFR-mediated cellular functions and that loss of mig-6 leads to abnormal EGFR activation and tumor formation.

In the first part of this study we aimed to determine the role of mig-6 in human melanoma development. Interestingly, *mig-6* knock-out mice are highly susceptible to carcinogen-induced melanoma formation. Based on these observations we intended to analyze mig-6 expression in primary human melanoma, metastases and cell lines and to investigate its relation to HER3.

In the second part of this thesis we planned to analyze the role of mig-6 in hepatocyte proliferation *in vivo* and in human hepatocellular carcinomas. In order to investigate the function of mig-6 during these processes we intended to use *mig-6* knock-out mice and to apply partial hepatectomy which allows a detailed analysis of hepatocyte proliferation *in vivo*. In addition, we wanted to analyze primary human liver tumors and liver cancer cell lines.

3. Material and Methods

3.1 Materials

3.1.1 Laboratory chemicals

5'-dc-azacytidine	Sigma, Taufkirchen
Acrylamide	Serva, Heidelberg
Agar	Difco, USA
Agarose	BRL, Eggenstein
Ampicillin	Roche, Mannheim
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
ATP (Adenosine triphosphate)	Amersham Pharmacia, Freiburg
BSA (Bovine serum albumin)	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Crystal violet	Sigma, Taufkirchen
Dacarbazine	Sigma, Taufkirchen
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
DTT (Dithiothreitol)	Sigma, Taufkirchen
EGF	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Formaldehyde	PolySciences, Eppenstein
Geneticin (G418, GibCo)	Invitrogen, Eggenstein
HEPES (N-(2-Hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid))	Serva, Heidelberg
Humaninsulin® Normal 40	Lilly, Giessen
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Biomol, Hamburg
L-Glutamine (GibCo)	Invitrogen, Eggenstein
Leupeptin	Sigma, Taufkirchen
Lipofectamine® (GibCo)	Invitrogen, Eggenstein
Lysozyme	Sigma, Taufkirchen
Oligofectamine®	Invitrogen, Eggenstein
Penicillin/Streptomycin	Gibco, Eggenstein
Phenol	Roth, Karlsruhe
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
Polyfect®	Quiagen, Hilden
Ponceau S	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'-Tetramethylethylenediamine)	Serva, Heidelberg
TPA (Tetradecanoyl-phorbol-13-acetate)	Sigma, Taufkirchen
Triton X-100	Serva, Heidelberg
Tween 20	Sigma, Taufkirchen
Xylol	Merck, Darmstadt

All other chemicals were purchased in analytical grade from Merck (Darmstadt).

3.1.2 Enzymes

Calf Intestine Alkaline Phosphatase	MBI Fermentas, St. Leon-Rot
DNAse I, RNAse free	Roche, Mannheim
Restriction Endonucleases	NEB, Frankfurt/ Main
	MBI Fermentas, St. Leon-Rot
	Boehringer, Mannheim
	Biolabs, New England
Pfu DNA Polymerase	MBI Fermentas, St. Leon-Rot
Platinum Taq Polymerase	Invitrogen, Eggenstein
RNase A	Sigma, Taufkirchen
T4-DNA Ligase	Roche, Mannheim
Taq DNA Polymerase	MBI Fermentas, St. Leon-Rot
Trypsin	Invitrogen, Eggenstein

3.1.3 "Kits" and other materials

BigDye® Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems /Foster City
Cell culture materials	Greiner, Solingen
	Nunclon, Dänemark
	Falcon, UK
	Corning Incorporated, USA
Cellulose nitrate 0.45 µm	Schleicher & Schüll, Dassel
Enhanced Chemi Luminscent (ECL) Kit	PerkinElmer/NEN, Köln
Hyperfilm MP	Amersham Pharmacia, Freiburg
Matrigel	BD Biosciences, Pharmingen
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Poly Prep® Chromatography columns	Bio-Rad, München
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 µm, cellulose acetate	Nalge Company, USA
Sterile filter 0.45 µm, cellulose acetate	Nalge Company, USA
Whatman 3MM	Whatman, Rotenburg/Fulda

3.1.4 Growth factors and ligands

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

3.2 Media

3.2.1 Bacterial media

LB or 2xYT media were used for cultivation of all *Escherichia coli* strains. If and as required 100µg/ml ampicillin or 70µg/ml kanamycin were added to the media after autoclavation. For the preparation of LB-plates 1.5% Agar was added.

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

3.2.2 Cell culture media

Cell culture media and additives were obtained from Invitrogen (Eggenstein). Media were supplemented to the requirements of each cell line. Freeze medium contained 90% heat-inactivated FCS and 10% DMSO.

Dulbecco's Modified Eagle Medium (DMEM) with 4,5 mg/ml Glucose, 10% FCS, 2 mM L-Glutamine, 1 mM sodiumpyruvate, 1% Penicillin/Streptomycin

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

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

3.3 Stock solutions and commonly used buffers

Acrylamide solution (30/0,8%)	30% (w/v) Acrylamid 0.8% (w/v) Bisacrylamid
HNTG	20mM HEPES, pH 7.5 150mM NaCl 0.1% TritonX-100 10% Glycerol 10mM $Na_4P_2O_7$
DNA loading buffer (6x)	0.05% Bromphenol blue 0.05% Xylencyanol 30% Glycerol 100mM EDTA pH 8.0

Laemmli buffer (2x)	65mM Tris/HCl pH 6.8 2% SDS 30% Glycerol 0.01% Bromphenol blue 5% ß-Mercaptoethanol
Laemmli buffer (3x)	100mM Tris/HCl pH 6.8 3% SDS 45% Glycerol 0.01% Bromphenol blue 7.5% ß-Mercaptoethanol
NET	50mM Tris/HCl pH 7.4 5mM EDTA 0.05% Triton X-100 150mM NaCl
PBS	137mM NaCl 27mM KCl 80mM Na_2HPO_4 1.5mM KH_2PO_4 pH 7.4
SD-Transblot	50mM Tris/HCl pH 7.5 40mM Glycine 20% Methanol 0.004% SDS
"Strip" buffer	62.5mM Tris/HCl pH 6.8 2% SDS 100mM β-Mercaptoethanol
TAE	40mM Tris/Acetate pH 8.0 1mM EDTA
TE10/0.1	10mM Tris/HCl pH 8.0 0.1 mM EDTA pH 8.0
Tris-Glycine-SDS	25mM Tris/HCl pH 7.5 200mM Glycine 0.1% SDS

Triton X-100 lysis buffer	50mM HEPES, pH 7.5 150mM NaCl 1mM EDTA 10% Glycerin 1% Triton X-100 10mM $Na_4P_2O_7$ 2mM VaO_5 10mM NaF 1mM PMSF 100µg/l Aprotinin

3.4 Cells

3.4.1 Eukaryotic cell lines

Cell line	Description origin	Reference
HT-144	Human malignant melanoma	ATCC, USA
Colo 829	Human malignant melanoma	gift from H. Hermeking
MM-358	Human malignant melanoma	gift from H. Hermeking
Mel Juso	Human malignant melanoma	gift from H. Hadam
Mel Gerlach	Human malignant melanoma	gift from L. Ziegler
WM-1341D	Human malignant melanoma	gift from M. Herlyn
Hs294.T	Human malignant melanoma	ATCC, USA
HepG2	Human hepatocellular carcinoma	ATCC, USA
Hs 817.T	Human hepatocellular carcinoma	ATCC, USA

All cell lines used in this study were grown as recommended by the supplier.

3.4.2 E. Coli strains

E. Coli strain	Genotype	Origin
DH5αF'	F' endA1 hsd17 (rk-mk+) supE44 recA1 gyrA (Nal) thi-1 Δ(lacZYA-argF196)	Genentech, USA
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB lacIqZΔM15 Tn10 (Tetr)]	Stratagene, NL

3.5 Antibodies

3.5.1 Primary antibodies

Antibody	Immunogen origin	Reference
Akt1/2	Rabbit, polyclonal; AA 345-480 of human Akt1	Santa Cruz, USA
p-Akt/PKB	Rabbit, polyclonal; phospho-Akt (Ser-473); recognizes p-Akt of human, rabbit and rat origin	NEB, Frankurt/M.
ß-actin	Rabbit, polyclonal; directed against a C-terminal peptide	Sigma, Taufkirchen
Cyclin A	Rabbit, polyclonal; recognizes the full length human Cyclin A protein	Santa Cruz, USA
Cyclin B1	Mouse, monoclonal; peptide of murine Cyclin B1	Cell Signaling, MA
Cyclin D1	Mouse, monoclonal; protein fragment corresponding to AA 1-200 of human Cyclin D1	Transduction Labs
p-c-Jun	Rabbit, polyclonal; synthetic phospho-peptide between AA 54-66 of human c-Jun	Upstate, NY
EGFR	Sheep, polyclonal; part of cytoplasmic domain of the human EGFR	UBI, Lake Placid
EGFR (108.1)	Mouse, monoclonal/ ectodomain of the human EGFR	(Daub et al., 1997)
p-EGFR (Y-1173)	Rabbit, monoclonal; recognizes endogenous EGFR phosphorylated at Y1173	Cell Signaling, MA
ERK2 (C-14)	Rabbit, polyclonal; peptide at C-terminus of rat ERK2	Santa Cruz, USA

p-ERK	Rabbit, polyclonal; recognizes phospho-p44/p42 (Thr-202/ Tyr-204) MAPK	NEB, Frankurt/M.
HER2	Rabbit, polyclonal; peptide between AA 1243-1255 of human HER-2	Upstate, NY
HER2 (4D5)	Mouse, monoclonal; directed against the extracellular domain of human HER-2	Boehringer Ingelheim
p-HER2 (Y1248)	Rabbit, monoclonal; synthetic phospho-peptide between AA 1243-155	Upstate, NY
HER3 (2F12)	Mouse, monoclonal; peptide between AA 1295-1323 of human HER3	Upstate, NY
HER3 (1B4C3)	Mouse, monoclonal; directed against the extracellular domain of human HER3	E. van der Horst
HER3 (C-17)	Rabbit, polyclonal; directed against a c-terminal peptide of human HER3	Santa Cruz, USA
p-HER3 (Y1289)	Rabbit, monoclonal; recognizes phospho-Y1289 of human HER3	Cell Signaling, MA
KI-67	Mouse, monoclonal; peptide between AA 1547-1742 of human KI-67	Transduction Labs
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
p38	Rabbit, polyclonal; peptide at the C-terminus of mouse p38	Santa Cruz, USA
p-p38	Rabbit, polyclonal; recognizes phospho-(Thr 180/Tyr 182) p38	Cell Signaling, MA
PI3K p85	Rabbit polyclonal; recognizes the SH2-domain of human p85	UBI, Lake Placid
p-Rb	Rabbit, polyclonal; recognizes phospho-S780 of human Rb	Cell Signaling, MA

Tubulin	Mouse, monoclonal; ascites	Sigma, Taufkirchen
p-Tyr (4G10)	Mouse, monoclonal; recognizes phospho-(3)-tyrosine residues	UBI, Lake Placid
VSV (P5D4)	Mouse, monoclonal; recognises an epitope of eleven amino acids derived from the vesicular stomatitis virus glycoprotein VSV-G	Roche, Mannheim

3.5.2 Secondary antibodies

For immunoblot analysis corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) were used.

Antibody	**Dilution**	**Origin**
Goat anti-mouse-HRP	1:10.000	Sigma, Taufkirchen
Goat anti-rabbit-HRP	1:50.000	Bio-Rad, München
Goat anti-sheep-HRP	1:25.000	Jackson ImmunoResearch Labs, USA
Goat anti-mouse Fab fragment	1:25.000	Amersham Biosciences, UK
Goat anti-rabbit Fab fragment	1:25.000	Amersham Biosciences, UK

3.6 Plasmids and oligonucleotides

3.6.1 Primary vectors

Vector description	**Origin**	**Reference**
pcDNA3	Mammalian expression vector, Amp_r, CMV promoter, BGH pA, high copy number plasmid	Invitrogen, USA

3.6.2 Constructs

Vector description	**Origin**	**Reference**
pcDNA3-mig-6-VSV	cDNA of human mig-6 in pcDNA3; c-terminal VSV tag	This study

3.6.3 Oligonucleotides

Cloning of mig-6 in pCDNA3 VSV

Mig-6 fwd 5’ CGCGGATCCGCGGCCACCATGTCAATAGCAGGAGTTGCTGC 3’
Mig-6 rev 5’ CCGGAATTCCGGAGGAGAAACCACATAGGATAAATG 3’

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’

RT-PCR primers

Mig-6 fwd	5’ GGAAGACCTACTGGAGCAGTCGCAG 3’
Mig-6 rev	5’ TGAGATGGACCATTTTCTGCAAAGC 3’
Tubulin fwd	5’ AAGTGACAAGACCATTGGGGGAGG 3’
Tubulin rev	5’ GGGCATAGTTATTGGCAGCATC 3’
HER3 fwd	5’ CTCCGCCCTCAGCCTACCAGTT 3’
HER3 rev	5’ TGCTCCGGCTTCTACACATTGACA 3’
GAPDH fwd	5’ ACCACAGTCCATGCCATCAC 3’
GAPDH rev	5’ TCCACCACCCTGTTGCTGTA 3’
MMP-14 fwd	5’ CGCTACGCCATCCAGGGTCTCAAA 3’
MMP-14 rev	5’ CGGTCATCATCGGGCAGCACAAAA 3’
MMP-2 fwd	5’ ATGGCAAGGAGTACAACAGC 3’
MMP-2 rev	5’ GCTGGTGCAGCTCTCATATT 3’
MMP-1 fwd	5’ CGACTCTAGAAACACAAGAGCAAGA 3’
MMP-1 rev	5’ AAGGTTAGCTTACTGTCACACGCTT 3’
MMP-9 fwd	5’ GACGCAGACATCGTCATCCAGTTT 3’
MMP-9 rev	5’ GCCGCGCCATCTGCGTTT 3’

Genotyping primers (*mig-6* KO mice)

Mig-6_1 5´AGCCCAAGCTGATCCTCTAGAGTCG 3´
Mig-6_2 5´ACTGTGACTGCCCCAGCAGGAC 3´
Mig-6_3 5´ TTCCTGGTAGAGCCATGCCATAG 3´

The genotyping of *mig-6* KO mice was done as described previously (Ferby et al., 2006).

3.7 Methods of molecular cloning

3.7.1 Plasmid preparation

Small amounts of plasmid DNA were prepared using the Qiagen Plasmid Mini Kit, larger amounts of DNA were obtained with the Qiagen Plasmid Maxi Kit following the manufacturer's recommendations.

3.7.2 Enzymatic manipulation of DNA

3.7.2.1 Specific digestion of DNA samples by restriction endonucleases

The ratio of Enzyme/DNA, the temperature, the buffer and the time of incubation were adjusted according to manufacturer's recommendations. Usually, incubations were done for 2 hours at 37°C with a calculated 5-fold overdigestion and the buffers as supplied by the manufacturer were chosen.

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

For dephosphorylation, 1µg of cut vector DNA was incubated with 5 units CIAP in adequate reaction buffer (e.g. 50mM Tris/HCl pH 8, 0.1mM EDTA pH 8.5) at 37°C for 10 minutes. Either reactions were stopped by heat inactivation at 85°C for 10 minutes or DNA was directly purified using the QIAquick PCR Purification Kit.

3.7.2.3 Ligation of vector and insert DNA

Purified, digested and dephosphorylated vector DNA (40ng), the designated insert DNA, 1µl 10x T4 DNA Ligase buffer (0.66M Tris/HCl pH 7.5, 50mM $MgCl_2$, 50mM DTT, 10mM 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 competent bacteria.

3.7.2.4 Agarose gel electrophoresis

Depending on the size of the fragments of interest 0.7-2% agarose gels were prepared in horizontal chambers. TAE buffer was used for the electrophoresis. Voltage was usually set to 4-10 V per cm width of the gel. After separation DNA fragments were stained by gently agitating gels in TAE containing 0.5µg/ml ethidium bromide and were subsequently viewed under UV light.

3.7.2.5 Isolation of DNA fragments from agarose gels

Following gel electrophoresis gel slices bearing DNA fragments of interest were cut out of the gel. Agarose was dissolved and DNA was purified using the QIAquick Gel Extraction Kit according to manufacturer's recommendations.

3.7.3 Introduction of plasmid DNA into E.coli

3.7.3.1 Preparation of competent cells

The preparation of competent cells was according to the procedure described by Chung and Miller (Chung and Miller, 1988). Competent cells were shock frozen in liquid nitrogen and stored for up to one year at –70°C. Transformation frequency ranged between 10^5 and 10^7 colonies/ µg DNA.

3.7.3.2 Transformation of competent bacteria

A 50µl aliquot of competent bacteria was added to a 50µl mixture of DNA, usually ligation cocktails, 10µl 5x KCM solution (500mM KCl, 150mM $CaCl_2$, 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 while constantly shaking. Bacteria were streaked out on appropriate agar plates containing ampicillin for the selection of the transformants.

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

Pfu Polymerase (MBI Fermentas) was used for long and accurate cDNA amplification:

1 µl template cDNA, 1-10ng
1 µl "forward" oligonucleotide, 10pmol/µl
1 µl "reverse" oligonucleotide, 10pmol/µl
5 µl 10x PCR buffer
5µl 20mM $MgCl_2$
5 µl dNTP-Mix, 2.5mM each
1 µl Pfu DNA Polymerase (2.5U/µl)
ad 50µl H_2O

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)
30 cycles:
1 minute 95°C (denaturation)
1 minute Tm (hybridization at appropriate temperature)
5 minutes 72°C (extension)

PCR products were either separated by agarose gel electrophoresis, excised and subsequently purified with QIAquick Gel Extraction or directly purified by the PCR Purification Kit.

7.5 DNA sequencing

Sequencing of DNA 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 Analyzer (ABI Prism).

3.8 Methods of mammalian cell culture

3.8.1 Calcium-Phosphate transfection

Cells were maintained in appropriate culture media at 7.5% CO2 and 37°C. Transfections were carried out using a modified calcium phosphate method. Briefly, $2x10^6$ cells were incubated overnight in 3ml of growth medium. 2µg of plasmid DNA was mixed with water and 0.25M $CaCl_2$ solution in a final volume of 500µl. The mixture was added to the same volume of 2x transfection buffer (HBS) and incubated for 15 minutes at room temperature before it was added drop wise to the cells. After incubation for 12 hours at 37°C, the medium was replaced.

3.8.2 Transfection of plasmid DNA using lipofectamine®

Target cells were transiently transfected using Lipofectamine® (Gibco-BRL) as described previously (Daub et al., 1997). Briefly, cells were seeded in 6cm plates. 350µl of serum-free medium containing 7µl of Lipofectamine and 2µg of total plasmid DNA per well 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.

3.8.3 Transfection of siRNAs using oligofectamine®

HER3 and *GL-2* siRNAs were transiently transfected in cells using Oligofectamine® (Gibco-BRL) according to the manufacturer's recommendations. Briefly, 20pmol siRNA was mixed with the appropriate amount of OPTI-MEM medium, mixed with the oligofectamine reagent and incubated for 20 minutes at room temperature. The cells were washed once in OPTI-MEM containing 0% FCS. The mixture was put on the cells for 4 hours and thereafter the medium was changed to normal growth medium containing 10% FCS. Silencing efficiency was tested at different time-points after transfection by Western blot analysis.

3.8.4 Stimulation of cells

Cells were seeded in cell culture dishes of appropriate size and grown overnight to about 80% confluence. After serum-starvation for 24 to 48 hours cells were stimulated with appropriate growth factors, washed with cold PBS and then lysed for 10 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.

3.9 Methods of Biochemistry and Cell Biology

3.9.1 Lysis of cells with Triton X-100 lysis buffer

Cells were washed with cold PBS and then lysed for 10 minutes on ice in buffer containing 50 mM HEPES pH 7.5, 150mM NaCl, 1% Triton X-100, 1mM EDTA, 10% glycerol, 10mM sodium pyrophosphate, 2mM sodium orthovanadate, 10mM sodium fluoride, 1mM phenylmethylsulfonyl fluoride, and 10µg/mL aprotinin. Lysates were precleared by centrifugation at 13000 rpm for 10 minutes at 4°C.

3.9.2 Determination of total protein concentration in lysates

The overall protein concentration was determined using the Micro BCA Protein Assay Kit (Pierce, Sankt Augustin) according to the supplied standard protocol.

3.9.3 Immunoprecipitation

An equal volume of HNTG buffer was added to the precleared cell lysates that have been adjusted for equal protein concentration. Proteins of interest were immunoprecipitated using the respective antibodies and 20-50µL of protein A- or G-Sepharose over night at 4°C.
Precipitates were washed three times with 0.5ml of HNTG buffer, suspended in 3x SDS sample buffer, boiled for 5 minutes, and subjected to Western blot analysis.

3.9.4 SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE)

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

Protein	MW (kD)	Protein	MW (kD)
Myosin	205	Ovalbumin	42.7
ß-Galactosidase	116.25	Carboanhydrase	29
Phosphorylase b	97.4	Trypsin-Inhibitor	21.5
BSA	66.2	Lysozym	14.4

3.9.5 Transfer of proteins on nitrocellulose membranes

For immunoblot analysis proteins were transferred to nitrocellulose membranes (Gershoni and Palade, 1982) for 3 hours at 0.8mA/cm2 using a "Semidry"-Blot device in the presence of Transblot-SD buffer. Following transfer proteins were stained with Ponceau S (2g/l in 2% TCA) in order to visualize and mark standard protein bands. The membrane was destained in water.

3.9.6 Immunoblot detection

After electroblotting the transferred proteins are bound to the surface of the nitrocellulose membrane, providing access for reaction with immunodetection reagents. Remaining binding sites were blocked by immersing the membrane in 1x NET, 0.25% gelatine or 5% milk, TBS-T for at least 4 hours. The membrane was then probed with primary antibody overnight at 4°C. Antibodies were diluted 1:500 to 1:2000 in NET, 0.25% gelatine or 1% BSA, TBS-T. The membrane was washed 3x 20 minutes in 1x NET, 0.25% gelatine or TBS-T, incubated for 1 hour with secondary antibody and washed again as before.
Antibody-antigen complexes were identified using horseradish peroxidase coupled to the secondary anti-IgG antibody. Luminescent substrates were used to visualize peroxidase activity. Signals were detected with X-ray films. Membranes were stripped of bound antibody by shaking in strip-buffer for 1 hour at 50°C. Stripped membranes were blocked and reprobed.

3.9.7 RNA isolation and RT-PCR analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden) and reverse transcribed using AMV Reverse Transcriptase (Roche, Mannheim). 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 5x 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). PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ) and 1µl RT-PCR products were used for PCR amplification according to the manufacturer's recommendations. PCR products were subjected to electrophoresis on 1.5-2% agarose gels and DNA was visualized by ethidium bromide staining.

3.9.8 Northern and Southern blot analysis

For analysis of *mig-6*, *EGFR*, *HER-2* and *HER3* messenger RNA and of *HER3* gene copy number in human melanoma cell lines a northern or southern blot standard protocol was used according to Sambrook et al. (1990). Loading of RNA and DNA samples was verified by rehybridization of the filters with a human *β-actin* probe.

3.9.9 Proliferation assay

75.000 or 250.000 cells were seeded in 24 well or 6cm plates and transfected with *HER3* or *GL-2* siRNAs using oligofectamine (Invitrogen). The cells were either grown in the presence of medium containing 10% FCS or serum-starved in medium containing 1% FCS and stimulated with 100ng/ml heregulin ß1. The cell number was counted (Coulter counter, Beckton Dickinson) at the indicated time points. The data are shown as mean ± SDM.

3.9.10 Migration assay

200.000 cells were seeded in 6 well plates and transfected with *HER3* or *GL-2* siRNAs the next day. The cells were serum-starved and 25.000 cells were seeded on to a membrane with 8µM pores of a modified boyden chamber (Schubert and Weiss) containing 400µl serum-free medium. The lower chamber was filled with 600µl medium containing 10% fetal calf serum or 100ng/ml heregulin ß1 serving as chemo attractant. The cells were allowed to migrate for 20 hours through the pores and were then stained by crystal violet. Stained cells were washed in PBS and pictures were taken on a Zeiss Axiovert 300 microscope (Carl Zeiss, Jena). For spectrophotometric measurement of stained cells the membranes were incubated for 1 hour in 5% acetic acid to dissolve the crystal violet and thereafter measured in an ELISA reader (Bio-Rad) at 570 nm.

3.9.11 Cell branching and Invasion assay

For cell branching assays, 200.000 cells were seeded in 6 well plates and transfected with *HER3* or *GL-2* siRNAs the next day. 96 well plates were coated for at least 3 hours with 50µl matrigel (contains laminin, collagen type IV, heparan sulfate proteoglycan and entactin; BD Biosciences) diluted in RPMI medium. 24 hours later 10.000 cells were seeded in triplicates on to the matrigel in medium containing 10% FCS. The cells were allowed to invade the matrix for one week. The medium was changed every second day during the assay. Bright field pictures were taken on a Zeiss Axiovert 300 microscope (Carl Zeiss, Jena). The invasion assays were done like the migration assays with one exception. The cells were seeded onto growth factor-reduced matrigel in a modified boyden chamber (Schubert & Weiss) and allowed to invade the matrix for 24 hours.

3.9.12 HER3 blocking antibody – Proliferation, Migration and Invasion assay

The HER3 blocking antibody (cl. 105.5) was purchased from Upstate, NY. The second HER3 blocking antibody (cl. 2D1D12) was generated in the Department of Molecular Biology at the Max-Planck Institute of Biochemistry. The HER2 blocking antibody (cl. 4D5) used was the non-humanized form of trastuzumab (Herceptin; Genentech/Roche).

The proliferation assay was performed using a MTT-Assay. Briefly, 7.500 cells were seeded in 48 well plates. The cells were serum-starved in medium containing 1% FCS for 24 hours, pre-incubated with either 10µg/ml HER3 blocking antibody (cl. 105.5) or an isotype control antibody for 1 hour and stimulated with 100ng/ml heregulin ß1. The cells were allowed to grow for 48 hours and at that time point, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolimbromide; thizolyl blue, Sigma, Taufkirchen) was added to each well at a final concentration of 1mg/ml. The plates were incubated for 2 hours. The yellow MTT dye is reduced by mitochondrial dehydrogenase activity to a purple formazan, which was then solubilized (SDS, 2-Butanol and HCl) and absorbance was measured at 570nm on a micro-

plate reader. MDA-MB 468 breast cancer cells were already described to be inhibited by the anti-HER3 antibody (cl. 105.5; van der Horst et al., 2005) and served as a positive control. The data are shown as percent of inhibition relative to control cells.
The migration and invasion assays were performed as described previously (Albini et al., 1987; van der Horst et al., 2005). Briefly, 300.000 cells were seeded in 6cm plates and serum-starved in medium containing 0.1% FCS for 24 hours. 200.000 cells per ml were incubated with 10µg/ml HER3 blocking antibody or an isotype control antibody for 1 hour and 50.000 cells were then seeded either on to a membrane or on to a growth factor-reduced matrigel coated membrane with 8 µM pores of a modified boyden chamber (Schubert and Weiss) containing 500µl serum free medium. Conditioned NIH 3T3 medium containing 0.01% ascorbic acid and heregulin ß1 (100ng/ml) were used as a chemo attractant. The cells were allowed to migrate or invade for 20 or 24 hours, respectively. Migrated or invaded cells were stained by crystal violet, washed in PBS and analyzed using a Zeiss Axiovert 300 microscope. For quantification at least 10 random fields were counted. The values for control cells were set to 100% and results are shown relative to controls. The data are presented as mean ± SDM.
To asses the HER3 phosphorylation state, cells were serum-starved for 24 hours, incubated with 10µg/ml blocking antibody for 1 hour, stimulated with 50ng/ml heregulin ß1 for 2 hours, lysed and subjected to immunoprecipitations using a specific HER3 antibody.

3.9.13 Apoptosis assay and cell cycle analysis by propidium Iodide staining

200.000 cells were seeded into 6 well plates and transfected with *HER3* or *GL-2* siRNAs the next day. 24 hours later apoptosis was induced by adding either 10 or 20µM dacarbazine in DMSO to the medium. After 48 hours the supernatant of each reaction was collected and the cells were trypsinized. After centrifugation the cells were incubated for 2 hours in a propidium-iodide buffer (0.1% Na-citrate, 0.1% Triton X-100, 20µM propidium-iodide) and thereafter subjected to flow cytometric analysis (Beckton-Dickinson Biosciences). Cell cycle profiles and apoptosis were determined using the Cell Quest Pro software (Beckton Dickinson Biosciences).

3.9.14 Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitations (ChIP) were performed as described (Villa et al., 2006; Buschbeck et al., 2007). Briefly, the cells were crosslinked with 1% formaldehyde at room temperature for 8 minutes and the reaction was stopped by the addition of 0.125M glycine. Cells where then rinsed two times with cold PBS, resuspended in lysis buffer (1% SDS, 10mM EDTA pH 8, 50mM Tris-HCl pH 8) and sonicated with the Bioruptor (Biogenide). Lysates were diluted ten times with the IP buffer (1% Triton X-100, 150mM NaCl, 2mM EDTA pH 8, 20mM Tris-HCl pH 8) and then incubated overnight with 5µg of each antibody. 40µl of protein A sepharose beads saturated with salmon sperm (Upstate) were added to the lysates for 2 hours and then washed for four times with Wash buffer 1 (1% Triton X-100, 0,1% SDS, 150mM NaCl, 2mM EDTA pH 8, 20mM Tris-HCl pH 8) and one time with Wash buffer 2 (1% Triton X-100, 500mM NaCl, 2mM EDTA pH 8, 20mM Tris-HCl pH 8). The immunoprecipitated DNA was quantified by real time quantitative PCR (Roche LightCycler). For the mig-6 promoter an 83 bp fragment located between -357 and -276 was amplified. Quantification of ChIP results was performed relative to the input amount according to (Frank et al., 2001), and setting the reference samples to the arbitrary value of 100. The sequences of the PCR primers for mig-6 were: mig-6_ChIP_fwd 5’ GCGATGTGCCCTCAGTTC 3’ and mig-6_ChIP_rev 5’ CTCTCAACGCCGAGCTTTC 3’.

3.9.15 5-dC-Azacytidine treatment of melanoma cells

HT-144, Colo 829, Hs294.T and WM-1341D melanoma cells were seeded in 6cm plates, treated with 1µM 5-dC-Azacytidine for 72 hours, lysed and 50µg of protein was subjected to Western blot analysis.

3.9.16 Indirect flow cytometry

500.000 cells were seeded in 10cm dishes and transfected with *HER3* or *GL-2* siRNAs the next day. 24 hours later the cells were collected using 10mM EDTA and dissolved in 1ml 3% FCS in PBS. The cell number was adjusted to 250.000 cells per reaction and cells were incubated for 30 minutes with 10µg/ml of each primary antibody at 4°C. The cells were washed 3 times in 3% FCS/PBS and incubated with a PE-labelled secondary (1:1000) antibody at 4°C for 30 minutes. After three more washes in 3% FCS/PBS the fluorescence intensity was measured in a flow cytometer (Beckton Dickinson Biosciences) and analyzed using the Cell Quest Pro software.

3.9.17 Stable isotope labelling by amino acids in cell culture (SILAC) and mass spectrometry

Mig-6 wildtype and knock-out mouse embryonic fibroblasts were grown in SILAC medium containing light or heavy amino acids for two weeks (6 passages). The cells were starved for 24 hours, stimulated with 100ng/ml EGF for 4 hours, lysed and filtered using a sterile 45µm filter. Next, mig-6 was immunoprecipitated using three different antibodies from wildtype and knock-out cells. The samples were pooled and prepared for mass spectrometry as described previously (Selbach et al., 2006). The samples were analyzed by online liquid chromatography–tandem mass spectrometry (LC-MS/MS) on a LTQ-Orbitrap mass spectrometer (Thermo Electron). The identified peptides were assigned to proteins using Mascot (Matrix Science) and quantified with MSQuant.

3.10 Methods of mouse genetics

3.10.1 Mice and gene targeting

The animals used in this study were kept in a barrier facility at the Max-Planck Institutes in Martinsried, Germany. The *mig-6* knock-out mice were generated using standard ES cell homologous recombination and blastocyst injection techniques as described previously (Ferby et al., 2006). Three different targeted ES cell clones were transmitted through the germline by crossing with wildtype C57BL/6 mice. Second to forth generation crosses into C57BL/6 were used for all the experiments.

3.10.2 2-stage skin carcinogenesis

5-7 week-old knock-out and littermate control mice were treated with DMBA and TPA as described previously (DiGiovanni et al., 1994).

3.10.3 Isolation of mouse embryonic fibroblasts (MEFs)

Mouse embryonic fibroblasts were isolated from E13.5 embryos as described previously (Conner et al., 2001). The cells were cultured in DMEM high glucose containing 10% FCS, 1% L-Glutamine and 1% Penicillin/Streptomycin.

3.10.4 Partial hepatectomy

For partial hepatectomy all mice were between 6 and 10 weeks of age. The surgery was done as described previously (Stepniak et al., 2006). Briefly, the mice were anesthetized with avertin. Next, the abdominal cavity was opened with a transverse incision below the rib cage

and the large left lateral and median lobes were ligated and removed. The abdominal wall was closed and the mice were recovered on a 37°C heat block. Livers were collected during partial hepatectomy (time point: 0 hours) and at indicated time points after the surgery. For RNA and protein isolation, liver pieces were snap frozen in liquid nitrogen. For histological analysis, livers were fixed in 4% para-formaldehyde overnight at 4°C and stored in 70% ethanol at 4°C.

3.10.5 KI-67 Immunohistochemistry on mouse liver sections

Fixed liver tissues were embedded in paraffin and 5µM sections were used for immunohistochemistry. The KI-67 staining was done as described previously (Stepniak et al., 2006). The percentage of KI-67 positive cells was quantified by counting hepatocytes in at least 15 random fields using a 20x objective. Sections from at least 5 individual animals for each genotype were used and the data are shown as mean ± SDM.

3.10.6 Primary hepatocytes/two-step collagenase perfusion

Primary hepatocytes were isolated using the two-step collagenase perfusion technique as described previously (Eferl et al., 2003).

3.10.7 DEN-induced liver carcinogenesis

Mig-6 knock-out and control animals were subjected to chemically-induced liver carcinogenesis as described previously (Maeda et al., 2005).
Briefly, fourteen-day-old knock-out mice and littermates on a mixed C57BL/6 x SV129 were injected intraperitoneally (i.p.) with 5 mg/kg DEN. After 6 and 8 months all mice were sacrificed and their livers removed and separated into individual lobes. Externally visible tumors were counted macroscopically. The large liver lobes were fixed in 4% para-formaldehyde for further investigations.

3.11 Patients

3.11.1 Melanoma patients

Formalin-fixed, paraffin embedded tissue of 130 primary cutaneous melanoma and 87 melanoma metastases were immunohistochemically analyzed for HER3 and mig-6. The patient age ranged from 19 to 90 years. Clinical follow-up was available in all of the patients (mean clinical follow up was 56 ± 25 months). There were 60 nodular (NMM), 42 superficial spreading (SSM), 3 lentiginous (LMM), 9 acral lentiginous (ALM) and 16 not otherwise specified (NOS) melanoma. All melanoma had a Breslow tumor thickness between 0.4 and 17 mm. 53 of 130 patients (41%) had metastases during follow up and 24 of 130 patients (18%) died. Matched tumor samples of primary melanoma and metastases were available for 20 patients. 54 of the 130 patients with primary cutaneous melanomas were previously reported in a sentinel lymph node study (Mihic-Probst et al., 2006).

3.11.2 Melanoma tissue microarray construction and immunohistochemistry

A morphologically representative region of a paraffin "donor" block was chosen to prepare the melanoma tissue microarrays. The representative region was taken with a core tissue biopsy (diameter: 0.6mm; height: 3-4mm) and precisely arrayed into a new "recipient" paraffin block using a customer built instrument (Kononen et al., 1998). After the block construction was completed, 4µm sections of the resulting tumor tissue microarray block were cut with a microtome and used for further analysis.
HER3, mig-6 and KI-67 immunohistochemistry was performed using a Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, Arizona). For antigen retrieval, slides were heated with cell conditioner 1 (standard procedure). Endogenous biotin was blocked with the appropriate kit. Primary antibodies against HER3 (Santa Cruz, clone C-

17; dilution 1:50), mig-6 (Hackel et al., 2001; dilution 1:150) and KI-67 proliferation antigen (clone MIB-1; dilution 1:20) were applied and revealed with the iVIEW DAB detection kit, yielding a brown reaction product. The signal was enhanced with the Ventana amplification kit. Slides were counterstained with haematoxylin prior to glass cover-slipping. The specificity of the staining was controlled by using iso-type antibody controls, secondary antibody controls or blocking peptides. The analysis of the tissue micro arrays was done using a Zeiss Axiovert 300 microscope.

3.11.3 Evaluation of HER3 and mig-6 expression in melanoma

To determine the expression frequencies of HER3 and mig-6, a semi-quantitative scoring system was applied following the German Immunohistochemical scoring (GIS) system in which the final immuno-reactive score equalled the product of the percentage of positive cells times the average staining intensity. Percentage of positive cells was graded as follows: 0 = negative, 1 = up to 10% positive cells; 2 = 11 to 50%; 3 = 51 to 80%; 4 = >80%. Staining intensity of 0 = negative; 1 = weakly positive; 2 = moderately positive; 3 = strongly positive (Shim et al., 2003; Khoury et al., 2006; Hutterer et al., 2007). The extent of KI-67 staining was recorded as the number of marked nuclei (cells) per 100 melanoma cells. All stainings were evaluated by two different investigators (M.R. and D.M.-P.).

3.11.4 Statistical analysis

The protein expression frequency for HER3 and mig-6 was analyzed by dividing the GIS score in three groups with GIS 1-4 = no/low, GIS 5-8 = moderate and GIS 9-12 = high expression. For statistical analysis, two groups were considered which were divided through GIS-dichotomization at the median (low: GIS ≤6 and high: GIS >6). The overall survival of melanoma patients was estimated by the Kaplan Meier method and differences between groups were assessed by the log-rank test. The overall survival was defined as the time of primary tumor diagnosis to the last follow-up visit or patient death. All p-values were calculated using the two-sided Fisher's exact test and p-values < 0.05 were considered statistically significant. A stepwise multivariable Cox regression model was adjusted, testing the independent prognostic relevance of HER3. The limit for reverse selection procedures was $p = 0.2$. The proportionality assumption for all variables was assessed with log-negative-log survival distribution functions. For the analysis of matched tumor-metastases pairs, the GIS values of HER3 and mig-6 in primary melanoma and melanoma metastases were directly compared and analyzed. The statistical analysis was performed with the SPSS 12.0 software (SPSS Inc., Chicago, IL).

3.11.5 HCC patients, tissue micro array construction and immunohistochemistry

Formalin-fixed, paraffin embedded tissue of 111 primary human hepatocellular carcinomas was immunohistochemically analyzed for EGFR and mig-6.

The tissue samples of hepatocellular carcinomas used in this study were from the archives of the Institute of Pathology of the Ludwig-Maximilian-Universtität München. Study outlines conformed to the guidelines of the local ethics committee. The tissue micro arrays were prepared using 1mm punch sets and a tissue arraying instrument (Beecher Instruments, Silver Spring, MD). Serial 5µm sections were prepared for immunohistochemical staining. For mig-6 immunohistochemistry, the sections were deparaffinized and pre-treated in Retrievit 4 (DCS) in a microwave at 750W for 30 minutes. Next, endogenous peroxidases were blocked with 7.5% H_2O_2 for 10 minutes at room temperature. The mig-6 primary antibody (rabbit polyclonal cl. 1573; homemade; dilution 1:200) was applied for 60 minutes at room temperature and revealed with the ImmPRESS anti-rabbit Ig detection system (Vector Laboratories) according to manufacturer's

recommendations. Slides were counterstained with haematoxylin (Vector Laboratories) and AEC (Zytomed Systems) was used as chromogen. The specificity of the staining was controlled by using iso-type antibody controls and non-immunized rabbit serum.
EGFR immunohistochemistry was performed using a Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, Arizona). For antigen retrieval, slides were pre-treated with Protease 1 (Ventana Medical Systems, Tucson, Arizona) for 4 minutes. The primary antibody against EGFR (Ventana Medical Systems; mouse monoclonal; cl. 36C) was applied and revealed with the XT ultra View DAB detection kit (Ventana Medical Systems, Tucson, Arizona), yielding a brown reaction product. Slides were counterstained with haematoxylin prior to glass cover-slipping.

3.11.6 Evaluation of EGFR and mig-6 expression in HCCs

Mig-6 and EGFR protein expression frequencies in HCCs were determined according to their staining intensities. Stained tumors were scored as follows: 0 = negative; 1 = weakly positive; 2 = moderately positive; 3 = strongly positive. The correlation between EGFR and mig-6 was analyzed by comparing the expression values of both proteins in each tumor directly. The correlations between mig-6 and EGFR were calculated using the Spearman's rank test. P-values were calculated using the two-sided Fisher's exact test or the paired Student's T-test and p-values < 0.05 were considered statistically significant. The statistical analysis was performed with the SPSS 12.0 software (SPSS Inc., Chicago, IL).

4. Results

4.1 The Role of Mig-6 and HER3 in Melanoma

4.1.1 *Mig-6* knock-out mice are highly susceptible to TPA/DMBA induced melanoma formation

Mig-6 is a negative regulator of EGFR-dependent skin morphogenesis and tumor formation *in vivo* (Ferby et al., 2006). Specifically, *mig-6* knock-out mice are highly susceptible to TPA/DMBA-induced skin carcinogenesis. Here, *mig-6* knock-out mice start to develop skin papillomas already two weeks after TPA/DMBA treatment when compared to wildtype control mice. Interestingly, developing and established papillomas can be efficiently reduced through the application of the EGFR small molecule inhibitor gefitinib (Iressa) suggesting that skin tumor development in *mig-6* knock-out mice is dependent on EGFR signalling (Ferby et al., 2006). Importantly, eight weeks after TPA/DMBA treatment *mig-6* knock-out mice start to develop large melanomas in back skin regions (Figure 13A and C). These melanomas only developed in knock-out mice and could never be seen in wildtype controls. Strikingly, developing melanoma cells already metastasized to the lymph nodes in *mig-6* knock-out mice (Figure 13B) suggesting that mig-6 may be involved in the suppression of melanoma development and progression.

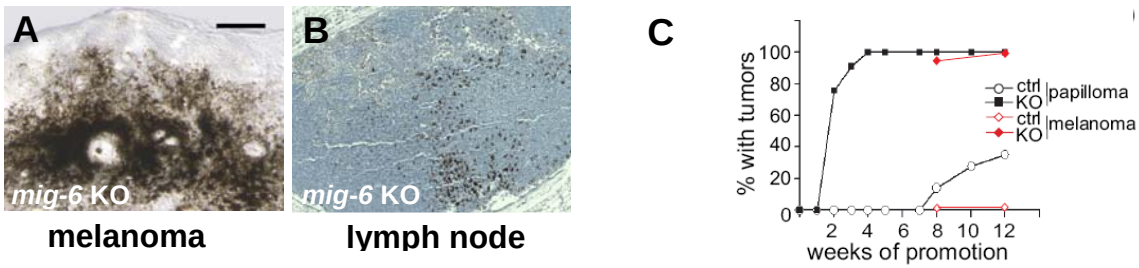


Figure 13. *Mig-6* knock-out mice are highly susceptible to TPA/DMBA induced melanoma formation
(A) Shaved skins of groups of mice (n=12) were treated once with 25nM DMBA and starting 1 week later every second day with 10nM TPA (in acetone) for the duration of the experiment. A skin section of a *mig-6* knock-out melanoma is shown.
(B) Melanoma cell metastasis to the lymph nodes in *mig-6* knock-out mice (note black cells). A cross section of a lymph node is shown.
(C) Time course of papilloma and melanoma formation in control (wildtype and heterozygotes; open circles) and knock-out mice (filled squares) represented as percentage of mice carrying visible papilloma and melanoma (The figure was adapted from Ferby et al., 2006).

4.1.2 Mig-6 is downregulated in human melanoma cell lines, primary malignant melanoma and metastases

Based on the data from *mig-6* knock-out mice we aimed to determine the role of mig-6 in human melanomagenesis. To do this we first analyzed human melanoma cell lines for the expression of *mig-6* by Northern blot. Strikingly, *mig-6* mRNA was absent or barely detectable in 39 out of 52 (75%) human melanoma cell lines and in 5 out of 6 mouse xenograft lines (83%; Figure 14) suggesting that mig-6 may be a tumor suppressor in melanoma. To further analyze mig-6 expression in primary human melanoma we analyzed mig-6 protein expression immunohistochemically in 130 primary melanoma and 87 metastases using tissue micro arrays. Strikingly, the characteristically cytosolic mig-6 staining (Figure 15A) was undetectable in the vast majority of primary melanoma (75%) and metastases (81%; Figure 15B and C) further supporting the conclusion that mig-6 might be a novel tumor suppressor of melanomagenesis.

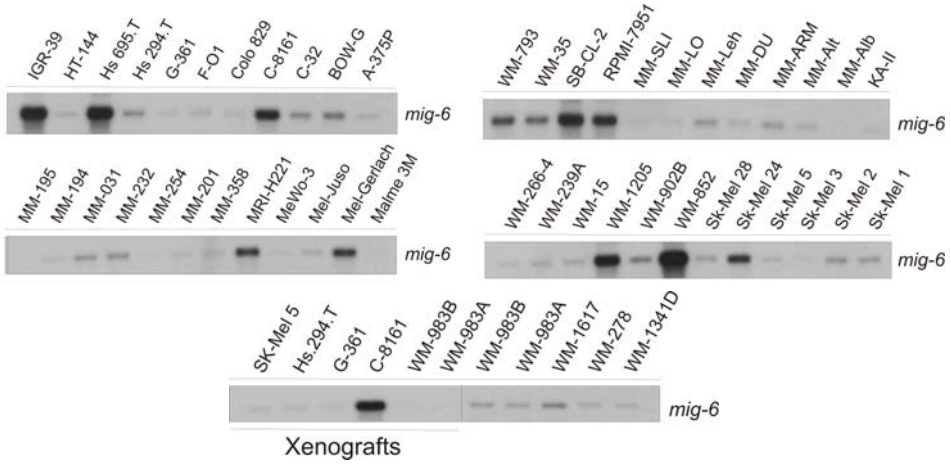


Figure 14. *Mig-6* is downregulated in human melanoma cell lines
Mig-6 mRNA expression was analyzed by Northern blot in a panel of 52 human melanoma cell lines and 6 mouse xenografts.

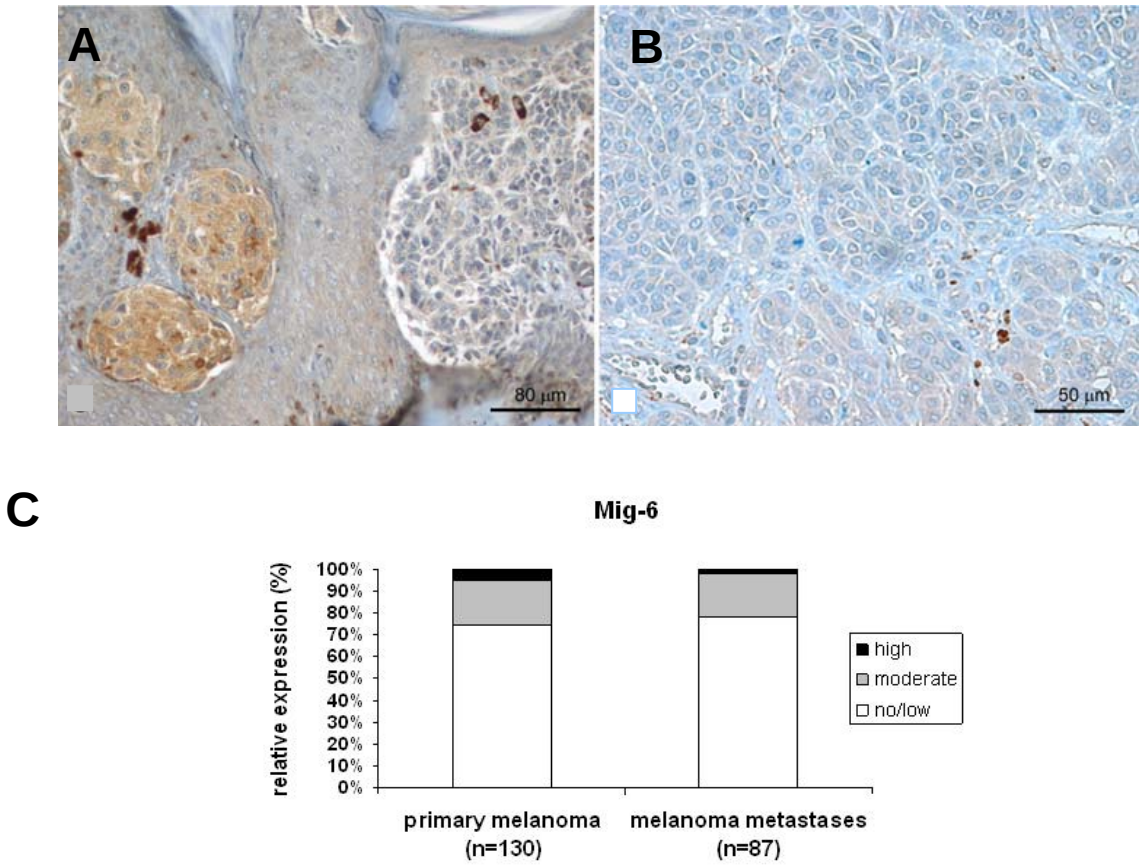


Figure 15. Mig-6 is downregulated in primary malignant melanoma and metastases
Mig-6 protein expression was analyzed immunohistochemically in 130 primary malignant melanoma and 87 melanoma metastases using a specific antibody and melanoma tissue micro arrays.
(A) Moderate and low mig-6 expression in primary malignant melanoma.
(B) Lack of mig-6 expression in melanoma metastases.
(C) Mig-6 expression frequencies in primary malignant melanoma and metastases.

4.1.3 *Mig-6* seems to be epigenetically silenced in melanoma cell lines

In order to address the mechanism of mig-6 inactivation in melanoma cells we analyzed cell lines with and without expression of the mig-6 protein. As shown in Figure 16A, mig-6 was expressed in Du-145 cells but barely detectable in HT-144, WM-1341D, MM-358 and Colo 829 melanoma cells. Since this expression pattern was also reflected on the level of *mig-6* mRNA, this pointed towards transcriptional regulation. During the last years it has become increasingly clear that epigenetic modifications such as histone and DNA methylation occur during gene repression and that they can account for the inactivation of tumor suppressor genes in cancer (Di Croce et al., 2004). First, we analyzed the proximal promoter of the *mig-6* gene for the presence of three histone H3 modifications: the repressive trimethylation of Lysines 9 and 27 (K9 and K27), and for the antagonizing acetylation of Lysines known to

facilitate transcription. Chromatin immunoprecipitation analysis showed that repressive K27 and K9 methylation marks where present at the *mig-6* promoter only in the two mig-6 negative cell lines HT-144 and WM-1341D, concomitantly with reduced levels of H3 acetylation (Figure 16B). In contrast, the mig-6 expressing cell line Du-145 exhibited strong H3 acetylation levels, and lacked repressive H3K9 and H3K27 methylation marks.

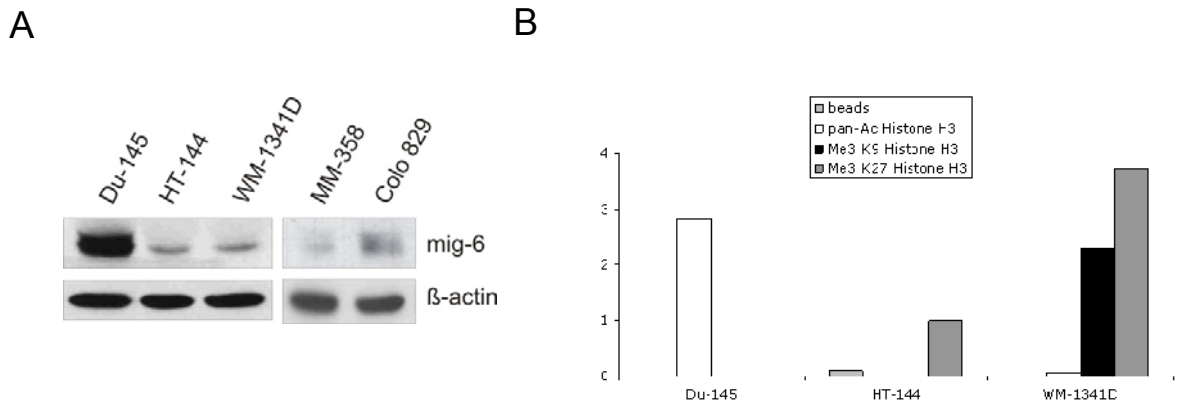


Figure 16. *Mig-6* seems to be epigenetically silenced in human melanoma cell lines
(A) The expression levels of mig-6 in Du-145, HT-144, WM-1341D, MM-358 and Colo 829 melanoma cells is shown by Western blot. ß-actin served as a loading control.
(B) Chromatin immunoprecipitation (CHIP) using either methylation specific antibodies (Histone H3 Lysine 9 and Histone H3 Lysine 27), a pan-Histone H3 acetylation specific antibody or beads alone were amplified with primers specific for the proximal *mig-6* promoter.

4.1.4 *Mig-6* silencing seems to be mediated by DNA methylation and the polycomb-repressive complex 2

Importantly, the methylation of K27 can be catalyzed by the polycomb-repressive complex 2 (PRC2; Villa et al., 2007) which has been implicated in cancer development (Sparmann et al., 2006). In order to address whether PRC2 contributes to the inactivation of *mig-6*, we knocked-down *Suz12*, an essential component of the PRC2 complex (Cao et al., 2004) by RNA interference in HT-144 and WM-1341D cells. As shown in Figure 17A, knock-down of *Suz12* correlated with a reduction of global K27 trimethylation levels. Under these conditions, we observed a strong reactivation of *mig-6* expression in HT-144 cells indicating that the PRC2 complex is potentially involved in the repression of the *mig-6* locus. In contrast, in WM-1341D cells we did not observe reactivation of *mig-6* expression upon *Suz12* suppression (Figure 17A). This could be partly due to the fact that we additionally detected trimethylation of lysine 9 on histone H3

which may add another layer of epigenetic regulation. DNA methylation is well established to contribute to the inactivation of tumor suppressor genes in a wide variety of cancers (Esteller M. 2007) and, importantly, has recently been linked to the function of PRC2 (Vire et al., 2006; Villa et al., 2007; Schlesinger et al., 2007). Since the *mig-6* promoter contains a cluster of potentially methylated CpGs we treated melanoma cell lines with the DNA methyltransferase inhibitor 5-dC-Azacytidine. Upon this treatment, HT-144, WM-1341D, Colo 829 and Hs294.T melanoma cells reactivated mig-6 expression (Figure 17B). In summary, our data provide some evidence that epigenetic mechanisms may account for the repression of the *mig-6* gene during melanoma development. Different epigenetic pathways including the polycomb-repressive complex 2 and DNA methylation seem to contribute to the loss of *mig-6* which in turn could facilitate HER3-mediated tumor development and progression.

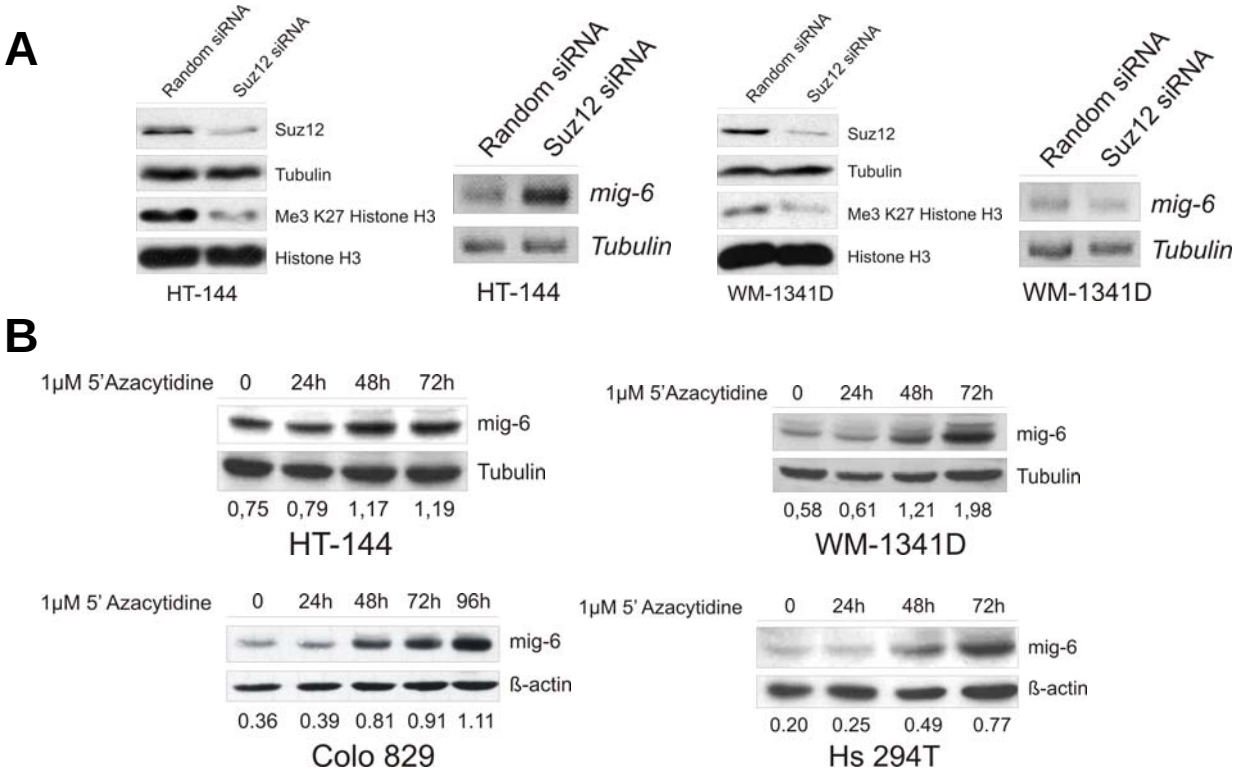
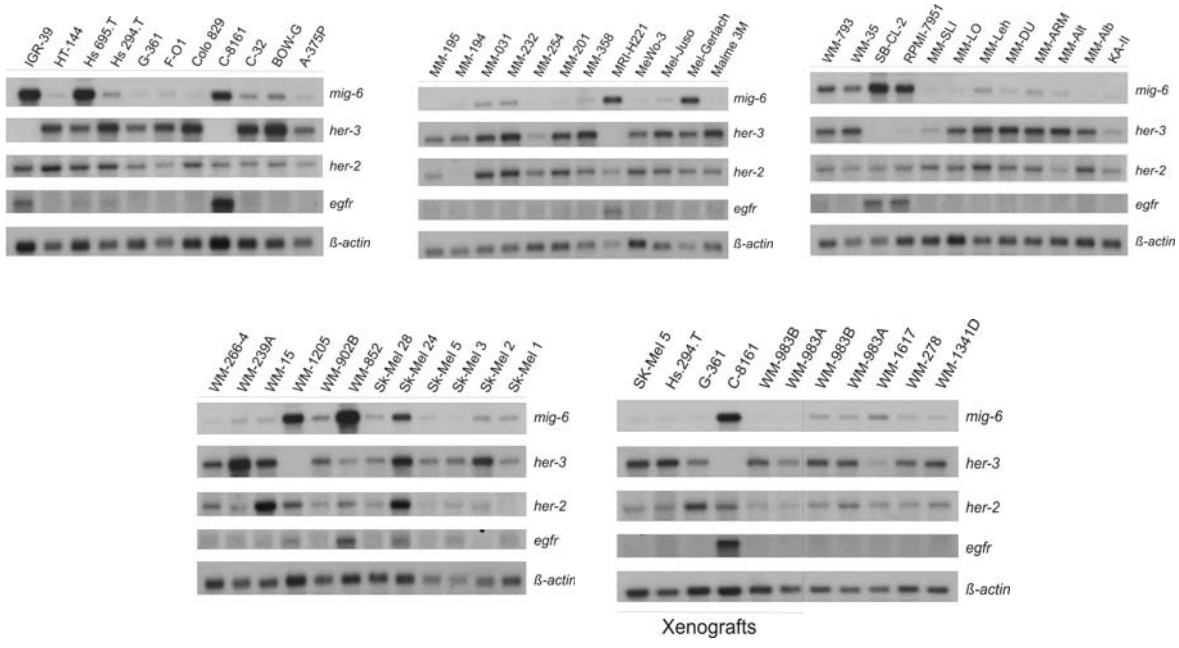


Figure 17. Epigenetic silencing of *mig-6* seems to involve DNA methylation and the polycomb-repressive complex 2 (PRC2)
(A) *Suz12* knock-down in HT-144 and WM-1341D melanoma cells. The cells were retrovirally infected with a *Suz12* short hairpin RNA construct and analyzed by Western blot. Immunoblots are shown for Suz12, trimethylated Lysine 27 on Histone H3, Histone H3 and Tubulin. The effect of *Suz12* knock-down on *mig-6* transcription is shown by RT-PCR for *mig-6* and *Tubulin*.
(B) Mig-6 expression upon 5-dc-Azacytidine treatment of WM-1341D, HT-144, Colo 829 and Hs294.T melanoma cell lines. All cell lines were seeded in 6cm tissue culture dishes and treated with 1µM 5-dc-Azacytidine for the indicated time points. Western blots are shown for mig-6 and Tubulin. The protein expression was quantified using the AIDA software and values normalized for Tubulin are shown.

4.1.5 Mig-6 levels inversely correlate with HER3 expression in melanoma cell lines

Mig-6 seems to be epigenetically inactivated in human melanoma cell lines. Based on these findings we aimed to clarify whether loss of *mig-6* could lead to the activation of the HER-family of receptor tyrosine kinases. To answer this question we analyzed 52 human melanoma cell lines and 6 mouse xenograft lines for the expression of *mig-6, EGFR, HER2* and *HER3* by Northern blot. Interestingly, we found that *HER3* is frequently expressed in melanoma cell lines (Figure 18A) whereas *HER2* and especially the *EGFR* were only marginally expressed. Strikingly, in the majority of cases *mig-6* expression inversely correlated with *HER3* expression (Figure 18B). These data suggest that loss of *mig-6* can lead to increased HER3 expression and thereby might facilitate tumor development. To further strengthen this hypothesis and to clarify whether loss of *mig-6* also leads to HER3 activation, we analyzed mig-6, p-HER3 and HER3 expression in 20 human melanoma cell lines by Western blot (Figure 18C). Yet again, we observed an inverse correlation of mig-6 and HER3 protein expression and, most importantly in some cell lines, an inverse correlation of mig-6 and phosphorylated HER3 indicating that loss of mig-6 may lead to HER3 activation. Interestingly, *mig-6* but not *HER3* is expressed in human epidermal melanocytes (Figure 18D) suggesting that loss of mig-6 and HER3 overexpression specifically occurs at some point during melanoma development. The mechanism of HER3 overexpression appears to be increased gene transcription since Southern blot analysis of 54 human melanoma cell lines did not reveal *HER3* gene amplification (Figure 19).

A



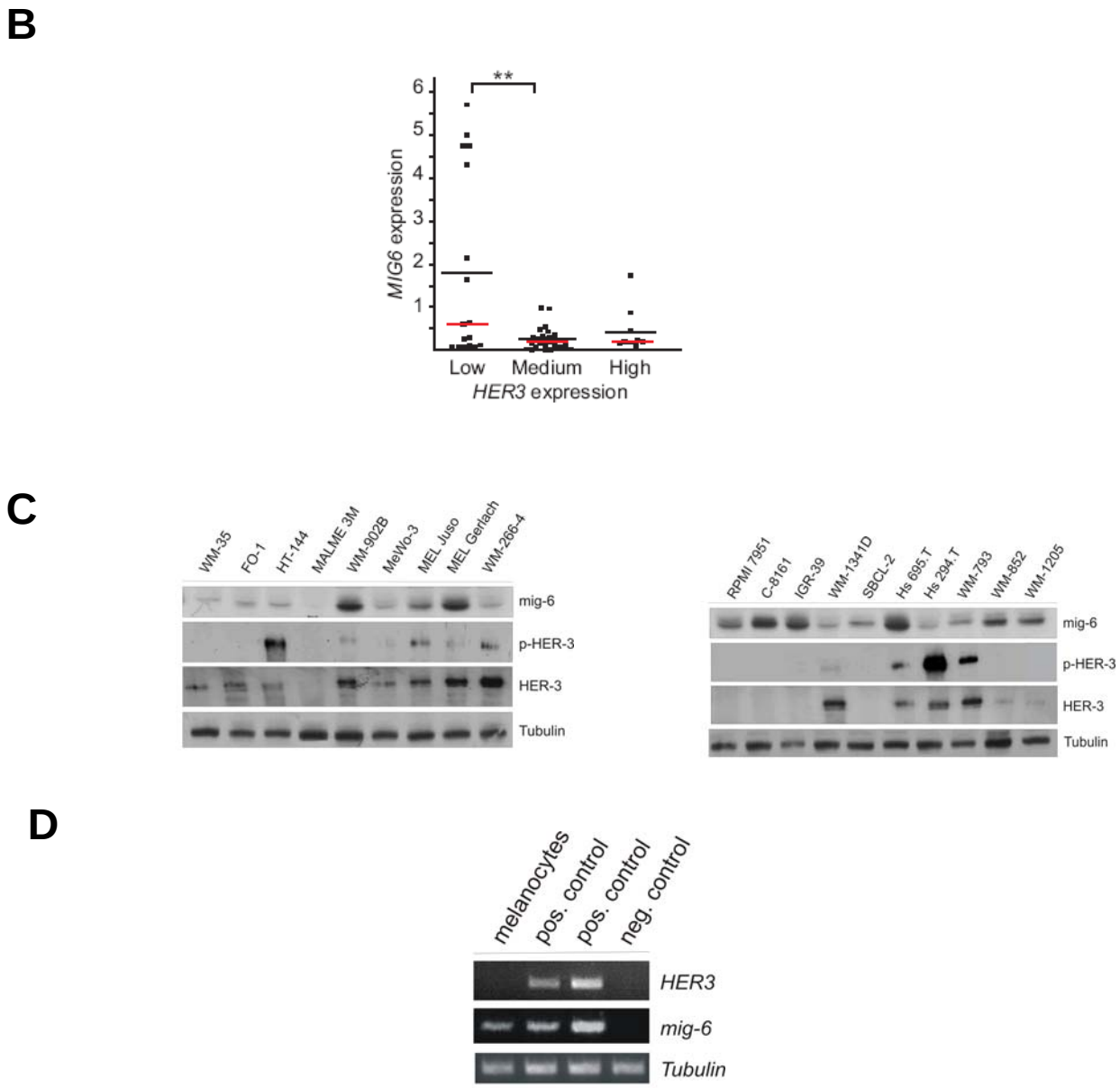


Figure 18. Mig-6 expression inversely correlates with HER3 expression in human melanoma cell lines

(A) *Mig-6, EGFR, HER2* and *HER3* mRNA expression was analyzed by Northern blot in a panel of 52 human melanoma cell lines and 6 mouse xenografts. *ß-actin* served as a loading control. The mRNA expression was quantified with the AIDA software and normalized to *ß-actin*.

(B) *Mig-6* expression inversely correlates to *HER3* expression. Every cell line was analyzed for its *mig-6* and *HER3* expression values and blotted in a diagram. The diagram shows a significant inverse correlation between *mig-6* and *HER3* expression levels in human melanoma cell lines.

(C) 20 human melanoma cell lines were analyzed by Western blot for mig-6, p-HER3 and HER3 levels. As shown on mRNA level, mig-6 protein levels inversely correlate with HER3 expression and activation. Tubulin served as a loading control.

(D) *HER3 and mig-6* expression in human epidermal melanocytes. RT-PCRs for *HER3* and *mig-6* are shown. *Tubulin* served as a loading control.

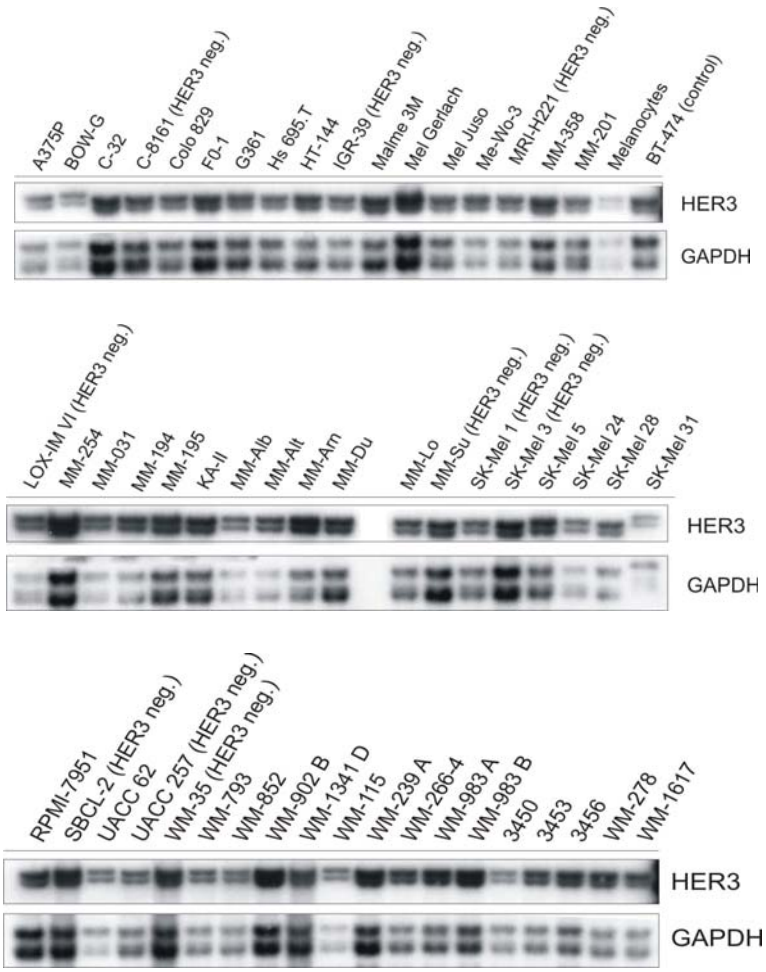


Figure 19. *HER3* is not amplified in human melanoma cell lines
HER3 gene copy number was analyzed in a panel of 54 human melanoma cell lines by Southern blot. Melanocytes, BT-474 breast cancer cells and HER3 negative cell lines served as controls. *GAPDH* served as a loading control.

4.1.6 HER3 is frequently expressed in primary malignant melanoma and metastases

As shown above, HER3 is frequently expressed in human melanoma cell lines suggesting an important role for this HER-family member in melanomagenesis. To further analyze the role of HER3 in primary tumors, we investigated HER3 expression in 130 primary malignant melanoma and 87 melanoma metastases using specific antibodies and tissue micro arrays. Importantly, in primary melanoma as well as in metastases we found a prominent immunoreaction for HER3 at the cell membrane (Figure 20A-D). Remarkably, in primary melanoma, moderate to high HER3 expression levels were found in 85 of 130 cases (65%; Figure 20E). Furthermore, HER3 was highly expressed in 35 of 87 melanoma metastases (40%; Figure 20E). Interestingly, moderate to high HER3 expression significantly correlated

with increased tumor cell proliferation (KI-67 Labelling Index) in primary malignant melanoma (p=0.008). In line with our previously published data on human melanoma cell lines (Ferby et al., 2006), HER3 expression in primary and metastatic melanoma inversely correlated with mig-6 expression (p=0.004; Figure 21A and B) confirming that loss of mig-6 correlates with an increase in HER3 expression.

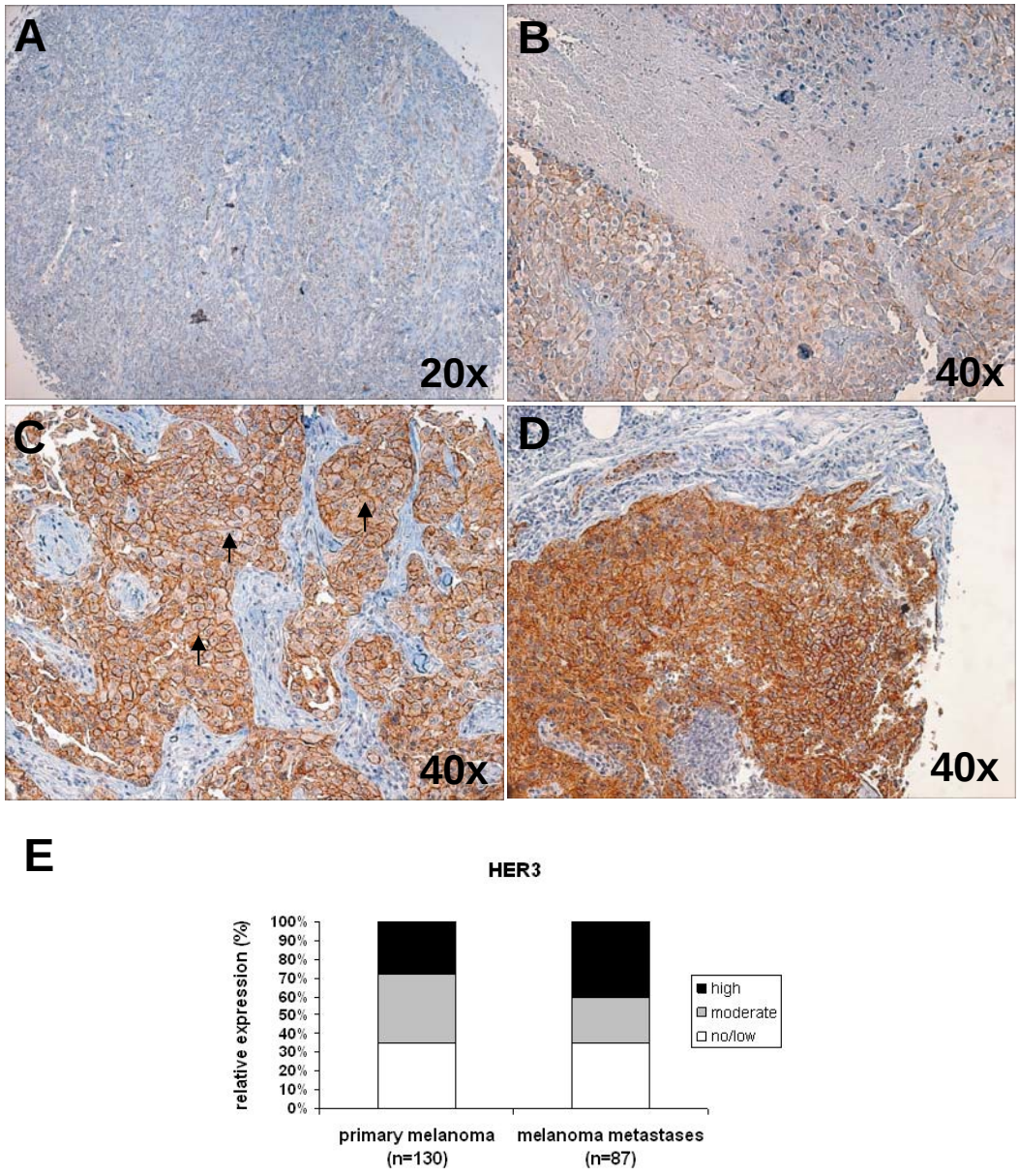


Figure 20. HER3 is frequently expressed in primary malignant melanoma and melanoma metastases

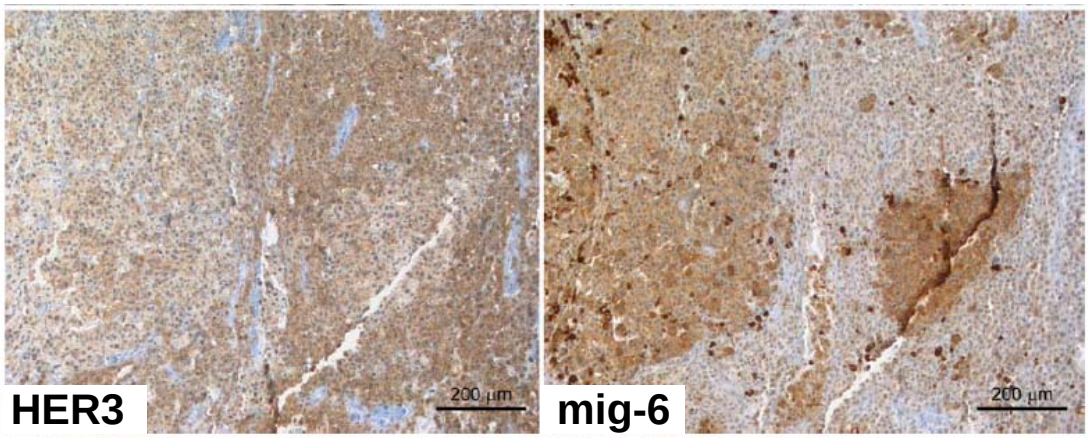
HER3 protein expression was analyzed immunohistochemically in 130 primary malignant melanoma and 87 melanoma metastases using specific antibodies and melanoma tissue micro arrays. All pictures were taken on a Zeiss Axiovert 300 microscope using 20x and 40x magnifications.

(A-C) Low, moderate and high HER3 protein expression in primary malignant melanoma. HER3 immuno reactivity is predominately accentuated at the cell membrane.

(D) HER3 protein expression in melanoma metastases.

(E) HER3 expression frequencies in primary malignant melanoma and melanoma metastases.

A



B

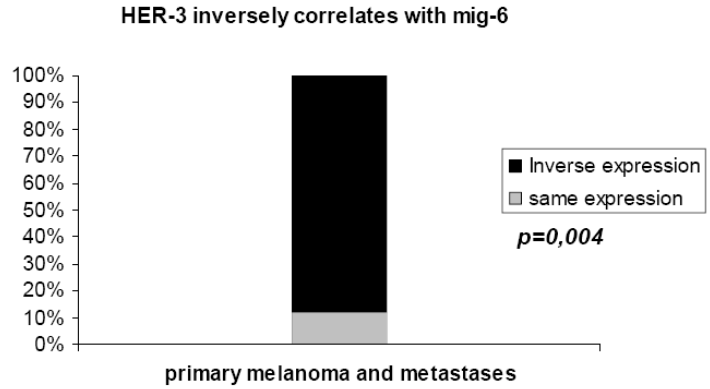


Figure 21. HER3 expression inversely correlates with mig-6 in primary malignant melanoma and metastases
HER3 and mig-6 expression was analysed according to the German Immunohistochemical score and values were directly compared for each tumor spot.
(A) Immunohistochemical staining of HER3 and mig-6 in a melanoma metastases showing inverse protein expression.
(B) HER3 inversely correlates with mig-6. The inverse correlation was statistically significant (Spearman's Test: p=0.004).

4.1.7 HER3 expression confers poor prognosis in melanoma patients

To investigate the relevance of HER3 expression for disease progression we analyzed the relationship between HER3 expression and established clinical parameters.

Strikingly, HER3 expression (p=0.014) was significantly associated with tumor specific survival (Figure 22). Notably, HER3 did not correlate with an increase in Breslow tumor thickness (p=0.067) or surface ulceration. In a multivariate analysis, two different Cox regression models were developed for the assessment of the overall survival rate. The characteristics of the variables are shown in Table 1. Because of model assumptions

(non-informative censoring and proportional hazards), only metastases during progression, tumor thickness, mig-6 and HER3 expression (mig-6 and HER3 Immunohistochemistry score) were considered. In the raw model, metastases (p=0.000) and HER3 expression (p=0.044) were correlated with poor prognosis. With reverse selection, the hazard ratio for death from melanoma concerning HER3 status was 2.636 (95% Confidence Interval: 1.042-6.671); accordingly, in cases with high HER3 expression, the probability of tumor-related death was almost three times higher than in cases with low HER3 staining. Because of the assumption of proportional hazards, the probability of melanoma-related death was consistently valid during the entire observation period.

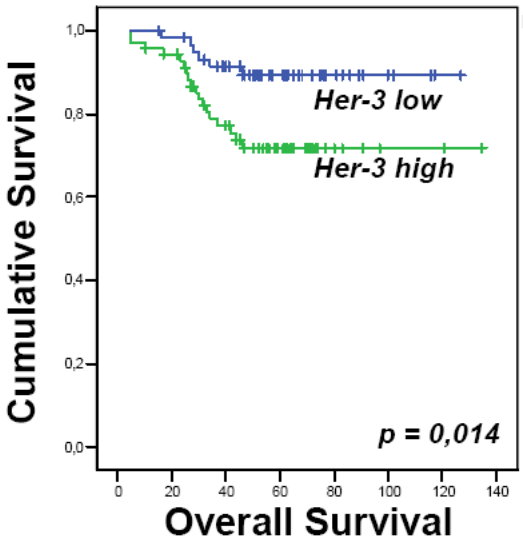


Figure 22. HER3 expression confers poor prognosis in melanoma patients
High HER3 expression is significantly associated with reduced overall survival of melanoma patients (p=0.014). The overall survival of melanoma patients was estimated by the Kaplan Meier method. The patient cohort was divided by GIS dichotomization at the median as described in Material and Methods. All p-values were calculated with the two-sided Fisher's exact test and p-values lower than 0.05 were considered statistically significant. All statistical calculations were performed with the SPSS 12.0 software (SPSS Inc., Chicago, IL).

Table 1. Multivariate analysis of factors possibly influencing overall survival

				Stepwise backward selection		
Name	**Variable**	**Characteristics**	**Global p**	**Hazard ratio**	**95% CI**	**p**
Raw Model						
tu_d	tumor thickness	0 ≤ 2mm 1 > 2mm	0.670	—		
mig-6	mig-6 IHC	0 low 1 high	0.469	—[b]		
HER3	HER3 IHC	0 low 1 high	**0.044**[a]	2.636	(1.042-6.671)	**0.041**
Metastases	Metastases during follow up	0 no metastases 1 metastases	**0.000**	22.251	(5.209-95.058)	**0.000**

[a] Bold type representing data with $p < 0.05$.

[b] Variable excluded from the model; limit for reverse selection procedures $p = 0.2$.

4.1.8 HER3 is associated with melanoma progression

In order to analyze the influence of HER3 on melanoma progression we studied matched tumor samples of primary melanoma and metastases in 20 patients. Remarkably, 10 of 20 (50%) patients showed an increase of HER3 expression in the metastases compared to the primary tumor. Six of 20 (30%) matched tumor samples showed a similar expression of HER3 in the primary melanoma as well as in the corresponding metastases. Only 4 of 20 (20%) patients showed less HER3 expression in the metastases when compared to the primary tumor (Figure 23). These results suggest that HER3 expression remains stable in the majority of melanoma metastases and may be involved in the progression of primary melanoma to the metastatic state. In contrast, mig-6 expression decreased in metastases compared to matching primary tumors in 40% of the patients (Figure 23). These data further confirm HER3 as a prognostic marker and target for therapy in a large number of melanoma patients.

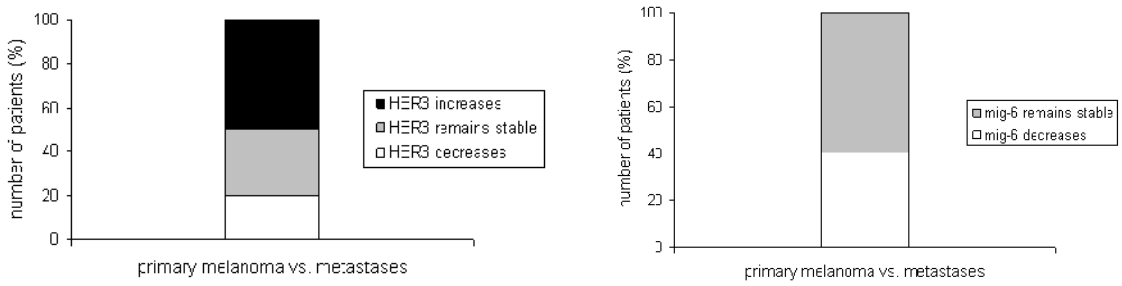
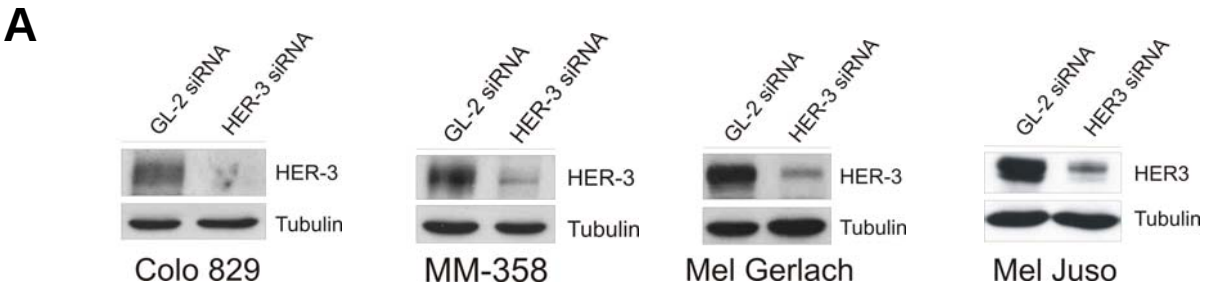


Figure 23. HER3 is associated with melanoma progression
HER3 expression increases during melanoma progression whereas mig-6 expression decreases. Matched tumor samples of primary melanoma and metastases of 20 patients were analyzed for HER3 and mig-6 expression. The GIS values of HER3 and mig-6 in primary melanoma and metastases were directly compared and blotted. HER3/mig-6 > 0: protein expression increases from primary tumor to metastases. HER3/mig-6 = 0: protein expression remains stable. HER3/mig-6 < 0: protein expression decreases from primary tumor to metastases.

4.1.9 *HER3* knock-down inhibits melanoma cell proliferation

In order to further characterize the role of HER3 in melanoma in more detail, we downregulated *HER3* by specific siRNAs in Colo 829, MM-358, Mel Gerlach and Mel Juso human melanoma cell lines (Figure 24A). Strikingly, depletion of *HER3* strongly inhibited proliferation in these cell lines (Figure 24B). Moreover, *HER3* knock-down specifically inhibits heregulin-induced melanoma cell proliferation suggesting that HER3 signalling is required for melanoma cell growth (Figure 24C). To further characterize the underlying mechanism of this growth inhibition we analyzed proteins implicated in cell cycle control. *HER3* knock-down led to an upregulation of p27 levels, reduced Cyclin B1 levels, and decreased Rb phosphorylation (Figure 24D) suggesting that ablation of *HER3* induces a G_1 cell cycle arrest. Importantly, HER3 predominantly signals via the PI3K-AKT pathway in the regulation of cell proliferation and -survival. Indeed, heregulin-induced AKT activation was impaired in the knock-down cell lines indicating that HER3 may signal via the PI3K-AKT pathway in melanoma cells (Figure 24D). In contrast, p-ERK and p-mTOR levels remained unchanged (Figure 24D). Our data suggest that HER3 regulates melanoma cell proliferation which involves upregulation of p27 and the PI3K-AKT pathway.


A
GL-2 siRNA
HER-3 siRNA
HER-3
Tubulin
Colo 829
GL-2 siRNA
HER-3 siRNA
HER-3
Tubulin
MM-358
GL-2 siRNA
HER-3 siRNA
HER-3
Tubulin
Mel Gerlach
GL-2 siRNA
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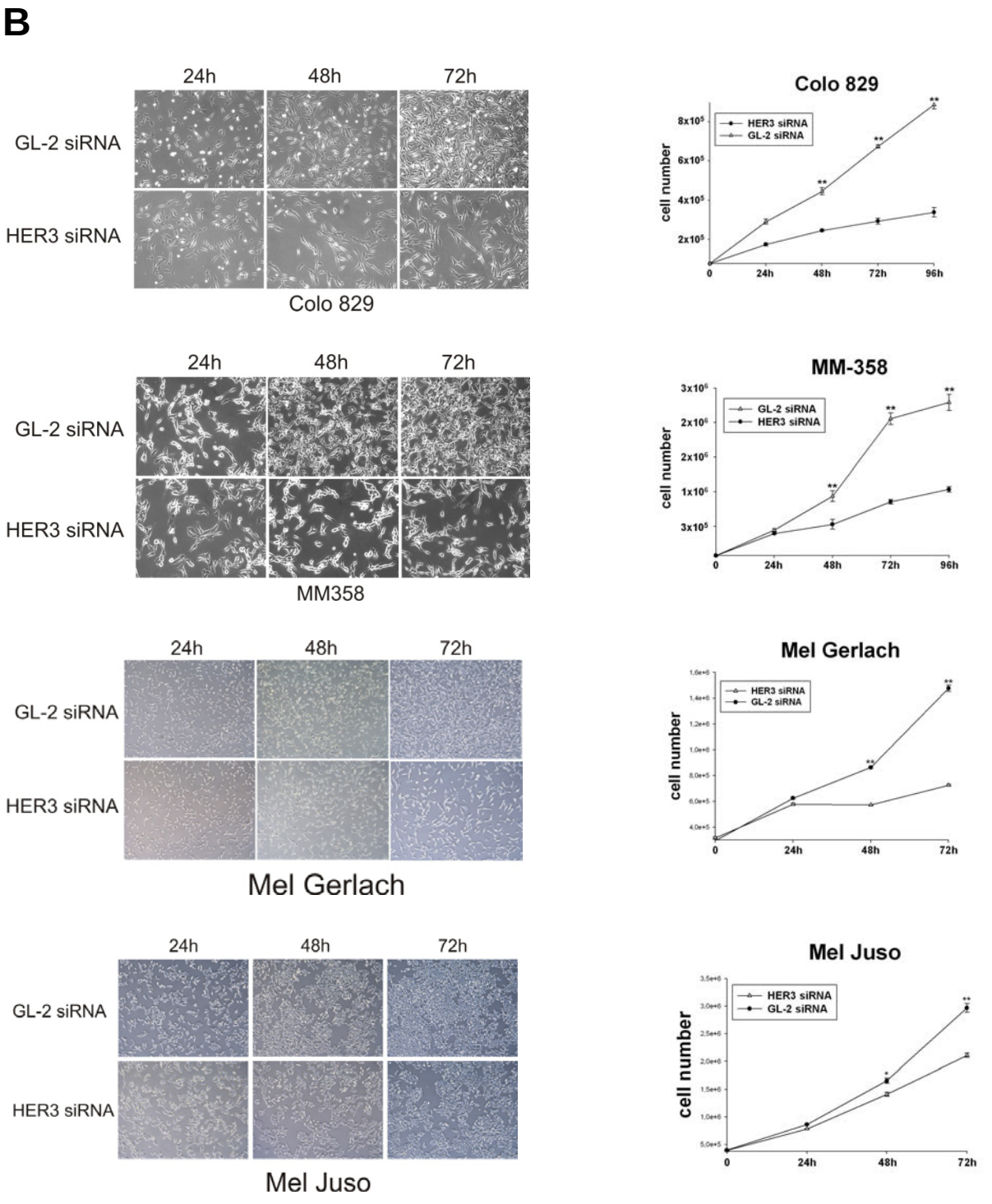
HER3
Tubulin
Mel Juso


B
24h
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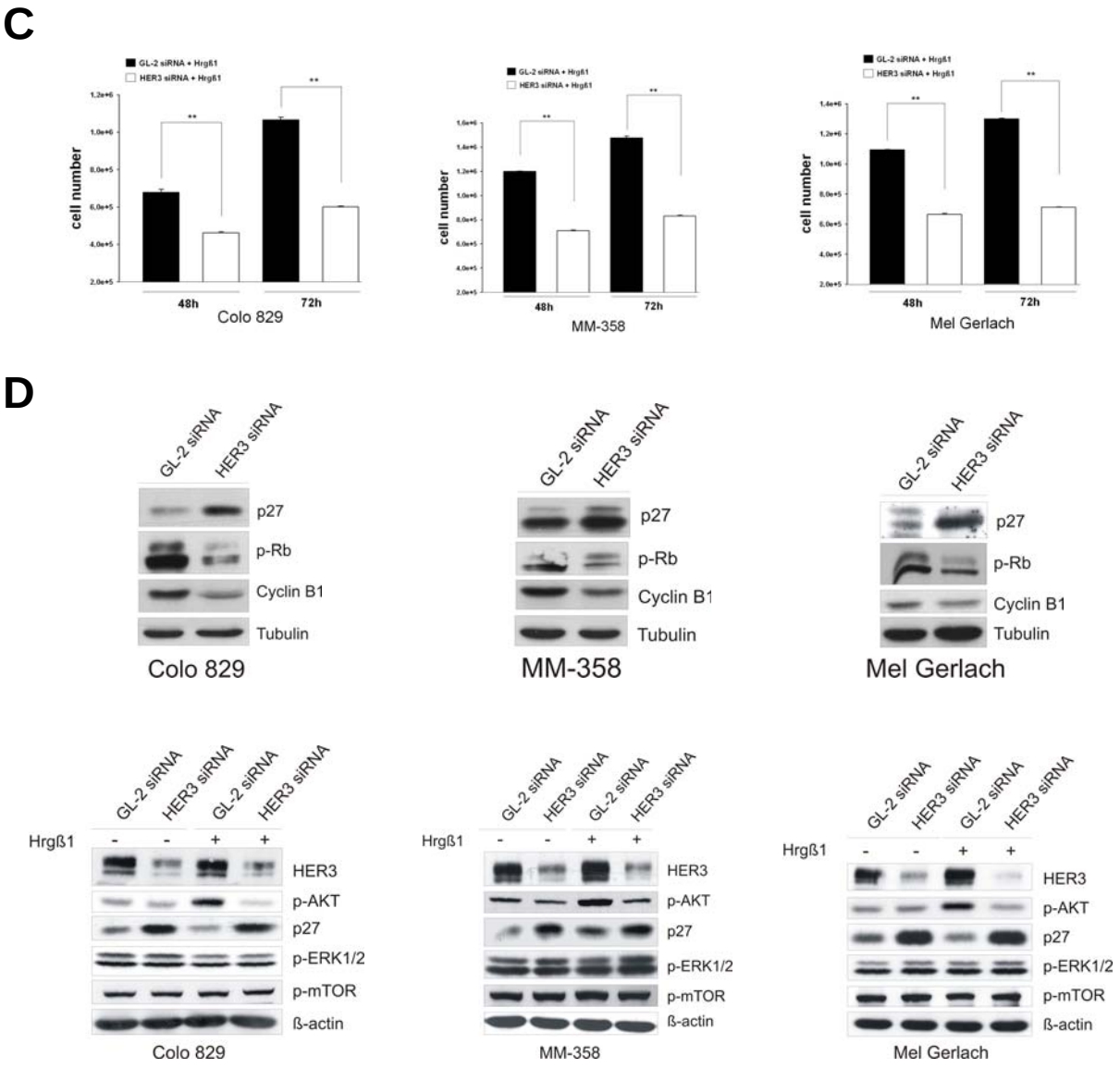


Figure 24. Role of HER3 in melanoma cell proliferation

(A) *HER3* knock-down in Colo 829, MM-358, Mel Gerlach and Mel Juso melanoma cells. The cells were seeded in 6cm tissue culture dishes and 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 Tubulin are shown.

(B) *HER3* knock-down inhibits proliferation of Colo 829, MM-358, Mel Gerlach and Mel Juso melanoma cells. The cells were seeded in 6cm tissue culture dishes, 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. Brightfield pictures were taken at the indicated time points using a Zeiss Axiovert 300 microscope at a 10x magnification.

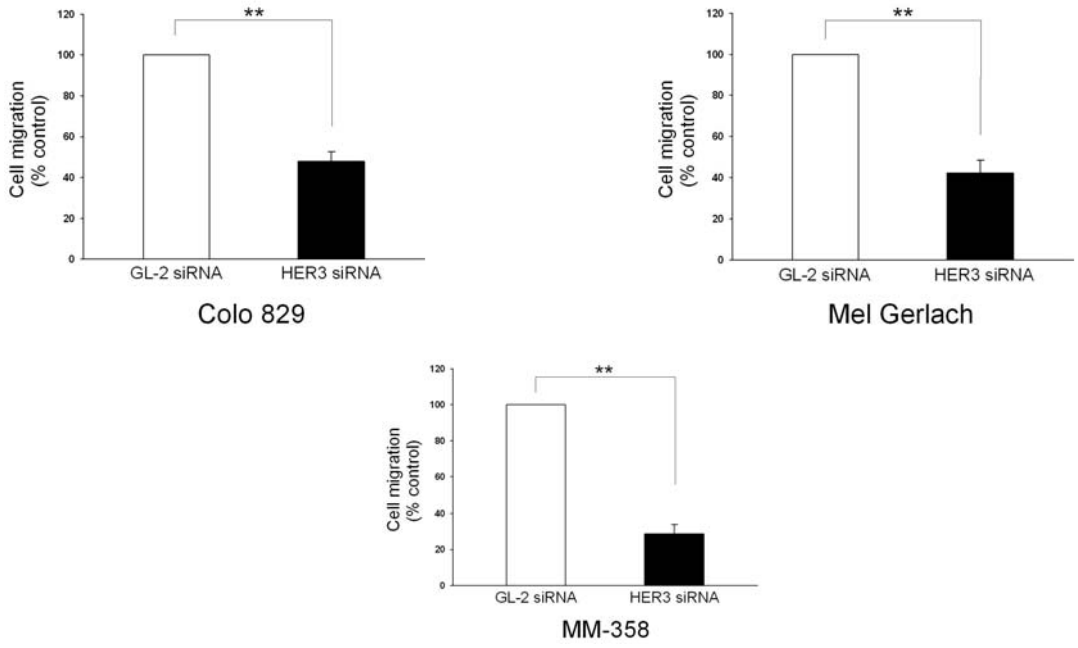
(C) *HER3* knock-down inhibits heregulin-induced proliferation of Colo 829, MM-358 and Mel Gerlach melanoma cells. *HER3* and *GL-2* knock-down cells were seeded in 6cm tissue culture dishes, serum-starved for 24 hours and stimulated with 100ng/ml Hrgß1 for the indicated time points. The cell number was determined as described in B.

(D) *HER3* knock-down impairs heregulin-induced AKT activity in Colo 829, MM-358 and Mel Gerlach melanoma cells. Western blots are shown for, p-AKT, AKT, p27, p-Rb, Cyclin B1, p-ERK1/2, p-mTOR and Tubulin.

4.1.10 *HER3* knock-down inhibits melanoma cell migration and invasion

As shown above, melanoma metastases frequently express high levels of HER3 suggesting that HER3 expression remains relatively stable during melanoma metastasis. Given the association between HER3 expression and poor survival, one might hypothesize that HER3 plays a role in melanoma progression. Increased tumor cell migration and invasion are important prerequisites for metastasis. In order to address this question, we analyzed the migration and invasion of Colo 829, MM-358 and Mel Gerlach melanoma cells upon interference for *HER3* expression. As shown in Figure 25A, *HER3* knock-down efficiently blocked melanoma cell migration of Colo 829, MM-358 and Mel Gerlach cells supporting the conclusion that HER3 is indeed a mediator of cell migration in melanoma. Furthermore, invasion of Colo 829 and Mel Gerlach cells was efficiently blocked while MM-358 cells did not invade the matrix even in untransfected controls (Figure 25B and data not shown). In addition, *HER3* knock-down did inhibit melanoma cell branching of Colo 829 and Mel Gerlach cells (Figure 25C).

A



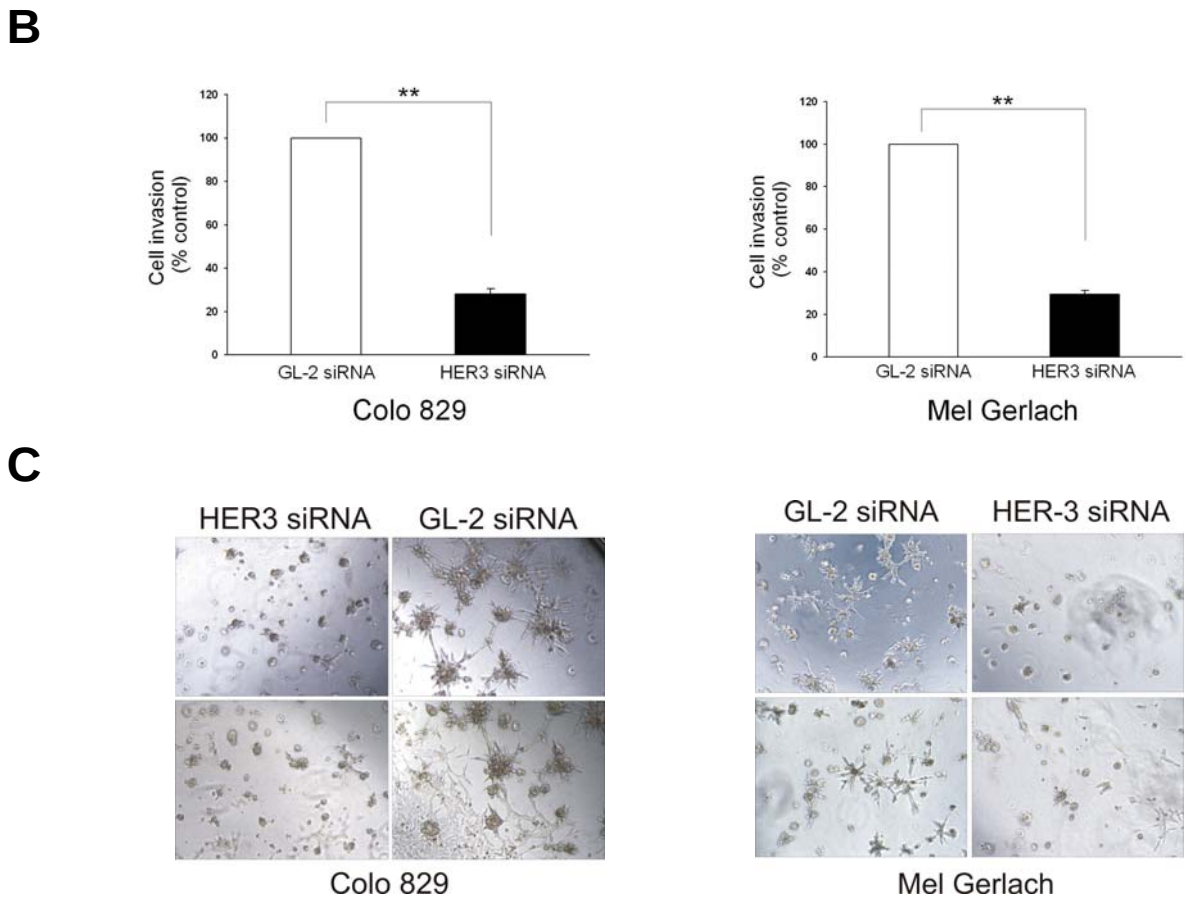


Figure 25. *HER3* knock-down inhibits melanoma cell migration and invasion
(A) *HER3* knock-down inhibits migration of Colo 829, MM-358 and Mel Gerlach melanoma cells. The cells were seeded in 6cm tissue culture dishes and transfected with *HER3* or *GL-2* siRNAs. 25.000 cells were serum-starved for 24 hours, seeded on to a membrane with 8 µM pores of a modified boyden chamber containing 400µl serum-free medium. The lower chamber was filled with 600µl medium containing 10% FCS serving as chemo attractant. The cells were allowed to migrate for 20 hours through the pores and were then stained by crystal violet. Stained cells were washed in PBS and pictures were taken on a Zeiss Axiovert 300 microscope. For quantification at least 10 random fields were counted and data are shown as mean ± SDM. P-values were calculated using the paired Students T-test and values below 0.05 were considered statistically significant.
(B) *HER3* knock-down inhibits invasion of Colo 829 and Mel Gerlach melanoma cells. The cells were seeded in 6cm tissue culture dishes and transfected with *HER3* or *GL-2* siRNAs. 25.000 cells were serum-starved for 24 hours, seeded on to a membrane coated with growth factor-reduced matrigel of a modified boyden chamber containing 400µl serum-free medium. The lower chamber was filled with 600µl medium containing 10% FCS serving as chemo attractant. The cells were allowed to invade the matrix for 24 hours and were then stained by crystal violet. Stained cells were washed in PBS and pictures were taken on a Zeiss Axiovert 300 microscope. For quantification at least 10 random fields were counted and data are shown as mean ± SDM. P-values were calculated using the paired Students T-test and values below 0.05 were considered statistically significant.
(C) *HER3* knock-down inhibits melanoma cell branching. Colo 829 and Mel Gerlach melanoma cells were seeded in 6cm tissue culture dishes and transfected with *HER3* or *GL-2* siRNAs. 72 hours later 10.000 cells were plated on to matrigel in 96 well plates. The cells were allowed to invade the matrix for 1 week and pictures were taken on a Zeiss Axiovert 300 microscope.

4.1.11 HER3 signalling regulates *MMP-14* and *MMP-2* expression in melanoma cell lines

We have shown that HER3 is frequently expressed in melanoma metastases and regulates melanoma cell migration and invasion. Based on these results we aimed to determine whether HER3 can regulate the expression of matrix-metallo proteinases which are key regulators of cancer cell motility and invasivity (Egeblad and Werb, 2002). Interestingly, heregulin stimulation in Colo 829, MM-358 and Mel Gerlach cells led to an upregulation of *matrix-metallo proteinase-14* expression (MMP-14; also known as membrane-type 1 matrix-metallo proteinase) and subsequent *MMP-2* (also known as gelatinase A) expression (Figure 26). In this context it is important to note that MMP-14 can proteolytically process proMMP-2 at the cell surface leading to MMP-2 activation and tumor cell invasion (Sato et al., 1994; Itoh et al., 2001). Thus, heregulin-induced *MMP-2* expression is likely to be a direct consequence of *MMP-14* activation. Notably, MMP-14 and MMP-2 seem to be the only MMPs to be regulated by HER3 signalling since neither *MMP-1* nor *MMP-9* expression was induced by heregulin. To further analyze the dependency of *MMP-14* and *MMP-2* expression on HER3 signalling we knocked-down *HER3* by RNA interference in Colo 829, MM-358 and Mel Gerlach cells (Figure 26). Strikingly, *HER3* knock-down completely abrogated heregulin-induced *MMP-14* and *MMP-2* expression. These data suggest that HER3 can regulate the expression of matrix-metallo proteinases in melanoma cell lines thereby possibly regulating melanoma cell invasivity.

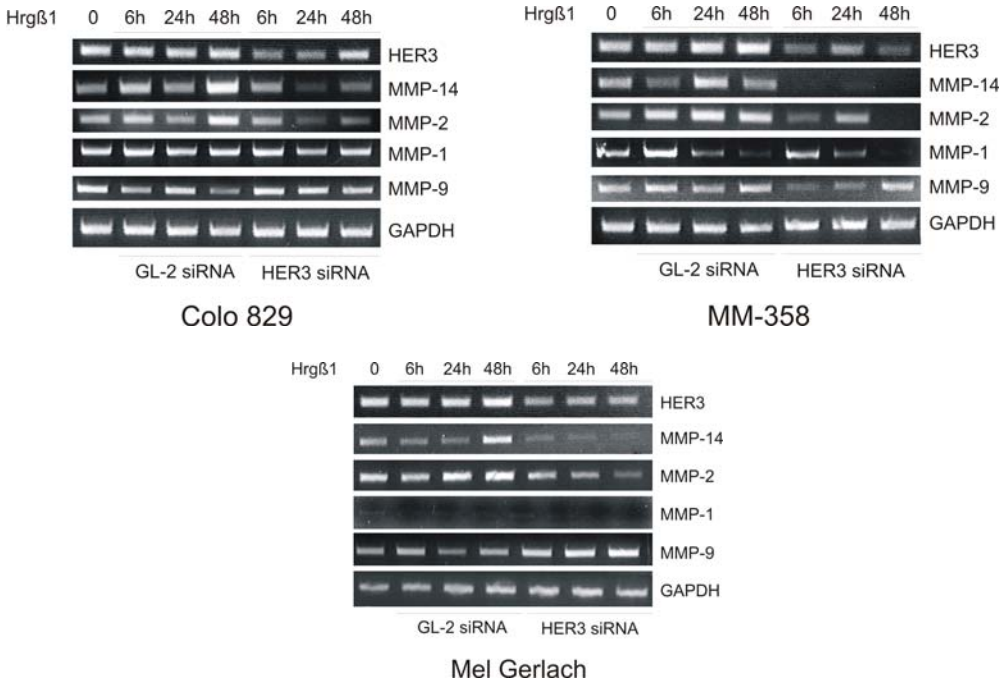


Figure 26. HER3 signalling induces *MMP-14* and *MMP-2* expression in melanoma cell lines
Colo 829, MM-358 and Mel Gerlach melanoma cells were transfected with *HER3* or *GL-2* siRNAs, stimulated with 50ng/ml heregulin ß1 for the indicated time points and subjected to RT-PCR analysis. RT-PCRs for *HER3, MMP-14, MMP-2, MMP-1* and *MMP-9* are shown. *GAPDH* served as a loading control.

4.1.12 Suppression of *HER3* sensitizes melanoma cells to dacarbazine-induced apoptosis

In contrast to A549 lung cancer cells which were shown to undergo apoptosis in the absence of HER3 (Sithanandam et al., 2005), suppression of *HER3* only mildly affected apoptosis in Colo 829 melanoma cells (Figure 27). Nevertheless we reasoned that inhibition of HER3 signalling might synergize with chemotherapy-induced apoptosis in melanoma cell lines. To date, dacarbazine is the only FDA-approved drug for melanoma therapy (Chudnovsky et al., 2005). Indeed, we found that dacarbazine-induced apoptosis was significantly increased in *HER3*-deficient melanoma cells as demonstrated for Colo 829, MM-358 and Mel Gerlach cells (Figure 28). This effect seems to be specific for dacarbazine since treatment of cells with an unrelated chemotherapeutic drug like doxorubicin did not significantly enhance melanoma apoptosis (data not shown). These results imply that combination therapies with HER3 and dacarbazine-like drugs might be useful for the treatment of advanced melanoma.
Importantly, all effects described above are specific for HER3 since HER2 surface expression and phosphorylation are unaffected in *HER3* knock-down cell lines (Figure 29).

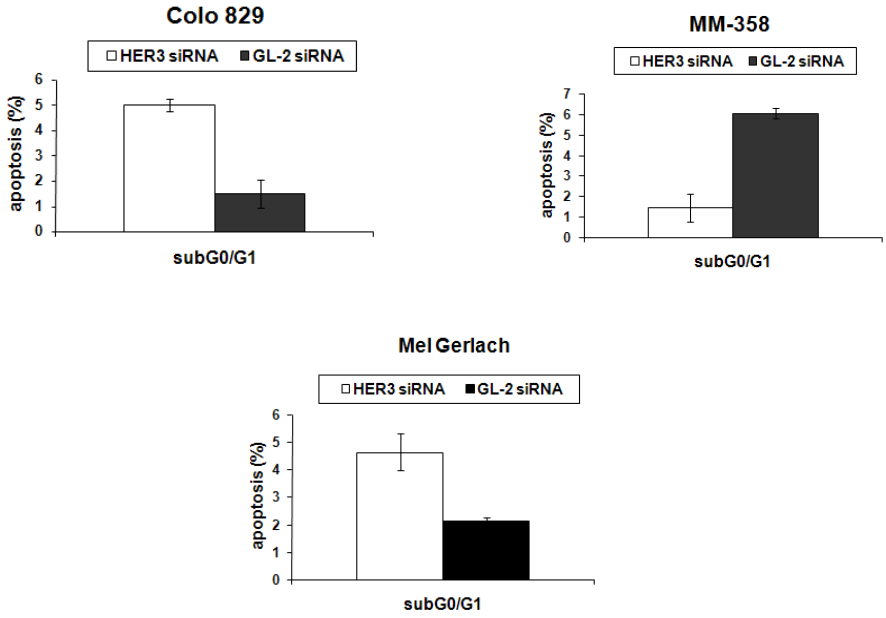


Figure 27. *HER3* knock-down does not induce apoptosis in melanoma cells
Colo 829, MM-358 and Mel Gerlach cells were transfected with *HER3* or *GL-2* siRNAs and grown for 48 hours. Apoptosis was measured by propidium iodide staining on a flow cytometer and quantified using the Cell Quest Pro software (Beckton Dickinson).

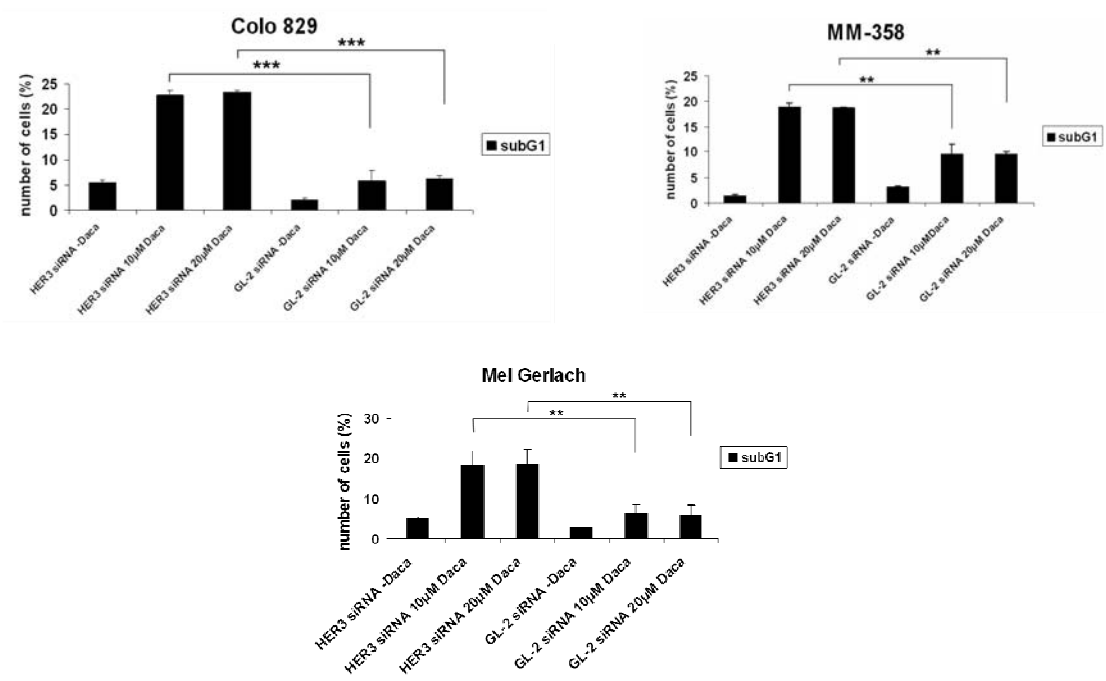


Figure 28. *HER3* knock-down sensitizes melanoma cells to dacarbazine-induced apoptosis
Colo 829, MM-358 and Mel Gerlach melanoma cells were seeded in 6 well plates and transfected with *HER3* or *GL-2* siRNAs. 24 hours later apoptosis was induced by adding either 10 or 20µM dacarbazine in DMSO to the medium. The cells were incubated for 72 hours and analyzed by propidium-iodide staining as described in Material and Methods. Cell cycle profiles and apoptosis were determined using the Cell Quest Pro software (Beckton Dickinson Biosciences).

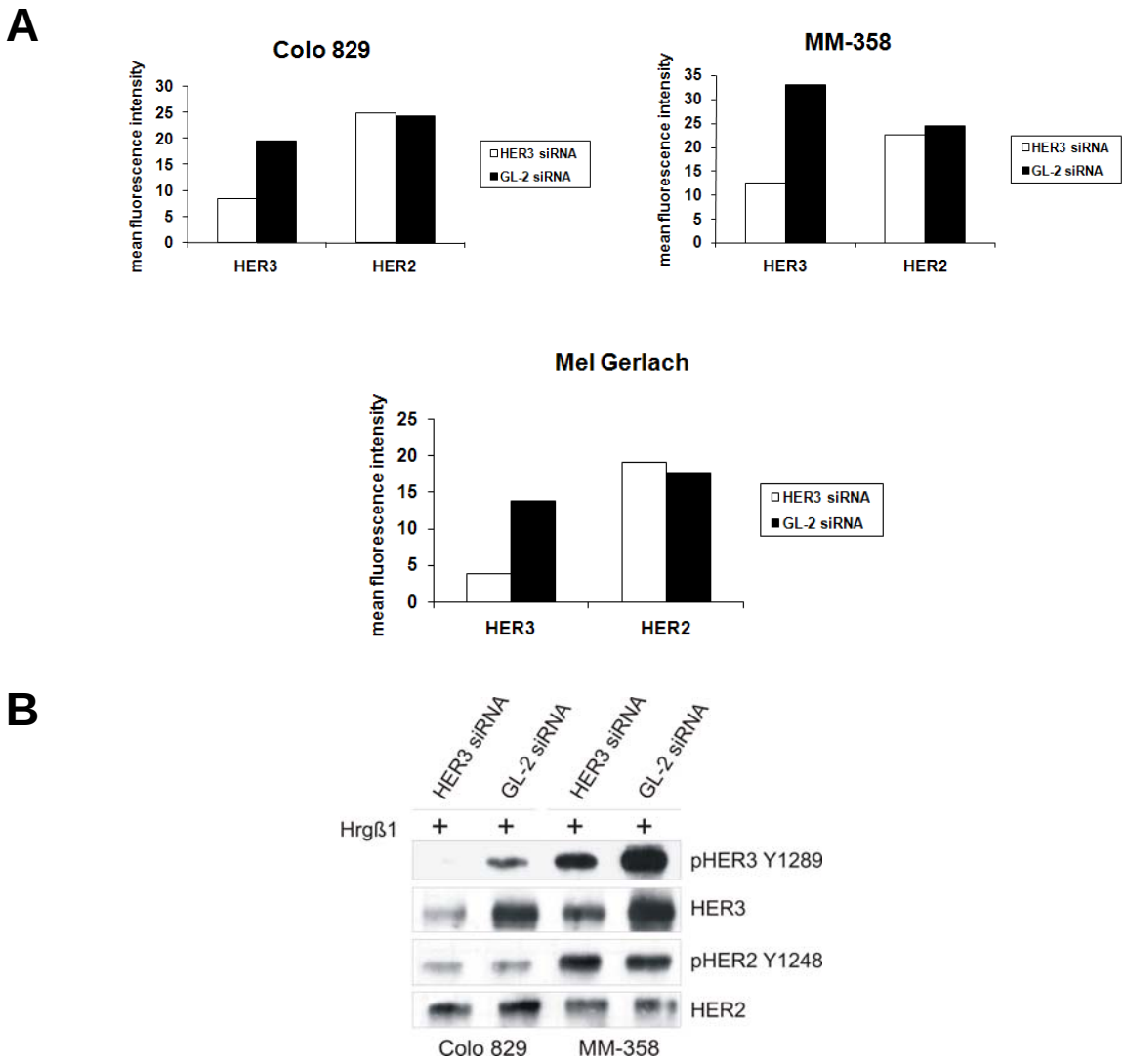


Figure 29. *HER3* knock-down does not alter HER2 surface expression nor HER2 activity
(A) HER3 and HER2 surface expression was measured by indirect flow cytometry. Colo 829, MM-358 and Mel Gerlach *HER3* knock-down cells were incubated with specific primary antibodies for HER2 and HER3 for 1 hour and afterwards with a PE-labelled secondary antibody followed by flow cytometry. The fluorescence intensities for HER3 and HER2 are shown.
(B) *HER3* knock-down does not alter HER2 activity. Colo 829 and M-358 melanoma cells were transfected with *HER3* and *GL-2* siRNAs. Upon heregulin stimulation HER3 and HER2 receptor phosphorylation were assessed by phospho-specific antibodies specific for tyrosine 1289 on HER3 and tyrosine 1248 on HER2.

4.1.13 Anti-HER3 monoclonal antibodies inhibit heregulin-induced HER3 activation and PI3-Kinase binding

We have shown above that HER3 is frequently overexpressed in primary melanoma and melanoma metastases and that high HER3 levels confer poor prognosis in melanoma patients. In addition, the RNA interference experiments suggest that HER3 may be a potential target for melanoma therapy development. To test this hypothesis *in vitro* we treated Colo 829, MM-358 and Mel Gerlach melanoma cell lines with an anti-HER3 monoclonal antibody (cl. 105.5). Remarkably, heregulin-induced activation of HER3 and its association with the PI3-K subunit p85 was completely abrogated in antibody-treated cells when compared to control cells (Figure 30). In addition, anti-HER3 monoclonal antibodies lead to receptor degradation or internalization similar to the results obtained with breast cancer cells (van der Horst et al., 2005).

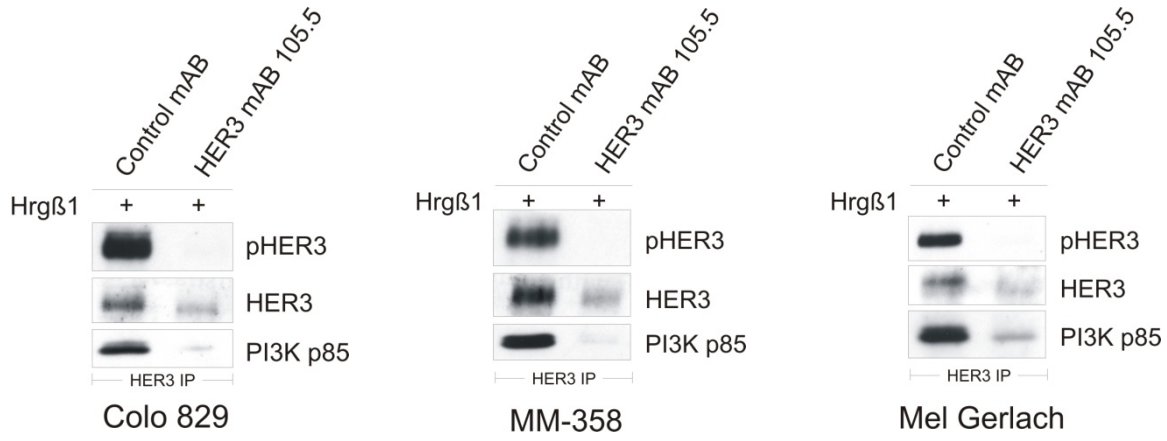


Figure 30. An anti-HER3 monoclonal antibody (cl. 105.5) inhibits HER3 activation and PI3-K binding
Anti-HER3 antibody treatment completely blocks HER3 activation, its association with p85 and leads to receptor internalization or degradation. Colo 829, MM-358 and Mel Gerlach melanoma cells were either incubated with 10µg/ml HER3 blocking antibody (cl. 105.5) or an isotype control antibody, stimulated with heregulin ß1, lysed and equal amounts of protein was subjected to immunoprecipitations using a specific HER3 antibody. Western blots for p-HER3 (Y1289), HER3 and p85 are shown.

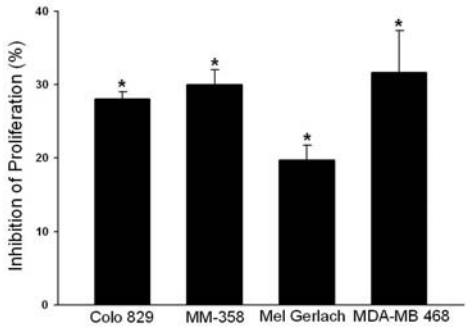
4.1.14 An anti-HER3 monoclonal antibody inhibits heregulin-induced melanoma cell proliferation, migration and invasion

As shown above the anti-HER3 monoclonal antibody inhibits heregulin-induced HER3 activation. Based on this effect we aimed to determine whether such antibodies can inhibit melanoma cell proliferation, migration and invasion. Strikingly, anti-HER3 monoclonal antibodies are able to block these heregulin-induced cell functions of human melanoma cell lines (Figure 31A, B and C) indicating that such antibodies may be

useful for melanoma therapy. In proliferation experiments we observed different sensitivities towards the HER3 blocking antibody. While Colo 829 and MM-358 cells displayed a strong inhibition, Mel Gerlach cells were only inhibited to about 20%. As a positive control we used MDA-MB 468 breast cancer cells since it was already published that these cells show a marked growth inhibition upon HER3 blocking antibody treatment (van der Horst et al., 2005). In Mel Juso melanoma cells we used a different anti-HER3 monoclonal antibody (cl. 2D1D12). In parallel we used an anti-HER2 monoclonal antibody (cl. 4D5) in order to exclude the possibility that inhibition of melanoma cell invasion is mediated by downregulation of HER2 signalling. As shown in figure 32, only the HER3 but not the HER2 antibody was able to inhibit heregulin-induced melanoma cell invasion clearly demonstrating that HER3 inhibition is sufficient to block melanoma cell invasivity.

Taken together, these results suggest that targeting HER3 may represent a promising possibility for melanoma therapy which could prove beneficial for clinical patient outcome.

A



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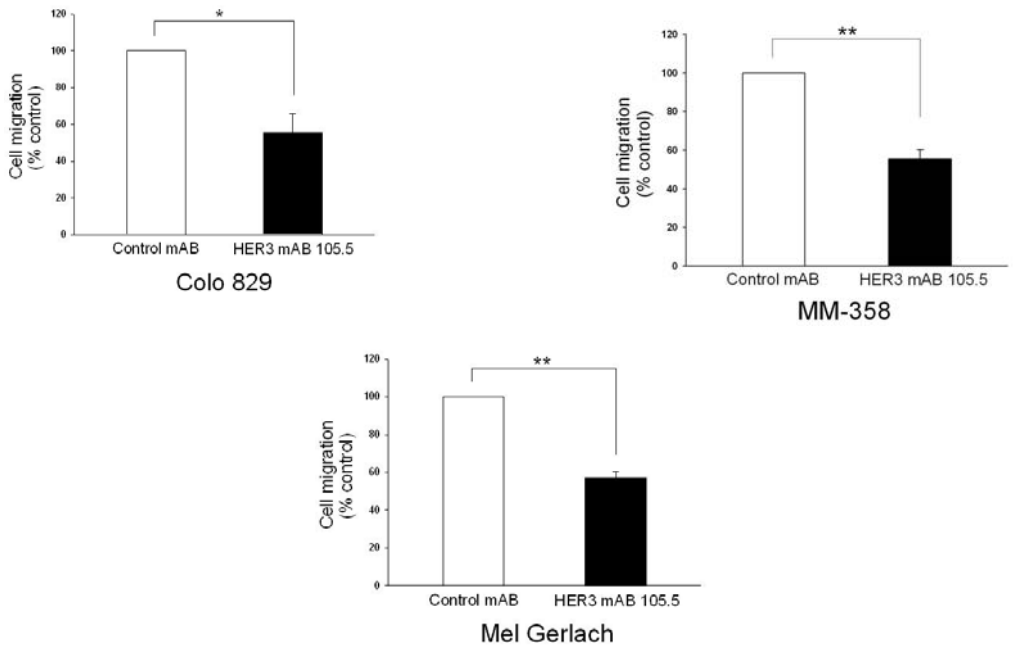

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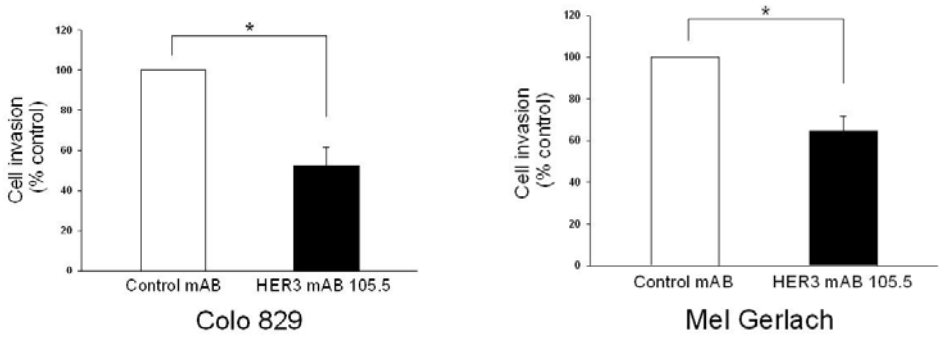

Figure 31. An anti-HER3 monoclonal antibody (cl. 105.5) inhibits heregulin-induced melanoma cell proliferation, migration and invasion

(A) An anti-HER3 antibody (cl.105.5) inhibits melanoma cell proliferation *in vitro*. The cells were serum-starved in medium containing 1% FCS for 24 hours, pre-incubated with either 10µg/ml HER3 blocking antibody or an isotype control antibody for 1 hour and stimulated with 100ng/ml heregulin ß1. The cells were grown for 48 hours and analyzed by a MTT-Assay as described in Material and Methods. MDA-MB 468 cells served as a positive control as described previously (van der Horst et al., 2005).

(B) An anti-HER3 antibody (cl. 105.5) blocks melanoma cell migration *in vitro.* The cells were either incubated with the anti-HER3 antibody or an isotype control antibody. The migration assay was done in a modified boyden chamber. Conditioned NIH 3T3 medium containing 100ng/ml heregulin ß1 was used as a chemo attractant. The quantification was done as described in Figure 25. The data are shown as mean ± SDM.

(C) An anti-HER3 antibody (cl. 105.5) blocks melanoma cell invasion *in vitro*. The assay was done as in B using growth factor-reduced matrigel in a modified boyden chamber.

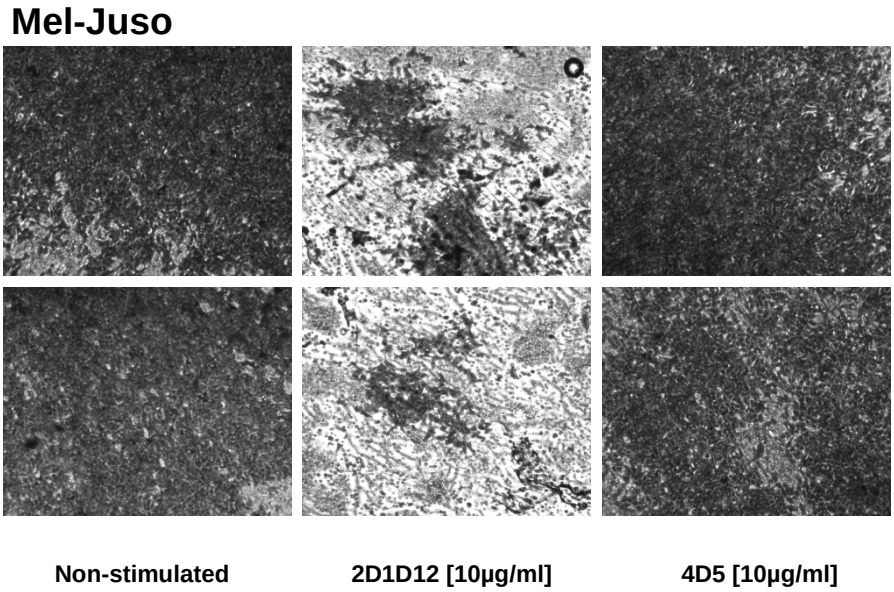

Figure 32. An anti-HER3 monoclonal antibody (cl. 2D1D12) inhibits the invasion of Mel Juso melanoma cells

Mel Juso melanoma cells were seeded in 6cm dishes, and pre-incubated either with an anti-HER3 mAB (cl. 2D1D12) or an anti-HER2 mAB (cl. 4D5) or left untreated for 1 hour at 37°C. Then 50.000 cells were seeded on to a growth factor-reduced matrigel coated membrane of a modified boyden chamber. The cells were allowed to invade the matrix for 24 hours (The experiment was performed by Dr. Edward Htun van der Horst).

4.2 Mig-6 is a Negative Regulator of Mouse Hepatocyte Proliferation

4.2.1 *Mig-6*- deficient primary hepatocytes show increased EGFR-AKT signalling upon growth factor stimulation

Mig-6 knock-out mice display multiple phenotypes in various organs (Ferby et al., 2006). In the liver, mig-6 is highly expressed; however, *mig-6* knock-out mice do not show obvious defects in liver development or function (Zhang et al., 2007). Notably, mig-6 is upregulated after partial hepatectomy suggesting a possible role of mig-6 in the control of liver regeneration (Mohn et al., 1990; Haber et al., 1993). Interestingly, *mig-6*-deficiency led to a marked increase in EGFR protein levels in the liver of two and five week-old knock-out mice suggesting a liver specific role for mig-6 in the regulation of the EGFR (Figure 33A). In order to investigate a possible function of mig-6 in the mouse liver, we isolated primary hepatocytes from adult *mig-6* knock-out and wildtype animals. *Mig-6*-deficient hepatocytes retained higher levels of basal EGFR, AKT and ERK1/2 phosphorylation than wildtype controls even in the absence of growth factor stimulation (Figure 33B). Interestingly, upon EGF stimulation, *mig-6*-deficient hepatocytes showed an increased induction of EGFR phosphorylation (Figure 33B). Concomitant, an increase in phosphorylated AKT levels was observed in *mig-6* knock-out hepatocytes (Figure 33B). In contrast, ERK1/2 activation remained comparable between wildtype and knock-out cells suggesting that loss of *mig-6* leads to an upregulation of EGFR signalling through the PI3K-AKT pathway.

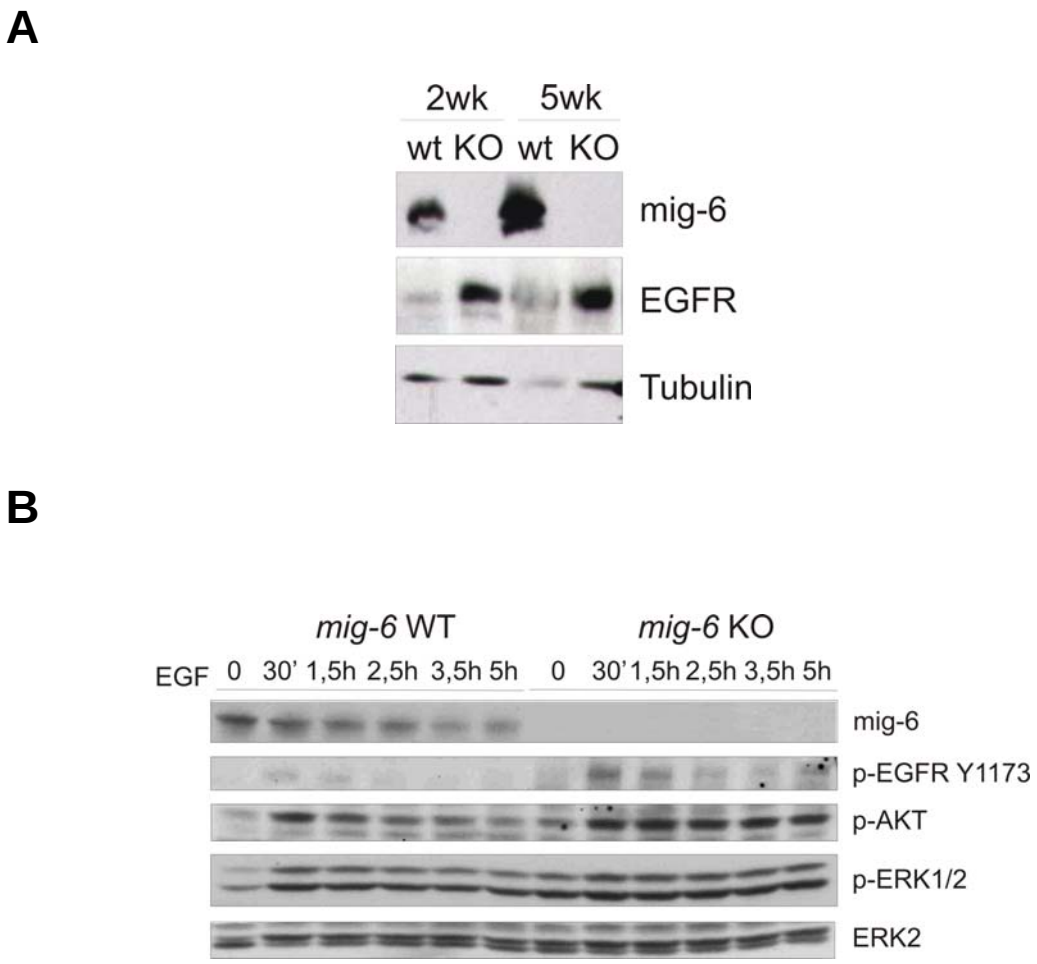

Figure 33. *Mig-6*-deficient primary hepatocytes show increased mitogenic signalling upon growth factor stimulation
(A) Increased levels of EGFR protein expression in *mig-6* knock-out livers. Livers from 2 and 5 week-old knock-out and wildtype control mice were lysed and subjected to Western blot analysis. Immunoblots for mig-6 and the EGFR are shown. Tubulin served as a loading control.
(B) Primary hepatocytes were isolated from 3 month-old *mig-6* knock out mice and wildtype littermates using a two-step collagenase perfusion technique. Isolated hepatocytes were then stimulated with 50ng/ml EGF, lysed at the indicated time points and subjected to Western blot analysis. Immunoblots for mig-6, p-EGFR (Y1173), p-AKT and p-ERK1/2 are shown. ERK2 served as a loading control.

4.2.2 *Mig-6* knock-out mice show increased hepatocyte proliferation after partial hepatectomy

Based on our observations in primary hepatocytes we wanted to study the effect of *mig-6*-deficiency on hepatocyte proliferation *in vivo*. In order to answer this question we subjected *mig-6* knock-out and wildtype control mice to a 70% partial hepatectomy and monitored liver regeneration in the absence of the *mig-6* gene. Liver samples were harvested at the indicated time points and protein lysates were subjected to Western blot analysis to examine the changes in mig-6 expression levels and to verify the suppression of *mig-6* during liver

regeneration in the knock-out mice. In agreement with previously published data (Mohn et al., 1990; Haber et al., 1993), mig-6 expression levels were found to be upregulated in control mice after partial hepatectomy (Figure 34A). Convincingly, *mig-6* knock-out mice displayed a marked increase in hepatocytes re-entering the cell cycle between 24 and 36 hours after partial hepatectomy (Figure 34B and C) indicating that mig-6 is a negative regulator of mouse hepatocyte proliferation. In contrast, only a few wildtype hepatocytes were able to enter S-phase at these time points.

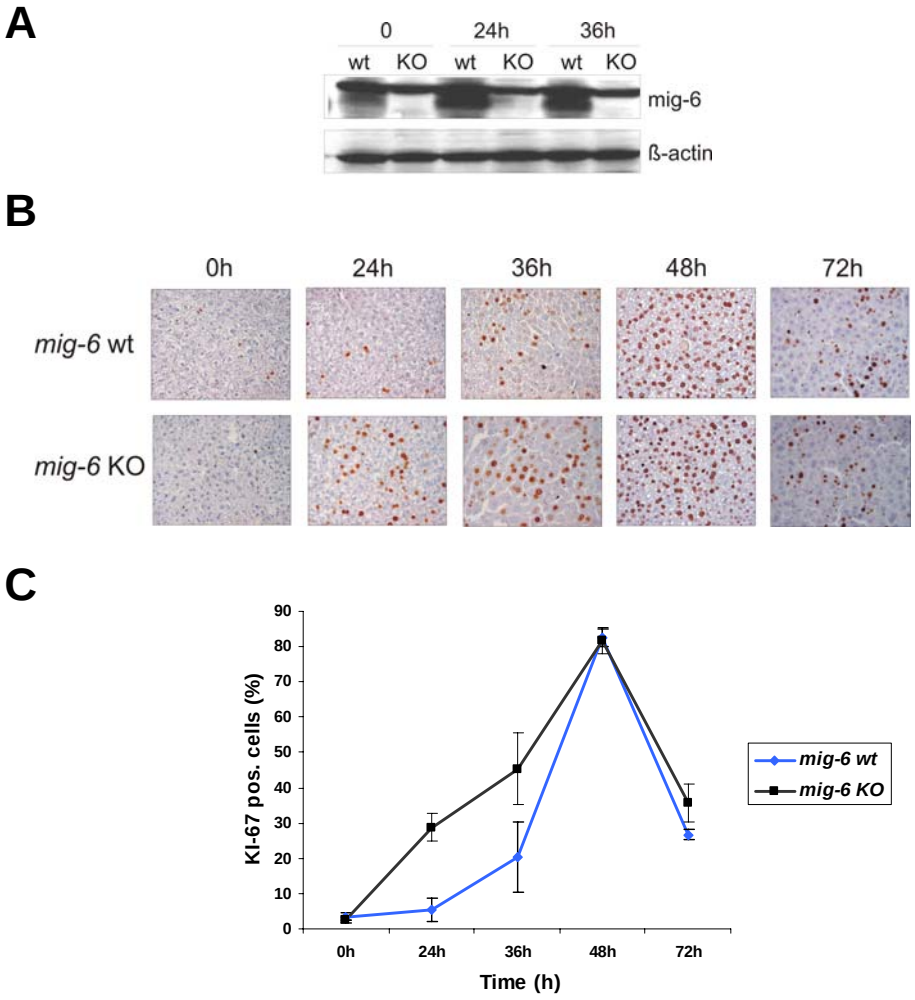

Figure 34. *Mig-6* knock-out mice display increased hepatocyte proliferation after partial hepatectomy (PH)
(A) Mig-6 protein expression is induced in the regenerating mouse liver. Livers from regenerating *mig-6* knock-out and wildtype controls were lysed at the indicated time points and analysed for mig-6 protein expression by Western blot. ß-actin served as a loading control.
(B) *Mig-6* knock-out mice and wildtype littermates (3-5 months old) were subjected to a 70% partial hepatectomy (PH). The livers were isolated at the indicated time points and paraffin sections of regenerating livers were immunohistochemically stained for the proliferation antigen KI-67. *Mig-6* knock-out mice showed significantly higher numbers of KI-67 positive hepatocytes at 24 and 36 hours after partial hepatectomy when compared to control mice.
(C) Quantification of KI-67 positive hepatocytes in C. The number of animals was at least 5 per time point analyzed. The values are shown as mean ± SDM.

4.2.3 Increased EGFR signalling in *mig-6* knock-out mice after partial hepatectomy

In order to investigate the underlying mechanism of the increased hepatocyte proliferation, we first analyzed the expression of cyclins in regenerating livers at the indicated time points. We found that Cyclin D1 was slightly elevated in *mig-6* knock-out livers at 24 and 36 hours after partial hepatectomy suggesting that cell cycle progression might be increased in *mig-6* knock-out mice (Figure 35A). Consistently, we found upregulated levels of phosphorylated Retinoblastoma (Rb) in *mig-6* knock-out livers (Figure 35B). In contrast, Cyclin A expression remained unchanged in knock-out and wildtype mice (Figure 35A). In order to dissect the underlying signalling mechanism that drives hepatocyte proliferation in regenerating *mig-6* knock-out livers, we analyzed members of the EGFR signalling pathway. As shown in figure 35B, we found a strong activation of the EGFR, AKT and ERK1/2 in *mig-6* knock-out livers at 24 and 36 hours after partial hepatectomy comparable to the effects we have seen in primary hepatocytes. Furthermore, we observed elevated activity of the AP-1 transcription factor c-Jun, which is known to be a key regulator of liver regeneration (Behrens et al., 2002). In line with this we found a downregulation of p38 phosphorylation. In this context it is important to note, that c-Jun directly controls hepatocyte proliferation by suppressing p38 activity (Stepniak et al., 2006). These data suggest that loss of *mig-6* in mice leads to increased hepatocyte proliferation after partial hepatectomy by hyperactivation of the EGFR through the PI3K-AKT and the MAPK pathway.

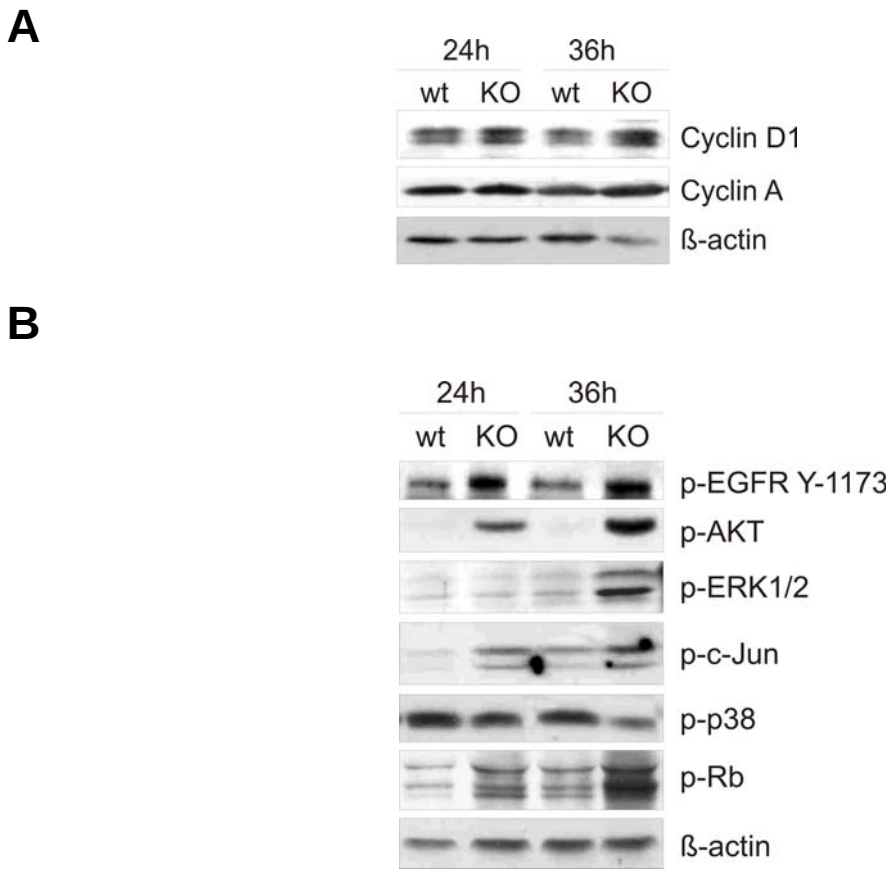

Figure 35. Increased mitogenic signalling in *mig*-6 knock-out mice after partial hepatectomy
Livers from *mig-6* knock-out and wildtype mice were lysed at the indicated time points after partial hepatectomy and protein lysates where subjected to Western blot analysis using the indicated antibodies.
(A) Cyclin D1 and Cyclin A expression in *mig-6* knock-out livers after partial hepatectomy. Immunoblots for Cyclin D1 and Cyclin A are shown. ß-actin served as a loading control.
(B) Marked upregulation of p-EGFR, p-AKT and p-ERK1/2 was observed in regenerating *mig-6* knock-out livers at 24 and 36 hours after partial hepatectomy when compared to wildtype control mice. Immunoblots for p-EGFR, p-AKT, p-ERK1/2, p-c-Jun, p-p38 and p-Rb are shown. ß-actin served as a loading control.

4.2.4 *Mig-6* knock-out mice are susceptible to DEN-induced liver carcinogenesis

Mig-6 knock-out mice develop no obvious liver abnormalities or defects in liver function. However, we have shown above that *mig-6*-deficient animals display accelerated liver regeneration after partial hepatectomy due to increased hepatocyte proliferation. Based on this result we asked the question whether *mig-6* knock-out mice might be susceptible to carcinogen-induced liver carcinogenesis. To do that, we treated *mig-6* knock-out, heterozygote and wildtype mice with the alkylating agent diethyl nitrosamine (DEN). DEN is a potent carcinogen which leads to the development of hepatocellular carcinomas (Maeda et

al., 2005). In young mice (2 weeks of age), DEN is a complete carcinogen whereas in older mice it requires tumor promoting agents in order to induce liver tumors (Sarma et al., 1986). In our experiments all mice were injected with 5mg/kg DEN on postnatal day 14 where a single DEN injection is sufficient to induce tumors. After 6 and 8 months, mice were sacrificed and the liver was macroscopically evaluated for developing hepatocellular carcinomas. It is important to note that female mice are less sensitive to DEN-induced liver carcinogenesis due to hormonal factors (Sarma et al., 1986). As shown in figure 36A, m*ig-6* knock-out mice seem to be highly susceptible to DEN-induced liver carcinogenesis. Importantly, all female *mig-6* knock-out mice develop hepatocellular carcinomas whereas no tumors were detected in *mig-6* wildtype or heterozygote controls (Figure 36B). In male mice, only one knock-out animal could be investigated. Two knock-out mice died during the course of the experiment due to yet unknown reasons. However, the remaining *mig-6* knock-out mouse did develop a large number of hepatocellular carcinomas (Figure 36B). Tumor development was also observed in male heterozygote and wildtype animals but to a lesser extent. Taken together, our results strongly suggest that mig-6 may act as a tumor suppressor in carcinogen-induced liver carcinogenesis.

A

Livers of DEN-injected mice (8 months)

B

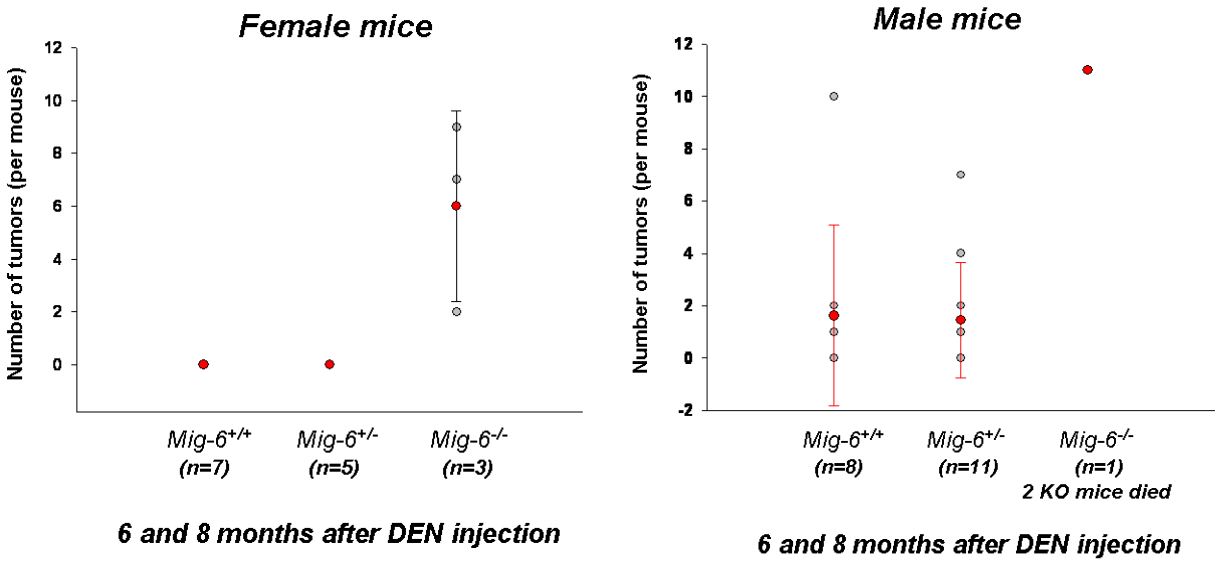

Figure 36. *Mig-6* knock-out mice are susceptible to DEN-induced liver carcinogenesis
(A) 14 days-old *mig-6* wildtype (WT), heterozygote (HET) and knock-out (KO) mice were injected with 5mg/kg Diethyl-nitrosamine (DEN) intraperitonally. After 8 months, the mice were sacrificed and livers were macroscopically evaluated for hepatocellular carcinoma development. Representative pictures of the large liver lobe 8 months after injection are shown.
(B) Quantification of the tumor number per mouse in DEN-injected male and female mice. Male and female mice were sacrificed 6 and 8 months after DEN injection. In the case of male mice, two *mig-6* knock-out animals died during the experiment. n = the number of animals per genotype.

4.2.5 Mig-6 is an endogenous inhibitor of EGFR signalling in human liver cancer cell lines

We have shown previously that mig-6 is downregulated in different human cancers, including breast, prostate, ovarian and skin cancer (Ferby et al., 2006). Furthermore, loss of mig-6 correlated with an increase in EGFR activation in human breast cancer cell lines suggesting that mig-6 is a specific tumour suppressor of EGFR-mediated carcinogenesis. Moreover, *mig-6* knock-out mice are highly susceptible to DEN-induced liver carcinogenesis suggesting a tumor suppressive function. However, the role of mig-6 in human liver cancer remains largely elusive. To study the effect of mig-6 on EGFR function in human liver cancer cell lines, we stimulated Hs 817.T and HepG2 liver carcinoma cells with EGF for the indicated time points (Figure 37). EGF stimulation led to a strong and continuous induction of mig-6 protein expression starting at 2 hours after growth factor addition (Figure 37). Interestingly, mig-6 induction led to a rapid decrease in EGFR phosphorylation and expression. Consistently, we observed a reduction in phosphorylated levels of ERK1/2 (Figure 37). These data demonstrate

that mig-6 is an endogenous inhibitor of EGFR signalling in human liver cancer cell lines and suggest a tumour suppressive role for mig-6 in EGFR-mediated liver carcinogenesis.

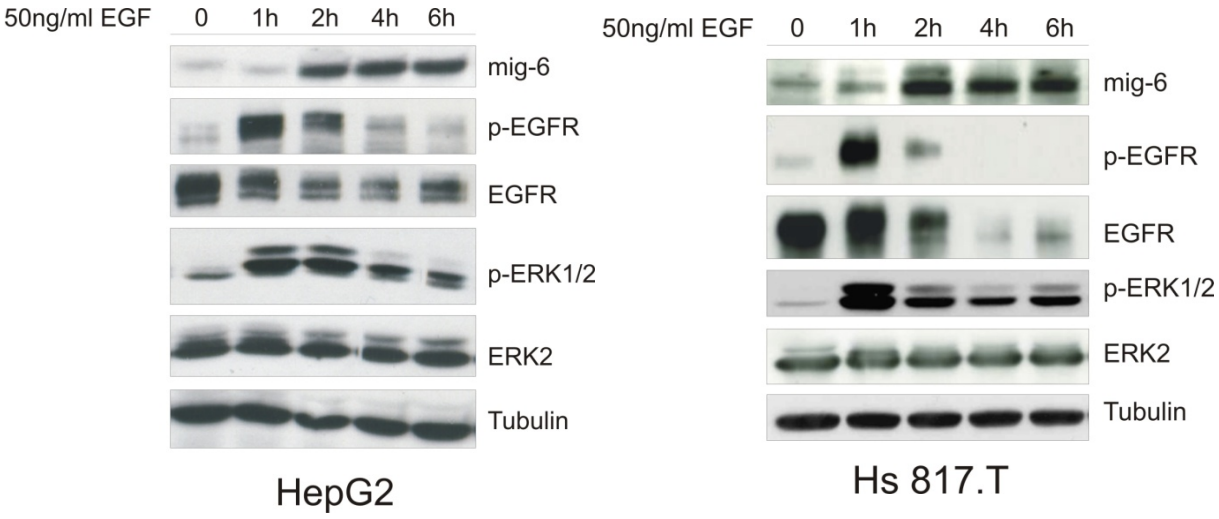

Figure 37. Mig-6 is an endogenous inhibitor of EGFR signalling in human liver cancer cell lines
Mig-6 induction leads to downregulation of EGFR signalling through the MAPK pathway and receptor internalization in human liver cancer cells. Hs 817.T and HepG2 liver cancer cells were stimulated with 50ng/ml EGF for the indicated time points, lysed and subjected to EGFR Immunoprecipitations or Western blot analysis. Immunoblots for mig-6, p-EGFR, EGFR, p-ERK1/2 and ERK2 are shown. Tubulin served as a loading control.

4.2.6 Mig-6 expression inversely correlates with EGFR expression in human hepatocellular carcinomas

Based on the results in liver cancer cell lines we aimed to determine if loss of mig-6 is sufficient to generate EGFR overexpression in human hepatocellular carcinomas (HCC). To address this question we analyzed the expression levels of EGFR and mig-6 immunohistochemically using a tissue micro array of 111 liver cancer patients. EGFR was found to be predominately expressed at the cell membrane whereas mig-6 expression was restricted to the cell cytoplasm. Interestingly, we found moderate to high EGFR expression in 36% of the patients suggesting that EGFR signalling may contribute to the development of a subset of human hepatocellular carcinomas (Figure 38). In contrast mig-6 expression was barely detectable in the vast majority of analyzed tumors (64%; Figure 38). Interestingly, we found that cells expressing EGFR did not show mig-6 expression and vice versa (Figure 39A). Overall, mig-6 expression levels inversely correlated with EGFR expression in 26% of all cases. However, EGFR overexpressing tumors (36%) displayed a significant down-regulation or loss of mig-6 expression in 64% of the cases ($p = 0.006$; Figure 39B). These

data suggest that mig-6 is a possible regulator of the EGFR in HCC and that loss of mig-6 may generate EGFR activation and thereby tumor development.

Taken together, our data define mig-6 as a negative regulator of EGFR signalling in human liver cancer cell lines and as a potential tumor suppressor of the EGFR in a significant number of human hepatocellular carcinomas.

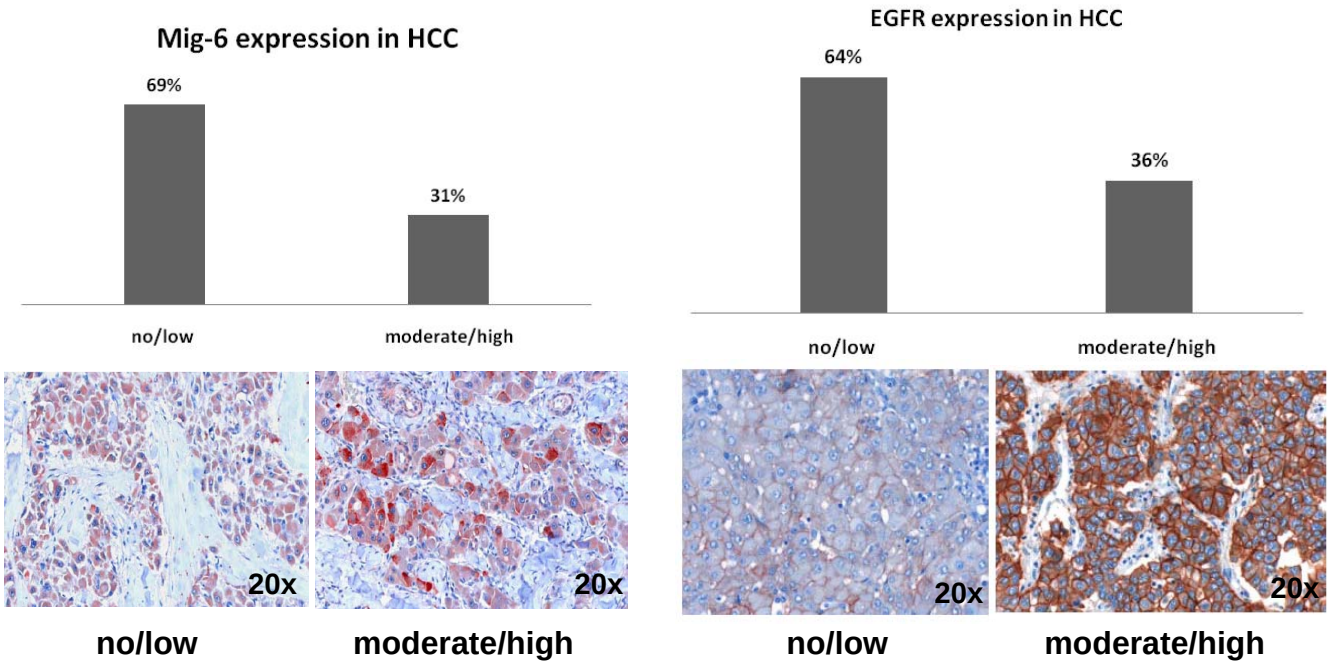

Figure 38. Mig-6 and EGFR expression in human hepatocellular carcinomas
Mig-6 and EGFR protein expression were analyzed immunohistochemically on tissue micro arrays of 111 human hepatocellular carcinomas. The expression intensities were scored as described in Material and Methods.

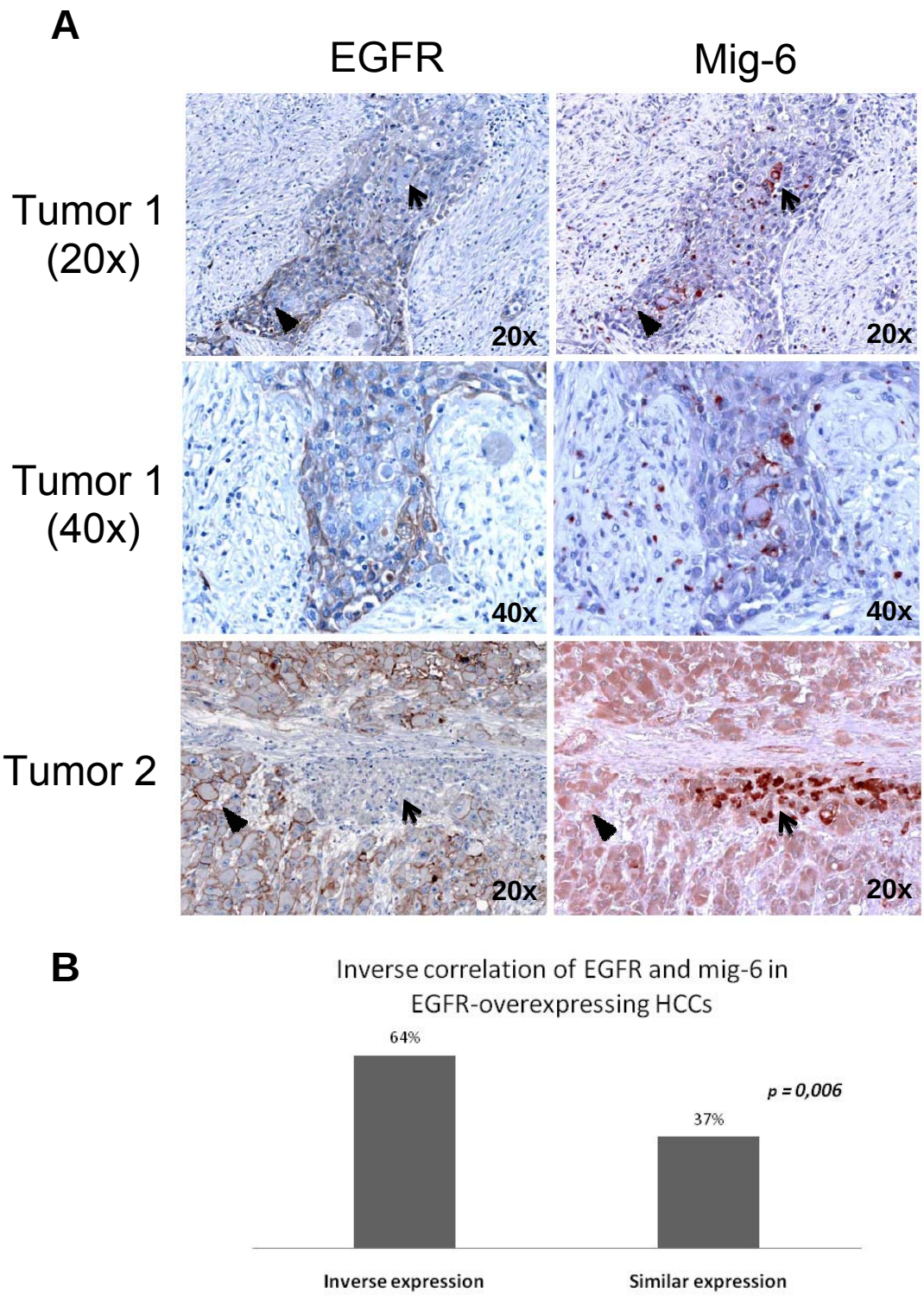

Figure 39. Mig-6 expression inversely correlates with EGFR expression in HCCs
Mig-6 and EGFR protein expression was analyzed immunohistochemically in 111 primary human hepatocellular carcinomas.
(A) Two tumors with inverse expression of mig-6 and EGFR are shown. Cells expressing EGFR but not mig-6 are highlighted with black triangles and cells expressing mig-6 but not EGFR with black arrows.
(B) In EGFR overexpressing tumors mig-6 is markedly downregulated in 64% of the cases (p = 0.006; Spearman's rank correlation).

4.3 Mig-6 Interaction Partners

4.3.1 Identifying mig-6 interaction partners with SILAC and mass spectometry

Mig-6 is a negative regulator of several receptor tyrosine kinases; however the exact mechanism of mig-6 action has not yet been elucidated. Based on the fact that mig-6 is a multi-adaptor protein that contains several protein interaction domains we aimed to identify mig-6 interaction partners that are recruited by mig-6 in order to regulate different cellular functions. To do that, we used stable isotope labelling of amino acids in cell culture (SILAC) combined with mass spectrometry (MS) in mouse embryonic fibroblasts (MEFs) derived from *mig-6* knock-out and wildtype control mice (Selbach et al., 2006). We differentially labelled the cells with light and heavy amino acids, and immunoprecipitated mig-6 with three different antibodies. The resulting lysates were then combined, further processed and subjected to MS analysis (Figure 40A). Under these experimental conditions, specific mig-6 interaction partners should only show a significant ratio in *mig-6* wildtype MEFs when compared to knock-out controls where such proteins should be undetectable (Figure 40B). Interestingly, we identified known interaction partners like 14-3-3gamma as well as novel mig-6 interaction partners like Talin-1 (Figure 40B). It will be of great importance in the future to analyze the biological function of these novel interactions in order to gain further insights into the mode of mig-6 action.

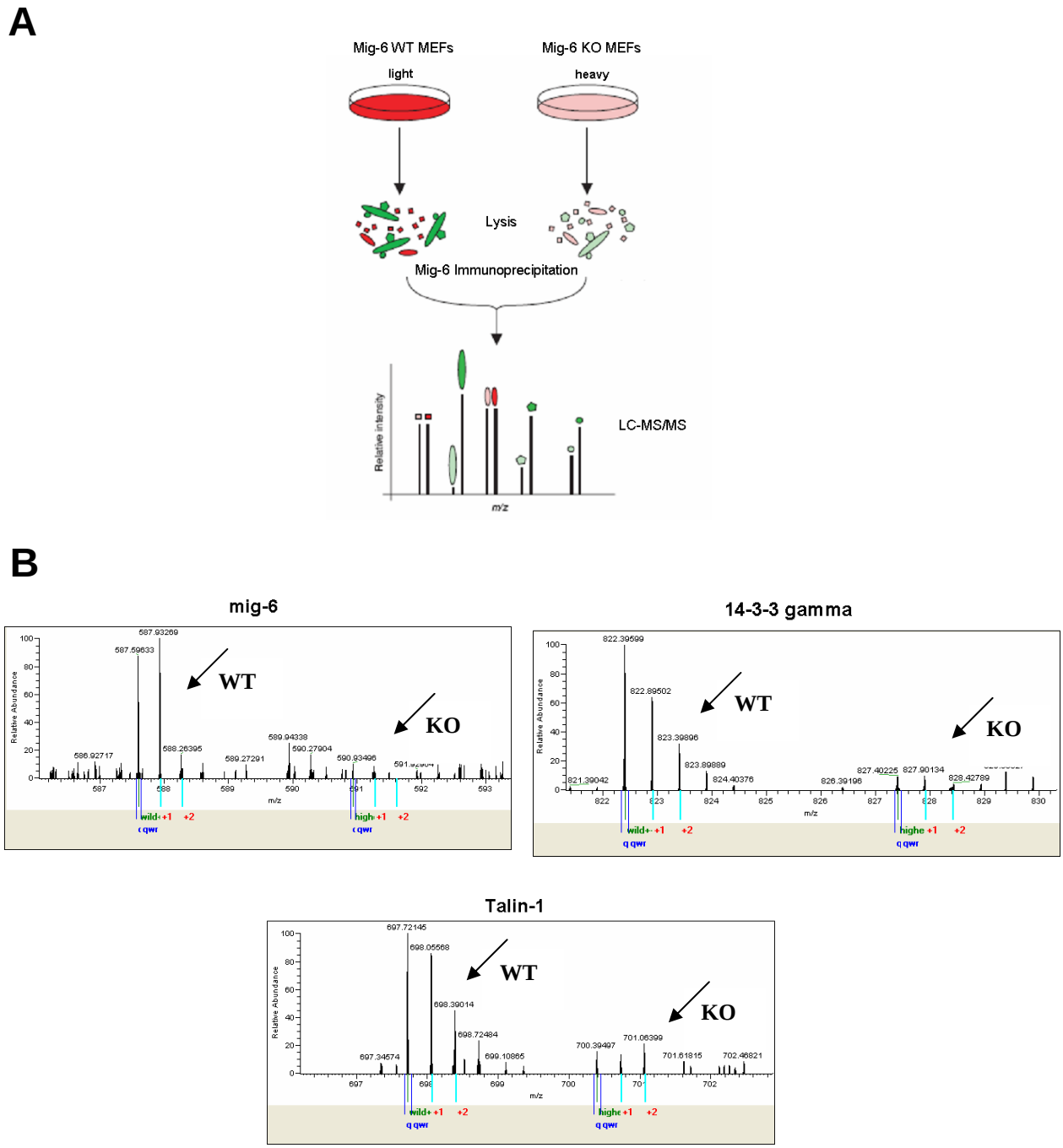

Figure 40. SILAC screen for mig-6 interaction partners
(A) *Mig-6* WT and KO mouse embryonic fibroblasts were labelled with light and heavy amino acids, respectively. After cell lysates, mig-6 was immunoprecipitated and the precipitates were subjected to mass spectrometry (The figure was adapted from Selbach et al., 2006).
(B) Examples for mig-6 interaction partners. Mig-6 could only be detected in wildtype MEFs (see ratios in the spectra) when compared to knock-out controls. Talin-1 and 14-3-3 gamma are potential mig-6 binding partners since these proteins were only detectable in wildtype immunoprecipitates and were absent in knock-out controls.

5. Discussion

5.1 HER3 is a potential target for melanoma therapy development

The understanding of the role of receptor tyrosine kinases in human cancer has led to the development of a variety of drugs which are successfully used in cancer therapy. However, in spite of significant advances in the development of efficacious, side-effect-poor therapies for major malignancies such as breast cancer, there is still a great unmet need for better, more effective therapies for other cancer types. Melanoma is a highly aggressive skin cancer and current therapies only show limited efficacy in patients with late stage disease (Chudnovsky et al., 2005; Gray-Schopfer et al., 2007). So far it is known that the Ras-Raf-MAPK and the PI3K-AKT pathways are frequently activated in malignant melanoma and that this contributes to tumor progression (Vivanco et al., 2002). However, the role of receptor tyrosine kinases in melanoma development remained largely elusive.

HER3 is highly expressed in a variety of human tumors and has been associated in some cases with poor prognosis (Tanner et al., 2006; Witton et al., 2003; Yi et al., 1997). Interestingly, recent studies suggest that breast tumors exposed to EGFR or HER2 targeted therapies escape this inhibition by persistent activation of HER3 and the PI3K-AKT pathway (Sergina et al., 2007; Liu et al., 2007; Engelman et al., 2007) suggesting that agents targeting HER3 could provide a novel and promising approach towards the treatment of some cancers. We have recently shown that *HER3* is frequently expressed in human melanoma cell lines (Ferby et al., 2006) and macro-array as well as northern blot analysis revealed that *HER3* is the most prominently expressed HER-family receptor in these cell lines. In the present study we show *HER3* expression in melanoma and its significant association with tumor cell proliferation. Furthermore, we demonstrate frequently higher HER3 expression in melanoma metastases compared to primary melanoma, suggesting that HER3 may be involved in disease progression. In addition, high levels of HER3 significantly correlated with decreased patient survival establishing HER3 as a novel prognostic marker in clinical melanoma. Importantly, HER3 expression is undetectable in primary melanocytes suggesting that HER3 overexpression specifically occurs during melanoma development. The mechanism of HER3 overexpression appears to be increased gene transcription since Southern blot analysis of 54 human melanoma cell lines did not reveal *HER3* gene amplification. *HER3* gene amplification could so far be detected in non-small cell lung and breast cancer as well as in synovial sarcomas (Cappuzzo et al., 2005; Sassen et al., 2008; Nakagawa et al., 2006)

whereas no amplification was reported for pancreatic, stomach, head and neck and brain tumors (Friess et al., 1995; Katoh et al., 1993; Issing et al., 1993; Reifenberger et al., 1994). However, the exact mechanism of *HER3* transcriptional upregulation remains to be investigated. To address the role of HER3 in melanoma development and progression, we analyzed human melanoma cell lines upon RNA interference for *HER3* expression. Inhibition of *HER3* resulted in reduced cell proliferation, migration and invasion. On the molecular signalling level, suppression of *HER3* led to an increase in p27 protein levels which can be the cause of the observed growth inhibition. Upregulation of p27 may be a direct consequence of the observed impairment of 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), however, in melanoma p27 regulation seems to be independent of AKT since p27 upregulation was also seen in unstimulated *HER3* knock-down cells. Interestingly, p27 proteolysis can be regulated by oncogenic signalling through the EGFR and HER2 (Chu et al., 2008). This mechanism may also apply to HER3-mediated p27 regulation in melanoma cell lines. Notably, *HER3*-deficiency did not affect the ERK1/2 and mTOR kinases suggesting that inhibition of other downstream pathways such as PI3K-AKT seems to be sufficient to block melanoma cell proliferation, migration and invasion.

Interestingly, *HER3* knock-down in lung cancer cells did lead to a significant increase in apoptosis (Sithanandam et al., 2005). However, interference with HER3 function was not sufficient to induce major apoptosis of melanoma cells. Based on these results we tested whether a combination of *HER3* downregulation with chemotherapeutic drug treatment would increase cell death. To date, dacarbazine is the only FDA-approved drug for the treatment of advanced melanoma with a tumor response rate of about 15-20% (Chudnovsky et al., 2005). Dacarbazine belongs to the family of alkylating antineoplastic agents that induce DNA damage and thereby interfere with cancer cell growth. Indeed, *HER3* knock-down cells were highly sensitive to dacarbazine-induced apoptosis suggesting that a combination with agents interfering with HER3 might prove effective in the treatment of malignant melanoma.

To further analyze whether HER3 can be a novel target in melanoma therapy, we treated human melanoma cells lines with anti-HER3 monoclonal antibodies *in vitro*. We found that such antibodies can inhibit heregulin-induced HER3 phosphorylation leading to receptor internalization or degradation. Furthermore, we show that binding of p85, the regulatory subunit of PI3K, to HER3 is abrogated upon antibody-incubation demonstrating that signalling via the PI3K-AKT pathway is inhibited in these cells. Importantly, melanoma cell migration and invasion are greatly reduced in antibody-treated cells when

compared to controls. These data demonstrate that anti-HER3 antibodies can inhibit HER3 signalling via the PI3K pathway in melanoma cell lines and thereby seem to block melanoma cell migration and invasion. It will be of great interest in the future to test the efficacy of such antibodies in preclinical mouse models in order to further validate HER3 as a therapeutic target for melanoma therapy.

Interestingly, recent work by Ueno and colleagues showed that HER3 is essential for heregulin-induced melanoma cell proliferation and metastasis *in vivo* (Ueno et al., 2008). The authors could show that interfering with HER3 function by siRNA could efficiently reduce tumor growth, angiogenesis and spontaneous metastasis to the lung in a xenograft mouse model. Moreover, Wimmer and co-workers could confirm our findings that HER3 is highly expressed in primary melanoma as well as metastases and this overexpression was associated with increased tumor thickness and hyperproliferative tumor stages (Wimmer et al., 2008). These results further support HER3 as a novel promising target for melanoma therapy development.

5.1.1 HER3 signalling regulates the expression of *MMP-14* and *MMP-2*

HER3 is frequently expressed in melanoma metastases and is likely involved in melanoma progression. Furthermore, we could show that HER3 is an essential mediator of melanoma cell migration and invasion *in vitro*. Based on these results we aimed to determine whether HER3 can activate the expression of matrix-metallo proteinases (MMPs) which may contribute to melanoma progression.

The family of human MMPs comprises over 20 zinc-dependent enzymes that are involved in extracellular matrix and basement membrane degradation (Overall et al., 2006). They are classified according to their structure into secreted and membrane-type MMPs. MMPs are often found upregulated in human cancer and contribute to tumor cell migration and invasion (Egelblad et al., 2002).

Recently, Ueno and colleagues reported that HER3 contributes to murine melanoma invasivity by regulating the expression of *MMP-9* (Ueno et al., 2008). However, as we show in human melanoma cell lines HER3 signalling induces the expression of the *MMP-14/MMP-2* axis. Importantly, both *MMP-14* and *MMP-2* expression are dependent on HER3 signalling since suppression of *HER3* by RNAi could block the observed induction.

MMP-14 (MT-1 MMP) belongs to the family of membrane associated MMPs and has several roles in cell migration, invasion and angiogenesis (Sato et al., 2005; Genis et al., 2006; Basile et al., 2007). Moreover, MMP-14 is able to activate proMMP-2 at the cell surface thereby

contributing to tumor cell invasion. Interestingly, MMP-14 promotes human melanoma cell growth and invasion and has been associated with poor prognosis as well as disease progression in melanoma patients (Iida et al., 2004; Kondratiev et al., 2008; Sounni et al., 2002; Hofmann et al., 2000). Thus, MMP-14 may represent an attractive target for melanoma therapy development. Along these lines, several broadband MMP inhibitors have been enrolled in clinical trials, however, none of them showed a significant anti-tumor response (Overall et al., 2002). It will be important in the future to improve the efficacy of anti-MMP inhibitors by generating monoclonal antibodies or small molecule inhibitors that specifically target MMPs like MMP-14 (Tu et al., 2008).

Interestingly, MMP-14 has already been linked to the HER receptor family since it has been shown that the EGFR can induce *MMP-14* expression and subsequent MMP-2 activation (Kheradmand et al., 2002) thereby contributing to lung morphogenesis *in vivo*. It has been shown before that HER3 is essential for the motility and intravasation of breast cancer cells; however, the underlying mechanism has yet not been elucidated (Xue et al., 2006). The fact that HER3 can regulate *MMP-14* and *MMP-2* expression in melanoma may help to understand the mechanism of HER3-mediated tumor cell migration and invasion. Several points have to be addressed in the future in order to strengthen this hypothesis. It remains to be shown that MMP-14 can indeed mediate heregulin-induced cell migration and invasion *in vitro* and *in vivo* for instance through suppression of *MMP-14* expression by RNA interference. Furthermore, it will be interesting to show if HER3-mediated MMP regulation also applies to other tumor types like breast cancer. Taken together, our experiments suggest that HER3 mediates melanoma cell migration and invasion at least in part through the regulation of MMPs and point out new possibilities for therapeutic intervention (Figure 41).

5.2 *Mig-6* is epigenetically silenced in human melanoma cell lines

In a previous study we found that mig-6 is deregulated in human melanoma cell lines and that this inversely correlates with HER3 overexpression (Ferby et al., 2006). Here we could show that there is a significant inverse correlation between HER3 and mig-6 in primary melanoma and metastases. It was shown before that loss of mig-6 leads to an upregulation and activation of the EGFR and enhanced HER2-dependent oncogenic signalling in breast cancer cells (Ferby et al., 2006; Anastasi et al., 2005). Moreover, mig-6 inversely correlates with the EGFR and the EGFRvIII in glioblastoma (data not shown). Here, mig-6 is expressed in

neurons and oligodendrocytes but seems to be largely downregulated in glioblastoma multiforme. In contrast the EGFR and the EGFRvIII are highly expressed in glioblastoma and thus may contribute to glioblastoma development and progression.

These results indicate that mig-6-deficiency is sufficient to promote oncogenic signalling by the HER-family of receptor tyrosine kinases. However, the mechanism of mig-6 downregulation remains unclear. A recent study suggested that *mig-6* is mutated in lung cancer cells leading to *mig-6* inactivation (Zhang et al., 2007); however, no mutations could be detected in a panel of 92 breast carcinomas (Anastasi et al., 2005). Interestingly, in neuroblastoma it was shown that mig-6 expression is induced upon Trichostatin A treatment suggesting that *mig-6* is epigenetically regulated in this tumor type (Carén et al., 2007). Moreover, it was speculated that the *mig-6* gene is lost by Loss of Heterozygosity (LOH) in different human cancers (Tseng et al., 2005; Nomoto et al., 2000; Ragnarsson et al., 1999). Here we show that in human melanoma cells *mig-6* is epigenetically inactivated rather than deleted. DNA methylation as well as the polycomb-repressive pathway seems to be involved in the repression of *mig-6*. In this context, DNA-methylation inhibitor treatment or knock-down of *Suz12*, a structural component of the polycomb-repressive complex 2 (PRC2), efficiently induced *mig-6* expression in melanoma cells. Importantly, components of the PRC2 were shown to be overexpressed in some cancers and to contribute to cancer cell proliferation (Bracken et al., 2003). In normal development as well as in cancer, different epigenetic pathways can contribute various layers of repressive modifications to the inactivation of genes. In WM-1341D melanoma cells, for instance, we additionally identified trimethylation of Lysine 9 (K9) which is caused by a PRC2-independent pathway. The ability to reactivate mig-6 with drugs targeting chromatin modifying enzymes indicates that such an epigenetic therapy is worth to be tested for the treatment of melanoma. In the future several questions have to be addressed in order to clarify the exact mechanism of *mig-6* downregulation as well as its ability to regulate HER3 (Figure 41). Detailed *mig-6* promoter analysis by bisulphite sequencing and subsequent methylation-specific PCRs and *mig-6* overexpressing cell lines may help to answer these points. In addition, in depth analysis of histone modifications at the *mig*-6 promoter are required to fully understand the epigenetic regulation of *mig-6* since it has been shown recently that methylation of histone H3 Lysine 27 can induce gene silencing in cancer cells independent of DNA methylation (Kondo et al., 2008). Taken together, our results establish HER3 as a target for melanoma therapy development and interference with its function may offer a new and promising approach to improve clinical patient outcome (Figure 41).

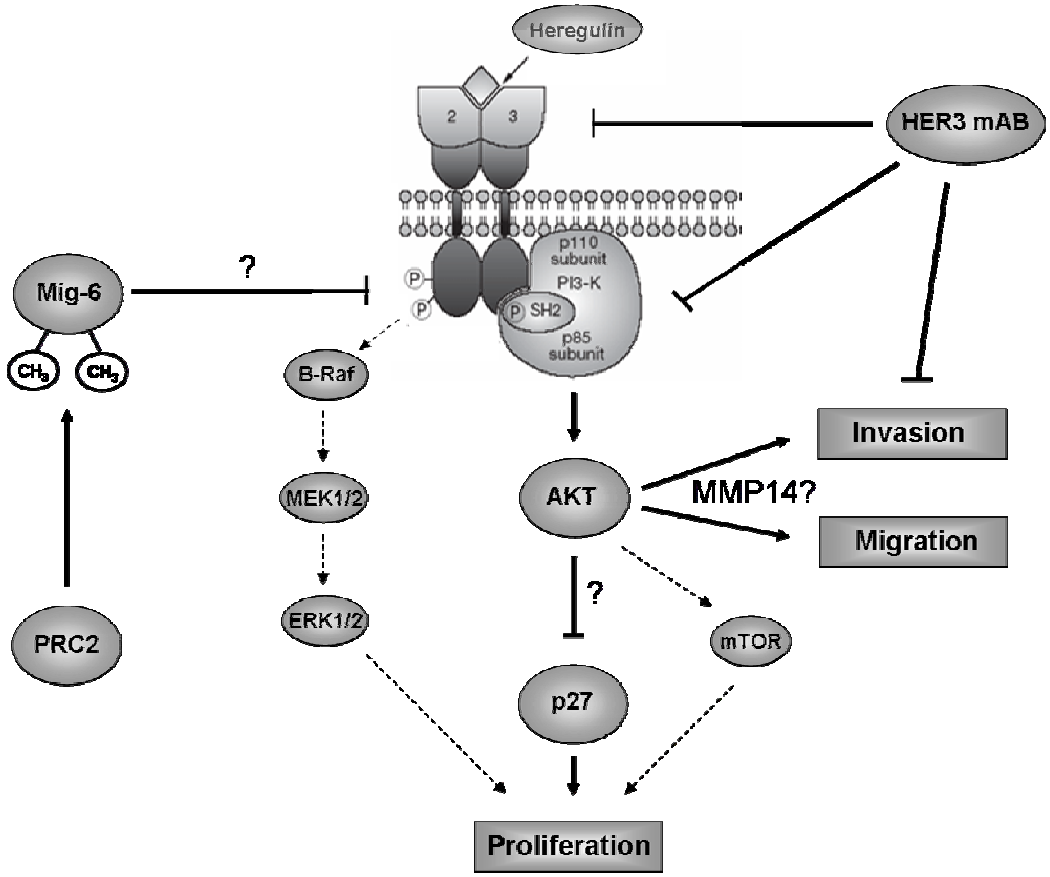

Figure 41. Model for the role of HER3 and mig-6 in human melanoma cells

5.3 Mig-6 is a negative regulator of mouse hepatocyte proliferation

In recent years several studies have significantly improved our understanding of the complex mechanisms underlying liver regeneration. In humans, liver regeneration often occurs upon liver damage by cirrhosis or hepatitis. Defects in proper liver regeneration can result in severe diseases like liver cancer or even death. Thus, it is of great importance to identify novel molecular mechanisms that control liver regeneration. Several studies in rodents, using gene knock-out or transgenic approaches, have identified different receptor tyrosine kinase signalling cascades to be key regulators of proper liver regeneration. In particular, members of the EGF and MET receptor pathways are critically involved in proper hepatocyte proliferation after liver injury. Recently, Natarajan and colleagues demonstrated in mice that the EGFR is a critical regulator of efficient liver regeneration after partial hepatectomy (Natarajan et al., 2007).

However, the role of negative regulators of receptor tyrosine kinase signalling in the regulation of hepatocyte proliferation remains largely elusive. As described above, mig-6 is a negative regulator of EGFR signalling (Hackel et al., 2001). In the liver, mig-6 is highly expressed; however, *mig-6* knock-out mice did not develop any obvious liver abnormalities. It has been shown previously that *mig-6* is an immediate early gene which is up-regulated in regenerating livers of wildtype mice suggesting that mig-6 may play a role in the initial phases of liver regeneration (Mohn et al., 1990; Haber et al., 1993). Here we show that mig-6 is a negative regulator of mouse hepatocyte proliferation *in vivo*. *Mig-6* knock-out mice display a marked increase in hepatocytes re-entering the cell cycle at early time points during liver regeneration. This increased proliferation is most likely due to hyperactivation of the EGF receptor through the serine/threonine kinase AKT pathway. Furthermore, we show that cell cycle regulators like Cyclin D1 are induced in regenerating knock-out livers whereas negative regulators like Rb are inactivated. Notably, we found elevated levels of the AP-1 transcription factor c-Jun and reduced levels of p38 phosphorylation. It is important to note that c-Jun directly controls liver regeneration by repressing p53, p21 as well as p38 MAPK activity (Stepniak et al., 2006). In summary, we have demonstrated that mig-6 is a negative regulator of the EGFR in mouse hepatocyte proliferation *in vivo* and that mig-6 is an essential factor for the initial phases of liver regeneration.

5.3.1 Mig-6 is a tumor suppressor of DEN-induced liver carcinogenesis

As mentioned above, *mig-6* knock-out mice do not develop any liver abnormalities or liver dysfunctions. Occasionally, *mig-6* knock-out mice did develop small hyperplastic liver nodules; however, this seems to be a rather infrequent event and such nodules never developed into hepatocellular carcinomas (data not shown). DEN-induced liver carcinogenesis in mice is a powerful tool to study the role of different genes in hepatocellular carcinoma development. It has been shown in the past that DEN-induced tumor development is dependent on different factors including cytokines like interferon gamma and growth factor receptors like the HER-family and MET (Matsuda et al., 2005; Lee et al., 2007; Horiguchi et al., 2000). Given the fact that mig-6 is a negative regulator of the EGFR and MET we aimed to determine the role of mig-6 in DEN-induced liver carcinogenesis. Interestingly, *mig-6* knock-out mice were highly susceptible to DEN-induced liver tumor development suggesting a tumor suppressive function. Strikingly, all DEN-treated female knock-out mice did develop hepatocellular carcinomas. This observation was rather surprising since it is known that female mice are highly resistant to DEN-induced carcinogenesis due to hormonal factors

(Sharma et al., 1986). One explanation for this might be that *mig-6* knock-out females have deregulated hormone signalling pathways which might cause the observed phenotype and thus would point to a novel role for mig-6 in the regulation of hormone signalling. In addition, it remains to be shown whether mig-6 can negatively regulate the EGFR or the MET receptor during the process of liver tumor development and whether inhibition of either receptor can rescue the observed phenotype.

5.3.2 Mig-6 is a potential tumor suppressor of EGFR-mediated liver carcinogenesis

Given its implication as a tumour suppressor in many different human cancers and its tumor suppressive function in DEN-induced liver carcinogenesis we analyzed the role of mig-6 in human liver cancer cell lines. Importantly, the EGF receptor and its ligands have been described to be frequently expressed in human liver cancer thereby leading to liver tumor development (Harada et al., 1999; Moon et al., 2006; Berasain et al., 2007). In this study, we show that mig-6 is as an endogenous inhibitor of EGFR signalling in human liver cancer cell lines. EGF stimulation of hepatocellular carcinoma cell lines strongly induced mig-6 expression. This induction led to a rapid decrease in EGFR phosphorylation and, in addition, receptor internalization. Concomitantly, we found a downregulation of phospho ERK1/2. These data suggest that mig-6 acts in a negative feedback loop to control EGFR signalling in human hepatocellular carcinomas. In contrast to the murine system where mig-6 seems to regulate the EGFR-AKT pathway, mig-6 did not affect AKT in human hepatocellular carcinoma cell lines (data not shown). One reason for this may be the fact that AKT is constitutively activated in HCCs and somehow rendered unresponsive to negative regulatory signals.

In primary human hepatocellular carcinomas, mig-6 is downregulated in a significant number of cases and that correlates with an increase in EGFR expression. These data suggest that loss of mig-6 in human liver tumors seems to be sufficient to generate increased EGFR signalling which may lead to tumor formation and progression. Interestingly, in EGFR-overexpressing tumors mig-6 expression is lost at high frequency (Figure 39). Several mechanisms including loss of herterozygosity and epigenetic silencing can account for *mig-6* repression in HCCs. It will be the aim of future studies to investigate the exact mechanism of mig-6 regulation in hepatocellular carcinomas and whether it can serve as a marker for EGFR-mediated liver carcinogenesis. Furthermore, it would be critical to elucidate the exact mechanism of mig-6 action towards the EGFR. Recently, Zhang and colleagues have

shown using crystallographic methods that mig-6 directly binds to the EGFR kinase domain thereby regulating EGFR activation (Zhang et al., 2007). This mechanism can also apply to HCCs; however, mig-6 may recruit additional proteins to the receptor which mediate receptor dephosphorylation and internalization.

To identify such interaction partners we performed a preliminary experiment using the SILAC method combined with mass spectrometry (Selbach et al., 2006) in mouse embryonic fibroblasts derived from *mig-6* wildtype and knock-out mice (Figure 40). Interestingly, several potential mig-6 binding partners could be identified. Among them we could identify known mig-6 interaction partners like 14-3-3 proteins as well as novel proteins like Talin-1 which is involved in integrin signalling (Albiges-Rizo et al., 1995; Tadokoro et al., 2003; Nieswandt et al., 2007). Along these lines, integrins are able to from complexes with the EGFR and thereby regulate EGFR downstream signalling (Cabodi et al., 2004). Thus, it is believable that mig-6 may either negatively regulate Talin-1 directly leading to integrin inactivation and subsequent EGFR inactivation or Talin-1 recruits mig-6 to integrin-EGFR complexes leading to its inhibition. It will be the aim of further studies to determine the biological function of these novel mig-6 interaction partners and whether these proteins contribute to the negative regulation of receptor tyrosine kinases. However, under the chosen experimental conditions we could not identify receptor tyrosine kinases like the EGFR suggesting certain limitations for this approach towards the identification of mig-6 binding partners. It will be critical to optimize the assay by using different mig-6 antibodies which bind to different mig-6 epitopes in order to identify the whole spectrum of interacting proteins. Moreover, since mig-6 is inducible by a variety of different extracellular stimuli (Zhang et al., 2007) it will be important to use these defined conditions in order to identify mig-6 binding partners which may bind only under certain physiological conditions thereby regulating different mig-6 functions. Such experiments could be performed in future studies and might help to extend our understanding about the role of mig-6 in different cellular processes.

6. Summary

The mitogen-inducible gene-6 (mig-6) is a multi-adaptor protein implicated in the negative regulation of the HER-family of receptor tyrosine kinases. In this thesis we investigated the role of mig-6 and its relation to the HER receptor family in melanoma development and progression as well as in hepatocyte proliferation *in vivo*.

In the first part of this study we demonstrate that HER3 is frequently expressed in malignant melanoma and metastases at super-normal levels. High HER3 expression may serve as a prognostic marker since it correlates with cell proliferation, tumor progression and reduced patient survival. Suppression of *HER3* expression by RNAi reduces the proliferation, migration and invasion of human melanoma cells and synergistically enhances dacarbazine-induced apoptosis *in vitro*. Importantly, HER3 signalling induces the expression of *MMP-14* and *MMP-2* which may mediate melanoma cell invasivity. Moreover, monoclonal antibodies specific for HER3 efficiently block the proliferation, migration and invasion of melanoma cells. We further show that the expression of the negative regulator mig-6 is reduced in malignant melanoma and metastases. *Mig-6* gene silencing in melanoma cells seems to occur by epigenetic mechanisms that involve DNA methylation and the polycomb-repressive complex 2.

In the second part of this thesis we demonstrate that mig-6 is a negative regulator of the EGFR in hepatocyte proliferation *in vivo* and in human liver cancer cell lines. Strikingly, *mig-6* knock-out mice display increased hepatocyte proliferation after a 70% partial hepatectomy. This phenotype correlates with increased activation of endogenous EGFR signalling predominantly through the AKT pathway. In line with this observation, primary *mig-6*-deficient hepatocytes display increased mitogenic signalling in response to EGF. In addition, we demonstrate that mig-6 is an endogenous inhibitor of EGFR signalling in human liver cancer cell lines. Moreover, mig-6 is downregulated in human hepatocellular carcinomas which correlates with increased EGFR expression.

7. Zusammenfassung

Das *mitogen-inducible gene -6* (mig-6) ist ein Adaptorprotein, dass eine wichtige Rolle bei der negativen Regulation von der Familie der HER Rezeptor Tyrosin Kinasen spielt. In der vorliegenden Doktorarbeit wurde die Funktion von mig-6 und dessen Einfluss auf die HER Rezeptoren sowohl in der Entstehung und der Progression von Melanomen als auch im Wachstum von Hepatozyten *in vivo* untersucht.

Im ersten Teil dieser Arbeit konnte eine hohe HER3 Expression in malignen Melanomen und Metastasen gezeigt werden. Dabei konnte die HER3 Expression als prognostischer Marker postuliert werden, da es mit dem Zellwachstum, der Tumor Progression als auch mit einer geringeren Lebenserwartung der Patienten korreliert. Weiter können Zellwachstum, Migration, Invasion und Insensivität gegen Chemotherapeutika von humanen Melanomzellen stark reduziert werden wenn man die Expressionslevels von *HER3* mittels RNAi senkt. Dabei wird die Zellinvasivität wahrscheinlich durch eine HER3-abhängige *MMP-14* und *MMP-2* Expression gesteuert. Diese physiologischen Effekte können auch durch die Behandlung mit einem HER3-spezifischen Antikörper beobachtet werden. Im Gegensatz zu HER3 ist die Expression des Negativ-Regulators mig-6 in Melanomen stark reduziert. Diese Inhibierung ist wahrscheinlich auf eine epigenetische Regulation zurückzuführen.

Im zweiten Teil dieser Arbeit konnte gezeigt werden, dass mig-6 ein Negativ-Regulator des EGF Rezeptors (EGFR) im Wachstum von Hepatozyten als auch in Leberkarzinomen ist. Dabei zeigen *mig-6* knock-out Hepatozyten ein erhöhtes Zellwachstum nach einer 70% partiellen Hepatektomie das durch eine verstärkte EGFR und AKT Aktivität charakterisiert ist. Auch in primären *mig-6* knock-out Hepatozyten konnte eine erhöhte mitogene Signalübertragung durch den EGFR gezeigt werden. In humanen Leberkarzinomzellen konnte nachgewiesen werden, dass mig-6 ein endogener Inhibitor des EGFR ist. Ein Zusammenhang zwischen mig-6 und dem EGFR konnte des Weiteren in primären Leberkarzinomen gezeigt werden. Dabei konnte festgestellt werden, dass EGFR exprimierende Tumore tendenziell keine mig-6 Expression aufweisen was auf eine tumor suppressive Funktion von mig-6 hindeutet.

8. References

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9. Appendix

Abbreviations

AR	Amphiregulin
ATCC	American type culture collection
bp	Base pairs
BSA	Bovine serum albumin
°C	Degree celsius
c-jun	Cellular homologue to v-jun (avian sarcoma virus 17 oncogene)
cDNA	Copy DNA
CDK4	Cyclin-dependent kinase 4
CRIB	Cdc42/Rac interaction and binding
DEN	Diethyl-nitrosamine
DMBA	Dimethylbenz-anthrazen
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dsDNA	Double-stranded DNA
E.coli	Escherichia coli
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamintetraacetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethyl)-N,N,N',N'- tetraacetic acid
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FDA	US Food and Drug Administration
FGF	Fibroblast growth factor
GRB 2	Growth factor bound 2
GST	Glutathion-S-transferase
GTP	Guanosintriphosphate
h	Hour
HCC	Human hepatocellular carcinoma
HEPES	N-(2-Hydroxyethyl)-piperazin-N‘-2-Ethansulfonic acid
HER2, 3, 4	Human EGF Receptor 2, 3, 4
Hrgß1	Heregulin ß1
HGF	Hepatocyte growth factor
IκBα	Inhibitor of kappa B alpha
IL-6	Interleukin-6
IP	Immunoprecipitation
JAK	Janus-Kinase
JNK	c-Jun N-terminal kinase
kb	Kilobase
kDa	Kilodalton

KO	Knock-out
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
MEFs	Mouse embryonic fibroblasts
MEK	MAPK/ERK Kinase
Mig-6	Mitogen-inducible gene 6
min	Minute
MMP	Matrix metallo proteinase
MT-MMP	Membrane-type matrix metallo proteinase
NF-κB	Nuclear factor-kappa B
PAGE	Polyacrylamide gel elektrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PH	Partial Hepatectomy
PI 3-Kinase	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
PMSF	Phenyl-Methyl-Sulfonyl-Fluoride
Raf	Homologue to v-raf (murine sarcoma viral oncogene)
Ras	Homologue to v-ras (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
Sos	Son of sevenless
SOCS	Suppressor of cytokine signalling
Src	Homologue to v-src (sarcoma viral oncogene)
STAT	Signal transducer and activator of transcription
Syk	Spleen tyrosine kinase
TEMED	N, N, N‘, N‘-Tetramethyletylendiamine
TGFα	Transforming growth factor alpha
TNFα	Tumor necrosis factor alpha
TPA	12-O-Tetradecanoyl-phorbol-13-acetate
Tween 20	Polyoxyethylensorbitanmonolaureate
V	Volt
VEGFR	Vascular endothelial growth factor receptor
Vol	Volume
VSV	Vesicular stomatitis virus glycoprotein VSV-G
WT	Wildtype

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